STRATEGIEN ZUR HERSTELLUNG VON REKOMBINANTEM HUMANEM PROINSULIN

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1 Zusammenfassung

Die rekombinante Herstellung von Proinsulin, seine anschließende Faltung und die Umwandlung zu Insulin sind Verfahren zur großtechnischen Produktion von humanem Insulin für die Therapie des *Diabetes mellitus*. Im Rahmen der Doktorarbeit wurden Möglichkeiten zur Gewinnung von humanem Proinsulin in seiner nativen Form in *Escherichia coli* sowie neue Strategien zur *in vitro* Faltung von Proinsulin untersucht.

Das geeignete Zellkompartiment für die Herstellung von nativem, korrekt disulfidverbrücktem Proinsulin in *E. coli* ist das Periplasma. Es wurden drei verschiedene Ansätze untersucht, um natives Proinsulin in möglichst hoher Ausbeute im Periplasma anzureichern: (1) als Fusionsprotein mit DsbA, (2) durch Kosekretion mit Chaperonen und (3) durch die systematische Variation der Kultivierungsparameter. DsbA ist eine Disulfid-Oxidoreduktase, die an der Disulfidverbrückung von Proteinen im Periplasma von *E. coli* beteiligt ist. Mit DsbA als Fusionspartner konnte natives Proinsulin mit den korrekten Disulfidbrücken hergestellt werden. Durch die Auswahl eines geeigneten *E. coli* Stammes und die Optimierung der Kultivierungsbedingungen konnte die Ausbeute an periplasmatischem Fusionsprotein mit nativem Proinsulinanteil weiter erhöht werden. Außerdem wurden die ATP-unabhängigen Chaperone DnaJ und Hsp25 für die Kosekretion mit Proinsulin verwendet. Chaperone sind Proteine, die durch nicht-kovalente Wechselwirkung mit anderen Proteinen diesen helfen, ihre native Konformation zu erreichen. Durch die Kosekretion von Proinsulin und DnaJ wurde die Ausbeute an nativem Proinsulin, im Vergleich zur Kultivierung ohne Kosekretion von DnaJ, um das 37-fache erhöht. Hsp25 hingegen hatte keinen Einfluß auf die Anreicherung nativen Proinsulins im Periplasma. Abschließend wurde die Gewinnung von nativem Proinsulin im Periplasma ohne Fusionspartner und ohne Kosekretion von Chaperonen weiter optimiert. Neben der systematischen Variation von Kultivierungsparametern wurde das Kultivierungsmedium durch niedermolekulare Zusätze und thiolhaltige Komponenten verändert und dadurch die Ausbeute an nativem Proinsulin drastisch erhöht. Die mit Hilfe der beschriebenen Ansätze erreichten Ausbeuten an nativem Proinsulin betrugen ca. 1 – 9 mg/g Trockenzellmasse. Diese Ausbeuten waren im Vergleich zu vorangegangenen Publikationen zur Herstellung von humanem Proinsulin im Periplasma ca. 100-fach höher. Wenn man die Ausbeuten jedoch mit denen bei der Herstellung von Proinsulin-Varianten mit verkürztem C-Peptid oder der Herstellung von Proinsulin in Form von *inclusion bodies* vergleicht, war die periplasmatische Produktion wesentlich weniger

effizient. Das zeigt deutlich, daß mit dem verwendeten Proinsulinkonstrukt unter den beschriebenen Bedingungen kein technisch relevanter Prozeß etabliert werden konnte. Neben der Maßstabsvergrößerung müßte auch eine intensive Optimierung insbesondere vermutlich des Proinsulinkonstruktes vorgenommen werden.

Neben der Herstellung von Proinsulin in nativer Form in *E. coli* wurde die *in vitro* Faltung von humanem Proinsulin untersucht und mittels *reversed phase* HPLC analysiert. Die Rückfaltung denaturierten und reduzierten Proinsulins war stark vom pH-Wert und dem Redoxpotential des Rückfaltungspuffers abhängig. Höchste Faltungsausbeuten wurden bei pH 9,5 – 11 und unter stark oxidierenden Bedingungen mit dem Redoxpaar Cystein/Cystin erreicht. Die Faltung des Proinsulins war bereits nach ca. 20 min abgeschlossen und es konnten Ausbeuten von 60 – 70 % nativem Proinsulin erreicht werden. Die Proinsulinfaltung war optimal bei Proteinkonzentrationen von 0,1 – 0,5 mg/ml. Bis 3 M Guanidinium-hydrochlorid bzw. 4 M Harnstoff konnte Proinsulin mit unverändert hoher Ausbeute rückgefaltet werden. Um die Volumen-Zeit-Ausbeute der Proinsulinfaltung zu erhöhen, wurde Proinsulin schrittweise rückgefaltet, d.h. Proinsulin wurde für 30 min renaturiert und anschließend sukkzessive denaturiertes, reduziertes Proinsulin zum Faltungsansatz zugegeben. Auf jeder Stufe der 30 Pulse umfassenden Pulsrenaturierung wurden Faltungsausbeuten um 60 % erreicht. Am Ende der Renaturierung betrug die Konzentration an nativem, korrekt disulfidverbrücktem Proinsulin im Faltungsansatz ca. 6 mg/ml. Das demonstriert, daß man mittels Pulsrenaturierung humanes Proinsulin in technisch relevanten Konzentrationen herstellen kann.

Weiterhin wurde die Proinsulinfaltung bei pH 7,5 mit Glutathion als Redoxsystem in Gegenwart von Proteindisulfidisomerase (PDI) untersucht. PDI ist ein Protein, das sowohl Chaperon- als auch Isomeraseaktivität besitzt. Es wurden PDI-Mutanten ohne Isomeraseaktivität (PDIΔC1,2) bzw. ohne Chaperonfunktion (PDI-aba'c) hergestellt und charakterisiert. Alle PDI-Varianten hatten vergleichbare Sekundärstrukturanteile und waren gleich stabil. Für Wildtyp-PDI (WT-PDI) und PDIΔC1,2 konnte mittels chemischer Quervernetzung die gleiche Bindungsfähigkeit für Peptide und Proteine gezeigt werden. Die Mutante PDI-aba'c hatte hingegen wie erwartet keine Peptidbindungsfähigkeit und keine Chaperonaktivität. Mittels eines Fluoreszenzassays wurde die Redoxaktivität der WT-PDI und der Mutante PDI-aba'c bezüglich Glutathion analysiert und für beide Varianten ein Redoxpotential von –0,12 V ermittelt.

Die nicht-katalysierte Proinsulinfaltung ist durch eine Geschwindigkeitskonstante von k_{app} = 0,002 s^{-1} charakterisiert und ermöglicht Ausbeuten an nativem Protein von ca. 20 %. In Gegenwart von WT-PDI bzw. PDI-aba'c wurde die Faltung signifikant beschleunigt sowie die Ausbeute auf maximal 55 % bzw. 40 % erhöht. Diese PDI-Varianten waren im Gegensatz zu PDIΔC1,2 katalytisch aktiv, d.h. sie beeinflußten die Proinsulinfaltung aufgrund ihrer Isomeraseaktivität. PDIΔC1,2 hingegen hatte keinen Einfluß auf die Geschwindigkeit der Proinsulinfaltung, aber erhöhte in mindestens equimolaren Konzentrationen die Ausbeute der Proinsulinfaltung auf ca. 40 %. Das Erfordernis von equimolaren Konzentrationen demonstriert, daß PDIΔC1,2 die Proinsulinfaltung als Chaperon beeinflußte. Mittels Lichtstreuung wurde der Einfluß der Chaperonfunktion der PDI auf die Aggregation des faltenden Proinsulins untersucht. Bereits 20 – 30 Sekunden nach dem Faltungsstart war die Aggregation des Proinsulins maximal. WT-PDI in katalytischen bzw. equimolaren Konzentrationen zu Proinsulin konnte ca. 10 – 20 % bzw. 40 % des faltenden Proinsulins vor Aggregation schützen. In mindestens equimolaren Konzentrationen konnte auch PDIΔC1,2 die Aggregation des Proinsulins um ca. 40 % verringern.

Um zu analysieren, ob die Chaperonfunktion bzw. Isomeraseaktivität der PDI während der gesamten Dauer der Proinsulinfaltung benötigt wird, wurden sogenannte *timed addition* Experimente durchgeführt, bei denen zu verschiedenen Zeiten nach Faltungsstart die PDI-Variante zugegeben wurde. PDIΔC1,2, die erst 7 Sekunden nach der Initiation der Faltung dem Faltungsansatz zugegeben wurde, konnte die Faltungsausbeute nicht erhöhen. Das demonstriert, daß die Chaperonfunktion der PDI nur in den ersten Sekunden der Faltung essentiell ist, um eine erhöhte Faltungsausbeute zu erreichen. Die Isomeraseaktivität hingegen beeinflußte die Proinsulinfaltung während der gesamten Faltungsdauer und war unabhängig von der Chaperonfunktion.

Da WT-PDI die Proinsulinfaltung deutlich beschleunigte und die Ausbeute an nativem Proinsulin erhöhte, wurde auch Vectrase™-P hinsichtlich ihres Einflusses auf die Faltungsausbeute und -geschwindigkeit untersucht. Vectrase™-P ist ein niedermolekulares Dithiol, das die Isomeraseaktivität von PDI nachahmt. Bereits katalytische Mengen an Vectrase™-P konnten die Ausbeute an nativem Proinsulin um ca. 30 % erhöhen. Die Geschwindigkeit der Proinsulinfaltung und die Abhängigkeit der Faltung vom Redoxpotential des Faltungspuffers wurden durch Vectrase™-P jedoch nicht beeinflußt.

2 Einleitung

2.1 Geschichte von Insulin und Proinsulin

Bereits 1892 beschrieben Mehring und Hedon die blutzuckersenkende Wirkung eines Pankreaspräparates. 30 Jahre später konnte durch Banting und Best erstmals Insulin isoliert und erfolgreich zur Behandlung eines diabetischen Hundes eingesetzt werden (Banting und Best, 1922). In den folgenden Jahrzehnten folgten die Sequenz- und Strukturanalyse des Insulins (Blundell et al., 1971a; Blundell et al., 1971b; Dodson et al., 1966; Harding et al., 1966; Adam et al., 1966; Sanger und Thompson, 1953; Sanger und Tuppy, 1951).

Nachdem die Bedeutung des Insulins für die Behandlung des *Diabetes mellitus* erkannt war, wurde verstärkt an der Herstellung von Insulin geforscht. Insulin oder Insulin-Analoga wurden synthetisch hergestellt und hinsichtlich ihrer biologischen Aktivität untersucht (Wollmer et al., 1981; Sieber et al., 1978; Sieber et al., 1974; Zahn et al., 1965; Pohlmeyer und Otto, 1965; Katsoyannis et al., 1964; Katsoyannis et al., 1963). Aufgrund der hohen Kosten und technisch unzureichender Produktionsmengen wurde das Augenmerk aber bald auf die Isolierung von Insulin aus Schweine- und Rinderpankreas sowie die gentechnische Herstellung von humanem Insulin gerichtet. Goeddel et al. beschrieben und patentierten als erste die Herstellung von Insulin in einem rekombinanten Organismus (Goeddel et al., 1982; Goeddel et al., 1979). Das darauf basierende, von Genentech Inc. umgesetzte, Verfahren war das erste zur rekombinanten Herstellung eines therapeutischen Proteins im technischen Maßstab. Es folgten viele weitere Beispiele zur rekombinanten Gewinnung von Insulin und Proinsulin (siehe Kapitel 2.4). In Hinblick auf eine verbesserte Therapie des *Diabetes mellitus* wurden Insulin-Analoga entwickelt, biophysikalisch charakterisiert (Weiss et al., 2001; Brange et al., 1990; Roy et al., 1990) und auf ihre biologische Aktivität untersucht (siehe Kapitel 2.2). Es wurden auch Möglichkeiten zur Behandlung des *Diabetes mellitus* in Ratten und Mäusen mittels Gentherapie untersucht (Yamaoka, 2001; Halvorsen und Levine, 2001; Demeterco und Levine, 2001; Lee et al., 2000; Olefsky, 2000). Heute werden sowohl Insulin als auch unterschiedliche Insulin-Analoga im technischen Maßstab hergestellt und in der Behandlung des *Diabetes mellitus* eingesetzt.

2.2 Eigenschaften, Biosynthese und Wirkung von Insulin

Insulin ist ein α-helikales Protein mit einem Molekulargewicht von 5700 Da. Es besteht aus der A-Kette und der B-Kette, die durch zwei intermolekulare Disulfidbrücken miteinander verbunden sind. Die A-Kette enthält außerdem eine intramolekulare Disulfidbrücke. Die biologische Vorstufe des Insulins ist das Proinsulin. Im Proinsulin sind die A- und B-Kette durch das C-Peptid (*connecting peptide*) verbunden. *In vitro* kann die Prozessierung des Proinsulins zum Insulin mittels Trypsin und Carboxypeptidase B bzw. mit Hilfe der Furin/ Prohormonconvertase-Proteasen durchgeführt werden (Mackin, 1998; Cowley und Mackin, 1997; Kaufmann et al., 1995; Kemmler et al., 1971).

Die Biosynthese des Prä-Proinsulins erfolgt in den β-Zellen der Langerhansschen Inseln des Pankreas. In den sekretorischen Granula der β-Zellen erfolgt die Umwandlung des Proinsulins zum Insulin, bei der das C-Peptid aus dem Proinsulin herausgespalten wird. Zwei für die Umwandlung des Proinsulins in seine biologisch aktive Form notwendige proteolytische Aktivitäten wurden in den sekretorischen Granula der β-Zellen identifiziert. Es handelt sich hierbei um Proteasen der Furin/Prohormonconvertase Familie, die für die Spaltung vieler Peptidhormon-Vorstufen verantwortlich gemacht werden (Mackin, 1998). Diese Proteasen werden durch den sauren pH-Wert in den reifen sekretorischen Granula aktiviert (Orci et al., 1987; Rhodes und Halban, 1987). Die Umwandlung des Proinsulins ist mit der Entstehung korrekt prozessierten Insulins in den sekretorischen Granula der β-Zellen abgeschlossen.

Insulin ist ein Peptidhormon mit blutzuckersenkender Wirkung. Als Antwort auf eine Erhöhung des Blutzuckerspiegels wird aus den sekretorischen Granula der β-Zellen der Langerhansschen Inseln das Insulin und das in equimolaren Konzentrationen anfallende C-Peptid exozytotisch freigesetzt. Dadurch kommt es zu einem erhöhten Umsatz der Glukose und somit zu einer Absenkung des Blutzuckerspiegels. Ein Übermaß an Insulin bewirkt Hypoglykämie, die negative Effekte u.a. auf den Hirnstoffwechsel hat. Bei Insulinmangel werden die Vorgänge zur Absenkung der Glukosekonzentration im Serum nicht induziert, sodaß der Blutzuckerspiegel steigt (Hyperglykämie). *Diabetes mellitus* ist eine Stoffwechselkrankheit, die mit Hyperglykämie und Glukosurie einhergeht. Man unterscheidet bei *Diabetes mellitus* verschiedene Formen (lt. Empfehlung der Weltgesundheitsorganisation von 1980):

Typ-I-*Diabetes*: Auch insulinabhängiger *Diabetes* genannt. Diese Form wird durch den Verlust insulinproduzierender β-Zellen aufgrund einer Autoimmunreaktion ausgelöst. Es gibt

Hinweise darauf, daß genetische Faktoren bei der Anfälligkeit für Typ-I-*Diabetes* eine Rolle spielen (Davies et al., 1994). Die Applikation von Insulin ist bisher die einzige Therapiemöglichkeit.

Typ-II-*Diabetes*: Auch nicht-insulinabhängiger *Diabetes* genannt. Er wird von zwei metabolischen Defekten hervorgerufen: (1) Insulinresistenz von Insulin-sensitivem Gewebe (z.B. Leber, Muskel, Fettgewebe) und (2) Defekte in der Insulinsekretion. Auch hier werden genetische Faktoren für eine Anfälligkeit für Typ-II-*Diabetes* verantwortlich gemacht. Man unterscheidet weiterhin zwischen Typ-IIa-*Diabetes* (nicht fettsüchtig) und Typ-IIb-*Diabetes* (fettsüchtig). Die Behandlungsmöglichkeiten beschränken sich auf eine zuckerreduzierte Diät und Medikamente zur Steigerung der Insulinsekretion. Unter Umständen kann auch durch Insulingabe die Kontrolle des Blutzuckerspiegels erreicht werden.

Durch Applikation von Insulin können beide Typen des *Diabetes* behandelt werden. Lösliches humanes Insulin („reguläres Insulin") erreicht ca. 1,5 bis 2 Stunden nach der subcutanen Injektion seine maximale Konzentration im Serum. Neutrales-Protamin-Hagedorn-Insulin („NPH") oder Zink-Insulin-Formulierungen haben eine verzögerte und mittel- bis langanhaltende Wirksamkeit (McCormick und Quinn, 2002). Um das Insulinprofil im Blut möglichst dem physiologischen Profil in gesunden Personen anzupassen, wurden Insulin-Analoga entwickelt. Derzeit befinden sich schnell wirksame sowie langanhaltend wirkende Insulin-Analoga auf dem Markt bzw. in klinischen Studien (Tabelle 1). Insulin-Analoga mit schnellerer Wirksamkeit enthalten Aminosäuresubstitutionen in der B-Kette des Insulins (z.B. Aspartat, Lysin, Prolin), durch die die Oligomerisierung des Insulins unterdrückt wird (Balschmidt und Brange, 1998; Brange et al., 1990; Sakata, 1985). Analoga mit längerer Verweilzeit im Organismus haben hingegen eine höhere Tendenz zur Selbstaggregation, da durch das Einfügen oder Anfügen von basischen Aminosäuren (z.B. Arginin) der isoelektrische Punkt des Insulin-Analogons erhöht wird. Nach subcutaner Injektion kommt es zur Kristallisation und somit zu einer verzögerten Freisetzung des Insulin-Analogons. Diese Insulin-Analoga können auch in Kombination miteinander oder mit regulärem oder NPH-Insulin eingesetzt werden und eine physiologischere Insulinkonzentration im Blut ermöglichen als es mit regulärem Insulin alleine möglich ist (Jacobsen et al., 2000). Eine Anwendung von Insulin-Analoga könnte die Nebenwirkungen der Therapie, die besonders durch schlecht regulierte Insulinprofile im Blut verursacht werden, verringern sowie die Lebensqualität der Patienten verbessern.

Tabelle 1 Übersicht über Insulin-Analoga, die in klinischen Studien zur Behandlung des *Diabetes mellitus* eingesetzt werden.

	Eigenschaften	**Referenz**
verlängert wirkendes Insulin:		
Modifikation: A21 Glycin, B31 Arginin und B32 Arginin angwendet von: Aventis Pharma (*Insulin glargine*)	im Vergleich zu NPH Insulin: - verzögerte Wirkung - verlängertes und verzögertes metabolisches Profil - keine ausgeprägte Maximalkonzentration im Serum - Wirkzeit 20 – 30 h - bessere Blutzuckerkontrolle in der Nacht - verringertes Auftreten von Hypoglykämia	McKeage und Goa, 2001; Campbell et al., 2001; Ashwell und Home, 2001
schnell wirkendes Insulin:		
Modifikation: B28 Aspartat bzw. B28 Lysin und B29 Prolin angewendet von: Novo Nordisk (*Insulin Aspart*), Lilli Co. Eli (inhalierbares *Insulin Aspart*), Humalog (*lispro*)	im Vergleich zu löslichem, humanem Insulin: - nach subcutaner Injektion schneller absorbiert - gleiche biologische Verfügbarkeit - 2-fach schnelleres Erreichen der Maximalkonzentration im Serum - 2-fach höhere maximale Serumkonzentration - 2-fach kürzere Verweilzeit im Blut - gleiche Rezeptorbindung - gleiche kardiovaskuläre Risiken - kann besser das endogene Insulinprofil im Blut nachahmen	Bode et al., 2002 Ertl, 2001; Heller et al., 2002; Ross et al., 2001; Lindholm und Jacobsen, 2001; Dimarchi, 1999; Balschmidt und Brange, 1998

2.3 Rekombinante Gewinnung von Proinsulin und Insulin

Neben der Gewinnung von Insulin aus Rinder-, Schweine- oder Schafpankreas und semi-synthetischen Methoden zur Konvertierung Schweineinsulins zu humanem Insulin können Insulin und Proinsulin mittels verschiedener Organismen rekombinant gewonnen werden. Die Mehrzahl industrieller Prozesse zur rekombinanten Herstellung von humanem Insulin nutzt *Escherichia coli* als Wirtsorganismus. In diesem gut charakterisierten Bakterium können rekombinante Proteine in Form von *inclusion bodies* effizient produziert werden (Rudolph,

1997; Rudolph und Lilie, 1996). Die Vorteile der Produktion unlöslichen Proteins in *E. coli* sind überzeugend: (a) meist kurze Prozeßführung, (b) das rekombinante Protein kann in Ausbeuten von bis zu 80 % des Gesamtzellproteins im Cytoplasma akkumuliert werden, (c) im *inclusion body* ist das Protein weitgehend vor proteolytischem Abbau durch Wirtsproteasen geschützt, (d) einfache und effiziente Isolierung der *inclusion bodies* aus dem Zellextrakt, (e) das isolierte rekombinante Protein enthält vielfach kaum Fremdproteine.

Die angewendeten bzw. patentierten Verfahren zur Herstellung von Insulin und Proinsulin sind nachfolgend aufgelistet.

(1) Herstellung der A- bzw. B-Kette des Insulins in *E. coli* in Form von *inclusion bodies*. Anschließend werden die isolierten Ketten des Insulins nach Sulfonierung der Cysteine vereinigt und in Gegenwart eines geeigneten Reduktionsmittels zum nativen Insulin rekombiniert (Chance und Hoffmann, 1983).

(2) Produktion von Proinsulin oder mini-Proinsulin in *inclusion bodies* in *E. coli*. Das Protein kann nach seiner Isolierung in Gegenwart von Redoxsubstanzen zum nativen Protein gefaltet und durch entsprechende Enzyme zum Insulin umgesetzt werden (Ertl et al., 2001; Vilela et al., 2000; Gorecki et al., 1996; DiMarchi et al., 1992; Dorschug et al., 1989; Goeddel et al., 1982). In einigen Fällen werden auch hier vor der Faltung die Cysteine mittels Sulfitolyse sulfoniert.

(3) Gewinnung nativen, humanen Proinsulins in *Streptomyces lividans*. Das bereits korrekt disulfidverbrückte Proinsulin wird aus dem Kulturmedium isoliert und enzymatisch zum Humaninsulin konvertiert (Koller und Riess, 1991).

(4) Gewinnung humanen Insulins oder humaner Insulin-Derivate durch chemische Modifikation von rekombinant produziertem Schweineinsulin (Obermeier et al., 1986).

(5) Nutzung rekombinanter Tabakpflanzen für die Gewinnung humanen Insulins (Daniell, 2001).

Neben den technischen Prozessen zur rekombinanten Produktion von Insulin und Proinsulin gibt es eine Vielzahl akademisch interessanter Ansätze zur Gewinnung von Insulin und Proinsulin in rekombinanten *E. coli* (Tikhonov et al., 2001; Schmidt et al., 1999; Chang et al., 1998; Cowley und Mackin, 1997; Shin et al., 1997; Castellanos-Serra et al., 1996; Samuelsson et al., 1996; Wei und Tang, 1995; Wei et al., 1995; Tang et al., 1993; Sung et al., 1986; Williams et al., 1982). Vielfach gelingt durch die Modifikation des N-Terminus, Austausch von Aminosäuren, das Anfügen eines *tag*s, die Fusion mit anderen Proteinen oder

durch Modifikation der Sequenz des C-Peptides eine verbesserte cytoplasmatische Akkumulation des rekombinanten Proteins. Die Ausbeuten an rekombinantem Insulin oder Proinsulin erreichen gewöhnlich 20 – 25 % des Gesamtzellproteins (Chang et al., 1998; Sung et al., 1986), es wurden aber auch Ausbeuten um 5 % (Wei and Tang, 1995) bzw. 90 % beschrieben (Tikhonov et al., 2001). Nach der Isolierung des rekombinanten Proteins folgt gegebenenfalls die Derivatisierung der Cysteine mittels Sulfitolyse (Katsoyannis et al., 1967) und anschließend die Faltung zur nativen Spezies in Gegenwart eines Reduktionsmittels wie z.B. β-Mercaptoethanol (Schmidt et al., 1999; Cowley und Mackin, 1997; Dai und Tang, 1996). Auch nicht-derivatisierte Cysteine können Disulfidbrücken formen, indem das reduzierte Proinsulin oder die reduzierte A- und B-Kette des Insulins in Gegenwart von Redoxsubstanzen inkubiert werden (Markussen, 1985; Katsoyannis und Tometsko, 1966). Typische Faltungsausbeuten liegen im Bereich um 50 % (Katsoyannis et al., 1967).

Die oben beschriebenen Verfahren beinhalten die Produktion unlöslichen, nicht-nativen Proteins im Cytoplasma von *E. coli* sowie die *in vitro* Rückfaltung zum nativen Insulin bzw. Proinsulin. Zusätzlich gibt es Untersuchungen zur Produktion des Proinsulins in *E. coli* in nativer Form. Dies kann im Periplasma von *E. coli* erreicht werden, da dort die zur Disulfidbrückenausbildung notwendigen oxidierenden Bedingungen herrschen (Rietsch und Beckwith, 1998). Außerdem ist, im Vergleich zum Cytoplasma, das native Proinsulin im Periplasma ca. 10-mal stabiler (Talmadge und Gilbert, 1982). Die beschriebenen Ausbeuten an nativem Proinsulin sind jedoch mit 0,002 – 0,01 mg/g Zellen mehrere Zehnerpotenzen geringer verglichen mit den oben beschriebenen Ausbeuten für die cytoplasmatische Produktion in *inclusion bodies* (Talmadge et al., 1981; Chan et al., 1981; Talmadge et al., 1980a; Talmadge et al., 1980b). Durch Modifikationen im C-Peptid des Proinsulins oder die Expression eines Konstruktes, das mehrere Kopien des Proinsulingens hintereinander enthält, kann die Ausbeute allerdings erhöht werden (Kang und Yoon, 1994; Shen, 1984).

Neben *E. coli* wurden auch andere Wirtsorganismen, z.B. *Bacillus amyloliquifaciens*, *Streptomyces lividans*, *Saccharomyces cereviseae* wurden für die Gewinnung nativen Proinsulins verwendet (Vertesy et al., 1991; Novikov et al., 1990; Thim et al., 1986). Die Ausbeute an nativem Proinsulin war jedoch auch hier sehr gering.

2.4 Der Faltungsweg von Insulin und Proinsulin

Insulin und Proinsulin enthalten in der nativen Struktur drei Disulfidbrücken (Abb. 1), die essentiell für deren native Konformation sind (Dai und Tang, 1996; Dai und Tang, 1994). Im nativen Insulin sind die folgenden Disulfidbrücken ausgebildet: [1] A7 – B7, [2] A20 – B19, [3] A6 – A11. Disulfidbrücke [3] ist intramolekular und die Disulfidbrücken [1] und [2] verbinden die A-und B-Kette des Insulins. Im einkettigen Proinsulin sind es entsprechend die Verknüpfungen [1] Cys^{7} – Cys^{72}, [2] Cys^{19} – Cys^{85}, [3] Cys^{71} – Cys^{76}.

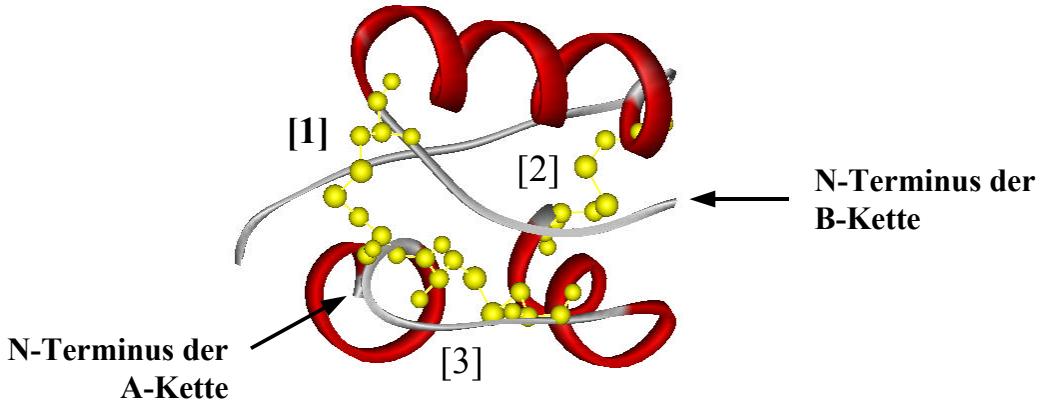

Abb. 1: Röntgenkristallstruktur von humanem Insulin mit einer Auflösung von 1,8 Å (Wittingham, 1996), PDB Eintrag 1XDA. Die α-Helices sind rot und die Cysteine gelb dargestellt. Die Disulfidbrücken sind analog zum Text mit [1], [2] bzw. [3] beschriftet.

Disulfidbrücke [3] wird während der Faltung des Insulins nach der Rekombination reduzierter A- und B-Kette bzw. während der Faltung des Proinsulins als erste und in einer sehr schnellen Reaktion ausgebildet (Yuan et al., 1999). In Untersuchungen mit einer Insulin-Variante aus Schwein, deren A- und B-Kette durch zwei Aminosäuren anstelle des C-Peptides verbunden sind, wurde festgestellt, daß auch dort Disulfidbrücke [3] sehr schnell ausgebildet wird. Hingegen wird Disulfidbrücke [2] wesentlich langsamer geformt (Qiao et al., 2001; Yuan et al., 1999). Diese Insulin-Variante faltet via verschiedener Zwei-Disulfid-Spezies, darunter auch Spezies mit nicht-nativen Disulfidbrücken. Qiao et al. (2001) postulierten, daß es mindestens zwei Faltungswege für die Insulin-Variante gibt:

(1) Einen direkten Weg.

Es wird in einer schnellen Reaktion Disulfidbrücke [3] ausgebildet. Dann folgt die langsame Ausbildung von Disulfidbrücke [1] oder [2] und anschließend die sehr schnelle Faltung zum nativen Protein.

(2) Einen selektiven Weg.

Im Gegensatz zum ersten Weg beinhaltet der selektive Faltungsweg Isomerisierungen von Disulfidbrücken. Es wird als erstes in einer langsamen Reaktion Disulfidbrücke [2] ausgebildet. Nahezu zeitgleich wird eine zweite Disulfidbrücke ausgebildet, die einer nativen oder nicht-nativen Verknüpfung entspricht. Durch langsame Isomerisierungen entstehen die nativen Zwei-Disulfid-Spezies, die dann zum nativen Proinsulin falten.

Insulin und Proinsulin scheinen somit auf ähnlichem Wege zu falten wie das homologe Protein *insulin-like growth factor* I (IGF-I, Yang et al., 1999; Milner et al., 1999; Rosenfeld et al., 1997). Der Hauptunterschied ist jedoch, daß beide Faltungswege des Insulins zum nativen Protein führen. Bei IGF-I werden hingegen zwei thermodynamisch ähnlich stabile Isoformen gebildet: natives IGF-I (60 %) und falsch-disulfidverbrücktes IGF-I (40 %) (Miller et al., 1993). Guo et al. untersuchten die Ursache für das unterschiedliche Faltungsverhalten von Insulin und IGF-I (Guo et al., 2002). Sie stellten einkettige Insulin / IGF-I Hybride her und konnten zeigen, daß IGF-I mit verkürztem C-Peptid (mini-IGF-I) sowie ein Hybrid aus Insulin-A-Kette und IGF-I-B-Kette ebenso wie IGF-I zu zwei thermodynamisch stabilen Isomeren falten. Im Gegensatz dazu folgt das Hybrid aus Insulin-B-Kette und IGF-I-A-Kette dem gleichen Faltungsweg wie Insulin und Proinsulin. Die Autoren folgerten, daß das unterschiedliche Faltungsverhalten von Insulin und IGF-I durch die B-Kette des Insulins bzw. B-Domäne des IGF-I kontrolliert wird (Guo et al., 2002).

2.5 An der Disulfidbrückenbildung beteiligte Enzyme – Die Familie der Disulfid-Oxidoreduktasen

Das Besondere an dieser Proteinfamilie sind ihre ähnlichen strukturellen und funktionellen Eigenschaften. Fast alle Disulfid-Oxidoreduktasen enthalten das Thioredoxinfaltungsmotiv und die Konsensussequenz -Cys-Xaa-Xaa-Cys-, die eine intramolekulare Disulfidbrücke ausbilden kann. Die biologische Aktivität der jeweiligen Disulfid-Oxidoreduktase ergibt sich aus dem Redoxpotential zwischen reduzierter und oxidierter Form. Zur Familie der Disulfid-Oxidoreduktasen gehören die prokaryontischen Dsb Proteine des Periplasmas, die cytoplasmatischen Proteine des Thioredoxin- und Glutaredoxin-Systems sowie die eukaryontischen Proteindisulfidisomerasen (Rietsch and Beckwith, 1998; Raina and Missiakas, 1997).

2.5.1 Proteindisulfidisomerase (PDI)

Proteindisulfidisomerase (PDI) ist ein multifunktionelles Mehrdomänenprotein des Endoplasmatischen Retikulums (Freedman et al., 2002; Ferrari und Soling, 1999). Sie ist an der Bildung, Isomerisierung und Reduktion von Disulfidbrücken beteiligt (Weissman und Kim, 1993; Creighton et al., 1980). Das Redoxpotential der PDI aus Hefe beträgt –0,11 V (Hawkins et al., 1991), d.h. es ist reduzierender als das von DsbA (-0,089 V, (Wunderlich und Glockshuber, 1993a)) und oxidierender als das von Thioredoxin (-0,27 V, (Holmgren, 1985)). Im Lumen des Endoplasmatischen Retikulums können sekretorische, ungefaltete Proteine durch Disulfidaustausch mit Glutathion oder mit PDI ihre native Konformation mit den korrekten Disulfidbrücken erreichen (Noiva und Lennarz, 1992; Roth und Pierce, 1987). Die Deletion des PDI-Gens in Hefe ist lethal (Laboissiere et al., 1995). Weiterhin wurde nachgewiesen, daß PDI Chaperonfunktion besitzt, d.h. unspezifisch Peptide und Proteine binden kann (Liang et al., 2001; Gillece et al., 1999; Klappa et al., 1998; Wilson et al., 1998; McLaughlin und Bulleid, 1998; Klappa et al., 1997; Yao et al., 1997; Song und Wang, 1995; Cai et al., 1994). PDI kann auch eine sogenannte Antichaperonaktivität ausüben, d.h. in sehr geringen (substoichiometrischen) Konzentrationen kann sie die Aggregation ungefalteter Proteine unterstützen (Song et al., 1997; Puig et al., 1997; Puig et al., 1994). Die Interaktion der PDI mit einem Protein kann vom Redoxzustand der PDI abhängen. Tsai et al. stellten *in vitro* die Bedingungen im Endoplasmatischen Retikulum nach und demonstrierten, daß reduzierte PDI das Cholera Toxin bindet, während oxidierte PDI nicht zur Interaktion befähigt ist und das Toxin freiläßt (Tsai et al., 2001). PDI wechselwirkt im Endoplasmatischen Retikulum mit Calretikulin, beide Proteine modulieren gegenseitig ihre Aktivitäten (Corbett et al., 1999; Baksh et al., 1995). Außerdem wurde PDI als Untereinheit der tetrameren Prolyl-4-Hydroxylase und des Triglyzerid-Transfer-Proteinkomplexes identifiziert (Pirneskoski et al., 2001; Bradbury et al., 1999).

PDI besteht aus den fünf Domänen **a**, **b**, **b'**, **a'** und **c** (Abb. 2). Dabei besitzen die ersten vier Domänen eine Sequenzhomologie zu Thioredoxin. Durch Strukturaufklärung mittels NMR wurde das Thioredoxinfaltungsmotiv für die Domänen **a** und **b** bestätigt (Kemmink et al., 1999; Kemmink et al., 1997; Kemmink et al., 1996; Kemmink et al., 1995). Für die Domänen **b'** und **a'** ist die Struktur bisher nicht geklärt, jedoch nimmt man aufgrund der Sequenzhomologie eine zu **a** und **b** ähnliche Struktur an. Die Domänen **a** und **a'** enthalten jeweils ein aktives Zentrum mit der Sequenz -Cys-Gly-His-Cys- und können als isolierte Domänen die Ausbildung von Disulfidbrücken katalysieren (Darby und Creighton, 1995a;

Darby und Creighton, 1995b). Die Funktion der Domäne **b** ist bisher nicht geklärt. Für die **b'** Domäne wurden Peptidbindungseigenschaften nachgewiesen (Cheung und Churchich, 1999; Darby et al., 1998; Klappa et al., 1998). Sie kann als isolierte Domäne jedoch nur Peptide bis zu einer Länge von 18 Aminosäuren binden und benötigt zur Bindung längerer Peptide oder Proteine Interaktionen mit weiteren Domänen der PDI (Sun et al., 2000; Darby et al., 1998; Klappa et al., 1998). Die Chaperonaktivität ist unabhängig von der Isomerasefunktion (Quan et al., 1995; Cai et al., 1994), aber die **b'** Domäne scheint essentiell für die Isomeraseaktivität der PDI zu sein (Darby et al., 1998). Nur durch das Zusammenwirken aller PDI-Funktionen können offenbar geschwindigkeitsbestimmende Disulfidisomerisierungen in Faltungsintermediaten effizient realisiert werden.

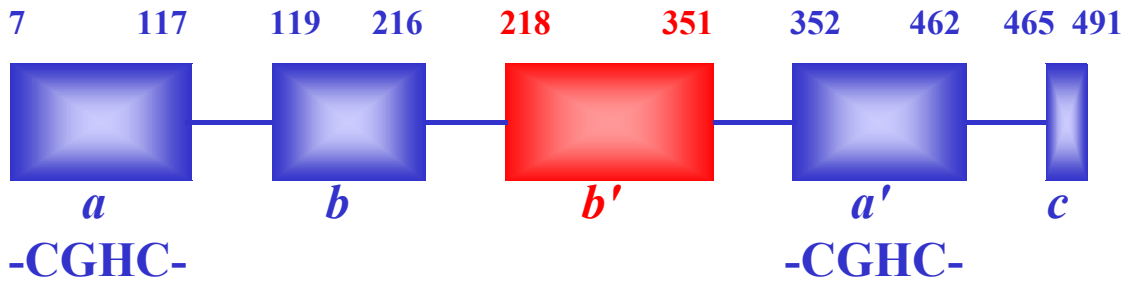

Abb. 2: Domänenstruktur der Proteindisulfidisomerase (PDI). „a" bis „c" bezeichnen die einzelnen Domänen. Die Grenzen der jeweiligen Domänen sind in Aminosäuren angegeben. Domäne **a** und **a'** enthalten jeweils das aktive Zentrum –Cys-Gly-His-Cys–. Die in rot dargestellte Domäne **b'** vermittelt Chaperoneigenschaften.

2.5.2 Dsb Proteine

Die Dsb Familie besteht aus den Proteinen DsbA bis DsbG. **DsbA** wurde erstmals 1991 von Bardwell et al. als ein an der Disulfidverbrückung im Periplasma von Prokaryonten beteiligtes Protein beschrieben (Bardwell et al., 1991). Es hat das Thioredoxinfaltungsmotiv (Guddat et al., 1998; Martin et al., 1993). In räumlicher Nachbarschaft zum aktiven Zentrum befindet sich ein ausgestreckter hydrophober Bereich, dem die Peptidbindung zugeschrieben wird (Zheng et al., 1997; Frech et al., 1996). Das aktive Zentrum enthält die Sequenz -Cys^{30}-Pro^{31}-His^{32}-Cys^{33}-. Dabei ist Cys^{30} dem Lösungsmittel besser zugänglich und aktiver als Cys^{33} (Nelson und Creighton, 1994). Das Redoxpotential des DsbAs beträgt -0,089 V (Wunderlich und Glockshuber, 1993a), das Cys^{30} hat einen pK_a Wert von 3,5 (Nelson und Creighton, 1994). Die reduzierte Form des DsbAs ist thermodynamisch stabiler als die oxidierte (Zapun et al., 1993; Wunderlich et al., 1993). Im nativen, oxidierten DsbA herrscht aufgrund der

hohen Energiedifferenz zur nativen, reduzierten Form eine konformationelle Spannung. Diese konformationelle Spannung begründet die hohe Oxidationskraft des DsbAs und seine hohe Reaktivität in Disulfidaustauschreaktionen (Wunderlich et al., 1993).

Die Redoxeigenschaften von DsbA sind von der Aminosäuresequenz des aktiven Zentrums abhängig. Substitutionen in den Aminosäuren zwischen Cys^{30} und Cys^{33} führen zu einer verringerten Oxidationskraft des DsbAs (Grauschopf et al., 1995). DsbA kann in einer extrem schnellen Reaktion seine intramolekulare Disulfidbrücke zwischen Cys^{30} und Cys^{33} auf faltende Proteine übertragen (Wunderlich et al., 1993). Dabei wird ein gemischtes Disulfid mit dem faltenden Protein ausgebildet (Frech et al., 1996). Disulfidaustausch-reaktionen zwischen DsbA und faltendem Protein laufen 100 bis 1000000 mal schneller ab als bei einer nicht-katalysierten Reaktion zwischen Thiolen/Disulfiden. Scheinbar stabilisiert DsbA den Übergangszustand von Thiolaustauschreaktionen (Darby und Creighton, 1995c).

DsbA wird durch **DsbB** reoxidiert (Bader et al., 1998; Missiakas et al., 1993; Bardwell et al., 1993). Die Reoxidation des DsbAs wird durch Interaktion der Cysteine der aktiven Zentren von DsbA und DsbB ermöglicht (Kishigami und Ito, 1996; Kishigami et al., 1995; Guilhot et al., 1995; Jander et al., 1994). Der Redoxzustand des integralen Membranproteins DsbB wird durch Elektronenübertragung auf Komponenten der Atmungskette und Ubichinon regeneriert (Xie et al., 2002; Kobayashi et al., 2001; Kadokura et al., 2000; Bader et al., 2000; Bader et al., 1999; Kobayashi und Ito, 1999; Bader et al., 1998; Kobayashi et al., 1997).

DsbC hat sowohl Disulfidisomeraseaktivität (Chen et al., 1999; Zapun et al., 1995; Shevchik et al., 1994) als auch eine Chaperonfunktion (Chen et al., 1999). Im Periplasma ist DsbC an der korrekten Faltung von Proteinen mit multiplen Disulfidbrücken beteiligt (Kobayashi et al., 1997; Joly und Swartz, 1997; Rietsch et al., 1997), *dsbC*-Null-Mutanten können keine korrekten Disulfidbrücken ausbilden (Rietsch et al., 1996). Während DsbA als Monomer vorliegt, ist DsbC ein Homodimer. Eine Punktmutation in der Kontaktfläche zwischen den Monomeren des DsbCs resultiert in einem monomeren Protein, das vergleichbar oxidierend ist wie DsbA (Bader et al., 2001).

Die Regeneration von DsbC erfolgt durch **DsbD**-katalysierten Elektronentransfer (Krupp et al., 2001; Goldstone et al., 2001; Chung et al., 2000; Katzen und Beckwith, 2000; Rietsch et al., 1997). Für den transmembranen Elektronentransfer sind 6 konservierte Cysteine des DsbDs notwendig (Stewart et al., 1999). DsbD ist essentiell für das Überleben bei Temperaturen über 42°C (Missiakas et al., 1995). *dsbD*-Null-Mutanten zeigen einen Defekt in

der Cytochrom c Biogenese (Crooke und Cole, 1995), der durch Supplementation mit Thiolkomponenten komplementiert werden kann (Sambongi und Ferguson, 1994). An der Reduktion des DsbDs können **DsbE** und **DsbF** beteiligt sein (Metheringham et al., 1996).

DsbG zeigt Disulfidisomeraseaktivität sowohl *in vivo* als auch *in vitro* (Bessette et al., 1999; van Straaten et al., 1998; Andersen et al., 1997). Außerdem hat DsbG eine Chaperonfunktion, die unabhängig von den Cysteinen des aktiven Zentrums ist (Shao et al., 2000). Interaktionen mit strukturierten, späten Faltungsintermediaten der Citratsynthase und der Luziferase wurden *in vitro* nachgewiesen. *In vivo* ist DsbG an der Regeneration des Redoxzustandes von DsbA, DsbB und DsbC beteiligt (Andersen et al., 1997).

2.5.3 Das Thioredoxin- und das Glutaredoxin-System

Beide Systeme sind u.a. an der Aufrechterhaltung des reduzierten Redoxzustandes im Cytoplasma beteiligt (Tabelle 2) (Rietsch und Beckwith, 1998). Ihre Redoxpotentiale sind weit reduzierender als die der Dsb Proteine oder der PDI. Das Thioredoxin- und Glutaredoxin-System wird für die Funktion von metabolischen Enzymen benötigt, die als Teil ihres katalytischen Zyklus eine Disulfidbrücke ausbilden, die reduziert werden muß damit das Enzym einen weiteren Katalysezyklus ausführen kann (Russel, 1995). Neben und unabhängig von seiner Funktion als Reduktase ist Thioredoxin in den Lebenszyklus von einigen Bakteriophagen (z.B. filamentöser Phage f1, Bakteriophage T7) involviert (Huber et al., 1987; Tabor et al., 1987; Russel et al., 1997).

Tabelle 2 Die Mitglieder des Thioredoxin- und des Glutaredoxin-Systems (Rietsch und Beckwith, 1998).

Protein	**Gen**	**Funktion**
Thioredoxin-System:		
Thioredoxin	*trxA*	Reduktase
Thioredoxin 2	*trxC*	Reduktase
Thioredoxin-Reduktase	*trxB*	Reduktion von Thioredoxin durch NADPH
Glutaredoxin-System:		
Glutaredoxine 1 bis 3	*grx*A bis *C*	Reduktase
Glutathion-Oxidoreduktase	*gor*	Reduktion von oxidiertem Glutathion durch NADPH

Die Cysteine des aktiven Zentrums von Thioredoxin liegen nach einem Zyklus Disulfidbrücken-Reduktion im disulfidverbrückten Zustand vor. Thioredoxin-Reduktase und NADPH realisieren die Reduktion des Thioredoxins, sodaß die Cysteine des aktiven Zentrums wieder reduziert vorliegen. Im Gegensatz dazu werden die Glutaredoxine durch Glutathion reduziert (Holmgren und Aslund, 1995). Das entstehende oxidierte Glutathion wird durch Glutathion-Oxidoreduktase und NADPH reduziert.

Thioredoxin kann als Oxidase und Reduktase fungieren, sobald es in das Periplasma von *E. coli* transloziert wird (Debarbieux und Beckwith, 2000; Debarbieux und Beckwith, 1998). Dort wird Thioredoxin von DsbB oxidiert (Debarbieux und Beckwith, 2000; Jonda et al., 1999). In das Periplasma sezernierte Thioredoxin-Varianten, die -Cys-Xaa-Xaa-Cys-Sequenzen anderer Mitglieder der Disulfid-Oxidoreduktasen im aktiven Zentrum aufweisen, können *dsbA*$^-$ Stämme komplementieren (Jonda et al., 1999; Mossner et al., 1999; Mossner et al., 1998). Eine Mutation im Thioredoxingen, die die Aminosäuresequenz im aktiven Zentrum von -Cys-Gly-Pro-Cys- zu -Cys-Gly-His-Cys- ändert, bewirkt eine Erhöhung des Redoxpotentials von -0,27 V (Holmgren, 1985) auf -0,235 V (Lundstrom et al., 1992). Damit einher geht auch eine signifikant höhere Isomerisierungsaktivität verglichen mit dem Wildtypprotein (Lundstrom et al., 1992; Krause et al., 1991).

Thioredoxine und Glutaredoxine scheinen überlappende Funktionen zu haben. Null-Mutationen in einem der Gene des jeweiligen Systems sind nicht lethal, d.h. eines der beiden Systeme reicht aus, um den reduzierten Zustand des Cytoplasmas zu erhalten. Doppelmutanten für *trxB* und *gshA* (Biosynthese von Glutathion) bzw. *trxB* und *gor* sind entsprechend nicht lebensfähig (Prinz et al., 1997). Die Bedingungen im Cytoplasma sind zu reduzierend, als daß strukturelle Disulfidbrücken in Proteinen ausgebildet werden könnten (Derman et al., 1993; Derman und Beckwith, 1991). Durch die Deletion von *trxB* bzw. *trxB* und *gor* wird das Redoxpotential des Cytoplasmas erhöht, wodurch die Ausbildung von Disulfidbrücken in Modellproteinen möglich wird. Die Deletion von *trxA* und *trxC* bzw. *trxA*, *trxB* und *trxC* ermöglicht hingegen keine Disulfidverbrückung in Modellproteinen (Prinz et al., 1997; Derman et al., 1993).

2.5.4 Einsatz von Faltungshelfern in *E. coli* und *in vitro*

Bei der Produktion rekombinanter Proteine in *E. coli* ist die Bildung korrekter Disulfidbrücken oft ein limitierender Faktor für das Erreichen der nativen Struktur. Daher wird versucht, die Faltung disulfidverbrückter Proteine durch die Variation der

Kultivierungsbedingungen zu verbessern. Während der Kultivierung von rekombinanten *E. coli* kann z.B. das Kultivierungsmedium mit redoxaktiven Substanzen (z.B. Glutathion) supplementiert werden. Dadurch kann die oxidative Faltung rekombinanter Proteine im Periplasma von *E. coli* unterstützt und z.B. die Ausbeute von basischem pankreatischem Trypsininhibitor (BPTI), IGF-I, Pektatlyase und α-Amylase/Trypsininhibitor (RBI) erhöht werden (Ostermeier et al., 1996; Samuelsson et al., 1996; Humphreys et al., 1995; Wunderlich und Glockshuber, 1993b).

In *E. coli* können DsbA im Periplasma und Thioredoxin im Cytoplasma als Fusionspartner von rekombinanten Proteinen deren Löslichkeit erhöhen (Collins-Racie et al., 1995; LaVallie et al., 1993). Die Koexpression verschiedener *dsb*s kann eine verstärkte Anreicherung nativer, rekombinanter Proteine ermöglichen (Kurokawa et al., 2001; Joly et al., 1998). Sone et al. zeigten, daß koexprimiertes *dsbC* aufgrund seiner Isomeraseaktivität zur Bildung nativer alkalischer Phosphatase führt (Sone et al., 1997). In *E. coli* Mutanten mit oxidierendem Cytoplasma (*trxB gor* Mutanten) kann bei simultaner Überproduktion von GroEL/ES, DnaK/J oder DsbC die Cytoplasmatische Produktion eines nativen Fab Antikörperfragmentes erreicht werden (Levy et al., 2001). Die Kosekretion mit PDI kann zur erhöhten Ausbeute korrekt disulfidverbrückter Proteine im Periplasma führen (Ostermeier et al., 1996; Humphreys et al., 1996; Humphreys et al., 1995).

Vielfältige Einsatzmöglichkeiten für Disulfid-Oxidoreduktasen und molekulare Chaperone *in vitro* sind beschrieben. In den meisten Fällen wurden Modellproteine verwendet, d.h. Proteine anhand derer die Eigenschaften der Faltungshelfer bezüglich der Proteinfaltung untersucht wurden (Shao et al., 2000; Chen et al., 1999; Joly und Swartz, 1997; Zapun et al., 1995; Frech und Schmid, 1995; Joly und Swartz, 1994; Zapun et al., 1993; Wunderlich et al., 1993). Unterschiedliche Modellproteine wurden *in vitro* auch in Gegenwart von PDI rückgefaltet. In Abhängigkeit vom verwendeten Protein wirkt PDI als Isomerase (Katiyar et al., 2001; Lilie et al., 1994; Weissman und Kim, 1993; Lyles und Gilbert, 1991; Wang und Tsou, 1991; Tang et al., 1988; Creighton et al., 1980) oder Chaperon (van den Berg et al., 1999; Song und Wang, 1995; Quan et al., 1995; Cai et al., 1994). Mit saurer Phospholipase A2 als Modellprotein kann PDI sowohl als Chaperon als auch als Isomerase die Rückfaltung beeinflussen (Yao et al., 1997). Im Gegensatz dazu hat PDI aus Hefe keine unabhängige Chaperonaktivität. Hefe-PDI erhöht die Rückfaltungsausbeute von Lysozym (Katiyar et al., 2001), kann aber nicht Rückfaltung von Proteinen, die keine Disulfidbrücken enthalten, unterstützen (z.B. Citratsynthase und GAPDH).

3 Zielstellung

Ziel der Doktorarbeit war die Optimierung der Herstellung humanen, nativen Proinsulins mit korrekten Disulfidbrücken. Dies sollte mit Hilfe zweier grundsätzlich verschiedener Techniken umgesetzt werden. Natives Proinsulin sollte im Periplasma von *E. coli* in möglichst großer Ausbeute produziert werden. Außerdem sollte die *in vitro* Faltung von Proinsulin analysiert und hinsichtlich einer hohen Produktausbeute verbessert werden.

Die Herstellung von Proteinen in *E. coli* ermöglicht hohe Ausbeuten an rekombinantem Produkt und wird für industrielle Verfahren angewendet. Im Periplasma von *E. coli* herrschen oxidierende Bedingungen und es sind Proteine vorhanden, die an der Ausbildung von Disulfidbrücken beteiligt sind. Damit sind Bedingungen gegeben um therapeutisch relevante Proteine, die Disulfidbrücken enthalten, im Periplasma von *E. coli* in ihrer nativen Form herzustellen.

Für humanes Proinsulin sollten die Produktionsbedingungen für rekombinante *E. coli* hinsichtlich einer maximalen Ausbeute an nativem, korrekt disulfidverbrücktem Protein variiert und optimiert werden. Es sollten verschiedene Möglichkeiten getestet werden:

(1) Einsatz der Disulfid-Oxidoreduktase DsbA als Fusionspartner für Proinsulin. Diese Fusion könnte die Ausbeute an nativem Proinsulin durch dessen verbesserte Löslichkeit und eine beschleunigte Disulfidverbrückung erhöhen.

(2) Kosekretion von Proinsulin mit molekularen Chaperonen. Die Anwesenheit zusätzlicher Chaperone im Periplasma von *E. coli* könnte die Faltung des Proinsulins ermöglichen bzw. verbessern.

(3) Optimierung der Kultivierungsbedingungen durch die Variation verschiedener Kultivierungsparameter. Durch den Einsatz von niedermolekularen Faltungshelfern sowie thiolhaltigen Substanzen könnte die Faltung des Proinsulins verbessert bzw. beschleunigt und die Ausbeute an nativem Proinsulin weiter erhöht werden.

Neben der Gewinnung in *E. coli* sollte auch die Faltung des Proinsulins *in vitro* untersucht werden. Das Ziel war, limitierende Schritte der Faltung des Proinsulins zu analysieren. Die Kenntnis dieser kritischen Punkte könnte der Ausgangspunkt für die Optimierung des Faltungsprozesses mit Schwerpunkt auf einer möglichst kurzen Prozeßführung und hohen Ausbeuten an nativem Protein sein. Dazu wurden zwei verschiedene Strategien verfolgt:

(1) Systematische Variation der Faltungsparameter, um eine verbesserte oxidative Faltung des Proinsulins zu erreichen.

(2) Einsatz von Varianten der Proteindisulfidisomerase und Untersuchung des Einflusses verschiedener PDI-Funktionen auf die Proinsulinfaltung. Diese Untersuchungen könnten weitere Einblicke in die Funktion und Wirkungsweise der PDI als Isomerase und Chaperon geben. Weiterhin sollte es mit Hilfe von PDI-Varianten möglich sein, limitierende Schritte der Faltung des Proinsulins zu analysieren. Nicht-produktive Nebenreaktionen könnten eventuell durch die Aktivität der PDI überwunden werden.

4 Ergebnisse und Diskussion

4.1 Sekretorische Expression von DsbA-Proinsulin in *Escherichia coli*

(Winter et al., 2000)

Die Kosekretion von DsbA mit rekombinanten Proteinen, die eine Disulfidverbrückung zum Erreichen ihrer nativen Struktur benötigen, kann zu einer erhöhten Ausbeute an korrekt gefaltetem rekombinantem Protein führen. DsbA kann löslich in großen Mengen im Periplasma angereichert werden (Wunderlich und Glockshuber, 1993a). Als Fusionspartner kann DsbA, ebenso wie Thioredoxin, löslichkeitsvermittelnd wirken und somit eine verbesserte Ausbeute an rekombinantem Protein ermöglichen. Diese Eigenschaften wurden eindrucksvoll anhand eines DsbA-Enterokinase Fusionskonstruktes dargestellt (Collins-Racie et al., 1995) und sollten für Proinsulin angewendet werden.

Es wurden DsbA-Proinsulin-Fusionskonstrukte mit folgendem Aufbau verwendet:

DsbA-Signalsequenz — DsbA-Sequenz — $(Gly)_5$-Arg-Linker — Proinsulin-Sequenz.

Das Fusionsprotein DsbA-Gly_5-Arg-Proinsulin konnte löslich in *E. coli* hergestellt werden. Um eine quantitative Analyse des Proinsulins im Fusionsprotein zu ermöglichen, wurden die entsprechenden Zellen einem Periplasmaaufschluß unterzogen (Kang und Yoon, 1994) und der Zellextrakt mit Trypsin behandelt. Aufgrund des Argininrestes im Linker zwischen DsbA und Proinsulin und der basischen Aminosäuren am C- und N-Terminus der B- bzw. A-Kette des Insulins, kann durch einen solchen Verdau Insulin vom restlichen Fusionsprotein abgetrennt werden. Das entstehende Insulin kann durch nachfolgenden Verdau mit Carboxypeptidase B zu korrekt prozessiertem Insulin umgesetzt werden. Die Menge an nativem Insulin in der Periplasmafraktion wurde mittels eines ELISAs bestimmt. Der ELISA erlaubt die Detektion nativen Insulins, wobei die Spezifität durch den im ELISA verwendeten sekundären Antikörper ermöglicht wird, der ausschließlich die native Konformation des Insulins bzw. Proinsulins erkennt.

Erwartet wurde, daß das Fusionsprotein aufgrund der DsbA-eigenen Signalsequenz effizient in das Periplasma transportiert wird und DsbA sowohl den Fusionspartner in Lösung hält, als auch eine verbesserte Disulfidverbrückung im Fusionspartner Proinsulin bewirkt. Es wurde nachgewiesen, daß das Fusionsprotein ausschließlich im Periplasma vorlag. Der erwartete Effekt des DsbAs auf den Fusionspartner hinsichtlich der Ausbeute an nativem Proinsulin

wurde jedoch nicht erreicht. Vermutlich reichte der positive Effekt von DsbA nicht aus, um andere limitierende Einflüsse bei der Faltung von Proinsulin in *E. coli* Zellen auszugleichen. Somit war eine weitere Optimierung des Expressionssystems erforderlich.

Die Produktion nativer, rekombinanter Proteine in *E. coli* wird maßgeblich von der Kultivierungstemperatur beeinflußt (Schein und Noteborn, 1988). Analog dazu wurde das Fusionsprotein am besten in Kultivierungen bei 25 °C angereichert. Weiterhin wurden verschiedene *E. coli* Stämme (C600, JM109, RB791, BL21) hinsichtlich einer optimalen Ausbeute an Fusionsprotein mit nativem Proinsulinanteil getestet. Aufgrund der besten Wachstumseigenschaften und Expressionsergebnisse wurde der Stamm RB791 gewählt. In begrenztem Maße konnten weitere Verbesserungen der Ausbeute nativen Proinsulins durch die Supplementierung des Kultivierungsmediums mit niedermolekularen Zusätzen wie L-Arginin oder Ethanol erreicht werden. Unter optimalen Bedingungen konnte Proinsulin mit einer Ausbeute von 9,2 mg/g Trockenzellmasse produziert werden.

Im Vergleich zu vorherigen Arbeiten zur Herstellung von nativem Proinsulin in Form eines Fusionsproteins war die Ausbeute an DsbA-Proinsulin vergleichbar hoch (Kang und Yoon, 1994; Murby et al., 1991). Allerdings sind die Ausbeuten mindestens Faktor Zehn geringer als die bei der Herstellung von Proinsulin in Form von *inclusion bodies*. Trotz der dort notwendigen anschließenden *in vitro* Faltung des Proinsulins sind die Endausbeuten signifikant höher als die hier erreichten Ausbeuten an nativem Proinsulin aus dem DsbA-Fusionsprotein.

4.2 Sekretorische Expression des Proinsulingens

Die Herstellung von Proinsulin im Periplasma in nativer Form und ohne Fusionspartner wurde bereits beschrieben, jedoch waren die Ausbeuten an Proinsulin mit maximal 0,01 mg/g Zellen sehr gering (Chan et al., 1981).

Humanes Proinsulin wurde in dem *E. coli* Stamm BL21(DE3) produziert und mit Hilfe der pelB Signalsequenz (Better et al., 1988) im Vektor pET20b(+)-PI effizient in das Periplasma von *E. coli* transportiert. Natives Proinsulin wurde nach dem Periplasmaaufschluß mit Hilfe eines ELISAs quantifiziert. Das Ziel der folgenden Untersuchungen war, Kultivierungsbedingungen zu finden, die eine maximale Anreicherung nativen Proinsulins im Periplasma ermöglichen.

4.2.1 Kosekretion von Chaperonen und Proinsulin

(Schäffner et al., 2001)

Chaperone sind eine funktionelle Klasse von nicht-verwandten Proteinfamilien, die anderen Proteinen beim Erreichen ihrer nativen Konformation helfen können, aber nicht selbst Teil der gefalteten Struktur sind (Ellis, 1993). Hitzeschockproteine (Hsp) bilden eine große Gruppe innerhalb der Chaperone. Da im Periplasma kein ATP verfügbar ist, wurden ATP-unabhängige Chaperone für die sekretorische Koexpression ausgewählt: DnaJ aus *E. coli* und Hsp25 aus Maus. Beide Chaperone können die Aggregation von Proteinen unter Hitzeschock unterdrücken (Ehrnsperger et al., 1997; Schroder et al., 1993). Als Kontrolle diente ein Nicht-Chaperon, ein *single chain* Fv Fragment gegen Oxazolon, („scFv", Fiedler und Conrad, 1995).

Dr. Jörg Schäffner führte Untersuchungen zur Kosekretion einer Variante des Gewebe-Plasminogenaktivators, der 9 Disulfidbrücken enthält, bzw. einem *single chain* Fv Fragment gegen Thyroid-stimulierendes Hormon mit DnaJ bzw. Hsp25 durch. In Analogie dazu wurde die sekretorische Koexpression von Proinsulin und DnaJ bzw. Hsp25 bei 25 °C durchgeführt. DnaJ bewirkte im Vergleich zur Kontroll-Kultivierung eine ca. 37-fache Steigerung der Ausbeute an nativem Proinsulin. Durch Zugabe von L-Arginin und GSH zum Kultivierungsmedium konnte die Ausbeute an nativem Proinsulin nicht weiter erhöht werden. Kosekretion von Hsp25 hatte im Gegensatz dazu keinen stimulierenden Effekt auf die Proinsulinausbeute. Die Kontrolle mit scFv läßt vermuten, daß es sich bei dem Einfluß von DnaJ auf die Proinsulinausbeute nicht um einen unspezifischen Effekt durch die Kosekretion

an sich sondern vielmehr um einen Chaperoneffekt handeln könnte. Jedoch wird dieses scFv in wesentlich geringerem Maße produziert und in das Periplasma sezerniert als DnaJ, sodaß die Koexpression des scFv möglicherweise keine geeignete Kontrolle für unspezifische Effekte ist. Ob die kosekretierten Proteine die *E. coli* Zellen auch in anderem Maße beeinflußten, wurde nicht untersucht.

Die Einflüsse von Chaperonen oder niedermolekularen Faltungshelfern auf die Faltung von rekombinanten Proteinen im Periplasma von *E. coli* sind für jedes Protein unterschiedlich. Entsprechend müssen die Kultivierungsbedingungen für jedes Protein separat ausgetestet werden. Es scheint kein generell gültiges Rezept zu geben, aus dem sich eine optimale, universell anwendbare Kultivierungsstrategie ableiten läßt. Weiterhin ist der Beweis einer realen Chaperonaktivität *in vivo* schwierig, da im Periplasma ein komplexes, dynamisches System vorliegt. Daher können wir aufgrund der experimentellen Befunde zwar annehmen, daß DnaJ eine tatsächliche Chaperonfunktion in unserem Expressionssystem ausübt, indirekte Effekte sind aber nicht ausgeschlossen.

4.2.2 Variation der Kultivierungsbedingungen zur Verbesserung der sekretorischen Expression des Proinsulingens

(Winter et al., 2002a)

Analog zum DsbA-Proinsulin ist auch die Bildung nativen Proinsulins ohne Fusionspartner in starkem Maße von den Kultivierungsbedingungen abhängig. Bei der Herstellung nativen humanen Proinsulins im Periplasma von *E. coli* erwies sich die Kultivierung bei 22 °C als optimal. Weiterhin wurde eine signifikante Abhängigkeit der Ausbeute an nativem Proinsulin vom Zeitpunkt der Induktion mit IPTG festgestellt. Unter optimalen Bedingungen (Kultivierung bei 22 °C, Induktion bei einer optischen Dichte der Kultur von ca. OD_{500nm} = 0,5) konnte eine Ausbeute an nativem Proinsulin von 2,4 ± 0,5 mg/g Trockenzellmasse erreicht werden.

Die Supplememtierung des Kultivierungsmediums mit Redoxsubstanzen (z.B. Glutathion, Vectrase™-P) kann die Anreicherung rekombinanter, nativer Proteine mit korrekten Disulfidbrücken im Periplasma von *E. coli* erhöhen. In rekombinanter *Saccharomyces cerevisiae* kann die Sekretion von saurer Phosphatase durch die Zugabe von Vectrase™-P zum Kulturmedium ca. dreifach erhöht werden (Woycechowsky et al., 1999). Vectrase™-P ist ein Dithiol mit geringem Molekulargewicht, das die Redoxeigenschaften der PDI

nachahmt. Vectrase™-P wurde während der Kultivierung von BL21(DE3)-pET20b(+)-PI zum Induktionszeitpunkt zugesetzt. Während 2 – 50 µM Vectrase™-P im Medium die Ausbeute an nativem periplasmatischen Proinsulin um ca. 60 % erhöhte, verringerte sich die Ausbeute drastisch in Gegenwart von 1 – 20 mM Vectrase™-P. Vermutlich bewirkten die Thiole der Vectrase™-P zu stark reduzierende Bedingungen, die die oxidative Faltung des Proinsulins im Periplasma negativ beeinflußten. Andere Redoxsubstanzen (Cystein/Cystin, β-Mercaptoethanol/Di(2-hydroxyethyl)-disulfid, Cysteamin/Cystamin) hatten einen leicht positiven Effekt auf die Ausbeute an nativem Proinsulin. Auch hier verringerte sich die Menge an Proinsulin in Gegenwart von mehr als 1 mM Redoxsubstanz im Medium. Ein möglicher synergistischer Effekt durch die kombinierte Zugabe von Vectrase™-P und oxidierten Redoxsubstanzen (z.B. Cystin, Glutathion) zum Kultivierungsmedium wurde nicht untersucht.

Die dargestellten Kultivierungen wurden im Schüttelkolbenmaßstab (20 ml) durchgeführt. Entsprechend ist für industrielle Anwendungen eine Maßstabsvergrößerung unabdingbar, die vermutlich eine Reihe weiterer Optimierungen erfordert. Obwohl eine enorme Steigerung im Vergleich zu den vorher publizierten Daten erreicht wurde, ist die Ausbeute sehr gering verglichen mit den Ausbeuten bei der Herstellung von Proinsulin in Form von *inclusion bodies*.

4.3 *In vitro* Faltung von humanem Proinsulin

(Winter et al., 2002b)

Die *in vitro* Faltung des Proinsulins wurde ohne den Einsatz von Faltungshelfern hinsichtlich hoher Faltungsausbeuten und hoher Volumen-Ausbeuten optimiert. Verschiedene Verfahren zur *in vitro* Faltung von Insulin und Proinsulin sind beschrieben und werden technisch angewendet. In der Mehrzahl der Verfahren beginnt der Faltungsprozeß mit der Aktivierung der freien SH-Gruppen des Proteins mittels Sulfitolyse, wodurch anschließend in Gegenwart eines Reduktionsmittels die Umsetzung zu nativem Protein erreicht werden kann. Die Faltung erstreckt sich gewöhnlich über einen Zeitraum von mehreren Stunden und ermöglicht Ausbeuten von ca. 50 % (Katsoyannis et al., 1967). Aus Proinsulin wird durch Proteolyse mit Trypsin und Carboxypeptidase B das biologisch aktive Insulin erhalten (Kemmler et al., 1971).

Hier wurde die Proinsulinfaltung ausgehend von denaturiertem und vollständig reduziertem His_8-Arg-Proinsulin untersucht und optimiert. Eine vollständige Denaturierung und Reduktion der Disulfidbrücken im Proinsulin wurde durch Inkubation des Proinsulins in 6 M Guanidiniumhydrochlorid (GdnHCl) bzw. 8 M Harnstoff und Zugabe von DTT in einem 100-fach molaren Überschuß bezüglich freier SH-Gruppen im Proinsulin erreicht. Der vollständige Verlust von Disulfidbrücken und Sekundärstrukturen wurde mittels *reversed phase* HPLC (RP-HPLC) und Circular Dichroismus (CD) nachgewiesen. Zur Renaturierung des Proinsulins wurde dieses 1:100 in Faltungspuffer verdünnt und die Faltung mittels RP-HPLC und ELISA verfolgt und quantifiziert.

Proinsulin konnte mit Ausbeuten von ca. 60 – 70 % an nativem Protein renaturiert werden. Das pH-Optimum der Proinsulinfaltung lag im stark basischen Bereich von pH 9,5 bis 11. Die Faltungsausbeute war maximal unter stark oxidierenden Bedingungen. Diese oxidierenden Bedingungen und der stark basische pH-Wert ermöglichen eine beschleunigte Disulfidverbrückung, durch die vermutlich aggregationsanfällige Faltungsintermediate schneller umgesetzt und somit vor Aggregation geschützt werden. Mit dem Redoxpaar Cystein/Cystin war die Faltung und Disulfidverbrückung des Proinsulins wesentlich effizienter als mit dem Tripeptid Glutathion (GSH/GSSG), das vermutlich aus sterischen Gründen die Cysteine im Proinsulin schlechter angreifen kann (Shaked et al., 1980). Weiterhin war die Proinsulinfaltung bis zu einer Proteinkonzentration von 0,5 mg/ml im Rückfaltungsansatz mit maximaler Ausbeute möglich. Proinsulin faltet weitgehend

unabhängig von der Konzentration an GdnHCl (bis 3 M) bzw. Harnstoff (bis 4 M) im Faltungsansatz. Der mittels CD gemessene GdnHCl-induzierte Übergang zeigte, daß bis ca. 1,5 M GdnHCl keine signifikante Änderung des Sekundärstrukturgehaltes auftrat. Die Abnahme des Sekundärstrukturgehaltes von 1,5 bis ca. 3 M GdnHCl wird, nach Brems et al., mit dem (partiellen) Strukturverlust des C-Peptides des Proinsulins erklärt (Brems et al., 1990). Somit ist es möglich, daß bis 3 M GdnHCl keine signifikant negative Beeinflussung der Faltung des Insulinanteils im Proinsulin stattfand. Mittels Pulsrenaturierung (Buchner et al., 1992; Rudolph und Fischer, 1990) konnte die Volumen-Ausbeute der Proinsulinfaltung auf 6 mg/ml natives Protein in 15 Stunden erhöht werden. Das renaturierte Proinsulin lag als Monomer vor und entsprach hinsichtlich der Disulfidverbrückung und der Konformation dem nativen Standard-Proinsulin.

Der deutliche Vorteil der beschriebenen optimierten Methode zur Renaturierung von Proinsulin gegenüber bestehenden Verfahren zur Faltung von Proinsulin liegt in der extrem schnellen Faltungskinetik mit Cystein/Cystin als Redoxsystem und der hohen Volumen-Ausbeute durch die angewendete Pulsrenaturierung. Das hier etablierte Verfahren scheint geeignet für die Produktion von Proinsulin im industriellen Maßstab.

4.4 Einfluß von Proteindisulfidisomerase auf die *in vitro* Faltung humanen Proinsulins

(Winter et al., 2002)

4.4.1 Herstellung von Varianten der humanen Proteindisulfidisomerase

Für die Untersuchungen der PDI-katalysierten Faltung des Proinsulins wurden verschiedene humane PDI-Varianten verwendet (Abb. 3). Wildtyp-PDI (WT-PDI) ist bezüglich ihrer Struktur, katalytischen Eigenschaften, Chaperonfunktion und ihres Einflusses auf die Faltung von Proteinen eingehend untersucht (siehe Kapitel 2.5.1). PDI-b'a'c ist eine Variante der WT-PDI, die nur die letzten drei der fünf PDI-Domänen besitzt. Diese drei Domänen stellen die Mindestlänge eines PDI-Konstruktes dar, das mit der gleichen Effizienz wie WT-PDI Peptide und Proteine binden kann (Darby et al., 1998; Klappa et al., 1998). Bisher wurde PDI-b'a'c nicht *in vitro* zur Rückfaltung von Proteinen eingesetzt. Weiterhin wurden PDI-Mutanten hergestellt, bei denen die beiden Cysteine in jeweils einem der beiden (PDIΔC1, PDIΔC2) bzw. in beiden (PDIΔC1,2) aktiven Zentren durch Serine ausgetauscht wurden. Letztere Mutante sollte, ebenso wie WT-PDI deren Cysteine mit Jodacetamid modifiziert wurden, keine Isomeraseaktivität besitzen (Yao et al., 1997; Lilie et al., 1994). Außerdem wurde eine PDI-Mutante (PDI-aba'c) hergestellt, die keine Chaperonfunktion, aber unveränderte Redoxaktivität und Stabilität besitzen sollte. Dafür wurden die Basenpaare 685 – 1000 der DNA-Sequenz der PDI entfernt. Die Deletion betrifft die für die Chaperonfunktion essentielle **b'** Domäne, sie wurde dadurch partiell entfernt. Bisher beschriebene PDI-Mutanten ohne Chaperonfunktion enthielten Punktmutationen in der **a'** Domäne (Klappa et al., 2000) oder es wurden die C-terminalen 51 Aminosäuren entfernt (Dai und Wang, 1997). Diese Mutationen führten zu einem Verlust der Chaperonaktivität, der jedoch eher auf eine verringerte Stabilität des Proteins bzw. einzelner Domänen zurückgeführt wird. Mögliche Begründungen hierfür wären 1) eine nicht-native Struktur einzelner Domänen, 2) eine veränderte Faltung der mutierten Domäne, sodaß die **b'** Domäne sterisch nicht zugänglich ist, 3) eine aktive **b'** Domäne, die permanent die nicht-nativen Strukturen der mutierten Domäne(n) bindet und somit nicht für andere Peptide oder Proteine zugänglich ist.

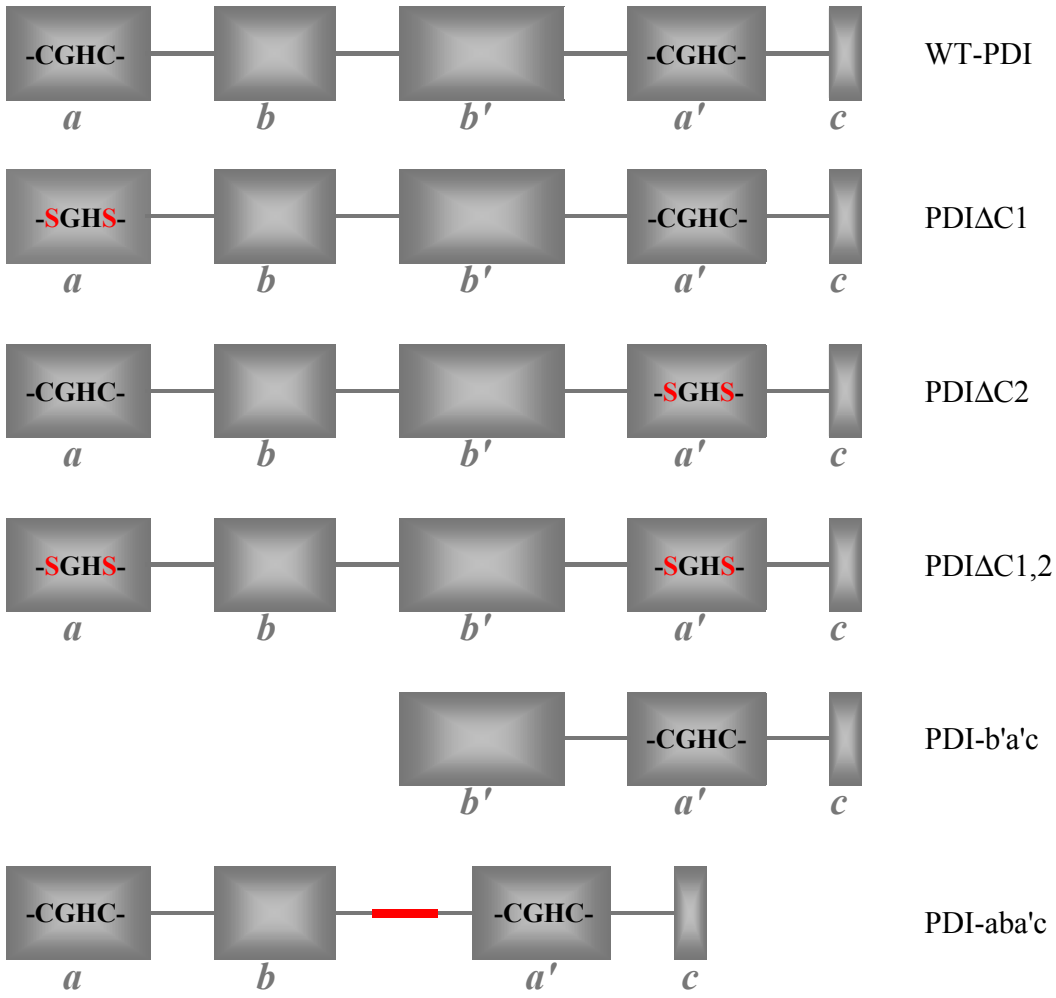

Abb. 3: Schematische Darstellung der verwendeten PDI-Varianten. Angegeben sind die aktiven Zentren (-CGHC-) bzw. die durch Substitution inaktivierten Zentren (-SGHS-) sowie die jeweiligen Domänen (***a*** bis ***c***). Die in PDI-aba'c verkürzte b' Domäne ist rot dargestellt.

4.4.2 Charakterisierung der Varianten der humanen Proteindisulfidisomerase

4.4.2.1 Stabilität und Struktur

Alle PDI-Varianten enthielten vergleichbare Anteile an Sekundärstrukturen (Abb. 4). Die Variante PDIΔC1,2 ist aufgrund der Punktmutation im Zellextrakt etwas anfälliger für proteolytischen Abbau als WT-PDI. Im gereinigten Zustand unterlag PDIΔC1,2, ebenso wie alle anderen PDI-Varianten, während der gesamten Inkubationszeit im Rückfaltungsansatz keinem nachweisbaren Abbau oder Aggregation. Gegenüber Proteinase K erwiesen sich WT-PDI und PDIΔC1,2 vergleichbar stabil und man kann davon ausgehen, daß beide PDIs eine kompakte Tertiärstruktur ausbildeten.

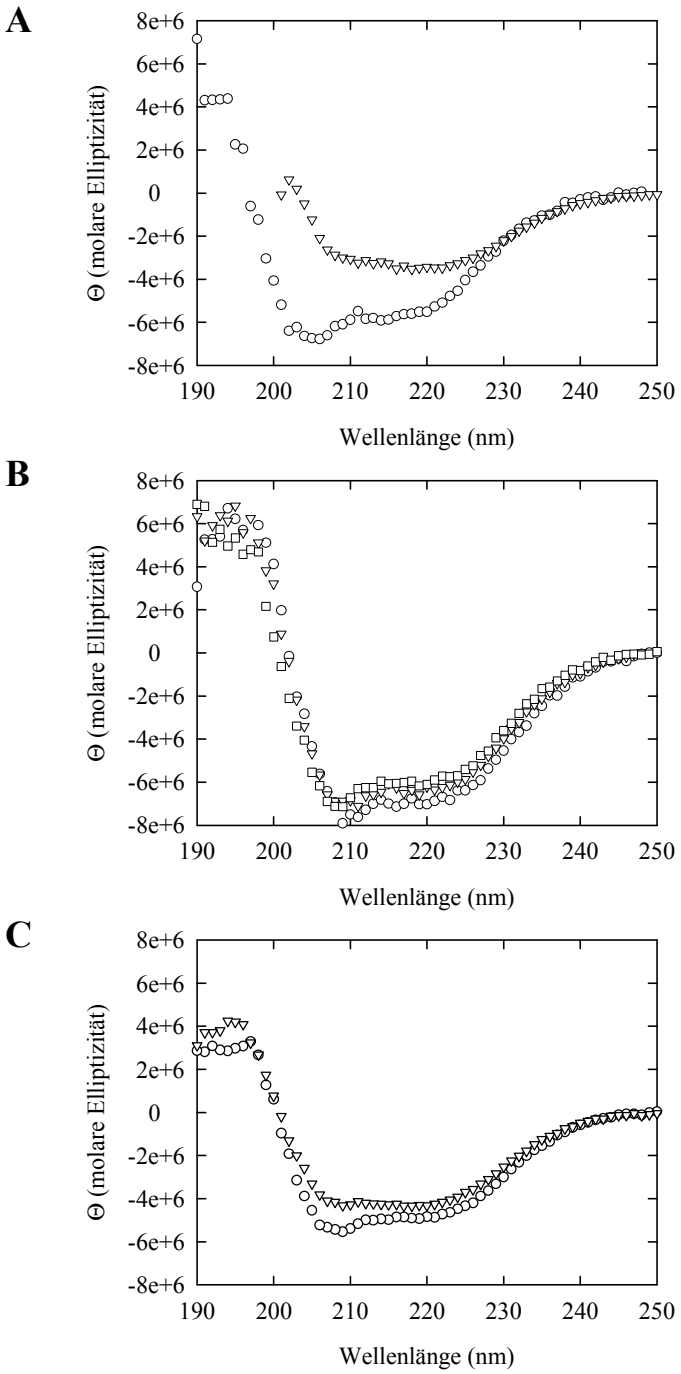

Abb. 4: Die Fern-UV-CD-Spektren von PDI-Varianten wurden von 190 – 250 nm bei 20 °C in 1 mM Tris / 1 mM Glycin pH 7,5, 0,1 mM EDTA gemessen.

A, CD-Spektren von WT-PDI (o) (0,1 mg/ml) bzw. PDI-b'a'c (∇) (0,37 mg/ml) gemessen in 0,1 cm Quartz-Küvetten an einem AVIV 62A DS Spektropolarimeter.

B, CD-Spektren von PDIΔC1 (o), PDIΔC2 (∇) bzw. PDIΔC1,2 (□),jeweils 1 mg/ml, gemessen in 0,01 cm Quartz-Küvetten am AVIV 62A DS Spektropolarimeter.

C, CD-Spektren von WT-PDI (o) (0,8 mg/ml) bzw. PDI-aba'c (∇) (0,87 mg/ml) gemessen in 0,02 cm Quartz-Küvetten am Jasco J-710 Spektropolarimeter.

4.4.2.2 Nachweis der Bindung von Peptiden an PDI-Varianten

Die PDI-Varianten wurden hinsichtlich ihrer Fähigkeit Peptide und Proteine zu binden untersucht. Die chemische Quervernetzung von Interaktionspartnern wurde von Dr. Peter Klappa durchgeführt und erfolgte mit dem homobifunktionellen Reagenz DSG (Klappa et al., 2001). Es wurden für WT-PDI und PDIΔC1,2 reversible Interaktionen mit ^{125}J-markierten Peptiden und Proteinen (z.B. Δ-Somatostatin, „*scrambled*" RNase, natives bzw. denaturiertes, reduziertes Proinsulin) nachgewiesen, d.h. die Interaktionen waren kompetierbar mit nicht-markierten Peptidsubstraten. Außerdem konnte die Bindung von ^{125}J-Δ-Somatostatin bzw. ^{125}J-Proinsulin an WT-PDI und PDIΔC1,2 in Gegenwart von Genistein, einem Inhibitor, der an die **b'** Domäne der PDI bindet, vollständig unterdrückt werden. Das zeigt, daß die Peptidbindungseigenschaften von PDIΔC1,2 durch die eingeführten Punktmutationen qualitativ nicht verändert wurden.

4.4.2.3 Nachweis der Chaperonaktivität von PDI-aba'c

Die partielle Deletion der **b'** Domäne in PDI (PDI-aba'c) hatte den vollständigen Verlust der Fähigkeit zur Bindung von ^{125}J-Δ-Somatostatin zur Folge. Es wurde eine zusätzliche Methode angewendet, um das Fehlen der Chaperonfunktion nachzuweisen. Glycerinaldehyd-3-Phosphat-Dehydrogenase (GAPDH) diente als Modellsystem für die Chaperon-vermittelte Aggregationsunterdrückung und Erhöhung der Faltungsausbeute. GAPDH enthält keine Disulfidbrücken und wurde bereits von Quan et al. (1995) und Cai et al. (1994) als Modellprotein für die Chaperonaktivität von PDI beschrieben.

Denaturierung von 0,14 mM GAPDH erfolgte in 6 M GdnHCl, 50 mM Tris pH 7,5, 5 mM EDTA, 1 mM DTT für 3 h bei 25 °C. Um eine Oxidation des Cysteins im aktiven Zentrum der GAPDH und einen Einfluß der Redoxaktivität der PDI auf die Reaktivierung von GAPDH auszuschließen, wurden Rückfaltung und Aktivitätstest unter reduzierenden Bedingungen durchgeführt. Die Rückfaltung von 2 µM GAPDH in Ab- oder Anwesenheit von PDI-Varianten erfolgte in 10 mM Na-Phosphat pH 7,2, 5 mM EDTA, 5 mM NAD^+, 5 mM DTT. Als Kontrolle diente BSA. Nach Inkubation der Proben für 10 min auf Eis, 30 min bei 4 °C und 3 h bei 25 °C wurde die Reaktivierungsausbeute mittels eines GAPDH-Aktivitätstests bestimmt. GAPDH katalysiert die oxidative Phosphorylierung von D-Glycerinaldehyd-3-Phosphat (GAP) zu 1,3-Bisphospho-D-Glycerat (BPG) unter Reduktion des Koenzyms NAD^+:

$$\mathbf{GAP + NAD^+ + H_2PO_4^{2-} \ll BPG + NADH + H^+.} \quad [1]$$

In Gegenwart von Na-Arsenat anstatt Na-Phosphat wird die Reaktion irreversibel, da das entstehende Endprodukt 1-Arseno-3-Phospho-D-Glycerat instabil ist und somit dem Gleichgewicht entzogen wird. Zur Messung der Aktivität rückgefalteter GAPDH wurde die entsprechende Rückfaltungsprobe 1:10 in 50 mM Tris pH 7,5, 5 mM EDTA, 20 mM Na-Arsenat, 1 mM GAP, 1 mM NAD^+, 5 mM DTT verdünnt, gemischt und die Entstehung von NADH über die Zeit als Extinktionsänderung bei 366 nm und 20 °C verfolgt. Die Aktivität der rückgefalteten GAPDH wurde nach folgender Formel berechnet:

$$\textbf{Spezifische Aktivität (U/mg)} = \frac{\mathbf{\Delta E/min \cdot Testvolumen}}{\mathbf{\varepsilon_{NADH} \cdot c \cdot d \cdot Probenvolumen}} \quad [2]$$

ΔE /min	Extinktionsänderung pro Minute bei 366 nm
Testvolumen	Volumen des Meßansatzes (ml)
ε_{NADH}	molarer Extinktionskoeffizient von NADH bei 366 nm ($3{,}3\ cm^2/\mu mol$)
c	Konzentration GAPDH (mg/ml)
d	Schichtdicke der Küvette (cm)
Probenvolumen	Volumen der eingesetzten GAPDH-Lösung (ml).

Übereinstimmend mit Cai et al. (1994), konnte WT-PDI konzentrationsabhängig die Reaktivierung der GAPDH von ca. 8 % auf 20 % erhöhen (Abb. 5). Die Chaperonfunktion von PDIΔC1,2 war signifikant geringer, das äußerte sich in einer Reaktivierungsausbeute von GAPDH von nur 14 %.

Quan et al. (1995) beschrieben, daß alkylierte PDI, d.h. PDI ohne Redoxaktivität, eine ca. 15 % verringerte Chaperonaktivität bezüglich GAPDH aufwies. Das deutet darauf hin, daß 1) die Chaperonaktivität unabhängig von der Redoxaktivität der PDI ist und 2) die Modifizierung der aktiven Zentren scheinbar auch einen Einfluß auf die Chaperonaktivität hat. Möglicherweise wurde mit Jodacetamid auch das Cystein in der **b'** Domäne kovalent modifiziert (Cheung und Churchich, 1999), sodaß die Peptidbindung negativ beeinflußt wird.

Die Mutante PDI-aba'c konnte, in Übereinstimmung mit den oben dargestellten Quervernetzungsdaten, die Reaktivierung der GAPDH nicht erhöhen. Reaktivierung von

GAPDH in Gegenwart von PDI-aba'c mit einem bis zu 256-fachen molaren Überschuß resultierte stets in einer Ausbeute von GAPDH von ca. 8 %. BSA und alkylierte PDI-aba'c verhielten sich analog (Abb. 5). Alkylierte PDI-aba'c diente als Kontrolle, um mögliche Einflüsse der Redoxaktivität auszuschließen.

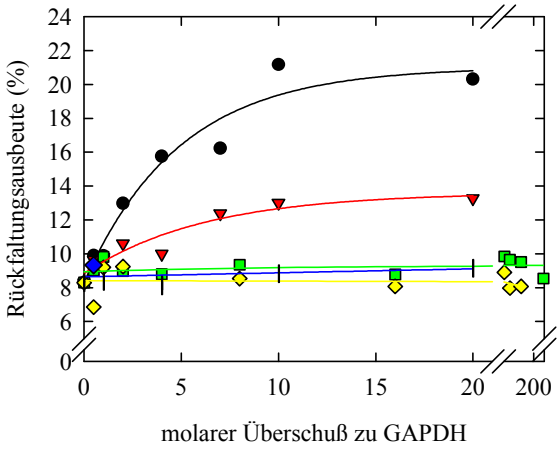

Abb. 5: Einfluß von PDI-Varianten auf die Rückfaltung von GAPDH. Denaturierung, Rückfaltung und Aktivitätsmessung der GAPDH erfolgten wie im Text beschrieben. Die Rückfaltung von GAPDH (2 µM) wurde in Gegenwart des angegebenen molaren Überschusses von WT-PDI (•), PDIΔC1,2 (∇), PDI-aba'c (■), alkylierter PDI-aba'c (◆) bzw. BSA (◆) durchgeführt.

4.4.2.4 Redoxaktivität von PDI

Für DsbA kann anhand unterschiedlicher spezifischer Fluoreszenz des reduzierten und oxidierten Proteins die Redoxaktivität bestimmt werden (Wunderlich und Glockshuber, 1993a). Inkubation von PDI in Gegenwart von reduziertem (GSH) bzw. oxidiertem Glutathion (GSSG) resultierte in reduzierter bzw. oxidierter PDI, die analog zu DsbA unterschiedliche Fluoreszenz-Emissionsspektren aufwies (Abb. 6). Die Differenz-Emissionsspektren zeigten ein Maximum der Emission bei 332 nm für WT-PDI und 335 nm für PDI-aba'c. Diese Eigenschaft wurde für die Messung der Redoxaktivitäten von WT-PDI und PDI-aba'c bezüglich Glutathion genutzt. Das Redoxgleichgewicht von PDI mit Glutathion ergibt sich aus folgender Reaktion:

$$\mathbf{PDI_{red} + GSSG \ll PDI_{ox} + 2\,GSH}. \qquad [3]$$

Nach Einstellung des Redoxgleichgewichtes wurden Emissionsspektren gemessen und die Änderung der Amplituden graphisch dargestellt (Abb. 7). Dabei wurde die durch

Luftsauerstoff bedingte Oxidation des GSH nach 24-stündiger Inkubation der Proben mittels Ellmans Test bestimmt und die Redoxabhängigkeit entsprechend korrigiert.

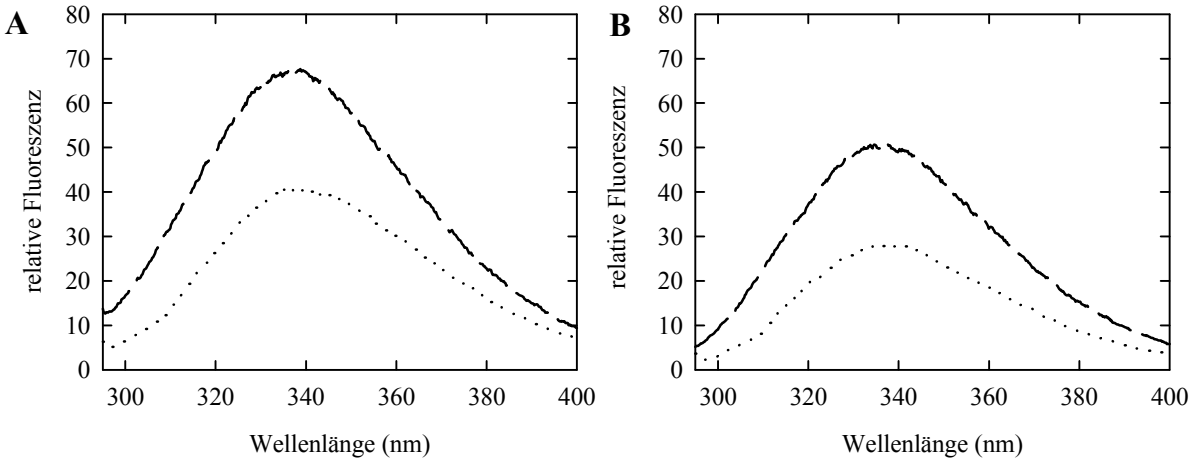

Abb. 6: Fluoreszenz-Emissionsspektren von oxidierter oder reduzierter WT-PDI und PDI-aba'c bei 25 °C. 0,178 µM WT-PDI (A) und PDI-aba'c (B) wurden in Anwesenheit von 2 mM GSH (----) für 1 h bzw. 2 mM GSSG (·····) für 24 h bei 24 °C inkubiert. Es wurde vollständige Reduktion bzw. Oxidation erreicht. Die Messungen erfolgten in 10 mM Tris/10 mM Glycin pH 7,5, 1 mM EDTA bei 20 °C. Anregungswellenlänge war 280 nm, Emission wurde von 295 – 400 nm gemessen.

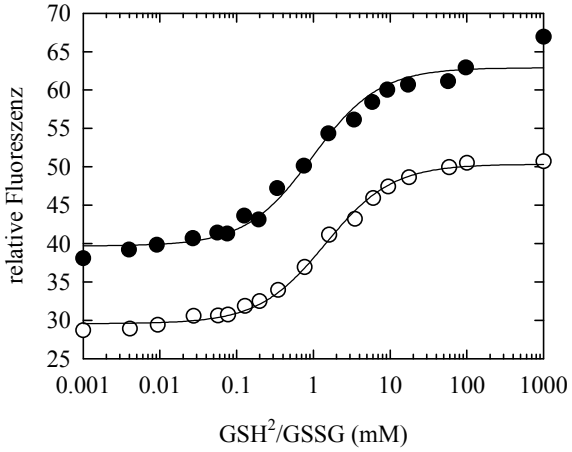

Abb. 7: Redoxgleichgewicht von WT-PDI (●) und PDI-aba'c (O) bei pH 7.5 und 25 °C. Die Fluoreszenzmessungen wurden analog zu Abb. 6 durchgeführt mit der Ausnahme, daß WT-PDI und PDI-aba'c mit verschiedenen molaren Verhältnissen GSH zu GSSG für 24 h inkubiert wurden. Die Gesamtkonzentration an Glutathion war 2 mM. Die durch Luftoxidation hervorgerufene Änderung der GSH-Konzentration wurde mittels Ellmans Test bestimmt und entsprechend korrigiert. Dargestellt sind die Fluoreszenzwerte bei 332 nm (WT-PDI) und 335 nm (PDI-aba'c).

Die oxidative Kraft von PDI bezüglich Glutathion wird durch die Gleichgewichtskonstante (K_{eq}) ausgedrückt:

$$\mathbf{K_{eq} = [PDI_{ox}] \cdot [GSH]^2 / [PDI_{red}] \cdot [GSSG]} \quad [4]$$

K_{eq}	Gleichgewichtskonstante
$[PDI_{ox}]$, $[PDI_{red}]$	Konzentration an oxidierter bzw. reduzierter PDI
[GSH], [GSSG]	Konzentration an reduziertem bzw. oxidiertem Glutathion.

Über die Nernst Gleichung kann das Standard-Redoxpotential aus den gemessenen Gleichgewichtskonstanten bestimmt werden (Wunderlich und Glockshuber, 1993a):

$$\mathbf{E°' = (E_{GSH}) - (R \cdot T / (z \cdot F)) \cdot \ln K_{eq}} \quad [5]$$

E°'	Standard-Redoxpotential in V
E_{GSH}	Redoxpotential von GSH (-0,23 V)
R	Gaskonstante (8,315 J K^{-1} M^{-1})
T	absolute Temperatur in K (297,15 K)
z	Anzahl übertragener Elektronen (2)
F	Farraday Konstante (96487 C mol^{-1}).

Für WT-PDI betrug die Gleichgewichtskonstante 0,97 mM und für PDI-aba'c 1,44 mM. Das Redoxpotential wurde für WT-PDI zu –0,116 V und für PDI-aba'c zu –0,121 V errechnet, d.h. beide PDIs haben ein identisches Redoxpotential. Das zeigt, daß die partielle Deletion der **b'** Domäne von PDI keine Auswirkungen auf die Redoxaktivität der Mutante PDI-aba'c hatte. Das ermittelte Redoxpotential ist nahezu identisch zu dem Redoxpotential von –0,11 V für Hefe-PDI.

4.4.2.5 Messung des Oligomerisierungszustandes

Mittels verschiedener Meßverfahren wurden unterschiedliche Assoziationszustände der PDI beschrieben. Rinderleber-PDI sedimentiert als Monomer mit einem Sedimentations-koeffizienten von s = 3,5 (Ultrazentrifugationsstudien, Lambert und Freedman, 1983). Hingegen scheint PDI als Dimer und Tetramer vorzuliegen, wenn man sich die Ergebnisse verschiedener Gelfiltrationsanalysen anschaut (Yu et al., 1994; Lambert und Freedman, 1983). Dabei erfolgt die Oligomerisierung nicht über Disulfidbrücken (Yu et al., 1994).

Es wurden analytische Ultrazentrifugationen durchgeführt, um einen evtl. Einfluß von Mutationen oder Domänendeletionen auf den Oligomerisierungszustand von PDI zu untersuchen. Die Sedimentationsgeschwindigkeit wurde bei 40000 rpm und 20 °C gemessen. Alle 10 min wurden Datenpunkte für die Absorption bei 230 nm und 280 nm aufgezeichnet. Das Sedimentationsgleichgewicht wurde bei 8000 rpm und 10000 rpm nach 70 Stunden erreicht. Die Messung wurde bei 230 nm und 250 nm durchgeführt. Alle PDI-Varianten populierten konzentrationsunabhängig ausschließlich die monomere Spezies (Tabelle 3). Dabei war das ermittelte Molekulargewicht stets etwas höher als das theoretische Molekulargewicht. Allerdings war in keinem Fall eine systematische Tendenz in Abhängigkeit der Proteinkonzentration festzustellen, sodaß kein Assoziationsgleichgewicht angenommen werden kann. Der Sedimentationskoeffizient war geringer als man für ein monomeres, globuläres Protein erwarten kann.

Tabelle 3 Mittels Ultrazentrifugation ermittelte Molekulargewichte und Sedimentationskonstanten von PDI-Varianten*.

Protein-konzentration	**PDI aus Rinderleber**		**Humane WT-PDI**		**Humane PDI-aba'c**		**Humane PDI-b'a'c**	
(µg/ml)	**s_{app} (S)**	**Mr_{app}**	**s_{app} (S)**	**Mr_{app}**	**s_{app} (S)**	**Mr_{app}**	**s_{app} (S)**	**Mr_{app}**
20	---	---	3,16	57440	---	---	---	---
40	---	---	---	---	2,42	44930	---	39680
100	2,94	65180	3,15	61530	---	---	2,42	40200
120	---	---	---	---	2,54	44780	---	---
200	---	---	3,13	60100	2,52	49330	2,34	40490
400	3,09	67950	3,20	55700	2,47	51460	2,54	42700
600	---	---	3,22	55350	---	---	2,46	41730
967	2,85	67210	3,10	58060	---	---	2,54	41540
Mittelwert	2,96	66780	3,16	57654	2,49	47625	2,46	41057

* Die theoretischen Molekulargewichte betragen für PDI (Rinderleber): 55000 Da, WT-PDI (human): 56400 Da, PDI-aba'c: 44300 Da, PDI-b'a'c: 32500 Da.

4.4.3 Einfluß von PDI-Varianten auf die Faltung von Proinsulin

Die Faltung von denaturiertem und reduziertem Proinsulin wurde bei pH 7,5 mit GSH/GSSG als Redoxpaar durchgeführt und mittels RP-HPLC analysiert. Die Proinsulinfaltung läßt sich als eine Reaktion erster Ordnung mit einer Geschwindigkeitskonstante von $k_{app} = 0{,}002\ s^{-1}$ beschreiben. Proinsulin faltet bei einer Proteinkonzentration von 100 µg/ml mit ca. 20 % Ausbeute. Obwohl diese Bedingungen kein Optimum bezüglich der Proinsulinfaltung darstellen, sind sie optimal hinsichtlich der Aktivität der eingesetzten PDIs. Glutathion ist das biologische Redoxäquivalent für PDI im Endoplasmatischen Retikulum (Hwang et al., 1992). Der Einfluß der verwendeten PDI-Varianten auf die Proinsulinfaltung kann folgendermaßen zusammengefaßt werden (siehe auch Tabelle 4):

(1) Die Proinsulinfaltung wurde von allen eingesetzten PDIs konzentrationsabhängig beeinflußt. PDI-Varianten mit zumindest einem aktiven Zentrum waren in katalytischen Mengen aktiv.

(2) Fehlen oder Inaktivierung eines aktiven Zentrums (PDI-b'a'c, PDIΔC1, PDIΔC2) resultierte in einer bezüglich WT-PDI geringfügig verringerten Faltungsgeschwindigkeit und –ausbeute von Proinsulin.

(3) Deletion aller Cysteine im aktiven Zentrum (PDIΔC1,2) führte zu einem Verlust der faltungsbeschleunigenden Eigenschaften. Zwei- bis dreifacher molarer Überschuß an PDIΔC1,2 war notwendig, um eine deutlich erhöhte Proinsulinausbeute zu erreichen.

(4) Die partielle Deletion der **b'** Domäne (PDI-aba'c) resultierte in einer im Vergleich zu WT-PDI signifikant verringerten Faltungsgeschwindigkeit und –ausbeute.

Es wurde vermutet, daß die partielle Deletion der Peptidbindungsdomäne von PDI die Isomerisierungsfähigkeit der Mutante (PDI-aba'c) komplett unterdrückt. D.h., die nicht-vorhandene Bindung von Faltungsintermediaten an PDI hätte zu einem Verlust der Isomeraseaktivität geführt. Offensichtlich war das nicht vollständig der Fall. PDI-aba'c in einem 2-fach molaren Überschuß zu Proinsulin erhöhte im Vergleich zur spontanen Faltung die Faltungsgeschwindigkeit ca. 3 bis 4-fach und verdoppelte die Ausbeute (siehe Abb. 8). Das ist ein Hinweis darauf, daß im Gegensatz zu früheren Annahmen die **b'** Domäne nicht essentiell für die Isomeraseaktivität der PDI ist. Ihr Fehlen bewirkt zwar eine drastische Verringerung der faltungsbeschleunigenden Eigenschaften bezüglich Proinsulin, jedoch kann eine signifikante Rest-Isomeraseaktivität festgestellt werden.

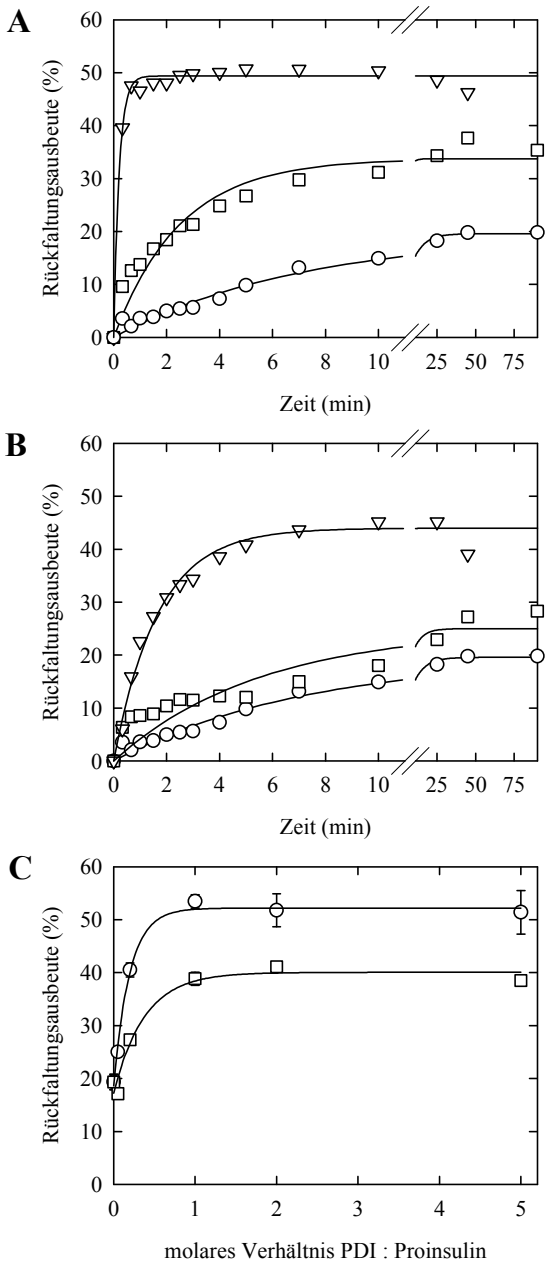

Abb. 8: Einfluß von WT-PDI und PDI-aba'c auf die Faltung von Proinsulin. Faltung von Proinsulin (100 µg/ml) wurde in 10 mM Tris/10 mM Glycin pH 7,5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG bei 25 °C durchgeführt.

A, Zeitliche Abhängigkeit der Proinsulinfaltung in Abwesenheit von PDI (o) und in Gegenwart von WT-PDI (∇) bzw. PDI-aba'c (□) in einem molaren Verhältnis zu Proinsulin von 2.

B, Zeitliche Abhängigkeit der Proinsulinfaltung in Abwesenheit von PDI (o) und in Gegenwart von WT-PDI (∇) bzw. PDI-aba'c (□) in einem molaren Verhältnis zu Proinsulin von 0,2.

C, Rückfaltung von Proinsulin in Gegenwart verschiedener molarer Verhältnisse von WT-PDI (o) bzw. PDI-aba'c (□). Angegeben sind die Mittelwerte aus einer 5-fach Bestimmung und die entsprechende Standardabweichung.

Tabelle 4 Einfluß von PDI-Varianten auf die Geschwindigkeit und die Ausbeute der Proinsulinfaltung bei einem 5-fach molaren Überschuß von Proinsulin bezüglich der PDI-Variante

PDI-Variante	**k_{app} [a] (s^{-1}) (n [c])**	**A_{max} [b] (%) (n)**
spontane Faltung	0,0022 ± 0,0008 [d] (5)	20,6 ± 2,3 (15)
WT-PDI	0,0120 ± 0,0039 (5)	45,0 ± 4,4 (12)
PDIΔC1	0,0054 ± 0,0009 (2)	41,5 ± 1,5 (2)
PDIΔC2	0,0038 ± 0,0001 (2)	43,7 ± 3,1 (2)
PDI-b'a'c	0,010 (1)	40,3 ± 2,9 (3)
PDIΔC1,2	0,002 (1)	26,6 ± 6,3 (3)
PDI-aba'c	0,005 (1)	27,3 ± 1,0 (5)

[a] k_{app} = apparente Geschwindigkeitskonstante

[b] A_{max} = maximale Ausbeute an nativem Proinsulin

[c] n = Anzahl der Bestimmungen

[d] Angegeben sind der Mittelwert und die Standardabweichung (n>2) bzw. die Schwankung um den Mittelwert (n=2).

4.4.4 Rückfaltung von Proinsulin in Gegenwart des Dithiols Vectrase ä -P

(Winter et al., 2002a)

Vectrase™-P kann die Isomeraseaktivität von PDI nachahmen (Woycechowsky et al., 1999). Da PDI die *in vitro* Faltung des Proinsulins deutlich beschleunigt und die Ausbeute an nativem Proinsulin erhöht, wurde auch Vectrase™-P hinsichtlich ihres Einflusses auf die Faltungsausbeute und –geschwindigkeit von Proinsulin untersucht. Im Vergleich zu PDI zeigte sich, daß wesentlich höhere Konzentrationen an Vectrase™-P nötig sind, um eine erhöhte Faltungsausbeute zu erzielen. Vectrase™-P in einem 30-fach molaren Überschuß zu Proinsulin verdoppelte die Ausbeute an nativem Proinsulin. Jedoch ist die Ausbeutesteigerung geringer als die in Gegenwart von PDI in stoichiometrischen Konzentrationen. Die Geschwindigkeit und auch die Redoxabhängigkeit der Proinsulinfaltung wurden durch

Vectrase™-P nicht beeinflußt. Das Optimum der Proinsulinfaltung lag stets bei einem Redoxverhältnis von GSH^2/GSSG von 0,5 bis 1,5 mM.

Es scheint, daß Vectrase™-P die Proinsulinfaltung auf nicht-geschwindigkeitsbestimmenden Stufen beeinflußt. Das könnte die unveränderte Faltungsgeschwindigkeit erklären. Jedoch wird scheinbar die Kompetition zwischen Faltung und Aggregation durch Vectrase™-P verändert, was zu einer erhöhten Ausbeute an nativem Protein durch Verringerung von unproduktiven Nebenreaktionen führt. Ein Nachteil der Vectrase™-P, im Vergleich zu PDI, ist das Fehlen der Chaperonfunktion.

4.4.5 Unterscheidung zwischen Chaperonfunktion und Isomeraseaktivität der PDI

Aggregation ist eine Reaktion zweiter oder höherer Ordnung, der mit der korrekten Faltung eines Proteins in Konkurrenz steht (Kiefhaber et al., 1991). Der Einfluß der Chaperonfunktion der PDI auf die Aggregation faltenden Proinsulins wurde mittels Lichtstreuung untersucht. Das durch Aggregation nach Verdünnung des denaturierten und reduzierten Proinsulins in Faltungspuffer entstehende Lichtstreusignal war linear abhängig von der eingesetzten Proteinkonzentration. Aggregation erfolgte in den ersten 20 bis 30 Sekunden der Rückfaltung und verlief damit genauso schnell wie die unproduktive Reaktion in den *timed addition* Experimenten (siehe unten). In Gegenwart katalytischer Mengen WT-PDI konnte die Aggregation faltenden Proinsulins bereits zu 10 – 20 % und in stoichiometrischen Konzentrationen zu ca. 40 % unterdrückt werden. Im Unterschied dazu wurde nur in Anwesenheit stoichiometrischer Konzentrationen von PDIΔC1,2 die Aggregation in ähnlichem Maße wie durch WT-PDI unterdrückt. Das verdeutlicht, daß PDI und PDIΔC1,2 eine Chaperonfunktion besitzen, die ausreicht, um die Aggregation von Proinsulin signifikant zu unterdrücken. Maximale Ausbeuten an korrekt gefaltetem Proinsulin können jedoch nur in simultaner Anwesenheit von Isomeraseaktivität und Chaperonfunktion erreicht werden. Es scheint, daß frühe Faltungsintermediate mit nativen oder nicht-nativen Disulfidbrücken extrem anfällig für Aggregation sind. Diese können, katalysiert durch WT-PDI, schneller zu weniger aggregationsanfälligen Intermediaten isomerisieren, wodurch eine beschleunigte Faltung und höhere Faltungsausbeuten erreicht werden können. Eine verfügbare Chaperonfunktion kann zwar aggregationsanfällige Faltungsintermediate in Lösung halten, deren Isomerisierung jedoch nicht katalysieren. Daraus resultieren geringere Faltungsausbeuten von Proinsulin in Gegenwart von PDIΔC1,2 im Vergleich zu WT-PDI.

Die Chaperonaktivität erfordert die Bindung von Peptiden oder Proteinen an eine Peptidbindungsstelle. Die Blockierung dieser Bindungsstelle kann zur Unterdrückung der Chaperonfunktion führen. Entsprechend verringerte die Inhibierung von WT-PDI bzw. PDIΔC1,2 mit Genistein die Rückfaltungsausbeuten für Proinsulin signifikant. Genistein ist ein hydrophobes Molekül und hatte keinen Einfluß auf die Proinsulinfaltung an sich. Die Faltungsgeschwindigkeit für Proinsulin in Gegenwart von Genistein-inhibierter WT-PDI war viermal geringer als in Gegenwart nicht-inhibierter WT-PDI, aber immer noch signifikant höher als in der spontanen Faltung. Das zeigt, daß für eine effiziente Isomerisierung die Peptidbindung notwendig ist. Weiterhin konnten Genistein-inhibierte WT-PDI bzw. PDIΔC1,2 nur noch ca. 10 % anstatt 40 % des faltenden Proinsulins vor Aggregation schützen. Die Chaperonaktivität der PDI wurde also durch Genistein nicht vollständig inhibiert, obwohl Genistein in einem 40-fach molaren Überschuß zu Proinsulin eingesetzt wurde. Möglicherweise kann Proinsulin in geringem Maße mit Genistein um die Peptidbindungsstelle konkurrieren, wodurch ein Teil des Proinsulins vor Aggregation geschützt wurde.

Die spontane Proinsulinfaltung war nach 60 min abgeschlossen. Dabei stellte sich die Frage, ob die Disulfidverbrückung bzw. –isomerisierung während der gesamten Faltungszeit stattfindet oder nicht. Um das zu analysieren wurden sogenannte *timed addition* Experimente durchgeführt, bei denen zu verschiedenen Zeiten nach Faltungsstart WT-PDI, PDIΔC1,2 oder der Inhibitor Genistein zugegeben wurde. Dabei stellte sich heraus, daß die Chaperonfunktion der PDI nur in den ersten Sekunden der Faltung für eine erhöhte Faltungsausbeute essentiell war. Die Isomeraseaktivität hingegen beeinflußte unabhängig von der Chaperonfunktion die Proinsulinfaltung während der gesamten Faltungsdauer. In Abwesenheit der Chaperon-funktion wurde die Isomeraseaktivität essentiell, um eine erhöhte Faltungsausbeute zu erreichen. Die verzögerte Zugabe der WT-PDI zu faltendem Proinsulin resultierte in einer verringerten Faltungsausbeute, deren Verlauf einer bi-phasischen Charakteristik entsprach. Dabei war die Geschwindigkeitskonstante für die erste, schnelle Phase ca. 20-fach größer als die der zweiten Phase. Die erste Phase verlief in einem ähnlichen Zeitfenster wie die Aggregation faltenden Proinsulins (siehe oben), die zweite Phase verlief in einem ähnlichen Zeitfenster wie die Bildung nativen Proinsulins. Daraus kann man ableiten, daß während der Renaturierung des Proinsulins eine Konkurrenz zwischen einem langsamen Faltungsprozeß und einer ca. 20-fach schneller verlaufenden unproduktiven Reaktion stattfindet. Dieser unproduktive Prozeß führte zur Aggregation faltenden Proinsulins.

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6 Abkürzungen

BPG	1,3-Bisphospho-D-Glyzerat
BPTI	basischer pankreatischer Trypsininhibitor
BSA	*bovine serum albumine*
CD	Circulardichroismus
C-Peptid	*connecting peptide*
Da	Dalton
Dsb	*disulfide binding protein*
DSG	Disuccinimidylglutarat
DTT	1,4-Dithiothreitol
E_0'	Standardredoxpotential bei pH 7,0
EDTA	Ethylendiamintetraessigsäure
ELISA	*enzyme-linked immunosorbent assay*
Fab	Antikörperfragment
GAP	D-Glyzeraldehyd-3-Phosphat
GAPDH	Glycerinaldehyd-3-Phosphat-Dehydrogenase
GdnHCl	Guanidiniumhydrochlorid
GSH	reduziertes Glutathion
GSSG	oxidiertes Glutathion
Hsp	*heat shock protein*
IGF-I	*insulin-like growth factor*-I
k_{app}	apparente Geschwindigkeitskonstante
IPTG	Isopropyl-β-D-thiogalactopyranosid
NAD	Nicotinamidadenindinukleotid
NADPH	reduziertes Nicotinamidadenindinukleotidphosphat
nm	Nanometer
NMR	*nuclear magnetic resonance*
OD_{500nm}	optische Dichte bei 500 nm
PDI	Proteindisulfidisomerase
pelB	Signalsequenz der Pektatlyase aus *Erwinia carotovora*
RBI	α-Amylase/Trypsininhibitor
RP-HPLC	*reversed phase high performane liquid chromatography*
rpm	*rounds per minute*
scFv	*single chain* Antikörperfragment
UV	Ultraviolett
WT	Wildtyp

7 Veröffentlichungen und zur Veröffentlichung eingereichte Manuskripte

1. Winter, J., Neubauer, P, Glockshuber, R., Rudolph, R. (2000) Increased production of human proinsulin in the periplasmic space of Escherichia coli by fusion to DsbA.
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3. Winter, J., Klappa, P., Freedman, R. B., Lilie, H., Rudolph, R. (2002) Catalytic Activity and Chaperone Function of Human Protein-Disulfide Isomerase Are Required for the Efficient Refolding of Proinsulin.
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4. Winter, J., Lilie, H., Rudolph, R. (2002a) The Low Molecular Weight Dithiol Vectrase™-P Stimulates Proinsulin Folding in vitro and Production of Native Proinsulin in the Periplasm of E. coli.
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Increased production of human proinsulin in the periplasmic space of *Escherichia coli* by fusion to DsbA

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Abstract

The production of human proinsulin in its disulfide-intact, native form in *Escherichia coli* requires disulfide bond formation and the periplasmic space is the favourable compartment for oxidative folding. However, the secretory expression of proinsulin is limited by its high susceptibility to proteolysis and by disulfide bond formation, which is rate-limiting for proinsulin folding. In this report we describe a method for the production of high amounts of soluble, native human proinsulin in *E. coli*. We fused proinsulin to the C-terminus of the periplasmic disulfide oxidoreductase DsbA via a trypsin cleavage site. As DsbA is the main catalyst of disulfide bond formation in *E. coli*, we expected increased yields of proinsulin by intra- or intermolecular catalysis of disulfide bond formation. In the context of the fusion protein, proinsulin was found to be stabilised, probably due to an increased solubility and faster disulfide bond formation. To increase the yield of DsbA-proinsulin in the periplasm, several parameters were optimised, including host strains and cultivation conditions, and in particular growth medium composition and supplement of low molecular weight additives. We obtained a further, about three-fold increase in the amount of native DsbA-proinsulin by addition of L-arginine or ethanol to the culture medium. The maximum yield of native human proinsulin obtained from the soluble periplasmic fraction after specific cleavage of the fusion protein with trypsin was 9.2 mg g^{-1}, corresponding to 1.8% of the total cell protein.

Keywords: Proinsulin; Secretory expression; DsbA fusion protein; Medium additives; Arginine

1. Introduction

Nearly 0.7% of the world population suffers from insulin-dependent diabetes with a continuously increasing number of patients. Consequently, the requirement for recombinant insulin is increasing. Insulin is a 51 amino acid polypeptide, consisting of two separate chains. Both A- and B-chain are connected by two intermolecular disulfide bonds. The A-chain contains an additional intramolecular disulfide bond. In the human pancreas, insulin is produced as a single

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polypeptide chain, proinsulin, with the A-chain and B-chain joined by the connecting peptide (C-peptide) (Mackin, 1998). After synthesis, folding and concomitant disulfide bond formation, native proinsulin is converted to insulin by enzymatic cleavage whereby the C-peptide is cleaved off and the carboxy-terminus of the B-chain is trimmed. Processing of proinsulin in vitro can be performed with trypsin and carboxypeptidase B (Kemmler et al., 1971).

Mature insulin is so far not producible in procaryotic host cells in its native conformation by recombinant techniques, essentially because correct disulfide bond formation only occurs at the level of proinsulin. Several strategies for production of proinsulin in the cytoplasm of *Escherichia coli* as insoluble inclusion bodies have been described and are commercially used (Tang and Hu, 1993; Kang and Yoon, 1991; Sung et al., 1986). The great advantage of these approaches is that proinsulin can be produced in high amounts, but the complex process of purification and the formation of the correct disulfide bonds during folding are critical cost factors. Alternatively, proinsulin has been produced in *E. coli* and secreted by routing the recombinant protein using appropriate signal sequences to the periplasmic space. In this case, however, the yield of correctly folded proinsulin was very low compared to the yield obtained by intracellularly production of proinsulin (Talmadge et al., 1981; Chan et al., 1981). Also alternative host organisms, such as *Bacillus subtilis*, *Streptomyces lividans*, and *Saccharomyces cereviseae* were used for secretory expression of the proinsulin gene (Novikov et al., 1990; Koller et al., 1989; Thim et al., 1986), but the yield was not significantly increased in comparison to that obtained in *E. coli*. The first report of an efficient secretory expression of a modified proinsulin gene in *E. coli* has been published by Kang and Yoon (1994). The authors constructed a so-called ZZ-proinsulin fusion construct in which the C-peptide was either totally deleted or drastically shortened (only 1–11 amino acids remained), which resulted in significantly increased expression yields.

The stability against degradation of a recombinant protein produced in *E. coli* is strongly dependent on its folding efficiency in vivo. Therefore, the bacterial periplasm principally represents the most favourable compartment for proinsulin production. The periplasm provides oxidising conditions and proteins of the Dsb family (DsbA, DsbC and DsbG), which are efficient catalysts of disulfide bond formation in the periplasm (Bessette et al., 1999; Raina and Missiakas, 1997). DsbA is the most important oxidase of free sulfhydryl groups in the periplasm. It has been shown to be useful for in vitro folding of disulfide-bonded proteins, such as BPTI, hirudin, α-lactalbumin, alkaline phosphatase, and bovine ribonuclease A (Zapun and Creighton, 1994; Wunderlich et al., 1993; Akiyama et al., 1992). A previous study in which enterokinase was fused to the C-terminus of DsbA showed that high amounts of soluble, native enterokinase can be produced in the periplasm (Collins-Racie et al., 1995). In contrast, coexpression of DsbA only yielded periplasmic IGF-I inclusion bodies (Joly et al., 1998). In the case of the secreted α-amylase/trypsin inhibitor RBI the yield was not improved by DsbA coexpression alone but could be strongly increased in combination with the addition of reduced glutathione to the cultivation medium (Wunderlich and Glockshuber, 1993).

In this study we investigate the periplasmic production of proinsulin in *E. coli* as a C-terminal fusion to DsbA. The rationale of this approach is the assumption that DsbA should stabilise unfolded proinsulin intramolecularly via its polypeptide binding site (Frech et al., 1996) and promote correct disulfide bond formation by intra- and intermolecular catalysis. We show that DsbA-proinsulin is efficiently secreted to the periplasm and that the proinsulin moiety in soluble fusion protein is correctly folded. Further significant improvements of production of the fusion protein could be obtained by optimisation of the bacterial growth conditions and by addition of low molecular weight folding enhancers to the cultivation medium.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strains used in this study are XLI blue (Stratagene) as host strain for routine cloning experiments, and the expression strains SF131 (Meerman and Georgiou, 1994), BL21 (Novagen), JM109 (Stratagene), C600, and RB791 (both *E. coli* Genetic Stock Center, New Haven). The plasmid pDsbA3 (Jonda et al., 1999) was used as cloning vector (Fig. 1). Vector pRK5-PI (Mather and Ullrich, 1987) was the source of the proinsulin gene.

2.2. DNA constructions

Plasmid preparation was carried out using a Qiagen plasmid miniprep kit according to the manufacturers instruction. Restriction enzyme digestion and ligation were carried out according to Sambrook et al. (1989). The vector pDsbA3-PI was constructed by inserting the human proinsulin gene into pDsbA3 downstream of the dsbA gene. Additionally, a sequence of 18 nucleotides was introduced between the 3′ end of dsbA and the 5′ end of the proinsulin gene, encoding a linker with five glycine and one arginine residue (Fig. 1). The human proinsulin gene was amplified using plasmid pRK5-PI as template, the forward primer 5′-d(GAAATATC-TGTCCGAGAAAA AAGGCGGTGGGGGTGGCCGCTTTGTGA-ACCAACACCTG), and the reverse primer 5′-d(CTCTTGATGACGTTGATCCCTAGGCTT-AAGCAC). The dsbA gene was amplified from pDsbA3 with the forward primer 5′-d(G-CGACTGGAATTCCATATGGCGCAGTAT-GAAGATG), and the reverse primer 5′-d(CTT-TATAGACAGGCTCTTTTTTCCG-CCA). PCR was carried out using Tfl polymerase (Promega) at 95, 55, and 72°C for denaturation, annealing, and synthesis, with 30 cycles. The am-

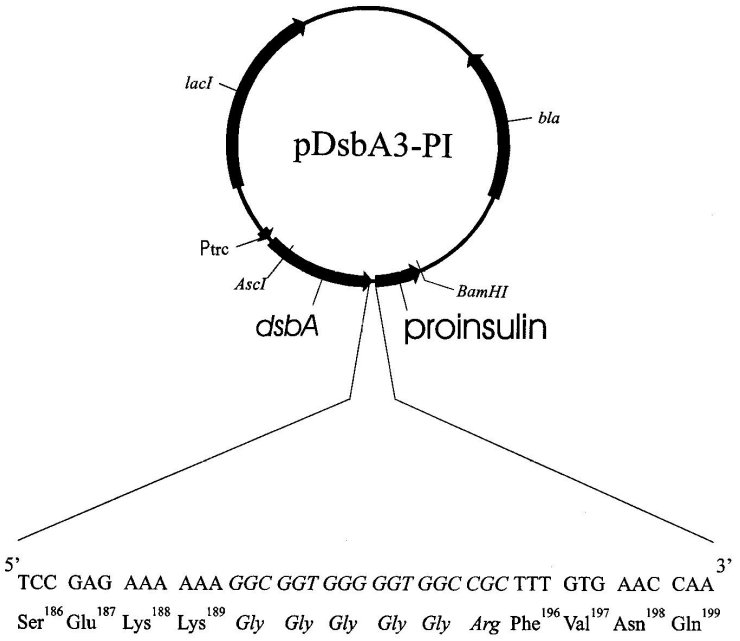

Fig. 1. Map of the vector pDsbA3-PI. The restriction sites used for cloning are indicated. The linker sequence between DsbA (residues 1–189) and proinsulin (residues 196–281) are shown in italics.

plified PCR products were used as templates for the following fusion PCR. First, to allow annealing of homologeous regions at the 3′ end of dsbA gene and the 5′end of proinsulin gene, both PCR products were incubated in a PCR reaction without primer at an annealing temperature of 40°C, with five cycles. The next 25 cycles were carried out at an annealing temperature of 62°C after addition of 5′-forward primer used for dsbA-PCR and 3′-backward primer used for proinsulin-PCR. The amplified PCR product was cloned between the AscI and BamHI sites of pDsbA3. The DNA sequence encoding the entire fusion protein was determined using a sequencing Kit (Biozym) and a Li-COR sequencer (MWG Biotech).

2.3. Expression of DsbA-proinsulin

E. coli cells harbouring pDsbA3-PI were grown at 25°C in shake flask cultures in LB or mineral salt medium containing glucose (10 g l^{-1}) and ampicillin (100 μg ml^{-1}). The composition and preparation of the mineral salt medium has been described previously (Teich et al., 1998). After the cells reached an optical density (OD_{500}) of 1, IPTG was added to 1 mM, the culture medium was supplemented with medium additives and the pH value was adjusted by adding HCl or NaOH, and the cells were grown for additional 6 h.

The preparation of periplasmic fractions was performed by osmotic shock according to Kang and Yoon (1994). Generally, 100 μl of extraction buffer were added to a cell pellet of 1 ml broth at $OD_{500} = 1$. The soluble periplasmic proteins and the residual insoluble proteins were analysed by tricine-SDS-PAGE (13%). For immunoblot analysis, the separated proteins were electroblotted onto a nitrocellulose membrane (Pall), incubated with a monoclonal mouse antibody against human insulin (Roche), and visualised by using the ECL detection system (Amersham).

2.4. Analysis of native proinsulin in periplasmic fractions

Trypsin converts the native DsbA-proinsulin fusion protein into DsbA (plus linker peptide), C-peptide, and human insulin. This human insulin contains an additional arginine residue at the C-terminus of the B-chain because further trimming by carboxypeptidase B digestion was omitted. The cleavage procedure was performed at pH 8.0 (10 mM Tris/HCl, 2 mM EDTA) on ice with different trypsin concentrations in the range of 1–200 μg trypsin per 100 μl of the periplasmic fraction. Optimal release of insulin from the fusion protein was obtained by incubation with 100 μg trypsin for 10 min (data not shown). Proteolysis was stopped by addition of soybean trypsin inhibitor (STI, Sigma) in a 10-fold molar excess over trypsin. The released insulin was analysed by a specific insulin-ELISA (Roche). It is a sandwich-ELISA with matrix-bound monoclonal anti-human insulin antibodies and peroxidase-conjugated monoclonal anti-human insulin antibodies recognising only native insulin or native proinsulin. Using a standard curve created with native insulin, the amount of proinsulin produced in *E. coli* was calculated. The concentration of native proinsulin obtained after cleavage with trypsin is given as mg native human proinsulin per g dry cell weight (mg g^{-1}) or per liter cultivation broth (mg l^{-1}). All experiments have been repeated at least twice and each sample was measured in three to five dilutions.

2.5. Purification of DsbA-proinsulin

Periplasmic fractions from 0.5 l of bacterial culture containing DsbA-proinsulin were adjusted to 1.2 M ammonium sulfate by gently adding ice-cold 3.5 M ammonium sulfate in 20 mM Tris/HCl (pH 7.4), incubated on ice for 30 min and centrifuged (48 000 × *g*, 4°C, 30 min). The pellet containing DsbA-proinsulin was resuspended in 5 ml ice-cold 10 mM MOPS/NaOH pH 7.0 (buffer A) and homogenised using an ultraturrax for 2 min. After dialysis over night against buffer A, the protein fraction was loaded onto a DEAE-Sepharose ff column (Pharmacia, 5 ml column volume). DsbA-proinsulin was eluted in a linear gradient from 0 M (buffer A) to 0.5 M NaCl in buffer A over 10 column volumes. Fractions of 2 ml were collected and the protein concentration was determined from UV spectra [ε(280 nm, M^{-1}, 1 cm = 34 140]. Fractions of

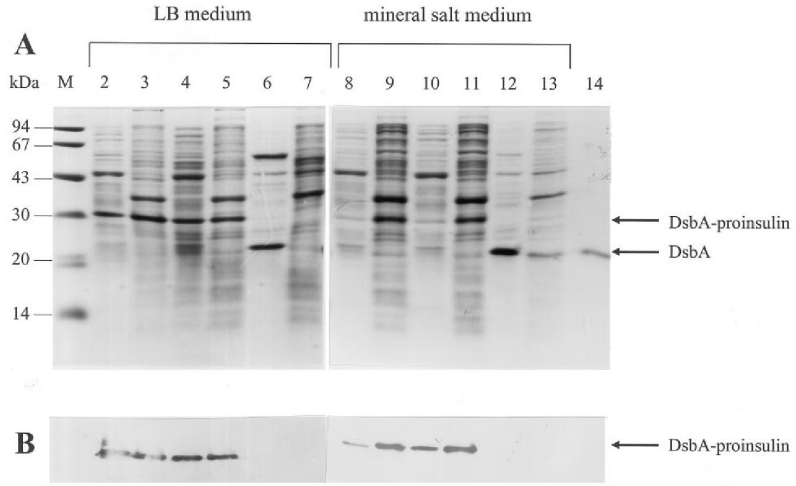

Fig. 2. Levels of the periplasmically expressed, soluble DsbA-proinsulin fusion protein and DsbA after growth in different media with different additives. A coomassie-stained tricine-SDS-gel (13%) (A) and the corresponding immunoblot with monoclonal anti-insulin antibodies (B) are shown. Cells overexpressing DsbA-proinsulin were grown in LB medium (lanes 2–3), LB with 0.4 M L-arginine (lanes 4–5), mineral salt medium (MSM) (lanes 8–9), and MSM with 1% ethanol (lanes 10–11). As a control *E. coli* RB791 overexpressing DsbA from the plasmid pDsbA3 was also cultivated in LB (lanes 6–7) and MSM (lanes 12–13). Lanes 2, 4, 6, 8, 10, 12 represent soluble periplasmic fractions. Lanes 3, 5, 7, 9, 11, 13 represent the residual cellular protein after removal of periplasmic proteins. Lane 1 shows the molecular mass standard (M), lane 14 shows purified DsbA.

each purification step were analysed by SDS-PAGE (12%). Trypsin digestions of purified DsbA-proinsulin were performed on ice at pH 7.5 (8 mM MOPS, 2 mM Tris/HCl, 180 mM NaCl) with a protein concentration of 100 μg ml^{-1} and a mass ratio DsbA-proinsulin: trypsin of 10: 1. Digestion products were analysed by RP-HPLC and tricine-SDS-PAGE (18%). Reversed phase HPLC was performed with a linear gradient from 20 to 50% (v/v) acetonitrile in 0.1% TFA on a C_{18} column (ET 125/3 NUCLEOSIL 100-5 C_{18} PPN, Macherey-Nagel) and the eluted digestion products were analysed by mass spectrometry. For SDS-PAGE analysis the digested fractions were precipitated by adding TCA to a final concentration of 15% (w/v), loaded onto tricine-SDS-gels (18%) under reducing conditions and blotted onto nitrocellulose for immunoblotting as described above, or on a PVDF membrane for N-terminal sequencing.

3. Results

3.1. Expression of DsbA-proinsulin and determination of proinsulin

For secretory expression of the human proinsulin gene, we constructed the plasmid pDsbA3-PI (Fig. 1). Using this vector dsbA-proinsulin expression is controlled by a trc promotor and the fusion protein is translocated to the periplasm by the dsbA signal sequence. The fusion protein was quantitatively secreted to the periplasm as the precursor form could not be detected by immunoblotting (data not shown). Unexpectedly, only ~10% of the fusion protein were found in the soluble fraction, while ~90% formed insoluble periplasmic inclusion bodies (Fig. 2(A–B), lanes 2 and 3). After trypsin digestion of the soluble periplasmic fraction the concentration of the generated insulin was mea-

sured by an insulin-ELISA and correlated to proinsulin based on a standard curve. Trypsin digestion of the fusion protein proved to be essential for quantitative determination of proinsulin, indicating that within the fusion protein the proinsulin part was not completely accessible to the ELISA antibody.

3.2. Expression of DsbA-proinsulin in different host strains at different growth temperatures

In order to achieve efficient production of the fusion protein in soluble form with a native proinsulin part, the expression was performed with *E. coli* SF131. In this strain, the genes degP, ompT, and ptr were deleted (Meerman and Georgiou, 1994). Ptr encodes Protease III, which has been shown to degrade insulin and other secreted fusion proteins. Thus, we assumed that in this strain proteolytic degradation of the soluble fusion protein should be lowered. LB was used as cultivation medium and the expression was analysed at growth temperatures from 20 to 30°C over 24 h after induction. Cultivation at 25°C for 4–6 h was found to be optimal for the production of soluble fusion protein with native proinsulin part (data not shown). The maximum amount of proinsulin was ~ 0.17 mg g^{-1}, corresponding to 0.56 mg g^{-1} of the full length fusion protein. Despite the use of the strain SF131 as a host, the yield of DsbA-proinsulin was still rather low. Therefore, we investigated expression of the fusion protein in other host strains, such as *E. coli* JM109, BL21, C600, and RB791. The yield of periplasmic proinsulin, detected after trypsin digestion (10 μg ml^{-1}) was highest in the *E. coli* strains C600 and RB791 (Table 1). In case of *E. coli* RB791 the cells grew much faster than C600 cells resulting in a higher cell density. Therefore, for further analysis, we compared the amount of soluble fusion protein produced in RB791 during growth at 25 and 37°C, respectively. The overall expression yield was highest at 37°C, but the highest amount of soluble fusion protein was found when cells were grown at 25°C (data not shown).

3.3. Conversion of purified DsbA-proinsulin to insulin by trypsin

In order to identify the digestion products of DsbA-proinsulin after trypsin treatment we purified the fusion protein from the periplasmic fraction (Fig. 3(A)). DsbA-proinsulin was first quantitatively precipitated with 1.2 M ammonium sulfate. The precipitate contained only two major impurities with an apparent molecular mass of ~ 43 and 50 kDa, which could be separated by ion exchange chromatography on DEAE-Sepharose. Further optimisation of the purification of DsbA-proinsulin was not attempted. Fractions containing homogeneous DsbA-proinsulin which consisted of $\sim 10\%$ of the total fusion protein were used for trypsinisation. The overall yield of purified protein was ~ 1 mg DsbA-proinsulin from 0.5 l of culture medium ($OD_{500} = 1$). After trypsin digestion, the cleavage products were separated on tricine-SDS-gels (18%) or reversed phase HPLC. Under optimal cleavage conditions, the fusion protein was rapidly converted to insulin (Fig. 3(B)). After 1 min, nearly the total fusion protein was digested and one major protein with an apparent molecular mass of 23 kDa appeared, which

Table 1
Levels of native DsbA-proinsulin in periplasmic extracts of different *E. coli* strains

E. coli host strains	Amount of proinsulin[a]	
	mg g^{-1}	mg l^{-1}
SF131	0.17	0.02
JM109	0.25	0.06
BL21	0.32	0.11
C600	0.83	0.24
RB791	0.83	0.29

[a] Cells were cultivated at 25°C, harvested 4 h after induction and the periplasmic proteins were obtained after osmotic shock. After trypsin digestion (1 μg trypsin per 100 μl periplasmic fraction), the released insulin was quantified by an insulin-ELISA, which was used to deduce the initial concentration of proinsulin. The given yields correspond to mg of native human proinsulin per g dry cell weight (mg g^{-1}) and mg of native human proinsulin per liter of cultivation broth (mg l^{-1}), respectively.

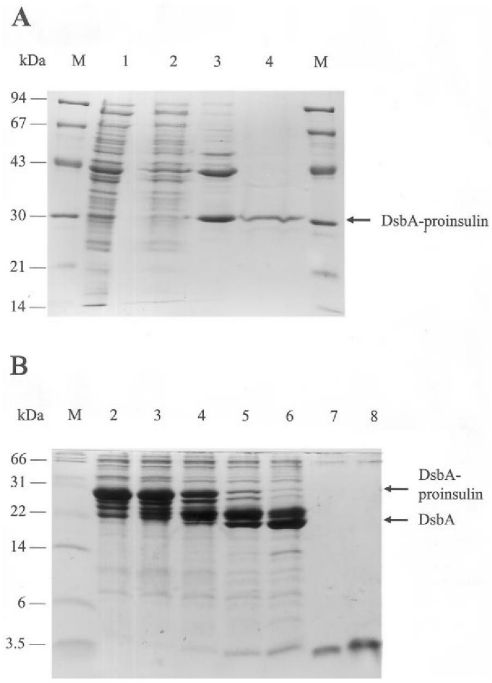

Fig. 3. DsbA-proinsulin purification and trypsin digestion products. A: SDS-PAGE (12%), analysis of recombinant DsbA-proinsulin purified from the periplasmic fraction of *E. coli* RB791. Soluble periplasmic fraction (lane 1), supernatant after ammonium sulfate precipitation (lane 2), and pellet after dialysis against 10 mM MOPS/NaOH pH 7.0 (lane 3), purified DsbA-proinsulin after ion exchange chromatography (lane 4). For details see the methods section. B: Trypsin digestion products of purified DsbA-proinsulin were analysed on reducing tricine-SDS-gels (18%). DsbA-proinsulin (20 μg) was incubated with trypsin in a mass ratio of 10: 1 for 0, 0.17, 1, 10, and 50 min (lanes 2–6); lanes 7 and 8, human insulin standard; molecular weight markers (M) are indicated in lanes 1 and 6 (A) and lane 1 (B).

is further degraded to a protein with ~20 kDa. The proteins correspond to C-terminally truncated fragments of DsbA-proinsulin and DsbA, respectively, as revealed by N-terminal sequencing. Simultaneously, a set of protein bands with an apparent molecular mass of 3–4 kDa, corresponding to the A- and B-chain of insulin and the C-peptide, could be observed. By reversed phase HPLC and mass spectrometry the digestion products of DsbA-proinsulin were identified as the expected insulin with an additional arginine residue at the C-terminus of the B-chain and the C-peptide.

3.4. Optimisation of the yield of native DsbA-proinsulin using medium additives

To further increase the yield of soluble fusion protein with a native proinsulin part we investigated the effect of different medium additives during cultivation of transformed RB791 in LB medium. We chose pH values of the culture medium from 4 to 9 and L-arginine (0.1–0.5 M), reduced glutathione (2–10 mM), and ethanol (1–5% v/v) as medium additives. For L-arginine it has been shown that it increases the refolding yield of recombinant proteins in vitro (Rudolph et al., 1997). Reduced glutathione allows disulfide bond isomerisation in disulfide-scrambled folding intermediates in vitro and in vivo (Zapun and Creighton, 1994; Wunderlich and Glockshuber, 1993; Akiyama et al., 1992). The addition of ethanol to *E. coli* cultures seems to induce the heat shock response and the production of molecular chaperones, and leads to an enhanced production of heterologeous proteins in *E. coli* (Kusano et al., 1999; Van Dyk et al., 1995). In addition, similarly to the in vitro folding of IGF-I, ethanol may influence the oxidative folding of proinsulin in the periplasmic space (Hejnaes et al., 1992). Since the pH value is a key factor in disulfide bond formation, we also investigated the production of native proinsulin at different pH values in the cultivation medium.

In all experiments *E. coli* RB791 was used as expression strain. Cells were cultivated at 25°C for 6 h after induction, and DsbA-proinsulin in the periplasmic extracts was digested with trypsin. SDS-PAGE and immunoblot analysis showed that L-arginine increased the amount of soluble periplasmic fusion protein 3–4 fold at a pH of ~6.5–7.0 in the medium (Fig. 2(A–B), lanes 2 and 4). The ratio of soluble to insoluble fusion protein was ~20:80% without additive and 60:40% with 0.4 M L-arginine. The maximum yield of proinsulin was 8.9–9.2 mg g^{-1} when 0.3 or 0.4 M L-arginine were added to the cultures

(Fig. 4). Although both concentrations of L-arginine increased the cell-specific yield of proinsulin to the same extent, the concentration of 0.4 M had a more negative effect on cell growth (see Table 2). Concentrations above 0.4 M L-arginine resulted in complete growth inhibition (data not shown). Interestingly, the amount of soluble and correctly folded DsbA-proinsulin produced in *E. coli* did not depend on the pH of the medium in a range of pH 5–7. At pH 8–9, the growth of the cells was strongly inhibited and hardly any native proinsulin was detectable in the corresponding samples.

3.5. Optimisation of DsbA-proinsulin expression in mineral salt medium

In order to analyse the influence of the medium composition on the yield of native fusion protein, cultivations were also performed in mineral salt medium. Generally, the yields of native proinsulin were ~ 10-fold lower compared to growth in LB medium and nearly all fusion protein seemed to appear in inclusion bodies. Supplementation of the

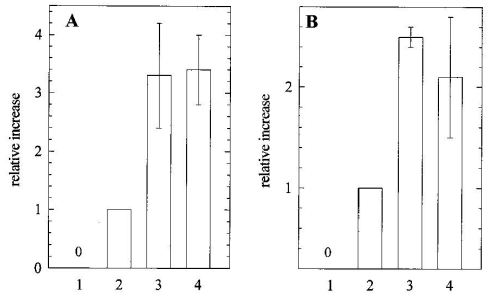

Fig. 4. Influence of medium additives on the yield of native DsbA-proinsulin produced in *E. coli* RB791 with plasmid pDsbA3-PI. Native insulin was measured by an insulin-ELISA after trypsin digestion with 100 μg trypsin per 100 μl periplasmic extract. Cells were cultivated in LB medium (panel A) or mineral salt medium (panel B). (A) supplementation of 0.3 or 0.4 M L-arginine (bars 3–4) and (B) supplementation with 1% ethanol (bar 3) or 3% ethanol and 10 g l^{-1} yeast extract (YE) (bar 4). The proinsulin content measured in cells grown in LB or mineral salt medium without supplement was set to 1 (bar 2) and the yield (as mg g^{-1}) from the cultivations in the corresponding medium was related to that. As a control, *E. coli* RB791 transformed with a control plasmid lacking the proinsulin gene was grown in LB medium and mineral salt medium, respectively (bar 1).

Table 2
Levels of the secreted native DsbA-proinsulin in periplasmic extracts of *E. coli* RB791 harboring pDsbA3-PI measured after growth in media with different supplements

Medium/supplement	Native proinsulin[a]	
	mg g^{-1}	mg l^{-1}
LB medium		
Without additives	2.7	2.5
0.3 M L-arginine	8.9	2.5
0.4 M L-arginine	9.2	1.1
Mineral salt medium		
Without additives	1.1	0.7
1% (v/v) ethanol	2.6	3.1
10 g l^{-1} yeast extract	1.5	1.6
3% (v/v) ethanol + 10 g l^{-1} yeast extract	1.6	1.8

[a] The amount of proinsulin was determined as described in Table 1. The results of one representative experiment are shown.

cultures with L-arginine, reduced glutathione, adjustment of the pH value and the combination of all parameters always resulted in lower yields compared to LB medium (data not shown). In contrast, the addition of yeast extract (5–20 g l^{-1}) and/or ethanol (1–5% v/v) to the mineral medium improved the yield of native fusion protein 2–3 fold to 2.6 mg g^{-1} (Table 2). These results are in accordance with investigations of Ben-bassat et al. (1984) who found that the addition of yeast extract to the growth medium improve the yield of soluble heterologous proteins produced in *E. coli*. In our case, the amount of insoluble DsbA-proinsulin was similar under both conditions (cultivation ± ethanol), but the ratio of soluble protein to total fusion protein was significant improved by the addition of ethanol (Fig. 2(A–B), lanes 8 and 10). Additionally, yeast extract or ethanol stimulated the growth of the bacteria leading to a higher final cell density, and thus to a higher amount of proinsulin per ml cultivation broth. Using this approach, the maximal yield of native proinsulin within the fusion protein in cultures with 1% (v/v) ethanol was 3.1 mg l^{-1}. This was the highest amount obtained in the whole optimisation series in mineral salt medium, although the specific yield in this case was only 2.6 mg g^{-1} (Table 2).

4. Discussion

We studied the secretory expression of a dsbA-proinsulin fusion construct in *E. coli* in dependence on medium additives and cultivation conditions. The yield of native proinsulin obtained from the DsbA-proinsulin was 1000-fold higher than the secretory expression yield described for isolated proinsulin so far (Chan et al., 1981). This indicates that DsbA is a very effective fusion partner for obtaining high amounts of soluble and correctly disulfide bridged proinsulin. Similarly, the fusion of the enterokinase gene to dsbA and its secretory expression resulted also in an increased yield of soluble protein in the periplasm of *E. coli* (Collins-Racie et al., 1995). A different approach to produce high amounts of proinsulin or modified proinsulin from a protein fusion has been described by Kang and Yoon (1994). The production of a ZZ-proinsulin fusion protein resulted in 0.8 mg l^{-1} proinsulin, which is four times less compared to our approach. However, for a modified ZZ-proinsulin the yield was approximately five times higher then in our case. Although this modified fusion protein was not completely secreted to the periplasm the authors showed that the secretion efficiency of the shortened protein was significantly increased compared to the full length protein. In our case the fusion protein was completely secreted to the periplasm. Possibly, the yield of DsbA-proinsulin could be further improved if the C-peptide is modified as described by Kang and Yoon.

The use of different medium additives during growth of *E. coli* expressing the dsbA-proinsulin gene resulted in an up to four-fold increase of the amount of native proinsulin. Much higher yields were achieved using LB medium as compared to mineral salt medium, indicating that the effect of supplements on the amount of native fusion protein depends also on many other components in the medium. LB medium is a rich medium which has the advantage that the cells show a higher growth rate than cells growing in mineral salt medium. On the other hand, mineral salt medium is a defined medium, which is commonly used in high cell density cultivations. For LB medium, only the addition of L-arginine increased the amount of soluble fusion protein with native proinsulin part. In case of mineral salt medium, only ethanol or yeast extract had a stimulating effect on the yield of native proinsulin. Since L-arginine is known to be an additive which increases the solubility of proteins and stimulates the refolding of proteins in vitro, we investigated the ability of L-arginine in the medium to increase the yield of soluble DsbA-proinsulin which is properly folded and contains the correct disulfide bond pattern. Cultivation in LB medium in the presence of 0.4 M L-arginine increased the cell specific yield of native proinsulin ~ 3.4 fold. Although this is a clearly positive effect, the results are low in comparison to in vitro studies on the refolding of other model proteins from inclusion bodies (De Bernardez Clark et al., 1999). Unexpectedly, the addition of glutathione had a negative effect on the yield of native proinsulin, although it had a positive effect in vivo and in vitro on the yield of BPTI, RBI, and alkaline phosphatase (Zapun and Creighton, 1994; Wunderlich and Glockshuber, 1993; Akiyama et al., 1992). This might indicate that formation of correct disulfide bonds is not the main problem upon formation of native proinsulin in the fusion protein. Surprisingly, the supplementation of the medium with yeast extract and ethanol had a positive effect on the amount of native proinsulin as well as on the cell growth in mineral salt medium. The amount of native proinsulin per liter cultivation broth was 2.5–5 times increased by yeast extract and ethanol, respectively. Ben-bassat et al. (1984) reported that the addition of yeast extract to cultures increases the production of soluble heterologous proteins in *E. coli*. Ethanol is a trigger of the heat shock response in *E. coli* and induces stress response proteins including molecular chaperones, such as both the DnaK–DnaJ–GrpE and GroEL–GroES chaperone machinery. It has been suggested earlier that the positive effect of ethanol on the expression of recombinant genes in the cytoplasmic space is connected to its stimulating effect on the heat shock response (Kusano et al., 1999; Van Dyk et al., 1995). However, ethanol may also stimulate the formation of native proinsulin in the periplasma in an analogous way as shown for the in vitro folding of IGF-I (Hejnaes et al., 1992).

In conclusion, our data indicate that the yield of native human proinsulin produced in *E. coli* can be significantly increased by fusion of the proinsulin gene to dsbA and by supplementation of the culture with medium additives. We could show that L-arginine, ethanol, and yeast extract are substances which can increase the yield of soluble fusion protein with a native proinsulin part. A great advantage of the fusion system is that the proinsulin part can be cleaved off from the DsbA part by trypsin which is commonly used for conversion of proinsulin into insulin, the therapeutically active form of this protein. By this proteolytic conversion we could directly show that the proinsulin part of the fusion protein was correctly folded.

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Cosecretion of Chaperones and Low-Molecular-Size Medium Additives Increases the Yield of Recombinant Disulfide-Bridged Proteins

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Attempts were made to engineer the periplasm of *Escherichia coli* to an expression compartment of heterologous proteins in their native conformation. As a first approach the low-molecular-size additive L-arginine and the redox compound glutathione (GSH) were added to the culture medium. Addition of 0.4 M L-arginine and 5 mM reduced GSH increased the yield of a native tissue-type plasminogen activator variant (rPA), consisting of the kringle-2 and the protease domain, and a single-chain antibody fragment (scFv) up to 10- and 37-fold, respectively. A variety of other medium additives also had positive effects on the yield of rPA. In a second set of experiments, the effects of cosecreted ATP-independent molecular chaperones on the yields of native therapeutic proteins were investigated. At optimized conditions, cosecretion of *E. coli* DnaJ or murine Hsp25 increased the yield of native rPA by a factor of 170 and 125, respectively. Cosecretion of DnaJ also dramatically increased the amount of a second model protein, native proinsulin, in the periplasm. The results of this study are anticipated to initiate a series of new approaches to increase the yields of native, disulfide-bridged, recombinant proteins in the periplasm of *E. coli*.

Most therapeutically relevant proteins contain disulfide bridges and cannot be produced in their native conformation in the bacterial cytosol. In vitro refolding of inclusion body material is often laborious and costly. An alternative strategy to obtain these proteins in their native forms is to use their secretion into the periplasmic space. Targeting of proteins to the periplasm has both advantages and disadvantages. A major drawback of the periplasm is that space is limited. Thus, yields of recombinant proteins generally never match those obtained upon cytosolic expression. Also, translocation into the periplasmic space can limit the final yields of recombinant proteins. However, in the case of those proteins that bear multiple disulfide bonds of nonlinear connectivities in their native conformations and that are resilient to renaturation of inclusion body material, expression in the periplasmic space may offer the method of choice. The periplasm is a compartment where oxidation of thiols can occur due to the activity of the disulfide oxidoreductase (Dsb) system (for a review, see reference 28). The overall milieu of the periplasm is strongly oxidizing, with the DsbA protein being the major oxidant. However, Dsb components with disulfide isomerase functions, DsbC and DsbG, have also been described (5, 40). Still, presumably disulfide bond isomerization is insufficient in the periplasm, given that recombinant proteins that carry multiple disulfide bonds in their native conformations have a pronounced tendency to aggregate. Considering this major drawback of the expression of disulfide-containing proteins, the following strategies were devised to optimize folding in the periplasm: (i) modification of the medium composition by the addition of low-molecular-size compounds known to stimulate folding in vitro, (ii) addition of a redox component to allow reshuffling of wrongly formed disulfide bridges, and (iii) cosecretion of ATP-independent chaperones.

As model proteins, a truncated version of tissue-type plasminogen activator, consisting of the kringle-2 and the protease domain (BM 06.022, also known as rPA [23]), proinsulin, and a single-chain antibody fragment were chosen.

Our objective was to improve the yield of native rPA in the periplasm of *Escherichia coli*. A beneficial effect was observed upon the addition of low-molecular-size folding enhancers and reduced glutathione (GSH) and also upon cosecretion of either DnaJ or Hsp25. The general applicability of an optimized periplasmic expression compartment was confirmed with the two additional model proteins.

MATERIALS AND METHODS

Genetic and protein analytic techniques. Cloning, transformation of *E. coli* cells, and DNA preparations were done by standard techniques (1). Oligonucleotides were purchased from Gibco BRL or MWG Biotech AG. Restriction enzymes were obtained from Roche Molecular Biochemicals GmbH, AGS GmbH, or New England Biolabs. Sequences of recloned DNA fragments were routinely confirmed by dideoxy sequencing (LiCor DNA-Sequencer 4000; LiCor, Lincoln, Nebr.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were carried out as described in reference 8.

Strains, plasmids, proteins, and chemicals. *E. coli* strain BL21(DE3) was obtained from Novagen and used for gene expression; *E. coli* N4830/pPL-dnaJ-23 as a DnaJ overexpression strain was kindly provided by Thomas Langer (University of Munich, Munich, Germany).

Plasmids of the pIN III ompA (16) series were kindly provided by Masayori Inouye (University of Medicine and Dentistry of New Jersey), pMC111 M1 as a source for *hsp25* DNA was provided by Matthias Gaestel (University of Halle-Wittenberg), pA27fd7 (23) as a source for the rPA gene and rPA standard was provided by Ulrich Kohnert (Roche Diagnostics), pUBS520 (6) was provided by Ulrich Brinkmann (Epidauros Biotechnology, Bernried, Germany), and pHEN-scFv-ox (12), containing a *pelB* signal sequence and a *lac* promoter as a source for the scFv-ox gene, was provided by Ulrike Fiedler (Scil Proteins). Plasmid pCANTAB5-TSH, a secretion construct for a single-chain Fv fragment against

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thyroid-stimulating hormone (scFv-TSH) containing a gene *3* signal sequence and the *lac* promoter, was provided by Alfred Engel (Roche Diagnostics). scFv-TSH is directed against the thyroid stimulating hormone.

Antibody for insulin was a gift of Konrad Kürzinger (Roche Diagnostics), and Hsp25 and DnaJ antibodies were kindly provided by Johannes Buchner (University of Munich, Munich, Germany) and Maciej Zylicz (University of Gdansk, Gdansk, Poland), respectively.

Chemicals were of analytical grade and purchased from Sigma, Roth GmbH, AppliChem GmbH, Biomol GmbH, Fluka, or ICN Pharmaceuticals. Cultivation medium substances were obtained from Becton Dickinson. Other substances and kits were bought from the suppliers as stated below.

Construction of expression plasmids. For cloning into pET20b(+) (Novagen), the coding sequence of rPA was PCR amplified from pA27fd7 (23) and inserted into pET20b(+). In this construct the second amino acid of rPA (Ser) is replaced by Ala. Proinsulin-encoding DNA was amplified by PCR from plasmid pRK-5-proinsulin (34) and ligated into pET20b(+). This vector mediates secretion via the *pelB* signal sequence. By QuikChange Mutagenesis (Stratagene), two surplus codons between signal sequence and proinsulin were removed. For coexpression of chaperones and model proteins, a two-plasmid expression system was chosen. After testing secretion of DnaJ and Hsp25, the genes were PCR cloned into pIN III ompA3 (16) and the coding sequences of DnaJ and Hsp25 with the regulatory sequences were recloned into plasmid pUBS520 (6), which bears the p15A replication origin and kanamycin resistance. This vector also carries the *dnaY* gene encoding the tRNA for the arginine codons AGA and AGG, which are rare in *E. coli* and thus often limit expression of genes with these codons (the gene for rPA contains seven of these rare arginine codons). The two-plasmid cosecretion system thus includes a vector for the secretion of the disulfide-bridged model protein on the ColE1-based pET vector and the chaperone on a p15A-based plasmid, which also carries the *dnaY* gene. Both the gene for the model protein and the chaperone were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Via PCR the coding and regulatory regions for scFv-ox were amplified from pHEN-scFv-ox (12) and cloned into pUBS520.

Cultivation of *E. coli* BL21(DE3) and rPA assay. In order to test rPA activity, cells were grown in Luria-Bertani medium at 24°C, induced with 1 mM IPTG at mid-log phase, and cultivated for a further 21 h. Medium additives (reduced GSH, 0 to 10 mM, and L-arginine, 0 to 0.4 M; formamide, 0 to 1 M; methylformamide, 0 to 1 M; acetamide, 0 to 1 M; methylurea, 0 to 1 M; or ethylurea, 0 to 1 M) were supplemented at the time of induction. After determination of the optical density at 600 nm (OD_{600}) (Pharmacia Ultrospec 3000; Pharmacia Biotech), 2-ml samples were collected and pelleted. For preparation of periplasmic extracts, the protocol described in reference 18 was downscaled to milliliter volumes. The soluble periplasmic fraction was assayed for rPA activity. For control purposes, cultures of *E. coli* BL21(DE3), transformed with pET20b(+) and pUBS520, were treated identically. Determination of functional rPA on microplates was performed according to a modified previously described protocol (38) with purified rPA as a standard. The concentration of rPA in the cellular extracts was determined by plotting the extinction against the square of the reaction time. The slope of a linear regression of this plot is directly proportional to the amount of rPA in the assay. The native state of rPA in extracts was tested in parallel assays after addition of 20 μl of 0.6-mg/ml fibrinogen fragments. The slope of the plot after addition of fibrinogen fragments divided by the slope in the absence of fibrinogen fragments defines the stimulation factor (23).

To obtain quantitative values of the influence of cellular components on the activity of rPA, purified rPA was diluted into periplasmic extracts of *E. coli* BL21(DE3)/pET20b(+)/pUBS520. The measured quenching of rPA activity (1.5-fold) was used as a correction factor for determinations of rPA activities. All determinations of rPA concentrations in the cellular extracts were normalized to 1 ml of cells at an OD_{600} of 1. Concentrations of L-arginine and glutathione in the cultivation medium were determined with diluted medium sample assays according to the methods described in references 13 and 17, respectively.

Expression studies and determination of scFv-TSH. *E. coli* BL21(DE3) transformed with pCANTAB5-TSH and pUBS520 was cultivated as described above in the presence of the indicated concentrations of reduced glutathione and L-arginine. Expression of scFv-TSH was determined via indirect enzyme-linked immunosorbent assay (ELISA) measurements (8) and detected using the ImmunoPure TMB substrate system (Pierce, Rockford, Ill.). The values of cell extracts without scFv-TSH were used for correction of background signal. scFv-TSH purified with the RPAS system (Amersham Pharmacia Biotech) was used as a standard.

Limited proteolysis of periplasmic DnaJ. *E. coli* XL1-blue cells, transformed with pIN III ompA3-dnaJ, secreting DnaJ, and N4830/pPL-dnaJ-23 cells, overexpressing DnaJ in the cytosol, were grown to mid-log phase and harvested 3 h after induction by centrifugation. The equivalent of 2 ml of bacteria of an OD_{600} of 1 were converted to spheroplasts according to the method described in reference 37. The spheroplasts were resuspended in 30 μl of 50 mM Tris-HCl (pH 8.0)–100 mM NaCl with or without 0.1% Triton X-100. For limited proteolysis, aliquots of these fractions were incubated with 25 μg of trypsin per ml. Proteolysis was stopped by the addition of 20 M excess soybean trypsin inhibitor. In a control experiment, 0.1 mg of purified DnaJ per ml was incubated with 6 μg of trypsin per ml and treated in the same way as the spheroplast samples. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed via Western blotting.

Cosecretion of proinsulin and DnaJ. *E. coli* BL21(DE3) cells harboring plasmids for cosecretion of proinsulin and chaperones were grown in Luria-Bertani medium at 25°C. One millimolar IPTG was added at an OD_{500} of 1, and cells were harvested 6 h after induction. Soluble periplasmic protein was released by osmotic shock according to the method described in reference 22. For analysis and quantification of native proinsulin, an ELISA that specifically detects native (pro)insulin (Enzymun Test Insulin; Roche Diagnostics) was carried out.

RESULTS

Yields of secreted rPA and scFv-TSH in the presence of medium additives. Tissue-type plasminogen activator (tPA) converts the zymogen plasminogen to plasmin, a serine protease that degrades fibrin networks in thrombi (9). The tPA variant rPA contains nine disulfide bridges and aggregates upon cytosolic synthesis in inclusion bodies. In vitro refolding of rPA from inclusion body material is routinely performed (A. Stern, U. Kohnert, R. Rudolph, S. Fischer, and U. Martin, June 1993, U.S. patent application 5,223,256). As the native state of rPA can easily be assessed, it was chosen as a model protein for expression in the native conformation in the periplasm. For secretion of rPA, plasmid vector pET20b(+) (Novagen), containing the signal sequence of PelB (pectate lyase from *Erwinia carotovora)*, was used. To determine the amount of functional rPA, protease activity was assayed according to the method described in reference 38 with minor modifications (see Materials and Methods).

The characteristic feature of rPA—the stimulation of the protease activity by fibrinogen fragments (23)—was used as an indication of the native state of the two-domain protein. rPA with correctly folded kringle and protease domains possessed proteolytic activity which could be stimulated by a factor of ca. 25 to 35 by fibrinogen fragments (23; Stern et al., October 1992, U.S. patent application 5,223,256). We first verified that stimulation by fibrinogen fragments was not affected when purified rPA was incubated with periplasmic extracts (data not shown), a prerequisite for testing native expression of the protease in the periplasm.

Periplasmic extracts were prepared from cells secreting rPA and control cells. Extracts from the control culture showed only low background protease activity which was not affected by fibrinogen fragments (data not shown). In the strain secreting rPA, 0.023 ng of active rPA per ml was determined in periplasmic extracts. As the activity could be stimulated 35-fold by fibrinogen fragments, rPA was assumed to be responsible for proteolytic activity.

The nine disulfide bridges of rPA are essential for the native conformation and consequently the activity of the protease. To facilitate reshuffling of incorrect disulfide bonds, GSH was added to the culture medium (39; R. Glockshuber, M. Wunderlich, A. Skerra, and R. Rudolph European patent application EPO 510 658) (Fig. 1A). The addition of 5 or 10 mM GSH resulted in a slight increase of protease activity. These results indicate that

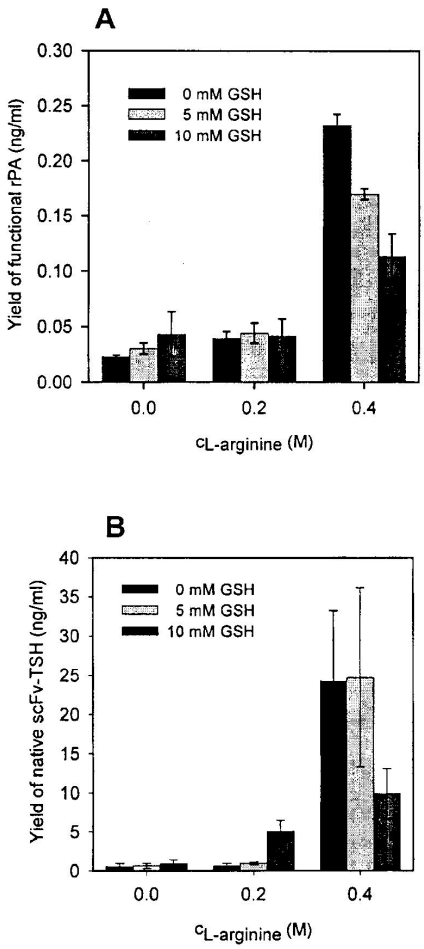

FIG. 1. Increases of the yields of secreted rPA and scFv-TSH upon addition of L-arginine to the cultivation medium. (A) Yields of native rPA in the periplasm of *E. coli* BL21(DE3)/pET20b(+)-rPA after cultivation (24°C) in the presence of the indicated concentrations of reduced GSH and L-arginine. Active rPA was determined as described previously (38). (B) Yields of native single-chain Fv (scFv-TSH) in the periplasm of *E. coli* BL21(DE3)/pCANTAB5-TSH after cultivation (24°C) in the presence of the indicated concentrations of L-arginine and GSH. Native scFv-TSH was determined using ELISA measurements. Mean values of at least three shake flask cultures and standard deviations are indicated.

disulfide shuffling is enhanced when reducing reagents are added to the culture medium.

L-Arginine is known to effectively improve the yield of native protein during in vitro refolding from inclusion body material (10, 25, 30). Thus, the in vivo effect of L-arginine on the yield of secreted native rPA was investigated. In the absence of GSH and at a concentration of 0.4 M L-arginine, the yield of active plasminogen activator increased about 10-fold (Fig. 1A). Interestingly, in the presence of L-arginine, the addition of GSH had no beneficial effect on the yield of rPA.

The yield of a second secreted model protein, a scFv-TSH (21), was also increased by the presence of L-arginine and reduced GSH. Addition of 0.4 M L-arginine led to the highest yield of native scFv-TSH (Fig. 1B), a 37-fold increase over the control expression. Though absolute yields with 25 ng/ml appear moderate, the results show that L-arginine is a compound that can be used to optimize folding of secreted proteins. A portion of the secreted scFv-TSH was detected in the medium supernatant, and the addition of 0.4 M L-arginine moderately increased the yield of scFv in the supernatant (data not shown). Concentrations of L-arginine higher than 0.4 M inhibited bacterial growth almost completely and led to reduced yields of scFv-TSH and rPA (data not shown). Taken together, these results demonstrate that in vivo structure formation of the two tested model proteins was significantly stimulated by the addition to the growth medium of L-arginine and, to a lesser extent, reduced GSH.

To determine whether GSH or L-arginine would be stably maintained during cell growth, the concentrations of GSH and L-arginine were determined by enzymatic analysis after extended culturing. Concentrations of L-arginine and total GSH in the culture medium remained constant during the entire culture process (20 h; Fig. 2A and B). However, the ratio of reduced GSH to oxidized GSH changed dramatically over 20 h at 24°C. During the first 5 h of cultivation almost all GSH was maintained in the reduced state. This ratio shifted to ca. 20% reduced GSH and 80% oxidized GSH after 20 h of cultivation, due to air oxidation of the thiol groups (Fig. 2B). These data confirm that a disulfide-shuffling system consisting of reduced and oxidized GSH can be maintained for 20 h during fermentation of the *E. coli* cells under aerobic conditions.

Construction of a two-plasmid system for cosecretion of DnaJ and Hsp25. In order to further increase the yield of secreted proteins, cosecretion of ATP-independent chaperones was tested. In a first experiment, the cosecretion of DnaJ was analyzed. This protein belongs to the Hsp70 (DnaK) system of *E. coli* and is known to suppress aggregation of nonnative proteins also in the absence of Hsp70 (7, 24, 31).

DnaJ, secreted by fusion to the OmpA signal peptide, was detected in the membrane fraction of periplasmic proteins (data not shown). This was expected, as DnaJ is known to associate with membranes (2). To confirm the native conformation of secreted DnaJ, limited proteolysis experiments were performed. Spheroplasts of *E. coli* XL1-blue/pIN III-dnaJ, secreting the chaperone, and N4830/pPL-dnaJ-23 (41), a control strain which overexpresses DnaJ in the cytoplasm, were prepared (37). Both spheroplast preparations were subjected to limited proteolysis with trypsin (Fig. 3). In intact spheroplasts, intracellular DnaJ of strain N4830 was completely protected from trypsin digestion, whereas secreted DnaJ, expressed in strain BL21(DE3)/pIN III-dnaJ, was susceptible to proteolysis (Fig. 3). The defined products of partial trypsinolysis were similar in size to those obtained by digestion of purified native DnaJ, a fact that suggests the native conformation of secreted DnaJ.

The effect of a second cosecreted chaperone, murine Hsp25 (14, 19), on the yield of recombinant proteins in the periplasm was investigated. Like DnaJ, Hsp25 has been demonstrated to prevent aggregation of nonnative proteins (11). Translocation of Hsp25 into the periplasm was also mediated by the OmpA

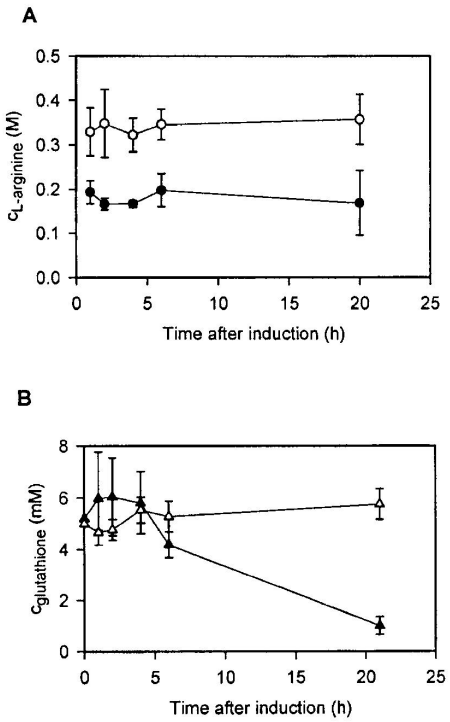

FIG. 2. Determination of the concentrations of L-arginine and GSH in the medium of *E. coli* BL21(DE3) after prolonged cultivation according to the methods described in references 13 and 17. (A) Determined L-arginine concentrations in the cultivation medium at the indicated time points after induction. At the time of induction L-arginine was added to the culture medium to final concentrations of either 0.2 M (filled circles) or 0.4 M (open circles). (B) Concentrations of GSH (closed triangles) and total GSH (GSH+GSSG; open triangles) in the cultivation medium after addition of 5 mM GSH.

signal peptide. Expression and secretion of Hsp25 were confirmed by Western blotting experiments (data not shown).

Yields of native rPA and proinsulin in the periplasm of *E. coli* upon cosecretion of DnaJ and Hsp25. Cosecretion of DnaJ yielded a fivefold increase of functional rPA in periplasmic extracts compared to what was observed with the clone without cosecretion. Upon addition of fibrinogen fragments, protease activity was stimulated 35-fold, indicating the native conformation of the secreted rPA. Under optimal expression conditions (0.4 M L-arginine and 5 mM GSH), the yield increased 170-fold (Table 1).

Similarly, cosecretion of Hsp25 increased the yield of native rPA in the periplasm ca. twofold. Under optimal expression conditions, i.e., 5 mM GSH and 0.4 M L-arginine (optimization data not shown), cosecretion of Hsp25 resulted in a 120-fold increase of active plasminogen activator (Table 1 and Fig. 4) compared to what was observed for the strain which did not secrete Hsp25 cultivated in the absence of medium additives.

The fact that both DnaJ and Hsp25 enhanced the yield of native rPA could be due either to the chaperone activities of these proteins or to indirect effects caused by the secretion of a second heterologous protein to the periplasmic space. To test the latter possibility, the effect of cosecretion of scFv-ox (12), a protein which lacks chaperone function, on the yield of native rPA was investigated. Under optimal conditions, cosecretion of scFv-ox yielded a fourfold increase of native rPA compared to the situation when rPA was expressed alone. However, the very low stimulation factor of 10 (Table 1) indicated incomplete folding of rPA. Thus, the huge increase of native rPA upon cosecretion of chaperones is very likely caused by the chaperoning activities of these proteins. Western blot experiments confirmed that the levels of DnaJ and Hsp25 remained constant at the concentrations of L-arginine and GSH tested here. In contrast, increases in scFv-ox levels were observed with increasing concentrations of L-arginine (data not shown).

As a second model protein for testing the effects of cosecreted chaperones, proinsulin was secreted to the periplasm.

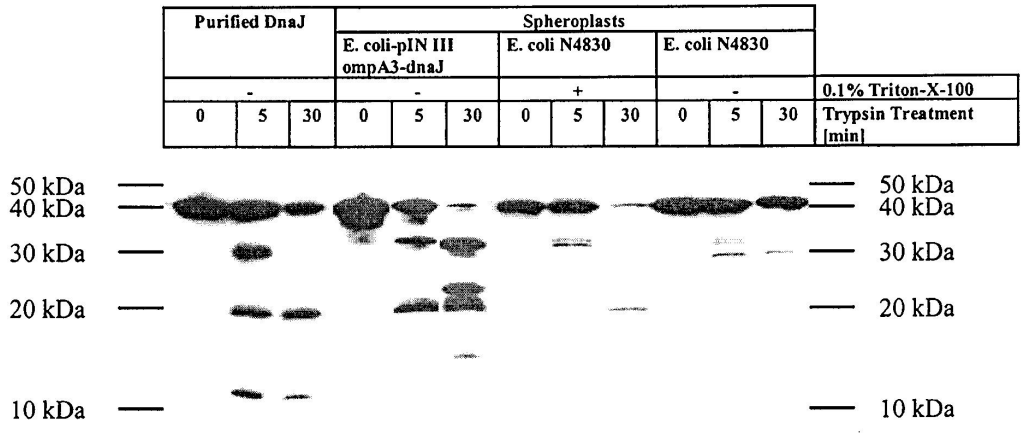

FIG. 3. Limited proteolysis for determination of native DnaJ. Spheroplasts were incubated with 25 μg of trypsin per ml and purified DnaJ was incubated with 6 μg of trypsin per ml for the indicated times. Proteolysis products of DnaJ and its fragments were detected with a rabbit anti-DnaJ antibody and subsequently with a donkey anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Amersham Pharmacia Biotech).

TABLE 1. Influences of cosecreted proteins and medium additive L-arginine on yield of native rPA[a]

Cosecreted protein	Without L-arginine			With 0.2 M L-arginine			With 0.4 M L-arginine		
	rPA[b] (ng/ml)	Stimulation factor[c]	OD_{600}[d]	rPA (ng/ml)	Stimulation factor	OD_{600}	rPA (ng/ml)	Stimulation factor	OD_{600}
None	0.030 ± 0.001	29	4.85	0.044 ± 0.090	20	5.05	0.170 ± 0.005	23	3.47
DnaJ	0.197 ± 0.019	29	4.31	0.730 ± 0.150	27	5.05	3.978 ± 1.000	18	2.12
Hsp25	0.053 ± 0.002	27	4.81	0.140 ± 0.001	17	4.56	2.850 ± 0.214	17	1.44
scFv-ox	0.041 ± 0.003	13	4.23	0.144 ± 0.047	8	3.76	0.713 ± 0.113	10	1.47

[a] Cultivations were carried out in the presence of 5 mM GSH; with control cells (no medium additive, no cosecretion), 0.023 ng of active rPA per ml was obtained.
[b] Values for rPA are means ± standard deviations for at least three shake flask culture experiments.
[c] Stimulation factor, stimulation of rPA activity by addition of fibrinogen fragments to the rPA assay (see Materials and Methods); stimulation factors of 25 to 35 are generally considered to indicate native rPA (23; Stern et al., U.S. patent application 5,223,256).
[d] OD_{600} values were determined at the time of harvest.

The amounts of native proinsulin in periplasmic fractions were assayed by ELISA using an antibody recognizing selectively native insulin. In the absence of cosecreted chaperones, 2 ng of native proinsulin per ml was detected (Fig. 5). When Hsp25 was cosecreted with proinsulin, no native proinsulin was detectable in the periplasm. In contrast, coexpression of DnaJ resulted in 74 ng of native proinsulin per ml, corresponding to a 37-fold increase of the yield. Upon cosecretion of the negative control, scFv-ox, only 0.3 ng of native proinsulin per ml was detected. Surprisingly, in this case, the presence of 0.4 M L-arginine decreased the amount of native proinsulin to 50% of that of cultivations in the absence of L-arginine (data not shown). With the third model protein, scFv-TSH, cosecretion of DnaJ or Hsp25 did not increase the yield of native scFv-TSH in the periplasm.

Influence of low-molecular-size additives on the yield of secreted rPA. In in vitro refolding experiments, several low-molecular-size additives, especially derivatives of formamide or urea, proved useful for increasing the yield of native rPA (29). We therefore examined the effects of formamide, methylformamide, acetamide, methylurea, and ethylurea on the yield of native rPA. Bacteria were able to grow in media which contained concentrations of up to 1 M formamide or acetamide but only up to 0.6 M methylformamide, methylurea, or ethylurea. The yield of native rPA was tested with the strain *E. coli* BL21(DE3)/pUBS520-dnaJ/pET20b(+)-rPA cosecreting DnaJ upon cultivation in the presence of these additives and 5 mM GSH. Although L-arginine, which was used for comparison, proved to be the most effective additive, acetamide or ethylurea also had significant beneficial effects on the yield of rPA (Fig. 6).

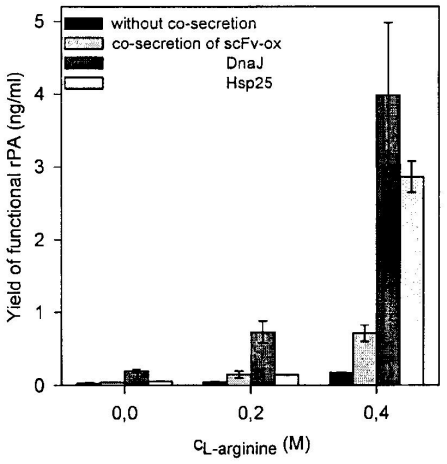

FIG. 4. Effects of cosecreted chaperones and scFv-ox (control) on the yields of native rPA at different concentrations of L-arginine. Cells were grown in the presence of 5 mM GSH. Active rPA was determined as described previously (38). Mean values of at least three shake flask culture experiments and standard deviations are indicated.

DISCUSSION

Native expression of disulfide-bridged proteins in prokaryotic host cells remains a scientific challenge (32). Though approaches have been taken to change the cytosolic milieu of *E. coli* to more oxidizing conditions to allow intracellular formation of disulfide bonds (4, 35), expression of disulfide-bridged proteins in the periplasmic space is an alternative strategy that has not been fully exploited. A major disadvantage of the periplasm as a folding compartment for proteins with multiple disulfide bonds is the strong oxidant DsbA. DsbA has been shown to introduce disulfide bonds into translocating polypeptides as soon as two cysteines have emerged into the periplasm (20, 33). Although disulfide isomerases exist in the periplasm, their function is obviously insufficient to correct

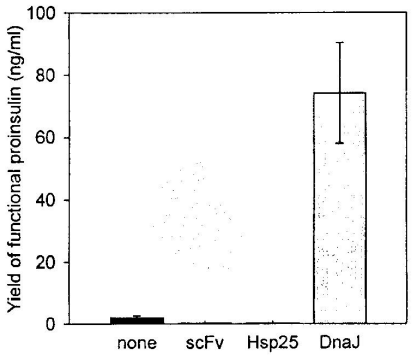

FIG. 5. Yields of proinsulin after cosecretion of DnaJ, Hsp25, and scFv-ox (control). Proinsulin was determined by ELISA (see Materials and Methods). Values represent mean values of at least three shake flask culture experiments. Standard deviations are indicated.

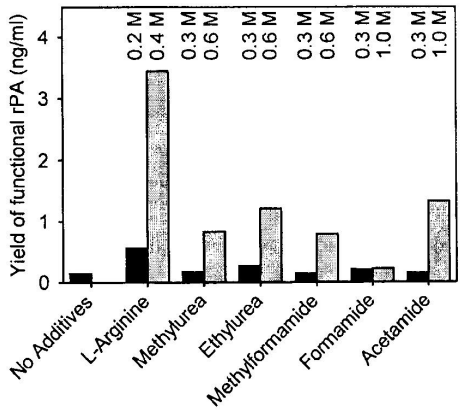

FIG. 6. Yields of native rPA in *E. coli* secreting DnaJ in the presence of 5 mM GSH and low-molecular-size additives at the indicated concentrations. Active rPA was determined as described previously (38).

wrongly paired cysteines of proteins containing multiple disulfide bonds of nonlinear connectivities (3, 26). The consequence is usually inclusion body formation of these misfolded proteins in the periplasm (15).

Our approach to overcome these problems was to suppress inclusion body formation in the periplasm by adding disulfide-reshuffling reagents and substances known to stabilize folding intermediates to the cultivation medium. Also, the effects of cosecreted ATP-independent molecular chaperones DnaJ and Hsp25, which have been shown to suppress aggregation of nonnative proteins in vitro (11, 31), were analyzed.

We were able to increase the yield of native rPA in the periplasm of *E. coli* up to 170-fold upon cosecretion of DnaJ and 125-fold upon cosecretion of Hsp25. This huge increase is, to our understanding, mainly due to a synergistic effect of the respective cosecreted chaperone and medium additives on the folding of rPA, as cosecretion of DnaJ or Hsp25 in the absence of medium additives gave rise to ca. fivefold or twofold increases, respectively, of rPA (Table 1). Improvement of the periplasm as an expression compartment for disulfide-bridged proteins has been reported earlier (27). For example, overexpression of DsbC considerably increased the yield of full-length tPA (27). Unfortunately, the yields of functional proteins published in reference 27 and those of our studies cannot be compared, as a variant of tPA has been used in the latter; furthermore, the data of the former study result from high cell density fermentations, whereas here shake flask cultures were used.

The fact that 5 mM GSH was optimal for the folding of rPA under almost all tested conditions confirms previous results that demonstrate that addition of GSH improved folding of an α-amylase-trypsin inhibitor in the periplasm (39).

With proinsulin, a 37-fold increase in the yield of native protein was obtained by cosecretion of DnaJ. Proinsulin secreted to the periplasm has been reported to be degraded by *E. coli* proteases (36). The presence of DnaJ may prevent the action of proteases and promote native structure formation. Cosecretion of Hsp25 or addition of L-arginine, however, did not improve the yield of native protein.

The increases of native rPA and proinsulin upon cosecretion of DnaJ and Hsp25 are likely to be due to the specific chaperoning activities of these proteins. Our interpretation that we are dealing with specific chaperone functions in cases where we observe increased amounts of folded proteins upon cosecretion of the chaperones is supported by the following observations. (i) If cosecretion of a heterologous protein should unspecifically enhance folding of rPA and proinsulin, cosecreted scFv-ox should have increased the yield. Furthermore, cosecretion of scFv-ox did not result in efficient stimulation of rPA activity by fibrinogen fragments by factors known for the completely folded protease. (ii) The chaperone requirement of a given protein is known to be relatively specific. In accordance with this notion, cosecretion of Hsp25 proved not to be effective in the case of proinsulin and neither DnaJ nor Hsp25 increased the yield of scFv-TSH. Thus, we propose that the beneficial effects of the secreted chaperones reflect the folding activities of DnaJ and Hsp25.

Besides L-arginine, a series of low-molecular-size reagents added to the cultivation medium increased the yield of active rPA. We limited our investigations to additives for which effects on the in vitro refolding of full-length tissue-type plasminogen activator had been demonstrated (29). Although L-arginine was the most effective compound in the case of rPA, other low-molecular-size additives may prove most efficient with different proteins. The effects of the tested additives on the yield of rPA are comparable to those obtained by in vitro refolding experiments (29). Therefore, we consider the effects to be due to the folding-enhancing activities of the compounds and not to their secondary osmolytic effects on cells.

Our results demonstrate that cosecretion of ATP-independent chaperones and the use of low-molecular-size medium additives to the culture medium can dramatically increase the yield of native eukaryotic proteins with complex disulfide patterns in the periplasm of *E. coli*. The mechanism by which the chaperones act in the periplasm remains unclear and needs further investigation. Still, this study may open new avenues for the production of disulfide-bridged proteins in their native conformation in prokaryotic organisms.

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Catalytic Activity and Chaperone Function of Human Protein-disulfide Isomerase Are Required for the Efficient Refolding of Proinsulin*

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Protein-disulfide isomerase (PDI) catalyzes the formation, rearrangement, and breakage of disulfide bonds and is capable of binding peptides and unfolded proteins in a chaperone-like manner. In this study we examined which of these functions are required to facilitate efficient refolding of denatured and reduced proinsulin. In our model system, PDI and also a PDI mutant having only one active site increased the rate of oxidative folding when present in catalytic amounts. PDI variants that are completely devoid of isomerase activity are not able to accelerate proinsulin folding, but can increase the yield of refolding, indicating that they act as a chaperone. Maximum refolding yields, however, are only achieved with wild-type PDI. Using genistein, an inhibitor for the peptide-binding site, the ability of PDI to prevent aggregation of folding proinsulin was significantly suppressed. The present results suggest that PDI is acting both as an isomerase and as a chaperone during folding and disulfide bond formation of proinsulin.

Protein-isulfide isomerase (PDI)[1] is a protein of the endoplasmic reticulum involved in the oxidative folding of many disulfide-bonded proteins (1–3). PDI consists of four domains arranged in the order **a-b-b′-a′** with an acidic extension at the C terminus (**c** domain) that contains the KDEL retention sequence. The first two domains show significant homology to thioredoxin (4, 5). Although there are no structural data available for the **b′** and **a′** domains, it can be infered from their sequence similarities to the **b** and **a** domain, respectively, that they also contain the thioredoxin fold. Both, the **a** and **a′** domains of PDI, contain the local sequence -WCGHCK- that is essential for the catalytic activity of PDI. Both isolated domains can operate as thiol:disulfide oxidoreductases, but all other PDI domains are required to assist protein refolding and formation of native disulfide bonds with maximum catalytic activity (6). No function has been assigned to the **b** domain so far, however, the **b′** domain provides the principle peptide-binding site (7). This domain alone is sufficient to bind peptides of 10–15 residues but binding of larger peptides or non-native proteins requires the contribution of either the **a** and **b** domains or the **a′** domain (7).

In vitro, PDI can assist folding of proteins that contain no disulfide bonds, demonstrating its function as a chaperone (8, 9). PDI can influence folding of proteins with multiple disulfide bonds as a chaperone and also as an isomerase (10–17). Yao *et al.* (11) proposed that PDI can fulfil both activities on the refolding of acidic phospholipase A_2. When using mitochondrial malate dehydrogenase (10) or rhodanese (8) as a substrate PDI influences refolding in a chaperone-like manner. PDI and also alkylated PDI displaying no isomerase activity can chaperone refolding of D-glyceraldehyde-3-phosphate dehydrogenase indicating that the active sites are not required for the chaperone activity of PDI (9, 18). The isomerase activity is involved in refolding of lysozyme (19), an antibody fragment (13), insulin (16), and proinsulin (17). Furthermore, under certain redox conditions PDI can reduce protein substrates with native disulfide bonds (13, 20). In addition, PDI can catalyze disulfide bond formation and rearrangements within kinetically trapped, structured folding intermediates (21). This demonstrates that the substrate specificity of PDI is not restricted to misfolded proteins containing no or non-native "scrambled" disulfide bonds.

It is possible to discriminate between isomerase and chaperone activity of PDI by using mutants or variants displaying only one of both activities. Active site mutants or alkylated PDI have been used to generate species that act as a chaperone only (11, 13). On the other hand mutants lacking chaperone activity do not exist as it is not clear which amino acid residues in the peptide-binding domain are involved and essential for substrate binding. Tsibris *et al.* (22) could show that isomerization of scrambled RNase and reduction of insulin are inhibited by estrogens which might be due to the similarity of PDI parts with estrogen receptor segments. Additionally, compounds with estrogenic activity can inhibit peptide binding to PDIp, a pancreas-specific member of the protein-disulfide isomerase family (23).

To dissect the different activities of PDI we used human proinsulin as model substrate. Human proinsulin consists of a single polypeptide which, after trypsin and carboxypeptidase B cleavage, can be converted to the biologically active insulin and the c-peptide *in vitro* (24). Proinsulin contains three disulfide bonds (1) Cys^{7}-Cys^{72}, (2) Cys^{19}-Cys^{85}, and (3) Cys^{71}-Cys^{76} which are essential for its native structure. In addition to the efficient spontaneous oxidative folding of reduced proinsulin at

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[1] The abbreviations used are: PDI, protein-disulfide isomerase; ELISA, enzyme-linked immunosorbent assay; GSH, reduced glutathione; GSSG, oxidized glutathione; iodoacetamide, iodoacetamide; RP-HPLC, reversed phase-high performance liquid chromatography; IGF-I, insulin-like growth factor I.

pH 10.5,[2] native proinsulin can also be generated at neutral pH starting from the scrambled molecule in the presence of PDI in a molar ratio PDI/substrate of 0.1 (17).

Here we investigated the mechanism of PDI function in proinsulin refolding. We show that PDI influences the refolding of denatured and reduced proinsulin both as an isomerase and as a chaperone. PDI in catalytic amounts is able to increase the refolding rate and the refolding yield while PDI variants devoid of isomerase activity influence the refolding yield only when present in stoichiometric amounts. The chaperone function is essential during the first seconds of refolding because aggregation of folding proinsulin is a major side reaction. Inhibition of the peptide-binding site of PDI leads to a suppression of the aggregation preventing role of PDI. Besides the chaperone function the isomerase activity is also required at the beginning of proinsulin folding, but the late refolding process does only depend on the isomerase activity.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant native human proinsulin was provided by BIOBRÁS, Montes Claros, Brazil. ELISA antibodies were obtained from Roche Molecular Biochemicals, Penzberg, Germany. The vectors pET23-PDI and pET23-PDI-b′a′c were received from Dr. L. W. Ruddock, University of Kent, Canterbury, UK. PDI and PDI variants were purified from *Escherichia coli* lysates as described below. Genistein and the homobifunctional cross-linking reagent disuccinimidyl glutarate were purchased from Sigma, iodoacetamide was obtained from ICN. X-ray films and Bolton-Hunter ^{125}I labeling reagent were obtained from Amersham Bioscience, Inc.

Unfolding and Refolding of Proinsulin—The human proinsulin, containing a N-terminal His_8-Arg-tag was subjected to reductive unfolding by adding 20 mg of the native proinsulin to 1 ml of 6 M guanidinium chloride, 10 mM Tris/HCl, pH 8.5, 1 mM EDTA, 1.1 M dithiothreitol. The sample was incubated for 8 h at 37 °C and then extensively dialyzed at 4 °C against 6 M guanidinium chloride, 5 mM EDTA, pH 3. For cross-linking, the denatured and reduced proinsulin was desalted to a final concentration of 2 M guanidinium chloride using a NAP-5 column (Amersham Bioscience, Inc.).

Refolding of proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C by diluting the unfolded and reduced proinsulin to a final concentration of 100 μg/ml, if not otherwise indicated. The sample was immediately mixed. At distinct time points aliquots were removed and acetonitrile (final concentration 20% (v/v)) and trifluoroacetic acid (final concentration 0.1% (v/v)) were added, if not otherwise indicated.

Refolding of proinsulin in the presence of genistein was performed with proinsulin at a final concentration of 27 μg/ml. Genistein (10 mM solution in Me_2SO) was added to a final concentration of 20 μM. Aliquots were removed and digested with trypsin and carboxypeptidase B (see below).

Quantification of Proinsulin Folding—Refolding of denatured and reduced proinsulin was monitored by reversed phase HPLC (RP-HPLC) using a C_{18} column (Macherey-Nagel) equilibrated with 100% solvent A (solvent A: 20% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid; solvent B: 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid). The protein was eluted at 20 °C with a flow rate of 0.5 ml/min of a linear gradient from 20 to 40% solvent B within 20 min. For samples containing genistein and for samples after digestion, the column was equilibrated with 10% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. The protein was eluted with a linear gradient from 10 to 38% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid with a flow rate of 0.5 ml/min within 40 min. Peaks were detected by absorbance at 214 nm and quantified according to a calibration curve.

Tryptic Digestion and ELISA—Refolding samples containing genistein were digested with trypsin and carboxypeptidase B (final concentration 1.35 μg/ml each) for 20 min at ambient temperature. Afterward acetonitrile and trifluoroacetic acid were added to a final concentration of 10 and 0.1% (v/v), respectively, and the samples were analyzed by RP-HPLC as described above.

To verify the native structure of the refolded proinsulin, a digestion was performed with native and refolded proinsulin at refolding conditions (100 μg/ml proinsulin). After incubation for 20 min at ambient temperature the digestion was stopped by addition of soybean trypsin inhibitor and EDTA. The final concentrations were 5 μg/ml trypsin, 5 μg/ml carboxypeptidase B, 25 μg/ml trypsin inhibitor, and 125 mM EDTA. Samples were analyzed by RP-HPLC as described above and by an insulin-ELISA as described previously (25).

Light Scattering—Light scattering due to aggregation was measured at 500 nm in a stirrable 2-ml cuvette using a fluorescence spectrometer (FLUOROMAX, Spec Instruments). For proinsulin concentrations of 10–100 μg/ml both excitation and emission slits were adjusted to 1 nm band width, for lower concentrations to 3 nm. The temperature of the refolding buffer (see above) was adjusted to 25 °C. The protein to be measured was added to the stirred refolding buffer and aggregation was recorded for 120 to 600 s. At the same conditions a calibration curve was generated for denatured and reduced proinsulin (from 0.2 to 10 μM). According to this calibration curve, the light scattering signal caused by the aggregation of proinsulin could be calculated to micrograms/ml aggregating proinsulin. Related to the amount of proinsulin used one can calculate the corresponding amount of proinsulin protected from aggregation.

Generation of PDI Variants—Point mutations to replace the codons for cysteine in the active sites of PDI by serine were generated using the vector pET23-PDI for the quick change procedure (Qiagen). The wild-type sequences 5′-TGTGGCCACTGC-3′ for the **a** domain (corresponding to -^{35}CGHC^{38}-) and 5′-TGTGGTCACTGC-3′ for the **a**′ domain (corresponding to -^{379}CGHC^{382}-) were changed to 5′-TCCGGTCACTCT-3′ encoding the amino acid sequence -SGHS-. The active site in either the **a** domain (PDIΔC1) or **a**′ domain (PDIΔC2) was mutated to generate a PDI-single mutant. The PDI-double mutant (PDIΔC1,2) was produced by replacing the cysteine codons of both active sites by serine. The correct sequence was verified by DNA sequencing.

Expression and Purification of PDI and PDI Variants—For the production of wild-type PDI, PDI-b′a′c, and the mutants PDIΔC1, PDIΔC2, and PDIΔC1,2, *E. coli* BL21(DE3)-pLysS was transformed with the corresponding plasmid-DNA. The cells were incubated at 37 or 30 °C on LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Three hours after induction with 1 mM isopropyl-thio-β-D-galactopyranosid, the cells were harvested by centrifugation. The cell pellet was suspended in buffer A (20 mM Na-phosphate, pH 7.3) and DNase was added to a final concentration of 10 μg/ml. After freezing and thawing the suspension twice, the lysate was centrifuged. After ultrafiltration (0.2 μm), the supernatant was loaded onto a Ni-NTA column (volume 12 ml, Qiagen), which, after activation with $NiSO_4$ and washing with double-distilled water, was equilibrated with buffer A. Proteins bound nonspecifically onto the matrix were removed by washing the column with buffer A containing 500 mM NaCl and 50 mM imidazole, followed by buffer A. Recombinant protein was eluted with buffer A containing 10 mM EDTA. The elution fraction was loaded onto a ResourceQ column (6 ml, Amersham Bioscience, Inc.) equilibrated with buffer A and eluted with a linear gradient from 0 to 0.5 M NaCl in buffer A. Fractions containing homogenous PDI or PDI variants were pooled and dialyzed against 1 mM Tris, 1 mM glycine, pH 7.5, 0.1 mM EDTA. The concentration of purified protein was determined by UV spectroscopy (molar absorbance coefficient 45,380 M^{-1} cm^{-1} (280 nm)). The molecular mass of the purified proteins was determined by mass spectrometry. PDIΔC1,2 was centrifuged (70,000 × *g*, 4 °C, 30 min) prior to use and afterward the correct concentration was determined by UV spectroscopy.

Alkylation of Cysteines—PDI was incubated with 5 mM dithiothreitol. After 20 min at 25 °C, iodoacetamide was added to a final concentration of 50 mM and the sample was incubated for 45 min at 25 °C. Excess dithiothreitol and iodoacetamide was removed by dialysis against 1 mM Tris, 1 mM glycine, pH 7.5, 0.1 mM EDTA and the alkylated PDI lyophilized and stored at −20 °C. For refolding experiments, lyophilized alkylated PDI was dissolved in double distilled water to a concentration of ~5 mg/ml. After centrifugation (70,000 × *g*, 4 °C, 30 min), the correct concentration was determined by UV spectroscopy.

Cross-linking—Bolton-Hunter ^{125}I labeling of denatured and reduced proinsulin (5 mg/ml, in 2 M guanidinium chloride, pH 3.0) was carried out as recommended by the manufacturer. Radiolabeled proinsulin was incubated with the *E. coli* cell extract that expressed PDI (10 μg/ml) on ice. After 10 min 0.1 volume of disuccinimidyl glutarate (5 mM in Me_2SO) was added and the sample incubated for additional 60 min. The reaction was stopped by the addition of SDS sample buffer (26). Proteins were separated on 12.5 or 15% polyacrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane and subsequently analyzed by autoradiography.

[2] J. Winter, unpublished results.

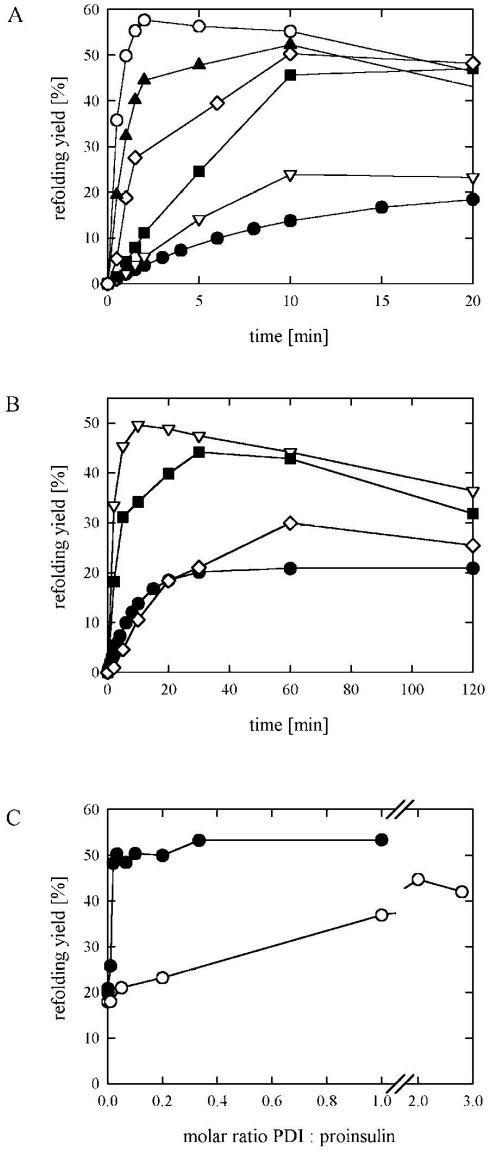

FIG. 1. **Influence of PDI on the kinetics and yield of proinsulin refolding.** Reactivation of denatured and reduced proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C. The final concentration was 10 μM for proinsulin and 70 mM for guanidinium chloride. At the times indicated an aliquot was withdrawn and acetonitrile (final concentration 20% (v/v)) and trifluoroacetic acid (final concentration 0.1% (v/v)) were added. The yield of refolded proinsulin was analyzed by RP-HPLC. *A*, influence of different concentrations of PDI on the kinetics. Refolding was performed in the absence of PDI (●), in the presence of PDI in a molar ratio (PDI/proinsulin) of 0.01 (▽), 0.033 (■), 0.1 (◇), 0.33 (▲), or 1 (○). The renaturation kinetic of spontaneous proinsulin folding (●) was fitted single exponentially with a rate constant of k_{app} = 0.0018 s^{-1}; *B*, influence of PDI variants on the kinetic of refolding. Refolding was carried out in the absence (●) or presence (▽) of PDI, or in the presence of the PDI mutants PDIΔC1 (■) and PDIΔC1,2 (◇). The final concentration of PDI and the PDI variants was 2 μM; *C*, influence of PDI and PDIΔC1,2 on the refolding yield. PDI (●) or PDIΔC1,2 (○) were added to the refolding proinsulin at the molar ratios indicated. Shown are the amounts of native proinsulin analyzed after the renaturation was completed (the samples were measured at least as duplicates).

RESULTS

Refolding of Proinsulin Is Catalyzed by PDI—PDI catalyzed the refolding of denatured and reduced proinsulin with two effects (Fig. 1*A*). First, PDI increased the rate of oxidative folding in a concentration-dependent manner. In the absence of PDI the apparent rate constant of oxidative refolding of proinsulin was k_{app} = 0.0018 s^{-1}. With equimolar amounts of PDI present the rate constant of folding was increased drastically to approximately k_{app} = 0.033 s^{-1}. Second, PDI enhanced the refolding yield (Fig. 1*C*). The yield reached a plateau of about 50–60% once PDI was present in a molar ratio of PDI/proinsulin of 0.033 and could not be increased further even in the presence of stoichiometric amounts of PDI. Under the same conditions bovine serum albumin did not affect proinsulin folding (data not shown). Refolding of proinsulin depended on the redox conditions; with 1 mM GSH and 2 mM GSSG the best yield of refolding was obtained for the spontaneous and the catalyzed reaction (data not shown).

A slow decrease in native proinsulin was observed after folding in the presence of high PDI concentrations (Fig. 1*A*). To analyze this effect native proinsulin was incubated under the same conditions in the absence and presence of PDI (data not shown). The amount of native proinsulin was unchanged under all conditions indicating that aggregation or proteolysis of native proinsulin did not occur under these conditions and that no isomerization of native disulfide bonds occurred, neither spontaneously nor catalyzed by PDI. In addition, native proinsulin at refolding conditions and refolded proinsulin were digested with trypsin and carboxypeptidase B and the digestion products were analyzed by an insulin-ELISA (25). At these conditions only correctly folded proinsulin can be converted to native insulin (24). The amount of insulin generated from native and refolded proinsulin, respectively, was identical indicating that the refolded proinsulin was indeed correctly folded and contained native disulfide bonds (data not shown).

PDI Variants Can Improve Refolding—PDI, PDI-b′a′c (a PDI variant containing only the last three domains), and mutants having one (PDIΔC1 and PDIΔC2) or both (PDIΔC1,2) active sites inactivated by cysteine to serine substitutions were tested with respect to refolding of denatured and reduced proinsulin. As shown in Fig. 1*B* only wild-type PDI was able to increase refolding to the maximum rate and yield. PDIΔC1 and PDIΔC2 increased the folding rate and yield, but not to the same extent as wild-type PDI, even when they were present at a two times higher concentration which corresponded to the same amount of active sites present in wild-type PDI. This effect was independent of which active site, either in the **a** domain or **a′** domain, was mutated. PDI-b′a′c had the same properties in proinsulin renaturation as PDIΔC1 and PDIΔC2 (data not shown). PDIΔC1,2 was not capable of increasing the folding rate, however, PDIΔC1,2 could improve the yield of refolding by about 30% (Fig. 1*C*). In contrast to wild-type PDI which increased the yield of refolding of proinsulin already at substoichiometric concentrations PDIΔC1,2 had to be present at least at stoichiometric concentrations. These results indicate that PDI acts both as an isomerase and as a chaperone when present during refolding of proinsulin.

PDI Is Essential during the First Seconds of Refolding—Next, we asked at which stages of the renaturation process the isomerase and the chaperone activity of PDI are required to allow efficient refolding of proinsulin. Refolding was performed in the presence of wild-type PDI or PDIΔC1,2 added to refolding proinsulin at different times of renaturation (Fig. 2). The chaperone activity was only effective when present during the first seconds of the refolding process. While PDIΔC1,2 could chaperone renaturation of proinsulin when present at the be-

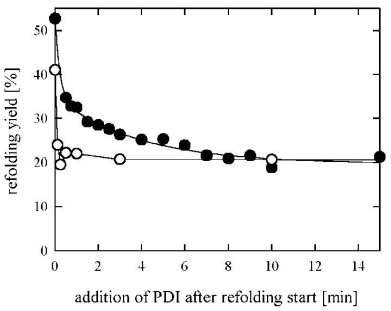

FIG. 2. **Timed addition of PDI to refolding proinsulin.** Reactivation was performed as described in the legend to Fig. 1, except that PDI (●) and PDIΔC1,2 (○), respectively, were added after refolding start at the times indicated. The final concentration was 2 μM for PDI and 28 μM for PDIΔC1,2. Refolding was analyzed after the renaturation was completed. The data (●) were fitted to a biphasic reaction with apparent rate constants of k_{app} = 0.075 min^{-1} and k_{app} = 0.0043 min^{-1}.

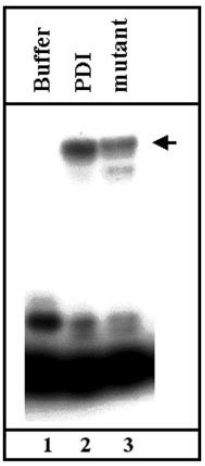

FIG. 3. **Interaction of PDI and PDIΔC1,2 with proinsulin.** Equal amounts of *E. coli* cell extracts that expressed wild-type PDI (*PDI*) and PDIΔC1,2 (*mutant*), respectively, were incubated with radiolabeled denatured and reduced proinsulin (final concentration 33 μM). For control, *E. coli* cell lysates that did not contain PDI or PDIΔC1,2 (*buffer*) were incubated at the same conditions. Incubation was performed in a total volume of 10 μl at 0 °C for 10 min. Samples were subsequently incubated with disuccinimidyl glutarate (final concentration 0.5 mM) for 60 min at 0 °C and analyzed on a 12.5% polyacrylamide gel with subsequent autoradiography. The cross-linking products are indicated by an *arrow*.

ginning of the process, no increase in yield was observed if PDIΔC1,2 was added to refolding proinsulin after 7 s of refolding. PDI inactivated by alkylation (13) was tested under the same conditions (data not shown) and its activity on refolding was identical to that of PDIΔC1,2 thus excluding unspecific effects of possible structural changes caused by the point mutations in PDIΔC1,2. Both, PDIΔC1,2 and alkylated PDI, showed identical results in the timed addition experiment.

In contrast, wild-type PDI, even when added 1 min after initiation of folding could improve renaturation. This improvement, however, was not as efficient as when present from the beginning. This indicates that the significant decrease in the refolding yield due to the absence of the chaperone function in the first seconds of refolding can be partly compensated by the isomerase activity of PDI. The observed decrease in the yield of refolding of proinsulin when PDI was added to the refolding sample at different time points exhibits a biphasic kinetic. A very fast first phase with an apparent rate constant k_{app} = 0.075 s^{-1} was followed by a slow phase with a k_{app} = 0.0043 s^{-1}. Compared with the rate constant of formation of native proinsulin (k_{app} = 0.0018 s^{-1}, Fig. 1*A*) the second phase occurred in a similar time range but the first phase was much faster. From this we conclude that upon renaturation of proinsulin there is a kinetic competition between an overall slow folding process and an ~20 times faster off-pathway reaction. This off-pathway seems to depend on redox reactions subsequently leading to aggregation.

Chaperone Function of PDI Prevents Aggregation of Proinsulin—Furthermore, PDI and PDIΔC1,2 were tested with respect to peptide binding. Chemical cross-linking with an amino-specific cross-linking reagent is a useful method to detect interaction partners for PDI (7, 26). Both variants of PDI were capable of binding radiolabeled denatured and reduced proinsulin (Fig. 3). Furthermore, the binding was reversible, as it could be competed with other substrate peptides and proteins (data not shown). This clearly demonstrates that PDI and PDIΔC1,2 were indistinguishable with respect to their interactions with substrates. PDIΔC1,2 shows two bands in the cross-linking (Fig. 3). The signal at lower molecular weight corresponded to a degraded PDIΔC1,2. Both, the full-length and the degraded PDIΔC1,2 were able to bind denatured and reduced proinsulin. This degradation was probably due to proteases present in the *E. coli* cell extract used for the cross-linking assay. No degradation of PDIΔC1,2 was observed with purified protein under refolding conditions as analyzed by SDS gels (data not shown). To demonstrate that the observed effects of the PDIΔC1,2 were not due to misfolding of one or more of its domains its stability was compared with that of wild-type PDI. Incubation of PDIΔC1,2 or the wild-type PDI with various concentrations of proteinase K showed that there was no significant difference in the protease sensitivity indicating that PDIΔC1,2 can adopt as compact a structure as the wild-type and that there was no difference in the structural stability of both proteins (data not shown).

Aggregation of folding proinsulin as detected by light scattering was proportional to the proinsulin concentration in the refolding sample (Fig. 4*B, inset*). It occurred in the first seconds of refolding, reaching a maximum after 20–30 s (Fig. 4*A*). This indicates that aggregation of proinsulin during renaturation occurred in the same time range as the fast phase of the unproductive reaction observed in the timed addition experiment of PDI (Fig. 2). PDI suppressed aggregation of refolding proinsulin substantially and was already effective in catalytic amounts (Fig. 4*B*) which is in agreement with the refolding data shown in Fig. 1*A* and the timed addition data from Fig. 2. PDI at a molar ratio of PDI/proinsulin of 0.05 could protect about 20% of the folding proinsulin from aggregation. In contrast, alkylated PDI in the same molar ratio could suppress only 10% of the aggregation (data not shown). Only when present in stoichiometric amounts could alkylated PDI prevent aggregation of folding proinsulin to the same extent as PDI. Under identical conditions bovine serum albumin did not suppress aggregation (data not shown).

This clearly indicates that PDI, present in catalytic amounts, possesses chaperone activity which alone is not sufficient to protect folding proinsulin from aggregation. Early intermediates in the proinsulin folding pathway, probably containing wrong disulfide bonds, seem to be highly susceptible to aggregation. In the presence of wild-type PDI these aggregation-prone intermediates can isomerize to folding intermediates that are less susceptible to aggregation. This results in reduced aggregation and, as a consequence, in an enhanced refolding yield. When present in very high concentrations alkylated PDI was similarly effective, indicating that a noncatalyzed isomer-

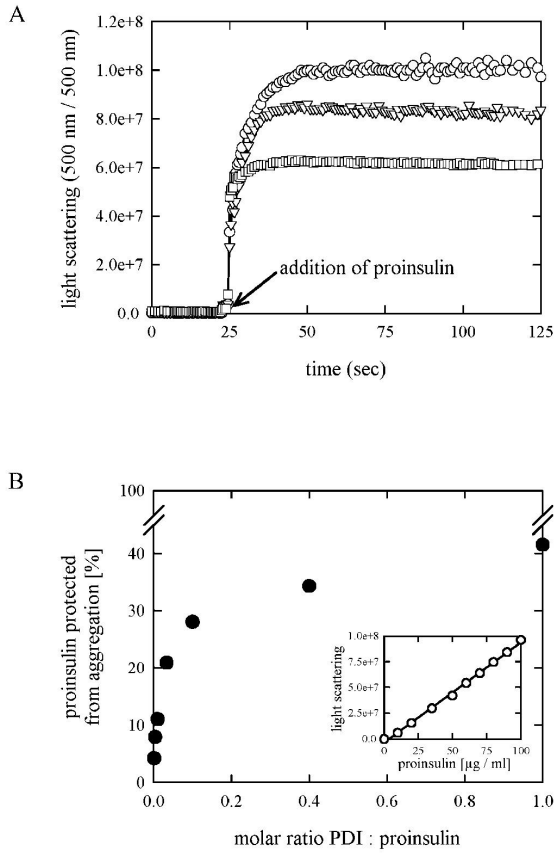

FIG. 4. **Influence of PDI on proinsulin aggregation.** Refolding of proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG, with different concentrations of PDI at 25 °C in a stirred cuvette. The final concentration was 10 μM for denatured and reduced proinsulin and 40 mM for guanidinium chloride. Aggregation was monitored by light scattering at 500 nm. *A*, kinetics of the aggregation of proinsulin in the absence of PDI (○), and in the presence of 2 μM PDI (▽), or 10 μM PDI (□); *B*, dependence of the aggregation of proinsulin on the concentration of PDI. The amount of proinsulin protected from aggregation was calculated according to the calibration curve shown in the *inset*. *Inset*, calibration curve of proinsulin aggregation from 10 to 100 μg/ml proinsulin.

ization of disulfides during proinsulin folding can occur if aggregation is sufficiently suppressed by the chaperone activity. However, independent of the presence of PDI a significant part of proinsulin aggregated during the refolding. This correlates with the maximum refolding yield of about 50%.

Proinsulin Binding to PDI Is Suppressed by the Inhibitor Genistein—To analyze whether the interaction of PDI and PDIΔC1,2 with denatured and reduced proinsulin can be suppressed by an inhibitor of the chaperone activity we used the small molecular weight substance genistein. It has been shown previously that genistein, a substance with estrogenic activity, can suppress the binding of Δ-somatostatin to PDIp, a member of the protein-disulfide isomerase family (23), and also PDI.[3] Both, PDI and PDIΔC1,2 showed the same genistein binding properties (Fig. 5*A*) indicating that the peptide-binding domain of PDIΔC1,2 was not affected by the mutations in both active sites. By titration of genistein we found that a 3-fold molar excess of genistein over proinsulin was required to completely suppress binding of denatured and reduced proinsulin to PDI as well as to PDIΔC1,2.

Genistein-inhibited PDI or alkylated PDI were significantly affected in their ability to prevent proinsulin from aggregation (Fig. 5*B*). In refolding samples analyzed by light scattering genistein-inhibited PDI and genistein-inhibited alkylated PDI could protect about 11% of the folding proinsulin from aggregation. This indicates that both PDIs still had some residual chaperone activity under the experimental conditions. Genistein inhibited 66% of the chaperone function of PDI. For comparison, the inhibitory effect of genistein on alkylated PDI was less pronounced (about 50% inhibition). The lower inhibition effect for alkylated PDI was due to its two times higher concentration in the refolding sample and therefore a two-times less excess of genistein over alkylated PDI compared with PDI.

In agreement with the results shown above, the inhibition of the chaperone function of PDI drastically changed the PDI-assisted refolding behavior of proinsulin (data not shown). In PDI-catalyzed refolding a 10-fold molar excess of genistein over proinsulin decreased the refolding rate four times and the yield of proinsulin refolding was only 37% compared with 50% in the presence of non-inhibited PDI. For refolding in the presence of genistein-inhibited PDIΔC1,2 or alkylated PDI the stimulating effect mediated by the variants with no isomerase activity decreased by 60% (corresponding to about 25% refolded proinsulin). The rate constant did not change compared with refolding without genistein. This shows that the inhibited PDIs still had some residual chaperone activity and that in the case of wild-type PDI the isomerase activity can facilitate a higher refolding yield. Similar results were obtained for refolding of proinsulin with a four times and a 100 times molar excess of genistein (data not shown). This is in agreement with the cross-linking data shown above. As a control we confirmed that the aggregation and refolding properties of proinsulin were not changed in the presence of genistein alone (see above). Additionally, Me_2SO used to solubilize the genistein did not influence proinsulin refolding, neither in the absence nor the presence of PDI or PDI variants (data not shown). This proves that by binding of genistein to the peptide-binding domain of PDI binding of folding proinsulin is suppressed. This inhibition of the chaperone function of PDI leads to enhanced aggregation of folding intermediates and to a reduction of the yield of refolding.

The Chaperone and Isomerase Activity of PDI Are Not Required during the Entire Refolding Process—To analyze whether both the chaperone and isomerase function of PDI were only required during the first seconds of refolding or whether they were essential during the entire refolding process, the refolding of proinsulin was monitored in the presence of PDI and PDIΔC1,2, and genistein was added at different times after initiation of refolding (Fig. 6*A*). Similar to these experiments we performed proinsulin refolding and added genistein-inhibited PDI at different time points after refolding started (Fig. 6*B*).

As described above, refolding of proinsulin in the presence of genistein-inhibited PDI or PDIΔC1,2 resulted in a significant decrease of the yield of refolding. In contrast, genistein added 25 s after initiation of refolding of proinsulin in the presence of PDI or PDIΔC1,2 did not influence the refolding yield. For both, refolding of proinsulin with PDI or PDIΔC1,2, the obtained refolding yield was similar to the yield of refolding without genistein. This indicates that at this time, 25 s after refolding started, the chaperone function was not longer required (Fig. 6*A*). These results are in agreement with the light scattering data (Fig. 4*A*) showing that aggregation occurred in the first few seconds of refolding. Timed addition of genistein-inhibited

[3] L. W. Ruddock and P. Klappa, unpublished observations.

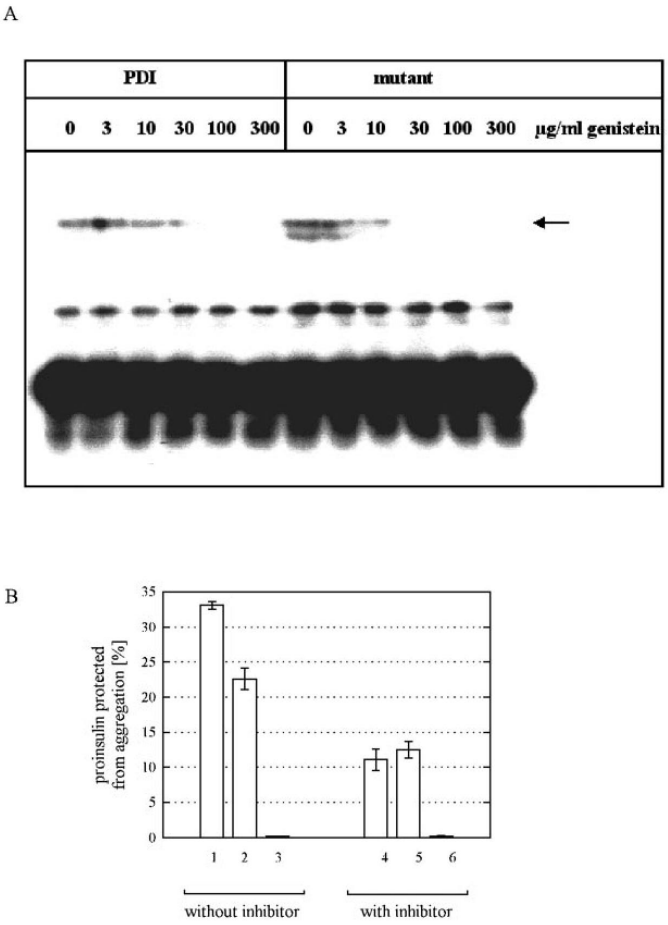

FIG. 5. **Influence of the inhibitor genistein on peptide binding of PDI.** *A*, inhibition of proinsulin binding to PDI and PDIΔC1,2 by genistein analyzed by cross-linking. *E. coli* cell lysates that expressed PDI (*PDI*) and PDIΔC1,2 (*mutant*), respectively, were incubated with 33 µM radiolabeled denatured and reduced proinsulin in the presence of the indicated concentrations of genistein. The samples were cross-linked with disuccinimidyl glutarate and analyzed on a 15% polyacrylamide gel with subsequent autoradiography. The cross-linking products are indicated by an *arrow*; *B*, influence of genistein-inhibited PDI and genistein-inhibited alkylated PDI on proinsulin aggregation. Refolding was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C in a stirred cuvette. The final concentration was 0.25 µM for denatured and reduced proinsulin, 1 mM for guanidinium chloride, 0.025 µM for PDI, 0.05 µM for alkylated PDI, 10 µM for genistein, and 0.009% (v/v) for Me_2SO. PDI or alkylated PDI were added to the refolding buffer after a preincubation with genistein or Me_2SO for 10 min. Afterward denatured and reduced proinsulin was added to the stirred sample. The amount of proinsulin protected from aggregation was determined according to a calibration curve. Aggregation of proinsulin was analyzed in the presence of PDI and Me_2SO (*bar 1*) or genistein (*bar 4*), in the presence of alkylated PDI and Me_2SO (*bar 2*) or genistein (*bar 5*), and in the absence of PDI but with Me_2SO (*bar 3*) or genistein (*bar 6*).

PDI to refolding proinsulin exhibited a decrease in the refolding yield with a biphasic characteristic and an apparent rate constant for the fast, first phase of $k_{app} = 0.029\ s^{-1}$. PDI with isomerase activity could increase the refolding yield significantly even when added 1 or 5 min after starting refolding. This catalytic effect was independent of the chaperone activity of PDI. The results of the above experiments clearly show that the isomerase activity could compensate for an impaired chaperone activity and that in this case the isomerase activity became essential to facilitate efficient proinsulin refolding. If the isomerase activity is available the chaperone activity seems to play an ancillary role.

DISCUSSION

Previous work on refolding activities of PDI *in vitro* showed that PDI has catalytic activity as a thiol:disulfide isomerase and can act as chaperone. In most cases PDI affects only one or the other of these functions (8, 9, 13). Yao *et al.* (11) proposed that for the reactivation of acidic phospholipase A_2 both functions are required. They stated that in their model system only a small part of PDI acts catalytically as an isomerase and the residual part functions as a molecular chaperone, which can be replaced by alkylated PDI. In our model system refolding of proinsulin is dependent on the redox conditions and influenced by aggregation and disulfide bond formation. A PDI variant with no isomerase activity can substantially suppress aggregation of refolding proinsulin. However, this PDI variant was not as efficient as wild-type PDI with respect to refolding yield and refolding rate, indicating that disulfide isomerization also plays an important role in the refolding of proinsulin. Maximum rate and yield of proinsulin refolding can only be achieved if both, the chaperone and the isomerase activity of PDI were present.

Although PDI exhibits isomerase and chaperone activity PDI seems to be more efficient as an isomerase than as a chaperone (13, 27). PDI acts only as an isomerase on refolding of an antibody fragment (13). This effect is limited to the first seconds of refolding. However, if the chaperone BiP is simultaneously present BiP can efficiently bind and re-bind folding intermediates. Thus it keeps the cysteines of the folding antibody fragment accessible to PDI over a much longer time scale and PDI can sequentially act as an isomerase (27). The active site sequence -WCGHC- of PDI enables very efficient disulfide isomerization as compared with wild-type thioredoxin. A Pro to His mutation (−CGP**C**− to −CGH**C**−) in the active site of thioredoxin increases the isomerization activity (28). For thioredoxin a flat and hydrophobic molecular surface area on one side of the redox active disulfide bond has been described that is suggested to be the binding area for redox interactions with other proteins (29, 30). On the basis of sequence homologies to thioredoxin, it was suggested that PDI also has protein binding capacity. The principle peptide-binding site is located in the **b′** domain of PDI, however, for efficient binding of proteins the contribution of the **a′** domain (**b′a′c**) or the **a** and **b** domains

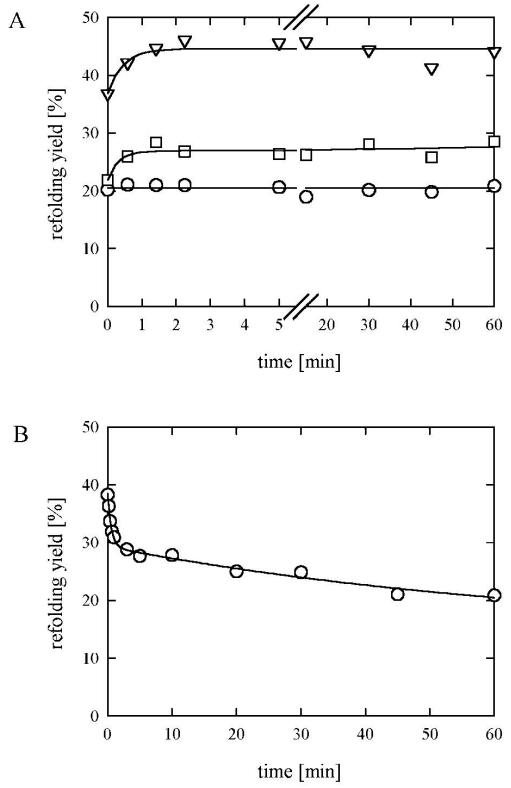

FIG. 6. **Influence of the inhibitor genistein on PDI-catalyzed refolding of proinsulin.** Refolding of denatured and reduced proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C. The final concentration was 2.5 μM for proinsulin and 15 mM for guanidinium chloride. After 60 min, refolded proinsulin was digested with trypsin and carboxypeptidase B for 20 min. Afterward acetonitrile and trifluoroacetic acid were added to a final concentration of 10 and 0.1% (v/v), respectively, and the samples were analyzed by RP-HPLC. *A*, timed addition of genistein. Genistein (10 μM) was added to refolding proinsulin in the absence of PDI (○), in the presence of 0.25 μM PDI (▽) and 0.5 μM PDIΔC1,2 (□) after renaturation start at the times indicated. The data were fitted single exponentially with apparent rate constants of $k_{app} = 0.038\ s^{-1}$ (for PDI), and $k_{app} = 0.055\ s^{-1}$ (for PDIΔC1,2). *B*, timed addition of genistein-inhibited PDI to refolding proinsulin. PDI and genistein were preincubated for 10 min and added to refolding proinsulin after the renaturation was initiated at the times indicated. Final concentration was 0.25 μM for PDI and 10 μM for genistein. The data were fitted to a biphasic reaction with apparent rate constants of $k_{app} = 0.029\ min^{-1}$ and $k_{app} = 0.00024\ min^{-1}$.

(**abb**′) are required (7). In most cases, aggregation is a nonproductive, off-pathway reaction, which competes with the correct folding of proteins (31). Consequently, chaperones (*e.g.* GroEL/GroES, DnaK/DnaJ/GrpE, SecB, and ClpB from *E. coli* or the Hsp families of eukaryotes) interacting with folding intermediates and thereby preventing or minimizing aggregation can increase the refolding yield (32–35). When folding intermediates escape the protective function of chaperones, they can form stable protein aggregates. Few cases are known where chaperones (*e.g.* the DnaK system of *E. coli*, sometimes sequentially acting with ClpB) have the potential to disaggregate protein aggregates (36–38) and most chaperones and also PDI cannot actively dissolve protein aggregates.

Here, wild-type PDI, the PDI domain construct **b**′**a**′**c**, and the full-length PDI mutants with only one active site (PDIΔC1 and PDIΔC2) were used to catalyze refolding of proinsulin. Although all non-wild-type variants are less effective than PDI with respect to proinsulin refolding they can significantly increase the refolding rate and yield. We demonstrated that (i) the **b**′**a**′**c** domain construct of PDI can catalyze the refolding of proinsulin, (ii) the presence of one active site was sufficient to accelerate proinsulin refolding, however, (iii) full-length PDI was needed for maximum catalytic activity. This is in agreement with the data of Darby *et al.* (6) who demonstrated that the **b**′ domain of PDI has an especially important role in catalysis, and that maximum catalytic activity in disulfide bond rearrangements requires the involvement of all PDI domains. It was proposed that all PDI domains participate in substrate binding and especially the binding of non-native proteins might require all domains of PDI (7, 39).

Using isomerase-inactive PDI variants and PDI with inhibited chaperone function, we now can clearly distinguish between both functions PDI provides. The mutation in the active site did change the enzyme characteristic but did not effect the chaperone activity. This was concluded as both, alkylated PDI and PDIΔC1,2, were equally active regarding peptide binding as shown by cross-linking and refolding of denatured and reduced proinsulin. Genistein, which can suppress Δ-somatostatin binding to the pancreatic protein-disulfide isomerase PDIp (23), is also able to bind to the peptide-binding site of PDI. Thus genistein can efficiently suppress binding of other substrates to PDI as shown by cross-linking experiments. By inhibition of the principal peptide-binding site the refolding yield of proinsulin was significantly decreased although not completely abolished indicating that other domains can contribute to the chaperone activity of PDI. However, in this case the interactions might be too weak to be detected in the cross-linking experiments. Genistein-inhibited PDI variants lacking isomerase activity that should not influence refolding were still able to increase proinsulin refolding, but not as efficient as the non-inhibited variant. This might indicate that proinsulin binding occurs not only at the **b**′ domain but extends through all PDI domains or that PDI contains more than one peptide-binding site with only one site as a target for genistein. Furthermore, neither for genistein nor for proinsulin the binding constant to PDI is known. Hence we cannot exclude that the binding of refolding proinsulin to PDI is possible with different affinities for different folding intermediates, even in the presence of an excess of genistein.

In spontaneous refolding of proinsulin about 20% of the denatured and reduced proinsulin was folding competent. The remaining part was excluded from productive refolding by very fast aggregation of completely reduced proinsulin or folding intermediates. Providing optimum conditions (*e.g.* refolding at pH 10.5)[2] aggregation of folding proinsulin is reduced and about 60% native proinsulin can be formed. Similarly, IGF-I that belongs to the insulin superfamily and contains three motif-specific disulfide bonds yields under optimized conditions about 60% native protein within 5 h (40). Proinsulin and also porcine insulin precursor (41) form no stable intermediate as possible intermediates seem to be either highly susceptible to aggregation or can fold to the native protein (41). In contrast, IGF-I yields two isoforms that are stable under refolding conditions (42–44). The first one is native IGF-I with the disulfide bonds: 1) Cys^{6}-Cys^{48}, 2) Cys^{18}-Cys^{61}, and 3) Cys^{47}-Cys^{52}, which correspond to Cys^{7}-Cys^{72}, Cys^{19}-Cys^{85}, and Cys^{71}-Cys^{76} in proinsulin. The second isoform is non-native IGF-I with the disulfide bonds 1 and 3 mismatched. These species occur in a ratio of 60% native to 40% mismatched.

The apparent rate constant determined in timed addition experiments with PDI showed that in the first seconds of proinsulin refolding very fast off-pathway reactions play an important role. These unproductive reactions are reduced by both

the chaperone and isomerase activity. The chaperone function of PDI was important during the first seconds but not at later stages of refolding. In the absence of the chaperone activity, however, the isomerase function of PDI became essential to ensure an increased refolding yield. The isomerase function of PDI is acting during the whole refolding process although 10 min after initiation of refolding its effect was less apparent. From the kinetics of spontaneous proinsulin refolding we know that refolding was completed after about 30–60 min. This shows that folding and possibly isomerization occurred even 10–30 min after refolding started when aggregation was already completed. These late intermediates were not particularly susceptible to aggregation and PDI could catalyze these reactions but did not enable a significant increase in the yield of refolding of proinsulin. In the presence of PDI, aggregation as the major side reaction was suppressed although not completely even in stoichiometric concentrations. Under these conditions the proinsulin species protected from aggregation (about 40%) became folding competent and refolded completely as the refolding yield during catalyzed refolding was increased to about 50–55%. This indicates that if side reactions can be efficiently suppressed, either by accelerated isomerization or reduced aggregation, denatured and reduced proinsulin can form the native molecule. However, off-pathway reactions occurred very fast and obviously they cannot be reversed by PDI. This might be the major reason why we cannot force the proinsulin to refold quantitatively.

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Manuskript zur Publikation eingereicht

The Low Molecular Weight Dithiol Vectrase-P Stimulates Proinsulin Folding in vitro and Production of Native Proinsulin in the Periplasm of *E. coli*

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Abstract: Folding of human proinsulin can be improved by protein disulfide isomerase (PDI) which acts both as an isomerase and as a chaperone. Here we show that a synthetic, low molecular weight dithiol (Vectrase™-P) mimics the redox activity of PDI during oxidative folding of proinsulin in vitro. Vectrase™-P is effective at catalytic concentrations; it does not change the folding kinetics but increases the yield of proinsulin refolding about two-fold. This effect on the yield of proinsulin refolding can also be observed upon in vivo folding. Upon growing *E. coli* cells producing periplasmic proinsulin in the presence of Vectrase™-P, the yield of correctly folded proinsulin in the periplasm was increased.

Key words: proinsulin, disulfide bond formation, refolding, PDI, periplasm, secretory expression

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Abbreviations: Vectrase™-P, (±)-trans-1,2-bis(2-mercaptoacetamido)cyclohexane; PDI, protein disulfide isomerase; GdnHCl, guanidinium hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; RP-HPLC, reversed phase HPLC; IPTG, isopropyl-β-D-thiogalactopyranoside; OD_{500nm}, optical density at 500 nm

1. Introduction

Proinsulin is the pro-form of biologically active insulin and can be produced recombinantly either as the inactive (aggregated) or the native protein. Refolding of proinsulin from the insoluble material (e.g. inclusion bodies from *E. coli*) in vitro is accompanied by the formation of the correct disulfide bonds. Oxidative folding of proinsulin is a widely used process with high yield [1,2]. Nevertheless, attempts have been made to produce proinsulin in vivo in its native conformation, e.g. by directing proinsulin or proinsulin fusion proteins to the periplasmic space of *E. coli* [3-5]. The periplasm of *E. coli* is an appropriate compartment to produce disulfide bonded proteins because of the more oxidizing conditions compared to those of the cytoplasm, the presence of proteins involved in disulfide bond formation (Dsb family), and the permeability of the outer membrane of *E. coli* to molecules smaller than 600 Da [6]. Therefore, redox components and other low molecular weight additives can enter the periplasmic space of *E. coli* from the medium to provide conditions which are favourable for the formation of the native structure of proteins [3,4,7-10].

Human proinsulin contains three disulfide bonds which are essential for its native conformation. The formation of the native disulfide bonds is the rate limiting step during proinsulin folding [11] and, therefore, acceleration of disulfide bond formation would lead to faster folding of the protein in vitro and also in vivo. PDI influences proinsulin folding as an isomerase as well as a chaperone resulting in a higher rate and yield of refolding [12]. PDI contains the active site motif –Cys–Xaa–Xaa–Cys– which is also found in other thiol:disulfide oxidoreductases such as thioredoxin and DsbA from *E. coli*. The redox potential (E°') of the active sites of DsbA, PDI, and thioredoxin are –0.089 V [13], –0.11 V [14], and –0.27 V [15], respectively. Consequently, DsbA is the most oxidizing enzyme. Previously, a synthetic dithiol, Vectrase™-P ((±)-trans-1,2-bis(2-mercaptoacetamido) cyclohexane, Fig. 1) with a redox potential (E°' = -0.24 V) more oxidizing than β-mercaptoethanol (E°' = -0.26 V, [16]) was described [17]. This redox potential is more reducing than that of PDI. Vectrase™-P catalyzed disulfide bond isomerization in scrambled bovine RNase A in vitro but with a clearly lower efficacy than PDI. Additionally, the secretion of *Schizosaccharomyces pombe* acid phosphatase containing eight disulfide bonds from *Saccharomyces cerevisiae* cells was increased three-fold when Vectrase™-P was present in the cultivation medium at a concentration of 0.4 – 1.5 mM.

In this report we describe the application of Vectrase™-P in proinsulin folding in vitro and its effect on the formation of native proinsulin in the periplasmic space of recombinant *E. coli*. In

vitro and in vivo Vectrase™-P is already effective at micromolar concentrations whereas millimolar concentrations of Vectrase™-P seem to decrease the amount of native proinsulin by keeping cysteines in the reduced form.

2. Materials and Methods

Recombinant human proinsulin was from BIOBRÁS, Brazil, the ELISA antibodies were from Roche Diagnostics, Germany, and Vectrase™-P was obtained from Bio Vectra, Canada. The C_{18}-reversed phase HPLC column was purchased from Macherey-Nagel, Germany.

2.1. Unfolding and refolding of proinsulin

His_8-Arg-proinsulin was denatured and reduced in 6 M guanidinium hydrochloride (GdnHCl), 10 mM Tris, 1 mM EDTA, 1 M DTT at pH 8.5 and a protein concentration of about 15 - 20 mg/ml. Afterwards the protein was dialyzed against 6 M GdnHCl, 5 mM EDTA, pH 3. Proinsulin was refolded in 10 mM Tris, 10 mM glycine, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at pH 7.5 and 25 °C if not otherwise indicated [12]. The final protein concentration was 100 µg/ml. The refolded protein was analyzed by reversed phase HPLC (RP-HPLC) with a linear gradient from 20 % to 55 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid [12].

2.2. Expression of the proinsulin gene

The construction of the plasmid pET20b(+)-PI was described previously [3]. Cultivation of BL21(DE3)-pET20b(+)-PI was performed at different temperatures on Luria-Bertani medium containing 100 µg/ml ampicillin. To obtain optimum expression of the proinsulin gene, the cells were cultivated at 22 °C and the main culture was inoculated to an optical density (OD) of $OD_{500nm} = 0.1$. Recombinant expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM after the cells reached $OD_{500nm} = 0.5$. After further 3 h of cultivation periplasmic extracts were prepared by osmotic shock and the amount of native proinsulin was determined by an insulin-ELISA as described before [4].

3. Results and Discussion

3.1. Refolding of His_8-Arg-proinsulin in vitro

Chemically denatured and reduced proinsulin (His_8-Arg-proinsulin) was used to study the in vitro refolding in the presence of Vectrase™-P. Vectrase™-P is a small chemical dithiol mimicking the active site of PDI (Fig. 1, [17]). As PDI has been shown to increase the rate and yield of proinsulin refolding [12] this chemical PDI mimeticum was tested with respect to its effect on proinsulin folding in vitro. For analysis of Vectrase™-P assisted folding, the same conditions as for the spontaneous and PDI-catalyzed proinsulin folding were employed (see materials and methods). Spontaneous refolding of proinsulin was completed after 30 to 60 min. Vectrase™-P present in the refolding sample at 10 µM did not change significantly the folding rate of GdnHCl-denatured and reduced proinsulin (Fig. 2A). Even a 30-times molar excess of Vectrase™-P over proinsulin did not change the refolding rate (data not shown). However, Vectrase™-P increased substantially the yield of native proinsulin (Fig. 2B). Within molar ratios of 0.03 – 1 of Vectrase™-P to proinsulin, refolding reached a yield of about 35 % of the theoretical amount. Higher concentrations of Vectrase™-P led to a decrease of the refolding yield since high concentrations of the free thiols of Vectrase™-P shifted the redox buffer to more reducing conditions. Surprisingly, addition of 10 µM Vectrase™-P did not change the redox dependency of proinsulin folding (Fig. 2C). The optimum redox buffer for proinsulin folding contained glutathione at a ratio of GSH^2 / GSSG = 0.5 - 1.5 mM (e.g. 1 mM GSH : 2 mM GSSG or 1.5 mM GSH : 1.5 mM GSSG), independent of the presence or absence of Vectrase™-P. The quantitative effect of Vectrase™-P on the yield of native proinsulin did not change upon varying the redox buffer from GSH^2 / GSSG = 0 - 7.6 mM, only at strongly reducing conditions the positive influence of Vectrase™-P was diminished.

Vectrase™-P did not significantly increase the rate of proinsulin refolding which indicates that Vectrase™-P cannot accelerate the overall slow folding by catalyzing a rate limiting step in proinsulin folding. Nevertheless, the competition of folding and aggregation of proinsulin seemes to be influenced by Vectrase™-P resulting in a higher final yield of native proinsulin. Vectrase™-P was described to unscramble non-native disulfide bonds in RNase (scrambled RNase) to form the native molecule which proofes the isomerase activity of this dithiol [17]. In this assay a much higher concentration of Vectrase™-P (1mM) was employed compared to our experiments. Similar to our results, Vectrase™-P did not increase the rate of scrambled

RNase folding but improved the yield of native RNase about two-fold compared to folding of scrambled RNase in the presence of glutathione alone. Compared to PDI-catalyzed refolding [12], Vectrase™-P showed less pronounced effects on the refolding rate and the yield of native proinsulin. Woycechowsky et al. [17] argued that the specific activity of disulfide bond isomerization of Vectrase™-P is about 500-fold less compared to that of PDI. This could result from the lower thiol pK_a value of PDI and a higher part of thiolate anion due to PDIs higher E°'. Thus, according to the redox potential (E°' = -0.11 V for PDI [14] and E°' = -0.24 V for Vectrase™-P [17]) it is expected that the effect of Vectrase™-P on proinsulin folding is less pronounced compared to that of PDI. Furthermore, proinsulin folding requires not only oxidation but also isomerization of disulfide bonds as well as a chaperone activity keeping intermediates in solution. PDI provides all these activities upon oxidative folding of proinsulin [12]. In contrast to PDI, Vectrase™-P has no peptide binding capability, therefore, the interactions of the active site thiols of Vectrase™-P with the cysteines in folding proinsulin are diffusion dependent. Vectrase™-P is not able to increase the local concentration of its active site cysteines to folding proinsulin which would allow faster disulfide bond formation or rearrangement. For PDI-catalyzed folding of an antibody fragment (Fab) it has been described that PDI could increase the yield of functional Fab without having a significant effect on the folding kinetics [18]. This effect was explained by the fast formation of a compact intermediate that shields the intradomain disulfide bonds from solvent. Therefore, the accessibility of these cysteines is rate limiting and cannot be influenced by PDI. In our case, Vectrase™-P seemed to influence disulfide bond formation or isomerization at not rate-limiting stages. However, Vectrase™-P probably acted at stages of proinsulin folding where intermediates occur that are especially susceptible to aggregation. By this, the kinetics of folding remain nearly unchanged but by decreased aggregation the final folding yield is improved.

3.2. Formation of proinsulin in the periplasm of E. coli

For secretion of proinsulin we fused the human proinsulin gene to the pelB signal sequence and the recombinant construct was expressed in *E. coli* BL21(DE3). Proinsulin was efficiently translocated to the periplasmic space and mature proinsulin could be detected in periplasmic extracts by western blot and N-terminal sequencing (data not shown). Different cultivation conditions were employed to allow efficient formation of correctly disulfide bonded proinsulin. Formation of native proinsulin showed a strong dependence on the cultivation

temperature (Table 1). At 22 – 28 °C the yield of native proinsulin reached a maximum after 3 h of induction and the proinsulin remained stable in the periplasm over a time range of about 2 h. In contrast, at 30 – 37 °C no increase of native proinsulin upon induction with IPTG could be observed. It was not examined if this was due to the formation of more insoluble protein or an overall decrease of the amount of proinsulin because of an increased activity of *E. coli* proteases at elevated temperatures. Furthermore, formation of native proinsulin depended on the time of induction. Addition of IPTG to cultures of OD_{500nm} = 1.5 to 2 resulted in ca. 0.2 - 0.5 mg native proinsulin per g cell dry weight (data not shown). Providing optimum conditions (22 °C, induction at OD_{500nm} = 0.5), 2.4 ± 0.5 mg native proinsulin per g cell dry weight could be obtained (Table 1).

3.3. Effect of Vectrase™-P on proinsulin folding in vivo

Vectrase™-P was tested on its effect on the yield of native proinsulin produced in the periplasm of *E. coli*. Vectrase™-P with a molecular weight of 262 Da should be able to pass the outer membrane of *E. coli*. Cultivation of proinsulin producing *E. coli* cells was performed under the optimized conditions described above. At the time of induction Vectrase™-P was added to the cultivation medium ranging from micromolar to millimolar concentrations (Fig. 3). No negative effect of Vectrase™-P on the cell growth was observed in the tested concentration range. Vectrase™-P present at 2 - 50 µM in the cultivation medium increased the formation of native proinsulin in the periplasm of *E. coli* by ca. 60 % (compared to cultivation without additives). In contrast, concentrations of 1 mM Vectrase™-P and higher resulted in a significant decrease in the yield of native proinsulin. This might be explained by the resulting reducing conditions that had been previously observed as not favourable for folding in vitro (compare Fig. 2B). The high similarity of the effects of Vectrase™-P on proinsulin folding in vitro and in vivo suggests that in vivo Vectrase™-P influenced the amount of native, periplasmic proinsulin by its redox activity i. e. its effect on oxidative folding. Another in vivo application of Vectrase™-P was previously described [17]. In this study the influence of Vectrase™-P (0.4 - 1.5 mM in the cultivation medium) on the secretion of recombinant acid phosphatase by *S. cerevisiae* was determined. A three-fold increase of native phosphatase per cell was observed. This increase corresponds to that of a *S. cerevisiae* strain overproducing yeast PDI 15-fold.

There exist a couple of examples where redox components were used to stimulate the formation of correct disulfide bonds in periplasmic proteins produced by recombinant *E. coli*. For instance, coexpression of DsbA and addition of GSH to the cultivation medium improved the yield of native, periplasmic α-amylase/trypsininhibitor in *E. coli* by facilitating faster folding and disulfide bond formation [10]. Similarly, for recombinant plasminogen activator, bovine pancreatic trypsin inhibitor, human insulin-like growth factor I, and pectate lyase increased yields of native protein have been reported upon addition of reduced or oxidized glutathione to the cultivation medium [3,4,7-9]. Thus, controlling the redox conditions in the periplasm of *E. coli* by adding low molecular weight thiols to the medium seems to be a generally applicable approach to produce disulfide bonded proteins in the bacterial periplasm. Also other small molecule thiols, namely para-substituted aromatic thiols [19], might be applicable to improve recombinant protein folding in the periplasm. These substances have a pK_a value below 7 and were previously described to reduce insulin in vitro with a higher rate compared to cysteine or glutathione. However, to achieve the same rate of reduction of insulin as described for PDI a 1000-times higher molar amount of these aromatic thiols compared to PDI was needed [19].

Vectrase™-P is a new thiol compound of very specific biophysical features. In contrast to all other thiols tested in recombinant protein production, Vectrase™-P is a dithiol. Upon reducing a protein it forms an intramolecular disulfide bond, mixed disulfides with the proteins should not be populated. In addition, one of the thiols of Vectrase™-P has a lower pK_a value of 8.3 making Vectrase™-P a kinetically very reactive compound. These characteristics might be the reason that optimum concentrations of Vectrase™-P used for recombinant protein production are in the micromolar range whereas other thiols such as glutathione or cysteine have to be used in millimolar concentrations.

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Table 1

Influence of the cultivation conditions on the level of native periplasmic proinsulin.

Cultivation Temperature [a]	native proinsulin [d] [mg/g cell dry weight]	
37 °C	0.8 ± 0.2	(3)
30 °C	0.8 ± 0.4	(2)
28 °C	1.4 ± 0.1	(2)
25 °C	1.5 ± 0.2	(2)
22 °C	1.9 ± 0.2	(3)
22 °C [b]	0.5	(1)
22 °C [c]	2.4 ± 0.5	(5)

[a] Expression of the recombinant gene was induced by addition of IPTG at an optical density of the culture of $OD_{500nm} = 1$, [b] $OD_{500nm} = 1.8$, or [c] $OD_{500nm} = 0.5$.

[d] Shown are the average (2 replicates) or the standard deviation (more than 2 replicates) of the amount of native proinsulin as determined by insulin-ELISA. The number of replicates are shown in brackets.

Figure legends

Fig.1. Structure of the small molecular weight dithiol Vectrase™-P (adapted from [17]).

Fig. 2. Influence of Vectrase™-P on proinsulin folding in vitro. Refolding of proinsulin was performed in 10 mM Tris, 10 mM glycine, 1 mM EDTA, pH 7.5, at 25 °C. The final concentration was 10 µM for proinsulin, 43 mM for GdnHCl, and 3 mM for glutathione. A: Influence of Vectrase™-P on the kinetics of the formation of native proinsulin. Proinsulin was renatured in refolding buffer containing 1 mM GSH and 2 mM GSSG in the absence of Vectrase™-P (o) and in the presence of 10 µM (∇) Vectrase™-P. At the times indicated an aliquot was withdrawn and the yield of refolded proinsulin was analyzed by RP-HPLC. The renaturation kinetics were fitted single exponentially with a rate constant of k_{app} = 0.002 s^{-1} for the spontaneous proinsulin folding and k_{app} = 0.003 s^{-1} for refolding in the presence of Vectrase™-P. B: Influence of different concentrations of Vectrase™-P on the yield of proinsulin refolding. Renaturation was carried out in the presence of 1 mM GSH and 2 mM GSSG, Vectrase™-P was added at the molar ratios indicated. Samples were analyzed by RP-HPLC after renaturation for 1 h. C: Influence of the redox potential on proinsulin refolding in the absence (o) and in presence of 10 µM Vectrase™-P (●). Glutathion was added at the indicated molar ratio of GSH to GSSG. Samples were analyzed by RP-HPLC after the renaturation for 1 h.

Fig. 3. Influence of Vectrase™-P on the yield of native proinsulin in the periplasm of *E. coli* cells. BL21(DE3)-pET20b(+)-PI was cultivated at 22 °C on Luria-Bertani medium. IPTG was added to a final concentration of 1 mM after the optical density of the culture reached OD_{500nm} = 0.5 and the cells were cultivated for an additional 3 hours. The amount of native proinsulin from two independent cultivations as determined by insulin-ELISA is shown.

Fig. 1

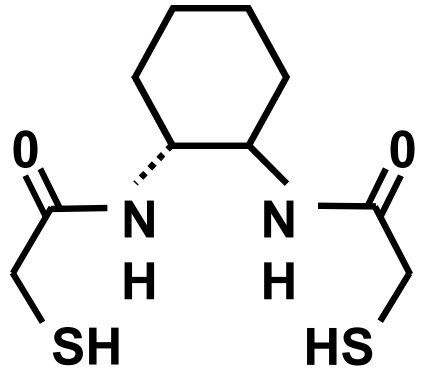


Fig. 2

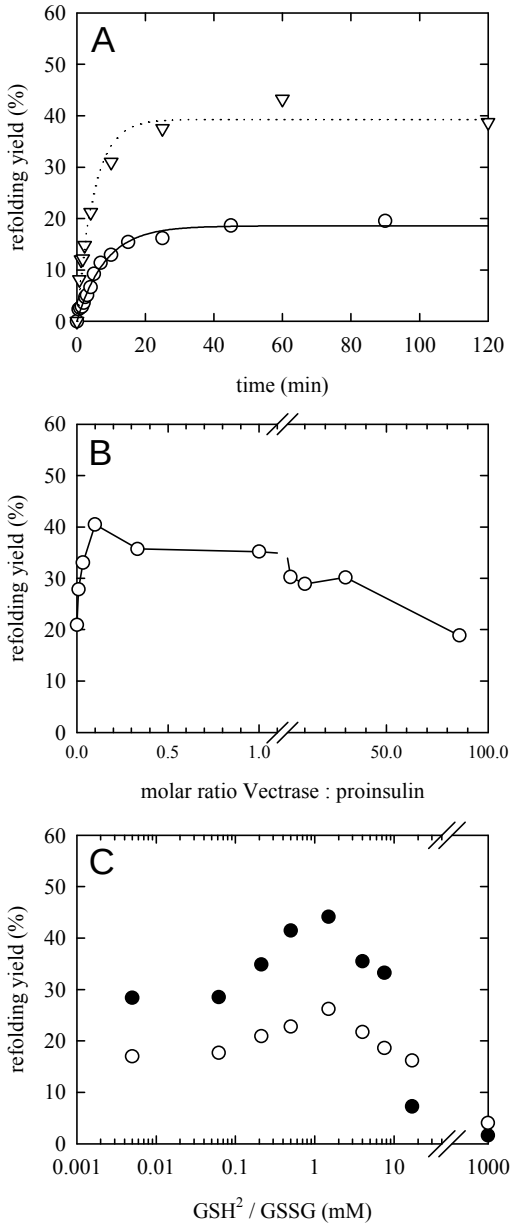
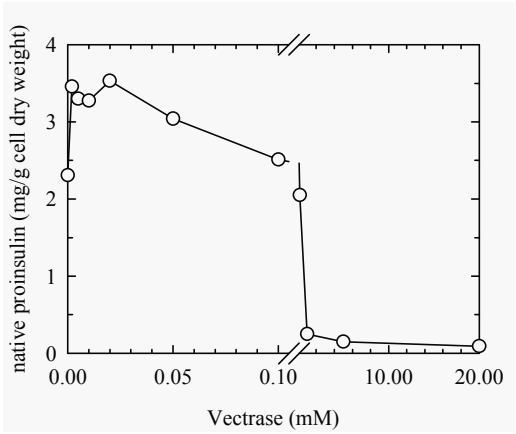

A
refolding yield (%)
time (min)
B
refolding yield (%)
molar ratio Vectrase : proinsulin
C
refolding yield (%)
GSH2 / GSSG (mM)

Fig. 3


native proinsulin (mg/g cell dry weight)
4
3
2
1
0
0.00
0.05
0.10
10.00
20.00
Vectrase (mM)

Manuskript zur Publikation eingereicht

Renaturation of human proinsulin – a study on refolding and conversion to insulin

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Subject category: protein structure and analysis

ABSTRACT

The production of human proinsulin in *Escherichia coli* usually leads to the formation of inclusion bodies. As a consequence, the recombinant protein has to be isolated, refolded under suitable redox conditions, and enzymatically converted to the biologically active insulin. In this study we describe a detailed *in vitro* renaturation protocol for human proinsulin that includes native structure formation and the enzymatic conversion to mature insulin. We used a His_8-Arg-proinsulin that was renatured from the completely reduced and denatured state in the presence of a cysteine / cystine redox couple. The refolding process was completed after 10 to 30 min and was shown to be strongly dependent on the redox potential and the pH value, but not the temperature. Refolding yields of 60 – 70 % could be obtained even at high concentrations of denaturant (3 M guanidinium HCl or 4 M urea) and protein concentrations of 0.5 mg/ml. By stepwise renaturation a concentration of about 6 mg/ml of native proinsulin was achieved. The refolded proinsulin was correctly disulfide-bonded, native, and monomeric as shown by RP-HPLC, ELISA, circular dichroism, and analytical gel filtration. Treatment of the renatured proinsulin with trypsin and carboxypeptidase B yielded mature insulin.

KEY WORDS

proinsulin, disulfide bond formation, proteolyse, pulse renaturation, oxidative folding

INTRODUCTION

Human proinsulin is an α-helical protein with a molecular mass of 9,500 Da. It can be enzymatically converted to the biologically active insulin used in the treatment of diabetes mellitus (1). Because of a steadily increasing number of people suffering from this desease the amount of insulin needed is rising continously. Several methods exist to produce either proinsulin or insulin in inclusion bodies using recombinant techniques. The recombinant protein has to be isolated, purified if necessary, and renaturated. Renaturation is limited by the formation of the correct disulfide bonds of the protein. Reasonably good refolding yields can be attained by oxidizing the reduced insulin precursor (2,3) or the reduced A and B chain to yield insulin upon renaturation (4). More commonly, proinsulin or the isolated insulin chains are converted into the S-hexasulfonates by sulfothiolysis and subsequently refolded in the presence of redox reagents (5-7).

Proinsulin contains three disulfide bonds [1] $Cys^{7} - Cys^{72}$, [2] $Cys^{19} - Cys^{85}$, [3] $Cys^{71} - Cys^{76}$. Disulfide bond [3] forms very fast and disulfide bond [2] forms more slowly during refolding of the fully reduced insulin precursor (2,5). The insulin precursor folds via different two-disulfide species, among them also non-native species, and it was proposed that there exist at least two different folding pathways (2). All three disulfide bonds are essential for the native conformation of proinsulin as well as for the biological activity of mature insulin (8,9). The A-chain and the B-chain of insulin contain structural information for the formation of the native molecule (10); the C-peptide (connecting peptide) folds autonomously (11).

Here we describe a detailed refolding protocol that allows the production of high amounts of native human proinsulin. Refolding was performed starting from the reduced and urea-denatured or guanidinium HCl-denatured state. All cysteines in proinsulin were completely reduced and were not activated by sulfothiolysis prior to refolding. Furthermore, this protocol demonstrates the efficient conversion of proinsulin to insulin. The described procedure might be of general use to establish a refolding protocol for other recombinant proteins.

MATERIALS AND METHODS

Unfolding and reduction of proinsulin

Recombinant human proinsulin (BIOBRÁS, Montes Claros, Brazil) containing a N-terminal His_8-Arg-tag was denatured in 6 M GdnHCl [2], 10 mM Tris, 1 mM EDTA, pH 8.5, and 6 M urea, 10 mM Tris, 1 mM EDTA, pH 8.5, respectively. Both buffers included DTT in a 100-fold molar excess over free cysteine residues in proinsulin. The samples were incubated for 8 h at 37 °C and then extensively dialyzed at 4 °C against 6 M GdnHCl, 1 mM EDTA, pH 3, and 6 M urea, 1 mM EDTA, pH 3, respectively.

Refolding of proinsulin

Refolding of proinsulin was performed by diluting the reduced and unfolded proinsulin in 10 mM Tris, 10 mM glycine, 1 mM EDTA, with the appropriate pH value, and cysteine / cystine as redox couple. The sample was immediately mixed. At distinct time points aliquots were removed. For reversed phase HPLC (RP-HPLC) analysis using a C_{18} column (Macherey-Nagel), acetonitrile and trifluoracetic acid (TFA) were added to final concentrations of 20 % (v/v) and 0.1 % (v/v), respectively. Refolding yields were determined by quantifying the peak area of the signal of the native species in a linear gradient from 20 % to 55 % (v/v) acetonitrile in 0.1 % (v/v) TFA (12).

The apparent pK_a of the cysteines was determined according to Equation (1):

$$f = \frac{a + b \cdot 10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \qquad (1)$$

where **a** and **b** are the initial and the final yield of refolding, respectively.

For gel filtration the refolded proinsulin was filtered through a 0.2 µm filter and then extensively dialyzed against 20 mM Na-phosphate pH 7.2 at 4 °C.

For CD measurements the RP-HPLC-peak of the native portion of the refolded proinsulin was collected manually and lyophilized. Afterwards, the protein was dissolved in 20 mM Na-phosphate pH 7.2 and extensively dialyzed against the same buffer.

Processing of proinsulin

Refolded proinsulin was converted to mature insulin by proteolysis using trypsin and carboxypeptidase B (1) with a ratio of proinsulin to enzyme of 300 : 1 and 600 : 1 (w/w), respectively. Digestion was performed in 0.1 M Tris/HCl, 1 mM $MgCl_2$, pH 7.5 at ambient temperature for 30 min. The reaction was stopped by addition of EDTA (0.1 M final concentration) and soybean trypsin inhibitor (STI) to a five-fold molar excess over trypsin and subsequently analyzed by an insulin-ELISA as described previously (13). For RP-HPLC analysis, proteolysis was stopped by addition of acetonitrile and TFA to a final concentration of 10 % (v/v) and 0.1 % (v/v), respectively. Digestion products were analyzed with a linear gradient from 10 % to 50 % (v/v) acetonitrile in 0.1 % (v/v) TFA (12).

Pulse renaturation

A step by step renaturation of denatured and reduced proinsulin was performed according to Rudolph and Fischer (14). Refolding of proinsulin was performed in 10 mM Tris, 10 mM glycine, 1 mM EDTA, pH 10.5, 0.5 mM cysteine, 4.5 mM cystine, at 15 °C, and a protein concentration of 0.5 mg/ml proinsulin. After 30 min an aliquot was removed for analysis by RP-HPLC, trypsin / carboxypeptidase B digestion, and ELISA. Subsequently, another pulse of 0.5 mg/ml reduced and denatured proinsulin was added. 30 pulses were performed. At each pulse, the redox substances, lost by the removal of aliquots for analysis, were replenished. The total concentration of GdnHCl and renatured proinsulin increased with each pulse.

CD measurements

The GdnHCl induced transition of proinsulin was measured at 232 nm with an AVIV Model 62ADS circular dichroism spectrometer. Native proinsulin (0.1 mg/ml) in 10 mM Tris, 10 mM glycine, 0.01 mM EDTA, pH 10.5, was incubated in a stirrable cuvette at 15 °C. Denatured proinsulin (0.1 mg/ml in 6M GdnHCl, 10 mM Tris, 10 mM glycine, 0.01 mM EDTA, pH 10.5) was titrated using a computer controlled Hamilton titrator (300 s incubation for each pulse, 99 s averaging time). The concentrations of GdnHCl were determined by refractometry (15).

CD spectra of the native and refolded proinsulin were determined in 20 mM Na-phosphate pH 7.2 at 20 °C using a Jasco J-710 circular dichroism spectrometer. Far-UV spectra of the native proinsulin were recorded at a protein concentration of 0.47 mg/ml in a 0.02-cm cell, averaged over 10 accumulations (50 nm/min). Far-UV spectra of the native portion of the

refolded proinsulin were recorded at a protein concentration of 0.04 mg/ml in a 0.1 cm cuvette, averaged over 10 accumulations (10 nm/min). Spectra were buffer-corrected and mean residue ellipticity values were calculated.

Analytical gel filtration

For analytical gel filtration a Superdex 75 column (bed volume 24 ml, Amersham Pharmacia) was used. Chromatography was performed with 20 mM Na-phosphate pH 7.2, 100 mM NaCl at 25 °C with a flow rate of 0.5 ml/min; 50 µg protein were injected. The column was calibrated with the following marker proteins: aprotinin from bovine lung (6,500 Da), cytochrome c from horse heart (12,400 Da), RNase A from bovine pancreas (13,700 Da), α-chymotrypsinogen A from bovine pancreas (25,000 Da), carbonic anhydrase from bovine erythrocytes (29,000 Da), ovalbumin from hen egg (43,000 Da), and bovine serum albumin (67,000 Da and 133,000 Da for the monomer and dimer, respectively).

RESULTS AND DISCUSSION

Refolding of His_8-Arg-proinsulin

Recombinantly produced proinsulin is the precursor in insulin production for medical utilisation. It's refolding to the native protein and subsequent conversion to insulin are the most critical and sumptuary steps. Therefore, improvements of these reactions would lead to a production process of insulin of a substantially higher efficiency. Recombinant human proinsulin containing an octa-histidin-tag and an additional arginine residue (His_8-Arg-proinsulin) was used to study and optimize the *in vitro* refolding of chemically denatured and reduced proinsulin.

Refolding was performed using the completely reduced proinsulin and not the S-sulfonated form that is often used for renaturation of recombinant proinsulin (5,7). To compare the refolding yield in dependence of the denaturant used for denaturation, refolding was performed starting from the urea-denatured and the GdnHCl-denatured state, respectively. In both cases the random coil state of the denatured and completely reduced protein was confirmed by CD spectroscopy (data not shown). The refolding yield for urea-denatured and GdnHCl-denatured proinsulin was similar in all experiments indicating that the kind of denaturant seems to have no influence on the folding (data not shown).

Refolding of proinsulin was analyzed by RP-HPLC and ELISA. As proinsulin contains six cysteines that form three disulfide bonds in the native molecule, refolding was performed in a buffered redox system. In a first approach, we analyzed the refolding in dependence of the redox potential using the biological redox mediator glutathione and the redox couple cysteine / cystine. In both cases the yield of native proinsulin depended on the redox potential of the refolding buffer (Fig. 1A). The yield of refolding raised the more oxidizing the refolding buffer was. This indicates that the oxidation of free SH groups as the rate limiting step was accelerated. Fast oxidation of disulfide bonds seems to prevent the formation of intermediates susceptible for aggregation (12). As, in most cases, aggregation is a non-productive, off-pathway reaction, which competes with the correct folding of proteins, the prevention of aggregation can increase the refolding yield (16). Refolding with cysteine / cystine yielded about two times more native proinsulin compared to folding in the presence of glutathione (Fig. 1A). Furthermore, optimum renaturation with cysteine / cystine occurred under more oxidizing conditions than with GSH / GSSG. Similar results were obtained for other small redox reagents (cysteamine / cystamine, β-mercaptoethanol / Di(2-hydroxyethyl)-disulfide) (data not shown). This shows that small redox substances as cysteine / cystine rather than the tri-peptide glutathione enabled efficient oxidation and formation of the native proinsulin.

pH- and temperature-dependent refolding of proinsulin

The pH value is a crucial factor in oxidative folding; at basic pH values, reduced and denatured proinsulin could be refolded to a yield of about 60 % (Fig. 1B). The apparent pK_a value was calculated to 8.1 and is therefore in a similar range as the pK_a for cysteine. Considering the pK_a value of the thiolate anion of 8.6 (17), the increased refolding yield at higher pH values is due to faster disulfide bond formation and / or shuffling. Even at pH 10.5 proinsulin could be refolded to a higher yield in the presence of cysteine / cystine compared to glutathione (data not shown). In addition, refolding with cysteine / cystine is completed after about 10 min whereas glutathione-mediated refolding needed about one hour for completion (data not shown). Under more reducing conditions the glutathione-mediated refolding takes about 16 h (2,7). Therefore, the usage of cysteine / cystine seems to enable more efficient refolding of proinsulin in general. Optimum renaturation of proteins at very basic pH values is relatively unusual. However, not only proinsulin but also another member of this protein superfamily, IGF-I, which has three motif-specific disulfide bonds as proinsulin, folds in a pH-dependent manner with a pH optimum around pH 9.0 (18). Although the folding pathway

of proinsulin and IGF-I shows significant differences (2,19), the pH dependencies of refolding seem to be comparable (18).

Usually also the refolding temperature is an important factor as aggregation, proteolytical degradation, or chemical modification are enhanced at elevated temperatures (20). Similar to IGF-I folding (18), proinsulin folding was not temperature-dependent. Although the change in temperature influenced the kinetic behavior of aggregation of folding intermediates and the kinetic of the formation of native proinsulin, it had no significant effect on the final yield (data not shown). Folding at 15 °C was chosen, although neither proteolytical degradation, modification, nor aggregation could be detected, even at 42 °C. In thermal unfolding experiments an engineered insulin monomer showed no significant change in secondary structure up to about 50 °C as measured by CD (21). The stability against thermal unfolding might be one reason why also proinsulin folds independently of the temperature in the range from 10 to 42 °C.

Effect of GdnHCl on refolding proinsulin

The production of proinsulin by renaturation on a technical scale requires high protein concentrations in order to minimize the reaction volume. To analyze if under the conditions described above proinsulin can be refolded to a high concentration of native protein, renaturation was performed with increasing concentrations of denaturant and, furthermore, with increasing concentrations of proinsulin, respectively. The refolding yield remained in a range of about 60 % when refolding was performed in the presence of up to 3 M GdnHCl (Fig. 2A) or 4 M urea (data not shown). This was surprising since under these conditions oxidized proinsulin does not exhibit its native structure. The GdnHCl-induced reversible unfolding transition of proinsulin showed that the protein is native in a range of 0 – 1 M GdnHCl, whereas it is completely unfolded at about 6 M GdnHCl (Fig. 2B). The main structural transition occurred between 3 and 6 M GdnHCl as measured by far-UV CD. However, a pre-transition region (1 – 3 M GdnHCl) could be observed. This first transition which had been previously described (11), was interpreted by Brems and co-workers as a structural change of the C-peptide without significant influence on the A-chain and the B-chain. This might explain that under partially denaturing conditions the native-like conformation of the A-chain and B-chain including all three native disulfide bonds was formed.

Refolding of proinsulin at high protein concentrations

Aggregation of folding proteins is usually enhanced by increasing protein concentrations (20,22). In our case, refolding yields of about 60 % were obtained at proinsulin concentrations of up to 0.5 mg/ml. Even at a protein concentration of 1 – 2 mg/ml, 30 – 40 % native proinsulin could be obtained (Fig. 3A). This might be due to GdnHCl present at 1.5 M in the refolding sample reducing aggregation of folding intermediates. At proinsulin concentrations of 2 – 3 mg/ml, part of the folding proinsulin seems to be trapped in intermediates as observed by RP-HPLC (data not shown). For sulfonated proinsulin Cowley and Mackin described refolding at a protein concentration of 2 mg/ml for 16 h but did not point out their refolding yield (7). In our case, the protein concentration was kept at 0.5 mg/ml to obtain maximum refolding yields; refolding was completed after 20 to 30 min at 15 °C.

To further increase the final concentration of renatured proinsulin, a stepwise renaturation of proinsulin was performed (Fig. 3B) (14,23,24). The protein can be refolded at a moderate concentration which allows maximum folding and after refolding is completed, additional denatured protein can be added as often as necessary. Here, 30 pulses were performed. With each pulse 0.5 mg/ml of reduced and denaturated proinsulin were added and refolded for 30 min. Due to the removal of an aliquot for analysis after each pulse, the total concentration of proinsulin and GdnHCl did not increase linearly. After 30 pulses, the theoretical final concentrations were 8 mg/ml for proinsulin and 3.8 M for GdnHCl (Fig. 3B, dotted line and dashed line). Pulse renaturation is a powerful method to yield a high final concentration of refolded protein as initially described by Rudolph and Fischer (14). In our case, this method enabled a final concentration of native proinsulin of about 6 mg/ml obtained within 15 h of refolding. From the trend of the refolding yield we conclude that even higher concentrations of native proinsulin could be obtained if more pulses would be performed.

Analysis of the refolded proinsulin

Eventually, proinsulin was refolded at 0.5 mg/ml, subsequently dialyzed against 20 mM Na-phosphate pH 7.2, and its native structure verified by different methods. About 70 % of the refolded protein was correctly disulfide-bonded and native as determined by RP-HPLC analysis (Fig. 4A and 4B) and ELISA (data not shown). Analytical gel filtration of the refolded proinsulin confirmed the monomeric form (data not shown). To validate the secondary structure of the native portion of the refolded proinsulin, the native proinsulin was separated from the non-native portion by RP-HPLC. After lyophilisation the protein was dialyzed against phosphate buffer, and far-UV CD spectra of this native proinsulin were

recorded. Both, the native standard and the native part of the refolded proinsulin, showed identical spectra (Fig. 5). They revealed a broad minimum at 206 – 208 nm, a maximum between 190 and 198 nm, and a local minimum around 220 nm. These characteristics indicate a significant α-helical content of proinsulin which can be expected as insulin has about 40 % α-helical structure.

According to Kemmler et al. (1), enzymatic conversion of native proinsulin with trypsin and carboxypeptidase B yielded mainly native insulin, the C-peptide, and only minor by-products (Fig. 4B). As revealed by mass analysis, apart from mature insulin also little amounts of Des-29,30-insulin and Des-30-insulin were obtained. This was due to partial non-specific cleavage of trypsin at the C-terminal side of lysin-29 followed by partial cleavage of this basic amino acid by carboxypeptidase B. The N-terminal tag (His_8-Arg) could be completely removed by trypsin.

Taken together, we showed that proinsulin can be refolded very efficiently starting from the denatured and reduced species. Refolding yields of about 60 to 70 % could be obtained and stepwise refolding allowed 6 mg/ml native proinsulin to be formed which could be converted to mature insulin by partial proteolysis with trypsin and carboxypeptidase B.

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ABBREVIATIONS

[2] The abbreviations used are: CD, circular dichroism; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; GdnHCl, guanidinium hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoracetic acid

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FIGURE LEGENDS

Fig. 1. Refolding of proinsulin. (A) Influence of the redox potential. Denatured and reduced proinsulin was refolded at 37 °C after dilution in 10 mM Tris, 10 mM glycine, 1 mM EDTA, pH 8.0, with different molar ratio of cysteine / cystine (open circle) or GSH / GSSG (black circle). The final concentrations were 50 µg/ml proinsulin, 25 mM GdnHCl, and 5 mM redox components. Samples were analyzed by RP-HPLC after 11 min (cysteine / cystine) or 100 min (GSH / GSSG). RSH corresponds to either cysteine or GSH, and RSSR corresponds to cystine or GSSG. (B) Variation of the pH value. Refolding was performed as described except that 0.5 mM cysteine and 4.5 mM cystine were used as redox couple and the pH values were varied. The data were fittet according to Equation 1 (see Matreials and Methods).

Fig. 2. Influence of the GdnHCl concentration on proinsulin refolding. (A) Variation of the GdnHCl concentration. Denatured and reduced proinsulin was refolded at a final concentration of 50 µg/ml at 15 °C in 10 mM Tris, 10 mM glycine, 1mM EDTA, pH 10.5, 0.5 mM cysteine, 4.5 mM cystine, with different concentrations of GdnHCl. Samples were analyzed by RP-HPLC after 1 h. (B) GdnHCl-induced transition of proinsulin at 15 °C in 10 mM Tris, 10 mM glycine, 0.01 mM EDTA, pH 10.5. The final concentration of proinsulin was 100 µg/ml.

Fig. 3. Influence of the proinsulin concentration on proinsulin refolding. (A) Variation of the concentration of proinsulin. Refolding was performed as described in the legend to Fig. 2A except that the final concentration of denatured and reduced proinsulin was varied and the final concentration of GdnHCl was 1.5 M. Samples were analyzed by ELISA. (B) Pulse renaturation. Refolding was carried out as described in the legend to Fig. 2A except that denatured and reduced proinsulin was added to the refolding buffer pulsewise with a final concentration of 500 µg/ml per pulse. After each 30 min samples were withdrawn and analyzed by RP-HPLC (black circle) and ELISA (open circle). 30 pulses were performed; the concentration of GdnHCl at each pulse (dashed line) and the theoretical amount of proinsulin (dotted line) are shown.

Fig. 4. Secondary structure content of native and refolded proinsulin. Far-UV CD spectra were recorded from 190 – 250 nm at 20 °C in 20 mM Na-phosphate pH 7.2. CD spectra of the native (standard) proinsulin (white circles) were recorded at a protein concentration of 0.47 mg/ml in a 0.02-cm cuvette; CD spectra of the native part of the refolded proinsulin (gray triangels) were recorded at a protein concentration of 0.04 mg/ml in a 0.1-cm cuvette.

Fig. 5. Processing of refolded proinsulin. Denatured and reduced proinsulin was refolded as described in the legend to Fig. 2A except that the final concentration was 500 µg/ml for proinsulin and 240 mM for GdnHCl. After 1 h samples were removed and (A) analyzed by RP-HPLC, and (B) digested with trypsin and carboxypeptidase B followed by RP-HPLC analysis. The digestion was performed with a final concentration of proinsulin of 0.3 mg/ml. As determined by mass spectrometry, peak 1 consists mainly of mature insulin and a small proportion of Des-29,30-insulin with an additional arginine residue. Peak 2 and peak 3 consist of the C-peptide, either mature (main part) or with an additional arginine residue or additional arginine and lysine residues.

Fig. 1

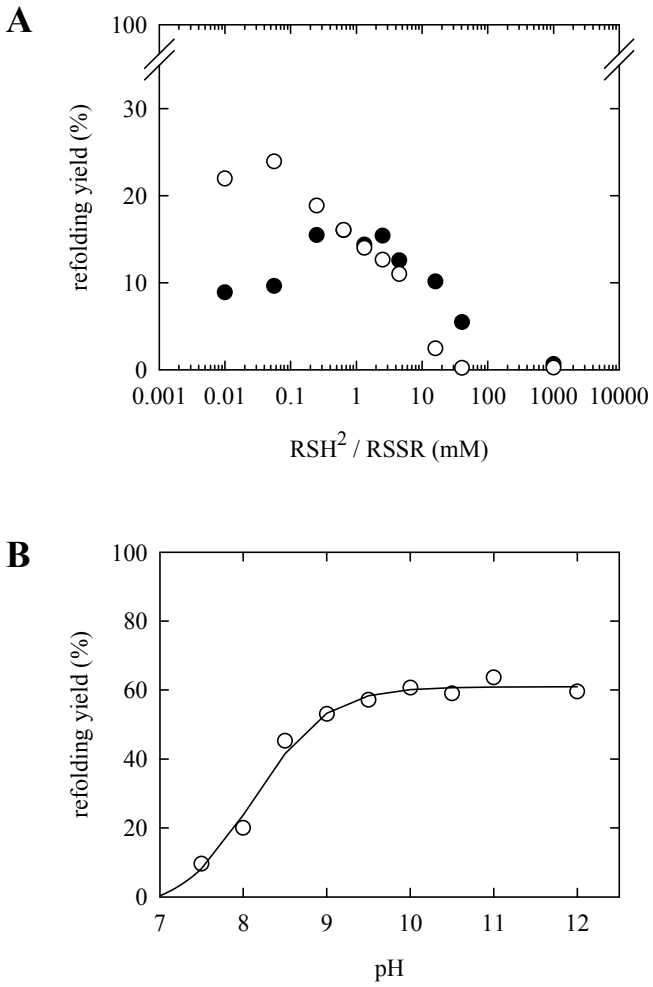

A
100
30
20
10
0
refolding yield (%)
0.001
0.01
0.1
1
10
100
1000
10000
RSH2 / RSSR (mM)
B
100
80
60
40
20
0
refolding yield (%)
7
8
9
10
11
12
pH

Fig. 2

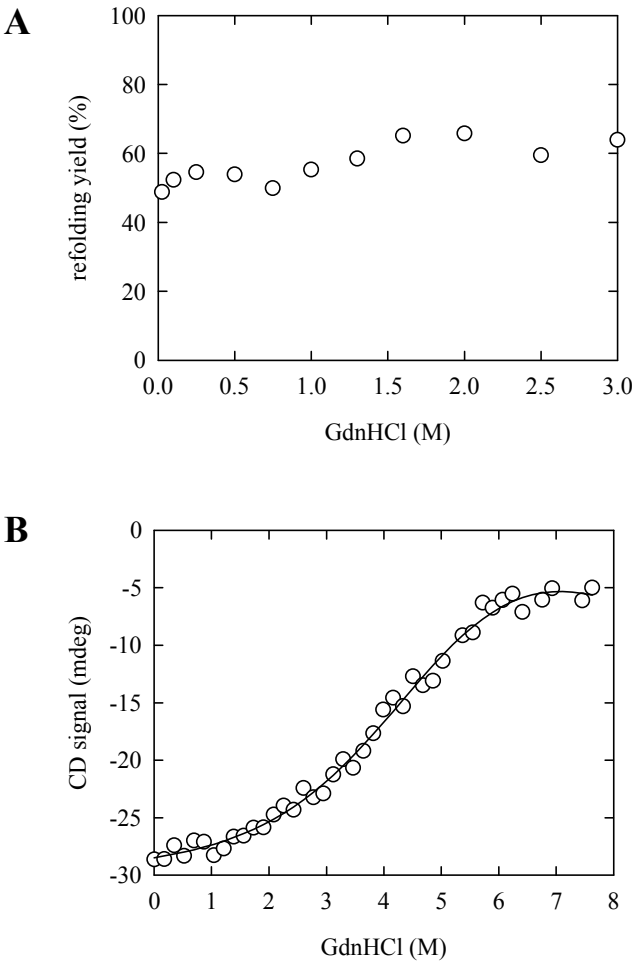

A
refolding yield (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
GdnHCl (M)
B
CD signal (mdeg)
0
-5
-10
-15
-20
-25
-30
0
1
2
3
4
5
6
7
8
GdnHCl (M)

Fig. 3

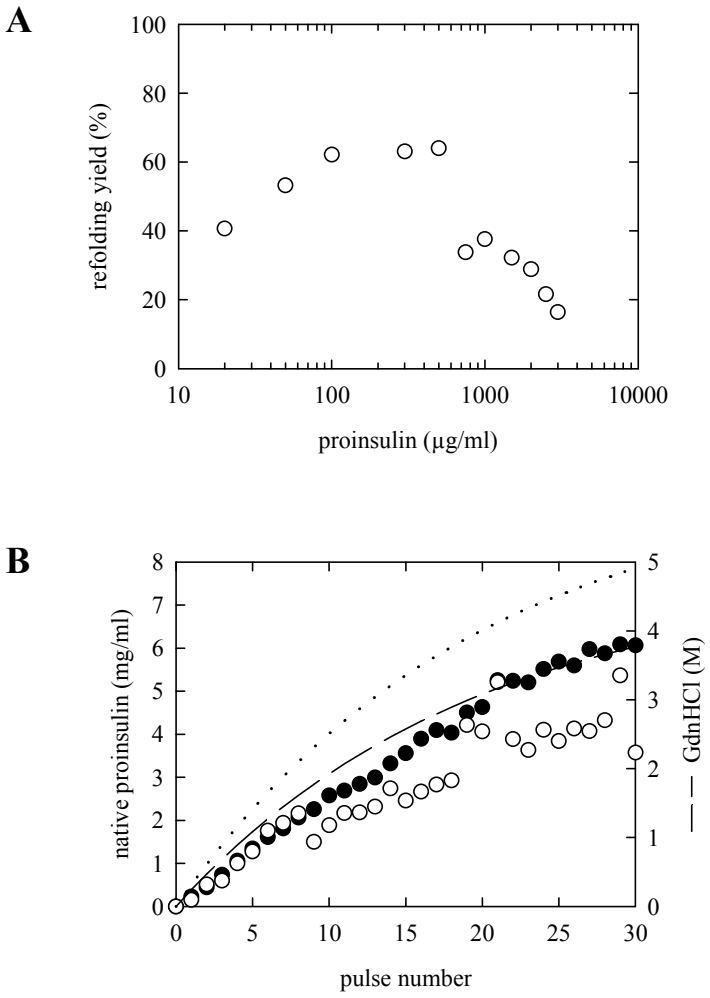

A
refolding yield (%)
proinsulin (µg/ml)
B
native proinsulin (mg/ml)
GdnHCl (M)
pulse number

Fig. 4

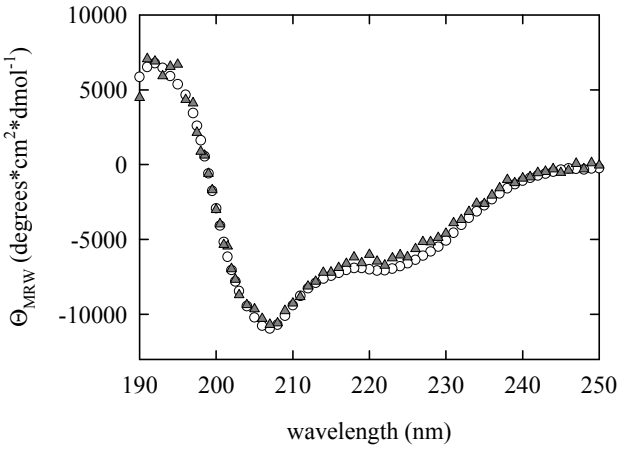
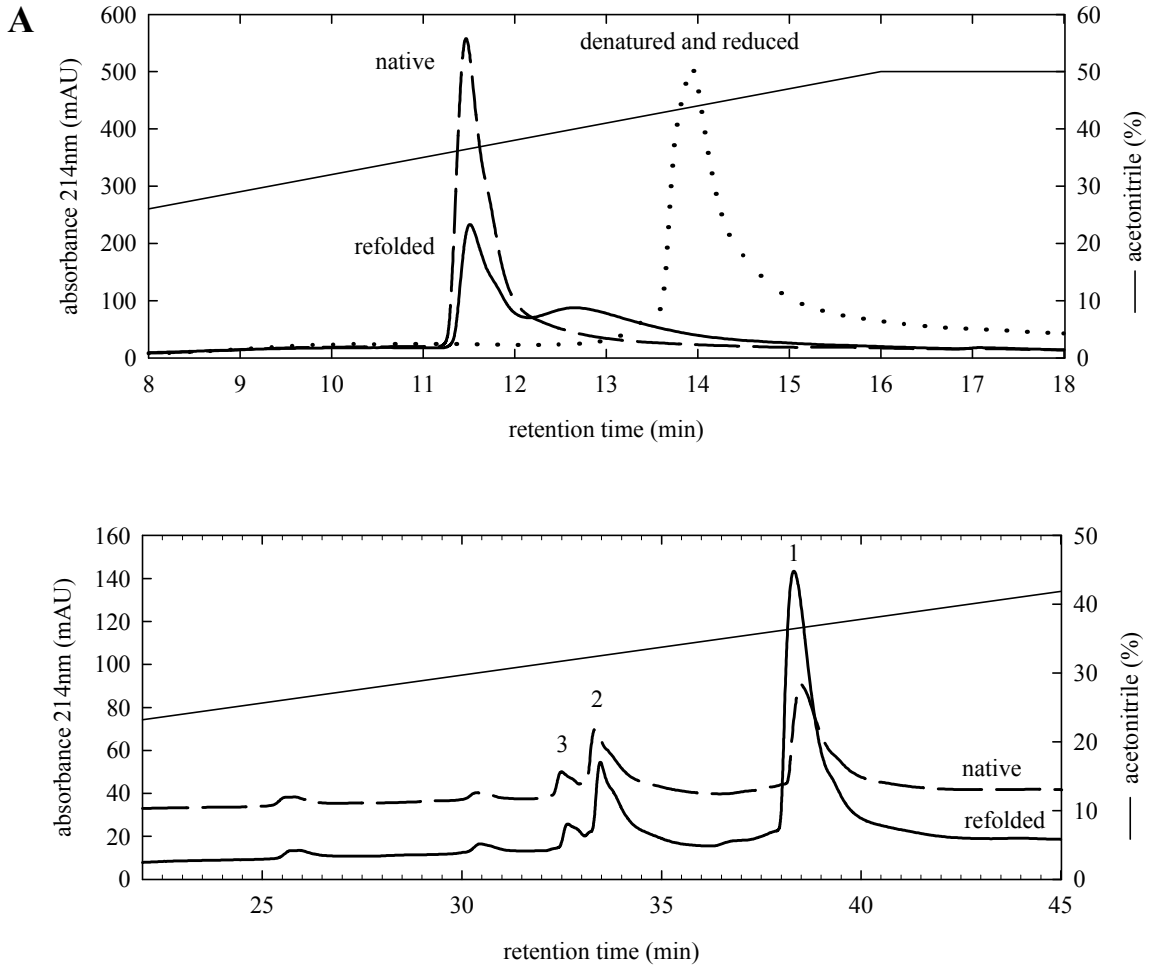

10000
5000
0
-5000
-10000
190
200
210
220
230
240
250
wavelength (nm)
Θ_{MRW} (degrees*cm^{-2}*dmol^{-1})

Fig. 5


A
absorbance 214nm (mAU)
acetonitrile (%)
retention time (min)
native
denatured and reduced
refolded
1
2
3
native
refolded

8 Anhang

Lebenslauf

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10/1993 bis 07/1998	Studium an der Martin-Luther-Universität Halle-Wittenberg im Fach Biochemie
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10/2000 bis 12/2000	Forschungsaufenthalt in der Arbeitsgruppe von Prof. Dr. Robert B. Freedman, University of Kent at Canterbury, Großbritannien; Stipendiat des DAAD

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, daß ich die vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.
Die aus fremden Quellen übernommenen Gedanken sind als solche kenntlich gemacht.
Die vorliegende Arbeit wurde noch keiner anderen Prüfungsbehörde vorgelegt.

Halle/Saale, den 05. April 2000