Protein disulfide isomerase isomerizes non-native disulfide bonds in human proinsulin independent of its peptide-binding activity

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Abstract: Protein disulfide isomerase (PDI) supports proinsulin folding as chaperone and isomerase. Here, we focus on how the two PDI functions influence individual steps in the complex folding process of proinsulin. We generated a PDI mutant (PDI-aba'c) where the b' domain was partially deleted, thus abolishing peptide binding but maintaining a PDI-like redox potential. PDI-aba'c catalyzes the folding of human proinsulin by increasing the rate of formation and the final yield of native proinsulin. Importantly, PDI-aba'c isomerizes non-native disulfide bonds in completely oxidized folding intermediates, thereby accelerating the formation of native disulfide bonds. We conclude that peptide binding to PDI is not essential for disulfide isomerization in fully oxidized proinsulin folding intermediates.

Keywords: oxidative protein folding; disulfide bond formation; chaperone; disulfide isomerization; folding intermediate

Introduction

Many proteins that are secreted into the endoplasmic reticulum require disulfide bond formation, which may be rate limiting in their folding process

Abbreviations: d/r-hPI, denatured, reduced human proinsulin; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; hPDI, human protein disulfide isomerase; IAA, iodoacetic acid; IAM, iodoacetamide.

Additional Supporting Information may be found in the online version of this article.

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and is catalyzed by the enzymes protein disulfide isomerase (PDI) and Ero1.^{1,2} PDI facilitates oxidative folding and disulfide isomerization in a large number of proteins *in vitro*^{3–7} and *in vivo* in the endoplasmic reticulum.^{8–10} Furthermore, PDI can catalyze oxidation and isomerization within kinetically trapped, structured folding intermediates showing that PDI's substrate specificity is not restricted to misfolded proteins containing no or non-native disulfide bonds.¹¹ PDI is a multifunctional and a multidomain protein (for review, see Ref. ¹²). It contains the catalytic domains **a** and **a'** carrying the CGHC active site, the noncatalytic domains **b** and **b'** with **b'** providing the principal peptide-binding site required for PDI's chaperone function, a linker **x** between the domains **b'** and **a'**, and an acidic C-terminal tail **c** in the domain order **abb'xa'c**.^{13–16} The linker **x** reversibly interacts with

b' thereby mediating access of substrates to the ligand-binding site.¹⁷ The structures of individual PDI domains and full-length PDI have been solved revealing that all domains contain the thioredoxin fold and that the active sites of **a** and **a'** lie close to each other supposedly allowing for efficient disulfide isomerization.¹⁴ Disulfide rearrangements in a substrate require reduced active-site thiols, which form mixed disulfides with substrate thiols and are released upon isomerization (see Ref. ¹²). Upon oxidation of substrate thiols and concomitant disulfide bond formation, however, PDI's active-site cysteines are left in a reduced state. They now require reoxidation to allow for another oxidation cycle, which is facilitated by the thiol oxidase Ero1.² Ero1 transfers electrons onto its cofactor FAD and eventually onto molecular oxygen as terminal electron acceptor.^{2,18,19} Both PDI and Ero1 are essential for the viability in yeast,^{2,20} emphasizing the importance of correct disulfide bond formation in secretory proteins.

PDI is a functionally versatile enzyme. Not all of its domains are essential for certain activities. Single catalytic domains of PDI (**a** or **a'**) can accomplish simple oxidation reactions,²¹ and PDI variants carrying only two or three domains are functionally active. The domain construct **b'xa'** interacts functionally with Ero1-L α ²² and serves as a functional subunit of prolyl-4-hydroxylase,²³ whereas **bb'xa'** is able to dissociate the cholera holotoxin.²⁴ However, if a high degree of conformational changes is involved in disulfide rearrangements within the substrate, all four domains of PDI are required.²¹ In the case of human proinsulin (hPI) folding, catalysis by full-length human PDI (hPDI) is significantly more efficient than catalysis by **b'a'c**,⁴ suggesting that both active sites are required for full disulfide isomerization.⁴ PI contains the three disulfide bonds Cys⁷–Cys⁷², Cys¹⁹–Cys⁸⁵, and Cys⁷¹–Cys⁷⁶, all of which are essential for biological activity.²⁵ The folding pathways for hPI and porcine insulin precursor have been proposed.^{26,27} Both involve formation of non-native disulfide bonds that spontaneously isomerize to form the native species. The existence of non-native disulfides during hPI folding emphasizes the requirement of catalyzed disulfide bond isomerization to allow fast and efficient folding. hPDI significantly catalyzes hPI folding; both hPDI's catalytic activity in combination with its peptide-binding ability are essential for rapid and efficient hPI folding.⁴ A hPDI variant lacking the active-site cysteines cannot accelerate the oxidative folding of hPI and hPDI with its peptide-binding site blocked by the inhibitor genisteine is unable to suppress aggregation, thus resulting in hPI folding that is similar to the noncatalyzed folding.⁴ In this report, we further dissect the requirement of chaperone and isomerase function of hPDI for hPI folding and generated the variant PDI-aba'c that completely lacks peptide

binding. hPI folding proceeds via many intermediates some of which are fully oxidized; their formation and isomerization to native hPI are catalyzed by hPDI and PDI-aba'c. This suggests that hPI binding to PDI is not essential for disulfide rearrangements in folding intermediates containing non-native disulfides.

Results

Generation of a PDI variant lacking peptide-binding activity

The domains of PDI are arranged in the order **abb'xa'c** with substrate binding occurring to **b'**. Thus, deletion of the **b'** domain should diminish substrate-binding activity of PDI. The crystal structure of yeast PDI¹⁴ suggests that deletion of **b'** should not affect the overall structure of the remaining domains with thioredoxin fold. Most of the **b'** domain of PDI was removed (amino acids 229–333) thereby maintaining the N-terminal amino acids 218–228 of **b'** and the C-terminal amino acids 334–351 corresponding to the linker **x**. This links **b** to the **a'** domain by a 27 amino acid linker allowing for a maximum of flexibility, which, in full-length PDI, is provided by the linker **x**.¹⁷ The mutant PDI-aba'c was solubly expressed in *E. coli*, purified to homogeneity, and the correct molecular mass confirmed by mass spectrometry (data not shown). Far-UV CD spectra of hPDI and PDI-aba'c were recorded (data not shown), and data were deconvoluted using the program CDNN.²⁸ This analysis revealed very similar secondary structural content of both proteins (30% α -helix, 20% β -sheet, 18% β -turn, and 32% random coil). Further, both proteins are monomeric in solution as shown by analytical ultracentrifugation [PDI-aba'c: $s_{app} = 2.49$ S, $M_{r app} = 47.6$ kDa (theoretically 44.2 kDa); hPDI: $s_{app} = 3.16$ S, $M_{r app} = 57.7$ kDa (theoretically 56.3 kDa)]. This indicates that PDI-aba'c constitutes a stably folded monomeric protein similar to hPDI.

PDI-aba'c shows no chaperone activity but a WT-like redox potential

To test if PDI-aba'c functionally interacts with protein substrates, we analyzed the chaperone activity of PDI-aba'c toward chemically denatured GAPDH. GAPDH is a suitable model protein because PDI assists its folding although it does not contain cysteine residues forming disulfide bonds.^{7,29} hPDI at a 10-fold molar excess to GAPDH increased the yield of active GAPDH from 8 to about 20% [Fig. 1(A)]. Also, PDI Δ C1,2, where the active-site cysteines are substituted by serine,⁴ chaperones GAPDH folding although to a lower extent than hPDI as shown before.⁴ In contrast, PDI-aba'c did not assist reactivation of GAPDH even at a 256-fold molar excess over GAPDH [Fig. 1(A)]. Thus, deletion of the **b'**

Figure 1. Comparison of the biophysical properties of hPDI and PDI-aba'c. (A) Refolding of GAPDH was analyzed in the presence of the indicated molar ratio of hPDI (○), PDIΔC1,2 (▼), and PDI-aba'c (△), respectively. BSA (□) and carbamidomethylated PDI-aba'c (◆) served as control. The average of at least two independent experiments is given. (B) Redox equilibria of hPDI and PDI-aba'c with glutathione. hPDI (○) or PDI-aba'c (□) was incubated in refolding buffer with different molar ratios of GSH to GSSG (total concentration 2 mM). Fluorescence values as recorded at 332 nm (hPDI) and 335 nm (PDI-aba'c) are shown. The redox potential of hPDI and PDI-aba'c was calculated to be -0.15 V.

domain in PDI resulted in complete loss of chaperone function toward GAPDH. Because the chaperone activity of PDI depends on its ability to bind to a substrate, we can exclude that PDI-aba'c shows peptide-binding activity. To test this, we analyzed the binding of the radiolabeled peptide Δ -somatostatin to PDI-aba'c. Binding of this peptide to hPDI was shown before by crosslinking experiments.^{4,15} In contrast to hPDI, no crosslinking products of PDI-aba'c with Δ -somatostatin were observed, thus confirming the loss of peptide-binding ability of PDI-aba'c (data not shown).

We then tested the redox activity of PDI-aba'c toward glutathione. An identical redox potential of $E^{\circ'} = -150$ mV was determined for hPDI and PDI-aba'c [Fig. 1(B)], which is similar to previous reports

(-163 mM to -175 mV)^{30,31} and to that of the bacterial disulfide isomerase, DsbC (-143 mV).³² This demonstrates that deletion of the b' domain of PDI does diminish chaperone activity but did not affect the redox activity toward glutathione as substrate.

PDI-aba'c exhibits redox activity in hPI folding

PDI increases the rate and yield of oxidative folding of hPI.⁴ We now used hPI to investigate the redox properties of PDI-aba'c in more detail. Oxidative folding of denatured and reduced hPI (d/r-hPI) was assayed in the absence of folding catalysts (spontaneous) and in the presence of either hPDI, PDIΔC1,2, or PDI-aba'c (Fig. 2). Spontaneous refolding of hPI to the native state proceeded with $k_{app} = 0.0022 \pm 0.0008$ s⁻¹. hPDI and PDI-aba'c at a 2:1

Figure 2. Influence of hPDI and PDI-aba'c on the kinetics and yield of hPI folding. Refolding of d/r-hPI was performed in refolding buffer with 1 mM GSH and 2 mM GSSG. Aliquots were removed at the indicated time points, folding stopped, and samples analyzed by RP-HPLC. (A) Spontaneous refolding of hPI (△) and in the presence of a twofold molar excess of hPDI (○), PDI-aba'c (□), and PDIΔC1,2 (◇). Data were fitted single exponentially. (B) Refolding of hPI with different molar ratios of hPDI (○), PDI-aba'c (□), and PDIΔC1,2 (◇). Shown is the yield of native hPI analyzed after the renaturation was completed; given is the average and error of two to five independent experiments.

Figure 3. Formation of folding intermediates. hPI was refolded and analyzed as described in Figure 2 with a 1:5 molar ratio of hPDI to hPI. Refolding was stopped after 20 s (lower panel) or 90 s (upper panel). Early intermediates, late intermediates (numbered consecutively), and native hPI are indicated.

molar ratio to hPI increased this rate by 36- and 3-fold, respectively, whereas PDI Δ C1,2 did not influence the rate because it lacks redox activity [Fig. 2(A)]. The overall yield of native hPI at the 2:1 molar ratio was increased by all three PDI variants, that is, 2.5-fold (hPDI), 2-fold (PDI-aba'c), and 1.4-fold (PDI Δ C1,2) [Fig. 2(B)]. The higher rate and yield of PDI-aba'c-catalyzed folding compared to PDI Δ C1,2 suggests that PDI-aba'c has retained a significant redox activity even though to a decreased extent compared to hPDI.

PDI accelerates formation and conversion of intermediates

Detailed analysis of the complex hPI folding process by RP-HPLC allowed detection of about 20 different folding intermediates (Fig. 3). The majority of these intermediates (early intermediates) are quickly converted to intermediates with a lower retention time in the RP-HPLC gradient (late intermediates), which are numbered consecutively as I₂, I₄, I₅, I₇, and I₈. The lower retention time indicates that these intermediates have a higher degree of disulfide linkage (see below).

Very similar (late) folding intermediates were observed during spontaneous and hPDI-, PDI Δ C1,2-, and PDI-aba'c-assisted hPI folding (Fig. 4). The main differences concern their kinetics of formation

and disappearance resulting in a different pattern and amount of intermediate populations in the catalyzed and noncatalyzed reactions. In spontaneous folding, early intermediates were detectable within the first 5 min and late intermediates showed a lag phase of accumulation of up to 2 min (Fig. 4, upper left panel). The kinetics of formation of late intermediates was slightly faster than the overall folding of hPI. The intermediate population was similar in the PDI Δ C1,2-assisted reaction and might explain the only slightly higher yield of native hPI observed for PDI Δ C1,2-assisted folding. Some intermediates persisted over a longer period of time probably because of prolonged binding to PDI Δ C1,2 (Fig. 4, upper right panel).

In contrast, the rate of formation of all intermediates was several-fold higher in the presence of catalytically active PDI. Early intermediates could not be detected in the hPDI- or PDI-aba'c-catalyzed reactions. They were converted to late intermediates or native hPI within the first 30 s. Thus, hPDI and PDI-aba'c significantly increased the kinetics of formation of late intermediates (see Fig. 4, lower two panels). Importantly, the hPDI-catalyzed formation of late intermediates is faster than the formation of native hPI, indicating that their formation is not rate limiting with hPDI present. For PDI-aba'c-catalyzed folding, especially the formation of the intermediates I₇ and I₈ was much faster than the overall folding process. This suggests that PDI-aba'c has a different preference for some intermediates than hPDI. Thus, the acceleration of the overall hPI folding by hPDI and PDI-aba'c is in part due to the shuffling of early into late intermediates.

Detailed analysis of folding intermediates

To better understand the difference in intermediate formation and their conversion to the native state catalyzed by hPDI and PDI-aba'c, respectively, we analyzed the intermediates in more detail. The late intermediates I₂, I₄, I₅, I₇, and I₈ were isolated by RP-HPLC. Their disulfide status was determined by thiol trapping followed by analysis on urea gels, which allows comparing the electrophoretic mobility of differentially modified intermediates or control samples. All five intermediates showed an identical mobility on urea gels, which corresponded to completely oxidized proinsulin (Fig. 5). Thus, all late intermediates contain three non-native disulfide bonds.

Next, we analyzed the refolding of I₂, I₄, I₅, I₇, and I₈ after denaturation. All intermediates could convert to native hPI (Fig. 6, circles) and into one another (Fig. 6, shown for all intermediates; note that the symbols for the respective intermediate are identical in all panels), which explains the similar kinetic behavior of the intermediates upon hPI folding. Because the intermediates appear to be in

Figure 4. RP-HPLC chromatograms of hPI refolding with PDI-variants. hPI was refolded and analyzed as described in Figure 2. For all figures, the same scale was chosen. The peaks for early and late intermediates and native hPI (N) are indicated. Time courses similar to Figure 3 are shown for hPI folding noncatalyzed and with a 2:1 ratio of PDI Δ C1,2 or a 1:5 ratio of hPDI or PDI-aba'c. The total recovery of hPI (sum of all peaks during folding) was comparable for all folding reactions.

equilibrium, it is impossible to dissect whether all or only a particular intermediate is able to convert directly to the native state.

The rate with which the intermediates spontaneously converted to native hPI was similar [$k_{app} = 0.0008\text{--}0.001\text{ s}^{-1}$; Figs. 6 and 7 (shown for I_4)]. This rate is comparable to the overall hPI folding reaction indicating that the conversion of intermediates to native hPI is the major rate-limiting step. hPDI and PDI-aba'c significantly increased the yield (2.5-fold)

and the rate of native hPI formation from the late intermediates (Fig. 7). Although hPDI was already effective at a 1:5 molar ratio to hPI ($k_{app} = 0.0018\text{--}0.0024\text{ s}^{-1}$), PDI-aba'c was required at higher concentrations to substantially accelerate formation of native hPI [$k_{app} = 0.0033 \pm 0.0006\text{ s}^{-1}$ (2:1 ratio)]. Importantly, both hPDI and PDI-aba'c catalyzed the conversion of late intermediates to the native state, strongly suggesting that PDI-aba'c exhibits isomerase activity independent of chaperone activity.

Figure 5. Determination of the number of disulfide bonds in hPI intermediates on an 18% urea gel. Bands corresponding to fully IAM-modified or IAA-modified hPI, corresponding to the fully reduced or oxidized species, respectively, are indicated. d/r-hPI was completely IAM modified (lane 1) or mixed with partially IAA-modified hPI (lane 2, * indicates d/r-hPI) or competitively modified with IAM and IAA (lane 8). I_2 , I_4 , I_5 , I_7 , and I_8 (lanes 3–7) were first IAM modified, reduced with DTT, and subsequently IAA modified. Note that I_2 to I_8 are completely IAA modified indicating that they are completely oxidized.

Figure 6. Spontaneous refolding of I_2 , I_4 , I_5 , I_7 , and I_8 . Intermediates were denatured and refolded as described in Figure 2. Peak areas were determined using peak fit. Note that intermediates convert into one another [I_2 , ∇ ; I_4 , $\square$; I_5 , $\diamond$; I_7 , $\triangle$; and I_8 (hexagon) and into native hPI ($\circ$)]. In the panel showing I_5 , please note that the curve describing the disappearance of I_5 was fitted to better visualize this intermediate.

Supposedly, the pronounced effect of hPDI on hPI folding compared to PDI-aba/c is due to binding of the protein substrate to hPDI, thus increasing the local concentration of catalytic activity in the vicinity of folding hPI. As a second possibility, hPDI might play a more active role by partially unfolding hPI upon binding and thus increasing the accessibility of shielded non-native disulfides in the late folding intermediates. We tested this hypothesis by analyzing I_4 folding in the combined presence of (i) PDI Δ C1,2 and hPDI thus adding an excess substrate-binding activity and (ii) PDI Δ C1,2 and PDI-aba/c thus combining substrate binding and isomerase activity in trans. The additional presence of substrate-binding activity did not affect either of the catalyzed reactions (Fig. 7, shown for I_4 with

PDI Δ C1,2 + PDI-aba/c). This indicates that either (i) PDI Δ C1,2 binds to intermediates thus making its thiols inaccessible to PDI or possesses no unfolding activity regarding the hPI folding intermediate or (ii) that unfolding of the intermediate is not rate limiting for isomerization to the native state.

Discussion

In this study, the PDI variant PDI-aba/c was generated that lacks the **b'** domain and thereby lacks substrate binding but retained redox activity. Both hPDI and PDI-aba/c significantly increased the kinetics and yield of the overall hPI folding reaction and the isomerization of late intermediates to the native state. However, only hPDI could fully support hPI folding, which is in line with previous studies

Figure 7. Refolding of I_4 spontaneously or with PDI variants. I_4 was denatured and refolded as described in Figure 2. The time courses of formation of native hPI (given as relative peak areas) during spontaneous folding (Δ), and with PDI Δ C1,2 ($\diamond$; 2:1 molar ratio to hPI), hPDI ($\circ$, 1:5 ratio), or PDI-aba'c ($\square$; 1:5 ratio), PDI-aba'c ($\blacksquare$; 2:1 ratio), and a combination of PDI Δ C1,2 and PDI-aba'c ($\blacklozenge$; 2:1 and 1:5 ratio) are shown.

showing that all domains of PDI are required for binding of protein substrates and complex disulfide arrangements.^{4,15,21} The results strongly suggest that PDI-aba'c possesses significant isomerase activity, which was apparently not completely abolished despite the lack of peptide binding. The presented data also indicate that hPI folding is accelerated by increasing the rate of oxidation and disulfide isomerization in fully oxidized intermediates (see Figs. 4 and 6). That quick disulfide shuffling increases formation of native hPI was also observed when d/r-hPI was refolded at increasing pH,³³ which increases the reactivity of the cysteine thiol and concomitant disulfide bond formation.

We performed refolding under conditions that allow detection of many disulfide intermediates, including early ones with likely one or two disulfide bonds and fully oxidized ones that carry non-native disulfides. Partially oxidized intermediates were observed for porcine insulin precursor²⁷ but not hPI before,²⁶ most likely because refolding of hPI was performed at alkaline pH²⁶ where hPI folding is completed very quickly.³³ In contrast, fully oxidized intermediates with non-native disulfide bonds were described for hPI²⁶ and insulin-like growth factor 1, whose folding is comparable to hPI folding.³⁴ We identified and isolated five fully oxidized folding intermediates; their isomerization to native hPI is rate limiting in the overall folding process. Isomerization to the native state proceeded for all late intermediates with similar rates. This suggests that either all intermediates directly convert to native hPI or that a very fast interchange between the intermediates precedes formation of the native state. Therefore, from our data, there is no indication that

hPI folding proceeds via one particular intermediate as suggested before.²⁶

The thiols and disulfides in hPI folding intermediates containing non-native disulfide bonds are presumably perfectly solvent exposed (see Ref. ³⁵). Thus, catalysis of disulfide shuffling may profit little from concomitant binding of PDI to hPI. Furthermore, fully oxidized hPI intermediates are not aggregation prone because aggregation only occurs in the first seconds of hPI refolding⁴ and late intermediates are detectable within the first minutes of folding (see Fig. 4). Thus, at the late stage of folding, even the low isomerase activity of PDI-aba'c could catalyze conversion of intermediates to the native state. The same argument would hold for the fact that we could not detect unfolding activity of PDI toward hPI. The difference between hPDI and PDI-aba'c in catalyzing protein folding might be more pronounced in case of more complex proteins where non-native disulfide bonds might be partially shielded from the solvent by residual protein structure and where such folding intermediates readily aggregate.

Taken together, we show here that hPDI and PDI-aba'c affect the folding process of hPI at the stage of conversion of early to late intermediates and isomerization of fully oxidized intermediates to native hPI. Catalysis of the first stage prevents aggregation and consequently increases the yield of refolding. The second step is overall rate limiting; its catalysis thus leads to an increase in rate of the overall folding process. We conclude that PDI-aba'c has significant isomerase activity and that peptide binding to PDI is not essential for its isomerase activity toward hPI.

Materials and Methods

Generation of PDI-aba'c

pET23-PDI encoding hPDI with N-terminal His₆-tag (hPDI) and the primer pair 5'CACCGCTTCCTGGAGGGCAAAATC^{3'} and 5'GGTCCTCGAGATCGTCATCATC^{3'} were used to amplify the complete plasmid apart from the base pairs 685–1000 in the His₆-PDI-encoding sequence, thus deleting amino acids 229–333 in His₆-PDI (corresponding to 246–350 in hPDI with signal sequence and 222–332 in mature yeast PDI). pET23-PDI-aba'c was generated following blunt end ligation, and the correct sequence was verified by DNA sequencing.

Expression and purification of PDI and PDI variants

Production, purification, and carbamidomethylation of hPDI variants were performed as described.⁴ Purified proteins were freeze dried if necessary. The concentration of purified protein was determined at 280 nm using the molar absorbance coefficient of

45,380 M⁻¹ cm⁻¹ (hPDI) and 42,400 M⁻¹ cm⁻¹ (PDI-aba'c). Ten micrograms of purified protein showed a single band on a reducing 12% SDS gel stained with Coomassie blue.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed using an analytical ultracentrifuge Optima XL-A, an An50Ti rotor, and double sector cells (Beckman Instruments). Sedimentation velocity was measured at 40,000 rpm, 20°C, and scans were taken every 10 min. The apparent molecular weight was calculated from sedimentation equilibrium at 8000 and 10,000 rpm. Data were analyzed at wavelengths of 230, 250, and 280 nm using the software provided by Beckman Instruments.

Crosslinking

Δ-somatostatin (Ala-Gly-Ser-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Ser) was synthesized as described¹⁵ and Bolton-Hunter-¹²⁵I-labeled as recommended by the manufacturer. Crosslinking of ¹²⁵I-Δ-somatostatin with the *E. coli* cell extract that produced hPDI or PDI-aba'c (10 μg/mL) was performed as described.⁴

GAPDH assay

Denaturation and reactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma) were performed according to Ref. ⁷. For details, please see the Supporting Information.

Determination of the redox potential

Redox activity was determined as described³⁶ with the indicated molar ratio GSH:GSSG (2 mM total concentration). For details, see the Supporting Information.

Unfolding and refolding of hPI

Denatured, reduced His₈-Arg-proinsulin (d/r-hPI) was prepared as described.⁴ Refolding of d/r-hPI was performed at 25°C in refolding buffer (10 mM Tris/10 mM glycine, pH 7.5, 1 mM EDTA) containing 1 mM GSH, 2 mM GSSG, and 100 μg/mL hPI. Aliquots were removed, acetonitrile/trifluoroacetate [final concentration: 20% (v/v)/0.1% (v/v)] was added, the samples were analyzed by RP-HPLC, and folding intermediates were quantified using the program peak fit (systat). For details, see the Supporting Information.

Refolding of intermediates

Folding intermediates were isolated from RP-HPLC, rechromatographed to ensure homogeneity, and dried. The dried protein was dissolved in 6M urea, 1 mM EDTA, pH 3, allowing complete solubilization of the intermediates. Then, refolding buffer was added, samples removed within 2 h, and analyzed by RP-

HPLC. Intermediates and native hPI were analyzed and quantified as described above.

Thiol trapping

Intermediates were successively modified with iodoacetamide (IAM), dithiothreitol (DTT), and iodoacetic acid (IAA) and analyzed on 18% urea gels as described in the Supporting Information.

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