Recognition of Protein Substrates by Protein-disulfide Isomerase

A SEQUENCE OF THE b' DOMAIN RESPONDS TO SUBSTRATE BINDING

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Refolding of partially folded mitochondrial malate dehydrogenase (mMDH) is assisted by protein-disulfide isomerase (PDI). The addition of a 20-fold molar excess of PDI over denatured protein (0.1 μM) accelerates the recovery of catalytic activity. PDI fluorescence measurements show that 1 mol of PDI binds 1 mol of denatured mMDH when their concentrations approach 1 μM. The binding of PDI, derivatized with the fluorescence probe iodoacetamide fluorescein, to partially folded mMDH is characterized by a dissociation constant of 0.2 μM.

It is shown that the fluorescence probe is covalently attached to a SH residue located in the b' domain. Based on the fluorescence measurements of native and derivatized PDI, it is suggested that recognition of the unfolded substrate involves conformational changes propagated to several domains of PDI.

Protein-disulfide isomerase (PDI) is a multifunctional enzyme that both catalyzes the formation of disulfide bonds (1, 2) and acts as a subunit of prolyl-4-hydroxylase (3). PDI has been proposed to function as a molecular chaperone by binding to unfolded protein species, thereby preventing aggregation and misfolding (4, 5). Despite these interesting studies, the situation is complicated because PDI, unlike other chaperones, catalyzes disulfide bond formation and reduction.

Several laboratories have attempted to show the site of binding of small molecular weight polypeptides that compete with misfolded protein substrates. Mutated PDI with the carboxyl terminus deleted shows neither peptide binding nor chaperone activity in assisting the refolding of denatured n-glyceraldehyde-3-phosphate dehydrogenase (6). On the other hand, it has been reported that deletion of the carboxyl-terminal domain (C domain) has no inhibitory effect on the assembly of recombinant prolyl-4-hydroxylase (7). Other investigators have reported that small molecular weight peptides bind to the b' domain of PDI (8).

The possibility exists that more than one site in the structure of PDI is involved in recognition and refolding of protein substrates. The binding and hydrolysis of ATP by PDI has been reported by Guthapfel et al. (9); strikingly, the ATPase reaction is stimulated in the presence of denatured polypeptides, whereas the disulfide oxidation of PDI is not influenced by ATP. However, the functional role played by ATP hydrolysis during the refolding of denatured proteins has not been investigated in detail.

The aim of the present work is 2-fold: first, to study regions of the primary structure of PDI involved in recognition of unfolded protein substrates and, second, to investigate whether the free energy of hydrolysis of ATP is required for unfolding of misfolded protein substrates.

EXPERIMENTAL PROCEDURES Purification of Proteins—PDI was purified by the method described in Ref. 10 with small modifications. Fresh porcine livers (600 g) were homogenized in 0.1 m M phosphate (pH 7.5) containing 1% Triton X-100 and 5 mM EDTA. After centrifugation, the supernatant was treated with ammonium sulfate, and the fractions obtained between 55–85% saturation were suspended in 25 mM citrate buffer (pH 5.3), dialyzed against the same buffer (buffer A), and applied to a DEAE-Sepharose fast flow column, which was eluted using a linear gradient of 0–0.7 mM NaCl in buffer B. Purified PDI was kept at 4 °C and used in subsequent studies.

The concentration of PDI was determined using absorbance at 280 nm = 1 for 1 mg of protein/ml (11). The activity of PDI was determined using the insulin reduction assay as described in Ref. 11.

The Escherichia coli GroEL-GroESL gene (12), inserted in a plasmid provided by Dr. F. Larimer (Oak Ridge Laboratories), was expressed in E. coli strain BL 21 (DE 3) cells. The protein GroEL was purified by modifications of the procedure included in Ref. 13. After ammonium sulfate fractionation, the protein was purified by means of three chromatographic steps: DEAE-Sephacel, gel filtration through Sepharose CL-4B, and affinity chromatography through red agarose (14). The last step removes contaminating proteins trapped by GroEL. The protein concentration was calculated using absorbance at 280 nm = 0.15 for 2.5 mg of protein/ml.

Purification of Proteins—Porcine heart mMDH was purchased from Roche Molecular Biochemicals. The enzyme was dialyzed against 20 mM Tris/HCl buffer, pH 7.5, at 4 °C, applied to a DEAE-cellulose column, and eluted by means of a linear gradient made with the equilibrium buffer (20 mM) and the same volume of 100 mM Tris/HCl (pH 7.5). The active fractions were concentrated by ultrafiltration. The enzyme concentration was calculated using A280 = 25 for a 1% solution, (15).

Denaturation of mMDH—A solution of MDH (5 mg/ml) was prepared in 100 mM Tris/HCl, pH 7.5, containing 3 mM GdnHCl and allowed to denature at 25 °C for 1 h. The protein solution was 5-fold diluted with 100 mM Tris/HCl, pH 7.5, and then passed through a Sephadex G-25 column to remove GdnHCl. Renaturation of MDH was initiated by diluting denatured protein (0.1 μM) in renaturation buffer and incubated for 3 h at 25 °C. The renaturation buffer consisted of 100 mM Tris/HCl, pH 7.5, dithiothreitol (1 mM), and KCl (0.1 mM) with and without chaperone proteins. Aliquots of this renaturing reaction were withdrawn at specific times and assayed for MDH activity. The assay buffer consisted of 100 mM Tris/HCl, pH 7.5, 0.5 mM oxaloacetate, and 0.2 mM NADH. The initial rate of conversion of NADH to NAD was determined by measuring changes in absorbance for 1 min at 25 °C.

Materials—Porcine liver was obtained from a local slaughterhouse. DEAE-Sephacel, DEAE-Sephrose CM-cellulose, DEAE-cellulose, and Sephadex-G-25 were purchased from Amersham Pharmacia Biotech. Insulin, GSH, NADPH, DTT, glutathione reductase, and scav-
blied RNase A were purchased from Sigma. The polypeptide mastoparan was obtained from Sigma. The reagent iodoacetamide fluorescein was purchased from Molecular Probes, Inc. (Eugene, OR).

**Labeling of PDI—**PDI (1 mg/ml) was reacted with iodoacetamide fluorescein (IAF) at a final concentration of 0.1 mM in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 M GdnHCl at 4 °C for 12 h. Under this set of experimental conditions, SH groups located in the central domain of the protein are exposed to the alkylating reagent.

The labeled protein was dialyzed against 0.1 M potassium phosphate (pH 7.4) to remove unreacted dye, followed by gel filtration chromatography on Sephadex-G-25. The labeled protein displayed catalytic activity when assayed using insulin as a substrate, suggesting that alkylation on the thioredoxin domain has not taken place. The degree of labeling (1.3 mol of IAF/mol of monomer) was determined by using an extinction coefficient of 4.9 × 10^4 M^-1 cm^-1 at 490 nm.

**Purification of the Peptide Containing the Modified Residue—**The modified protein was denatured in 0.8 ml of 6 M guanidinium chloride containing dithiothreitol (1 mM) for 1 h at 37 °C. A freshly prepared solution of 20 mM iodoacetic acid was then added, and the mixture was incubated in the dark at room temperature for 30 min. The mixture was then dialyzed against 2 liters of 0.1 M ammonium bicarbonate.

The labeled protein (100 nmol) was suspended in 0.8 ml of 0.1 M ammonium bicarbonate, pH 8, and digested with trypsin for 24 h at 37 °C at a substrate/trypsin ratio of 40:1 (by mass). To 0.5 ml of tryptic digest, dithiothreitol (1 mM) was added, and the precipitate was removed by centrifugation. The solution was then lyophilized, and the peptides were separated by reverse-phase HPLC (Vydac C_18 column). The separation was performed with a linear gradient of 10–80% B over 70 min at a flow rate of 0.5 ml/min. Eluant A was 0.1% trifluoroacetic acid, and eluant B was 0.1% trifluoroacetic acid in 80:20 acetonitrile/H_2O. Absorbance was monitored at 220 nm, and fluorescence was monitored at 535 nm for the detection of labeled peptides. The fluorescent peptides were further purified with a linear gradient of 5–60% B over 30 min at a flow rate of 0.5 ml/min. The sequence of the isolated peptide, labeled with fluorescein, was determined by Edman degradation using an Applied Biosystems model sequencer (model 492 cLC).

**Fluorescence Spectroscopy—**Emission spectra were recorded in a Perkin-Elmer LS-50B spectrofluorimeter. For fluorescein-labeled protein, the excitation was at 480 nm, whereas for unlabeled proteins the excitation was set at 295 nm. Excitation and emission slits were set at 2.5 nm. The results of the fluorescence titration experiments were fitted to Equation 1 as follows,

\[
a/1 - a = [\text{mMDH}]F/K_D
\]

Where \(a = (F - F_0)/F_0\), \(F_0\) is the observed fluorescence, \(F\) and \(F_0\) the fluorescence intensities of free and bound IAF-PDI, respectively. In the analysis of the results, it was assumed that the stoichiometry of binding is 1 mol of IAF-PDI/1 mol of denatured mMDH.

**RESULTS**

**Reactivation of Partially Folded mMDH—**Since PDI is a multifunctional enzyme endowed with ATPase and chaperone activities, it was thought to be of interest to investigate whether both catalytic activities are linked during the process of refolding of a protein substrate. Is the ATPase activity enhanced during the refolding of the protein substrate? As a protein substrate of PDI, we have chosen an intermediate of mMDH generated by GdnHCl denaturation of the wild type protein. Partially folded mMDH, containing 12% α-helix, exhibits exposed hydrophobic amino acid residues and is devoid of catalytic activity (15). Moreover, the unfolded conformations of mMDH recognizes GroEL and regains a good deal of its catalytic activity (90%) in the presence of Mg ATP (16–18).

The results included in Fig. 1 show the time course of recovery of dehydrogenase activity in the presence of 1 mM DTT. Under this set of experimental conditions, the recovery of catalytic activity is due to spontaneous refolding of the protein after reduction of disulfide bonds generated during GdnHCl treatment (15). A significant recovery of catalytic activity was observed in the presence of increasing concentrations of PDI. Maximum recovery of dehydrogenase activity, which amounts to approximately 50% of the wild-type protein, takes place when the concentration of PDI (2.2 μM) was 20-fold higher than the concentration of the protein substrate. A further increase in PDI concentration has no effect either on the rate or extent of recovery of catalytic activity.

No antichaperone activity was detected at PDI concentrations above 2.0 μM.

When similar reactivation experiments were performed in the presence of Mg-ATP (1 mM) at the optimal mixing molar ratio of PDI/protein substrate of 20:1, the final recovery of catalytic activity was practically identical to that observed in the absence of Mg-ATP (Fig. 1).

To ascertain whether the binding of the protein substrate influences ATPase activity displayed by PDI, the hydrolysis of ATP (1 mM) in the absence and presence of partially folded mMDH was measured using a coupled enzymatic assay consisting of pyruvate kinase and lactate dehydrogenase.

As shown by the results included in Fig. 2, the ATPase activity (k_{CAT} = 0.26 min^{-1}) characteristic of PDI remains practically invariant upon increasing the concentration of partially folded mMDH from 2 to 8 μM. In marked contrast to the lack of inhibition of ATPase activity caused by the binding of mMDH, the glutathione-dependent reduction of insulin is strongly inhibited by small concentrations of partially folded mMDH as shown in Fig. 2. This finding suggests that insulin and denatured mMDH might share common binding sites on PDI.

Although partially folded mMDH has been used in most of the studies reported in this work, it should be emphasized that the rate of reactivation of malic dehydrogenase is not affected by the nature of the initial unfolded species used in the refolding experiments. Indeed, the kinetics of reactivation of partially folded mMDH, prepared as described under “Experimen- tnal Procedures,” is similar to that observed when 3 M guanidinium chloride-treated enzyme is immediately diluted into the renaturation buffer containing PDI (Fig. 1).

**Recognition of PDI by Denatured mMDH—**PDI contains tryptophanyl residues, which exhibit a structureless emission band centered at around 340 nm when excited at 295 nm. The intensity of the emission band is enhanced by the addition of equimolar amounts of denatured mMDH (Fig. 3). mMDH does not contain tryptophanyl groups; and under the experimental
conditions chosen for the luminescence experiments, the enzyme does not display any emission band centered at 340 nm.

A gradual increase in tryptophanyl fluorescence is observed when the concentration of denatured protein varies from 0 to 1.5 mM. As shown in the inset of Fig. 3, the fluorescence intensity reaches a maximum value at a mixing molar ratio of the proteins of approximately 1:1, taking the molecular masses of mMDH and PDI as 35 and 55 kDa, respectively.

In order to determine the affinity of mMDH for PDI at a concentration comparable with those used in the reactivation experiments, it was desirable to label PDI with a fluorescent probe characterized by high fluorescence yield at protein concentrations ranging from 0.05 to 0.1 mM. Derivatized IAF-PDI exhibits a maximum of fluorescence at around 535 nm due to the presence of the fluorescence chromophore. The fluorescence quantum yield of the probe (Q = 0.35), excited in the spectral region (460–480 nm) located far away from the aromatic amino acid residues tyrosine and tryptophan, can be used to detect the presence of protein-protein complexes in solution. Moreover, IAF is bound to cysteinyl residues, which can be localized on the polypeptide chain by sequencing the protein.

The emission of the chromophore excited at 480 nm is increased upon the addition of increasing concentrations of denatured proteins. Fig. 4 shows the emission spectra of IAF-PDI (0.96 mM) recorded after the addition of denatured mMDH and scrambled RNase A. To measure the affinity of IAF-PDI for partially folded mMDH, mixtures containing IAF-PDI (0.05 mM) and increasing concentrations of denatured protein were allowed to equilibrate for 2 min at 25 °C, and their spectra were immediately recorded. As shown in Fig. 4, inset, the fluorescence emitted by IAF-PDI is increased until it remains practically constant when the concentration of partially folded mMDH is greater than 0.6 mM. Assuming a stoichiometry of binding of 1:1, the dissociation constant of the protein-protein complex was estimated to be 0.2 μM.

Sequence of the Labeled Peptide—To ascertain whether labeling of PDI by the extrinsic probe IAF has taken place in the...
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Fig. 5. Separation of tryptic peptides from modified PDI by reverse-phase HPLC. Separation was performed with a linear gradient of 10–80% B over 70 min at a flow rate of 0.5 ml/min. Elution was monitored at 220 nm. Fractions of 0.5 ml were collected, and each fraction was analyzed by fluorescence measurements using an excitation wavelength of 480 nm and emission wavelength of 530 nm. The fractions showing fluorescence (•) were pooled, lyophilized, and purified by reverse-phase HPLC with a linear gradient of acetonitrile 5–60% B over 30 min at a flow rate of 0.5 ml/min. The purified labeled peptide was used for amino acid sequencing.

Fig. 6. HPLC separation of PDI. Elution profiles are shown of PDI in the absence (solid line) and presence of the peptide mastoparan (1 mM) (•). Each 50 μl of a solution of 2 mg/ml of PDI was applied to a TSK 3000 SW gel filtration column equilibrated with 0.1 M Tris/HCl (pH 7). Flow time was 0.3 ml/min. The elution profile of the standard bovine serum albumin (dashed line) is shown. Two elution bands are detected in bovine serum albumin corresponding to species of 134 and 67 kDa, respectively. One elution profile is detected in PDI (55 kDa).

Central domains of the protein, derivatized PDI was subjected to trypsin digestion, and the peptides were separated by HPLC as outlined under "Experimental Procedures." Upon separation of tryptic peptides by HPLC using a reverse-phase column, one major labeled peptide was identified (Fig. 5).

Rechromatography of the labeled peptide with the same column using a different solvent composition resulted in further purification. The fractions, characterized by a fluorescence maximum at 535 nm, due to the presence of the extrinsic chromophore fluorescein, were pooled, lyophilized, and sequenced. Edman degradation gave the following amino acid sequence: Ile-Thr-Glu-Phe-X-His-Arg. Comparison of the determined sequence of the modified peptide with the reported primary structure of human PDI (19) indicated that the labeled peptide corresponds to amino acid residues 322–328 of the human enzyme. The position indicated as X in the peptide sequence corresponds to cysteine 326 in the human enzyme. The absence of any labeled peptide covering the sequence Trp-Cys-Gly-His-Cys-Lys pertaining to the active site region of thioredoxin, strongly suggests that the reaction with IAF is restricted to free cysteine groups in the central domain b′ of PDI.

Aggregation State of PDI—It has been reported that monomeric species of PDI are in dynamic equilibrium with dimeric and tetrameric species at pH 7.4 in 0.3 M NaCl (9). The possibility that high molecular “clusters” of PDI preferentially bind small molecular weight peptides was investigated by gel filtration chromatography using 0.1 M Tris/HCl, pH 7, as the mobile phase. When pure PDI, stored at 4 °C, was submitted to gel filtration, one major peak was detected. The retention time of PDI is slightly different from bovine serum albumin (monomeric) characterized by a molecular mass of 67 kDa (Fig. 6). The addition of the peptide mastoparan (Ile-Asn-Leu-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu) does not cause any change in the monomeric state of PDI, even at concentrations required for saturation of the enzyme (20). Hence, PDI does not undergo a reversible process of association in the absence or presence of low molecular weight peptides. When the same experiments were performed with IAF-PDI mixed with mMDH and the elution profiles were monitored by fluorescence measurements at 535 nm, we were unable to detect macromolecular species corresponding to tetramers of PDI (results not shown).

Our results do not show dimerization or aggregation of PDI at neutral pH. The discrepancy with other published data must be attributed to the conditions of storage of purified PDI, since it has been reported that a metastable dimer is induced by freezing the samples in phosphate buffer (21).

Discussion

Mitochondrial MDH, a dimeric protein, undergoes structural transitions in solutions containing GdnHCl. In the presence of 3 M GdnHCl, the protein shows little residual secondary structure; and dilution of the denatured protein with buffer restores a fraction of the α-helix content (15). The partially folded species recover only 16% of the catalytic activity after the addition of 1 mM DTT. The rate of recovery of catalytic activity of the dehydrogenase is influenced by the presence of PDI in the renaturation buffer. Under optimal conditions of temperature (25 °C) and PDI concentration (2.2 μM), the recovery of activity amounts to 50% of the wild-type protein. Like other well-characterized chaperone proteins, i.e. GroEL, PDI functions primarily by unfolding misfolded protein intermediates; but it appears that binding of the protein substrate to the chaperon would provide the free energy required for unfolding. Two lines of experimental evidence are consistent with this hypothesis: first, the rate of reactivation of partially folded mMDH is not influenced by the presence of Mg-ATP together with PDI in the refolding buffer, and second, the intrinsic ATPase activity of PDI is not perturbed by its interaction with the protein substrate.

In agreement with the results published by other laboratories (16, 17), it was found that unfolded mMDH recovers a good
deal of its catalytic activity (90%) when the denatured protein substrate interacts with GroEL and Mg-ATP at equimolar concentrations of the proteins.

In view of these results, it is pertinent to ask why PDI is less efficient than GroEL in assisting the refolding of partially folded mMDH. Based on the studies reported by other laboratories on the binding of small molecular weight peptides to PDI, it has been suggested that weak affinity of PDI for denatured proteins might explain low recovery of catalytic activity. Our studies on mMDH indicate that derivatized IAF-PDI binds denatured mMDH with a dissociation constant of 0.2 μM, which is 5-fold higher than the dissociation constant determined for denatured mMDH bound to GroEL in the absence of Mg-ATP (17). Hence, a 20-fold molar excess of PDI over the denatured protein substrate (0.1 μM) would be sufficient to ensure binding and reactivation of mMDH. However, the reactivation experiments conducted in the presence of a 20-fold excess of PDI have shown partial recovery of catalytic activity.

There are some aspects of the interaction of chaperone proteins with unfolded substrates that should be considered in the analysis of the behavior of PDI. GroEL interacts with denatured mMDH, and the binding of Mg-ATP drives the chaperone complex through a functional state in which refolding of the protein substrate occurs inside the cavity of GroEL (18). Encapsulation of the protein substrate occurs at a folding environment that facilitates conformational arrangements and prevents all unproductive interactions with other nonnative proteins during the chaperone cycle.

PDI does not possess a cavity for encapsulation of a protein substrate of the size of mMDH. On the other hand, the binding of small molecular weight polypeptides does not promote association of PDI into “clusters” of large molecular weight that would prevent exposure of the protein substrate to the surrounding solvent. Several laboratories have reported that the interaction of misfolded proteins (i.e. scrambled RNase A) with PDI competes with the binding of peptides (11, 20). Based on these observations, it has been suggested that the peptide binding site corresponds to a site at which PDI interacts with unfolded regions of proteins during its action in the cell. Although the hypothesis is attractive, the domain associated with peptide binding has not been completely elucidated. Initially it was reported that deletion of 51 amino acid residues of the C-terminal domain totally prevented peptide binding and catalytic activity (6). Subsequent studies have shown that the deletion includes amino acid residues pertaining to the C-terminal domain and a fraction of the α domain (7). More recently, it has been reported that the b’ domain provides the principal peptide binding site of PDI (8). In view of these observations, it seems very likely that amino acid residues outside the C domain might contribute to the interaction of PDI with the protein substrate. Our fluorescence results have shown that binding of the protein substrate causes conformational changes in PDI propagated to different domains of its structure. Not only the fluorescence probe positioned in the b’ domain, but also tryptophanyl residues distributed along the α and β domains, sense the conformational changes induced by the protein substrate. It is conceivable that the flexibility of the protein plays an important role in reactions such as isomerization of disulfide bonds and refolding of protein substrates.

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