Hormone Binding by Protein Disulfide Isomerase, a High Capacity Hormone Reservoir of the Endoplasmic Reticulum*

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Protein disulfide isomerase (PDI)† is a versatile and multifunctional folding assistant of the eukaryotic endoplasmic reticulum (1–6). Its essential function in eukaryotes (7) is as a catalyst of disulfide rearrangements (isosomerase activity) during oxidative protein folding (ER). There are two remarkable facts about PDI. It is a very abundant protein in the cell; its concentration in the ER lumen has been estimated to be 100–200 μM (8). The other remarkable fact about PDI is its uncanny ability to bind things. Ligands range from peptide/protein substrates to hormones. Remarkable fact about PDI is its uncanny ability to bind things. Peptide/protein interactions are essential for effective catalysis of disulfide formation and isomerization. PDI interacts covalently with substrate cysteines (9), but it also binds a variety of peptide/protein substrates (10). There are few strict sequence/structure requirements for binding (11, 12), although there is some effect of sequence on reactivity with PDI (13). PDI activity can be inhibited by a number of peptides (11), including the cyclic peptide antibiotic, bacitracin (14, 15). A tripeptide affinity probe, which originally identified PDI as a glycosylation site binding protein (16) localized one peptide binding site near the C terminus of PDI (12), but deletion of the C-terminal binding site has little effect on the oxidase and isomerase activities (17). Binding of radiolabeled peptides to various fragments of PDI has recently suggested that multiple sites exist that may interact with various peptides/proteins (10) and that a distinct structural domain (b') that is N-terminal to one of the two active sites contributes significantly to peptide binding.

Noncovalent interactions with protein substrates can suppress substrate aggregation during protein refolding. The chaperone activity of PDI is observed with proteins that form disulfides (18) and proteins that do not (19–21). By interacting with multiple sites on aggregation-prone substrates, PDI can facilitate substrate aggregation under certain conditions, a behavior we have termed anti-chaperone activity (18, 20, 22).

In addition to unfolded protein substrates, PDI associates tightly with some folded proteins. It serves as the β subunit of prolyl 4-hydroxylase (23) and as a subunit of the microsomal triglyceride-transfer complex (24). The level of PDI present in the ER is considerably higher than either of these complexes, suggesting that PDI is involved in the folding, stability, or assembly of the complex and in its retention in the ER through the C-terminal KDEL signal of PDI (25).

In addition to peptides and proteins, PDI binds hormones. It was identified by photoaffinity labeling as a thyroid-hormone binding protein (26). Displacement of radiolabeled T3 from PDI purified from liver suggested a high affinity for T3 (27). However, Guthapfel et al. (28) have recently shown that the high affinity binding of T3 (Kd = 21 nM) has very low capacity (0.002 mol of T3/mol of PDI), implying that high affinity binding is due to an impurity rather than PDI itself. PDI also binds β-estradiol (E2), and although the number of estrogen binding sites has not been determined, estrogen has been reported to inhibit isomerase activity (29). A short segment of the PDI sequence exhibits some similarity to the estrogen receptor, but this sequence does not appear to constitute an independent structural domain (30). Finally, like many ER-resident proteins, PDI binds calcium with low affinity (2–5 mM) and high capacity (19 mol Ca2+/mol protein) (31). Calcium affects the chaperone activity of PDI (20) but has no influence on the isomerase function (20, 32).

The relationships between the hormone and peptide/protein binding sites are not at all clear. The interactions of PDI with...
hormone ligands might be due to specific binding sites, or it may be a reflection of nonspecific binding site(s) that are normally involved in PDI/substrate interactions. There have been no systematic attempts to determine the number of binding sites for individual ligands, nor have there been any attempts to examine overlap in the interaction sites. We have used competitive ligand binding of 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS), radiolabeled E2, and T3 along with various unlabeled peptide inhibitors to probe the various interactions these ligands make with PDI. The results show that PDI has multiple binding sites for hormones. Binding occurs at two different hormone binding sites. Both sites bind T3, whereas only one of them interacts with estradiol. These sites are distinct from peptide/protein binding sites. Thus, the hormone binding capacity of PDI appears to be a discrete function of the molecule and not coupled to its catalytic activity in disulfide isomerization. Because of its high local concentration in the ER, PDI could serve as an intracellular reservoir for these hormones despite its relatively low affinity. Based on the relative concentrations and affinities of PDI and the estrogen receptor, more than 90% of the intracellular T3 and estradiol relative concentrations and affinities of PDI and the estrogen hormone binding capacity of PDI appears to be a discrete function of the molecule and not coupled to its catalytic activity in disulfide isomerization. Because of its high local concentration in the ER, PDI could serve as an intracellular reservoir for these hormones despite its relatively low affinity. Based on the relative concentrations and affinities of PDI and the estrogen receptor, more than 90% of the intracellular T3 and estradiol would be expected to be bound to PDI. This high capacity intracellular reservoir would tend to raise the cellular levels of hormone that are necessary to cause receptor occupancy, even when the estrogen and T3 receptors have nm affinity for hormones.

EXPERIMENTAL PROCEDURES

Materials—Recombinant rat PDI was expressed and purified as described previously (34). The concentration of PDI monomers (55 kDa) was determined by absorbance at 280 nm (ε 1% = 0.94 cm -1 mg/ml) (35). bis-ANS was obtained from Molecular Probes, and its concentration was determined by absorbance at 385 nm using an extinction coefficient of 16,790 cm -1 M -1 (36). E2, T3, DTT, HEPES, equine liver alcohol dehydrogenase (ADH), bacitracin, insulin chain B (oxidized, sulfonated), calcium chloride, and magnesium chloride were purchased from Sigma. ADH concentration was determined by absorbance at 280 nm using an extinction coefficient of 0.455 cm -1 mg/ml (37). Bacitracin is a mixture of multiple peptides, with the prominent species being bacitracin-A (58, 39). The molecular weight of bacitracin-A (1421) was used for estimating bacitracin concentration. Tritiated E2 (2,4,6,7-[3H]E2, specific activity, 84 Ci/mmol) and labeled T3 (L-5-[125I]T3, 230 Ci/mmol) were from PerkinElmer Life Sciences with purity checked by reverse phase high pressure liquid chromatography.

Fluorescence Measurements—Measurements were made in 50 mM HEPES, pH 7.0, containing 10 mM DTT at 25 °C using various concentrations of PDI and bis-ANS. The excitation wavelength was 370 nm, and emission was scanned from 425 to 575 nm using a PerkinElmer LS-5 fluorescence spectrometer.

Thermal Aggregation Assay—ADH aggregation upon thermal denaturation was followed by measuring the apparent absorbance at 360 nm caused by light scattering because of increasing turbidity (20). Temperature was controlled at 40 °C using a circulating water bath connected to a Beckman DU 7500 spectrophotometer. Initiation of the assay was by addition of ADH (final concentration, 11 μM dimer) to a 50 mM HEPES-NaOH buffer, pH 7.0, containing 10 mM DTT and PDI at various concentrations. The apparent velocity of aggregation (Aoc/min) was measured after any lag as the maximum change in absorbance with time.

Isomerase Activity Assay—Oxidative refolding of RNase in the presence and absence of inhibitors was followed by an established continuous assay (8) where the formation of native RNase was measured spectrophotometrically by monitoring hydrolysis of cCMP, an RNase substrate, at 296 nm. Reduced, denatured RNase (final concentration, 8 μM) was added to PDI (1.4 μM) in a buffer containing 4.5 mM cCMP, 1 mM glutathione, 0.2 mM glutathione disulfide, 2 mM EDTA, 100 mM Tris-HCl, pH 8. RNase was reduced and denatured by overnight incubation at 10 mg/ml in denaturing buffer (140 mM DTT, 2 mM EDTA, 6 mM GdnHCl, 100 mM Tris-HCl, pH 8). Denaturing buffer was exchanged for 0.1% acetic acid using a Bio-Gel P6 spin column. Reduced, denatured RNase concentration was determined by absorbance at 280 nm using an extinction coefficient of 9300 cm -1 M -1.

Equilibrium Dialysis—Microdialysis cells from Bel-Art Products were used with two 100 μl cavities separated by 12–14 kDa molecular mass cut-off dialysis membrane (Spectra/Por). Experiments were performed at 25 °C and incubated for 18–24 h, sufficient time to ensure equilibrium as determined by following the time course for approach to equilibrium in control experiments. Dialysis buffer contained 10 mM DTT, 10 mM NaCl, and 50 mM HEPES-NaOH, pH 7.0. Bis-ANS concentration after equilibrium dialysis was determined by absorbance at 385 nm, [3H]E2 and [125I]T3 concentrations were determined by scintillation counting calibrated against standard concentrations of [3H]E2 and [125I]T3. There was no detectable degradation of PDI during equilibration as detected by SDS-polyacrylamide gel electrophoresis analysis.

RESULTS

Ligand Binding—PDI binds a diverse set of ligands (Fig. 1), interacting with unfolded and folded proteins, E2, T3, peptide-like antibiotics such as bacitracin, and peptides such as oxidized insulin chain B (InB). Despite this promiscuity in interacting with potential ligands, the relationships among these sites have not previously been determined. PDI has been shown to bind hydrophobic ligands such as β-estradiol (27) and T3 (28); however, the numbers of E2 and T3 binding sites have not been determined. Equilibrium dialysis using purified, recombinant rat PDI (Fig. 2) shows that there is a single E2 binding site but two T3 sites, both of which have modest affinity (Table I). Individual Kd values cannot be resolved for the two T3 binding sites, suggesting that the Kd values are similar.

Nonspecific, hydrophobic surfaces and/or binding sites can often be detected by fluorescence changes on binding the environmentally sensitive fluorescent probe, bis-ANS (40, 41). When bound to hydrophobic sites, bis-ANS exhibits enhanced fluorescence and a characteristic blue shift in the emission maximum. PDI binds bis-ANS with μM affinity, and the fluorescence of the bound probe is enhanced and blue-shifted (~30 nm shift in maximum emission), indicating that the bis-ANS is binding to a hydrophobic site(s) on the protein (Fig. 3 and Table I). The fluorescence intensity of bis-ANS bound to PDI was determined by titrating a limited amount of bis-ANS (0.5 μM) with increasing concentrations of PDI (0–20 μM) (40). When calibrated in this way, the fluorescence intensity observed at excess, saturating bis-ANS suggests the binding of 0.9 ± 0.2 mol of bis-ANS/mol of PDI (Table I). Calcium, yet another PDI ligand (31), enhances the fluorescence of PDI-bound bis-ANS by ~1.6-fold with half-maximal enhancement occurring at 0.9 ± 0.1 mM calcium (data not shown). Magnesium, which like calcium affects PDI-chaperone/anti-chaperone activity, has a similar effect, but sodium chloride does not.

Competition experiments show that T3 inhibits bis-ANS binding (Fig. 4). The maximum concentration of T3 is limited by...
solubility; however, extrapolation by fitting the data to a hyperbola suggests that T₃ displaces (>87%) bis-ANS fluorescence with half-maximal inhibition occurring at 69 ± 5 µM at a bis-ANS concentration of 10 µM. None of the other known PDI ligands compete for this site; neither E₂ (up to 64 µM), InB (up to 250 µM) nor bacitracin (up to 500 µM) significantly affects bis-ANS binding to PDI. Thus, PDI has one hydrophobic site that binds bis-ANS or T₃, and the other ligands must bind at different sites.

Equilibrium dialysis shows that there are two T₃ binding sites (Fig. 2). Although one of these also binds bis-ANS, the other accommodates E₂. Of all the ligands tested, only T₃ affects E₂ binding (Fig. 5). Half-maximal inhibition occurs at 8.5 ± 3.2 µM T₃, and T₃ can displace E₂ completely (≤95%). By contrast, neither bis-ANS, bacitracin, nor InB has any effect on E₂ binding. T₃ displaces both E₂ and bis-ANS from their binding sites, but E₂ and bis-ANS do not compete with each other. This also implies that there must be at least two independent binding sites for T₃, consistent with the observed stoichiometry of T₃ binding.

Effects of Ligands on PDI Catalytic Activity—E₂ inhibits the PDI-catalyzed refolding of reduced RNase; however, the inhibition in only partial, and saturating E₂ inhibits the isomerase activity by only half (Fig. 6). Although T₃ and E₂ bind to the same site, T₃ has no significant effect on PDI activity up to a concentration of 70 µM. Guthapfel et al. (28) reported that T₃ also partially inhibits PDI with a Kᵢ of 1.3 µM; however, under very similar conditions, we repeatedly failed to observe significant (±10%) inhibition of the isomerase activity. InB has previously been shown to inhibit the isomerase activity with an inhibition constant of 230 ± 40 µM (11). Bacitracin, at concentrations that have previously been reported to inhibit PDI (42), does inhibit PDI-catalyzed RNase refolding. However, at the same concentrations bacitracin also inhibits spontaneous RNase A refolding in the absence of PDI to the same extent (data not shown). The effects of bacitracin on PDI activity may reflect its interactions with the RNase substrate and/or its interaction with PDI.

Effect of Ligands on PDI Chaperone and Anti-chaperone Activities—At high concentrations, PDI inhibits the aggregation of unfolded proteins. This chaperone activity, which has been attributed to the interaction of PDI and the unfolded protein substrate (8, 19, 43), is observed during the refolding of proteins...
that have disulfides as well as proteins that do not form disulfides. Under certain conditions and with certain substrate proteins, PDI exhibits what has been termed anti-chaperone activity. When it functions in its anti-chaperone mode, PDI facilitates rather than inhibits substrate aggregation (18, 20). We have suggested that the chaperone/anti-chaperone functions of PDI result from multiple, noncovalent interactions between PDI and an aggregation-prone substrate (20). These interactions cross-link substrate aggregates until the PDI concentration is sufficiently high to inhibit cross-linking, much like the inhibition of classical immunoprecipitation under conditions of antibody excess. Both chaperone and anti-chaperone activity are observed during the thermal aggregation of ADH (20). At lower concentrations of PDI, increasing PDI enhances substrate aggregation, but at higher PDI concentrations, increasing PDI inhibits substrate aggregation. At concentrations that should result in significant occupancy of their binding sites, neither E2 nor T3 have significant effects on the chaperone or anti-chaperone activity (Fig. 7). These ligands also have no effect on PDI chaperone activity using citrate synthase as a substrate (20) (data not shown). Both InB and bis-ANS inhibit ADH aggregation in the absence of PDI, so that their effect on PDI chaperone and anti-chaperone activities could not be determined. By contrast, relatively low concentrations of bacitracin inhibited the chaperone activity without having a large effect on substrate aggregation in the absence of PDI (Fig. 7).

**DISCUSSION**

PDI has been repeatedly isolated from cells based on its propensity to bind a surprisingly diverse group of ligands. It is the \( \beta \)-subunit of prolyl hydroxylase (23) and a subunit of an ER triglyceride transferase (24), although its catalytic activity is not required for the function of these complexes (25). PDI was also identified in a screen for proteins that interact with a potential glycosylation binding site present on a small peptide photo-affinity label (44), although it was subsequently found that PDI does not participate directly in glycosylation (45). In a search for thyroid hormone binding proteins, PDI was identified through photoaffinity labeling as a major T3 binding protein (46).

PDI, like some other folding assistants (47), must interact with a large number of different substrates with few recognizable sequence cues except for a small preference for peptides that contain cysteine (10, 11). Recently, Winther and colleagues (13) found that peptide sequence can affect the rate at which PDI reacts with peptide disulfides with some preference for a sequence that has the features, small/helix breaker-Cys-X-hydrophobic/basic-hydrophobic. Peptide binding sites have been difficult to localize on PDI. Early indications with a photo-affinity label suggested that a peptide binding site was located near the C terminus (12). Deletion of this region has little effect on isomerase activity, but it does eliminate chaperone activity (17). Klappa et al. (10) have recently found that interaction between PDI and peptides involves a single domain (b'), but other sites are involved in the binding of large, protein substrates. Thus, multiple sites with limited specificity are involved in the interaction of PDI with its protein substrates.

The competition binding experiments reported here show that PDI has multiple ligand binding sites (Fig. 8), some of which display overlapping specificity. However, the sites involved in hormone binding are distinct from those involved in peptide and substrate binding. Peptides and peptide analogs do not displace hormones from their binding sites, and the binding of hormones is noncompetitive with protein substrate. The partial inhibition by E2 means that protein substrates can still interact with PDI, even when E2 is bound. The lack of an observable effect on chaperone/anti-chaperone behavior also argues that the hormone sites are not utilized during the interaction between PDI and its unfolded protein substrates.

Previous measurements of T3 and E2 binding to PDI have been performed by displacement of a small amount of labeled hormone by unlabeled competitor. Affinities estimated in this way suggested that PDI binds both hormones with high affinity (26, 29), although lower affinity sites were observed as backgrounds. Guthapfel et al. (28) has recently shown that high affinity (nm) T3 binding is not stoichiometric (<0.01 mol hor-
mone/mol PDI), implying that tight binding is from impurities rather than from PDI itself. By contrast, the low affinity (μM) binding we observe for T₃ and E₂ (Table I) is stoichiometric. Although PDI does not exhibit high affinity for these hormone ligands, it does have a limited number of reasonably specific sites for their interaction that do not interfere significantly with the catalytic or chaperone activities of the molecule. Thus, hormones are not binding to sites that interact with protein substrates.

With dissociation constants of 2–4 μM (Table I), it would be tempting to conclude that hormone binding by PDI does not have any physiological relevance because the Kᵦ values for hormone binding to their cellular receptors are in the low μM range (Kᵦ = 10–300 μM) (48–50). However, the local concentration of PDI in the ER is quite high, estimated at ~200 μM (51). Thus, the binding of hormone to PDI will be driven by the high, local concentration of PDI despite the low Kᵦ value. A specific example illustrates how the presence of a high capacity, low affinity reservoir will affect the location of hormone binding (Fig. 9). With a Kᵦ for hormone binding to PDI of 2 μM and a concentration of 200 μM PDI, simple equilibrium binding tells us that ratio [HR-PDI]/[HR].sources will be 100, and 99% of the E₂ in the endoplasmic reticulum will be bound to PDI. If the ER represents 10% of the cell volume, then about 90% of the intracellular E₂ would be bound to PDI at any given free concentration of hormone. At a free cytosolic concentration of hormone (10 pM) that is sufficient to half-saturate the estrogen receptor, hormone binding to PDI would require a total intracellular concentration of 100 μM to generate a free hormone concentration of 10 pM. The total concentration of hormone bound to PDI would be ~90 μM, whereas the free concentration of hormone would be 10 μM. With a high capacity reservoir for hormone binding, the concentration of hormone necessary to generate a physiological response would be significantly higher than the Kᵦ for equilibrium hormone binding. Physiological concentrations of T₃ range from about 10 pM in serum (52) to 75 pM in brain cytosol (53). The physiological E₂ concentrations are larger, from 0.2–2 nM in human serum (54), to 0.4 nM in placenta (55), and up to 3–5 μM in developing follicles in the ovary (56). All of these are significantly higher than the Kᵦ measured in vitro (48–50), suggesting that there must be some mechanism for modulating the response of hormone receptor to physiological concentrations of hormone.

The function of high capacity reservoirs of low affinity may be to balance the need for high specificity and regulation. Tight binding is essential for high specificity and for discriminating between similar structures. For very high affinity receptors, such as the estrogen and T₃ receptors, cell volume may limit the number of molecules present in a given cell. For example, with a cell volume of 6000 μM³ (32), at a concentration of 10 pM, there would be only 40 molecules of hormone/cell. A stochastic fluctuation in the distribution of hormone molecules in the cells would raise the possibility that some cells would not have any hormone molecules. By increasing the number of molecules in the cell by 10-fold or more, a low affinity, high capacity reser-
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