TITLE

Red/Ox States of Human Protein Disulfide Isomerase Regulates Binding Affinity of 17-Beta-Estradiol

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ABSTRACT

Human protein disulfide isomerase (hPDI) is a key redox-regulated thiol-containing protein operating as both oxidoreductase and molecular chaperone in the endoplasmic reticulum of cells. hPDI thiol-disulfide interchange reactions lead to the adoption of two distinct red/ox conformations with different substrate preferences. hPDI also displays high binding capacity for some exogenous steroid hormones including 17-Beta-Estradiol (E2) and thus contributes to the regulation of their intracellular concentration, storage and actions. The primary focus of this study is to investigate the impact of E2 binding on functional activity of recombinant hPDI. Then, we examine the effect of E2 binding on structural alteration of hPDI red/ox conformations and its influence on affinity and position of interaction using experimental and computational analysis. Our results revealed that interaction of one E2 per each hPDI molecule leads to the inhibition of hPDI reductase activity and conformational changes in both oxidation states. Mutually, E2-binding position are also redox-regulated with higher affinity in oxidized hPDI compare to the reduced form. The importance of histidine-256 protonation states in distinct binding preferences of E2 were also demonstrated in hPDI red/ox conformations. These findings might pave the way for better understanding of the mechanisms behind the redox-dependent hormone-binding activity of hPDI.

KEYWORDS

oxidative stress; disulfide exchange; estrogen; 17-Beta-Estradiol (E2)

Chemical compounds studied in this article:

17- β -Estradiol (PubChem CID: 5757); Glutathione disulfide (PubChem CID: 65359); Glutathione (PubChem CID: 124886); Dithiothreitol ((PubChem CID: 446094)

INTRODUCTION

Preserving cellular redox balance is strongly regulated by the thiol-containing proteins that are sensitive to redox status [1]. The human protein disulfide isomerase (hPDI) is an essential redox-sensitive enzyme, associated with maintaining cellular redox poise by possessing two couples of catalytic cysteines [2,3]. hPDI is composed of four thioredoxin-like domains, a, b, b' and a' in a U-shaped architecture. The a and a' work as the catalytic domains by accommodating pairs of cysteines in conserved –CGHC– motifs. The central domains (bb') are the base of the protein, which mainly involve in substrates binding and coordination of adjacent catalytic domains [4]. Structural plasticity of hPDI is typically under the influence of disulfide bonds that leads to the formation of two distinct oxidized (ox-hPDI) and reduced (red-hPDI) states [5,6]. Red-hPDI exhibits a “closed” conformation, which provides less accessible area for substrate binding on the b' domain. By contrast, oxidized form forms an “opened” structure with more accessible cleft for target acquisition [4,7,8]. hPDI redox-associated conformational changes lead to the recognition of different substrates for further oxidation, reduction and/or isomerization reactions [2].

The balance between the concentration of ox- and red-hPDI in endoplasmic reticulum is under the influence of folding load of reduced substrates (i.e. nascent or misfolded polypeptides) that regulates the redox homeostasis [2]. During the higher rate of protein synthesis in growing cells or cellular stress, hPDI-redox balance shifts into red-hPDI for oxidative protein folding/refolding [2]. Therefore, hPDI red/ox ratio varies upon different environmental and/or internal alterations that could sense cellular state.

Apart from enzymatic and chaperone-dependent activities, hPDI moderates the concentration and storage of some hormones including the Triiodothyronine (T3) and 17- β -Estradiol (E2)

[9]. Hormone-binding capacity of hPDI also decreases the intracellular half-life of E2 elimination by inhibiting the accessibility of estrogen-metabolizing enzymes and thus regulates its mechanisms of action in different tissues [10]. E2 not only affects development of reproductive system, but also modulates many biological processes in a variety of organs and tissues [11,12]. Therefore, regulating the E2 storage directly alters cellular activities in health and disease.

During the last two decades, some studies have been focusing on the molecular aspects of E2-hPDI interaction and its implication on different hPDI redox-dependent activities [9,13–15]. Taken together, these evidences indicated that E2-binding capacity of rat and human microsomal-derived PDI modulates the isomerization and chaperone functions [9,13,14], but detailed mechanisms remained unclear. Due to the essential roles of redox conformational exchanges in hPDI functions, a key question is what would be the effect of different redox states of hPDI on its E2 interaction capacity and how it affects hPDI functions. Our primary focus is to investigate the impact of E2 binding on reductase activity of recombinant hPDI. Then, E2 binding properties of hPDI are analyzed and compared under the different redox states using fluorescence spectroscopy. Possible interaction sites of E2 on ox- and red-hPDI are investigated by considering structural dynamics of hPDI using ensemble docking approach.

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURES

hPDI Cloning, Expression and Purification

The human P4HB coding DNA sequence (CDS) that encodes hPDI was cloned into pET19b (Novagen) using BamHI/NdeI restriction sites with N-terminal HIS-tag and the sequence was verified by DNA sequencing. Recombinant hPDI was expressed in Rosetta-gami 2(DE3) pLysS (Novagen) and purified using ion metal affinity chromatography (IMAC) by HIS-Select Nickel affinity gels (Sigma) followed by ion-exchange chromatography using Q Sepharose Fast Flow (GE Healthcare Life Sciences) as described previously [16]. Before further investigations, the purity of the protein was analyzed by Coomassie Brilliant Blue-stained 12% SDS-PAGE. Protein identity was verified by Western blotting using P4HB antibody (Abcam). Protein concentrations were determined by the Bradford method or spectrophotometrically at 280 nm with the absorption coefficient of $45.38 \text{ mM}^{-1} \text{ cm}^{-1}$ [5].

Reductase Activity Assay

The 0.1 M Potassium phosphate buffer containing 2.5 mM EDTA and 60 μM human insulin (pH 7.5) in the absence and presence of 0.42 μM recombinant hPDI was prepared and reactions were initiated by adding 0.1 mM dithiothreitol (DTT) (Sigma). The turbidity of the participated insulin chain B was immediately recorded by measuring the absorbance at 650 nm at 2 min interval for total 90 min at 25 °C using microplate reader. Recombinant hPDI activity was calculated by maximal slope of the curve (V_{max}) relative to the lag time for the start of precipitation (??) as previously reported [17]. The assay was repeated with different concentrations of E2 (0.2, 0.4, 0.6, 0.8 and 1 μM) to achieve the half maximal inhibitory concentration (IC₅₀) of E2. All experiments were carried out by three technical replicates.

Intrinsic Fluorescence Measurements

Reduced and oxidized recombinant hPDI (0.42 μ M) were prepared by adding 1 mM GSH (Sigma) and 1 mM GSSG (Sigma) in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl at 25 °C for 30 min, respectively, as described earlier [6]. Intrinsic fluorescence spectra were recorded at 300-400 nm at two different temperature of 27 °C and 37°C, with excitation at 290 nm using a 1cm path length fluorescence cuvette with the slit widths of 10 nm and 20 nm for the excitation and emission, respectively.

Fluorescence Quenching

The quenching experiment was initiated by repetition of 12 additions of 2 μ l of E2 (10 μ M) to 200 μ l buffer (50 mM Tris-HCl supplemented with 150 mM NaCl, pH 7.5) containing either reduced or oxidized recombinant hPDI up to final concentration of 1 μ M. This process was also repeated in the absence of hPDI as a blank. Thermodynamic parameters of E2 binding were calculated using Stern-Volmer (eq. 1), modified Stern-Volmer (eq. 2) and van't Hoff (eq. 3) equations, as follows [18]:

$$\frac{F_0}{F} = 1 + K_{sv}[E2] = 1 + k_q\tau[E2] \quad (\text{eq. 1})$$

$$\frac{F_0}{(F_0 - F)} = \frac{1}{f_a K_{sv}} \times \frac{1}{[E2]} + \frac{1}{f_a} \quad (\text{eq. 2})$$

$$\log \left[\frac{F_0 - F}{F} \right] = n \log [E2] + \log K_{binding} \quad (\text{eq. 3})$$

where the F_0 and F are the fluorescence intensities in the absence and presence of E2, respectively; K_{sv} , k_q and $K_{binding}$ correspond to Stern–Volmer, bimolecular quenching and binding constants, respectively; And τ , f_a and n refer to the fluorophore lifetime in the

absence of quencher, the intensity of the accessible fraction and binding stoichiometry, respectively.

COMPUTATIONAL PROCEDURES

Molecular Dynamics Simulations

Crystal structures of ox-hPDI (PDB ID: 4EL1) and red-hPDI (PDB ID: 4EKZ) were used as the starting conformations for computational analysis [4]. Molecular dynamics (MD) simulation was carried out with CHARMM 27 force field in the NAMD 2.10 package [19] to sample alternative conformations of each static X-ray structure and create receptor ensemble for subsequent ensemble docking. Missing residues and hydrogens were retrieved using Modeller 9.13 [20] and Reduce 3.23 [21], respectively. Four initial systems were prepared by considering two hPDI redox states, each has two different protonation states on imidazole ring of HIS256 (HSE and HSD). These systems were denoted as ox- or red-HSE/HSD. Each conformation was solvated in a rectangular TIP3P water box with box padding of 15 Å. Then, 23 Sodium ions were added to neutralize systems. MD simulations were started with optimization of systems for 20000 steps using conjugate gradient method while harmonic restraints were applied on heavy atoms with a force constant of 100 kcal mol⁻¹ Å⁻². Simulations were continued by 500 ps isothermal–isobaric (NPT) dynamics while temperature of systems were gradually increased from 10 to 300 K with a time step of 1 fs while harmonic restraints were gradually relaxed. Temperature and pressure of systems were controlled using Langevin thermostat and Nosé-Hoover Langevin piston at 1 atm, respectively [22,23]. Non-bonded interactions beyond 12 Å were truncated with an smooth switching starting from 10 Å. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method with a grid point spacing of 1 Å [24]. 12 ns production

run were carried out after 2 ns equilibration phase, while time step was set to 1 fs. SHAKE algorithm was used to constrain all water bonds. The system configurations were recorded every 2 ps. First 2 ns of all recorded snapshots were excluded and remaining structures were used for subsequent analysis. From 40 ns MD simulations of all systems, 4000 structures (1000 per each system) were clustered into 152 groups based on backbone RMSD via quality threshold (QT) algorithm in the VMD 1.9 package [25,26]. 77 representative structures from the most populated clusters (>90%) of the ox-hPDI (ox-HSE/HSD) and red-hPDI (red-HSE/HSD) systems were then used for ensemble docking.

Ensemble Docking

The structure of 17- β -Estradiol was downloaded from ZINC database (<http://zinc.docking.org/>). Ligand and receptor ensembles of the ox- and red-hPDI (HSE and HSD) were prepared for docking via AutoDockTools 1.5.7rc1 [27]. Search space and docking parameters were identical for all ensemble structures. Box dimension was defined as 40 $\times$ 42 $\times$ 38 Å³ with grid spacing of 0.375 Å. Ensemble docking was performed for all structures via AutoDock Vina [28]. Exhaustiveness parameter was set to 1000. For each receptor ensemble ~350 complexes were generated. To identify different binding sites of E2 on the ox- and red-hPDI (HSE and HSD) complex ensembles, all complexes were superimposed from central domains of protein. Quality threshold (QT) clustering was then applied to cluster ligands on the basis of their positions at two levels. To locate different E2 interaction sites, ligands were clustered with root mean square deviation (RMSD) cutoff of 6 Å and ranked according to their affinity scores. Then, best modes of binding within each interaction site were collected by additional clustering of ligands with RMSD cutoff of 3 Å.

The best affinity binding mode of each system was selected for subsequent MD simulation to investigate their binding properties and stability using the same protocol as described in previous section. The CHARMM General Force Field (CGenFF) program was used to generate force field parameters of E2 (<https://cgenff.paramchem.org/>). 12 ns production run was performed for each complex and its corresponding receptor. This includes 96 ns simulation for all systems. Non-covalent interaction energies and hydrogen bonds between the ligand and its surrounding residues (within the 4.5 Å) were calculated during the MD trajectories. The cutoff for calculation of hydrogen bonds were considered as 3.5 Å for donor-acceptor distances and 30° for donor-hydrogen-acceptor angles, respectively. All snapshots were superimposed from residues within 4.5 Å of ligand in docking structure and the time series of RMSD of ligand relative to the first frame was monitored to analyze dynamical variations in binding modes of the complexes.

All analyses were implemented using R statistical language version 3.2.2. Molecular graphics were prepared by VMD 1.9.2.

RESULTS AND DISCUSSION

The recombinant hPDI was expressed, purified as a single band at ~60 kDa on Coomassie Brilliant Blue-stained SDS-PAGE and verified by Western blotting for further enzymatic and structural analysis (Figure S1a). Initial evaluation of Native-PAGE followed by Western blotting using hPDI specific antibody indicated at least two main redox isoforms with different electrophoretic mobilities which refer to the oxidized (opened) and reduced (compact) states at upper and lower bands, respectively (Figure S1b).

Insulin turbidity assay was carried out to evaluate the effect of E2 on recombinant hPDI reductase activity. As shown in Figure 1a, during this process, hPDI reduces two inter-chain disulfides of insulin and becomes oxidized through the ping-pong reaction, while B chain precipitate in the reaction buffer [29]. The cycle is restarted by reducing hPDI via DTT as the recovering agent. Figure 1b shows the absorbance of precipitated B chain at wavelength of 650 nm at six different concentrations of E2. Since there is a delay time between the production and complete precipitation of free B chain aggregates, the maximal slopes of the absorbance curves and the lag times for starting precipitation at different concentrations of E2 were used to evaluate hPDI reductase activity [29]. As shown in Figure 1b and 1c, E2 interaction prevents hPDI reductase activity. IC₅₀ was calculated by rescaling all values at different E2 concentrations against the 0 μ M of E2. The required concentration of the ligand for inhibiting 50% of hPDI activity (IC₅₀) was determined as 0.71 μ M (Figure 1c).

To investigate whether the E2 binding is associated with hPDI conformational changes and functional inhibition, redox-dependent conformational dynamics of hPDI was studied by intrinsic fluorescence spectroscopy. The fluorescence spectra of ox- and red-hPDI are shown in Figure 2a. As shown in this figure, ox-hPDI spectrum displayed a red shift of the

maximum emission wavelength (~ 2.35 nm) with 14.76% decrease in fluorescence intensity with respect to red-hPDI, which confirms the higher solvent accessibility of tryptophan residues in the average conformations of ox-hPDI in buffer solution.

Next step was to use fluorescence quenching to investigate the influence of E2 interaction on conformational shifting of ox- and red-hPDI. Figure 2b and 2c shows that the titration of 10 μ M E2 for 12 additions reduced fluorescence intensities in both red- and ox-hPDI with much more effect of ox-hPDI, which indicated that the tryptophan residues in both states become accessible due to the E2 interaction. hPDI has five tryptophans, two of them are adjacent to the catalytic sites (i.e. TRP52 and TRP396) and therefore become quenched [30]. To answer the question of which of the three remaining tryptophans plays the most in quenching experiments, solvent accessible surface area (SASA) of ox- and red-hPDI were computationally calculated during the MD simulation in ox- and red-hPDI in the presence and absence of E2. As shown in Figure 2d, TRP364 is more accessible to the solvent compare to the TRP128 and TRP407. The significant higher solvent accessibility of TRP364 was also detected between the ox-hPDI and red-hPDI which might explain the importance of this residue as a fluorescence probe to evaluate hPDI conformational changes. Moreover, the SASA values in ox- and red-hPDI in the presence of E2 indicated the significant higher solvent accessibility of TRP364 compare to the unliganded state (Figure 2e and 2f). TRP364 is located in the X-linker between the b' and a' domains, which mostly contribute to adjustment of hPDI inter-domain motions during the catalytic and chaperone activities [7]. Therefore, E2 might inhibit hPDI reductase activity by disturbing domain rearrangements.

Binding features of E2 to ox- and red-hPDI were analyzed by Stern-Volmer/modified Stern-Volmer equations at two different temperatures within the range of IC₅₀ and shown in Figure

3a-3c. In Both states, the bimolecular quenching constant of the quencher (K_{sv} ; slopes of the Figure 3a plots) were decreased by elevating the temperature confirming the formation of ligand-protein complex at the ground state (i.e. static quenching) (Table 1). The fraction of accessible initial fluorescence (f_a) for the E2 were derived from modified Stern-Volmer equation for ox- and red-hPDI (as the intercept of Figure 3b plots) and listed in Table 1. These values indicated that the f_a is much more in oxidized compare to the reduced state (Figure 3b and Table 1). The number of binding sites (n) and binding constant ($K_{Binding}$) at 300 and 310 K were determined as the slope and the intercept of Figure 3c plots in ox- and red-hPDI. Binding stoichiometry is one ligand per each ox- or red-hPDI ($n \sim 1$). Moreover, comparative evaluation between the two redox states showed the higher affinity of E2 binding for ox-hPDI due to the higher binding constant relative to the red-hPDI at 37°C (Figure 3c and Table 1). Since the enthalpy and entropy remained constant or not significantly changed over the studied temperatures, standard enthalpy changes (ΔH°) for both redox states were calculated by solving the van't Hoff equation [18]. Other thermodynamic parameters were obtained using the equation 4 [18] and listed in Table 1.

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K_{Binding} \quad (\text{eq. 4})$$

Where ΔG° , ΔS° , T and R are the standard Gibbs free energy, entropy changes of binding, temperature and gas constant, respectively. As shown in Table 1, the negative signs of ΔG° with positive signs of ΔH° and ΔS° specify that the process of E2 interaction is spontaneous, endothermic and entropy-driven in both red and ox-hPDI. Since positive values of ΔH° and ΔS° are associated with hydrophobic interactions [31], E2 mostly interacts hydrophobically with ox- and red-hPDI (Table 1). Taken together, experimental analyses indicated that E2 binding is under the influence of hPDI oxidation states and this might have impact on

reductase activity of hPDI. This is also in agreement with recent study which highlighted the redox-dependent ligand binding of hPDI since the ligand model (α -synuclein) could only interact with ox-hPDI rather than reduced form [32].

Detailed characterization of E2 interaction sites in ox- and red-hPDI was obtained via computational studies. Docking analysis of E2 with NMR structure of bb' domains of hPDI indicated that HIS256 in the core of hydrophobic pocket of b' domain mainly contribute to E2 binding [15]. Imidazole ring of the histidine has two nitrogen atoms (ND1 and NE2) and thus, different protonation states are possible for this residue (i.e. HSD, HSE, HSP, which depends on the protonation of ND1, NE2 or both, respectively). As shown in Figure S2, initial results on examination of MD trajectories indicated that HIS256 become more solvent accessible in HSD form in both ox- and red-hPDI. Due to the localization of this residue within the hydrophobic pocket, environmental variations upon ligand and/or substrate binding might have influence on its protonation states and regulation of interactions.

To assess whether the protonation state of HIS256 is associated with E2 interaction sites, E2 binding under the different HIS256 protonation states in ox- and red-hPDI were analyzed. Total 48 interaction sites were identified by clustering of complex ensembles according to their positions. Top three binding sites with highest affinity in each system were displayed in Figure 4. The results of additional clustering for identification of binding modes within the best affinity interaction sites were also depicted in colored boxes of Figure 4. As shown in panel a, the cluster within the hydrophobic pocket (S1) exhibits the best affinity in both states in HSE form with higher score in ox-hPDI (9.2 kcal/mol) compared to the red-hPDI (8.4 kcal/mol) which is in agreement with our fluorescence studies. Lower affinity interaction sites (S2 and S3) are located in the bb' cleft. Analysis of physicochemical properties of

surrounding residues within 4.5 Å of ligand in each interaction site indicated that 80% of residues in the S1 are hydrophobic. This value goes down to the 46% in S2 and S3, 41% in S4 and only 18% in S5. Therefore, it seems that S1 provides hydrophobic interactions in comparison with other possible binding sites which explains the hydrophobic nature of E2 interaction in both ox- and red-hPDI.

As shown in Figure 4b, displacement of a single hydrogen atom of HIS256 into ND1 resulted in alteration of top three affinity clusters of E2 interaction sites in HSD group. The highest affinity cluster is associated with S2 between the bb' domains in both oxidation states. By contrast to the HSE group, the third position belongs to the hydrophobic pocket (S1) in ox- and red-hPDI. This result also indicated the significant effect of HIS256 and its protonation state on relative preferences of E2 binding sites.

For better characterization of E2 binding and its dynamics under the different HIS256 protonation states of ox- and red-hPDI, best mode of E2 binding in each system were analyzed during the MD simulations. Figure 5 shows time-course pattern of hydrogen bonds formation between the E2 and its surrounding residues within the S1 pocket. As shown in Figure 5a and 5b, HSE256 and HIS438 play important role as the hydrogen acceptors and TRP396 as the hydrogen donor in red-hPDI, while HSE256 and ILE248 contribute as the hydrogen donors and acceptor in ox-hPDI, respectively. ARG300 works as hydrogen donor in both oxidation state. The hydrogen bond propensities in the S1 pocket during the MD trajectories are listed in Table S1a.

The RMSD fluctuations of E2 in S1 in ox- and red-hPDI were depicted in Figure 6a. The comparative overview between the RMSD and time-course hydrogen bonding pattern of E2 indicated that in ox-hPDI, HSE256 and ILE248 mainly form hydrogen bonds with 17-

hydroxyl group of E2 as the hydrogen donor and acceptor, respectively (Figure 6b). As shown in Figure 6a and 6b, the cation- π interaction and hydrogen bond of ARG300 also regulates E2 binding. These interactions induce two poses of binding during the simulation. In red-hPDI, the C-ring of the E2 become stable via hydrogen bond of HSE256 and 17-hydroxyl group of E2 (pose-I). Formation of extra hydrogen bonds via HIS438 and TRP396 of the a' domain with 3-hydroxyl group of E2 induce another binding pose for E2 (pose-II). Therefore, E2 binding might affect inter-domain arrangements in red-hPDI. Interestingly, HSE256 act as both acceptor and donor of hydrogen during the E2 binding in red- and ox-hPDI, respectively. Results of interaction energies of S1 residues are also complemented to the pattern of hydrogen bonds. Some of these residues are typically involved in electrostatic interaction energy (e.g. ILE248, HIS256, ARG300, TRP396 and HIS438), while others e.g. PHE304, ARG300, PHE249 and MET324 mostly contribute to the van der Waals interaction energy (see Figure S3). The overall interaction energies in S1 is indicated in Figure S4 and Table S2. Among residues of S1 pocket, ILE248 and ARG300 show more contribution in overall electrostatic interaction energy in ox-hPDI compared to the reduced form, which is also in agreement with their hydrogen bond formation in this state (Figure S3, S4 and Figure 5a).

Figure 7 indicated the analyzing results of hydrogen bonds of S2 cleft in HSD group. Residues with dominant hydrogen bonds during the simulations are PHE288, LYS230, GLU252, GLY284 ASP181 and LEU236. The frequency of the formation of hydrogen bonds are also listed in Table S1b. The RMSD values indicated that there are at least two binding poses for E2 within the bb' cleft of ox-hPDI (Figure 8a). Presence or absence of dual hydrogen bonds (ASP181 with 17-hydroxyl group of E2 and GLY284 with 3-hydroxyl E2) induced binary poses for E2 binding in this state (Figure 7 and 8). In red-hPDI, another

pattern of hydrogen bonds formed which keeps E2 in a steady state condition (e.g. PHE288 and LEU236) (Figure 8). Interaction energy analysis of surrounding residues also highlighted the importance of unfavorable electrostatic interactions of LYS247 and LYS283 in ox-hPDI which mostly increased potential energy of interaction in ox-hPDI (see Figure S5, S6 and Table S2).

Previous investigation on characterization of E2 binding site indicated that this site is located between the b and b' domains. While they only used truncated bb' domains for docking analysis, their experimental analysis on hPDI indicated the importance of both b and b' domains on E2 interaction without considering the redox-conformational changes [15]. In conjugation with this study, we revealed that at least three conformational factors should be considered for E2 binding: i) hPDI tetra-domain arrangements and their dynamical behavior, ii) red/ox conformations of hPDI and iii) protonation states of HIS256 within the hydrophobic pocket. These factors not only affect binding environment but also regulate interaction affinity and position.

In summary, our study highlighted the possible roles of red/ox conformations in regulation of hormone-binding capacity of hPDI. The position and the affinity of E2 interaction are affected by hPDI oxidation cycle and also the protonation states of HIS256 within the core of S1 pocket. In HSE256 group, the conformation of S1 pocket is preferred by E2 in both redox states of hPDI, while in HSD256 group, The S2 cleft—between the b and b' domains—becomes more favored. In both cases of HSE256 and HSD256 conditions, the highest interaction affinities were detected in oxidized rather than reduced hPDI. Different cellular localization of hPDI in plasma membrane and endoplasmic reticulum along with its unique redox environmental sensation suggested that different binding affinity of E2 in red- and ox-

hPDI might have regulatory impact on its functional activities. This feature not only might control the redox balance of ox-/red-hPDI in the endoplasmic reticulum but also adjust the storage and downstream actions of E2 within the cell.

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FIGURE LEGENDS

FIGURE 1. The effect of 17- β -estradiol (E2) binding on hPDI reductase activity **(a)** Redox-dependent reductase activity of human protein disulfide isomerase (hPDI) with the insulin (INS) precipitation assay. Red van der Waals representations denotes cysteines **(b)** Absorption spectra of Insulin chain B at 650 nm at different concentrations of E2. Slope and lag time represent the $V_{\max}$ and t_{lag} , respectively. **(c)** The half maximal inhibitory concentration (IC₅₀) of E2 for hPDI reduction activity (Standard errors are presented by error bars).

FIGURE 2. Redox-associated conformational changes of hPDI upon E2 interaction. **(a)** Fluorescence spectra of reduced (blue) and oxidized (red) hPDI at temperature of 310 K. **(b)** and **(c)** Fluorescence quenching spectra of reduced (blue) and oxidized (red) hPDI in presence of E2. Total concentrations of E2 started from 0 μ M (A) to 1 μ M (L) in **(b)** red- and **(c)** ox-hPDI at temperature of 310 K (Standard errors are presented as error bars). **(d)** Solvent accessible surface area (SASA) of tryptophans in ox- and red-hPDI during the MD simulations. Comparative SASA of **(e)** reduced and **(f)** oxidized hPDI in the presence and absence of E2 during the MD trajectories (Wilcoxon signed-rank test significance: (*) p -value = 2.681e-13, (**) p -value = 2.834e-15, (***) p -value < 2.2e-16).

FIGURE 3. Stern–Volmer plots (**a** and **b**), and modified Stern–Volmer plots (**c**) of ox- (red) and red- (blue) hPDI by E2 interaction at temperature of 300 K (circle) and 310 K (triangle) (Standard errors are presented as error bars). F_0 and F are the fluorescence intensities in the absence and presence of E2, respectively; K_{sv} Stern–Volmer constant; kq : bimolecular quenching constant; τ : the fluorophore lifetime in the absence of quencher; f_a : the intensity of the accessible fraction; $K_{binding}$: binding constant; n : binding stoichiometry.

FIGURE 4. E2 interaction sites and binding modes of hPDI in oxidized (**a**) and reduced (**b**) states under the ND1 (HSD) and NE2 (HSE) protonation states of HIS256. The binding sites were ranked according to their affinities into S1-S5. Red boxes represent top affinities of binding modes within each interaction site. All values are in kcal/mol.

FIGURE 5. Time-course pattern of hydrogen bonds of E2 within the S1 pocket. (**a**) Violin plots of Hydrogen bonds between the E2 and surrounding residues as the acceptor (left) and donor (right) of hydrogens. The probability density of the hydrogen bonds at different time points are indicated in the plots (**b**) Mutual exclusivity of hydrogen bonds occurrence in residues in the S1 as the acceptors (left) and donors (right) of hydrogens.

FIGURE 6. Characterization of E2 binding within the S1 pocket. (**a**) RMSD fluctuation of E2 in ox- and red-hPDI. (**b**) 3D structure of E2 binding in S1 at different redox states and binding poses. E2 colored as yellow. b' and a' domains are shown in grey and blue, respectively. Oxygen, nitrogen, sulfur and carbon atoms were in red, blue, yellow and cyan, respectively. Hydrogen bonds are indicated by magenta dash-lines.

FIGURE 7. Time-course pattern of hydrogen bonds of E2 within the S2 cleft. (**a**) Violin plots of hydrogen bonds between the E2 and surrounding residues as the acceptors (left) and donors (right) of hydrogens. The probability density of the hydrogen bonds at different time

points are indicated in the plots **(b)** Mutual exclusivity of hydrogen bonds occurrence in residues in the S2 as the acceptors (left) and donors (right) of hydrogens.

FIGURE 8. Characterization of E2 binding within the S2 cleft. **(a)** RMSD fluctuation of E2 in ox- and red-hPDI. **(b)** 3D structure of E2 binding in S2 at different redox states and binding poses. E2 colored as yellow. a, b, b' and a' domains are represented in pink, green, grey and blue, respectively. Oxygen, nitrogen, sulfur and carbon atoms were in red, blue, yellow and cyan, respectively. Hydrogen bonds are indicated by magenta dash-lines.

FIGURES

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TABLES

Table 1. Thermodynamic parameters of E2 interactions in ox- and red-hPDI.

Redox state	Temperature (K)	K_{sv} ($10^6 \times M^{-1}$)	f_a	n	$K_{binding}$ ($10^6 \times M^{-1}$)	ΔG^0 ($kJ \cdot mol^{-1}$)	ΔH^0 ($kJ \cdot mol^{-1}$)	ΔS^0 ($J \cdot mol^{-1} \cdot K^{-1}$)	Interaction Mode
O x - hPDI	300	0.52 ± 0.07 6	1.03	1.09	0.50 ± 0.09 6	-32.74	+61.42	+313.86	Hydrophobic
	310	0.40 ± 0.02 7	0.45	0.93	0.40 ± 0.03 3	-33.23			
R e d - hPDI	300	0.45 ± 0.07 3	0.55	0.94	0.50 ± 0.07 0	-32.74	+38.75	+238.30	Hydrophobic
	310	0.30 ± 0.03 1	0.38	0.80	0.25 ± 0.03 5	-32.05			