Thermodynamics of the folding of d-glyceraldehyde-3-phosphate dehydrogenase assisted by protein disulfide isomerase studied by microcalorimetry

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Thermodynamics of the refolding of denatured d-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) assisted by protein disulfide isomerase (PDI), a molecular chaperone, has been studied by isothermal microcalorimetry at different molar ratios of PDI/GAPDH and temperatures using two thermodynamic models proposed for chaperone-substrate binding and chaperone-assisted substrate folding, respectively. The binding of GAPDH folding intermediates to PDI is driven by a large favorable enthalpy decrease with a large unfavorable entropy reduction, and shows strong enthalpy-entropy compensation and weak temperature dependence of Gibbs free energy change. A large negative heat-capacity change of the binding, $-156 \text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, at all temperatures examined indicates that hydrophobic interaction is a major force for the binding. The binding stoichiometry shows one dimeric GAPDH intermediate per PDI monomer. The refolding of GAPDH assisted by PDI is a largely exothermic reaction at 15.0–25.0 °C. With increasing temperature from 15.0 to 37.0 °C, the PDI-assisted reactivation yield of denatured GAPDH upon dilution decreases. At 37.0 °C, the spontaneous reactivation, PDI-assisted reactivation and intrinsic molar enthalpy change during the PDI-assisted refolding of GAPDH are not detected.

Keywords: GAPDH folding; microcalorimetry; molecular chaperone; protein disulfide isomerase; thermodynamics.

Protein disulfide isomerase (PDI) is an unusual multifunctional protein mainly located in the endoplasmic reticulum at high concentration [1]. It has been characterized to be a physiological catalyst for the formation of the native disulfide bonds of nascent polypeptides [2] and a part of the quality-control machinery in the endoplasmic reticulum [3]. In recent years, more and more [4–6] and in vitro [7,8] experimental data have supported the theory that PDI functions as both an enzyme and a molecular chaperone [9]. The intrinsic chaperone activity of PDI, which is independent of its isomerase activity, was first identified in this laboratory by its actions to increase PDI, which is independent of its isomerase activity, was first identified in this laboratory by its actions to increase PDI-assisted folding has nothing to do with the formation of disulfide bonds and can only be accounted for by the chaperone activity of PDI. Unlike most chaperones, such as heat-shock protein (Hsp)60 and Hsp70, which require ATP to stimulate the release of bound substrate for further folding [10], PDI binds with folding intermediates formed in an early stage after dilution of denatured GAPDH to form transient complexes, and dissociation is independent of ATP [4].

GAPDH is a homotetrameric enzyme and has been used as a model protein for studies on unfolding, refolding, dissociation and association of oligomeric proteins [11,12] and on the intrinsic chaperone activity of PDI [4]. A stable cold-folding intermediate of GAPDH as a dimer has been characterized for the interaction with GroEL [13].

Microcalorimetry, including differential scanning calorimetry and isothermal microcalorimetry, has been widely used to study the thermodynamic and kinetic properties of interactions between biological macromolecules by virtue of its general applicability and high precision [14–16]. The thermal unfolding of GAPDH has recently been investigated by differential scanning calorimetry [17]. The binding reactions of chaperones, such as GroEL [18–20], SecB [21–23], SecA [24] and Hsp90 [25], with substrates, but no folding reactions assisted by chaperones have been studied by microcalorimetric methods. The mechanism by which PDI interacts with folding intermediates is little known and no information on the thermodynamics of PDI-assisted protein folding by microcalorimetry is available so far, although PDI has now been widely recognized as a chaperone [26–28].

In this communication, the thermodynamics of GdnHCl-denatured GAPDH refolding assisted by PDI at various temperatures and molar ratios of chaperone to substrate has been studied by microcalorimetry using two thermodynamic models proposed for chaperone-substrate binding and chaperone-assisted folding, respectively. The results

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Abbreviations: GAPDH, d-glyceraldehyde 3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; Hsp, heat-shock protein; PDI, protein disulfide isomerase.

Enzymes: d-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); protein disulfide isomerase (EC 5.3.4.1).

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indicate that the binding of GAPDH folding intermediates to PDI is driven by a large favorable enthalpy decrease with a large unfavorable entropy reduction, showing strong enthalpy–entropy compensation and weak temperature dependence of Gibbs free energy change.

**MATERIALS AND METHODS**

**Materials**

PDI prepared from bovine liver [29] showed one band on SDS/PAGE with a specific activity higher than 800 U g⁻¹. The preparation and activity assay of rabbit muscle GAPDH were as described by Liang et al. [11]. GroEL was prepared as described by Zhang and Wang [13].

Protein concentrations were determined by measuring the absorbance at 280 nm with the absorption coefficients (Aₓₐₜₐₜ) of 1.00 for GAPDH and 0.9 for PDI, and by the method of Bradford [30] for GroEL with BSA as a standard. Homotetrameric GAPDH and homodimeric PDI were both considered monomers and GroEL a tetradecamer in the calculations of concentrations and molar ratios. In all experiments, 0.1 M potassium phosphate buffer containing 2.5 mM EDTA, 5 mM dithiothreitol, pH 7.5, was employed and is referred to as phosphate buffer.

**Unfolding and refolding of GAPDH**

GAPDH at 139 μM was fully denatured in phosphate buffer containing 3.0 M GdnHCl at 4 °C overnight. The refolding of denatured GAPDH was initiated by 50-fold dilution at 15.0, 20.0, 25.0 and 37.0 °C in phosphate buffer in the presence or absence of different concentrations of PDI or GroEL. Recovery of activity was determined 3.5 h after dilution, and the reactivation yield was defined as percentage of native GAPDH activity [4]. Although preincubation of GAPDH-refolding solution at 4 °C for 30–60 min followed by incubation at certain temperatures for 2 h increases the reactivation yields significantly [13], it is impossible in the present study of the isothermal microcalorimetric measurements to carry out the preincubation. Nevertheless, the interaction between PDI and GAPDH folding intermediates characterized by microcalorimetric measurements and the activity assay of refolded GAPDH were accurate and reliable.

**Isothermal microcalorimetric measurements**

Samples were centrifuged to remove particles before calorimetric measurements using an LKB-2107 batch microcalorimeter comprising a microbatch reactor and a heat-conduction isothermal calorimeter [31,32]. Compartments I and II of the reaction and reference cells contained 20.0 μL denatured-GAPDH solution and 980 μL refolding solution, respectively. There was no chaperone in the refolding solution in compartment II of the reference cell, in order to deduct the heats of the dilution, mixing, aggregation and reactivation of GAPDH during the spontaneous refolding. When the microcalorimetric system reached a thermal equilibrium with a steady baseline, the refolding was initiated by rotation of the calorimeter unit 360 degrees clockwise and then anticlockwise to mix thoroughly the solutions in the two compartments of dilution of denatured protein. Little heat was observed on the spontaneous refolding of denatured GAPDH on dilution, and was therefore ignored. The heat released by dilution of PDI or GroEL was determined to be negligible. The pH value of the refolding solution was determined to be unchanged after the calorimetric measurements.

**THERMODYNAMIC MODELS**

**Thermodynamic model for chaperone–substrate binding**

For the binding reaction of a chaperone to its substrate with n identical and independent binding sites, the intrinsic binding constant, Kᵣᵣ, is defined by Eqn (1):

$$Kᵣᵣ = \frac{x}{(1 - x)(C_C,0 - nxC_S,0)}$$

Here, C_C,0 and C_S,0 are concentrations of the chaperone and the substrate after dilution, respectively; n is binding stoichiometry (i.e. the number of chaperones bound by one substrate molecule); and the part of the substrate bound by chaperone, x, is determined by the formula in Eqn (2):

$$x = \frac{\Delta H_{m,a}^{b}}{\Delta H_{m,0}^{b}}$$

where ΔH_{m,0}^{b} is the intrinsic enthalpy change for chaperone–substrate binding, and ΔH_{m,a}^{b}, the apparent molar binding enthalpy of substrate, which is calculated from the peak area of the experimental calorimetric curves [32].

For Eqn (3):

$$r = \frac{nC_C,0}{nS,0} = \frac{C_C,0}{C_S,0}$$

Here, r is molar ratio of chaperone to substrate.

Substituting Eqns (2 and 3) into Eqn (1), we get

$$r = \frac{(C_S,0Kᵣᵣn + 1)\Delta H_{m,a}^{b} - C_S,0Kᵣᵣn(\Delta H_{m,a}^{b})^2}{C_S,0\Delta H_{m,0}^{b} - C_S,0\Delta H_{m,a}^{b}}$$

The above thermodynamic model, an isotherm for chaperone–substrate binding, is used to perform a nonlinear least-squares analysis of the molar ratio r as an explicit function of the apparent molar binding enthalpy, ΔH_{m,a}^{b}, using Microcal ORIGIN software (version 5.0), and the three unknown binding parameters, Kᵣᵣ, ΔH_{m,0}^{b}, and n, are thus obtained.

**Thermodynamic model for chaperone-assisted substrate folding**

For the folding of a substrate assisted by a chaperone with molar ratios to the substrate higher than the corresponding binding stoichiometry, the heat determined by using isothermal microcalorimetry represents the apparent molar enthalpy change (ΔH_{m,a}), which is calculated from the peak area of the experimental calorimetric curves [32]. The value of ΔH_{m,a} includes contributions of the intrinsic enthalpy change for chaperone–substrate binding, ΔH_{m,0}^{b}, and the apparent molar enthalpy change during chaperone-assisted folding of the substrate, ΔH_{conf}^{b}. Thus, in Eqn (5) we have:

$$\Delta H_{m,a} = \Delta H_{m,0}^{b} + \Delta H_{conf}^{b} b(r - n)^{b} \frac{1}{1 + b(r - n)^{b}}$$

Here, b is molar ratio of substrate to chaperone.
where $\Delta r e f H^0_m$ is the intrinsic molar enthalpy change during chaperone-assisted folding, and $b$ is an empirical coefficient correlated to the chaperone activity. The larger the value of $b$, the more pronounced the chaperone activity. The above thermodynamic model is an empirical isotherm for chaperone-assisted substrate folding and is used to perform a nonlinear least-squares analysis of the total apparent enthalpy change $\Delta H_{m,a}$ as an explicit function of the difference between $r$ and $n$. Three unknown parameters for folding and binding, $\Delta r e f H^0_m$, $\Delta r e f H^0_m$, and $b$, are thus obtained. The value of the power in the Eqn (5) has been optimized to be eight.

**RESULTS**

Thermodynamics of binding of GAPDH folding intermediates with PDI

As shown in Fig. 1, no calorimetric change was detected for the dilution of native GAPDH in the presence of PDI at 25.0 °C (same data at 15.0, 20.0 and 37.0 °C are not shown), indicating no interaction between the native GAPDH and PDI. The calorimetric curves for the refolding of denatured GAPDH at 25.0 °C in the presence of PDI at molar ratios to GAPDH of 0.32 and 0.63 returned to the baseline within 5 min. The calorimetric data within 5 min referred to the binding of GAPDH folding intermediates to PDI, as little activity of GAPDH was recovered within the first 5 min of the refolding (Fig. 2). At higher molar ratios of 3.2 and 6.3, it took <1 h for calorimetric curves to return to the baseline. The heat produced in 3.5 h referred to the refolding of denatured GAPDH, as the reactivation of GAPDH reached a maximal level in 3.5 h (Fig. 2). The heat of GAPDH folding included the heat of binding of GAPDH folding intermediates to PDI.

As shown in Fig. 3, in the presence of PDI with molar ratios to GAPDH of 0.16–3.2, the apparent molar enthalpy changes ($\Delta H_{m,a}$) for the binding of GAPDH folding intermediates with PDI at 15.0, 20.0, 25.0 and 37.0 °C were all negative and reached a maximal value at a molar ratio of 0.63. The thermodynamic parameters obtained according to Eqn (4) were summarized in Table 1. The small standard deviations indicated that the thermodynamic model for...
Table 1. Thermodynamic parameters for the binding of GAPDH folding intermediates with PDI and GroEL. Refolding of denatured GAPDH was carried out as described in the legend to Fig. 3 in the presence of different concentrations of PDI or GroEL. Thermodynamic parameters, $K_b$, $\Delta H_m^0$ and $n$, were determined using Eqn (4) as described in ‘Thermodynamic models’. The intrinsic molar binding free energy ($\Delta G_m^0$) and the intrinsic molar binding entropy ($\Delta S_m^0$) for the binding reaction were calculated using $\Delta G_m^0 = -RT\ln K_b$ and $\Delta S_m^0 = \frac{\Delta H_m^0 - \Delta G_m^0}{T}$, respectively. Data are expressed as mean $\pm$ SD ($N = 2$–4).

<table>
<thead>
<tr>
<th>Chaperone</th>
<th>Temperature (°C)</th>
<th>$K_b \times 10^{-7}$ (m$^{-1}$)</th>
<th>$n$</th>
<th>$\Delta H_m^0$ (kJ·mol$^{-1}$)</th>
<th>$\Delta G_m^0$ (kJ·mol$^{-1}$)</th>
<th>$\Delta S_m^0$ (kJ·mol$^{-1}$·K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDI</td>
<td>15.0</td>
<td>6.37 $\pm$ 1.93</td>
<td>0.435 $\pm$ 0.083</td>
<td>$-6840 \pm 10$</td>
<td>$-43.0 \pm 0.7$</td>
<td>$-23.6 \pm 0.04$</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>4.92 $\pm$ 0.55</td>
<td>0.509 $\pm$ 0.031</td>
<td>$-7440 \pm 10$</td>
<td>$-43.2 \pm 0.3$</td>
<td>$-25.2 \pm 0.04$</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>3.24 $\pm$ 0.88</td>
<td>0.447 $\pm$ 0.096</td>
<td>$-8620 \pm 20$</td>
<td>$-42.9 \pm 0.7$</td>
<td>$-28.8 \pm 0.1$</td>
</tr>
<tr>
<td></td>
<td>37.0</td>
<td>1.93 $\pm$ 0.46</td>
<td>0.442 $\pm$ 0.097</td>
<td>$-10500 \pm 30$</td>
<td>$-43.3 \pm 0.6$</td>
<td>$-33.7 \pm 0.1$</td>
</tr>
<tr>
<td>GroEL</td>
<td>25.0</td>
<td>19.4 $\pm$ 7.2</td>
<td>0.552 $\pm$ 0.025</td>
<td>$-9020 \pm 30$</td>
<td>$-47.3 \pm 0.9$</td>
<td>$-30.1 \pm 0.1$</td>
</tr>
</tbody>
</table>

Chaperone–substrate binding was appropriate. The values of $n$ at the four temperatures examined were $\approx 0.5$, which is close to the molar ratio of 0.63 at which $\Delta H_m^0$ reached the maximum. This suggests a stoichiometry of one PDI subunit binding with two GAPDH subunits. A plot of $\Delta H_m^0$ vs. $T\Delta S_m^0$ for the binding reaction at different temperatures showed a slope of 0.989 with a linear correlation coefficient of 1.00 and an enthalpy intercept of $-121$ kJ·mol$^{-1}$, indicating strong enthalpy–entropy compensation.

As shown in Fig. 4, the molar heat-capacity changes of binding, $\Delta C_m^PDI$, were $-170$ kJ·mol$^{-1}$·K$^{-1}$ for the plot of $\Delta H_m^0$ vs. $T$ and $-141$ kJ·mol$^{-1}$·K$^{-1}$ for the plot of $\Delta S_m^0$ vs. lnT with linear correlation coefficients of $-0.9962$ and $-0.9933$, respectively. The values of $\Delta C_m^PDI$ obtained from the above two plots squared with each other. The above results indicated that the molar heat-capacity change of binding was independent of temperature.

In order to test further the validity of Eqn (4), the thermodynamic parameters for the binding of GroEL, a well-characterized chaperone [33,34], to GAPDH folding intermediates at 25.0 °C were also determined using the same method (Table 1). The experimental data (not shown) corresponded closely to the values predicted using Eqn (4).

**Thermodynamics of PDI-assisted folding of GAPDH**

As shown in Fig. 5, in the presence of PDI with molar ratios of 0.63–9.5 the apparent molar enthalpy changes for PDI-assisted refolding of denatured GAPDH at 15.0, 20.0, 25.0 and 37.0 °C were also negative. The solid lines predicted by using Eqn (5) and parameters in Table 2 represented the apparent molar enthalpy changes for the folding reaction, which accorded well with the experimental data. At 37.0 °C, the values of $\Delta H_m^{\text{obs}}$ did not change and remained the same as the value of $\Delta H_m^{\text{act}}$ at molar ratios of PDI/GAPDH higher than 0.5 in Fig. 3, suggesting that no refolding occurred. The small standard deviations for the thermodynamic data (Table 2) indicated that the thermodynamic model for chaperone-assisted substrate folding was also appropriate.

**Effect of temperature on PDI-assisted reactivation of denatured GAPDH**

As shown in Fig. 6, the reactivation yield of denatured GAPDH of 2.78 µM at 15.0, 20.0 and 25.0 °C increased...
with an increasing ratio of PDI to GAPDH. Either the spontaneous or the PDI-assisted reactivation of GAPDH decreased with the increase of temperature and was not detected at 37.0 °C. The above results were in agreement with the thermodynamic data in Fig. 5.

**DISCUSSION**

The two thermodynamic models developed in this study are adaptable to contemporary isothermal titration calorimetry. The validity of the two models has been demonstrated by the following facts. (a) The experimental data are in excellent accordance with the theoretical curves predicted using the two models, with small standard deviations for adaptable to contemporary isothermal titration calorimetry. The two thermodynamic models developed in this study are determined using Eqn (5) in ‘Thermodynamic models’. Data are expressed as mean ± SD (N = 2–4), b is an empirical coefficient correlated to the ability of a chaperone; z is the relative increase of the PDI-assisted reactivation yields of GAPDH compared to the spontaneous reactivation yields.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>i &gt; j &gt; b</th>
<th>H_{in}^b (kJ·mol(^{-1}))</th>
<th>i &gt; j &gt; ref</th>
<th>H_{in}^b (kJ·mol(^{-1}))</th>
<th>b × 10^5</th>
<th>z (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.0</td>
<td>−6900 ± 140</td>
<td>−3710 ± 190</td>
<td>3.00 ± 0.98</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>−7440 ± 160</td>
<td>−3110 ± 220</td>
<td>1.00 ± 0.38</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>−8580 ± 150</td>
<td>−4410 ± 220</td>
<td>0.904 ± 0.235</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37.0</td>
<td>−10300 ± 50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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</tr>
</tbody>
</table>

When the molar ratios of PDI to GAPDH are lower than the binding stoichiometry of 0.5, the PDI-assisted refolding of GAPDH is not significant, and the apparent molar enthalpy changes result mainly from binding. When the molar ratios of PDI to GAPDH are higher than 0.5 but lower than 3.2, the apparent molar enthalpy changes plateau, which fits with both equations, as the binding is saturated but no significant reactivation appears. When the concentration of PDI increases further, the PDI-assisted refolding of GAPDH becomes dominant, and the apparent molar enthalpy changes increase until the ratio of PDI to GAPDH reaches 6.3. Therefore the apparent molar enthalpy change is PDI-concentration dependent and appears to be bi-phasic, with a binding phase and a refolding phase. The above findings indicate that PDI functions as a chaperone much more efficiently at higher concentrations [4,6,35].

The binding of PDI to GAPDH folding intermediates is driven by a large favorable enthalpy change but with a large unfavorable entropy decrease, both of which depend strongly on the temperature, whereas the Gibbs energy of association is almost temperature independent. The binding reaction is an exothermic process in the temperature range 15.0–25.0 °C with marked enthalpy–entropy compensation. The temperature independence of $\Delta_b C_m^0$ and the strong enthalpy–entropy compensation result from the large negative value of $\Delta_b C_p^m$ [24,36], $−156 ± 12$ kJ·mol\(^{-1}\)K\(^{-1}\) (the average of the two values of $\Delta_b C_p^m$ in Fig. 4), which indicates that hydrophobic interactions are the major driving force of the binding reaction [18,19,36]. It is of note that the value of $\Delta_b C_p^m$ for PDI–GAPDH binding is two orders of magnitude higher than that for the binding reactions of GroEL [18,19] or SecB [23] with substrates.

Like the binding of SecB with carboxymidomethylated RNase A or carboxymidomethylated α-lactalbumin [23], the binding constant, $K_b$, of the PDI–GAPDH interaction decreases with temperature increases in the range 15.0–37.0 °C, indicating an exothermic reaction. With decreasing temperature from 37.0 to 15.0 °C, both the empirical coefficient for the chaperone activity of PDI, b, and the percentage of the increase of the PDI-assisted reactivation yield of GAPDH, z, increase in a parallel way. At 37.0 °C, neither the intrinsic molar enthalpy change during the refolding of denatured GAPDH nor the reactivation assisted by PDI was detected. The above suggest that in the temperature range 15.0–37.0 °C, PDI shows more pronounced *in vitro* chaperone activity in

![Fig. 6. Effect of temperature on the PDI-assisted reactivation of denatured GAPDH. Refolding of denatured GAPDH was carried out as described in the legend to Fig 1 in the presence of different concentrations of PDI at: (■) 15.0 °C; (○) 20.0 °C; (▲) 25.0 °C; and (▼) 37.0 °C. The activity of recovered GAPDH was assayed 3.5 h after dilution. The data are the averages of two independent experiments.](image-url)
assisting the refolding of GAPDH at lower temperatures than at higher temperatures. It has been reported that the values of $K_0$ for the binding of GroEL with the unfolded mutant of subtilisin, BPN$^\prime$ [18] or denatured Cys-Ala double mutant $\beta$-lactamase [37] increase with increasing temperature in the measured range, and higher temperatures favor the GroEL-assisted reactivation of denatured Cys-Ala double mutant $\beta$-lactamase in an endothermic reaction [37]. Considering the fact that the binding of GroEL with GAPDH folding intermediates is driven by a large favorable enthalpy change with a large unfavorable entropy change, and the spontaneous reactivation yield of denatured GAPDH decreases with increasing temperature in the range 10–25 °C [13], the present temperature dependence of the PDI-assisted folding of GAPDH may be ascribed to the nature of GAPDH folding itself. However, the possibility that PDI has higher chaperone activity at lower temperatures cannot be excluded. Crowding has been reported to enhance the chaperone activity of PDI [35]. Higher temperatures increase the activity of some heat shock proteins [37]. For different chaperones, there may be different stress dependence of chaperone activity.

The values of binding stoichiometry of PDI/GAPDH at all temperatures examined are close to 0.5, indicating that one PDI monomer binds with two GAPDH monomers or one GAPDH dimer. It has been suggested that PDI binds with early GAPDH folding intermediates [4] and the dimeric form of GAPDH is the early folding intermediate recognized and bound by the chaperone GroEL [13,33]. Therefore the present thermodynamic data provide the first evidence that each PDI monomer binds with one dimeric GAPDH folding intermediate formed in the early refolding stage.

The dissociation constant, $K_{d}$, for the binding reaction of GAPDH folding intermediates with PDI at 25.0 °C, 30.9 nm, is six-fold that with GroEL at the same temperature. Recently, Cheung and Churchich [38] reported that PDI binds with the malate dehydrogenase folding intermediate with a $K_0$ of 0.2 $\mu$m, which is fivefold the dissociation constant for the interaction of GroEL with the same substrate. Generally, GroEL binds with its substrate to form a stable complex, which does not dissociate unless ATP is added. In contrast, the disulfide bond-independent binding of PDI with GAPDH intermediates appears to be transient [4]. The binding of GroEL with its substrate would result in the conformational adjustment with space change of its central cavity [39], which could ‘clamp’ the substrate to form a stable complex in the absence of ATP. The PDI molecule may have only a relatively exposed hydrophobic cleft rather than a central cavity as in, for example, the GroEL molecule, for substrate binding.

It is noted that for chaperones GroEL [18–20], SecB [21–23], SecA [24] and Hsp90 [25], binding reactions but not the assisted substrate folding have been studied using microcalorimetry. Those chaperones are all ATP dependent, and the refolding of substrates only happens with ATP hydrolysis, which must produce heat changes to disturb the measurement. The present model for chaperone–substrate binding is suitable not only for PDI but also for GroEL and other chaperones, and the model for chaperone-assisted substrate folding is successful with ATP-independent chaperones. PDI assists the folding of physiological substrates containing disulfide bonds with higher efficiency than nonphysiological substrates [6], as PDI functions as both chaperone and isomerase in these cases. However, the mechanism with which PDI assists the folding of physiological substrates is much more complicated than that in which PDI functions as a disulfide bond-independent chaperone with GAPDH. The present thermodynamic findings have provided further insight into the mechanism with which PDI functions as a molecular chaperone.

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