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On the non-respect of the thermodynamic cycle by DsbA variants

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Abstract

The mechanism of the disulfide-bond forming enzyme DsbA depends on the very low pK_a of a cysteine residue in its active-site and on the relative instability of the oxidized enzyme compared to the reduced one. A thermodynamic cycle has been used to correlate its redox properties to the difference in the free energies of folding ($\Delta\Delta G^{red/ox}$) of the oxidized and reduced forms. However, the relation was proved unsatisfied for a number of DsbA variants. In this study, we investigate the thermodynamic and redox properties of a highly destabilized variant $DsbA_{P151A}$ (substitution of *cis*-Pro151 by an alanine) by the means of intrinsic tryptophan fluorescence and by high-sensitivity differential scanning calorimetry (HS-DSC). When the value of $\Delta\Delta G^{red/ox}$ obtained fluorimetrically for $DsbA_{P151A}$ does not correlate with the value expected from its redox potential, the value of $\Delta\Delta G^{red/ox}$ provided by HS-DSC are in perfect agreement with the predicted thermodynamic cycle for both wild-type and variant. HS-DSC data indicate that oxidized wild-type enzyme and the reduced forms of both wild-type and variant unfold according to a two-state mechanism. Oxidized $DsbA_{P151A}$ shows a deviation from two-state behavior that implies the loss of interdomain cooperativity in DsbA caused by Pro151 substitution. The presence of chaotrope in fluorimetric measurements could facilitate domain uncoupling so that the fluorescence probe (Trp76) does not reflect the whole unfolding process of $DsbA_{P151A}$ anymore. Thus, theoretical thermodynamic cycle is respected when an appropriate method is applied to DsbA unfolding under conditions in which protein domains still conserve their cooperativity.

Keywords: calorimetry; DsbA; redox potential; stability; thermodynamic cycle

Formation of disulfide bonds is a crucial step in the folding, assembly and stabilization of secreted proteins. Deficiency in their formation generally entails misfolding and aggregation or proteolysis of polypeptide chains. In vivo, disulfide bonds formation is catalyzed by specialized disulfide oxidoreductases: protein disulfide isomerase in eukaryotes (Freedman et al., 1994), and several Dsb enzymes in prokaryotes (DsbA, DsbC . . . ; for a review see Bardwell (1994)).

All these oxidoreductases possess a thioredoxin-like domain. They share the same active-site containing two cysteine residues, in the sequence Cys-Xaa_1-Xaa_2-Cys, that form a reversible disulfide bond. Each enzyme possesses a characteristic Xaa_1-Xaa_2 dipeptide sequence (PH for DsbA, GH for protein disulfide isomerase, GP for thioredoxin). This sequence was found to be critical in determining both the stability of the active-site disulfide bond and the oxidizing power of the enzymes (Grauschopf et al., 1995). The change of one dipeptide sequence for another induces a shift of the redox potential in the direction of the protein from which the dipeptide sequence originated (Lundstrom et al., 1992; Kortemme et al., 1996; Chivers et al., 1997). The biophysical characterization of DsbA has shown that this enzyme is the strongest oxidant in the disulfide oxidoreductase family (Wunderlich et al., 1993; Zapun et al., 1993). At least two physical properties can explain this unusual oxidizing power. First, the pK_a of the N-terminal cysteine thiol of the active-site (Cys30) is extremely low (3–3.5) which makes it highly reactive toward the cysteine residues of the protein substrates. Second, the oxidized form of DsbA is more unstable than the reduced form that favors thermodynamically the transfer of its disulfide bridge to substrate proteins during their folding.

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Abbreviations: $\Delta(\Delta G_{ss})$, denaturational increment of disulfide stability; $\Delta\Delta G^{red/ox}$, difference in Gibbs free energy between reduced and oxidized states; ΔC_p, denaturation heat capacity increment; ΔG, Gibbs energy of denaturation; ΔH_{cal}, calorimetric denaturation enthalpy; ΔH_{VH}, van't Hoff enthalpy of denaturation; ΔS, denaturation entropy; DTT, 1,4-dithio-DL-threitol; GdmCl, guanidinium chloride; GSH/GSSG, reduced/oxidized glutathione; HS-DSC, high-sensitivity differential scanning calorimetry; K_{ox}, equilibrium constant for the thiol-disulfide exchange with glutathione; K_{ss}^{u}, equilibrium constant of disulfide bond formation in the denatured state; T_d, denaturation temperature.

Existence of a consistent thermodynamic cycle (Fig. 1) linking the relative stability of both states of DsbA and the redox properties of the protein was proposed (Wunderlich & Glockshuber, 1993; Wunderlich et al., 1993; Zapun et al., 1993, 1994). The difference in stability between the reduced and oxidized forms, $\Delta\Delta G^{red/ox\ 1}$, should be equal to the difference in the stability of the disulfide present in the native and unfolded protein, $\Delta(\Delta G_{ss}) = -RT\ln(K_{ox}/K_{ss}^{u})$. Although the interdependence of the thermodynamic difference and the redox potential was demonstrated for DsbA and a series of variants with substitutions in the dipeptide sequence between the active-site cysteines (Grauschopf et al., 1995), the situation appeared to be more complex for variants with substitution of charged residues in the vicinity of the active-site (Hennecke et al., 1997; Jacobi et al., 1997). Several suggestions can be made to explain these deviations from theory. In this paper, we explore the possibility that the widely used experimental method to determine the free energies of unfolding (fluorescence), may reflect the denaturation process in a biased way. Crystallographic data for oxidized DsbA show the existence of two structural domains in this protein: the thioredoxin domain (domain A) and a compactly folded helical domain (domain B) (Martin et al., 1993). The intrinsic probe of fluorescence (Trp76) is located in the helical B-domain of the protein and may not be affected by structural modification occurring in the thioredoxin domain (Fig. 2). We chose for this work a variant showing large discrepancy between its oxidizing properties and the $\Delta\Delta G^{red/ox}$ value measured by fluorescence. This variant has an alanine in the position 151 instead of a *cis*-proline highly conserved in the thioredoxin family. Interestingly, this proline is the closest residue to the active disulfide bond and is part of a hydrophobic cluster surrounding the active-site which may constitute the unfolded protein binding site (Martin et al., 1993; Guddat et al., 1997). Unfolding processes of wild-type DsbA and $DsbA_{P151A}$ were investigated both by the classical fluorimetric method and by high-sensitivity differential scanning calorimetry (HS-DSC), and compared.

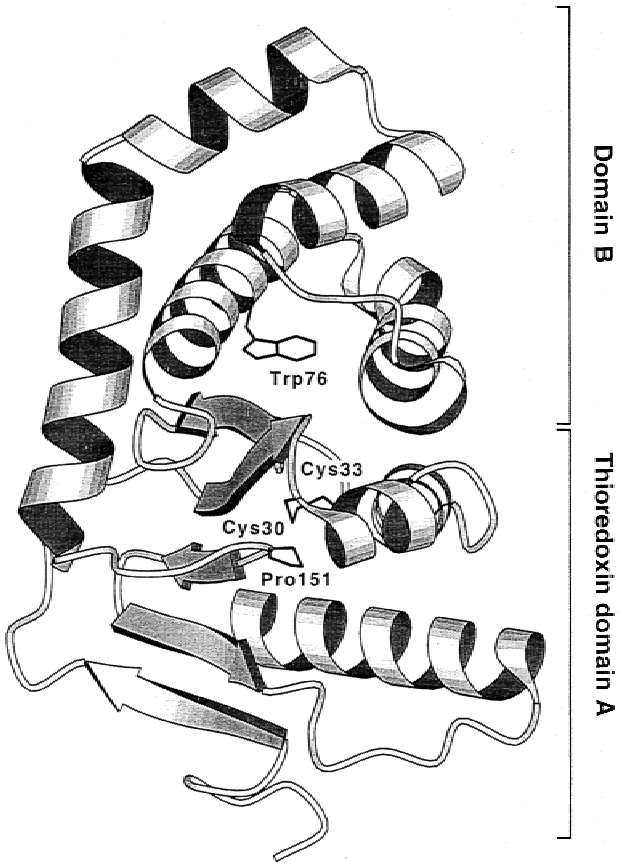

Fig. 2. Ribbon representation of *Escherichia coli* DsbA. The figure was drawn from X-ray coordinates of the protein (PDB entry 1DSB) using Molscript (Kraulis, 1991). The side chains of Cys30, Cys33, Trp76, and Pro151 are highlighted.

Results

Spectroscopic and redox properties of $DsbA_{P151A}$

Secondary and tertiary structures of $DsbA_{P151A}$ and DsbA were compared by circular dichroism and fluorescence spectroscopy. Far-UV CD spectra of reduced and oxidized $DsbA_{P151A}$ were indistinguishable from those of the wild-type DsbA (data not shown) signifying that the secondary structure of the protein was maintained. This result indicates that the Pro → Ala replacement did not alter significantly the α-helix and β-sheet contents of DsbA. Both reduced proteins displayed very similar tryptophan fluorescence emission spectra with λ_{max} = 322 nm after excitation at 280 nm, at pH 7.0. Oxidized DsbA showed a 3.3-fold decrease in fluorescence intensity at 322 nm when compared to its reduced form, whereas the oxidized mutant protein only presented a 1.5-fold decrease and no λ_{max} shift. However, this difference was sufficient to determine both its thermodynamic stability and redox equilibrium with glutathione by fluorescence spectroscopy.

The relative oxidizing power of $DsbA_{P151A}$ was determined by measuring the equilibrium constant (K_{ox}) for the thiol-disulfide exchange reaction with glutathione (GSH/GSSG). Measurements were made by taking advantage of the difference in the intensity of fluorescence emission at 322 nm for oxidized and reduced species. The K_{ox} of DsbA was found to be 0.145 mM in agreement with previous measurements (Wunderlich & Glockshuber, 1993; Grauschopf et al., 1995). $DsbA_{P151A}$ proved slightly more reducing than the wild-type enzyme (K_{ox} = 0.82 ± 0.06 mM) (Fig. 3).

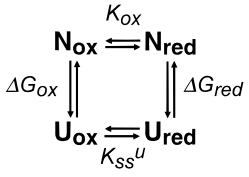

Fig. 1. The relationship proposed to link redox potential and free energies of reduced and oxidized DsbA: $\Delta G_{red} - \Delta G_{ox} = -RT\ln(K_{ox}/K_{ss}^{u})$.

Thermodynamic stabilities of $DsbA_{P151A}$ as determined by fluorescence

The thermodynamic stabilities of oxidized and reduced forms of DsbA and $DsbA_{P151A}$ were first investigated by GdmCl-induced unfolding/refolding experiments. Transition curves were obtained from the variation in fluorescence intensity at 355 and 322 nm for oxidized and reduced DsbA, respectively, as previously described

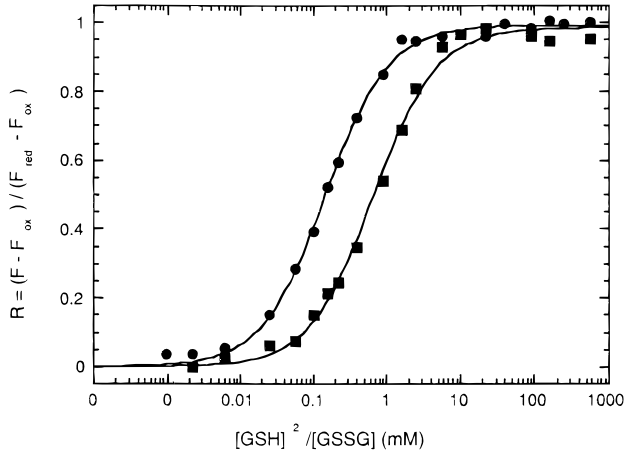

Fig. 3. Redox equilibrium of DsbA (●) and $DsbA_{P151A}$ (■) variant with glutathione. The relative amount of reduced protein was determined using the specific DsbA fluorescence at 322 nm, in 100 mM sodium phosphate, 1 mM EDTA, pH 7.0 (excitation at 280 nm).

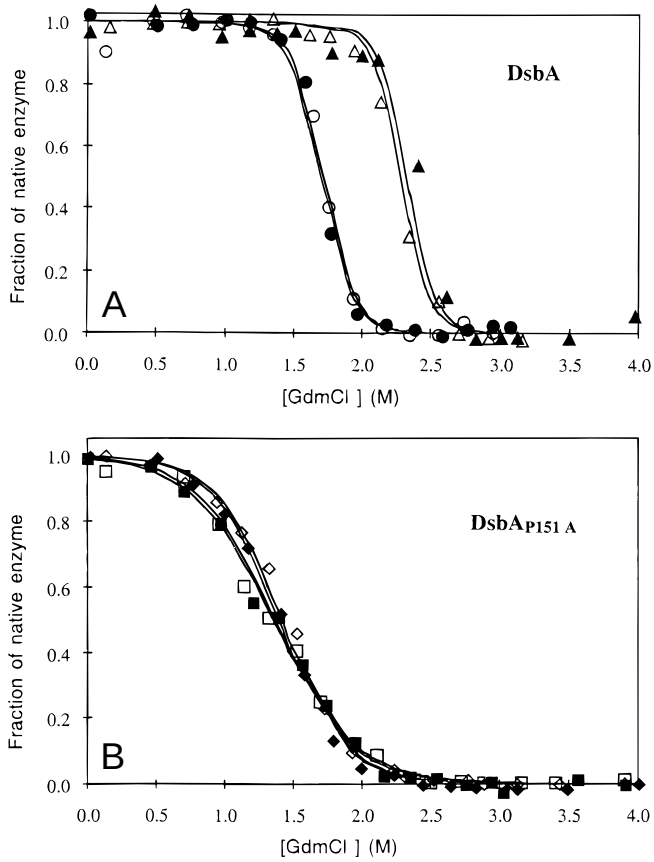

Fig. 4. GdmCl-dependent unfolding/folding of oxidized and reduced (**A**) DsbA (○, △) and (**B**) $DsbA_{P151A}$ (□, ◇). All fluorescence spectra were recorded in 100 mM phosphate, 1 mM EDTA, pH 7.0, at 30 °C in the presence of 1 mM DTT for reduced proteins. The protein concentrations were 0.42 μM. Excitation was measured at 280 nm. Normalized transition profiles are shown. Unfolding and refolding experiments are represented by closed symbols and open symbols, respectively. The folding transitions and curve fitting were done as described in Materials and methods.

(Wunderlich et al., 1993). For $DsbA_{P151A}$, the difference between spectra for native and unfolded states was maximal at 322 nm for both redox states of the protein. In every case, highly cooperative and reversible transitions were obtained (Fig. 4). Free energies of unfolding were calculated by extrapolation of ΔG to zero GdmCl concentration assuming a two-state model of unfolding. As previously reported, the disulfide bond strongly destabilizes DsbA ($\Delta G = 52.8 \pm 2.0$ kJ/mol for reduced DsbA compared to 34.7 ± 1.9 kJ/mol for oxidized DsbA). Both redox states of $DsbA_{P151A}$ are less stable than the wild-type ($\Delta G = 14.8 \pm 0.3$ and 11.9 ± 0.4 kJ/mol, respectively), the free energy difference between both states being only 3 kJ/mol. Furthermore, both forms of the variant exhibit a strongly reduced cooperativity m ($m = 8.6 \pm 0.4$ and 10.3 ± 0.4 kJ/mol compared to 20.3 ± 1.1 and 24.1 ± 0.9 kJ/mol for oxidized and reduced DsbA, respectively) suggesting that they may not fold/unfold according to a two-state model (Myers et al., 1995).

HS-DSC studies

Calorimetric curves obtained for DsbA and variant $DsbA_{P151A}$ in oxidized and reduced states are shown in Figure 5. Corresponding denaturation parameters are presented in Table 1. Reversibility of thermal unfolding of DsbA was tested by reheating of the sample in the calorimetric cell after completing the first scan at 100 °C (Fig. 5, inset). The thermogram of the second heating shows denaturation transition at the same position but with lower enthalpy as compared with the first scan. Overall, this indicates 75% reversibility of thermal unfolding of DsbA. Partial irreversibility seen from enthalpy decrease can be assigned to secondary processes, as for example, aggregation. Aggregation could be reduced by optimization of the heating conditions (decreasing final temperature and duration of protein incubation in the unfolded state).

Calorimetric measurements of reduced DsbA and $DsbA_{P151A}$ were carried out both in the presence of DTT in the cell and after elimination of reducing agent by gel-filtration. This was done to check if the presence of DTT does not influence thermal denaturation generating discrepant calorimetric and fluorimetric data. No perturbations of the heat capacity curves and transition parameters by DTT were observed. For this reason, the values of calorimetric parameters from repeated measurements for reduced DsbA and $DsbA_{P151A}$ in the presence and absence of DTT were averaged (Table 1).

As can be seen in Table 1, reduced forms of DsbA and $DsbA_{P151A}$ show remarkable increase in both transition temperature (T_d) and enthalpy (ΔH_{cal}) as compared with corresponding oxidized forms: $\Delta T_{d,\mathrm{DsbA}}^{red/ox} = 8.4\,°\mathrm{C}$; $\Delta T_{d,\mathrm{P151A}}^{red/ox} = 8.7\,°\mathrm{C}$; $\Delta H_{cal,\mathrm{DsbA}}^{red/ox} = 102.2$ kJ/mol and $\Delta H_{cal,\mathrm{P151A}}^{red/ox} = 95.2$ kJ/mol. These increments of T_d and ΔH_{cal} are of the same order for DsbA and variant $DsbA_{P151A}$. Additionally, calorimetric data reveal substantial destabilization of both oxidized and reduced forms of the studied variant as compared with that of DsbA: $\Delta T_d^{\mathrm{P151A/DsbA}} = -11.0\,°\mathrm{C}$ and $\Delta H_{cal}^{\mathrm{P151A/DsbA}} = -186.3$ kJ/mol for oxidized and $\Delta T_d^{\mathrm{P151A/DsbA}} = -10.7\,°\mathrm{C}$ and $\Delta H_{cal}^{\mathrm{P151A/DsbA}} = -193.3$ kJ/mol for reduced forms, respectively.

The values of the denaturation heat capacity increment ΔC_p are presented in the fourth column of Table 1. Within the errors of estimation, the values of ΔC_p for $DsbA_{P151A}$ are close to that of DsbA and do not depend significantly on the redox-state of the protein. The large errors of ΔC_p for $DsbA_{P151A}$ are related to some

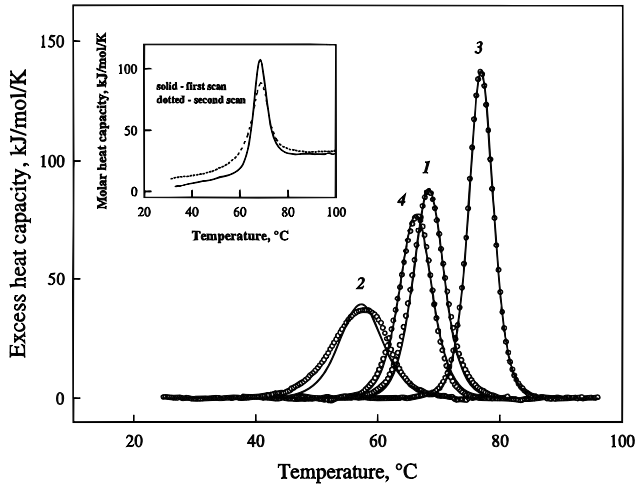

Fig. 5. Excess heat capacity curves for DsbA (curves 1 and 3) and variant $DsbA_{P151A}$ (curves 2 and 4) in oxidized (curves 1 and 2) and reduced (curves 3 and 4) states (100 mM phosphate, 1 mM EDTA; pH 7.0). Circles, experimental; solid lines, two-state fitting. Inset: calorimetric raw data for oxidized DsbA.

instability in the baseline in the post-denaturation segment of the thermogram probably because of aggregation of unfolded protein. Baseline instability did not, however, affect significantly such parameters as transition temperature and enthalpy. The average value of ΔC_p over all the data presented in Table 1 is $\Delta C_p = 8.98 \pm 1.36$ kJ/mol/K. This is in agreement with the value of this parameter for oxidized DsbA $\Delta C_p = 9.28 \pm 2.09$ kJ/mol/K. The attempt was made to estimate ΔC_p for DsbA from the slope of temperature dependence of calorimetric enthalpy by measuring at different pH varying from 7.0 to 9.5, but it failed because of distortions of thermograms obtained at pH >8.5. At pH 8.0 and 8.5 the following values of transition parameters of oxidized DsbA were obtained: $T_d = 68.04$ °C, $\Delta H_{cal} = 592.3$ kJ/mol, $\Delta C_p = 9.25$ kJ/mol/K at pH 8.0, and $T_d = 66.8$ °C, $\Delta H_{cal} = 565.6$ kJ/mol, $\Delta C_p = 9.33$ kJ/mol/K at pH 8.5. Although these data are not fully sufficient to evaluate the dependence $\Delta H_{cal}(T_d)$, one can see that ΔC_p values do not depend on pH between pH 7.0 and 8.5. The value $\Delta C_p = 9.28 \pm 2.09$ kJ/mol/K = 0.44 J/g/K for oxidized DsbA (Table 1) agrees well with the average value of this parameter for small globular proteins (Privalov, 1979; Gómez et al., 1995).

The values of cooperative ratio $\Delta H_{cal}/\Delta H_{VH}$ are presented in the last column of Table 1. According to this parameter, the reduced forms of both DsbA and variant respect a two-state transition mechanism, while oxidized forms seem to deviate from it. However, more precise analysis of transition mechanism by fitting the excess heat capacity curves according to the two-state model (Fig. 5) shows that there is no systematic deviation of a calculated curve from an experimental one for oxidized DsbA. In contrast, a clear deviation from the two-state approximation is found for oxidized $DsbA_{P151A}$ suggesting the existence of few folding intermediates.

Tables 2A and 3 contain values of thermodynamic unfolding parameters of DsbA and $DsbA_{P151A}$: Gibbs energy (ΔG), enthalpy (ΔH), and entropy ($T\Delta S$) of unfolding calculated at 30 °C by extrapolation of calorimetric parameters (Table 1) assuming $\Delta C_p = 9.28 + 2.09$ kJ/mol/K for both redox-states of DsbA and its variant. It was also assumed that uncertainty of thermodynamic parameters for oxidized $DsbA_{P151A}$ due to the complexity of its unfolding transition could be neglected. In the last column of Table 2 the denaturation increment of disulfide stability ($\Delta(\Delta G_{ss})$) is presented, calculated from equation $\Delta(\Delta G_{ss}) = -RT \ln (K_{ox}/K^u_{ss})$.

Table 1. *Denaturation parameters at pH 7.0 of DsbA and $DsbA_{P151A}$ according to HS-DSC*

Protein	T_d (°C)	ΔH_{cal} (T_d) (kJ/mol)	ΔC_p (kJ/mol/K)	$\Delta H_{cal}/\Delta H_{VH}$
Oxidized				
DsbA $n = 5$[a]	68.40 ± 0.03	618.0 ± 25.0	9.28 ± 2.09	1.08 ± 0.01
$DsbA_{P151A}$ $n = 3$	57.40 ± 0.80	431.7 ± 5.1	10.77 ± 4.88	1.21 ± 0.05
Reduced				
DsbA $n = 3$	76.77 ± 0.16	720.2 ± 31.2	8.15 ± 2.18	0.98 ± 0.02
$DsbA_{P151A}$ $n = 4$	66.06 ± 0.23	526.9 ± 40.0	7.72 ± 4.10	0.98 ± 0.06

[a] n, Number of repetitions of measurements.

Table 2. *Gibbs energy of denaturation of DsbA and $DsbA_{P151A}$ (pH 7.0; $t = 30$ °C)*

A. HS-DSC measurements				
Protein	ΔG^{ox} (kJ/mol)	ΔG^{red} (kJ/mol)	$\Delta\Delta G^{red/ox}$ (kJ/mol)	$\Delta(\Delta G_{ss})$[a] (kJ/mol)
DsbA	48.7	65.93	17.23	17.8
$DsbA_{P151A}$	24.96	37.6	12.64	13.44
B. Fluorimetric measurements				
Protein	ΔG^{ox} (kJ/mol)	ΔG^{red} (kJ/mol)	$\Delta\Delta G^{red/ox}$ (kJ/mol)	
DsbA	34.7	52.8	18.1	
$DsbA_{P151A}$	11.9	14.8	2.9	

[a] $\Delta(\Delta G_{ss}) = -RT \ln(K_{ox}/K^u_{ss})$. The redox potential in the denatured state K^u_{ss} was considered to be identical to that measured for the wild-type enzyme, i.e., 170 mM (Zapun et al., 1993).

Table 3. *Enthalpy and entropy of denaturation of DsbA and variant $DsbA_{P151A}$ (pH 7.0; $t = 30$ °C)*

Protein	$(\Delta H)^{ox}$ (kJ/mol)	$(T\Delta S)^{ox}$ (kJ/mol)	$(\Delta H)^{red}$ (kJ/mol)	$(T\Delta S)^{red}$ (kJ/mol)
DsbA	261.6	213.0	286.2	220.3
$DsbA_{P151A}$	177.4	152.4	192.3	154.5
Increment	−84.2	−60.6	−93.9	−65.8

Discussion

A concept of thermodynamic cycle was used to explain the oxidizing properties of DsbA linking the difference in stability of oxidized and reduced forms and the stability of the disulfide bond expressed as a redox equilibrium with glutathione. This relationship was confirmed for DsbA and a number of variants with substitution in the dipeptide sequence within the active-site cysteines. However, large deviations from this cycle appeared for DsbA variants modified either in the kink of the active-site helix or in variants with altered electrostatics around their active-site (Hennecke et al., 1997; Jacobi et al., 1997). Until now, thermodynamic studies of DsbA and its variants have been performed only by fluorimetry. Under these experimental conditions, the $\Delta\Delta G^{red/ox}$ value of 3 kJ/mol obtained for $DsbA_{P151A}$ allows to expect a large increase of the redox potential of the active-site disulfide ($K_{ox} \sim 50$ mM). Surprisingly, the studied variant was only slightly more reducing than the wild-type ($K_{ox} = 0.82$ mM). This variant is one of the most radical examples of discrepancy between the value of K_{ox} predicted from the cycle and that measured experimentally. Hence, it is certainly a good candidate to study in greater details the thermodynamic properties of DsbA. Additionally, the substitution P151A occurs in a region which was shown by NMR to display significant differences in the electronic structure of oxidized and reduced states (Couprie et al., 1998). In the present study, we focused on the following question: does this DsbA variant (and potentially, others) really escape from the theoretical thermodynamic cycle or does the fluorimetric method used to determine free energy values introduce a bias in the results? The later possibility may result from the location of the intrinsic fluorophore (Trp76) in the helical B-domain of the protein, far away from the active-site cysteines and the thioredoxin domain.

ΔG values fluorimetrically obtained for $DsbA_{P151A}$ are largely underestimated compared to the values obtained by HS-DSC (Table 2). Substitution of Pro in position 151 of DsbA may cancel some interactions maintaining the integrity of folded structure of the protein. Consequently, the helical B-domain of the protein would acquire more freedom, uncoupling the changes in the vicinity of Trp76 from unfolding of the polypeptide chain. This is consistent with recent concepts on folding intermediates where intermediate states are considered to involve defined domains of the protein molecule rather than representing misfolding states of the whole polypeptide chain (Freire, 1995; Privalov, 1996). Such intermediates, identified with independent portions or subdomains of protein, were found in α-lactalbumin, equine lysozyme, staphylococcal nuclease and myoglobin (for review see Privalov (1996)). These domains reveal different degrees of folding and/or interaction with the rest of the molecule depending on the conditions (pH, detergent, endogenous ligand, etc.). In some specific conditions, these subdomains unfold independently (deviation from two-state unfolding), in other cases two parts may interact positively forming a single cooperative unit (two-state unfolding) (Freire et al., 1992; Privalov, 1996). The last phenomenon seems to be observed for thermal unfolding of $DsbA_{P151A}$ in reduced state since it follows strictly a two-state transition, while a non-two-state transition is observed for oxidized $DsbA_{P151A}$. Moderate concentrations of GdmCl, used in fluorimetric study, can act not only as a destabilizing factor, like temperature in calorimetric measurements, but they can also favor domain uncoupling. This may explain why Trp76 stops functioning as an intrinsic probe of global unfolding for both redox states of variant $DsbA_{P151A}$ when the unfolding is induced by GdmCl.

As a consequence, the underestimation of ΔG values provide a wrong estimation of $\Delta\Delta G^{red/ox}$. According to the thermodynamic cycle, the $\Delta\Delta G^{red/ox}$ expected for DsbA and $DsbA_{P151A}$ from the measured K_{ox} are 17.80 and 13.44 kJ/mol, respectively. The $\Delta\Delta G^{red/ox}$ values at 30 °C observed in the case of the wild-type are in the same range by both fluorimetric and calorimetric methods (18.1 and 17.23 kJ/mol, respectively), while the values measured for $DsbA_{P151A}$ differ substantially ($\Delta\Delta G^{red/ox} = 2.9$ and 12.64 kJ/mol, respectively) (Table 2). Using the appropriate method, these results show that the variant does fulfill the predictions of the thermodynamic cycle. This might also be true for other variants reported not to respect the cycle (Hennecke et al., 1997; Jacobi et al., 1997).

Another relation has been proposed to explain the oxidizing power of DsbA: the Brönsted-like correlation between K_{ox} and the pK_a of the active-site thiol (Szajewski & Whitesides, 1980; Grauschopf et al., 1995; Bulaj et al., 1998). $DsbA_{P151A}$ also respects this correlation ($pK_a = 4.03$; Charbonnier et al., 1999) but unlike the variants previously mentioned (Hennecke et al., 1997, Jacobi et al., 1997), this mutation has no large electrostatic effect in the vicinity of the active-site.

Replacement of a *cis*-proline by a non-proline residue is expected to produce either a *cis* non-proline peptide bond with no major impact on the structure or a *trans* conformer with large modifications in the geometry of the backbone. Both events have already been observed in *cis*-Pro variants of various proteins. For instance, the Pro202 → Ala human carbonic anhydrase II retains a *cis* conformation (Tweedy et al., 1993), whereas its isomerization to a *trans* bond was observed in staphylococcal nuclease variants (Evans et al., 1987; Hodel et al., 1995). In the case of thioredoxin, for which *cis*-Pro76, the structural analog of Pro151 was mutated in alanine, no conclusion was drawn concerning the conformation of Ala76 in the variant P76A (Kelley & Richards, 1987; Gleason, 1992). Whatever their conformation, *cis*-Pro → Xaa variants are generally strongly destabilized. The observed decreases in stability caused by the substitution of a *cis*-proline by an alanine range from ~20 kJ/mol for the RNase T1 P39A (Mayr et al., 1993) and carbonic anhydrase II P202A, to 8–9 kJ/mol for RNase A variants (Schultz & Baldwin, 1992) with an intermediate value of 16.3 kJ/mol for thioredoxin P76A. In our case, the substitution of *cis*-Pro151 decreases the stability of oxidized DsbA by about 24 kJ/mol and the reduced state by about 28 kJ/mol. These values are significantly higher than those mentioned previously.

To analyze energetic changes of DsbA caused by Pro151 substitution, deeper insights in the contribution of the entropic and enthalpic factors can be helpful (Table 3). It is known that Pro → Ala substitution results generally in an increase of configurational entropy of the protein in unfolded state (Matthews et al., 1987). Thus, one could expect the destabilization of variant $DsbA_{P151A}$ mainly due to the increase of its unfolding entropy. However, as can be seen in Table 3, substitution of Pro151 in DsbA leads to entropy decrease both in oxidized and reduced form indicating that effects other than change of configurational entropy of the unfolded state are prevalent. Comparison of the mutation increments of ΔH and $T\Delta S$ (Table 3) show that destabilization of $DsbA_{P151A}$ is enthalpically driven. Taking into account the location of Pro151 in a hydrophobic cluster close to the active-site (Martin et al., 1993), one can suggest that its substitution leads to the loss of favorable packing interactions of the side chains in folded DsbA.

Decrease of packing density was shown to increase abruptly configurational entropy of folded conformation and, thus, should contribute negatively to the entropy of unfolding (Bromberg & Dill, 1994; Richards & Lim, 1993). Unpacking should also result in decrease of unfolding enthalpy due to disruption of van der Waals contacts (Makhatadze & Privalov, 1995). This is consistent with the observed decrease of ΔH for $DsbA_{P151A}$ (Table 3). It was already shown for bacterial ribonuclease barnase that the loss of tight packing in the hydrophobic core reduces by a half the net energy of folding (Kellis et al., 1989), even if this unpacking does not alter the three-dimensional structure of the protein. Analysis of crystallographic structure of $DsbA_{P151A}$ (Charbonnier et al., 1999) points up a local reorganization of the structure around position 151 and a larger accessibility to the solvent of the active disulfide bridge. Structural changes may introduce some uncertainty in the thermodynamic analysis of destabilizing factors because in this particular case a contribution of hydration effects into enthalpy and entropy of unfolding can not be totally excluded. Hydration effects contribute negatively both to the unfolding enthalpy and entropy (Makhatadze & Privalov, 1995). This agrees well with the observed tendencies (Table 3). Finally, it can be concluded that Pro151, which is a well conserved residue in the thioredoxin super-family, plays a key role in maintaining the integrity of the folded structure of DsbA. Substitution of this residue breaks the native packing in hydrophobic cluster and has a major impact on the cooperativity of the protein folded structure.

Materials and methods

Materials

1,4-Dithio-DL-threitol (DTT), reduced glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma. Guanidium chloride (GdmCl) was from Fluka. DEAE-Sephacel and phenyl-Sepharose CL-4B were obtained from Pharmacia-Biotech. $DsbA_{P151A}$ was purified by the same protocol as the wild-type DsbA: anion exchange chromatography on DEAE-Sephacel followed by hydrophobic chromatography on phenyl-Sepharose CL-4B (Wunderlich & Glockshuber, 1993).

Spectroscopic methods

Circular dichroism measurements were made with a Jobin Yvon CD6 dichrograph. Fluorescence spectra were recorded on a Spex 0.34 spectrometer. UV absorptions were measured with a Hewlett Packard 8453 spectrophotometer. DsbA and $DsbA_{P151A}$ protein concentrations were determined spectrophotometrically using an absorption coefficient of $A_{280,1mg/mL,1cm} = 1.10$ (Wunderlich & Glockshuber, 1993).

Determination of redox properties of Cys30

The redox equilibrium measurements were performed as described by Wunderlich and Glockshuber (1993) in a 100 mM phosphate buffer, pH 7.0 containing 1 mM EDTA at 30 °C, assuming no significant equilibrium concentrations of DsbA/glutathione mixed disulfides. Equilibration of enzymes at different GSH/GSSG ratios was performed under argon atmosphere, in degassed buffers for 16 h.

Unfolding-refolding fluorescence studies

All the unfolding-refolding experiments were carried out as previously described (Wunderlich et al., 1993) in a 100 mM phosphate buffer pH 7.0 containing 1 mM EDTA at 30 °C. Measurements involving reduced proteins were performed under argon atmosphere, with degassed buffers. The transitions were measured fluorimetrically. The exact GdmCl concentrations were calculated from the refractive index (Nozaki, 1972). Experimental data were analyzed according to a two-state equilibrium, using the model of linear dependency of ΔG upon denaturant concentration (Pace, 1986). Experimental data were fitted with a simplex procedure based on the Nelder and Mead algorithm (Press et al., 1986).

High-sensitivity differential scanning calorimetry (HS-DSC) studies

Calorimetric measurements were carried out with high-sensitivity differential scanning microcalorimeter VP-DSC (MicroCal Software, Inc., Northampton, Massachusetts) within the temperature range 6–110 °C, at the heating rate 60 deg/hour and excess pressure 2 atm. Solutions of DsbA and variant in oxidized form for calorimetric measurements were dialyzed against corresponding buffer at 4 °C overnight. 100 mM phosphate and 40 mM glycine-NaOH containing 1 mM EDTA were used as buffer solutions at pH 7.0 and pH 8.0–9.5, respectively. Protein concentration after dialysis was determined spectrophotometrically and varied from 0.12 to 0.36 mg/mL. Reduction of the disulfide bond of DsbA and $DsbA_{P151A}$ was carried out by addition of 1 mM DTT into the dialyzed protein solution degassed and kept under argon. When needed, DTT was eliminated prior to measurements by gel-filtration using HiTrap Desalting column (5 mL, Pharmacia Biotech). All calorimetric measurements of reduced proteins were carried out in strictly degassed solutions, saturated with and kept under argon. Primary data processing and fitting of heat capacity curves were carried out using Origin 4.1 software (MicroCal Software, Inc.). Molar heat capacity of DsbA and $DsbA_{P151A}$ was calculated assuming a molecular weight of 21.1 kDa. Thermodynamic functions of unfolding at reference temperature T were calculated using the following equations:

$$\Delta H(T) = \Delta H_{cal}(T_d) + \Delta C_p(T_d)(T - T_d) \quad (1)$$

$$\Delta S(T) = \Delta H_{cal}(T_d)/T_d + \Delta C_p(T_d)\ln(T/T_d) \quad (2)$$

$$\Delta G(T) = \Delta H(T) - T\Delta S(T). \quad (3)$$

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