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On the role of the cis-proline residue in the active site of DsbA

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Abstract

In addition to the Cys-Xaa-Xaa-Cys motif at position 30–33, DsbA, the essential catalyst for disulfide bond formation in the bacterial periplasm shares with other oxidoreductases of the thioredoxin family a cis-proline in proximity of the active site residues. In the variant $DsbA_{P151A}$, this residue has been changed to an alanine, an almost isosteric residue which is not disposed to adopt the cis conformation. The substitution strongly destabilized the structure of DsbA, as determined by the decrease in the free energy of folding. The pK_a of the thiol of Cys30 was only marginally decreased. Although in vivo the variant appeared to be correctly oxidized, it exhibited an activity less than half that of the wild-type enzyme with respect to the folding of alkaline phosphatase, used as a reporter of the disulfide bond formation in the periplasm. $DsbA_{P151A}$ crystallized in a different crystal form from the wild-type protein, in space group $P2_1$ with six molecules in the asymmetric unit. Its X-ray structure was determined to 2.8 Å resolution. The most significant conformational changes occurred at the active site. The loop 149–152 adopted a new backbone conformation with Ala151 in a trans conformation. This rearrangement resulted in the loss of van der Waals interactions between this loop and the disulfide bond. His32 from the Cys-Xaa-Xaa-Cys sequence presented in four out of six molecules in the asymmetric unit a gauche− conformation not observed in the wild-type protein. The X-ray structure and folding studies on $DsbA_{P151A}$ were consistent with the cis-proline playing a major role in the stabilization of the protein. A role for the positioning of the substrate is discussed. These important properties for the enzyme function might explain the conservation of this residue in DsbA and related proteins possessing the thioredoxin fold.

Keywords: cis-proline; crystallography; disulfide formation; DsbA; thioredoxin-like oxidoreductases

DsbA is the specialized enzyme that oxidizes disulfide bonds in exported proteins in the periplasm of *Escherichia coli* (Raina & Missiakas, 1997). It is the most efficient oxidant of reduced protein substrates yet characterized (Wunderlich et al., 1993; Zapun et al., 1993). It works independently of glutathione, the usual partner of thiol-disulfide redox reactions in the cytoplasm and the endoplasmic reticulum, and needs DsbB, an inner membrane protein, for its reoxidation (Bardwell et al., 1993). DsbA belongs to a family of enzymes including thioredoxins and eukaryotic protein-disulfide-isomerases (PDI) that share the consensus sequence Cys-Xaa-Xaa-Cys in their active site. The crystal structure of DsbA was determined at 2.0 Å (Martin et al., 1993) and later refined to 1.7 Å (Guddat et al., 1997b). It consists of a helical domain (residues 60–136) and a thioredoxin-type domain (residues 1–59 and 137–189). The thioredoxin-fold is also observed in other members of the thiol-disulfide redox proteins (thioredoxin, glutaredoxin, fragments a and b of PDI) and in two glutathione interacting enzymes (glutathione peroxidase and glutathione transferases). The common structural motifs have been reviewed and consists of a central five-stranded β-sheet with four flanking α-helices (Martin, 1995).

Several structure-based studies have been carried out by site-directed mutagenesis to explore the mechanism and determine the structure-function relationships of DsbA. The active site contains two cysteine residues within the Cys30–Pro31–His32–Cys33 sequence that form a reversible disulfide bond in the oxidized state. The role of the dipeptide Xaa-Xaa in the redox potential was demonstrated in several ways. The characteristic Xaa-Xaa dipeptide sequence, Pro-His in DsbA, is Gly-His in PDI and Gly-Pro in thioredoxin. The change of one dipeptide sequence for another produces a shift of the redox potential toward that of the protein from which the dipeptide originated (Lundstrom et al., 1992; Kortemme et al., 1996; Chivers et al., 1997). Another series of mutations of the two central residues in the active site of DsbA

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Abbreviations: $\Delta\Delta G^{red/ox}$, difference in Gibbs energy between reduced and oxidized states; AP, alkaline phosphatase; hTrx, human thioredoxin; K_{ox}, equilibrium constant for the thiol-disulfide exchange with glutathione, RMSD, root-mean-square deviation; Spc^R, resistance to spectinomycin; Str^R, resistance to streptomycin; TrxA, *Escherichia coli* thioredoxin.

established the interdependence of oxidizing power, measured as the redox equilibrium K_{ox} with glutathione, and the pK_a value of the active thiol of Cys30 (Grauschopf et al., 1995). After replacement of His32 with Tyr, Leu, or Ser, it had been proposed that this residue makes an electrostatic contribution to the enzyme stability (Guddat et al., 1997a). The role played by the kink in the active-site helix and that of charged residues in the vicinity of the active site, Glu24, Glu37, Glu38, and Lys58, in the determination of the oxidizing power was recently investigated (Hennecke et al., 1997b; Jacobi et al., 1997). These residues hardly affect the properties of DsbA and it appears that more drastic changes are needed to destabilize the enzyme. Our current work aims to determine the contribution of the Pro151 that is in close proximity of the essential Cys30. This residue is in the cis conformation and is part of a loop that is conserved in proteins with the thioredoxin fold. The structures of thioredoxin, DsbA, and others, indicated that the corresponding proline side chain makes numerous van der Waals interactions with the disulfide bond. Which is the main factor that leads to the conservation of the cis-proline in the thioredoxin fold? Is it the reactivity of the disulfide bond, the substrate binding, the stability, or the folding? At present, information is only available for thioredoxin. The NMR structures of human thioredoxin (hTrx) with two target peptides derived from Ref-1 and NF-κB place the cis-Pro ring in a similar position relative to the mixed disulfide bond and show that the two hydrogen bonds made by the main chain of the cis-Pro loop with the peptide main chain are conserved (Qin et al., 1996). Biophysical studies of the *E. coli* thioredoxin and of its variant $TrxA_{P76A}$ indicated that pro76 plays a significant role in the stability of the enzyme and in the folding of the protein (Kelley & Richards, 1987; Georgescu et al., 1998).

We have further investigated the role of this conserved residue, in the stabilization of the protein in a variant of DsbA with the cis-Pro151 amino acid changed into Ala. We report the periplasmic expression of the variant ($DsbA_{P151A}$), its in vivo stability and activity, as well as the difference between the in vitro stability of the oxidized and the reduced forms ($\Delta\Delta G^{red/ox}$) and the oxidizing properties of $DsbA_{P151A}$ (K_{ox}, pK_a). The X-ray structure at 2.8 Å of the $DsbA_{P151A}$ variant, the rearrangement of the cis-Pro loop, and its consequence on the active-site structure are discussed.

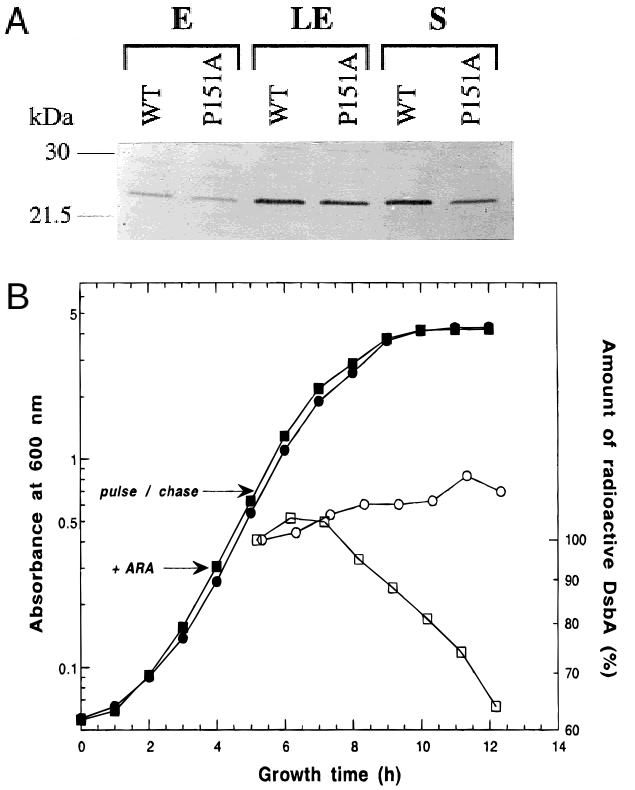

Fig. 1. (**A**) In vivo expression and (**B**) stability of DsbA and $DsbA_{P151A}$. **A:** Aliquots of the cultures of strains SBS2916 carrying pPB2959 (WT) and SBS2916 carrying pPB3141 (P151A) grown in TYE + 0.5% glycerol were withdrawn. The total fractions were prepared and analyzed by SDS-PAGE and immunoblot. E, exponential phase ($A_{600} \sim 1$); LE, late exponential phase ($A_{600} \sim 4$); S, stationary phase ($A_{600} > 5.5$). **B:** Cultures of strain SBS2916 carrying either pPB2959 or pPB3141 were grown in M9 minimal medium, induced with arabinose (ARA), pulse-labeled and chased as described in Materials and methods. Aliquots of the cultures were withdrawn during the chase and the crude extracts were analyzed by 12% SDS-PAGE and autoradiography. The growth curves of SBS2916 carrying either pPB2959 (●) or pPB3141 (■) are represented in the left *Y* axis. The autoradiograms were densitometrically scanned and the values for each time point were normalized and reported for DsbA (○) and $DsbA_{P151A}$ (□) in the right *Y* axis.

Results

Functional properties of DsbA and $DsbA_{P151A}$ in vivo

The levels of expression for both proteins were compared by subcloning the *dsbA* and *$dsbA_{P151A}$* genes into the pBAD24 vector (Guzman et al., 1995) downstream from the tightly regulated and arabinose inducible P_{BAD} promoter, resulting in plasmid pPB2959 and pPB3141, respectively. The expression of *dsbA* and *$dsbA_{P151A}$* could thus be modulated over a wide range of arabinose concentrations, from very low levels obtained in the absence of arabinose to very high levels upon arabinose induction (see Fig. 4B, lanes 3–6). The plasmids pPB2959 and pPB3141 were introduced into a *dsbA* null strain (SBS2916) for further studies. Cells were grown in rich medium without arabinose and the production of DsbA and $DsbA_{P151A}$ during growth were compared (Fig. 1A). Whereas similar amounts of both proteins were detected during the exponential phase of growth, $DsbA_{P151A}$ was present in much less extent than DsbA during the stationary phase of growth. This suggests an altered in vivo stability of $DsbA_{P151A}$, which was also observed by pulse-chase experiments (Fig. 1B). No significant degradation of DsbA could be observed over the first 7 h of chase, whereas $DsbA_{P151A}$ was only stable for the first 2 h, which corresponded to the end of the exponential phase of growth. The amount of $DsbA_{P151A}$ then decreased regularly with a half-time estimated to 10 h.

The capacity of DsbB to reoxidize DsbA and $DsbA_{P151A}$ was analyzed. Oxidized and reduced forms of DsbA could be discriminated by electrophoresis after chemical modification of free SH with biotin-maleimide. In a *dsbB* strain both DsbA and $DsbA_{P151A}$ were fully reduced while a mixture of oxidized and reduced protein was visible in the *$dsbB^+$* strain for both wild-type and mutant proteins (Fig. 2), showing that DsbB was an efficient catalyst of $DsbA_{P151A}$ reoxidation.

Biophysical properties of $DsbA_{P151A}$ in vitro

Both reduced $DsbA_{P151A}$ and DsbA displayed very similar tryptophan fluorescence emission spectra with λ_{max} = 322 nm after

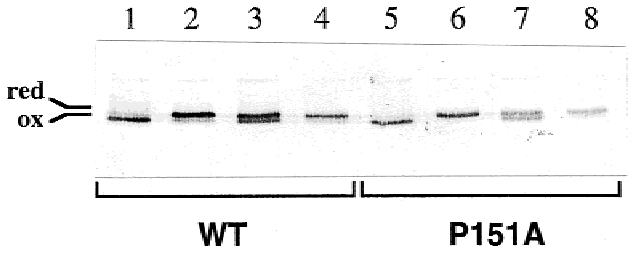

Fig. 2. In vivo redox states of DsbA and $DsbA_{P151A}$. Cells of SBS2916 (*dsbA*::Ω *dsbB*$^+$; lanes 1–3 and 5–7) and SBS3261 (*dsbA*::Ω *dsbB*::Tn*10*; lanes 4 and 8) containing plasmid pPB2959 (DsbA; WT, lanes 1–4) or pPB3141 ($DsbA_{P151A}$; P151A, lanes 5–8) were grown in rich medium supplemented with 0.033 mM arabinose. At $A_{600} \sim 2.5$, proteins were precipitated with trichloracetic acid, solubilized and treated differently before SDS-PAGE under reducing conditions and western-blotting with an anti-DsbA serum. Lanes 1 and 5: proteins were incubated 30 min with 20 mM oxidized DTT before addition of 5 mM biotin-maleimide. Lanes 2 and 6: proteins were incubated 30 min with 2 mM reduced DTT before addition of 5 mM biotin-maleimide. Lanes 3–4 and 7–8: proteins were incubated with 5 mM biotin-maleimide. Red and ox indicate the position of reduced and oxidized DsbA and $DsbA_{P151A}$.

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 decrease and no λ_{max} shift (Fig. 3). It was previously demonstrated that the fluorescence of Trp76 was quenched by the disulfide bond via Phe26 (Hennecke et al., 1997a). Because the identical spectra obtained for reduced forms are consistent with no major reorganization in the Trp76 environment, the effect might be very local. The substitution P151A might have affected the quenching of Trp fluorescence by the disulfide bond, indicating that Pro151 could be involved in the orientation of this bond relative to the rest of the molecule or could restrict its mobility.

Biophysical properties of both DsbA and $DsbA_{P151A}$ are summarized in Table 1. $DsbA_{P151A}$ proved slightly more reducing than the wild-type as shown by its six-fold increased K_{ox} value. In agreement with the correlation between K_{ox} and pK_a of Cys30 established by Grauschopf et al. (1995), Cys30 in $DsbA_{P151A}$ exhibited a higher pK_a value (4.03 ± 0.10) than DsbA. The pK_a for the wild-type protein was found to be <3 and could not be precisely determined because of denaturation below this pH. The amplitude of the absorbance change at 240 nm was 4,550 M^{-1} cm^{-1}, which is in the range expected for the titration of a single thiol residue (ΔA_{240} = 5,000 M^{-1} cm^{-1} (Benesch & Benesch, 1955)). The major consequence of the replacement of Pro151 by an alanine is the large stability decrease observed for both oxidized and reduced form (destabilized by 23.5 and 28.3 kJ/mol, respectively). The resulting smaller difference in free energy between the oxidized and reduced states ($\Delta\Delta G^{red/ox}$) correlates with the value that could be predicted from the measured redox equilibrium constants of Moutiez et al. (1999).

Enzymatic activity of $DsbA_{P151A}$

The oxidase activities of DsbA and $DsbA_{P151A}$ were tested by the in vitro refolding of denatured reduced ribonuclease A at different $[GSH]^2/[GSSG]$ ratios. At 5 mM $[GSH]^2/[GSSG]$ ratio, $DsbA_{P151A}$ was less efficient than DsbA and displayed only 70–75% of its activity (data not shown). Such a reducing environment might not favor the glutathione-dependent reoxidation of DsbA or $DsbA_{P151A}$ and this might account for the low catalysis of RNase refolding. In a more oxidizing environment, $[GSH]^2/[GSSG]$ ratio of 0.45 mM, the activity of both proteins increased slightly. From K_{ox} curves (Moutiez et al., 1999), it was expected that at lower value of $[GSH]^2/[GSSG]$, the ratio of oxidized to reduced $DsbA_{P151A}$ should be higher than the corresponding oxidized to reduced ratio in the wild-type. However, under such conditions, $DsbA_{P151A}$ only retained 60–64% of the wild-type activity (data not shown).

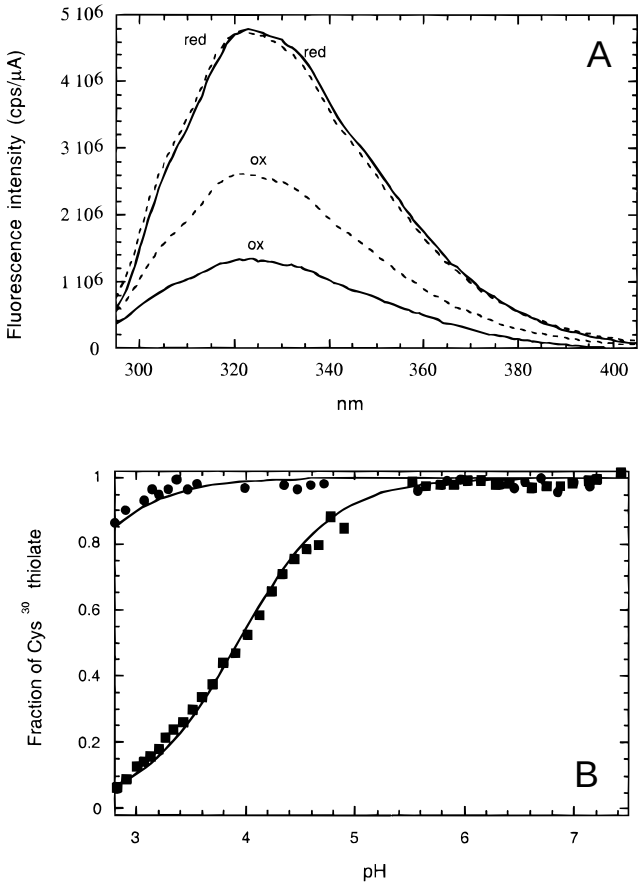

Fig. 3. Physical properties of $DsbA_{P151A}$. **A:** Fluorescence emission properties of native oxidized and reduced DsbA (——) and $DsbA_{P151A}$ (- - -). All spectra were recorded in 100 mM phosphate, 1 mM EDTA, pH 7.0, at 30 °C in the presence of 1 mM DTT for reduced proteins. The protein concentrations were 0.42 μM. Excitation was at 280 nm. **B:** Determination of the pK_a values of the active-site cysteine Cys30 in DsbA (●) and $DsbA_{P151A}$ (■). The thiolate was detected by UV absorbance at 240 nm. Normalized transitions are shown. The pK_a values were calculated from the Henderson–Hasselbach equation.

Table 1. *Biophysical features of $DsbA_{P151A}$ and DsbA*

	DsbA	$DsbA_{P151A}$
K_{ox} (mM)	0.145 ± 0.005	0.82 ± 0.06
pK_a of Cys30	≤3	4.03 ± 0.1
ΔG_{ox} (kJ mol^{-1})	−48.5 ± 3.1	−25.0 ± 2.9
ΔG_{red} (kJ mol^{-1})	−65.9 ± 3.2	−37.6 ± 3.6

A better picture of the activity of $DsbA_{P151A}$ toward a physiological substrate is obtained by comparing the capacity of DsbA and $DsbA_{P151A}$ to catalyze disulfide bond formation in vivo, by following the appearance of a periplasmic disulfide-bonded protein, the alkaline phosphatase (AP) (Fig. 4A). AP was expressed either from the chromosomal gene fusion *agp*::*phoA* (low level of AP (Pradel & Boquet, 1989)) or from the *phoA* chromosomal gene in a *phoR* strain (higher level of AP (Wanner, 1987)). At very low levels of *dsbA* or $dsbA_{P151A}$ expression (no arabinose), little AP activity was detected with $DsbA_{P151A}$ whatever the *phoA* system used. Thus, at low catalyst concentration it appears that $DsbA_{P151A}$ is less active in oxidizing AP than DsbA. Increased $DsbA_{P151A}$ production under arabinose induction resulted in an increase in the AP activity. Even under such conditions where the amount of $DsbA_{P151A}$ produced largely exceeded that of DsbA (Fig. 4B, compare lanes 6 and 2), the activities obtained did not reach the levels observed with non-induced wild-type DsbA. In all cases, $DsbA_{P151A}$ displayed less disulfide formation activity than DsbA in vivo. Grauschopf et al. (1995) described two DsbA variants of the Cys-Xaa-Xaa-Cys motif with K_{ox} in the same range as that of $DsbA_{P151A}$, i.e., $DsbA_{CSVC}$ and $DsbA_{CSFC}$ (K_{ox} = 0.9 and 1.2 mM, respectively (Grauschopf et al., 1995)). We compared their capacity to catalyze disulfide bonds formation in AP when expressed under the same conditions as $DsbA_{P151A}$ (Fig. 4A). $DsbA_{CSVC}$ and $DsbA_{CSFC}$ gave similar results as those obtained with $DsbA_{P151A}$.

Structure determination

Wild-type DsbA crystals (Martin et al., 1993) belong to the C2 space group with two molecules in the asymmetric unit, corresponding to a density V_m of 2.8 Å^3/Da. Crystals for $DsbA_{P151A}$ were obtained under similar conditions but in the presence of 10% (v/v) dimethylsulfoxide instead of 1% 2-methyl-2,4-pentanediol. These crystals were grown in a new crystal form: $P2_1$; a = 64.9 Å, b = 190.7 Å , c = 67.1 Å , β = 111.9° with six molecules (which will be called A to F) in the asymmetric unit and higher solvent content (V_m = 3.1 Å^3/Da) (Stura et al., 1998). By cryo-cooling the crystals, no improvement in resolution was achieved and the mosaicity was significantly higher (Table 2).

The asymmetric unit of $DsbA_{P151A}$ is nearly three times larger than that of DsbA ($V(DsbA_{P151A})$ = 385,265 Å^3 and V(DsbA) = 117,410 Å^3). The structure was solved by molecular replacement with the program AMoRe (Navaza & Saludjian, 1997) with data from 8 to 3.5 Å. The refined structure of DsbA at 1.7 Å (PDB code 1fvk) was used as the search model, and the final model consisting of six molecules gave an *R*-value of 37.1%. Clear peaks were obtained for the first five molecules both at the rotation and the translation function stages. The sixth molecule could be positioned with confidence only after the six-body translation, giving an increase in the coefficient correlation of 3.5%. Packing analysis showed no overlap between the backbone of the six molecules. Non-crystallographic symmetry restraints were applied on the six molecules except around the cis-Pro loop, the Cys-Pro-His-Cys motif and at some of the crystals contacts. The Gly149–Ala152 loop was rebuilt into an "omit" electron density map. The side chains of Val150 and Ala152 are unambiguous in the electron density. This defines a trans conformation and is inconsistent with a cis conformation between Val150 and the mutated residue Ala151.

The new crystal form for $DsbA_{P151A}$ described here contained six molecules per asymmetric unit. In wild-type DsbA the two molecules A and B in the asymmetric unit are essentially identical.

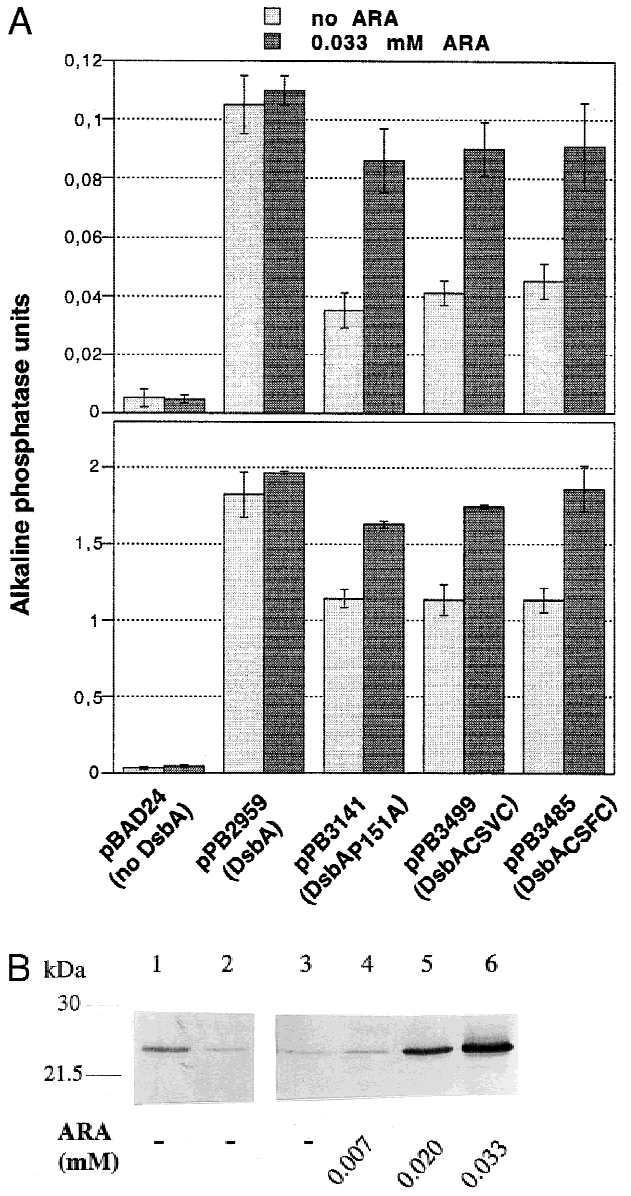

Fig. 4. The in vivo disulfide bond forming activity of $DsbA_{P151A}$ is correlated to its expression level. **A:** The effects of the DsbA P151 → A151, CPHC → CSVC, and CPHC → CSFC substitutions on the activity of AP were studied. AP was expressed from an *agp*::*phoA* gene fusion (upper panel) or from the *phoA* gene (lower panel). Data were obtained from exponentially growing cultures of *dsbA* strains SBS2916 (*agp*::*phoA*; upper panel) and SBS3136 ($phoA^+$ *phoR68*; lower panel) carrying either plasmid pBAD24 (no *dsbA* gene; first column) or plasmids encoding different variants of DsbA: pPB2959 (DsbA; second column), pPB3141 ($DsbA_{P151A}$; third column), pPB3499 ($DsbA_{CSVC}$, fourth column), and pPB3485 ($DsbA_{CSFC}$, fifth column). Bacteria were grown in rich medium either without arabinose or with 0.033 mM arabinose to induce the expression of the *dsbA* genes. Data originated from three independently made experiments. **B:** The production of $DsbA_{P151A}$ from pPB3141 was studied under different level of arabinose induction. Bacterial cultures were grown in TYE + 0.5% glycerol medium containing variable amounts of arabinose (ARA). The total cell fractions were prepared when the cultures reached an $A_{600} \sim 3.5$. These fractions were analyzed by 12% SDS-PAGE and immunoblot. In lane 1, SBS2915 carrying pBAD24 (DsbA produced from the chromosome); lane 2, SBS2916 carrying pPB2959 (DsbA produced from the plasmid); lane 3–6, SBS2916 carrying pPB3141 ($DsbA_{P151A}$ produced from the plasmid).

Table 2. *Crystallographic data and refinement statistics*

Beamline	LURE, W32	DESY, BW7A
Temperature	4 °C	−150 °C
Data collection statistics		
Unit cell		
a, *b*, *c* (Å)	64.9, 190.7, 67.1	64.0, 188.9, 66.3
α (=γ), β (°)	90, 111.9	90, 112.7
Resolution limit (Å)	2.8	2.8
Mosaicity	0.15	0.75
Observations	117,916	219,788
Unique reflections	31,364	33,548
Completeness (%)[a]	84.6 (87.9)	86.5 (78.8)
R_{sym}[b] (%)[a]	7.8 (48)	7.8 (56)
$I/\sigma(I)$[a]	13.3 (1.7)	11.0 (1.7)
Refinement statistics		
Resolution (Å)	10.0–2.8	
R[c] (%)	21.8	
R_{free} (%)	28.9	
RMSDs from ideality		
Bond lengths (Å)[d]	0.013	
Bond angles (°)[d]	1.43	
Ramachandran core (%)[e]	88.5	

[a]The number in parentheses is the value in the highest resolution shell.
[b]$R_{sym} = \Sigma\Sigma_j|I_j - \langle I\rangle|/\Sigma|\langle I\rangle|$ where I_j is the intensity measurement for reflection j and $\langle I\rangle$ is the mean intensity for multiply recorded reflections.
[c]$R = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$.
[d]Evaluated with Xplor (Brünger, 1992).
[e]Evaluated with Procheck, most favored region (Laskowski et al., 1993).

A slight difference of helical domain orientation relative to the thioredoxin fold domain was reported for DsbA (Guddat et al., 1997b) and in the *Vibrio cholerae* homolog, TcpG (Hu et al., 1997). Given the moderate resolution of the $DsbA_{P151A}$ X-ray structure, the analysis of the structural differences between the six molecules must be treated with more caution than those for the wild-type protein. For the mutant molecules A to E, the deviations in the Cα positions of the helical domains or on the thioredoxin fold domains are comparable to those measured for all DsbA residues ($\langle$RMSD$\rangle$ = 0.31, 0.20, and 0.33 Å for 112 Cα, 77 Cα, and 189 Cα, respectively). This suggests that there are no significant differences in orientation of the two domains in the five molecules. However, monomer F does not superimpose as well (RMSD = 0.7 Å for 189 Cα). This monomer is less clearly defined in the electron density map with an average *B*-factor of 63 Å^2 for main-chain atoms, compared to 47 Å^2 for the other five.

One of the critical questions addressed by this study is the conformation of the Val150–Ala151 bond. Omit maps, calculated with phases from the final model without the cis-Pro loop (Gly149 to Ala152), clearly indicate that this loop adopts a different conformation from that of the wild-type DsbA. The deviation in the main-chain atoms between $DsbA_{P151A}$ and DsbA is significantly higher in this part of the molecule than in the rest of the protein, with an RMSD of 1.9 Å for the Gly149 to Ala152 loop compared to 0.47 Å for the whole protein (Fig. 5A). The main differences occur in this loop where the conformation of the polypeptide deviates up to 3.2 Å from the wild-type at carbon position of the Val150 carbonyl. The rearrangement starts with a significant change in the Phi-Psi values of Gly149 and Val150 (Table 3).

Though the carbonyl of Ala151 cannot be unambiguously positioned in the electron density, three main elements favor a trans-conformation for the Val-Ala bond: (1) side chains of Val150 and Ala152 are well defined in the electron density and the positions of these preclude a cis conformation, (2) the loop 149–152 makes no intramolecular hydrogen bonds that could constrain the local backbone geometry of the alanine that replaces the proline, (3) a cis-conformation for Ala151 would be inconsistent with the general rearrangement observed of the backbone of this loop. This site is where the protein undergoes the most significant structural differences in its backbone compared to the wild-type enzyme.

What are the consequences of the Pro151 → Ala substitution on the structural properties of DsbA active site? In DsbA, Pro151 is the residue closest to the oxidized disulfide bond. Due to a global rearrangement of the loop, the positions occupied by the proline residue and the carbonyl of Val150 in DsbA are left accessible to solvent in the $DsbA_{P151A}$. Consequently, the van der Waals contacts made between this portion of the loop and the disulfide bond are effectively lost (seven contacts in A and B monomers of DsbA and 1 or 2 in $DsbA_{P151A}$ six molecules). Interestingly, this correlated with the observed decrease in the quenching efficiency of Trp76 in the oxidized $DsbA_{P151A}$ (Fig. 3). No rearrangement is induced on the main chain of the Cys-Pro-His-Cys motif, nor on the position of the disulfide bond by this release of constraint on the disulfide bond.

The main differences in the conformation of the Cys-Pro-His-Cys segment were in the position of the side chain of His32. In four of six molecules, the histidine residue adopts a gauche− (g−) conformation that places the imidazole ring near the place occupied by the proline in DsbA and above the disulfide bond (Fig. 5B). In the two other molecules, it adopts the gauche+ (g+) conformation that orients the side chain toward the solvent. The electron density maps and the *B*-factor suggest that the side chain is better ordered in the g− conformation than in the exposed g+ conformation ($\langle B\rangle$ = 55 Å^2 for g+ and B = 90 Å^2 for g−). Analysis of the crystal contacts near the His32 indicates that three of the g− rotamers were restricted to this conformation due to crystal contacts with other monomers. The fourth g− rotamer (monomer B) could also adopt the two other positions, trans or g+ without steric hindrance from crystal packing. In the two monomers where His32 is in the g+ conformation, the side chain is free to adopt other rotamers, as in both cases it makes no interactions with side chains from other monomers. This analysis suggested that the proportion of g+ and g− observed, 33 and 66%, respectively, is an artifact of crystal packing. It is difficult to estimate the thermodynamically relevant proportion of His32 directed toward the disulfide bond and that directed toward the solvent, but it is apparent that the g− conformation is sufficiently stable to be clearly defined in one of the nonrestricted environments (monomer B).

Discussion

The replacement of the conserved cis-proline in the active site of DsbA lowers significantly the activity of the enzyme in vivo, resulting in a decrease of the AP activity. The DsbA-dependent disulfide bond formation in the periplasm involves a cyclic process where (1) reduced protein substrates are oxidized by DsbA and (2) reduced DsbA is reoxidized by DsbB to regenerate a competent enzyme. Taking this into consideration, several factors could account for the observed loss of $DsbA_{P151A}$ activity: a lesser amount of active catalyst, a less oxidizing enzyme, and/or a weaker substrate binding.

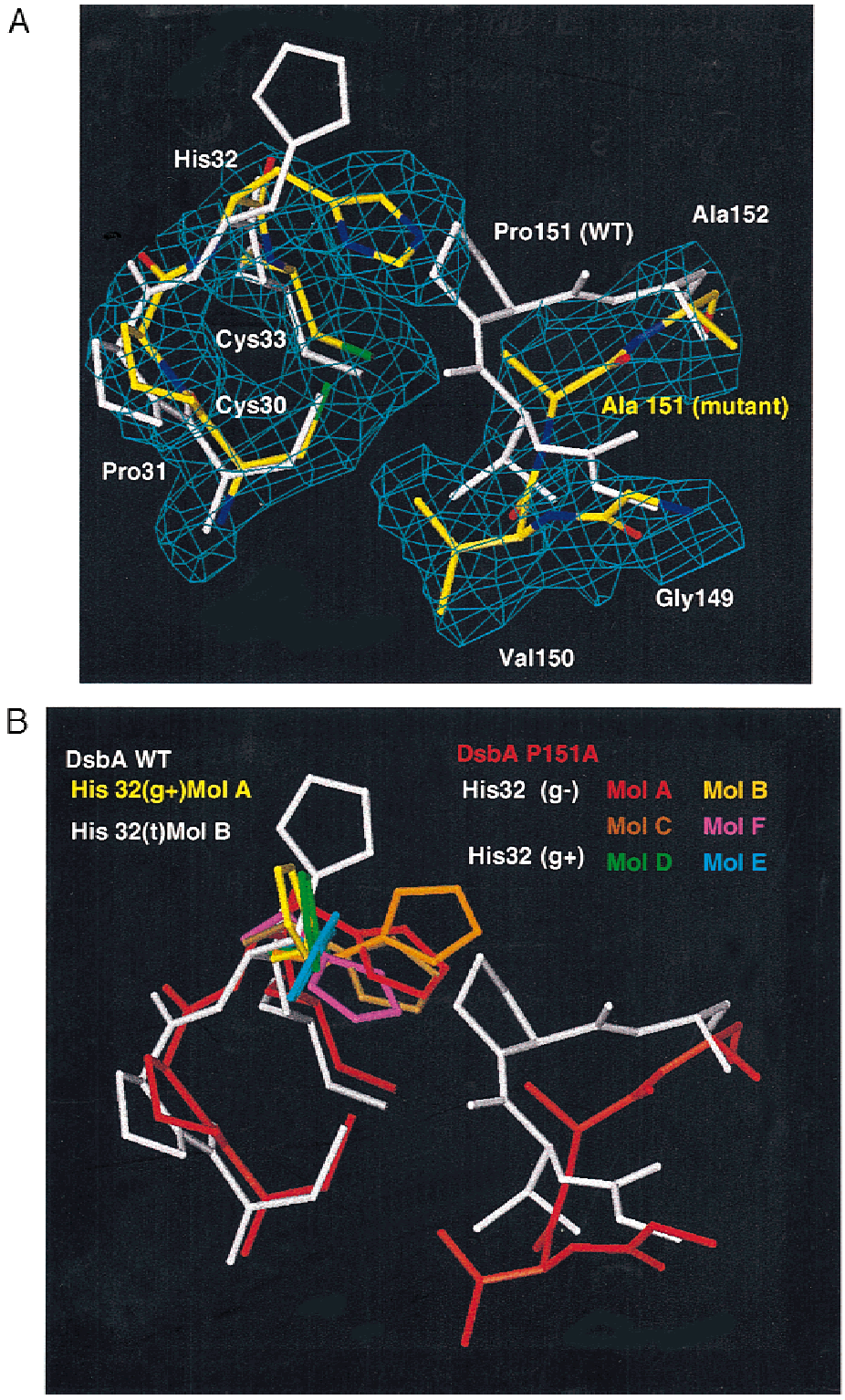

Fig. 5. Comparison of DsbA and $DsbA_{P151A}$ active site. **A:** $2F_o - F_c$ electron density map (at 2.8 Å resolution) in the active site of $DsbA_{P151A}$ contoured at 1.5σ. The structure of wild-type DsbA (white) has been superimposed on the mutant model (yellow). **B:** Comparison of the His32 positions in the six monomers of $DsbA_{P151A}$ and in the two monomers of wild-type DsbA. The side chain uses the gauche− rotamer, pointing toward Ala151. This is observed in four out of six of the $DsbA_{P151A}$ monomers. This rotamer cannot occur in wild-type DsbA due to steric hindrance with the cis-Pro151.

$DsbA_{P151A}$ was quantitatively purified from the periplasm of *E. coli* implying that the protein was correctly expressed and secreted. The purified enzyme is well-folded and active in vitro. $DsbA_{P151A}$ is resistant with respect to proteolysis with a long half-life in vivo. No degradation was observed during the exponential growth phase, but proteolysis did occur during the post-exponential

Table 3. *Conformation of the 149–152 loop in DsbA (WT) and in DsbA (P151A)*[a]

	Gly149		Val150		Pro/Ala151[a]		Ala152	
	Phi	Psi	Phi	Psi	Phi	Psi	Phi	Psi
WT	−164	−133	−72	157	−72	157	−152	148
P151A	−97	174	−79	−21	−133	150	176	159

[a]Phi and Psi values are average values over two molecules in the asymmetric unit for DsbA WT and over six molecules for $DsbA_{P151A}$. The angular standard deviation over the different molecules in the asymmetric unit is in all cases less than 7°.

[a]Residue 151 is a proline in DsbA WT and an alanine in $DsbA_{P151A}$.

growth phase. The AP activity was determined during the exponential growth phase where, at the same induction level, there are very few differences between the expression of *dsbA* and *dsbA*$_{P151A}$ from the plasmids. At 15 μM arabinose, $DsbA_{P151A}$ is expressed at the same level as DsbA from its chromosomal gene (not shown) and the AP activity in vivo is only 50–60% of that observed naturally. At 33 μM arabinose, the $DsbA_{P151A}$ level is five-fold more than chromosomal DsbA and AP activity is only 70–80% of that measured naturally. Also, these results clearly indicate that $DsbA_{P151A}$ was correctly expressed and that the low level of AP activity obtained could not be attributed to a poor expression of $DsbA_{P151A}$. The redox status of DsbA and $DsbA_{P151A}$ were compared when both proteins were expressed under 33 μM arabinose induction. Approximately half of both proteins were found in the oxidized form. Moreover, the relative proportion of oxidized $DsbA_{P151A}$ was slightly higher than for DsbA, showing that the DsbB-mediated reoxidation of $DsbA_{P151A}$ was not impaired. It is noteworthy that $DsbA_{P151A}$ remained a good substrate for DsbB as an additional proof of its correct folding. From our results, it is clear that the difference in the in vivo AP activity cannot be attributed to the smaller amount of $DsbA_{P151A}$ present in the bacteria nor to an inefficient reoxidation of $DsbA_{P151A}$.

The second set of reasons for the decreased activity of $DsbA_{P151A}$ can be ascribed to its intrinsic biophysical properties. Compared to the set of DsbA variants previously studied (Guddat et al., 1997b; Hennecke et al., 1997b; Jacobi et al., 1997), $DsbA_{P151A}$ can be included in the subset of variants poorly affected regarding K_{ox} and pK_a. Although less oxidizing than DsbA, it can still be considered as an efficient oxidase from this point of view. The analysis of $DsbA_{P151A}$ active-site structure showed no rearrangement of the main chain of the Cys-Pro-His-Cys segment. The side chain of His32, a residue postulated to contribute electrostatically to the oxidizing power of DsbA (Warwicker & Gane, 1996; Guddat et al., 1997a), was found to have greater conformational flexibility in $DsbA_{P151A}$ compared to DsbA. The trans-conformation of the Val150–Ala151 bond and the resulting positioning of this loop made room for movement of the His32 side chain, and possibly for a water molecule when this side chain is out of the cavity. Although it has not been possible to estimate the occupancy in solution of the cavity by the histidine side chain, this might result in a change of the electrostatics in the active site leading to an increased pK_a and K_{ox}. To estimate the consequences of a slight K_{ox} increase on the in vivo activity, two variants exhibiting similar K_{ox} values than $DsbA_{P151A}$ were tested under the same experimental conditions. Interestingly, the modifications introduced in these variants, $DsbA_{CSVC}$ and $DsbA_{CSFC}$, did not take place in the same region of the protein as P151A and had smaller consequences on the protein stability (Grauschopf et al., 1995) and probably on the protein structure also. The three variants were similarly affected regarding disulfide bond activity on AP. The K_{ox} value per se appears to be a major determinant in the loss of enzyme function and can explain at least partially the observed variations of activity in vivo.

The third factor to consider is substrate binding. How could the substitution have influence on the interaction with the substrate? It can be shown that the substitution largely destabilizes the overall structure of the protein, as depicted by the small ΔGs in vitro, for both reduced and oxidized $DsbA_{P151A}$ (Table 1). A similar observation was made for $TrxA_{P76A}$, which lost 16.3 kJ mol^{-1} in its free energy of folding (Kelley & Richards, 1987). The high number of monomers in the $DsbA_{P151A}$ crystal lattice and the partially disordered monomer F correlates well with an overall destabilization of the protein. The increased mobility of $DsbA_{P151A}$ might indirectly result in less efficient substrate binding and poor positioning with respect to the disulfide bond. At the level of the local structure, a dramatic change occurred in the loop beyond Ala151 in the trans-conformation. The peptide binding in DsbA can be modeled from the complexes between human Trx and the peptides from NF-κB and Ref-1 (Guddat et al., 1997b). In both Trx-peptide structures, the cis-Pro loop makes two hydrogen bonds with peptide main-chain atoms (Qin et al., 1996) (Fig. 6). Despite different orientations of the two peptides, the same main-chain atoms of Trx are implicated in these hydrogen bonds: Thr75-O and Thr75-NH. Superposition of the DsbA thioredoxin domain with Trx suggests that these interactions may also be conserved in DsbA and the homologous main-chain atoms would be Val150-O and Val150-NH. The rearrangement of the 149–152 loop in the X-ray structure of $DsbA_{P151A}$ resulted in a reorientation of both Val150-O and Val150-NH and a potential loss of both hydrogen bonds with the peptide.

In conclusion, we have shown that cis-Pro151 is a key residue for the function and stability of both oxidized and reduced forms of DsbA. Although its replacement by an alanine had little influence on the biophysical properties of the active-site disulfide, it significantly affected the enzyme activity. This altered activity can largely be explained by variation in the K_{ox} although a less efficient substrate binding is also likely. Sequence homologies in the thioredoxin structural family are very low except at the level of the active-site cysteines and this peculiar cis-residue. Our results support the idea that there may be very high evolutionary pressure on maintaining the cis-Pro in the structures of thioredoxin-like oxidoreductases, and that this might be directly related to the fine balance between the determinants controlling their function.

Materials and methods

Bacterial strains and media

The *E. coli* K-12 strains used in this study were BW494 (F$^-$ Δ*lacU169 proC*::Tn*5 phoM451 phoR68 rpsL aroB,* obtained from Barry Wanner), SBS1554 (HfrC *relA pit-10 tonA22 thi garB10 ompF627* Δ*phoA20 agp*::*phoA*-IS*50*L-*neo*; (Pradel & Boquet, 1989)), SBS2280 (HfrC *relA pit-10 tonA22 thi garB10 ompF627 dsbB*::Tn*10*; (Belin & Boquet, 1993)), SBS2451 (HfrC *relA pit-10 tonA22 thi garB10 ompF627 dsbA*::Ω), MC1061 (F$^-$ Δ*(ara-leu)*

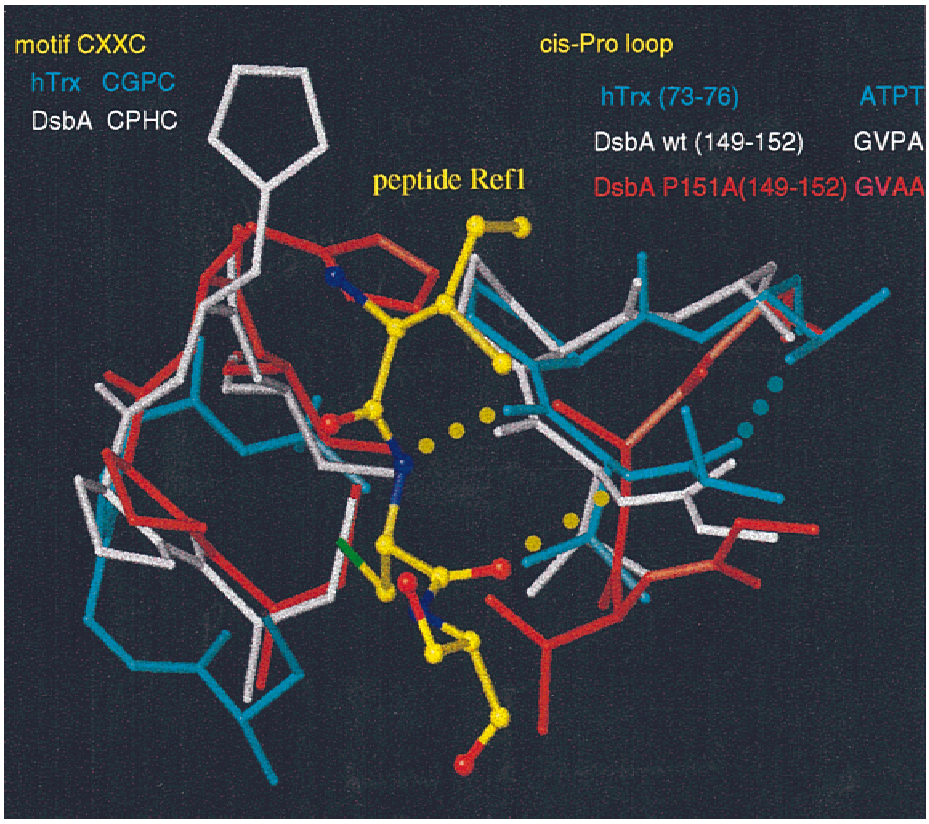

Fig. 6. Superposition of hTrx, DsbA, and $DsbA_{P151A}$. The thioredoxin-fold domain of wild-type DsbA and $DsbA_{P151A}$ has been superimposed on human thioredoxin (hTrx) complexed with peptide from Ref-1 (Qin et al., 1996). Comparison of the three active sites shows the conserved conformation of the cis-Pro loop in hTrx (cyan) and wild-type DsbA (white) despite different sequences besides the cis-proline. The numbering of the Trx residues in the figure corresponds to the human sequence. The Pro151 (75 in hTrx) to Ala mutation induces a major rearrangement of the active site (red). Hydrogen bonds and van der Waals contacts observed between peptides and the hTrx cis-Pro loop may be conserved in wild-type DsbA but lost in the mutant.

araD139 Δ*lacX74 galE15 galK16 mcrA mcrB1 rpsL hsdR2*), SBS2915 (MC1061 *agp*::*phoA*-IS*50*L-*neo*), SBS2916 (MC1061 *agp*::*phoA*-IS*50*L-*neo dsbA*::Ω), SBS3136 (MC1061 *proC*::Tn*5 phoR68 dsbA*::Ω), and SBS3261 (MC1061 *agp*::*phoA*-IS*50*L-*neo dsbA*::Ω *dsbB*::Tn*10*). Strain SBS2451 contained a *dsbA*-null mutation constituted by a chromosomal insertion of a *Bam*HI-*Bam*HI 2 kb-longΩ(Str^R/Spc^R) fragment in the *Bgl*II site of *dsbA*. Strain SBS2915, SBS2916, SBS3136, and SBS3261 were obtained by transduction with P1 (Miller, 1992).

TYE medium (bactotryptone 10 g/L, yeast extract 5 g/L, NaCl 8 g/L, pH 7.5) + 0.5% glycerol was used as a rich medium, M9 medium (Miller, 1992) + 0.5% glycerol as a minimal medium. Ampicillin was added at 200 μg/mL, streptomycin and spectinomycin at 20 μg/mL, and kanamycin at 30 μg/mL.

Plasmids constructions

Plasmids manipulations were performed according to Sambrook et al. (1989) and recommendations of molecular biology enzymes suppliers (New England Biolabs, Beverly, Massachusetts). A 0.8 kb *Bsp*HI-*Ssp*I fragment encoding *dsbA* was obtained from pPB2196 (Belin & Boquet, 1994). This fragment was inserted between the *Nco*I and *Sma*I sites of pBAD24 (Guzman et al., 1995) and pTrc99A (Amersham Pharmacia Biotech, Uppsala, Sweden), resulting in pPB2959 and pPB2190, respectively. pPB3141, pPB3485, and pPB3499 derived from pPB2959 and encoded variants of DsbA, respectively, $DsbA_{P151A}$ (the Pro151 residue was changed to Ala151), $DsbA_{CSFC}$ (the Pro31–His32 residues were changed to Ser31–Phe32) and $DsbA_{CSVC}$ (the Pro31–His32 residues were changed to Ser31–Val32) were obtained by site-directed mutagenesis using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, California). The coding deoxyoligonucleotides used were, respectively:

5′GCAATTGCGTGGCGTT**GCT**GCGATGTTTGTTAACGG3′,

5′CTCTTTCTTCTGC**TCTTTC**TGCTATCAGTTTGAAG3′,

5′CTCTTTCTTCTGC**TCTGTT**TGCTATCAGTTTGAAG3′.

The complementary deoxyoligonucleotides were used according to the instructions of the manufacturer. The cycles used for the polymerase chain reaction were the following: 30 s at 95 °C, one time; 30 s at 95 °C, 1 min at 55 °C, 10 min at 72 °C, each 16 times. The entire nucleotide sequence of the mutated *dsbA* genes was determined on double-stranded DNA plasmid using a Pharmacia T7 sequencing kit. Plasmids pPB2190 and pPB3140 were used for high level production of DsbA and $DsbA_{P151A}$, respectively, in strain SBS2916 (*dsbA*::Ω). pPB3140 derived from pPB2190, as

described above, for pPB3141 and pPB2959. pPB3140 contained the same CCG → GCT mutation in the *dsbA* gene. The protocol used for pPB2959 was applied to pPB2190, from mutagenesis to nucleotide sequence determination.

Alkaline phosphatase (AP) activity determination

AP activity was measured within bacterial suspensions. All cultures were grown at 37 °C in 250 mL flasks filled with 25 mL of medium, inoculated to 1/200th from an overnight culture grown in the same conditions. To determine the PhoA activity, aliquots of the culture were centrifuged and the cells were suspended in 500 μL of 150 mM Tris-HCl pH 8.8. A drop of chloroform and 0.1% SDS was used to disrupt the cell membrane (Miller, 1992). After 5 min at 37 °C, 25 μL of 0.5 M *para*-nitrophenyl phosphate was added to start the assay. The reaction was stopped after 5 min by the addition of 1 mL cold 1 M NaOH. After pelleting the cells, the A_{410} of the supernatant was read against a blank. The AP activities of bacterial cultures were expressed as $\Delta A_{410}\ \mathrm{min}^{-1}$ (mL of culture)$^{-1}$.

Cell fractionation and western blot analysis

The periplasmic fractions were obtained from bacteria after lysozyme spheroplast formation according to the method described by Malamy and Horecker (1964). The samples were analyzed by SDS-PAGE and western blot analysis (Sambrook et al., 1989). Proteins were electroblotted on nitrocellulose. DsbA and $\mathrm{DsbA}_{\mathrm{P151A}}$ were detected with an anti-DsbA rabbit serum and a peroxidase-conjugated $\mathrm{F(ab')}_2$ fragment goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pennsylvania). The peroxidase activity was revealed using diaminobenzidine.

Pulse-chase analysis

Bacterial cultures were grown in M9 minimal medium + glycerol supplemented with 50 μg/mL each amino acid (except methionine and cysteine) and 200 μg/mL ampicillin. At $A_{600\mathrm{nm}} \sim 0.3$, 13.3 mM arabinose was added to the cultures. At $A_{600\mathrm{nm}} \sim 0.7$, the cells were labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 s (Pro-Mix™ from Amersham Pharmacia Biotech, 30 μCi/mL). Unlabeled cysteine and methionine (each 100 μg/mL) were added and aliquots of the cultures (500 μL) were withdrawn at different time points. Cells were collected by centrifugation and suspended in reducing SDS-PAGE loading buffer. Samples were boiled for at least 20 min and analyzed by 12% SDS-PAGE and autoradiography. Autoradiograms were scanned using a CCD-camera and Densylab software (Microvision Instruments, France).

Determination of in vivo redox states

Cells were grown in TYE medium + glycerol and 33 μM arabinose. At $A_{600\mathrm{nm}} \sim 2.5$, 100 μL of culture was mixed with 100 μL of cold 20% trichloracetic acid. Precipitated proteins were collected by centrifugation and washed once with cold 10% trichloracetic acid. Proteins were then solubilized in 100 μL of 100 mM Tris pH 8.0, 1.5% SDS, and 1 mM EDTA containing 10 mM biotin-maleimide and incubated at room temperature for 2 min. Oxidized and reduced samples were prepared by solubilization of precipitated proteins in 90 μL of 100 mM Tris, pH 8.0, 1.5% SDS, and 1 mM EDTA containing either 20 mM oxidized DTT or 2 mM reduced DTT, respectively. Incubation was performed at room temperature during 30 min before addition of 10 μL of a 100 mM biotin-maleimide solution. Samples were analyzed by SDS-PAGE under reducing conditions and western-blotting with anti-DsbA serum.

Enzyme purification

$\mathrm{DsbA}_{\mathrm{P151A}}$ variant was purified by the same protocol as the wild-type DsbA: anion exchange chromatography on DEAE-Sephacel (Pharmacia) followed by hydrophobic chromatography on phenyl-Sepharose CL-4B (Pharmacia) (Wunderlich & Glockshuber, 1993). DsbA and $\mathrm{DsbA}_{\mathrm{P151A}}$ protein concentrations were determined spectrophotometrically using an absorption coefficient of $A_{280,1\mathrm{mg/ml,1cm}} = 1.10$ (Wunderlich & Glockshuber, 1993).

Determination of redox properties, pK_a of Cys30 and stability

The relative oxidizing power of $\mathrm{DsbA}_{\mathrm{P151A}}$ was determined by measuring the equilibrium constant (K_{ox}) for the thiol-disulfide exchange reaction with glutathione (GSH/GSSG), taking advantage of the differences in the fluorescence emission spectra of oxidized and reduced species. 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. Equilibration of enzymes at different GSH/GSSG ratios was performed under argon atmosphere, in degassed buffers for 16 h. Fluorescence spectra were recorded on a Spex 0.34 spectrometer.

The pK_a values of the thiol group were determined from the increase in absorbance at 240 nm upon formation of the thiolate (Benesch & Benesch, 1955; Nelson & Creighton, 1994). Reduced DsbA and $\mathrm{DsbA}_{\mathrm{P151A}}$ (1 mg/mL in distilled water) were prepared by incubation with 1 mM DTT for several minutes. DTT was removed by gel filtration on G-25 (Pharmacia) previously washed with water. The pH titration was performed with an initial protein concentration of 24 μM, in an initial volume of 2 mL in 10 mM phosphate, 10 mM citrate, 10 mM borate, pH 8.5. The pH was lowered by stepwise addition of 10–20 μL of a 0.1 M HCl solution also containing 10 mM of each acid mentioned above. Oxidized protein was used as a reference and treated in the same conditions. The values of $(A_{240}/A_{280})_{red} - (A_{240}/A_{280})_{ox}$ represent the specific absorption of the thiolate and were fitted according to the Henderson–Hasselbach equation. UV absorptions were measured with a Hewlett Packard 8453 spectrophotometer.

Thermodynamic stability was determined from the measurement of the free energies of folding by high-sensitivity differential scanning calorimetry (HS-DSC) as described elsewhere (Moutiez et al., 1999).

Crystallization and data collection

The oxidized form of $\mathrm{DsbA}_{\mathrm{P151A}}$ was prepared as described for the wild-type enzyme (Martin et al., 1993). Before crystallization the protein was oxidized with 1.5 mM copper(II)[1,10-phenanthroline]$_3$ and dialyzed against 10 mM HEPES (pH 7.8). The sitting drop procedure was used to grow crystals by vapor diffusion. Under the conditions of crystallization reported for wild-type DsbA, $\mathrm{DsbA}_{\mathrm{P151A}}$ gave urchin-like crystals that were not suitable for data collection. As a control, wild-type DsbA prepared according to the same

protocol yielded good diffracting crystals, isomorphous with that already reported (Martin et al., 1993). A screen for additives led to a modified crystallization procedure where larger crystals could be obtained in the presence of 10% (v/v) DMSO and 100 mM NaCl (Stura et al., 1998). Diffraction data were collected on these crystals at the W32 station of the LURE synchrotron (Orsay, France) at 7 °C and at BW7A station of the DESY synchrotron (Hamburg, Germany) with cryo-cooling system. Intensities were evaluated using Denzo and Scalepack (Otwinowski & Minor, 1997). The atomic model was established alternating cycles of reconstruction with program O (Kleywegt & Jones, 1997) and refinement with X-Plor (Brünger, 1992). No water molecules have been included in the refinement. The structure of the $DsbA_{P151A}$ was refined to a final *R*-value of 21.8% and a free *R*-value of 28.9%. The model had standard stereochemistry, as evaluated with PROCHECK (Laskowski et al., 1993) (Table 2). Superposition of the proteins of the thioredoxin superfamily was done with program TOP (Guoguang, 1996). Coordinates of $DsbA_{P151A}$ have been deposited at the Brookhaven Protein Data Bank (PDB entry 1BQ7).

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References

Bardwell JCA, Lee J-O, Jander G, Martin N, Belin D, Beckwith J. 1993. A pathway for disulfide bond formation in vivo. *Proc Natl Acad Sci USA 90*:1038–1042.

Belin P, Boquet PL. 1993. A second gene involved in the formation of disulfide bonds in proteins of the *Escherichia coli* periplasmic space. *C R Acad Sci Paris 316*:469–473.

Belin P, Boquet PL. 1994. The *Escherichia coli dsbA* gene is partly transcribed from the promoter of a weakly expressed upstream gene. *Microbiology-UK 140*:3337–3348.

Benesch RE, Benesch R. 1955. The acid strength of the -SH group in cysteine and related compounds. *J Am Chem Soc 77*:5877–5881.

Brünger AT. 1992. *X-PLOR (version 3.1) manual*. New-Haven, Connecticut: Yale University

Chivers PT, Prehoda KE, Raines RT. 1997. The CXXC motif: A rheostat in the active-site. *Biochemistry 36*:4061–4066.

Grauschopf U, Winther JR, Korber P, Zander T, Dallinger P, Bardwell JC. 1995. Why is DsbA such an oxidizing disulfide catalyst? *Cell 83*:947–955.

Guddat L, Bardwell J, Glockshuber R, Huber-Wunderlich M, Zander T, Martin J. 1997a. Structural analysis of three His32 mutants of DsbA: Support for an electrostatic role of His32 in DsbA stability. *Protein Sci 6*:1893–1900.

Guddat LW, Bardwell JCA, Zander T, Martin JL. 1997b. The uncharged surface features surrounding the active-site of *Escherichia coli* DsbA are conserved and are implicated in peptide binding. *Protein Sci 6*:1148–1156.

Guoguang L. 1996. A WWW service system for automatic comparison of protein structures. *PDB Quarterly Newsletter 78*:10–11.

Guzman L-M, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation and highlevel expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol 177*:4121–4130.

Hennecke J, Sillen A, Huber Wunderlich M, Engelborghs Y, Glockshuber R. 1997a. Quenching of tryptophan fluorescence by the active-site disulfide bridge in the DsbA protein from *Escherichia coli*. *Biochemistry 36*:6391–6400.

Hennecke J, Spleiss C, Glockshuber R. 1997b. Influence of acidic residues and the kink in the active-site helix on the properties of the disulfide oxidoreductase DsbA. *J Biol Chem 272*:189–195.

Hu SH, Peek JA, Rattigan E, Taylor RK, Martin JL. 1997. Structure of TcpG, the DsbA protein folding catalyst from *Vibrio cholerae*. *J Mol Biol 268*:137–146.

Jacobi A, Huber-Wunderlich M, Hennecke J, Glockshuber R. 1997. Elimination of all charged residues in the vicinity of the active-site helix of the disulfide oxidoreductase DsbA. *J Biol Chem 272*:21692–21699.

Georgescu RE, Li JH, Goldberg ME, Tasayco ML, Chaffotte AF. 1998. Proline isomerization-independent accumulation of an early intermediates and heterogeneity of the folding pathways of a mixed a/b protein, *Escherichia coli* thioredoxin. *Biochemistry 37*:10286–10297.

Kelley R, Richards F. 1987. Replacement of proline-76 with alanine eliminates the slowest kinetic phase in thioredoxin folding. *Biochemistry 26*:6765–6774.

Kleywegt GJ, Jones TA. 1997. Model building and refinement practice. *Methods Enzymol 277*:208–230.

Kortemme T, Darby NJ, Creighton TE. 1996. Electrostatic interactions in the active-site of the N-terminal thioredoxin-like domain of protein disulfide isomerase. *Biochemistry 35*:14503–14511.

Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr 26*:283–291.

Lundstrom J, Krause G, Holmgren A. 1992. A Pro to His mutation in active-site of thioredoxin increases its disulfide-isomerase activity 10-fold. New refolding systems for reduced or randomly oxidized ribonuclease. *J Biol Chem 267*:9047–9052.

Malamy MH, Horecker BL. 1964. Release of alkaline phosphatase from cells of *Escherichia coli* upon lysozyme spheroplast formation. *Biochimie 3*:1889–1893.

Martin JL. 1995. Thioredoxin: A fold for all reasons. *Structure 3*:245–250.

Martin JL, Bardwell JCA, Kuriyan J. 1993. Crystal structure of the DsbA protein required for disulphide bond formation in vivo. *Nature 365*:464–468.

Miller JH. 1992. *A short course in genetics: A laboratory manual and handbook for Escherichia coli and related bacteria*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Moutiez M, Burova TV, Haertlé T, Quéméneur E. 1999. On the non-respect of the thermodynamic cycle by DsbA variants. *Protein Sci 8*:106–112.

Navaza J, Saludjian P. 1997. AMoRE: An automated molecular replacement program package. *Methods Enzymol 216*:581–593.

Nelson JW, Creighton TE. 1994. Reactivity and ionization of the active-site cysteine residues of DsbA, a protein required for disulfide bond formation in vivo. *Biochemistry 33*:5974–5983.

Otwinowski Z, Minor W. 1997. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol 276*:307–326.

Pradel E, Boquet PL. 1989. Mapping of the *Escherichia coli* acid glucose-1-phosphatase gene *agp* and analysis of its expression in vivo by use of an *agp-phoA* protein fusion. *J Bacteriol 171*:3511–3517.

Qin J, Clore GM, Kennedy WP, Kuszewski J, Gronenborn AM. 1996. The solution structure of human thioredoxin complexed with its target from Ref-1 reveals peptide chain reversal. *Structure 4*:613–620.

Raina S, Missiakas D. 1997. Making and breaking disulfide bonds. *Annu Rev Microbiol 51*:179–202.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Stura ES, Charbonnier JB, Taussig M. 1998. Epitaxial jumps. *J Cryst Growth*. In press.

Wanner BL. 1987. Phosphate regulation of gene expression in *Escherichia coli*. In: Neidhardt FC, ed. *Escherichia coli and Salmonella typhimurium: Cellular and molecular biology*. Washington, DC: American Society for Microbiology. pp 1326–1333.

Warwicker J, Gane PJ. 1996. Calculation of Cys 30 delta pK_a's and oxidizing power for DsbA mutants. *FEBS Lett 385*:105–108.

Wunderlich M, Glockshuber R. 1993. Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. *Protein Sci 2*:717–726.

Wunderlich M, Jaenicke R, Glockshuber R. 1993. The redox properties of protein disulfide isomerase (DsbA) of *Escherichia coli* result from a tense conformation of its oxidized form. *J Mol Biol 233*:559–566.

Zapun A, Bardwell JCA, Creighton TE. 1993. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation in vivo. *Biochemistry 32*:5083–5092.