Description of the topographical changes associated to the different stages of the DsbA catalytic cycle

FLORIANA VINCI,[2] JOËL COUPRIE,[1] PIERO PUCCI,[2] ERIC QUÉMÉNEUR,[1] AND MIREILLE MOUTIEZ[1]

[1]CEA, Département d'Ingénierie et d'Etudes des Protéines–Bâtiment 152, C.E. Saclay, F-91191 Gif-sur-Yvette, France
[2]Dipartimento di Chimica Organica e Biochimica, Università di Napoli "Federico II," 80126 Napoli, Italy
[3]CEA Valrho, DSV/DIEP/SBTN, F-30207 Bagnols-sur-Cèze, France

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Abstract

This paper provides a description of the surface topography of DsbA, the bacterial disulfide-bond forming enzyme, in the different phases of its catalytic cycle. Three representative states, that is, oxidized and reduced protein and a covalent complex mimicking the DsbA-substrate disulfide intermediate, have been investigated by a combination of limited proteolysis experiments and mass spectrometry methodologies. Protease-accessible sites are largely distributed in the oxidized form with a small predominance inside the thioredoxin domain. Proteolysis occurs even in secondary structure elements, revealing a significant mobility of the protein. Many cleavage sites disappear in the reduced form and most of the remaining ones appear with strongly reduced kinetics. The protein within the complex shows an intermediate behavior. This variation of flexibility in DsbA is probably the determining factor for the course of its catalytic cycle. In particular, the great mobility of the oxidized protein might facilitate the accommodation of its various substrates, whereas the increasing rigidity from the complexed to the reduced form could help the release of oxidized products. The formation of the complex between PID peptide and DsbA does not significantly protect the enzyme against proteolysis, reinforcing the results previously obtained by calorimetry concerning the weakness of their interaction. The few cleavage sites observed, however, are in favor of the presence of the peptide in the binding site postulated from crystallographic studies. As for the peptide itself, the proteolytic pattern and the protection effect exerted by DsbA could be explained by a preferential orientation within the binding site.

Keywords: DsbA; catalytic cycle; peptide-enzyme complex; limited proteolysis; mass spectrometry

Most of the secreted proteins contain disulfide bridges that play a key role in the folding and stability of the tertiary structure. The formation of these bonds is generally a slow and rate-limiting step in the folding process that requires enzymatic catalysis in vivo. The bacterial DsbA is such an enzyme that oxidizes randomly reduced unfolded proteins in an extremely rapid thiol-disulfide exchange reaction. It belongs to the thiol-disulfide oxidoreductase family structurally related to thioredoxin. This family includes many members of various sizes and various biophysical properties (Ferrari and Söling 1999). These enzymes share a thioredoxin domain, which contains the active site motif Cys-Xaa-Xaa-Cys. All of them are involved in thiol-disulfide exchange processes but display different specialties. Although thioredoxin, the 12-kD group leader, is a very powerful reductase, the 57-kD eukaryotic protein disulfide

Reprint requests to: Mireille Moutiez, CEA, Département d'Ingénierie et d'Etudes des Protéines–Bâtiment 152, C.E. Saclay, F-91191 Gif-sur-Yvette, France; e-mail: mireille.moutiez@cea.fr; fax: 33-1-6908-9071.

Abbreviations: Asp-N, endoproteinase Asp-N; DTT, dithiothreitol; ESIMS, electrospray ionization mass spectrometry; GdmCl, guanidinium chloride; Glu-C, endoproteinase Glu-C; IAM, iodoacetamide; MALDIMS, matrix assisted laser desorption ionization mass spectrometry; PDI, protein disulfide isomerase; PID, peptide derived from DsbB sequence; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; thioredoxin domain "lower" part, strands β1, β4, β5, helix α7, and the loop connecting β5 and α7; thioredoxin domain "upper" part, strands β2 and β3.

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isomerase (PDI) is endowed with disulfide isomerase activity. DsbA is by far the most oxidizing member of the family. Despite the availability of several structures for thioredoxin (first of them reported by Holmgren et al. 1975; Forman-Kay et al. 1991), PDI domains (Kemmink et al. 1996; Kemmink et al. 1997; Kemmink et al. 1999), Dsb proteins (Martin et al. 1993; Hu et al. 1997; Guddat et al. 1998; Schirra et al. 1998; McCarthy et al. 2000), and extensive biochemical experimentation, the molecular basis for their different properties is still puzzling. The active site sequence Cys-Xaa-Xaa-Cys was shown to have a determinant role on the oxidizing power (Grauschopf et al. 1995). In the case of DsbA, the redox equilibrium constant with glutathione was found to be approximately 0.1 mM for the wild-type protein (Cys-Pro-His-Cys) and increased to 1 M when the two central residues were replaced by the sequence Gly-Pro found in thioredoxin (Huber-Wunderlich and Glockshuber 1998). Similar results were obtained in experiments performed on thioredoxin (Mössner et al. 1998) and PDI (Kortemme et al. 1996; Holst et al. 1997).

The very high oxidizing potential of DsbA is accompanied with two particular features. First, the pKa of the first cysteine of the active site, Cys30, is especially low (3–3.5) (Wunderlich and Glockshuber 1993; Nelson and Creighton 1994). At physiological pH, Cys30 is hence completely ionized, which makes it highly reactive for thiol-disulfide reactions. Second, although disulfides generally stabilize proteins, the reduced form of DsbA is much more stable than the oxidized one (Moutiez et al. 1999). Structural studies have pointed out the essential role of the electrostatic stabilization of the thiolate of Cys30 for the stability of the reduced form (Gane et al. 1995), but these same studies do not address the role of the dynamics of the protein in the activity, particularly the role of domain motions. Data still remain to be investigated to explain the different steps of the catalytic cycle: the specific binding of reduced, denatured substrates to the oxidized form; the oxidation and release of substrate; and the binding of the reduced protein to its membrane partner DsbB, followed by reoxidation and dissociation of the complex DsbA-DsbB (Bardwell et al. 1993; Bader et al. 1998).

The three-dimensional structure of DsbA can be divided into two domains: the family consensus domain that possesses a thioredoxin fold and an additional helical domain usually named B-domain. Although the structure of the oxidized enzyme has been solved since 1993 (Martin et al. 1993), the structure of the reduced form has been unavailable for a long time. In 1998, both a nuclear magnetic resonance (NMR) and an X-ray structure of reduced DsbA have been published (Guddat et al. 1998; Schirra et al. 1998). However, these structures do not bring all the expected explanations about the properties of the protein and are even contradictory on some points (e.g., the relation between domain motions and redox state).

The results presented in this paper aim at providing a description of the conformational variations associated to the different phases of the DsbA catalytic cycle. We focused on the two extreme stages of the enzyme cycle, the oxidized and the reduced forms, and on an intermediate stage, a stable complex between DsbA and a peptidic ligand. In a previous work, we have designed a stable ligand-enzyme complex in which the labile cystine bond that normally forms during the catalytic cycle has been replaced by a cysteine-homoalanine thioether bond (Couprie et al. 2000). This approach enables the direct use of the wild-type form of the protein, which is the most relevant one to obtain precise insights into the enzymatic mechanism. Moreover, the chemical stability of such a complex makes possible studies in physiological conditions of pH and temperature. As an investigative tool, we focused on a strategy that combines limited proteolysis with mass spectrometry methodologies. This procedure had been developed to probe the surface topography of proteins and to investigate interface regions in protein complexes (Zappacosta et al. 1996; Scaloni et al. 1998). Only the protein regions both exposed and flexible are accessible to proteolytic cleavages. Stereochemical barriers prevent the occurrence of cleavages within the highly structured core of the protein or at least slow down their kinetics.

The results reported here show that reduction essentially results in a loss of mobility in both domains of DsbA, which is probably a determining factor for the course of the catalytic cycle. The complex shows an intermediate flexibility between the oxidized and the reduced states, a tendency that can be connected with the results previously obtained by calorimetry (Couprie et al. 2000).

Results

Oxidized DsbA and DsbA-PID peptide complex were separately analyzed by high-performance liquid chromatography (HPLC) and characterized by electrospray ionization mass spectrometry (ESIMS). Both samples showed the occurrence of a single symmetric peak. The molecular masses of the corresponding species determined by ESIMS (21130.33 ± 1.21 Da for the oxidized protein and 22817.87 ± 1.13 Da for the complexed protein) were in perfect agreement with the expected mass values (21130.07 and 22816.87 Da, respectively). Reduced DsbA was used immediately after reduction by dithiothreitol (DTT) for limited proteolysis experiments. To show that the DsbA sample was reduced throughout the limited proteolysis experiments, the reduced protein was incubated in the experimental conditions used for proteolysis experiments and then carboxyamidomethylated. The experimental conditions used for the alkylation were highly selective toward cysteine residues, as previously shown (Couprie et al. 2000). The ESIMS analysis of the reduced protein after carboxyamidomethylation showed

the presence of a single species at 21189.80 ± 1.63 Da corresponding to the reduced DsbA with a single alkylated cysteine (expected molecular mass 22189.07 Da). This result is in perfect agreement with our previous data showing that complete chemical modification of the two cysteine residues can only be achieved after full denaturation of the protein (Couprie et al. 2000).

Topographical study of oxidized DsbA

The surface topography of oxidized DsbA was probed using the limited proteolysis/mass spectrometry approach based on the mass spectral analysis of the fragments released from DsbA by controlled proteolysis experiments. Limited proteolysis experiments were performed using trypsin, chymotrypsin, Asp-N, Glu-C, and subtilisin as proteolytic probes according to the strategy previously described (Zappacosta et al. 1996). Oxidized DsbA was incubated with each protease at pH 7.5 and 37°C with an appropriate enzyme-to-substrate ratio (see Materials and Methods). The extent of the enzymatic hydrolysis was monitored on a time-course basis by taking samples from the incubation mixture at different interval times. After quenching of the hydrolysis reaction by freezing, each aliquot was fractionated by reversed phase high-performance liquid chromatography (RP-HPLC) on a SMART system (Pharmacia). The mass values of proteolytic fragments were determined by mass spectrometric techniques with sufficient accuracy to enable unambiguous identification of the peptides, hence, the assignment of the cleavage sites in the amino acid sequence of DsbA. Only the low specificity of subtilisin made necessary further MS/MS analyses of the proteolytic fragments to confirm assignment of the peptide identity.

Special care was taken to define the appropriate conditions in terms of time of incubation and enzyme-to-substrate ratio. For the investigation of protein topography by limited proteolysis, a prerequisite is the conformational homogeneity of this protein. In the case of DsbA, the protein has been largely studied by many biophysical techniques. NMR studies (Couprie et al. 1998), chromatographic behavior, and calorimetric studies (Couprie et al. 2000) all converge on the existence of a unique monomeric conformation of DsbA in solution (within the experimental limits of detection related to each of these methods). Each protease used in this study was investigated with different ratios of protease-to-oxidized DsbA. The use of higher protease/DsbA ratios than those finally retained resulted in a sharp decrease in the selectivity of hydrolysis and a consequential increase of proteolytic fragments. Indeed, for specific proteases as well as for unspecific ones, the proportion of peptides originated from subdigestion of larger fragments greatly increased with the ratio of protease/DsbA, as already described in other studies (Zappacosta et al. 1996). On the basis of the preliminary experiments, the conditions of limited proteolysis were selected to maximize the selectivity of cleavages to preferred sites.

As an example, Figure 1 shows the HPLC chromatograms of the aliquots withdrawn at 15, 30, and 60 min of tryptic digestion. Mass spectral identification of the tryptic peptides observed within the proteolysis experiment is reported in Table 1. The occurrence of few fragments in the HPLC profiles and the presence of a large amount of undigested protein (peak 14) clearly indicated that under the experimental conditions used, the activity of the proteases was restricted to few specific sites. Three fragments rapidly appeared and were identified as peptides 184–189, 159–183, and 104–132 (peaks 1, 6, and 8, respectively), indicating the occurrence of early cleavage sites at Arg103, Lys132, Lys158, and Lys183. At longer hydrolysis time additional fragments were detected. Special attention was brought to the analysis of these new fragments to distinguish between peptides resulting from subdigestion of larger ones and fragments released from native DsbA with slower proteolysis kinetics. This analysis revealed the presence of four minor cleavage sites at Lys7, Lys48, Lys58, and Lys70, as inferred from the identification of the peptides 8–48 and 59–70 (peaks 12 and 5, respectively).

The overall data from the limited proteolysis experiments are summarized in Table 2 and Figures 2 and 3. DsbA proved to be highly resistant to the proteolysis regardless of the protease used as shown by the high enzyme/substrate ratio needed to observe proteolysis and by the low number of fragments released in the chromatograms. Preferential cleavage sites were classified as "major" or "minor" on qualitative evaluation according to their kinetics of appearance (at early or longer times of incubation) during the time-course experiments. Most of the major cleavages occurred in the thioredoxin domain, especially in the "lower" part (strands β1, β4, β5, and helix α7). Minor cleavage sites accumulated in the "upper" part of the β-sheet (strands β2 and β3) and in helix α2, a region described as the hydrophobic core of the protein (Hennecke et al. 1999). No region of the protein appeared to be really protected against proteolysis with the exception of helix α3 in which no cleavage sites could be detected.

Topographical study of reduced DsbA

The surface topography of reduced DsbA was monitored using the same limited proteolysis-mass spectrometry approach. Comparative experiments were performed on the reduced protein in the conditions previously described for the oxidized form.

Figure 4A and B show the HPLC profiles corresponding to the 60-min aliquots of the tryptic hydrolysis of the oxidized and the reduced form of DsbA, respectively. Peak identifications are reported in Table 1. The comparison of both chromatograms indicates clearly that reduced DsbA is

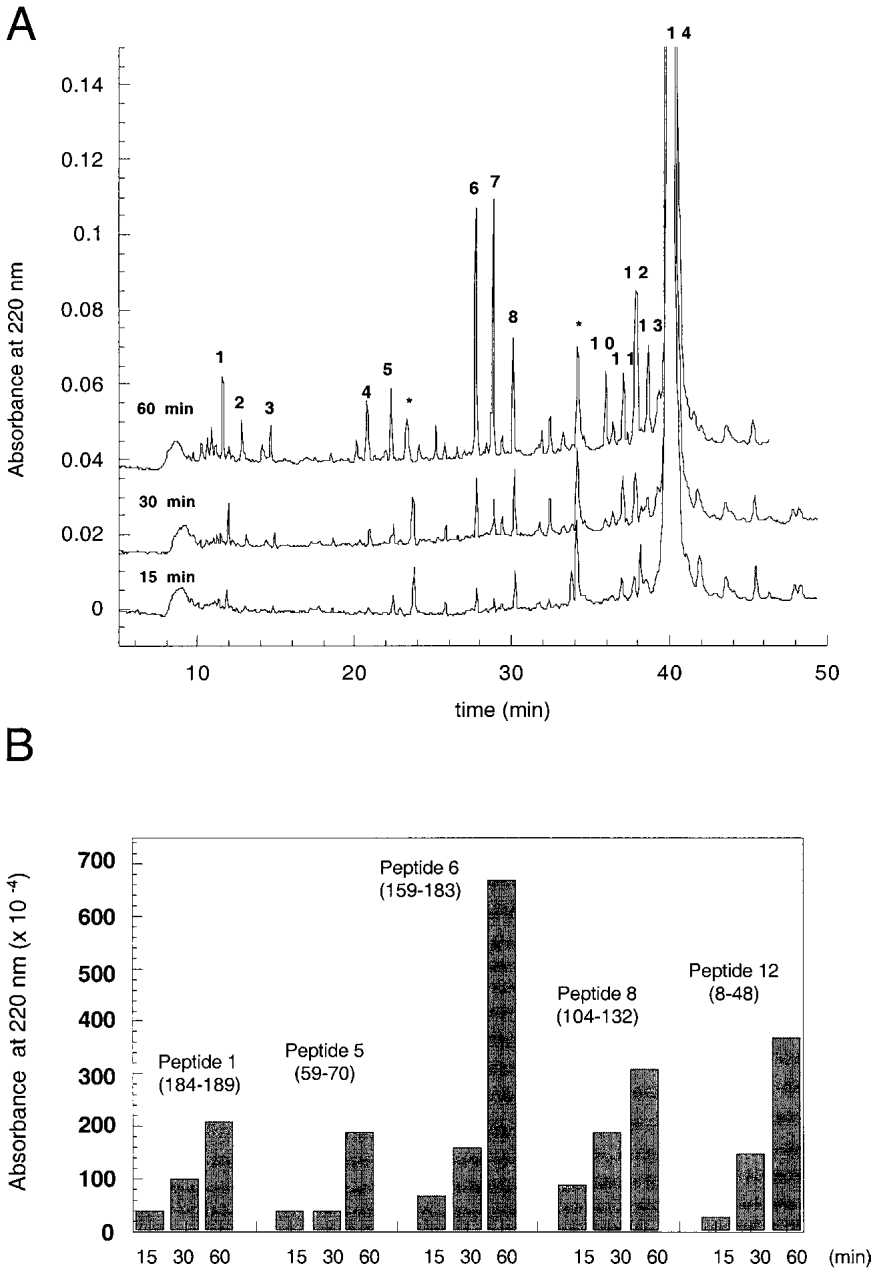

Fig. 1. (*A*) Time-course analysis of oxidized DsbA digested by trypsin, at pH 7.5 37°C using an enzyme/substrate ratio of 1 : 70. High-performance liquid chromatography (HPLC) chromatograms of the aliquots withdrawn at 15 min, 30 min, and 60 min. Individual fractions were collected and analyzed by electrospray ionization mass spectrometry (ESIMS). Peak numbering is consistent within all the tryptic digestion experiments (Fig. 4 and Table 1). Peaks nonrelated to the protein are labeled with an asterisk. (*B*) Histograms depicting the amount of a selection of tryptic peptides at different times.

much more resistant to proteolysis than oxidized DsbA. The time-course analysis of the tryptic experiment on the reduced protein showed again the early occurrence of cleavages at the level of Arg103, Lys132, Lys158, and Lys183. The identification of peptides showed the increased accessibility of Lys7 as shown by the early appearance of the peptide 1–7. Conversely, all the other peptides corresponding to minor cleavage hydrolysis of the oxidized form have disappeared (peaks 12 and 5). A new cleavage site appeared at Arg148, as indicated by peak 9 (peptide 149–189), that increased at a rate comparable to peaks 1 or 8.

The data summarizing the results of the proteolysis experiments on reduced DsbA with the five proteases are reported in Table 2 and Figure 5. Regardless of the protease used, the kinetics of appearance of proteolytic fragments were dramatically slowed down, indicating an increase in

Table 1. *Peptides released from oxidized DsbA after proteolysis experiments with trypsin*

Peak	MH^+ theoretical value	MH^+ experimental value (ox)	MH^+ experimental value (red)	MH^+ experimental value (complex)	Assignment
	810.83	—	810.29	—	1–7
1	767.89	767.15	767.17	767.30	184–189
2	903.01	903.74	902.32	902.34	133–140[a]
3	843.95	843.50	843.27	843.33	141–148[a]
4	1020.22	1021.03	1019.23	1019.77	149–158[b]
5	1338.51	1338.97	—	—	59–70
6	2894.19	2895.23	2894.13	2893.49	159–183
7	2573.86	2574.83	2574.80	2573.56	110–132
	3894.40	—	—	3895.10	149–183[b]
8	3203.53	3204.64	3202.50	3203.64	104–132
9	4643.28	4644.30	4643.90	4645.10	149–189[b]
10	3017.5	3018.60	—	—	71–98
11	5584.29	5582.26	—	—	1–48
12	4790.47 ox 4792.47 red	4790.82	—	—	8–48
13	4664.30	4664.90	—	—	8–47

[a] Small peptides probably resulting from subdigestion.
[b] Very late cleavage site in the oxidized form.

the overall compactness of the protein. In the case of the Glu-C endoproteinase hydrolysis, no fragments could even be detected with the enzyme-substrate ratio 1/20 previously used with oxidized DsbA. It was necessary to increase the ratio up to 1/10 to observe significant hydrolysis reactions during the usual experiment duration.

The comparative analysis of the results obtained with oxidized and reduced DsbA showed that the differences in the proteolytic sites were mainly located in the hydrophobic core of the protein (strands β2, β3, and helix α2) where a disappearance of the minor cleavage sites was observed in the reduced form. Of special interest is the disappearance of the hydrolysis site at the level of Phe129, a residue located at the hinge between helices α5 and α6, indicating a loss of flexibility in that region. It should also be noticed that proteolysis at Asp110 (helix α4) tends to be slower, whereas no cleavage was observed at the neighboring residue Phe112. Finally, all the chymotryptic cleavages occurring in strands β4, β5, and helix α7 appeared later in the time-course experiment, indicating a decreased accessibility of the "lower" part of the thioredoxin domain. Although most of the differences between oxidized and reduced DsbA concerned the disappearance of proteolytic sites, two new hydrolysis sites were identified in the reduced protein at Arg148, as mentioned previously, and at Glu94 where a cleavage by Glu-C was observed.

Topographical studies of the DsbA-PID complex

The experimental approach described previously was finally used to investigate the surface topography of the covalent

Table 2. *Preferential cleavage sites detected on the different forms of DsbA*

Enzyme	Oxidized	Reduced	Complexed
Trypsin	**R103 K132 K158 K183** K7 K48 K58 K70	**K7 R103 K132 K158 K183** R148	**R103 K132 K158 K183** **R148**
Asp-N	**D5 D172 D180** D123 D144 D167 D110	**D5 D172 D180** D123 D144 D167 D110	**D5 D172 D180** D123 D144 D167 D110
Glu-C	**E24 E38 E52 E121 E139**	E24 E38 E52 E121 E139 E94	E24 E38 E52 E121 E139 E94
Chymotrypsin	**Y9 F154 Y159 F174 Y184** F26 Y34 Y59 W76 F112 F129	**Y9** F154 Y159 F174 Y184	**Y9 F174 Y184** F112[a] F129[a] F154 Y159
Subtilisin	**Y9 L12 F26 T57 N62 A125 F129** T168 Y178	**Y9 L12 A125** T57 N62 F129 T168 Y178	**Y9 L12 A125** T57 N62 F129 T168[a] Y178[a]

Sites were listed as "major" (bold style) when they appear at early times of incubation, as "minor" (normal style) when they appear at longer hydrolysis times.
[a] These sites occur at very late time of incubation.

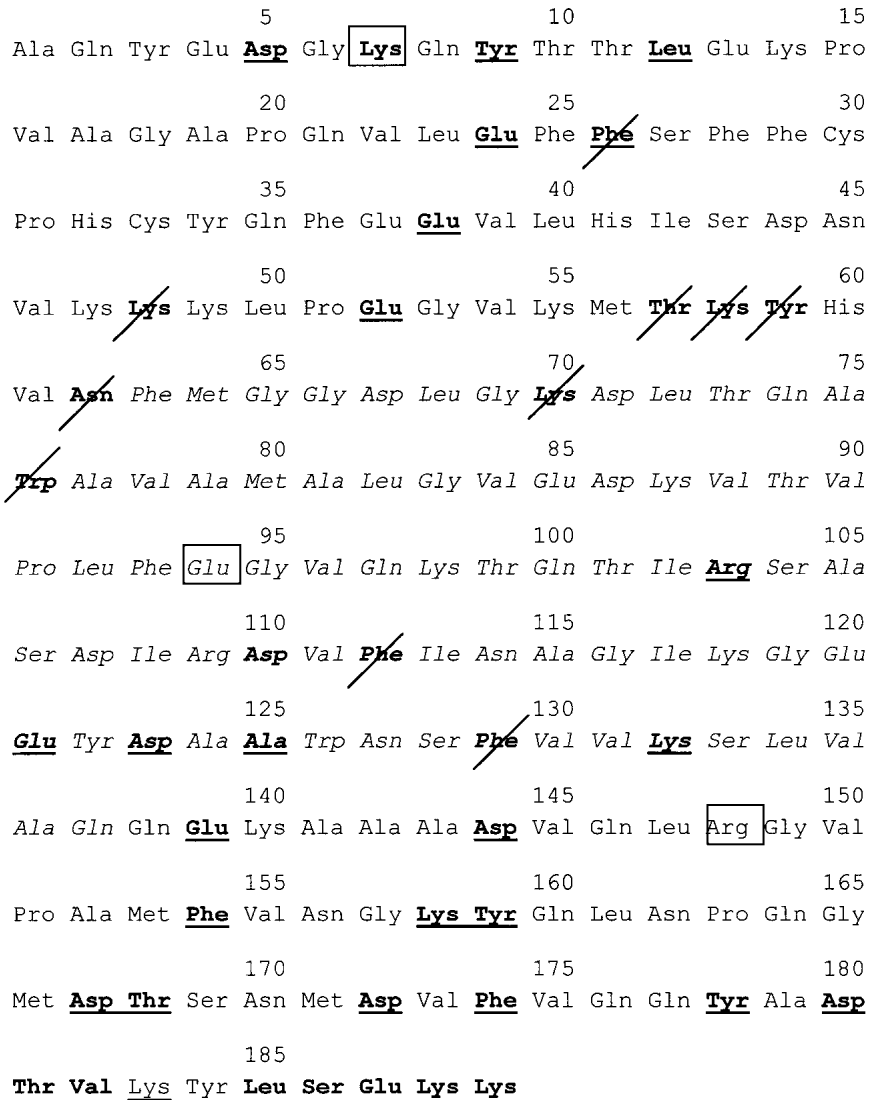

Fig. 2. Amino acid sequence of DsbA. B-domain is in italic. Proteolytic sites in oxidized DsbA are in bold; major sites are underlined. The sites that appear on reduction are surrounded by a square; those that disappear are crossed out.

complex between DsbA and the peptide PID (Fig. 6) as a mimic of the DsbA-substrate disulfide intermediate (Couprie et al. 2000).

Figure 4C shows the HPLC profile of the aliquot withdrawn after 60 min of tryptic hydrolysis of the DsbA-PID complex. The comparison of this profile with the chromatograms obtained for oxidized and reduced DsbA (Fig. 4A, B) indicated that the complex was resistant to proteases with a proteolytic behavior more resembling the reduced form of DsbA than the oxidized species. Analysis of the peptidic fragments indicated again the occurrence of major cleavages at residues 103, 132, 158, and 183. Moreover, as for reduced DsbA, all the peptides generated by minor cleavages occurring within the hydrophobic core of the protein were missing. Nevertheless, several features distinguished the conformation of the complex from the reduced protein: Lys7 was not recognized by proteases anymore and an increased rate of appearance of peptides resulting from cleavage after Arg148 (peptide 149–189—peak 9—and its subdigested fragment 149–183) was detected. Further information brought by this set of experiments concerned the sensitivity to proteolysis of the peptide PID itself within the protein complex. After 1-hr incubation with trypsin, most of the peptide lost the C-terminal residue, the major proteic species being henceforth the complex between full DsbA and PID 1′-13′.

The overall results of the limited proteolysis investigation of the DsbA-PID complex are reported in Table 2 and Figure 5. When these data are compared with those obtained on the oxidized and reduced forms of the protein, a number of

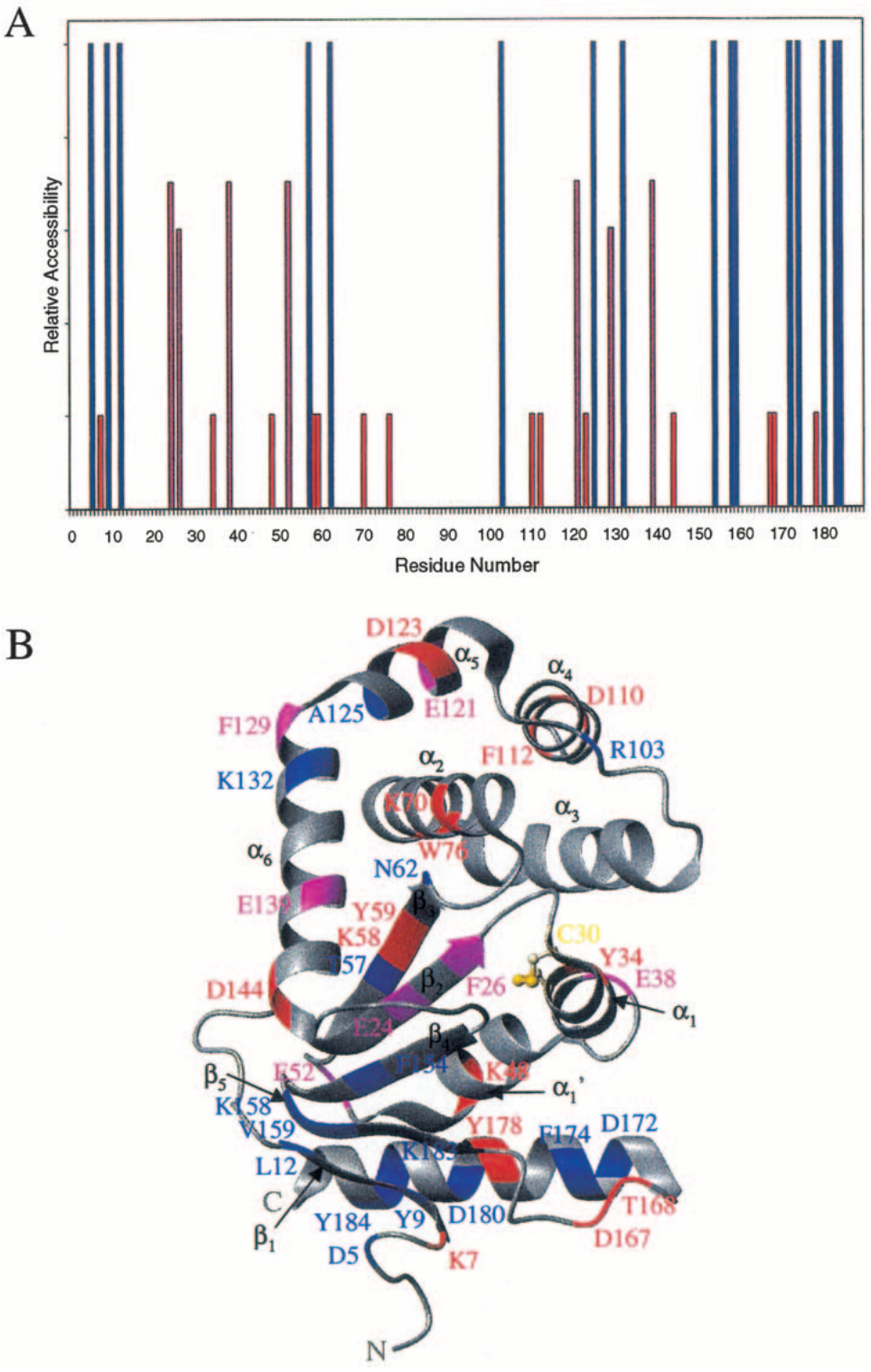

Fig. 3. (*A*). Relative accessibility to proteases of oxidized DsbA on the basis of the qualitative analysis of proteolytic fragments generated during the time-course experiments. (*B*) Proteolytic cleavage sites represented on the three-dimensional structure of oxidized DsbA (Guddat et al. 1997), pdb code number: 1FVK. Major cleavage sites are indicated in blue; minor cleavage sites appear in red. Residues F26 and F129 that show different rates of appearance depending on their cleavage by chymotrypsin or subtilisin are in magenta, as well as cleavage sites caused by Glu-C that require a very high amount of protease. The active site disulfide is colored in yellow.

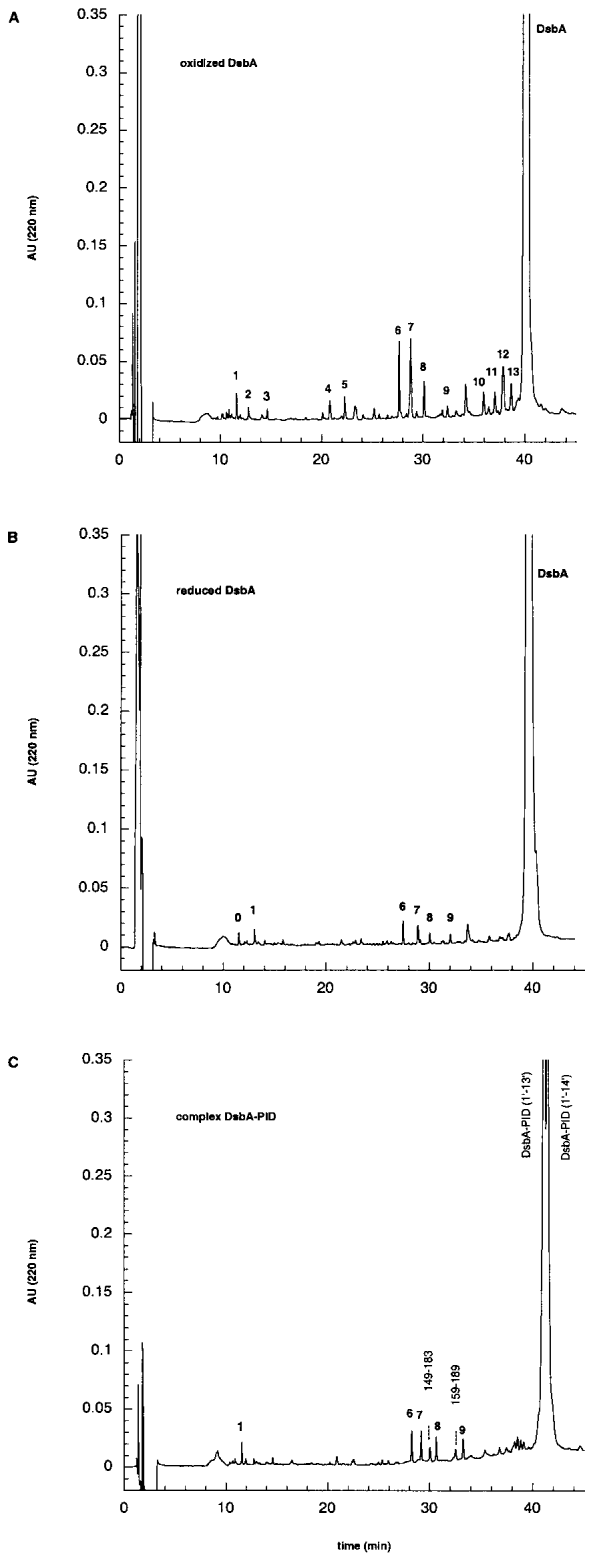

Fig. 4. Comparative HPLC profiles of the tryptic digests of (*A*) oxidized DsbA, (*B*) reduced DsbA, and (*C*) complex DsbA-PID after 60 min incubation using an enzyme/substrate ratio of 1 : 70. Individual fractions were collected and analyzed by ESIMS.

considerations can be drawn. For all the proteases used, the HPLC profiles obtained for the proteolyzed complex are similar to those obtained for the reduced enzyme (data not shown). As for the reduced species, the minor proteolytic sites present in oxidized DsbA and mainly located in strands β2, β3, and helix α2 disappeared in the complex. The kinetics of appearance of most of the proteolytic fragments analyzed, as judged from the HPLC profiles, were intermediate between those observed for the oxidized and reduced form. In the case of Glu-C, as for reduced DsbA, it was necessary to increase the enzyme–substrate ratio up to 1/10 to obtain exploitable data. In addition to the differences already underlined for the tryptic experiments, other differences concerning residues Glu94, Thr168, Tyr178, and Phe129 could be inferred from the data of Table 2. Hydrolysis at the level of Glu94 by Glu-C (peptide 95–121) occurred very early in the time-course experiments, indicating that this residue is more sensitive to proteolysis than in the reduced protein. Contrary to the overall kinetic trend of the complex that seemed intermediate between those of the redox forms, hydrolysis at the level of Thr168 and Tyr178 by subtilisin was very slow. Finally, hydrolysis at the level of Phe129 that had completely disappeared in reduced DsbA occurred in the complex, although at very late stages of the experiments.

Analysis of the data obtained for the PID peptide within the complex showed a very high sensitivity of the peptide bond Arg13′-Phe14′ that was easily cleaved by trypsin and in a very nonspecific manner by both chymotrypsin and Asp-N. Control experiments performed on the isolated peptide with these latter proteases did not show any cleavage at this specific position. As expected, Asp-N cleaved the isolated peptide at Asp9′, whereas chymotrypsin cleaved PID at Phe5′ and Phe10′. The comparison of the proteolytic patterns obtained for isolated and complexed PID peptide indicated that Asp9′ and Phe10′ were protected against proteolysis in the complex. No cleavages at these sites were in fact observed when PID was complexed to DsbA, whereas hydrolysis at Phe5′ remained unchanged.

Discussion

The factors determining the catalytic properties of DsbA are still not clearly identified. The determination of the three-dimensional structures of both redox states of DsbA has not shed all the expected light on the catalytic mechanism of the enzyme; indeed, both structures are rather similar. This reinforces the hypothesis that the highly oxidizing properties of DsbA result from hydrogen bonds and electrostatic interactions that favor the thiolate over the disulfide at the active site (Guddat et al. 1998). A recent study performed on Grx3, another member of the thioredoxin oxido-reductase family, has also indicated a major role of direct hydrogen bonding in the stabilization of the thiolate in the whole

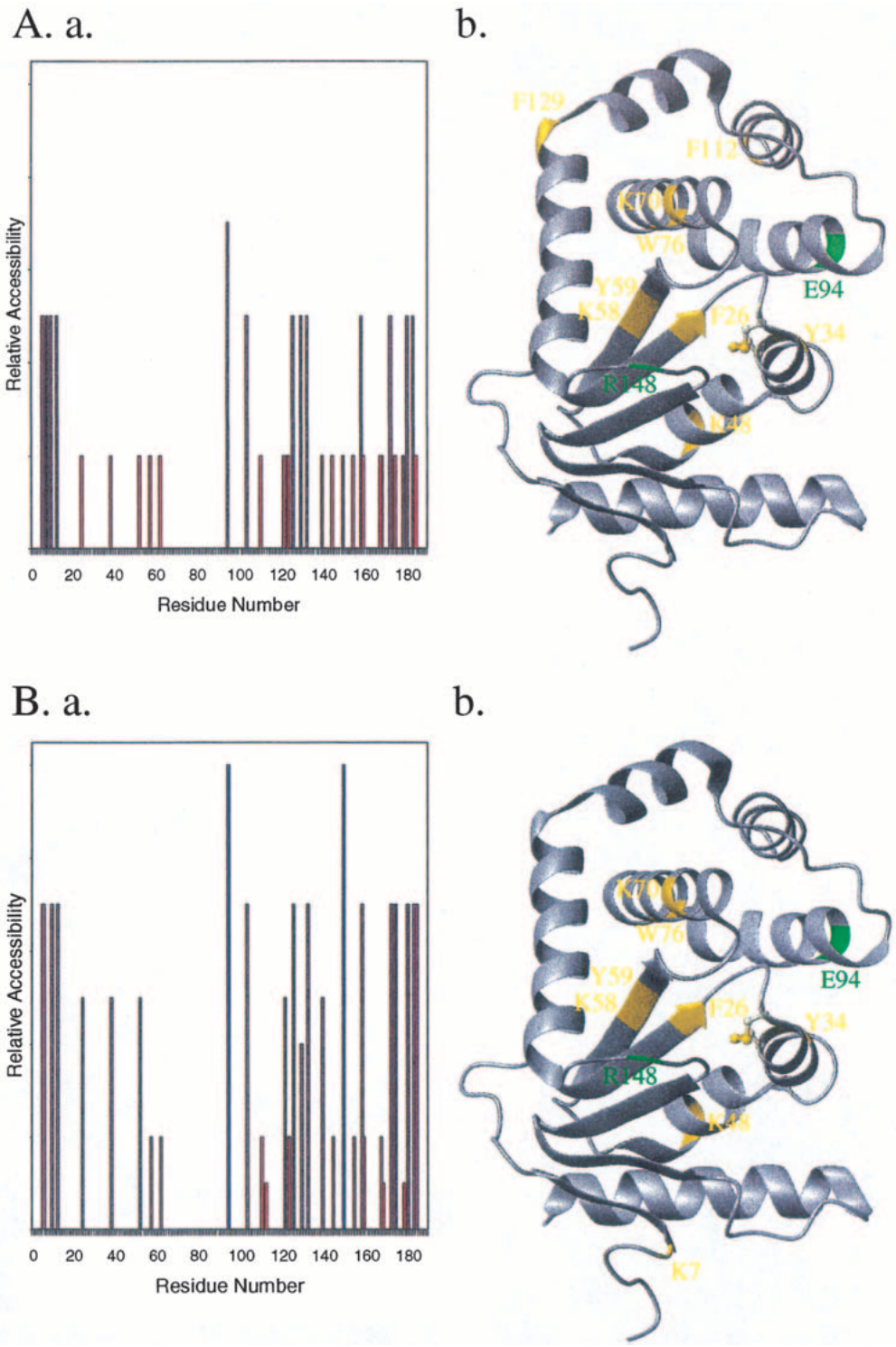

Fig. 5. Relative accessibility to proteases of reduced DsbA (*A.a*) and complex DsbA-PID (*B.a*) on the basis of the qualitative analysis of proteolytic fragments generated during the time-course experiments. The color code is the same as Figure 3. Proteolysis sites modified on reduction (*A.b*) or complexation with peptide PID (*B.b*). Sites that disappear are in yellow and new sites appear in green.

family (Foloppe et al. 2001). Despite these results, our understanding of the catalytic cycle of DsbA is still far from complete. Several questions remain unanswered, especially the involvement of domain motions throughout the cycle and structural data about the interaction of DsbA with its different partners (either unfolded polypeptide substrates or

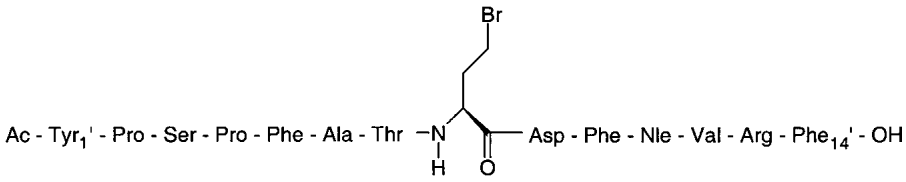

Fig. 6. Sequence of peptide Br-PID. The bromo-homoalanine reacts with the cysteine 30 of the active site to form a thioether bond.

DsbB). Our present work aims at bringing some pieces of information on these points. Three states of DsbA representative of most of the catalytic cycle have been investigated by a combination of limited proteolysis experiments and identification of the resulting peptidic fragments by mass spectrometry. Proteolysis of a protein substrate only occurs if the peptidic chain can bind and adapt to the specific stereochemistry of the active site of the protease. It means that in folded proteins, the amino acids likely to be hydrolyzed must be located within exposed and/or flexible parts of the structure. Of course, the data brought by this technique do not have the resolution of x-ray or NMR data; they just provide a kind of imprint of the molecule studied. However, when the three-dimensional structure is available, as it is the case for DsbA, these experiments give information on the dynamics within the protein that is likely to expose some proteolytic sites. These movements take place on a time-scale that can be qualified as an "enzymatic" time-scale, that is, compatible with the time required for protein–protein interactions. The comparison of the proteolytic sites between the different states of the protein shows the evolution of these movements within the protein and hence provides data on the conformational changes that occur throughout the catalytic cycle.

The most immediate observation for DsbA concerns the high stability of the protein. In agreement with thermodynamic measurements (Moutiez et al. 1999; Couprie et al. 2000), whatever its state, DsbA proved to be highly resistant to proteolysis. The protease to substrate ratios effective in causing the appearance of preferred predominant cleavage sites were particularly high. The kinetics of cleavage of the different forms of the enzyme globally follow the same tendency as ΔG values (Couprie et al. 2000), namely the reduced form is much more stable against proteolysis than the oxidized protein, whereas the complex shows an intermediate stability.

In the conditions in which proteolysis occurs, the analysis of the preferred cleavage sites in the oxidized state shows that, although these sites are spread on most of the protein (with the exceptions previously mentioned), they are especially concentrated in the "lower" part of the thioredoxin domain (as defined in Results). They occur not only in loops but more surprisingly also in defined secondary structure elements such as helix α7 and β-strands (β1, β4, and β5). After prolonged incubation time, some cleavages also appear in the "upper" part of the β-sheet (β2 and β3), a region poorly accessible to the solvent according to three-dimensional structures. In the β-sheet, cleavage sites are found particularly at the extremities of β-strands. The exact position of the secondary structural elements is usually somehow uncertain, and in the case of DsbA these elements have been noticed to differ sometimes by one or two residues between NMR and x-ray studies (Couprie et al. 1998). However, the finding of recurring cleavages in these regions may be more important than being merely a question of delimitation of secondary structure elements. The most likely interpretation of our data is that the whole β-sheet region is mobile enough to enable interaction and reaction with the active site of proteases. Nevertheless, the fact that some of the observed cleavages could result from a modification of the topographical properties of the protein after a first proteolytic event cannot, of course, be ruled out. It is interesting to notice that the "lower" part of the thioredoxin domain contains most of the putative substrate-binding site (Guddat et al. 1997). The physiological role of DsbA in its oxidized state is to introduce disulfide bonds into secreted or exported proteins. The great mobility of this region in the oxidized form could facilitate the processes of recognition and binding of its large panel of proteic substrates that show various size, chemical properties, and perhaps degrees of folding.

When considering the reduced form, the kinetics of cleavage were found more or less reduced at nearly all positions. The minor cleavage sites located in β-strands β2 and β3 even completely disappear. The related loss of mobility can reasonably be attributed to a tighter packing of the β-sheet, especially in the "upper" part. We can assume that β2 and β3 probably have a more ordered structure resulting in stronger contacts between the strands. These observations corroborate well with the results obtained by NMR from the comparison of oxidized and reduced ^{1}H, ^{15}N, and ^{13}C chemical shifts. This region is one of the four that undergoes large chemical shift variations (Couprie et al. 1998). Previous work of Frech et al. (1996) indicated that the folding of the substrate would decrease its affinity for DsbA and facilitate its dissociation from the enzyme. Our results offer new insights on the catalytic mechanism. The rigidity of the thioredoxin domain increases from the oxidized form to the reduced one, with an intermediate situation found in the complex. Although the great mobility of the oxidized form

in this region probably enables accommodation of various substrates, the increasing rigidity from the complex to the reduced form could help the release of oxidized substrates. Moreover, the rigidity of the thioredoxin domain in the reduced state could be a major determinant for the specific recognition by DsbB, the DsbA oxidase, that presumably interact at least partially at the same binding site. The alternative null hypothesis cannot, however, be excluded. In that case, the flexibility would play no role in the intermolecular interactions of DsbA but would be strictly linked to the lower stability of the oxidized form in which some intramolecular interactions are lost or weakened to accommodate the active site disulfide bond.

The rigidifying of the thioredoxin domain after reduction is not an isolated phenomenon; the whole DsbA protein is more or less affected. In the helical domain, we also observe that helices α2, α4, and α6 probably have tighter interactions. The proteolytic sites of these regions either disappear or appear at longer incubation times. In particular, in the reduced enzyme no more cleavage occurs at Phe129, the residue that makes the junction between helices α6 and α7. It is noteworthy that this residue had already been pointed out by NMR experiments as undergoing significant chemical shift variation on reduction (Couprie et al. 1998). The loss of mobility that occurs on both domains of the protein may lead to a larger exposure to proteolysis of the last turns of helix α3 with the appearance of a cleavage site at Glu94 in both reduced and complexed forms.

At the junction between B-domain and thioredoxin domain, the turn formed by residues 63 to 66 constitutes a hinge around which domain motions occur. All structural studies agree today on the fact that domain movements do happen in DsbA (Guddat et al. 1997; Guddat et al. 1998; Schirra et al. 1998). The conflicting point is what causes these movements and when. The resolution of the reduced structure by NMR has indicated that these motions were related to the redox state (Schirra et al. 1998); however, this hypothesis was based on the comparison of their structure with the oxidized structure obtained from x-ray experiments in different experimental conditions (Martin et al. 1993; Guddat et al. 1997). The crystallographic data proposed from the comparison of a panel of structures obtained for both reduced and oxidized DsbA that domain motion is a consequence of substrate binding (Guddat et al. 1998). Our data are clearly insufficient to bring a definite answer to this question; nevertheless, several observations can be made. First, the hinge region is surrounded by helix α2 (B-domain) and strand β3. Despite the apparent poor accessibility to the solvent of these elements, proteolysis does occur in these regions in the oxidized form. This area becomes clearly less sensitive to proteases in the other states with the cleavage sites disappearing in both the complex and the reduced forms. The second observation is the increased accessibility to proteolysis of Glu94, a residue of helix α3 (B-domain), close in space to the thioredoxin domain, once more in both the complexed and reduced forms. We can hypothesize that domain movements are an integral part of the overall mobility of the oxidized form and also contribute to the process of interaction with substrates. This comes close to the conclusion drawn from the x-ray studies and is supported by the large variation of domain orientations in the different oxidized structures (Guddat et al. 1998). In both the complexed and reduced states, the loss of mobility is sufficient to explain the absence of hydrolysis in this part of the molecule and no hypothesis can be drawn about the occurrence of domain movements or not.

Preliminary analyses performed on the DsbA-PID complex by NMR have indicated that the peptide was in an extended conformation and that preferential noncovalent interactions do exist between both partners. However, calorimetric experiments have shown that these interactions were relatively weak (Couprie et al. 2000). Our present data reinforce this conclusion: No zone of DsbA is significantly protected by its interaction with PID. The putative binding site is bounded by the active site helix α1/α1′ and the loop connecting strand β5 and helix α7. A hydrophobic pocket is located inside this hydrophobic groove and contains residues of α1/α1′, β4, β5, and α7 (Guddat et al. 1997). The disappearance/lower rate of hydrolysis at residues Lys7 and Thr168 (loop connecting β5 and α7) is in favor of the actual presence of the peptide in this area. The other favorable argument is relative to the behavior of Arg148. Schirra et al (1998) have pointed out that the side chain of Arg148 was protruding over the entrance of the hydrophobic pocket in the reduced form and could shield the entrance of substrates into the groove in this redox state. The reduction of DsbA makes possible the hydrolysis at the level of Arg148 and, more interestingly, the cleavage at Arg148 is even easier in the complex. We can assume that the presence of the peptide in the groove can completely push the side chain of Arg148 toward the solvent.

As for the peptide itself, the great sensitivity of the bond Arg13′-Phe14′ to hydrolysis, regardless of the protease used, indicates that its size exceeds the size of the binding site of the protein. The interaction with DsbA protects residues 9′ and 10′ against proteolysis. Because Phe5′ and Phe10′ are at the same distance in regard to the homoserine involved in the covalent linkage with DsbA, the occurrence of a proteolytic event only at Phe5′ could be explained by a preferential orientation of the PID peptide in the binding site. However, this hypothesis requires more precise data to be confirmed; data that could not be obtained by the method used in this study. The resolution of the structure of the complex by NMR is currently in progress and will give a complete picture of both the peptide-binding site and the set of interactions between PID and the enzyme.

Materials and methods

Reduced dithiothreitol, trypsin, and subtilisin were obtained from Sigma Chemical Company. Chymotrypsin, endoproteinase Asp-N, and endoproteinase Glu-C were purchased from Boehringer Mannheim GmbH. Bio Gel P2 fine 45–90 μm (wet) was from Bio-Rad Laboratories. Solvents were HPLC grade from SDS. The concentration of solution of oxidized, reduced, and complexed DsbA was determined using an absorption of 1.1 at 280 nm for 1 mg/mL solution (Wunderlich and Glockshuber 1993).

Formation of the DsbA–DsbB peptide complex

DsbA was purified as previously described (Wunderlich and Glockshuber 1993). DsbA-PID peptide complex was obtained as described (Couprie et al. 2000).

Protein reduction

Protein reduction was performed in 50 mM sodium phosphate buffer pH 7.5 using a 50 molar excess of DTT over the number of cysteine residues for 60 min at 37°C under argon atmosphere. DTT was removed by fast desalting on a Bio Gel P2 fine 45–90 μm column (Bio-Rad Laboratories) using an isocratic gradient with degassed 50 mM sodium phosphate buffer pH 7.5. Reduced protein was immediately used for limited proteolysis experiments performed under argon atmosphere. To show that the DsbA sample is reduced throughout the limited proteolysis experiments, the reduced protein was incubated in the conditions used for hydrolysis experiments for 1 hr and 3 hr at 37°C under argon atmosphere. It was then carboxyamidomethylated using a 10 molar excess of iodoacetamide (IAM) over the number of cysteine residues. The alkylation reaction was performed for 20 min at room temperature in the dark to minimize photolytic production of iodine that is a very potent oxidizing agent for thiols. Samples were desalted by reversed-phase chromatography by use of a μRPC C_2/C_{18} SC 2.1/10 column (Pharmacia) on a SMART System (Pharmacia). The elution system consisted of 0.1% TFA (solvent A) and 0.07% TFA in 95% acetonitrile (solvent B) from 15% to 95% B at a flow rate of 200 μL/min. Eluted compounds were monitored at 220 nm and 280 nm and analyzed by ESIMS.

Proteolysis experiments

Limited proteolysis experiments were conducted by treating the isolated protein in the reduced, oxidized, or complexed form separately with an appropriate ratio of protease (trypsin, chymotrypsin, endoproteinase Glu-C, endoproteinase Asp-N, or subtilisin). Enzymatic digestions were all performed in 50 mM sodium phosphate buffer pH 7.5 at 37°C with a final protein concentration of 10 μM. The proteolysis experiments performed on the reduced DsbA were performed under argon atmosphere. For the limited proteolysis experiments performed with trypsin, an enzyme/substrate ratio of 1/70 was used; for those performed with chymotrypsin and Glu-C an enzyme/substrate ratio of 1 : 100 and 1 : 10 (or 1 : 20) were used, respectively. The hydrolysis with subtilisin and Asp-N were all performed with an enzyme/substrate ratio of 1 : 300 and 1 : 90, respectively. The extent of digestion was monitored on a time-course basis by sampling the reaction mixture at different reaction times ranging from 15 min to 3 hr. Proteolytically digested protein samples were analyzed and fractionated by reversed-phase chromatography with a μRPC C_2/C_{18} S.C 2.1/10 column (Pharmacia) on a SMART System. Peptides were eluted by means of a linear gradient of 5% to 60% acetonitrile in TFA (0.1%) over 45 min; elution was monitored at 220 and 280 nm. Individual fractions were collected and identified by mass spectrometry (matrix assisted laser desorption ionization [MALDI] or ESIMS).

Mass spectrometry analyses

Matrix assisted laser desorption ionization mass spectrometry analyses (MALDIMS)

Peptides were analyzed with a reflectron MALDI-TOF mass spectrometer, Voyager DE (Perseptive Biosystem). The mass range was calibrated with bovine insulin (average molecular mass 5734.6 Da) and a matrix peak (379.1 Da) as internal standards. One μL of the peptide fractions collected after chromatographic separation was applied to a sample slide and allowed to air dry before applying 1 μL of α-cyano-4-hydroxycinnamic acid (10 mg/mL) in ethanol/acetonitrile/0.1% TFA 1 : 1 : 1 (v:v:v). The matrix was allowed to air dry before collecting spectra. Mass spectra were generated from the sum of 50 laser shots and were performed by use of an accelerating voltage of 20 KV and a delay time of 100 ns. Raw data were analyzed by using Grams/386 software provided by the manufacturer.

Electrospray mass analyses

ESIMS and MS/MS analyses were performed by use of a QUATTRO II triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass). Protein and peptide samples derived from the chromatographic separation were injected directly into the ion source by Harward Apparatus with a flow rate of 6 μL/min. Data were acquired at 10 sec/scan and elaborated by MassLynx software provided by the manufacturer. Mass scale calibration was performed by means of multiply charged ions from a separate injection of horse heat myoglobin (average molecular mass 16951.5).

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