pH-dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol:disulphide oxidoreductase) and protein disulphide-isomerase: studies with a novel simple peptide substrate

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A decapeptide containing two cysteine residues at positions 3 and 8 has been designed for use in monitoring the disulphide bond-forming activity of thiol:disulphide oxidoreductases. The peptide contains a tryptophan residue adjacent to one of the cysteine residues and an arginine residue adjacent to the other. Oxidation of this dithiol peptide to the disulphide state is accompanied by a significant change in tryptophan fluorescence emission intensity. This fluorescence quenching was used as the basis for monitoring the disulphide bond-forming activity of the enzymes protein disulphide-isomerase (PDI) and DsbA (a periplasmic protein thiol:disulphide oxidoreductase) in the pH range 4.0–7.5, where the rates of spontaneous or chemical oxidation are low. Reaction rates were found to be directly proportional to enzyme concentration, and more detailed analysis indicated that the rate-determining step in the overall process was the re-oxidation of the reduced form of the enzyme by GSSG. The pH-dependence of the enzyme-catalysed reaction reflected primarily the pK_a of the reactive cysteine residue at the active site of each enzyme. The data indicate a pK_{app} of 5.6 for bovine PDI and of 5.1 for *Vibrio cholerae* DsbA.

INTRODUCTION

Protein disulphide-isomerase (PDI) and DsbA (periplasmic protein thiol:disulphide oxidoreductase) are eukaryotic and prokaryotic enzymes found in the lumen of the endoplasmic reticulum and in the bacterial periplasm respectively. Each of these enzymes has been implicated in the formation of native disulphide bonds at protein biosynthesis, based on a range of findings both *in vivo* and *in vitro* [1–8].

PDI was originally defined on the basis of its ability to catalyse the oxidative refolding of reduced unfolded proteins [9]; it acts by catalysing thiol:disulphide exchange reactions which can lead to net formation, reduction or isomerization of protein disulphide bonds. DsbA was originally detected genetically and *dsbA*$^-$ mutants were found to exhibit a defect in the formation of disulphide bonds in newly synthesized periplasmic proteins [8,10–12]. PDI is found in all eukaryotic systems studied and has been characterized in most detail in mammals. DsbA has been found in many Gram-negative bacteria, including *Escherichia coli* and *Vibrio cholerae* [8,10–12].

There is considerable interest, both mechanistic and biological, in defining the activities of these enzymes in more detail. Their sequences are known and there is a growing body of chemical information about their active sites, and about the chemical properties of the thiol groups of the Cys-Xaa-Yaa-Cys residues which represent their consensus active site sequences (for a review see [4]).

A wide range of assays has been used to characterize the catalytic properties of these enzymes. Assays based on catalysis of reduction of insulin by GSH [13,14] are simple but the overall reaction is the net reduction of protein disulphides, contrary to the physiological role of these enzymes. Assays based on the re-oxidation of reduced bovine pancreatic trypsin inhibitor (BPTI) and the interconversion of specific intermediates in the BPTI refolding pathway [15,16], or on the rearrangement of disulphide bonds in 'scrambled' ribonuclease [17], are more physiologically meaningful. These assays have provided information on the activity of the enzymes in complex reactions involving both thiol:disulphide chemistry (protein disulphide formation and isomerization) and protein folding but preparation of the substrates is complicated. At the other extreme, DsbA has also been used to catalyse the simple redox reaction between GSSG and the non-protein dithiol, dithiothreitol; this assay requires extremely constraining low-pH conditions [18].

There are considerable benefits in being able to isolate the catalysis of thiol:disulphide redox reactions from effects connected with protein folding. The ideal substrate would be a peptide with two cysteine residues which can be interconverted between the dithiol and disulphide states and where the formation of the disulphide bond is neither constrained nor favoured by the formation of other interactions (folding). Creighton and co-workers [19,20] introduced such a peptide and analysed the kinetics of its oxidation by GSSG, in both the absence and presence of PDI and DsbA. Unfortunately, this assay requires time-consuming liquid chromatographic techniques to resolve the various forms of substrate peptides (as examples see [19,20]). An analogous system allowing direct observation of peptide oxidation would be advantageous.

Previous work on the oxidation of the heat-stable enterotoxin produced by some enterotoxinogenic strains of *E. coli* showed that the oxidation of certain disulphide bonds could be monitored by the quenching of the intrinsic fluorescence of the substrate (S. Eaglestone, L. W. Ruddock, R. B. Freedman and T. R. Hirst, unpublished work). This idea was adapted here to produce a peptide substrate which would allow for the rapid determination of disulphide bond-forming activity.

The criteria for the peptide design were that it should contain two cysteine residues separated by a flexible linker region, and

Abbreviations used: DsbA, periplasmic protein thiol:disulphide oxidoreductase from *Vibrio cholerae* or *Escherichia coli*; PDI, protein disulphide-isomerase; BPTI, bovine pancreatic trypsin inhibitor; TFA, trifluoroacetic acid.

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should have a fluorescent group on one side of one cysteine residue and a protonatable group on the other side of the second cysteine residue. The linker should be long enough to permit formation of an unstrained disulphide bond and should have no marked propensity to form secondary structure. The peptide would also need to be small (for ease of synthesis) and water soluble. Upon oxidation the fluorescent group and the protonatable group would be brought closer together and quenching of the fluorophore would occur. In order to eliminate any pH-dependence of reaction rates from effects on the peptide, tryptophan was chosen as the fluorophore, arginine as the charged quencher and no other residues with charged side chains were introduced into the peptide. The peptide synthesized was NRCSQGSCWN.

We now report the synthesis and properties of this decapeptide and its use as a substrate for studies of the oxidative activity of PDI and DsbA.

EXPERIMENTAL

Peptide synthesis

The substrate peptide NRCSQGSCWN was prepared using a Shimadzu PSSM8 automated peptide synthesizer with Fmoc chemistry, using reagents purchased from Nova Biochem. The peptide was prepared on pre-loaded Fmoc-Asn-TGA resin and was cleaved from the resin using standard Fmoc protocols. The peptide was extracted with ether, resuspended in 5 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) and purified by reverse-phase chromatography (Pharmacia FPLC). Samples were applied to a 3 ml Resource RPC column (Pharmacia) pre-equilibrated with 5 % acetonitrile in 0.1 % TFA and eluted with a linear gradient of 5–95 % acetonitrile in 0.1 % TFA with a flow rate of 1 ml/min. Species identification was confirmed by mass spectrometry using a matrix-assisted laser desorption time-of-flight mass spectrometer (VG Instruments). The purified peptide (M_r 1154) eluted in a single peak and was stored at −20 °C in the elution buffer (approx. 30 % acetonitrile in 0.1 % TFA). The peptide concentration was determined spectrophotometrically using an absorption coefficient of 5600 $M^{-1} \cdot cm^{-1}$ at 278 nm. The stock peptide solution was 1.05 mM.

Protein purification

V. cholerae DsbA was prepared by the method of Lowe et al. [21]. The concentration of DsbA was determined spectrophotometrically using a calculated absorption coefficient of 11900 $M^{-1} \cdot cm^{-1}$ at 278 nm. Bovine PDI was prepared by the method of Freedman et al. [17]. The concentration of PDI was determined spectrophotometrically using a calculated absorption coefficient of 43000 $M^{-1} \cdot cm^{-1}$ at 278 nm.

Spectroscopic determination of the oxidation of the substrate peptide

McIlvaine buffer (0.2 M disodium hydrogen phosphate/0.1 M citric acid; pH 3.0–7.5) was placed in a fluorescence cuvette; to this was added as appropriate, GSH (stock solution 60.1 mg/ml, in McIlvaine buffer), GSSG (stock solution 30.7 mg/ml, in McIlvaine buffer), DsbA (stock solution 0.82 mg/ml, in 10 mM Tris/50 mM NaCl) and PDI (stock solution 2.11 mg.ml, in PBS). The final assay volume was 1.05 ml. After mixing, the cuvette was placed in a Perkin-Elmer LS50 spectrophotometer for 3 min to allow thermal equilibration of the solution to 25 °C. Substrate peptide (1.05 mM, in 30 % acetonitrile/0.1 % TFA) was added (usually 5 μl to give a substrate concentration of 5.0 μM, but see Figure 5), mixed and the change in fluorescence intensity (excitation 280 nm, emission 350 nm, slits 5/5 nm) was monitored over an appropriate time (5 min to 1 h) and 600 data points were collected.

With increasing concentrations of GSSG at a fixed redox potential, a decrease in the fluorescence intensity of the reduced and oxidized substrate was observed. This decrease was consistent with the increased absorbance at 280 nm of the solution with increasing GSSG concentration.

Analysis of the rate of oxidation

Fluorescence data were transferred to Igor v1.21 (Wavemetrics Ltd., Lake Oswego, OR, U.S.A.) running on a Power Macintosh 7100. Owing to the complicated nature of the potential reaction scheme, the data were analysed as half-times of the reaction. To eliminate discrepancies due to noise in the signal, the half-time was calculated by the following procedure. The initial fluorescence intensity was calculated from a linear fit of the first 30 time points extrapolated to zero time. The final fluorescence intensity was calculated from an average of the last 30 time points. The mid-point fluorescence was calculated as the mean of the initial and final fluorescence intensities. The half-time was calculated from a linear fit of the 30 data points around the mid-point fluorescence. A measure of the rate of the reaction was determined from the inverse of the half-time of the reaction.

RESULTS

Fluorescence properties of the peptide

The peptide, as designed, was relatively easy to synthesize and showed fluorescence properties characteristic of an exposed tryptophan group, with an emission maximum at 357 nm. Upon oxidation, a 19 % decrease in the fluorescence intensity of the substrate was observed (Figure 1), with no significant change in λ_{max}. This decrease was probably due to the expected quenching of the tryptophan residue as the arginine residue was brought into close proximity by disulphide bond formation.

The fluorescence intensity of the reduced peptide showed negligible pH-dependence over the range pH 4.5–6.5. At lower pH values a decrease in the fluorescence intensity was observed (14 % lower at pH 3.0), whereas at higher pH values an increase in the fluorescence intensity was observed (10 % higher at pH 7.5). This slight pH-dependence of the intrinsic fluorescence probably arose from the protonation equilibria of the N- and C-termini.

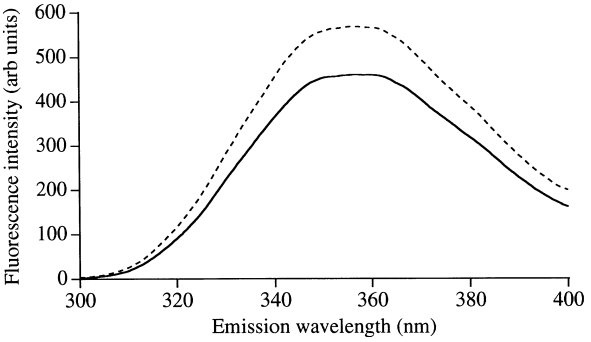

Figure 1 Typical emission spectra for oxidized (——) and reduced (– – – –) peptide NRCSQGSCWN (S) at pH 6.0 and [S] = 5 μM

Abbreviation: arb, arbitrary.

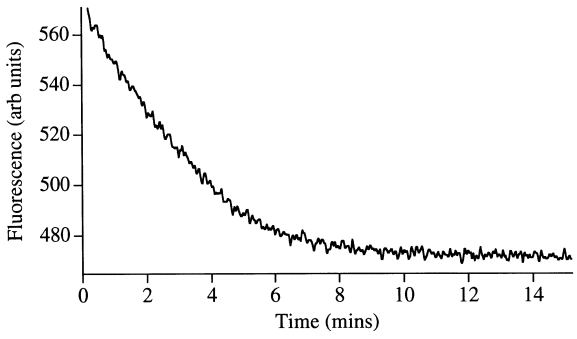

Figure 2 Typical time-dependent fluorescence profile during the oxidation of the peptide NRCSQGSCWN (S) catalysed by DsbA

pH 6.0, [S] = 5 μM, [DsbA] = 0.38 μM, [GSH] = 2 mM and [GSSG] = 0.5 mM. Abbreviation: arb, arbitrary.

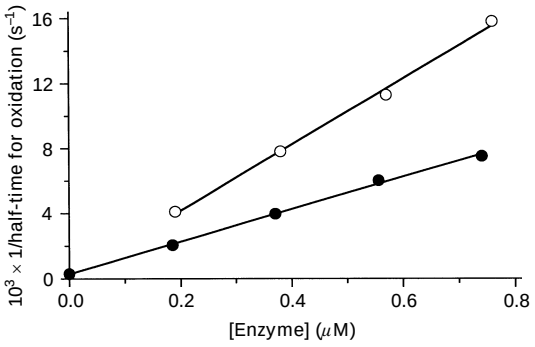

Figure 3 Linear dependence on the enzyme concentration of the inverse half-time of oxidation of the peptide NRCSQGSCWN catalysed by DsbA (○, pH 6.0) or PDI (●, pH 6.5)

$[S]_0$ = 5.0 μM, [GSH] = 2 mM and [GSSG] = 0.5 mM.

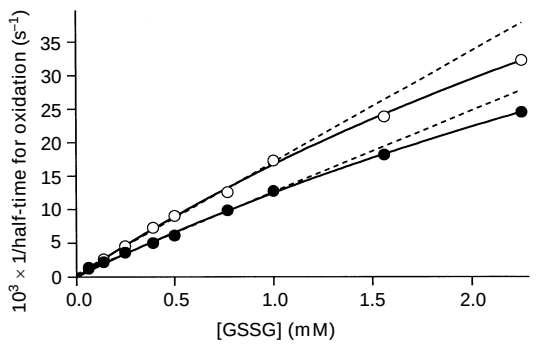

Figure 4 Variation of the inverse half-time of oxidation of the peptide NRCSQGSCWN with GSSG concentration catalysed by DsbA (○, [DsbA] = 0.38 μM, pH 6.0) or PDI (●, [PDI] = 0.56 μM, pH 6.5)

Lines of best fit to hyperbolic function (——, DsbA $R^2 = 0.999$, PDI $R^2 = 0.999$) and extrapolation of linear fit up to 1 mM GSSG (– – – –, DsbA $R^2 = 0.996$, PDI $R^2 = 0.997$) are shown. [S] = 5.0 μM, $[GSH]^2$:[GSSG] = 4 mM.

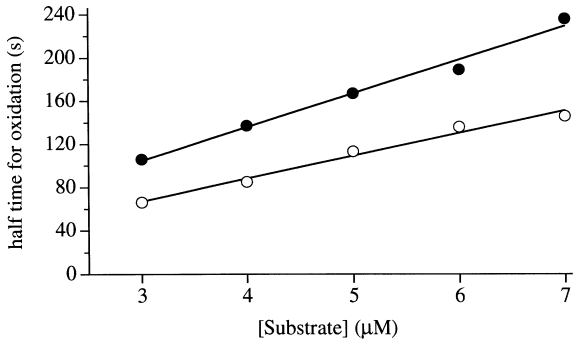

Figure 5 Linear dependence of the half-time of oxidation of the peptide NRCSQGSCWN catalysed by DsbA (○) or PDI (●) with substrate concentration

[GSH] = 2 mM and [GSSG] = 0.5 mM. ○, [DsbA] = 0.38 μM, and pH 6.0. ●, [PDI] = 0.56 μM, and pH 6.5.

Oxidation of the peptide

The change in fluorescence on oxidation of the dithiol form of the peptide was used to monitor the formation of the intramolecular disulphide bond. At pH values of 8.0 or greater, spontaneous oxidation of the substrate was observed, presumably due to air oxidation. Over the pH range 3.0–7.5 negligible oxidation of the peptide was observed in the absence of a glutathione buffer. At pH values of 6.0–7.5, in a glutathione buffer, chemical oxidation of the substrate was observed in the absence of enzyme catalysts, with the rate of oxidation increasing with pH. By contrast, over the pH range 4.0–5.5 negligible oxidation of the substrate was observed in the presence of a glutathione buffer alone, but an enzyme-catalysed rate was observed in the presence of bovine PDI or *V. cholerae* DsbA (see below).

Catalysis of peptide oxidation by DsbA and PDI

In the presence of catalytic amounts of DsbA or PDI and a glutathione buffer, measurable oxidation of the peptide was observed at neutral or moderately acidic pH. Oxidation was monitored throughout the time-course of the reaction (Figure 2). The kinetics showed a moderate fit to a first-order process ($R^2 \geqslant 0.98$) but with non-random residuals (see detailed analysis below). In order to analyse the kinetics as a function of enzyme and substrate concentrations, the half-time of reaction was determined from each time-course and the rate of reaction was expressed in terms of the inverse half-time. The rate of oxidation expressed in this manner was directly proportional to the enzyme concentration for both DsbA and PDI (Figure 3; $R^2 = 0.997$ for DsbA, $R^2 = 0.998$ for PDI).

In order to examine the relationship between reaction rate and [GSSG], it was necessary to eliminate the effects of redox equilibria; hence [GSSG] was varied while maintaining constant $[GSSG]/[GSH]^2$. At a fixed redox potential, the rate of oxidation of the substrate increased with increasing [GSSG] over the range 0.0625–2.25 mM; this increase fitted a hyperbolic function (Figure 4; $R^2 = 0.999$ for DsbA, $R^2 = 0.999$ for PDI).

The half-time of oxidation of the substrate was linearly dependent on the peptide concentration, implying that the rate of oxidation was independent of the peptide concentration (Figure 5; $R^2 = 0.981$ for DsbA, $R^2 = 0.987$ for PDI). Since the rate is independent of substrate concentration, we would expect the initial decrease in fluorescence observed to be linear. For the PDI-catalysed reactions the decrease in fluorescence was linear for approx. 90% of the oxidation reaction. The DsbA-catalysed

Table 1 Comparison of derived rate constants for the oxidation of the peptide NRCSQGSCWN catalysed by *V. cholerae* DsbA (pH 6.0) and bovine PDI (pH 6.5) with values for *E. coli* DsbA catalysis (pH 7.4) of a BPTI-derived peptide [22]

Derived rate constants	Hyperbolic fit for *V. cholerae* DsbA	Hyperbolic fit for bovine PDI	Literature values for *E. coli* DsbA
k_4	$238\ s^{-1}\cdot M^{-1}$	$115\ s^{-1}\cdot M^{-1}$	$41\ s^{-1}\cdot M^{-1}$
k_1k_3	$1.3\times10^7\ s^{-2}\cdot M^{-1}$	$1.3\times10^7\ s^{-2}\cdot M^{-1}$	$3.0\times10^7\ s^{-2}\cdot M^{-1}$
$k_1[\text{Pep(red)}]+k_3+k_2$	$41\ s^{-1}$	$69\ s^{-1}$	$148\ s^{-1}$

reactions deviated more from linearity. This difference could possibly be due to the time taken for the system to reach a steady state.

Analysis of mechanism and determination of rate parameters

The reaction scheme for oxidation of the substrate peptide is potentially very complicated, with the possible formation of a variety of mixed disulphide intermediates. This scheme can be simplified by eliminating the reduction of the oxidized peptide and the formation of mixed disulphides between glutathione and the peptide to give:

$$\text{Enz(ox)}+\text{Pep(red)}\underset{k_2}{\overset{k_1}{\rightleftharpoons}}\text{Enz–Pep}\xrightarrow{k_3}\text{Enz(red)}+\text{Pep(ox)}$$

$$\text{Enz(red)}+\text{GSSG}\xrightarrow{k_4}\text{Enz(ox)}+2\,\text{GSH}$$

Under steady-state conditions the rate of oxidation of the substrate peptide becomes:

$$\text{Oxidation rate} = \frac{k_1k_3k_4[\text{Enz}][\text{Pep(red)}][\text{GSSG}]}{k_1k_4[\text{Pep(red)}][\text{GSSG}]+k_1k_3[\text{Pep(red)}]+k_3k_4[\text{GSSG}]+k_2k_4[\text{GSSG}]}$$

Although the dependence of the rate of oxidation on [GSSG] does not saturate, a hyperbolic fit to the data obtained does allow for the approximate determination of the terms $k_1k_3[\text{Pep(red)}]$, $(k_1k_4[\text{Pep(red)}]+k_3k_4+k_2k_4)$ and $k_1k_3k_4[\text{Pep(red)}][\text{Enz}]$. The values determined for the rate constants by this method are given in Table 1 for PDI at pH 6.5 and for DsbA at pH 6.0. The values show a good correlation with those reported by Darby and Creighton [22], who used gel-filtration chromatography to follow the catalysis of disulphide bond formation in a model peptide by *E. coli* DsbA at pH 7.4.

pH-dependence of the catalysis of disulphide bond formation by DsbA and PDI

The rate of DsbA-catalysed oxidation increased with increasing pH over the pH range 4.0–7.5 to an apparent plateau in the pH range 6.5–7.5; no oxidation was seen below pH 4.0 (Figure 6, upper panel). The mid-point for the rate of oxidation lay at a pH value of 5.1. Since the observed rate comprises the sum of the enzyme-catalysed rate and the non-catalysed rate, which increased with pH, the enzyme-catalysed rate is bell-shaped, with a maximum at pH 6.5.

The rate of PDI-catalysed oxidation also increased with increasing pH (Figure 6, lower panel). The pH-dependence of the enzyme-catalysed rate (corrected for the non-catalysed rate)

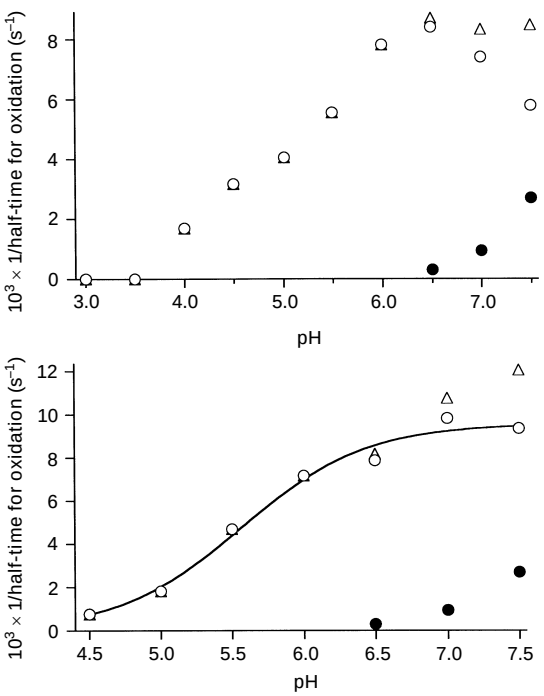

Figure 6 Variation of the inverse half-time of oxidation of the peptide NRCSQGSCWN with pH

[GSH] = 2 mM and [GSSG] = 0.5 mM. Upper panel: catalysed by DsbA (△), uncatalysed (●) and corrected (DsbA-uncatalysed) (○). [DsbA] = 0.38 μM. Lower panel: catalysed by PDI (△), uncatalysed (●) and corrected (PDI-uncatalysed) (○). [PDI] = 0.74 μM. The line of best fit is to a single pK_a-dependent event for the corrected values.

reached a plateau at higher pH values. The mid-point for the enzyme-catalysed rate occurred at pH 5.6.

DISCUSSION

Fluorescence spectroscopy using the substrate peptide NRCSQGSCWN proved suitable as a fast and reproducible means of determining the activity of the disulphide bond-forming enzymes, bovine PDI and *V. cholerae* DsbA, under a range of physical conditions. It thereby permitted the rapid determination of the pH-dependence of the activity of both enzymes.

Analysis of the enzyme-catalysed rate of oxidation showed an independence of peptide substrate concentration and a near linear dependence on [GSSG] (up to 1 mM GSSG), implying that:

$$k_1k_3[\text{Pep(red)}] \gg k_1k_4[\text{Pep(red)}][\text{GSSG}]+k_3k_4[\text{GSSG}]+k_2k_4[\text{GSSG}]$$

Under these conditions the rate of oxidation simplifies to:

$$\text{Oxidation rate} = k_4[\text{Enz}][\text{GSSG}]$$

Thus for both PDI and DsbA, the kinetic analysis demonstrates that the rate-limiting step in the catalysed oxidation of the substrate peptide is the re-oxidation of the enzyme, as had previously been reported [20,22]. If this holds true across the pH range studied, then the pH-dependence of the overall oxidation reaction directly represents the pH-dependence of the enzyme recycling step in which the reduced (dithiol) form of the enzyme reacts with GSSG. Since this step involves a S_N2 attack by the active-site thiol of the enzyme, the pH-dependence

will primarily reflect the pK_a of the active-site thiol. However, it will also indicate any pH-dependent factors deriving from ionizations of other groups which affect the reactivity of the active-site thiolate group and any pH-dependent conformational changes in the protein. For all members of the PDI family reported so far, the pK_a of the active-site thiol is significantly lower than would be expected for a conventional cysteine residue [23–26].

The pH dependence of the PDI-catalysed reaction, when corrected for the pH-dependence of the uncatalysed reaction, shows a single pK_a-dependent event (Figure 6, lower panel). The calculated pK_{app} is 5.6 ± 0.1 and it is probable that this represents the pK_a of the reactive cysteine in the catalytic site. This value is significantly lower than that of 6.7, determined by pH-dependence of the rate constant for the reaction of the cysteine with iodoacetate reported by Hawkins and Freedman [24].

The DsbA-catalysed reaction shows a more complicated pH-dependence. Below pH 4.0 activity is lost completely and this is accompanied by a large decrease in the intrinsic fluorescence of the protein, indicating that a pH-dependent conformational change occurs (results not shown). The bell-shaped pH-dependence of the enzyme-catalysed reaction above pH 4.0 is indicative of at least a two-pK_a-dependent event. Analysis in terms of two ionizations gives pK_{app} values around 5.1 and 7.5. As for PDI, the lower value probably represents the pK_a of the active-site cysteine, although the pK_a of the active-site cysteine of *E. coli* DsbA has been reported to be around 3.5 by UV spectroscopy [25]. The higher pK_{app}, around 7.5, may represent a group having an effect on the pK_a of the active-site cysteine. There are three possible residues in *V. cholerae* DsbA which might have such an effect. This group may be the side chain of Glu-24 which has been reported to alter the activity of *E. coli* DsbA [26]. The homologous group in thioredoxin, Asp-26, has a pK_a of 7.5 in the oxidized form [27] and a pK_a of 7.0 in the reduced form of the enzyme [28]. Alternatively, the group may be the side chain of His-32. This residue has been implicated in influencing the redox potential of *E. coli* DsbA ([26]; P. J. Gane and J. Warwicker, unpublished work). Protonation of His-32 stabilizes the thiolate form of Cys-30, increasing the oxidizing activity of *V. cholerae* DsbA. The stabilizing effect would be expected to be mutual, leading to His-32 having a higher pK_a than that usually expected for histidine residues. Thirdly, the group may be the side chain of His-94. This group is homologous to Gln-97 in the helical domain of *E. coli* DsbA. This residue has also been implicated in influencing the redox potential of *E. coli* DsbA [26]; protonation of His-94 would stabilize the thiolate and, as for His-32, this effect would be expected to be mutual, leading to His-94 having an unusually high pK_a.

Systematic structural studies combined with analysis of the enzymic activities (including pH-dependence) of mutant forms of DsbA are currently in progress. This will allow for the elucidation of the residues responsible for the observed pH-dependency and an increased understanding of the mode of action of these enzymes.

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