Bacterial Protein Disulfide Isomerase: Efficient Catalysis of Oxidative Protein Folding at Acidic pH†

Martina Wunderlich, Angelika Otto, Robert Seckler, and Rudi Glockshuber*

Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Universitätsstrasse 31, D-93040 Regensburg, Germany

Received June 8, 1993; Revised Manuscript Received August 2, 1993®

ABSTRACT: Periplasmic protein disulfide isomerase (DsbA) is essential for disulfide formation in *Escherichia coli*. The tryptophan fluorescence of DsbA measures the redox state of the enzyme during catalysis of the oxidative folding of hirudin, a thrombin inhibitor containing three disulfide bonds and lacking tryptophan. With stoichiometric amounts of DsbA, reduced hirudin is rapidly oxidized in a process initially leading to random disulfides. Disulfide reshuffling involving reduced DsbA yields completely native inhibitor within 1 h, even at pH 4. Catalytic amounts of DsbA become essential for hirudin folding in the presence of redox buffers at acidic pH. The second-order rate constants of disulfide exchange reactions involving DsbA are several orders of magnitude above the known values for alkyl dithiols and disulfide oxidoreductases. DsbA preferably reacts with reduced, unfolded polypeptides. The reduction of DsbA by hirudin is faster by 1 order of magnitude than its reduction by the strong reductant dithiothreitol. Together, unusually fast disulfide interchange reactions and a preference for folding polypeptides appear to be responsible for the catalytic efficiency of DsbA and for disulfide formation *in vivo* at acidic pH.

Protein disulfide isomerase from the periplasm of *Escherichia coli* (DsbA)[1] is a monomeric protein with a molecular mass of 21.1 kDa (Bardwell et al., 1991; Kamitani et al., 1992; Wunderlich et al., 1993). The enzyme contains two cysteine residues that are involved in the active-site disulfide bridge, Cys30-Pro31-His32-Cys33. DsbA is the strongest oxidant of the family of known protein disulfide isomerases (PDIs) with a redox potential of –0.089 V (Wunderlich & Glockshuber, 1993; Zapun et al., 1993) compared to –0.11 V for eukaryotic PDI (Hawkins et al., 1991). The oxidative force of DsbA may result from a tense conformation of its oxidized form (Wunderlich et al., 1993; Zapun et al., 1993). The intrinsic fluorescence of DsbA increases more than 3-fold upon reduction, presumably because the catalytic disulfide quenches the fluorescence emission of a neighboring tryptophan residue (Wunderlich & Glockshuber, 1993; Zapun et al., 1993). Therefore, the redox state of DsbA can be observed directly during the catalysis of oxidative protein folding when a substrate protein without tryptophan is used. Here, we use hirudin, a small inhibitor of thrombin with 65 amino acids and known three-dimensional structure (Folkers et al., 1989; Haruyama & Wüthrich, 1989; Rydel et al., 1990; Vitali et al., 1992; Szyperski et al., 1992), as a substrate protein. Its three intramolecular disulfide bridges connect residues 6–14, 16–28, and 22–39 (Dodt et al., 1985) and reform spontaneously and quantitatively *in vitro* after complete reduction and unfolding (Otto & Seckler, 1991; Chatrenet & Chang, 1992).

In the present study, the folding of reduced hirudin in the presence of DsbA was analyzed by two different approaches: First, as in previous studies on the interaction of the eukaryotic enzyme with RNaseA (Lyles & Gilbert, 1991a,b), DsbA was added in stoichiometric amounts as the oxidizing agent in order to measure the redox properties of DsbA toward hirudin and the reaction rates of hirudin oxidation. Second, using catalytic amounts of DsbA in the presence of a glutathione redox buffer (Saxena & Wetlaufer, 1970; Rudolph & Fuchs, 1983), the disulfide isomerase activity of DsbA was investigated.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals. GSH, GSSG, DTT, and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic peptides were kindly provided by S. Modrow (University of Regensburg). All other chemicals were from Merck (Darmstadt) and of highest purity available. Oxidized DsbA was purified from an overproducing *E. coli* strain as described (Wunderlich & Glockshuber, 1993), bovine RNaseA was from Boehringer (Mannheim), and recombinant hirudin was kindly provided by the Hoechst A. G. (Frankfurt/Main).

Protein Concentrations. Protein concentrations were determined using the extinction coefficients at 280 nm ($A_{280,1cm,0.1\%}$). Values of 1.10, 0.70, and 0.44 were used for oxidized DsbA (Wunderlich & Glockshuber, 1993), native RNaseA (Gill & von Hippel, 1989), and native hirudin (Otto & Seckler, 1991), respectively.

Preparation of Reduced, Unfolded Hirudin and RNaseA. Reduced, unfolded hirudin and RNaseA were prepared by treatment with 0.1 M DTT in 6 M GdmCl, 1 mM EDTA, 0.1 M Tris-HCl (pH 8.5) (16 h, 25 °C) followed by gel filtration in 10 mM HCl using a PD10 column (Pharmacia) as described (Otto & Seckler, 1991). The quantitative reduction of the proteins was verified by the determination of free sulfhydryls according to Ellman (1959). Extinction coefficients at 280 nm ($A_{280,1cm,0.1\%}$) of reduced, unfolded hirudin (0.37) and

† This work was supported by the Deutsche Forschungsgemeinschaft (Gl 159/1-2) and the Fonds der Chemischen Industrie.

* Author to whom correspondence should be addressed. Phone: 49-941-9433013. FAX: 49-941-9432813. E-mail: GLOCKSHUBER@VAX1.RZ.UNI-REGENSBURG.DBP.DE.

® Abstract published in *Advance ACS Abstracts,* October 1, 1993.

[1] Abbreviations: DsbA, periplasmic protein disulfide isomerase from *E. coli*; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; OD, optical density; PDI, protein disulfide isomerase; RNaseA, ribonuclease A; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/93/0432-12251$04.00/0

dRNaseA (0.56) were calculated according to Gill and von Hippel (1989).

Fluorescence Measurements. Fluorescence measurements were performed on a Perkin-Elmer MPF-3L spectrometer. To measure the kinetics of reduction of DsbA, oxidized DsbA (2.0 μM) was rapidly mixed with the reductants, and the increase of the relative amount of reduced DsbA (R) was determined by fluorescence spectroscopy. Reactions were performed at 30 °C at pH 3 in 0.1 M sodium phosphate, at pH 4 in 0.1 M formic acid/NaOH containing 0.2 M KCl, at pH 5 in 0.1 M acetic acid/NaOH, at pH 6 in 0.1 M sodium phosphate, and at pH 8.7 in 0.1 M Tris-HCl containing 0.2 M KCl. It was verfied that the measured rate constants were independent of ionic strength. The redox state of DsbA was calculated from the intrinsic fluorescence at 322 nm, which increases 3.2-fold upon reduction of the catalytic disulfide (Wunderlich & Glockshuber, 1993). An excitation wavelength of 295 nm was used throughout to selectively detect the tryptophan fluorescence of DsbA. All buffers were degassed, flushed with nitrogen, and contained EDTA (1 mM).

Oxidative Refolding of Hirudin and HPLC Analysis of Folding Intermediates. Oxidative refolding of hirudin (final concentration 28–91 μM) was performed at 25 °C either in 0.2 M KCl, 1 mM EDTA, and 0.1 M Tris-HCl (pH 8.7) or in 0.2 M KCl, 1 mM EDTA, and 0.1 M formic acid/NaOH (pH 4.0). For spontaneous refolding of hirudin and for experiments involving catalytic amounts of DsbA, GSH (1 mM) and GSSG (1 mM) were included. To exclude air oxidation, all buffers were degassed and flushed with nitrogen. To stop oxidative folding of hirudin, $^1/_3$ vol of 30% (v/v) formic acid was added to the samples, resulting in a final pH of <2. This effectively quenches uncatalyzed disulfide formation and reshuffling (Weissman & Kim, 1991) and was also sufficient in the presence of DsbA, which unfolds below pH 3 (M.Wunderlich and R. Glockshuber, unpublished results). Acid-quenched folding intermediates were separated by reversed-phase HPLC on a Beckman Ultrasphere ODS column (5 μm, 4.6 mm × 25 cm) heated to 55 °C. A gradient of 23% (v/v) acetonitrile at 0 min to 25% (v/v) acetonitrile at 60 min in 0.1% (v/v) trifluoroacetic acid was used. Active hirudin was quantified by thrombin inhibition assays as described (Otto & Seckler, 1991).

Competition Experiments. The following peptides were used in attempts to compete binding and fast reduction of DsbA by reduced hirudin and reduced RNaseA: (peptide A) MMDPNSTSEDVKFT; (peptide B) EDVKFTPDPYQVPF; (peptide C) VAAAKSSENDRLRLL; (peptide D) VPQPA-PVAAPARRTR. DsbA (1 μM) was reduced by hirudin (0.33 μM) or RNaseA (0.25 μM) at 30 °C in 0.1 M formic acid/NaOH (pH 4.0), containing 0.2 M KCl, 1 mM EDTA, and peptides in concentrations ranging from 1 to 100 μM. In all experiments, DsbA was equilibrated with the peptides for 30 min before the addition of the reducing polypeptide. Likewise, competition studies with reduced, carboxamidomethylated hirudin and RNaseA (used at 3–10-fold molar excess over the unmodified proteins) were performed. For carboxamidomethylation, solutions of reduced hirudin and RNaseA in 10 mM HCl were mixed with of $^1/_4$ vol of 2 M iodoacetamide in 0.5 M Tris-HCl (pH 8.5) and 5 mM EDTA and incubated for 2 min at 25 °C. The reagents were removed by size exclusion chromatography in 10 mM HCl, and carboxamidomethylated proteins were stored in 10 mM HCl at 4 °C.

RESULTS

Stoichiometric Oxidation of Hirudin by DsbA. In the first set of experiments, hirudin was reoxidized by 3 molar equiv of oxidized DsbA (corresponding to a 1:1 ratio of cysteine residue concentrations). The reaction was monitored by the increase in tryptophan fluorescence of DsbA between pH 8.7 and pH 4 at a protein concentration of 2 μM. Above pH 6,

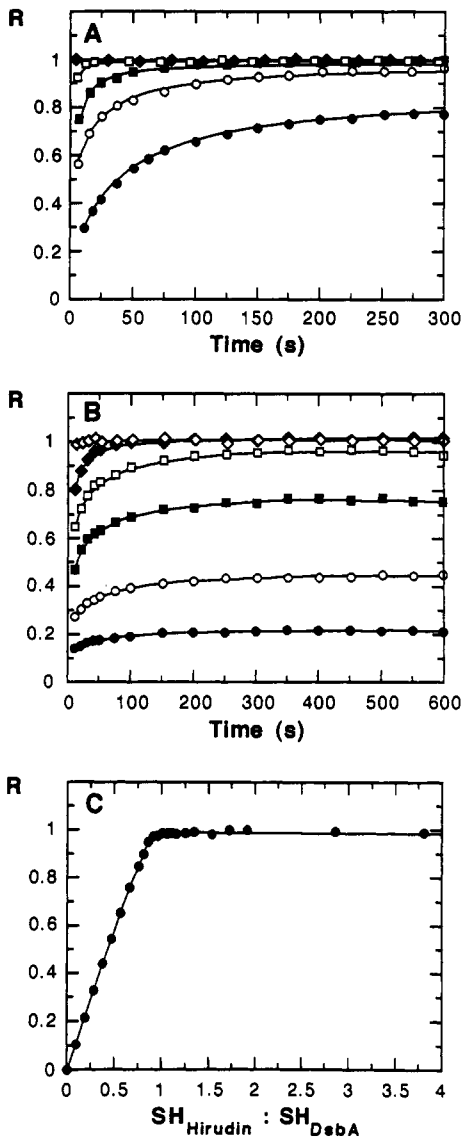

FIGURE 1: pH dependence (A) and stoichiometry (B, C) of the reduction of DsbA by hirudin. (A) Oxidized DsbA (2.0 μM) was rapidly mixed with reduced hirudin (0.67 μM), and the increase in the fraction of DsbA reduced (R = $[DsbA]_{reduced}/[DsbA]_{total}$) was determined by fluorescence spectroscopy. Reactions were performed at pH 3 (●), pH 4 (○), pH 5 (■), pH 6 (□), and pH 8.7 (◆) at 30 °C (buffers are given under Experimental Procedures). The redox state of DsbA was calculated from the intrinsic fluorescence at 322 nm, which increases 3.2-fold upon reduction of the catalytic disulfide. This change is essentially independent of pH (Wunderlich & Glockshuber, 1993). An excitation wavelength of 295 nm was used to detect selectively the tryptophan fluorescence of DsbA. The solid lines were derived from nonlinear regressions according to second-order kinetics, assuming identical initial concentrations of dithiol and disulfide and negligible concentrations of mixed disulfides between the two proteins. (B) Reduction of DsbA by different amounts of reduced hirudin. DsbA (2.0 μM) was reduced by 0.2 (●), 0.43 (○), 0.75 (■), 1.0 (□), 1.5 (◆) and 3.0 (◇) molar equiv of hirudin thiols in 0.1 M formic acid/NaOH (pH 4), 1 mM EDTA, and 0.2 M KCl at 30 °C. (C) Fluorescence titration of DsbA with reduced hirudin. A solution of reduced hirudin (10 μM) was added stepwise to a solution of oxidized DsbA (2.0 μM in 0.1 M formic acid/NaOH (pH 4), 1 mM EDTA, and 0.2 M KCl) at 30 °C. The tryptophan fluorescence at 322 nm was recorded 15 mins after each addition and corrected for the volume increase, and the relative amount of reduced DsbA (R) was calculated as described.

Table I: pH Dependence and Apparent Second-Order Rate Constants (k_{red}) of the Reduction of DsbA by Reduced, Unfolded Polypeptides and Dithiothreitol[a]

	k_{red} (M^{-1} s^{-1})			
reductant	pH 3	pH 4	pH 5	pH 6
hirudin	8.2×10^3	8.2×10^4	6.4×10^5	3×10^6
RNaseA	1.4×10^3	1.7×10^4	6.9×10^4	5.3×10^5
DTT	8.0×10^2	5.3×10^3	3.7×10^4	1.2×10^5

[a] All reactions were performed at 30 °C using DsbA at a concentration of 2 μM. The reaction buffers are given under Experimental Procedures. For all reactions, stoichiometric amounts (at the thiol level) of the reductants were used (cf. Figure 5). The kinetics was evaluated according to an apparent second-order dithiol/disulfide reaction with identical initial concentrations of the reactants (correlation coefficients >0.995).

the reduction of DsbA by stoichiometric quantities of hirudin is complete within the time scale of manual mixing (Figure 1A). Below pH 6, the kinetics can be followed after manual mixing and is well described by a second-order reaction with identical initial concentrations of reactants, assuming negligible concentrations of mixed disulfides. Therefore, the reactivities of fully reduced hirudin and of hirudin reoxidation intermediates toward oxidized DsbA appear very similar. The rate constants of the reaction are independent of the protein concentration (data not shown) and decrease almost 10-fold per pH unit (Figure 1A, Table I). The pH dependence of the reaction (Table I) indicates that the thiolate form of the cysteine residues of hirudin is the nucleophilic species. The stoichiometry of the reduction of DsbA by hirudin was confirmed by varying the $SH_{hirudin}/SH_{DsbA}$ ratio (Figure 1B,C).

The disulfides formed in hirudin immediately after its stoichiometric oxidation by DsbA at pH 8.7 are not the native ones. Thrombin inhibition studies revealed only about 15% native inhibitor molecules after a reaction time of 1 min at pH 8.7. This value is close to that expected for a complete, random oxidation (15 possible disulfide patterns corresponding to a 7% probability of native hirudin).

To investigate oxidative folding in more detail, reduced hirudin and oxidized DsbA were mixed at pH 8.7, the reaction was stopped after different times by adding formic acid (final pH <2), and the acid-quenched hirudin species were separated by HPLC (Figure 2A). In accordance with the fluorescence and thrombin inhibition data, only oxidized hirudin species (probably all containing three disulfide bonds), including about 5% native hirudin, were observed 10 s after the onset of the reaction. Essentially all hirudin molecules reached the native, active state, however, within 1 h. As nonnative, fully oxidized hirudin can only be converted to native hirudin by intermolecular disulfide exchange reactions, DsbA must also isomerize nonnative hirudin disulfides. There was no evidence from the HPLC traces for a significant population of DsbA/hirudin mixed disulfides during the reshuffling reactions.

At the relatively high protein concentrations necessary for the HPLC analysis, the stoichiometric oxidation of hirudin by DsbA occurs in the dead-time of manual mixing also at pH 4.0. Like at pH 8.7, the rapid oxidation is followed by disulfide isomerizations, leading to fully native hirudin within 1 h (Figure 2B). The distribution of folding intermediates at pH 4 is not significantly different from the one observed at pH 8.7 (Figure 2), and the difference becomes negligible when DsbA is used in a 1.5-fold molar excess over hirudin (data not shown). As cysteine thiols become significantly deprotonated at pH 8.7 and thus destabilize native folding intermediates (Weissman & Kim, 1991; Creighton, 1992; Goldenberg, 1992), the observed pH-independent distribution of hirudin intermediates may be surprising. However, only fully oxidized hirudin isomers with at least two nonnative disulfide bonds are expected as intermediates in the stoichiometric oxidation experiment. These intermediates should be largely unfolded at either pH, and all disulfides in these intermediates should have similar reactivities toward DsbA.

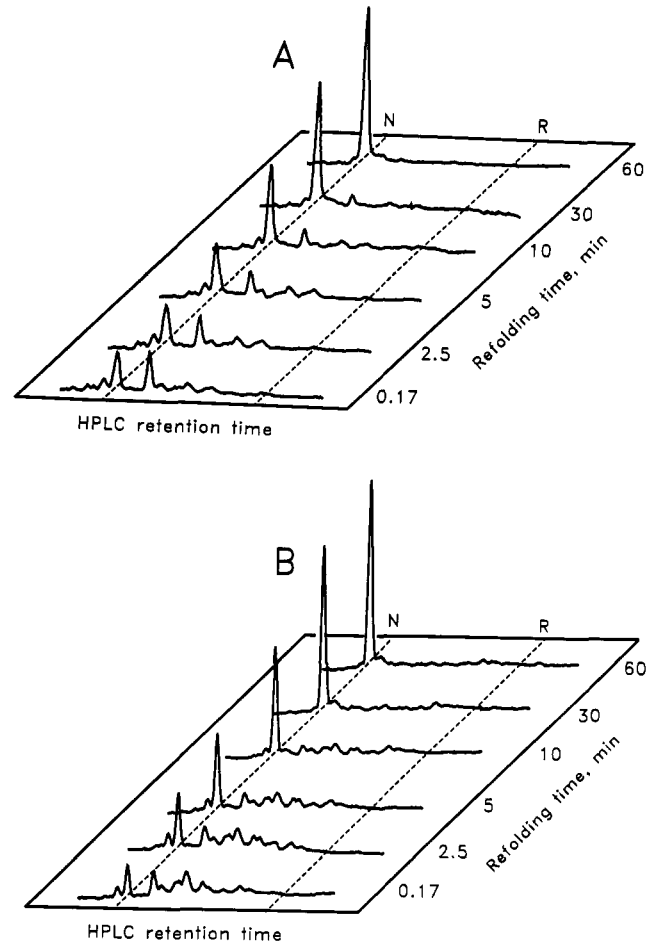

FIGURE 2: HPLC analysis of hirudin folding intermediates obtained after various folding times in the presence of stoichiometric amounts of DsbA at pH 8.7 (A) or pH 4.0 (B). Reoxidation and refolding of hirudin was initiated at 25 °C by the addition of reduced hirudin to the buffers containing oxidized DsbA. The protein concentrations were 28 μM hirudin and 84 μM DsbA, corresponding to a 1:1 ratio of cysteine residues. At the times indicated by the labels, a portion of the sample was removed, quenched with acid, separated by reversed-phase HPLC, and detected by their absorbance at 275 nm (full scale ≈ 10 mOD). The dashed lines labeled R and N denote the elution positions of fully reduced hirudin (44 min) and native hirudin (22 min), respectively.

Catalytic Function of DsbA. In order to investigate the *catalytic* function of DsbA during oxidative folding of hirudin, the inhibitor was reconstituted in the presence of a redox buffer and substoichiometric amounts of DsbA. When reduced hirudin is refolded at pH 8.7 in a 1:1 mixture of reduced and oxidized glutathione (1 mM GSH and 1 mM GSSG), completely native hirudin is obtained within 4 h (Figure 3A). Catalytic amounts of DsbA (SH_{DsbA}:$SH_{hirudin}$ = 1:10) significantly accelerate the overall folding reaction and decrease the half-time of formation of native hirudin by 2–3-fold without changing the relative distribution of partially oxidized folding intermediates (Figure 3B).

The catalytic efficiency of DsbA is more obvious at acidic pH (Figure 3C,D). Below pH 6, no hirudin folding is detectable even after several hours of incubation in the GSH/GSSG redox buffer. However, in the presence of substoi-

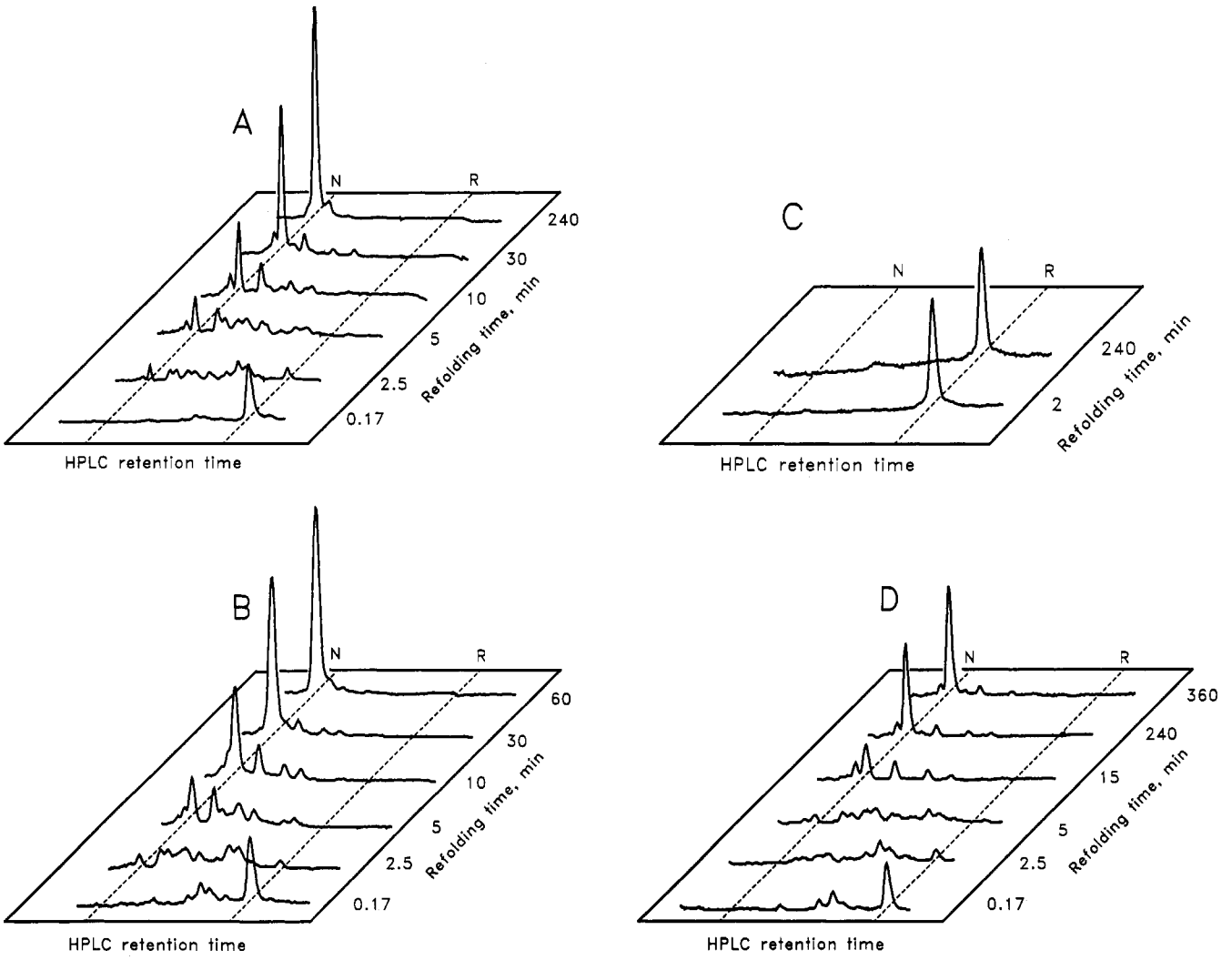

FIGURE 3: HPLC analysis of hirudin refolding using a glutathione redox buffer in the absence or presence of catalytic amounts of DsbA. Hirudin refolding (final hirudin concentration 91 μM) was initiated at pH 8.7 (A, B) or pH 4.0 (C, D), and intermediates were trapped and separated as described in the legend to Figure 2, except that the reoxidation buffers contained 1 mM GSH and 1 mM GSSG. The refolding in the absence (A, C) and in the presence (B, D) of catalytic amounts of DsbA (using a DsbA/hirudin stoichiometry of 1:10 at the level of cysteine residues) is compared.

chiometric amounts of DsbA, fully native hirudin is formed, even at pH 4 (Figure 3D), on a time scale similar to that of the reaction at pH 8.7 (Figure 3B). Thus, DsbA becomes essential for hirudin folding at pH 4, and the relative acceleration of hirudin folding by DsbA increases strongly with decreasing pH.

As in the experiments with stoichiometric amounts of DsbA (Figure 2), the distribution of folding intermediates of hirudin is not significantly different at pH 8.7 and pH 4, when catalytic amounts of DsbA are used, and is similar to the distribution observed during uncatalyzed folding at pH 8.7 (Figure 3). That the distribution of hirudin folding intermediates is largely independent of pH even during oxidation in the presence of a redox buffer may be understood, if intermediates with a subset of native disulfides are only marginally stable (Chatrenet & Chang, 1992). Because no hirudin folding occurs below pH 6 in the absence of DsbA, GSSG (and probably also GSH) cannot react directly with folding hirudin at pH 4. Only the direct interaction between DsbA and hirudin and the equilibrium between DsbA and GSH/GSSG (Wunderlich & Glockshuber, 1993) are involved in the overall folding of hirudin at acidic pH. Therefore, the redox state of DsbA used in catalytic amounts during hirudin folding was investigated.

Initiation of hirudin folding at pH 4 in the presence of GSH/GSSG by the addition of catalytic amounts of oxidized DsbA results in a fast, complete reduction of DsbA, as indicated by the increase in tryptophan fluorescence during the mixing period (Figure 4). During the next 15 min, the fluorescence burst is followed by relaxation of the signal to a value corresponding to 41% of reduced DsbA. This value is expected for the redox equilibrium of DsbA with the glutathione redox buffer at pH 4 (Wunderlich & Glockshuber, 1993), corrected for the amount of reduced hirudin initially present, as indicated by the time course of DsbA fluorescence observed in the absence of hirudin (Figure 4). At the time when the redox equilibrium is reached, only 10–15% native hirudin has been formed, but the distribution of hirudin folding intermediates indicates that the inhibitor has been largely oxidized (Figure 3D). As the direct reaction between hirudin and DsbA is very rapid even at pH 4 (Figure 2B), the reoxidation of DsbA by GSSG is rate-limiting for hirudin oxidation in the presence of catalytic amounts of DsbA. Still, the overall oxidation of hirudin precedes disulfide reshuffling. This conclusion must also hold at pH 8.7, as revealed by comparison of the HPLC profiles (Figure 3B,D). Overall, DsbA-catalyzed hirudin refolding is not much slower at pH 4 than it is at alkaline pH. If one takes into account the pH dependence of the redox

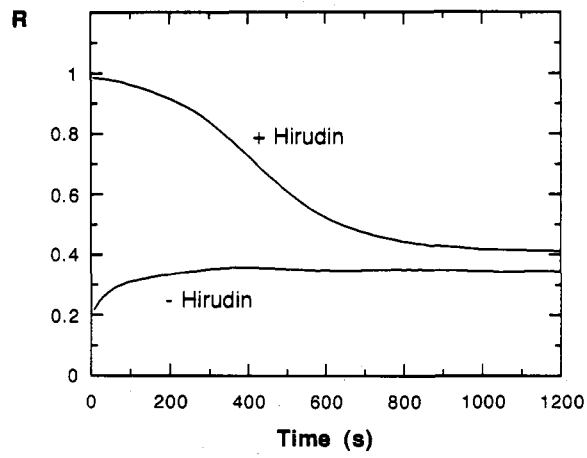

FIGURE 4: Redox state of DsbA present in catalytic amounts during oxidative folding of hirudin at pH 4. Reduced hirudin (91 μM) was incubated at 25 °C for 30 min in 0.1 M formic acid/NaOH (pH 4.0) and 1 mM EDTA containing 1 mM GSH and 1 mM GSSG. Hirudin refolding was started by the addition of oxidized DsbA (27 μM), and the fraction of DsbA reduced (*R*) was recorded using its tryptophan fluorescence at 322 nm (3-mm fluorescence cuvettes). The reaction conditions correspond to those used in the analysis of hirudin folding intermediates given in Figure 3D. For the analysis of the DsbA/glutathione equilibrium, oxidized DsbA was added to a redox buffer containing 1.55 mM GSH and 0.73 mM GSSG, corresponding to the redox conditions in the catalyzed reaction after complete reactivation of hirudin.

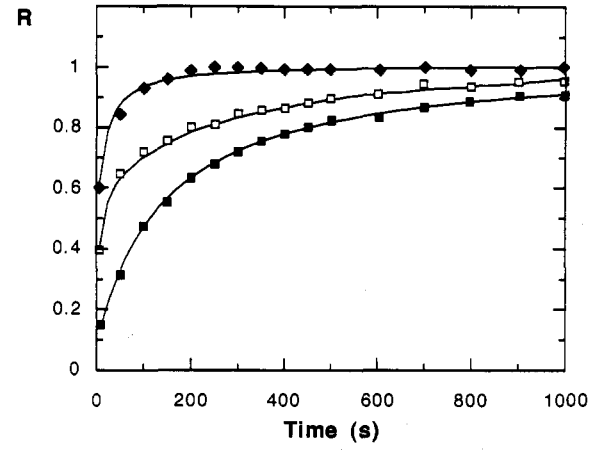

FIGURE 5: Polypeptide specificity of DsbA. Oxidized DsbA (2 μM) was reduced by stoichiometric amounts of reduced, unfolded hirudin (0.67 μM) (◆), reduced, unfolded RNaseA (0.5 μM) (□), and DTT (2 μM) (■) in 0.1 M formic acid/NaOH (pH 4.0), 0.2 M KCl, and 1 mM EDTA at 30 °C. The fraction of DsbA reduced (*R*) was monitored by the increase in the tryptophan fluorescence of DsbA, and the reaction was fit according to an apparent second-order reaction (solid lines).

equilibria (Wunderlich & Glockshuber, 1993), indicating that >90% of DsbA is reduced at pH 8.7 at equilibrium with the glutathione redox buffer compared to only 30–40% at pH 4.0, it becomes evident that DsbA catalyzes disulfide reshuffling at acid pH essentially as well as in the vicinity of the normal p*K* of an organic thiol (Szajewski & Whitesides, 1980).

Kinetics of DsbA Reduction by DTT and Reduced Polypeptides. The fast reduction of DsbA by hirudin prompted us to investigate the rates of reduction of DsbA between pH 3 and pH 6, where the reaction can be followed after manual mixing (Table I, Figure 5). Surprisingly, reduced, unfolded hirudin is 10–25 times more effective in reducing DsbA than the strong reductant dithiothreitol (DTT). Therefore, DsbA may exhibit a peptide binding site, increasing the local concentration of the reducing polypeptide thiol group and thus accelerating DsbA reduction. This view is supported by the fact that reduced bovine RNaseA that contains four disulfide bonds and also lacks tryptophan (Wlodawer & Sjölin, 1983; Smyth et al., 1963; Kim et al., 1992) reduces DsbA 2–5 times faster than DTT (Table I, Figure 5).

An inhibitory effect of peptides on disulfide isomerase activity has been observed in the case of the eukaryotic enzyme (Morijana & Gilbert, 1991). Thus, the peptide specificity of DsbA was probed with synthetic peptides in an attempt to compete DsbA reduction by hirudin or RNaseA. A number of peptides 14–15 residues in length, which lacked cysteine residues but otherwise varied in amino acid composition (see Experimental Procedures), were used at concentrations of up to 100 μM (300–400-fold molar excess over reduced hirudin or RNaseA). However, none of the peptides significantly inhibited the reaction. The reaction rates were also unaffected by hirudin or RNaseA with thiols blocked by carboxamidomethylation used in a 10-fold molar excess over the unmodified proteins. Therefore, a possible polypeptide binding site in DsbA appears to be of rather low affinity (K_D > 1 mM). This is consistent with the catalytic function of DsbA, as rapid release of the folding polypeptide appears to be necessary for efficient catalysis of protein folding. The rates of reduction of DsbA are in all cases strongly dependent on pH and increase about 5–10-fold per pH unit (Table I). In summary, although we have not obtained direct evidence from competition experiments, a low-affinity polypeptide binding site on DsbA is suggested by the observed reaction rates.

DISCUSSION

The efficiency of bacterial protein disulfide isomerase is based on extremely fast disulfide exchange reaction rates (Zapun et al., 1993), about 3 orders of mangnitude above known values for alkyl mono- and dithiols which are normally in the range of 1–100 M^{-1} s^{-1} at pH 7.0 (Szajewski & Whitesides, 1980). The enzyme is also significantly more reactive than other disulfide oxidoreductases. As an example, DTT reduces DsbA about 1000 times faster than *E. coli* thioredoxin at neutral pH (k_{red} = (1–2) × 10^3 M^{-1} s^{-1} at pH 7.2; Holmgren, 1979). Since the p*K* values of alkyl thiols and cysteine residues are in the range of 9 and the thiolate form is the nucleophilic species (Szajewski & Whitesides, 1980), the proton of the thiol attacking the DsbA disulfide may be transferred to a side chain of DsbA near the active site, thereby lowering the thiol p*K* and increasing the local thiolate concentration. Alternatively, the high reaction rates may be an intrinsic property of the tense disulfide bond of DsbA, which lowers the free energy of stabilization of the protein by about 23 kJ/mol at 30 °C (Wunderlich et al., 1993; Zapun et al., 1993). The former explanation, however, is supported by the observation that DsbA also catalyzes disulfide reshuffling very efficiently during oxidative hirudin folding at pH 4.

The high reaction rates and the efficiency of DsbA at acidic pH may indeed be necessary for disulfide formation in the periplasm of *E. coli* at low pH values. The outer membrane of the bacterium is permeable for molecules smaller than 500 Da (Payne & Gilvarg, 1968; Decad & Nicaido, 1976), and hence the pH in the periplasm must be identical with the pH in the medium. As the lower limit of *E. coli* growth is around pH 4.4 (Prescott et al., 1990), where uncatalyzed disulfide exchange reactions do not occur to a significant extent, DsbA appears to be required for disulfide formation under these growth conditions and may become essential for *E. coli* at acidic pH.

We believe that the constant redox state of DsbA during the catalysis of protein folding under well-defined redox conditions (Figure 4) resembles the situation in the lumen of the endoplasmic reticulum (ER) of higher cells. The redox conditions in this cellular compartment are determined by excess GSH and GSSG (Hwang et al., 1992), and hence it can be assumed that the redox state of eukaryotic PDI in the ER is indeed identical with the value calculated from the known PDI/glutathione equilibrium (Hawkins et al., 1991; Lyles & Gilbert, 1991; Wunderlich & Glockshuber, 1993) and the concentrations of GSH and GSSG in the ER (Hwang et al., 1992). As discussed previously (Wunderlich & Glockshuber, 1993), the reduced form of eukaryotic PDI is likely to be present in a significant molar excess over the oxidized form in the ER. This molar excess appears essential for the reduction of nonnative disulfides by PDI.

In conclusion, the possibility to study the redox state of protein disulfide isomerases during catalysis simultaneously with folding of substrate proteins may greatly facilitate the elucidation of the catalytic mechanism of this class of enzymes, as well as the elucidation of protein folding pathways.

ACKNOWLEDGMENT

Discussions with H. Lilie, R. Jaenicke, R. Rudolph, and F.-X. Schmid are gratefully acknowledged. We thank S. Modrow for supplying us with synthetic peptides.

REFERENCES

Bardwell, J. C. A., McGovern, K., & Beckwith, J. (1991) *Cell 67*, 581–589.

Chatrenet, B., & Chang, J.-Y. (1992) *J. Biol. Chem. 267*, 3038–3043.

Creighton, T. E. (1992) *BioEssays 14*, 195–199.

Decad, G. M., & Nikaido, H. (1976) *J. Bacteriol. 128*, 325–336.

Dodt, J., Seemüller, U., Maschler, R., & Fritz, H. (1985) *Biol. Chem. Hoppe-Seyler 366*, 379–385.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Folkers, P. J. M., Clore, G. M., Driscoll, P. C., Dodt, J., Köhler, S., & Gronenborn, A. M.(1989) *Biochemistry 28*, 2601–2617.

Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319–326.

Goldenberg, D. P. (1992) *Trends Biochem. Sci. 17*, 257–261.

Haruyama, H., & Wüthrich, K. (1989) *Biochemistry 28*, 4301–4312.

Hawkins, H. C., de Nardi, M., & Freedman, R. B. (1991) *Biochem. J. 275*, 341–348.

Holmgren, A. (1979) *J. Biol. Chem. 254*, 9627–9632.

Hwang, C., Sinskey, A. J., & Lodish, H. F. (1992) *Science 257*, 1496–1502.

Kamitani, S., Akiyama, Y., & Ito, K. (1992) *EMBO J. 11*, 57–62.

Kim, E. E., Varadarajan, R., Wyckoff, H. W., & Richards, F. M. (1992) *Biochemistry 31*, 12304–12314.

Lyles, M. M., & Gilbert, H. F. (1991a) *Biochemistry 30*, 613–619.

Lyles, M. M., & Gilbert, H. F. (1991b) *Biochemistry 30*, 619–625.

Morjana, N. A., & Gilbert, H. F. (1991) *Biochemistry 30*, 4985–4990.

Otto, A., & Seckler, R. (1991) *Eur. J. Biochem. 202*, 67–73.

Payne, J. W., & Gilvarg, C. (1968) *J. Biol. Chem. 243*,6291–6299.

Prescott, L. M., Harley, J. P., & Klein, D. A. (1990) *Microbiology*, pp 122–123, Wm. C. Brown Publishers, Dubuque, IA.

Rudolph, R., & Fuchs, I. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 813–820.

Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., & Fenton, J. W., II (1990) *Science 249*, 277–280.

Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015–5023.

Smyth, D. G., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 227–234.

Szajewski, R. P., & Whitesides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011–2026.

Szyperski, T., Güntert, P., Stone, S. R., Tulinsky, A., Bode, W., Huber, R., & Wüthrich, K. (1992) *J. Mol. Biol. 228*, 1206–1211.

Vitali, J., Martin, P. D., Malkowski, M. G., Robertson, W. D., Lazar, J. B., Winant, R. C., Johnson, P. H., & Edwards, B. F. (1992) *J. Biol. Chem. 267*, 17670–17678.

Weissman, J. S., & Kim, P. S. (1991) *Science 253*, 1386–1393.

Wlodawer, A., & Sjölin, L. (1983) *Biochemistry 22*, 2720–2728.

Wunderlich, M., & Glockshuber, R. (1993) *Protein Sci. 2*, 717–726.

Wunderlich, M., Jaenicke, R., & Glockshuber, R. (1993) *J. Mol. Biol.* (in press).

Zapun, A., Bardwell, J. C. A., & Creighton, T. E. (1993) *Biochemistry 32*, 5083–5092.