Redox Properties of Protein Disulfide Bond in Oxidized Thioredoxin and Lysozyme: A Pulse Radiolysis Study†

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ABSTRACT: We have studied the one-electron reduction of oxidized *Chlamydomonas reinhardtii* thioredoxin and compared it to that of hen egg white lysozyme, using $CO_2^{\bullet -}$ free radicals as reductants. This comparison shows that the thioredoxin disulfide/thiol redox couple has different properties than that of lysozyme: the disulfide radical pK_a is much lower (around 5 for small disulfides, 4.62 for lysozyme, <3 for thioredoxin). To get a better understanding of the modulation of the thioredoxin redox properties we have constructed the mutants W35A and D30A. Their reduction by pulse radiolysis indicates that W35 strongly controls both the disulfide radical acidity (the pK_a in W35A is equal to ca. 4), and the thiol reactivity. Asp30 is also involved in the control of proton transfer to the disulfide free radical. In addition, its removal seems to increase the reduction potential of the thioredoxin thiyl/thiol couple. Overall, the reduction properties of thioredoxin confirm its nature as a unique reductant.

Several proteins are able to catalyze dithiol/disulfide exchange reactions in prokaryotic or eukaryotic cells. Many of these catalysts [glutaredoxin, protein disulfide isomerase (PDI) or its bacterial equivalent DsbA] share a large 3D homology with a ubiquitous model protein called thioredoxin. Thioredoxin (Trx)[1] is a small thermostable protein of about 12 kDa or ca. 110 amino acids with the highly conserved active-site sequence W_{35}-C-[G/P]-P-C-[R/K]$_{40}$ (*Chlamydomonas reinhardtii* thioredoxin *h* numbering used throughout this manuscript).

Although glutaredoxin and PDI (DsbA) share significant 3D homology with thioredoxin, they exhibit very different reduction potentials (PDI ca. −180 mV vs ca. −270 mV for thioredoxin (*1*) and substrate specificity (reduced glutathione is the reductant for glutaredoxin and NADPH + thioredoxin reductase for thioredoxin). These pronounced differences are mostly due to small variations in the active sites of the proteins (W-C-P-Y-C for glutaredoxin and W-C-G-H-C repeated twice for PDI). As a consequence, PDI acts as an oxidant, being involved in protein folding processes, and thioredoxin as a reductant, although there seems to be some interchangeability (*2*). Actually, thioredoxin is the best disulfide reductant known so far, being more rapid and quantitative than dithiothreitol, the best disulfide reductant available (*3*). A simple mutation transforming the WCGPC active site into a WCGHC site results in an increase of the redox potential by 35 mV and is accompanied by the appearance of more oxidizing properties (*1*). Thus, subtle variations in the redox active site of biocatalysts can have very pronounced effects on their reactivity, and especially so in the case of thioredoxin.

Disulfide/dithiol redox systems are involved in the regulation of cell growth and proliferation (*4*, *5*) and human cancer development (*6*, *7*), and one of the most important proteins in this field is Trx. It intervenes in cellular controls involving free radical species, such as DNA radiosensitivity (*8*), DNA synthesis through ribonucleotide reductase activity (*9*, *10*), apoptosis (*11*, *12*), reactive oxygen species and stress signaling (*13*), and transcription factor activation [NF-κB and AP-1 (*14*−*18*), AP-2 (*18*)]. It is also involved in cytoprotective function of prostaglandin E1 (*19*) and embryo development (*20*). Trx also plays an important role in antioxidant defense (*21*−*23*), and in the development of postirradiation effects (*24*).

The two cysteines responsible for the redox properties of thioredoxin (Cys36 and Cys39) do not have the same behavior. Cys36 is solvent-accessible and has a pK_a value close to the physiological pH (*25*), whereas Cys39 has a pK_a that is at least 4 units higher than physiological pH (*26*−*28*), making this residue relatively unreactive. Asp30 is well positioned to be a good candidate for activating Cys residues by general acid−base catalysis (*29*): it could protonate (during substrate oxidation) or deprotonate (during substrate reduction) the thiol function of Cys39.

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[1] Abbreviations: Trx, thioredoxin h of *Chlamydomonas reinhardtii* (CH1); Lys, hen egg white lysozyme; Trx/SS, thioredoxin oxidized form; Trx $(SH)_2$, thioredoxin reduced form; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTNB, dithionitrobenzoic acid; $Trx/SS^{\bullet -}$ thioredoxin disulfide radical; Trx/$(SH)S^{\bullet}$, thioredoxin thiyl radical; (HS)Trx/SS/Trx(SH) thioredoxin dimer. Similar abbreviations are used for proteins and for lysozyme, such as $Lys/SS^{\bullet -}$ lysozyme disulfide radical anion, etc.

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Scheme 1: Proposed Kinetic Scheme for the Chain Reduction of Protein Disulfide Bonds by $COO^{\bullet -}$ Free Radicals

$$CO_2^{\bullet -} + P/SS \rightarrow CO_2 + P/SS^{\bullet -} \quad (1)$$

$$P/SS^{\bullet -} + H^+ \rightleftarrows P/SSH^{\bullet} \quad (2)$$

$$P/SS^{\bullet -} + H^+ \rightleftarrows P/(SH)S^{\bullet} \quad (3)$$

$$P/SSH^{\bullet} \rightarrow P/(SH)S^{\bullet} \quad (4)$$

$$2\ P/SS^{\bullet -} \rightarrow P/SS + P/(SH)_2 \quad (5)$$

$$P/(SH)\ S^{\bullet} + HCOO^- \rightleftarrows CO_2^{\bullet -} + P/(SH)_2 \quad (6)$$

$$2\ P/(SH)\ S^{\bullet} \rightarrow (HS)P/S\cdots S/P(SH) \quad (7)$$

$$P/SS^{\bullet -} \rightarrow P^{\bullet}/SS \quad (8)$$

$$2\ CO_2^{\bullet -} \rightarrow C_2O_4^{2-} \quad (9)$$

$$P^{\bullet}/SS \rightarrow \text{products} \quad (10)$$

All prokaryotic and eukaryotic thioredoxins contain a conserved tryptophan residue, W35, exposed at the active-site disulfide/dithiol. Site-directed mutagenesis experiments indicate that this residue is important in Trx activity (*30*). Stone et al. (*31*, *32*) characterized the backbone and tryptophan side-chain dynamics: the W35 side chain is very mobile and exposed on the protein surface.

In the regulation of all events involving free radicals such as oxidative stress, irradiation by ionizing radiations, etc., one-electron redox processes take place and sulfur free radicals are created. Their properties play a crucial role in the course of the reactions leading to the cellular effect of the radiation treatment. The most important sulfur free radicals are thiyl and disulfide radicals. Both can be studied easily by pulse radiolysis especially through reduction of disulfide bonds. Kinetic studies of the reduction process has been investigated in small molecules (*33*−*36*) as well as in some proteins (*37*−*41*). From γ and pulse radiolysis analysis, $CO_2^{\bullet -}$ proved to be able to induce a rapid and specific one-electron reduction of disulfide bonds. The elementary reactions induced during this reduction process are quite similar to those observed in small molecules (Scheme 1). The first step is the formation of the disulfide radical noted $P/SS^{\bullet -}$ (reaction 1). Its protonated form $P/SSH^{\bullet}$ appears under acidic conditions (equilibrium 2). The free radical pK_a seems to lie between ca. 4.5 and 6 (*38*). The disulfide radical decay may involve several reactions: disproportionation of the disulfide radical anion (reaction 5) and/or SS bond cleavage leading to the thiyl radical and a thiol or thiolate function [$P/(SH)S^{\bullet}$] (reaction 3). This latter reaction is usually much faster with the protonated radical (reaction 4). However the relative importance of these reactions seems to vary from one protein to another. In lysozyme, we have demonstrated that reduction of disulfide 6−127 leads also to cleavage of the polypeptidic backbone (*42*). Since the reductant is specific for disulfide bonds, this cleavage can only be initiated by electron migration through the protein, symbolized by reaction 8.

The thiyl radical can dimerize, leading to intermolecular disulfide bond, i.e., protein dimer (reaction 6). In the presence of formate ions, it can also oxidize formate ions (reaction 6): the whole process is thus a chain reaction whose length would depend on the pK_a of the disulfide radical and on the reduction potential of the thiyl/thiol couple. It has been also suggested that thiyl radicals are able to oxidize tyrosine residues in an inter- or intramolecular process (*43*, *44*). However, up to now, there is no evidence for this process in proteins. The key reaction of this system is thus the protonation equilibrium (2) of the disulfide radical since it allows the transformation of a mild reductant (disulfide radical) to a rather strong oxidant (thiyl radical) (*43*). In addition, the chain length of this reduction process allows an evaluation of the one-electron reduction potential of the thiyl/thiol redox couple compared to that of the $CO_2^{\bullet -}/HCOO^-$ couple [1.07 V at pH 7 (*45*)].

The aim of this study is thus to characterize the reactivity of sulfur free radical in thioredoxin, which has unique properties as a reductant and plays an important role in many cellular regulation reactions. Since W35 and D30 seem to control electron- and proton-transfer reactions in the two-electron process, one might expect that they also play a role in the modulation of free radical reactions. We investigated by γ and pulse radiolysis the reduction by $CO_2^{\bullet -}$ free radicals of thioredoxin *h* of *Chlamydomonas reinhardtii* (Trx) and its two mutants: W35A and D30A. However since knowledge about protein free radical reactivity is poor, the results are compared to those obtained with a model of known structure and reactivity. Therefore we compare the behavior of thioredoxin sulfur radicals to those formed in lysozyme (Lys), a very good model for several reasons. Its three-dimensional structure is well-known, it contains four disulfide bonds (positioned respectively at 6−127, 30−115, 64−80, and 76−94 residues) and no thiol functions. We have demonstrated that only one out of the four disulfide bridges, i.e., 6−127, can be reduced under steady-state conditions (*42*) and shown that the disulfide free radical stability was due to interaction with the charged end of Arg5. Partial information about the pulse radiolysis reduction of Lys by $CO_2^{\bullet -}$ and e^-_{aq} is also available (*45*−*48*).

MATERIALS AND METHODS

Reagents. Sodium formate and potassium hydrogen phosphate were of the highest quality available (Prolabo Normatom or Merck Suprapure). Nitrous oxide was delivered by Alpha Gaz. Its purity is higher than 99.99% (<20 ppm O_2). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, $C_{14}H_8N_2O_8S_2$) was provided by Sigma. Water was purified on an Elga Maxima system (resistivity 18.2 MΩ).

Lysozyme. Hen egg white lysozyme (Sigma) was purified to homogeneity by ion-exchange chromatography (20 mM Tris-HCl buffer, pH 7.8, 400−1000 mM NaCl linear gradient) over Macro-Prep High S resin (Bio-Rad) followed by concentration, gel filtration over Sephadex G-75 (10 mM phosphate buffer, pH 7.0), total dialysis against distilled water, and lyophilization. Purity was checked by homogeneous 12.5% polyacrylamide gel electrophoresis with 0.2% sodium dodecyl sulfate (SDS−PAGE). The concentration of protein was measured spectrophotometrically with $\epsilon_{278nm} = 37.9\ mM^{-1}\ cm^{-1}$ (*49*).

Thioredoxin. Recombinant *Chlamydomonas reinhardtii* thioredoxin *h* was purified from *E. coli* as previously

described (*50*) (heat treatment, ammonium sulfate fractionation, and Sephadex G50 gel filtration, followed by ion-exchange chromatography on DEAE-Sephacel). Purity was checked by SDS−PAGE electrophoresis. Samples to be irradiated were dialyzed against a phosphate−formate buffer and the concentration was assessed by absorbance where $\epsilon_{278nm} = 14\,500\ M^{-1}\ cm^{-1}$ except for the W35A, mutant for which it was $8900\ M^{-1}\ cm^{-1}$.

Site-Directed Mutagenesis. The cytosolic thioredoxin cDNA was modified by PCR as in ref *51* with cloning and mutagenic oligonucleotides derived from the cDNA sequence. Site-directed mutagenesis was performed directly on the constructed pET-CH1 (*50*) by PCR to replace the codon of Trp35 or that of Asp30 in the wild type by a codon for alanine (giving W35A or D30A, respectively). The mutations were confirmed by DNA sequencing using the dideoxy chain-termination method (kit T7 sequencing, Pharmacia), and the Trx cDNA sequence was controlled at each subcloning step.

The mutated Trx cDNA (300 bp) was cloned in the expression vector pET-3d into the *Nco*I and *Bam*HI sites, then the recombinant protein was expressed in an *Escherichia coli* strain. This protein had a catalytic site characteristic of thioredoxin (WCGPC) (*52*).

Titration of Sulfhydryl Groups. Free sulfhydryl groups were determined by optical titration with dithionitrobenzoic acid (DTNB) at pH 8.0 (100 mM Tris-HCl buffer), with $\epsilon_{(410nm)} = 13.6\ mM^{-1}\ cm^{-1}$ for the 3-carboxylato-4-nitrothiophenolate anion (*53*) (verified by us with glutathione). Thiol groups in the reduced proteins were alkylated prior to electrophoresis by reaction with 10 mM iodoacetamide (10 min, room temperature).

Pulse Radiolysis Experiments. The doses per pulse (2−60 Gy) were calibrated by the absorption of the stable semiquinone form of methyl viologen (MV^{2+}) obtained by radiolysis of methyl viologen in N_2O-saturated phosphate−formate buffer [0.1 M formate, 10 mM phosphate, 400 μM methyl viologen, pH 8, $G(MV^{\bullet +}) = 0.62\ \mu mol\ J^{-1}$, $\epsilon_{600nm} = 13.7\ mM^{-1}\ cm^{-1}$) (*54*).

The reducing agent used in our experiments is $CO_2^{\bullet -}$ free radical. This radical is obtained in pure form in less than 1 μs by scavenging the radicals formed in the course of water radiolysis with formate ion and nitrous oxide. Unless otherwise stated, samples to be irradiated were made up in 20 mM phosphate and 100 mM sodium formate buffer, adjusted to the required pH with sulfuric acid or sodium hydroxide and saturated with N_2O. The doses per pulse were ca. 10 Gy ($[CO_2^{\bullet -}] \approx 6\ \mu M$), unless otherwise stated.

RESULTS

Formation of Disulfide Free Radical in Lysozyme. Upon pulse radiolysis of lysozyme solutions ([lys] = 250 μM−1.8 mM, phosphate buffer 20 mM, $[HCOO^-] = 100$ mM, dose = 5−50 Gy, $[CO_2^{\bullet -}] \approx 3-30\ \mu M$), disulfide free radicals ($Lys/SS^{\bullet -}$) are created. At pH 8, this reaction is completed in 150−200 μs and the resulting spectrum, shown in Figure 1A, is stable for at least 100 ms. The absorption spectrum is characterized by a broad band showing a maximum at 425 nm, in agreement with the findings of Hoffman and Hayon (*46*) and as for many proteins (*39*). The extinction coefficient at the maximum (425 nm) is equal to

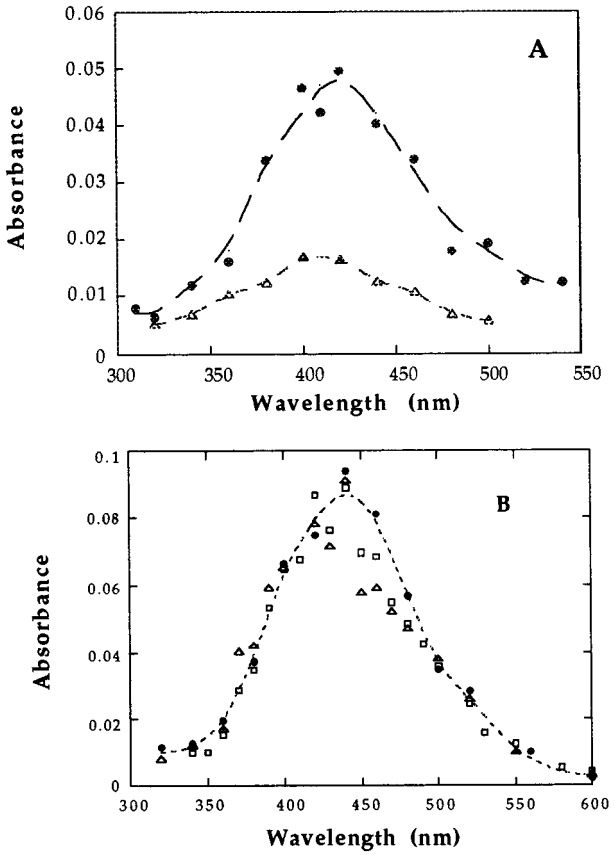

FIGURE 1: Absorption spectrum of disulfide radicals formed in lysozyme and thioredoxin following pulse radiolysis. (A) Lysozyme 250 μM, formate 0.1 M, phosphate 0.02 M, N_2O-saturated, optical path 2 cm. (●) pH 8.0, dose 8 ± 1 Gy, 100 μs after the pulse. (△) pH 4.5, dose 15 ± 1 Gy, 20 μs after the pulse. (B) Thioredoxin, D30A, and W35A measured 80 μs after the pulse at pH 7. Protein 100 μM (Trx, D30A, or W35A), formate 0.1 M, phosphate 0.02 M, N_2O-saturated, dose 10 ± 1 Gy, optical path 2 cm. (●) Trx; (△) W35A; (□) D30A.

$6800 \pm 250\ M^{-1}\ cm^{-1}$ (average over 15 measurements in pseudo-first-order conditions, 300 $\mu M \le [lys] \le 1.8$ mM, $[CO_2^{\bullet -}] \sim 3\ \mu M$).

In acidic medium (pH 4) the absorbance reaches a maximum in ca. 25 μs, followed by a rapid decrease. The spectrum obtained is typical of the protonated form of the disulfide free radical (maximum at ca. 400 nm) (Figure 1A) ($\epsilon_{400nm} = 1250 \pm 40\ M^{-1}\ cm^{-1}$). The absorbance at a fixed wavelength (420 nm) changes with pH following a sigmoidal relationship (Figure 2A). This phenomenon is attributed to the disulfide radical protonation equilibrium (reaction 2), established at the end of the build-up phase. Average extinction coefficients were determined at various pH values. They were fitted by a sigmoidal expression:

$$\epsilon_T = \frac{\epsilon_A 10^{-pH}}{10^{-pH} + K_A} + \frac{\epsilon_B K_A}{10^{-pH} + K_A}$$

In this expression ϵ_A is the extinction coefficient of the acidic form of the free radical $Lys/SSH^{\bullet}$ and ϵ_B is that of its basic form $Lys/SS^{\bullet -}$. The best fit is obtained for pK_a equal to 4.62 ± 0.5.

The kinetic analysis of the reduction reaction was performed with an excess of protein vs free radical (300 $\mu M \le$ [lys] $\le$ 1.8 mM, $[CO_2^{\bullet -}] \sim 3\ \mu M$), at several pH values

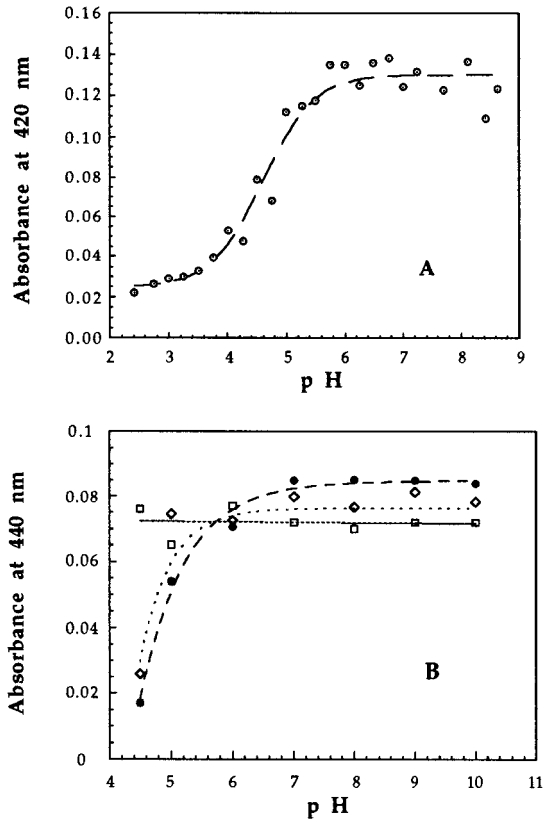

FIGURE 2: Variation in absorbance of disulfide free radicals in lysozyme and thioredoxin with pH. (A) Lysozyme 250 μM, formate 0.1 M, phosphate 0.02 M, N_2O-saturated, dose 40 Gy, wavelength 420 nm (B) Thioredoxin and mutants; protein 100 μM, formate 0.1 M, phosphate 0.02 M, N_2O-saturated, dose 11 Gy. (●) W35A; (◇) D30A; (□) Trx.

between ~4 and 11. At all pH values, whenever the pseudo-first-order conditions were not fulfilled, the kinetics were always consistent with the competition between reactions 1 and 9 (Scheme 1). At constant formate concentration, the rate constant $k_{1,lyz}$ appears to increase with decreasing pH. For instance, at 20 mM phosphate and 100 mM formate, we found $k_{1,lys} = (7.2 \pm 0.4) \times 10^8$ M^{-1} s^{-1} at pH 4.5 and $(2.66 \pm 0.3) \times 10^8$ M^{-1} s^{-1} at pH 8.

Free Radical Decay in Lysozyme. At several pH values between 4.5 and 9.5 it is possible to analyze the decay of lys/$SS^{\bullet -}$ as a sum of a first-order process and a second-order process. The second-order rate constant $2k_{lys}$ depends on the pH value [$2k_{lys} = (4.9 \pm 1.8) \times 10^6$ M^{-1} s^{-1} at pH 8.2, from an average of 20 measurements]. This rate constant does not depend on the irradiation dose nor the lysozyme concentration, but it does depend on salt (formate or sodium chloride) concentration, as a result of ionic strength effect. The first-order process has a rate constant of $k_{lys} = 24.7 \pm 13$ s^{-1} (from an average of 50 measurements). This rate constant does not depend on the pH value, the dose (5−50 Gy), the protein concentration, nor formate concentration. In acidic medium (pH ≤ 4), the radical disappearance occurs as a first-order reaction with a rate constant equal to $k_{lys} = (1.97 \pm 0.25) \times 10^4$ s^{-1}. Lysozyme and formate concentrations do not affect its value. Final products are the reduced protein, dimers, and fragments (*42*, *55*).

One-Electron Reduction of Oxidized Thioredoxin and Its Mutants. Upon pulse radiolysis of Trx, W35A, and D30A (protein 100 μM, phosphate buffer 20 mM, $[HCOO^-] = 100$ mM, dose = 10 ± 1Gy, $[CO_2^{\bullet -}] \approx 6$ μM), disulfide free radicals are created. At pH 8, the reaction is completed in ca. 100 μs in the three proteins. The absorption spectra of Trx/$SS^{\bullet -}$, W35A/$SS^{\bullet -}$, and D30A/$SS^{\bullet -}$ are shown in Figure 1B. They are made up of a broad band with a maximum at 440 nm for the three proteins, similar to lysozyme (Figure 1A). The molar extinction coefficient at 440 nm of the radical P/$SS^{\bullet -}$ and the rate constants of free radical formation at various pH between 5 and 9, were determined under pseudo-first-order conditions, as for lysozyme. Values at pH 8 are given in Table 1. They increase with decreasing pH, similar to lysozyme.

Table 1: Extinction Coefficients of Disulfide Radical Anions and Formation Rate Constants of Thioredoxin and Mutants D30A and W35A

protein	ϵ_{440nm} of the disulfide radical anion (mol^{-1} dm^3 cm^{-1})	k_1 at pH 4.5 (mol^{-1} dm^3 s^{-1})	k_1 at pH 8 (mol^{-1} dm^3 s^{-1})
thioredoxin	8200 ± 500	$(4.84 \pm 0.2) \times 10^8$	$(6.4 \pm 0.2) \times 10^8$
D30A	7800 ± 500	$(2.5 \pm 0.2) \times 10^9$	$(5.4 \pm 0.2) \times 10^8$
W35A	8900 ± 500	$(2.5 \pm 0.2) \times 10^9$	$(3.9 \pm 0.2) \times 10^8$

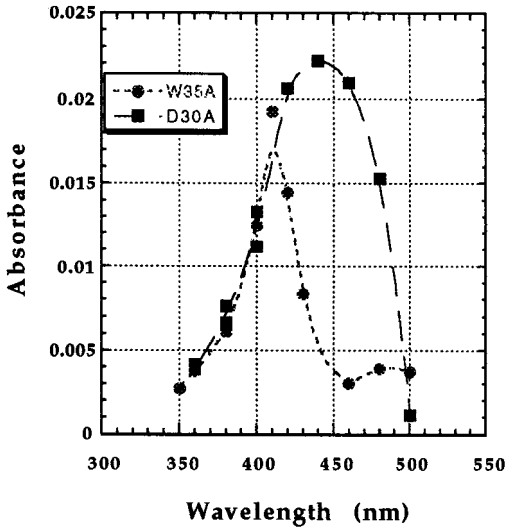

FIGURE 3: Absorption spectrum of disulfide radicals in D30A and W35A at pH 4.5. Protein 100 μM (Trx, D30A, or W35A), formate 0.01 M, phosphate 0.02 M, N_2O-saturated, dose 9 Gy, 20 μs after the pulse, optical path 2 cm. (●) W35A; (■) D30A.

In acidic medium (pH 4.5), a striking difference appears between the wild-type protein and the mutants. The absorption spectrum of Trx free radical and the rate constant of its formation (Table 1) do not change. The absorption spectra of W35A and D30A free radicals are shown in Figure 3. The transient spectrum for D30A is similar to that at pH 8−10 although the maximal absorbances are much smaller. That of W35A free radical is shifted to the UV region. It presents a relatively narrow band centered at 410 nm. The maximal absorbances at 440 nm are reported as a function of pH in Figure 2B for the three proteins. A drastic decrease of absorbances can thus be visualized for the mutants in acidic medium.

To calculate the rate constants of the free radical formation at pH 4.5, kinetic traces such as those shown in Figure 4 were fitted with a two-exponential function, to deconvolute formation from decay. The rate constants are reported in Table 1. They are higher than that of wild-type protein.

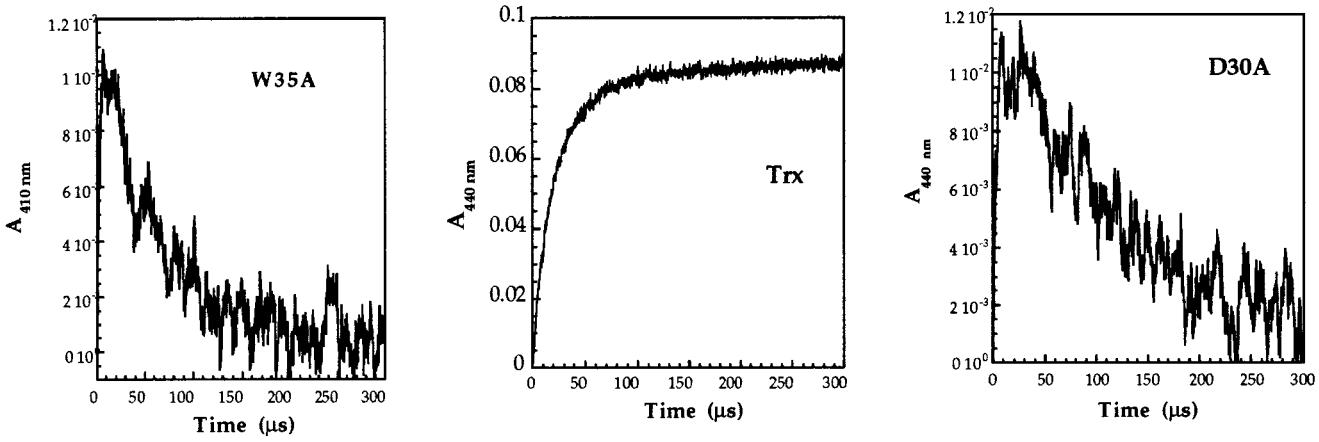

FIGURE 4: Kinetic traces of disulfide radical evolution in acidic medium up to 300 μs after the pulse. Protein 100 μM, formate 0.1 M, phosphate 0.02 M, N_2O-saturated, dose 10 ± 1 Gy, optical path 2 cm.

Table 2: Results of the Kinetic Analysis of Disulfide Radical Decay in the Proteins Studied

protein	pH	decay (rate constant)
lysozyme	≥5	second-order [$(4.9 \pm 1.8) \times 10^6$ M^{-1} s^{-1} at pH 8.2] first-order (24.7 ± 13 s^{-1})
	<5	first-order [$(1.97 \pm 0.25) \times 10^4$ s^{-1}]
Thioredoxin	4−10	second-order [$2k = (4.0 \pm 0.2) \times 10^5$ mol^{-1} L s^{-1} at pH 10] first-order (6.7 ± 0.2 s^{-1})
D30A	6−10	first-order (11 ± 1 s^{-1})
	4.5	first-order [$(5.7 \pm 1) \times 10^3$ s^{-1}]
W35A	6−10	first-order (56.3 ± 0.2 s^{-1})
	4.5	first-order [$(6.7 \pm 1) \times 10^3$ s^{-1}]

Free Radical Decay in Trx, W35A, and D30A. The disulfide free radical decay was examined for the three proteins between pH 4.5 and 10 and the results are summarized in Table 2. The processes appear markedly different: for thioredoxin, at all pH values, the main process is second-order with a contribution of a first-order reaction. For both mutants, the main processes are first-order at all pH values. The difference is even more striking in acidic medium. As examples, some kinetic traces at pH 4.5 are reported in Figure 4. The lifetime of the disulfide radical appears on the millisecond time scale for Trx whereas for both mutants the decay takes place at the microsecond time scale. The final products were detected by SDS−PAGE analysis with and without alkylation of thiol functions after steady-state γ radiolysis (*55*) and pulse radiolysis (this work). At all pH values, oxidized thioredoxin leads only to the reduced protein without any dimerization or fragmentation. This was confirmed by HPLC analysis (P. Deccotignies, Institut de Biotechnique der Plantes, Orsay, France). Conversely the two mutants undergo partial dimerization due to intermolecular disulfide bonds in alkaline medium.

DISCUSSION

The experimental results can be interpreted by use of the reaction set given in Scheme 1.

Disulfide Radical Formation. The reaction of $CO_2^{\bullet -}$ with the disulfide bond in all proteins leads to the formation of the disulfide radical (reaction 1). This rate constant is little affected by mutation. The rate constants are of the same order of magnitude (ca. 10^8 M^{-1} s^{-1}), although slower in Lys than in Trx at pH 7. The pH dependence of the reaction shows the same pattern as previously described with other proteins (*39*): this process is acid-catalyzed for both proteins.

At pH ≥ 7, the radical obtained is in its anionic form $P/SS^{\bullet -}$ for all proteins studied. Disulfide radical anion absorption spectra do not change much with the protein, except that one may notice a small shift in the maximum wavelength (425 nm in lysozyme, 440 nm in thioredoxin and mutants). Similarly, extinction coefficients for the free radicals are around ca. 5000−7000 M^{-1} cm^{-1}, reflecting a similar environment, probably of a polar nature.

Disulfide Anion Radical Decay. The free radical decays vary with the protein. For Trx, it is mostly due to reaction 5 (dismutation), as shown by the kinetic analysis and the final products. Dismutation does occur for lysozyme as well, but this step is not apparent for Trx mutants since no second-order component of the decay appears. The first-order component present in all radical decays, is attributed to reaction 3 (disulfide bond opening) for all proteins. In lysozyme it may also involve electron migration to the cleavage site, symbolized by reaction 10 (*42*).

At constant pH and protein and formate concentrations, the reduction yields are as follow: W35A <Trx < D30A < Lys, in the whole pH range (4−10) (*56*). These yields are strongly dependent on pH and protein and formate concentration. The chain length, defined as $G(-SH)/G(CO_2^{\bullet -})$, is equal to 1 for Trx and W35A at pH ≥ 7, indicating that the chain does not propagate. Thus reaction 3 drives different processes according to the protein. For Trx and W35A, since in this pH region there is no chain, reaction 6 (formate oxidation) does not take place. Opening of the disulfide bond can only lead to dimerization (reaction 7). Since no dimer is found for Trx, we suggest that they are reduced by $Trx/(SH)_2$:

$$Trx/(SH)_2 + (HS)Trx\ SS\ Trx(SH) \rightarrow Trx/SS + 2\ Trx/(SH)_2$$

Free Radical Protonation. The major difference between Lys and Trx appears to be the free radical protonation. In

lysozyme this protonation is clearly observed by the change of absorption spectrum in acidic medium (Figure 1A). The free radical pK_a for Lys is equal to 4.62, assuming that the acido-basic thermodynamic equilibrium is settled even in acidic medium.

All experimental results agree in demonstrating that the disulfide radical protonation does not take place in thioredoxin: (i) the absorption spectrum does not change in acidic medium, (ii) the decay kinetics is the same (mostly second-order process), and (iii) the final compounds are the same (reduced protein only). Obviously the pK_a of the Trx/SSH$^{\bullet}$ − Trx/SS$^{\bullet -}$ couple is below 3. This absence of protonation cannot be attributed to solvent inaccessibility, thus to kinetic control. We propose a thermodynamic modulation of the protonation equilibrium constant due to structure, similarly to what happens to Cys36 in the reduced protein (*26*).

D30 and W35 act in this protonation, however, in different ways. In acidic medium, W35A free radical absorption spectrum is shifted to the UV region and the free radical decay is accelerated, like for lysozyme. Both facts are consistent with formation of protonated disulfide radical. The first-order decay would then be attributed to reactions 4 and 6, reaction 6 being much faster than reaction 4 since we do not see the appearance of the thiyl radical, characterized by an absorbance maximum around 330 nm.

D30A free radical absorption spectra have similar shapes in basic and acidic media, but the free radical decay is as fast as for W35A. We propose that reaction 2 (protonation of the free radical) happens slowly and is followed by reaction 4, which is much faster. Reaction 2 would be the rate-determining step whose rate constant is in Table 2.

Redox Properties of Thiyl/Thiol Couple. The chain length is ca. 10-fold higher for Lys than for Trx and mutants at all pH values (*56*). In addition, in basic medium the reaction is no longer a chain for Trx and W35A (*56*). However, thiyl radicals are formed, since we do observe dimers for W35A and since decays always have first-order steps that cannot be interpreted without reaction 3. It seems that thiyl radicals do not react with formate. Two interpretations can be proposed: either the thiyl radical is located on Cys39, which seems to have a low reactivity, perhaps due to inaccessibility, or it is located on Cys36 which is reactive in biological conditions, but the one-electron reduction potential of the thiyl/thiol couple is much lower than that of COO$^{\bullet -}$/HCOO$^-$ couple [1.07 V at pH 7 (*45*)]. For comparison, the reduction potential of GS$^{\bullet}$/GSH couple is believed to be equal to 0.92 V (*45*), thus slightly lower than that of formate. Yet the chain length is high at all pH values for glutathione as it is for lysozyme (*41*, *56*) due to the velocity of reaction 1, which drives reaction 6 out of equilibrium. In our case a total absence of propagation would be consistent with a reduction potential very low, probably lower than ca. 0.5 V, making the displacement of equilibrium 6 very difficult.

Some Roles of W35 and D30. The aim of this study was to get some insight on the modulation of the one-electron redox properties of thioredoxin by sequence. The originality of this protein is indeed enlightened by the use of our experimental conditions. We show that, in the wild-type protein, the free radical species, disulfide and thiyl, are both rather unreactive compared to other proteins such as lysozyme. This lack of reactivity is indeed a key aspect of the biological properties of the enzyme: side effects such as pro-oxidant action of thiyl radicals are thus avoided. It may have a structural, i.e., kinetic origin, but a thermodynamic reason seems highly probable.

D30 and W35 do control the thioredoxin redox properties, although in different ways. W35 removal (i) makes the thiol groups unreactive toward Ellman's reagent (*56*); (ii) allows fast protonation of the disulfide radical, and (iii) allows dimerization of the thiyl radical and/or makes the protein unreactive toward its own dimer. D30 removal (i) allows protonation of the disulfide radical, although probably too slowly to reach thermodynamic equilibrium, (ii) renders the protein more oxidant toward formate ions, and (iii) also allows dimerization of the thiyl radical and/or makes the protein unreactive toward its own dimer.

Thus D30 and W35 residues participate in giving thioredoxin very unusual redox properties, different from those of glutathione. These redox properties are potentially very important to the signal transduction function of Trx. Many transcription factors (most notably AP1 and NF-κB) display a DNA binding activity that is abolished by oxidation of critical Cys residues (*57*). DNA binding is enhanced by reduction through either glutathione or the thioredoxin system. Thus such mutations have impacts upon signal transduction since they act in a series of redox-sensitive sulfhydryl switches that govern activation of nuclear transcription factors.

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