Thioredoxin. A Localized Conformational Change Accompanying Reduction of the Protein to the Sulfhydryl Form*

Lubert Stryer, Arne Holmgren, and Peter Reichard

ABSTRACT: Thioredoxin is a low molecular weight protein which acts as a hydrogen donor in the enzymatic reduction of ribonucleoside diphosphates. The oxidized form, which contains two tryptophan residues and one disulfide bridge, is reduced by reduced triphosphopyridine nucleotide (TPNH) and a specific enzyme, thioredoxin reductase, to the sulfhydryl form. Reduction led to a 2.5-fold increase in the quantum yield of the tryptophan emission of thioredoxin, whereas the wavelength of the emission maximum was unaltered. The fluorescence polarization of a dimethylaminonaphthalenesulfonyl derivative of thioredoxin did not change on reduction of the protein, nor was there any appreciable difference in the optical rotatory dispersion or circular dichroism of the oxidized and reduced forms. These findings suggest that the conformational change which accompanies the reduction of thioredoxin is localized in nature and that one or both tryptophan residues are located near the active site of the protein.

Thioredoxin is a protein of molecular weight 12,000, which acts as the hydrogen donor in the enzymatic reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates in *Escherichia coli* (Laurent *et al.*, 1964). The protein contains two tryptophan residues and one disulfide bridge. This oxidized form of thioredoxin (thioredoxin-S_2) is reduced by TPNH[1] and a specific enzyme, thioredoxin reductase (Moore *et al.*, 1964), to the sulfhydryl form (thioredoxin-$(SH)_2$) (reaction 1).

$$\text{thioredoxin-}S_2 + \text{TPNH} + H^+ \longrightarrow \text{thioredoxin-}(SH)_2 + \text{TPN}^+ \quad (1)$$

The sulfhydryl form then reduces a ribonucleotide (*e.g.*, CDP) in the presence of two additional protein fractions from *E. coli* enzymes B1 and B2 (Holmgren *et al.*, 1965) (reaction 2).

$$\text{thioredoxin-}(SH)_2 + \text{CDP} \longrightarrow \text{thioredoxin-}S_2 + \text{deoxyCDP} \quad (2)$$

Thus thioredoxin can be regarded as a cofactor in ribonucleotide reduction; it is alternately reduced by thioredoxin reductase and oxidized by enzymes B1 and B2.

A spectrofluorimetric and spectropolarimetric study of the reduced and oxidized forms of thioredoxin was carried out to determine whether any conformational changes accompany the reduction of thioredoxin-S_2. The reduction was carried out either enzymatically according to reaction 1 or chemically by dithioerythritol. The present studies suggest that reduction is accompanied by a localized conformational change near the active site involving one or both tryptophan residues of the protein.

Experimental Section

Materials. TPNH and DTE were obtained from Sigma. Potassium phosphate buffer (0.1 M, pH 6.8) was used throughout this study. Two different preparations of thioredoxin were used. Most of the experiments reported here were carried out with material of an estimated purity of 85% prepared according to Laurent *et al.* (1964). A second preparation of better than 96% purity was obtained by preparative polyacrylamide electrophoresis (Jovin *et al.*, 1964; A. Holmgren and P. Reichard, to be published). This material was used to check the results obtained with the less pure preparation. Both preparations were lyophilized with a tenfold excess (by weight) of mannitol. Lyophilization and storage of the protein did not decrease its activity, as measured in reaction 1.

Thioredoxin reductase was prepared according to Moore *et al.* (1964), as modified by L. Thelander (to be published). This enzyme was shown to be homo-

* From the Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California, and the Department of Chemistry II, Karolinska Institute, Stockholm, Sweden. *Received November 18, 1966.* Supported by research grants from the National Institutes of Health (GM-11783-02) and the National Aeronautics and Space Administration (NGR-05-020-137) to L. S. and by the National Institutes of Health (CA-7897-02), the Damon Runyon Foundation (DRG 470G), and the Swedish Medical Research Council (B66-57) to P. R.

[1] Abbreviations used: TPNH, reduced triphosphopyridine nucleotide; TPN^+, oxidized triphosphopyridine nucleotide; DTE, dithioerythritol; dansyl, 1-dimethylamino-5-naphthalenesulfonyl; $[M']$, mean residue rotation; $[\theta]$, mean residue ellipticity; q, fluorescence quantum yield; CDP, cytidine diphosphate.

geneous.

Dansylation of Thioredoxin. Thioredoxin (0.023 μmole), containing 4 mg of mannitol, was dissolved in 0.15 ml of buffer. 1-Dimethylamino-5-naphthalenesulfonyl chloride (0.015 ml of a 10-mg/ml solution in acetone) was added slowly and the mixture was shaken at room temperature for 4 hr and then kept at 4° overnight. After centrifugation, the supernatant solution was passed through a column of Sephadex G-25 (0.24 × 18 cm), which was equilibrated with buffer. Fractions containing the dansylated protein were localized by their fluorescence, combined, and used for the fluorescence polarization experiments. From the optical density at 340 and 280 mμ it was estimated that an average of two dansyl groups were attached to each molecule of thioredoxin.

Reduction of Thioredoxin. ENZYMATIC REDUCTION. Thioredoxin (2.6 mμmoles) and TPNH (8.5 mμmoles) were dissolved at 5° in 3 ml of buffer. The reduction started on addition of 1.4 μg of thioredoxin reductase and was followed by the decrease in the fluorescence of TPNH at 460 mμ, on excitation at 365 mμ. In a separate experiment under identical conditions, the increase in the fluorescence at 342 mμ on excitation at 280 mμ was measured.

CHEMICAL REDUCTION. Thioredoxin (4.5 mμmoles) was dissolved at 5° in 3 ml of buffer. Dithioerythritol (0.01 ml of a 0.5 M solution) was added and the increase in fluorescence at 342 mμ on excitation at 280 mμ was measured. The reaction was complete after 15 min. In a fluorescence polarization experiment, 5.0 mμmoles of dansylated thioredoxin, dissolved in 0.28 ml of buffer, was reduced by the addition of 1 μl of 0.5 M dithioerythritol.

Fluorescence. Fluorescence emission spectra were obtained on a recording spectrofluorimeter (Stryer, 1965). The intrinsic fluorescence of thioredoxin was excited at 280 mμ. The emission was focused on a Bausch and Lomb grating monochromator, Model 33-86-45-69, operated at a half-band-width of 3.3 mμ. The amplified output of an RCA 1P21 phototube was recorded on a Varian X-Y recorder which was coupled to the emission monochromator wavelength drive. The emission spectra shown in Figure 1 are the direct recorder tracings which have not been corrected for the variation with wavelength in the sensitivity of the detection system. The relative sensitivity of the detection system was found to 0.94, 1.13, 1.15, 1.18, 1.06, 1.04, 0.98, 0.90, and 0.76 at 10-mμ intervals from 320 to 400 mμ. The absolute quantum yield was determined by integrating the emission spectrum, using L-tryptophan in water as a standard of quantum yield 0.20.

A direct measure of the fluorescence polarization was obtained by use of a ratio amplifier (Stryer, 1965). The sample in a 2-mm path-length cell was excited with x-polarized light, and the emission was observed at right angles by two phototubes, one admitting x-polarized light, the other y-polarized light. The ratio of these emissions, $r = F_x/F_y$, is related to the customary measure of polarization p by the expression $p = (F_x - F_y)/(F_x + F_y) = (r - 1)/(r + 1)$. The light output of a mercury arc filtered by a Corning CS 7-60 filter and a Polaroid type HNB sheet polarizer was used to excite the emission of the dansyl chromophore attached to thioredoxin. The emission was isolated by a Corning CS 3-71 filter.

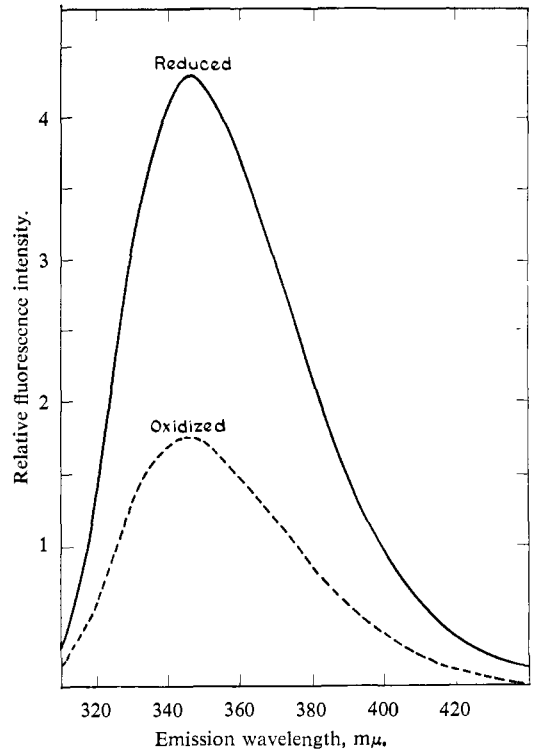

FIGURE 1: Fluorescence emission spectra of the oxidized (- - - -) and reduced (———) forms of thioredoxin. The fluorescence was excited at 280 mμ. There is a 2.5-fold increase in quantum yield upon reduction of thioredoxin, whereas the emission maximum is unaltered. The corrected emission peak is at 342 mμ. Spectra were taken at 5°.

Optical Rotatory Dispersion and Circular Dichroism. A Durrum-Jasco ORD/UV5 instrument was used to measure optical rotatory and circular dichroism spectra. The cell path was 1 cm and the protein concentration was 0.16 mg/ml. The mean residue weight of thioredoxin is 115. $[M']$ is the mean residue rotation in degrees per centimeters squared per decimole. The mean residue ellipticity $[\theta]$ is defined as $[\theta] = (2.303 \cdot (4500)/\pi)(\epsilon_L - \epsilon_R)$, where $(\epsilon_L - \epsilon_R)$ is the difference in extinction coefficient (expressed in centimeters squared per millimole) for left and right circularly polarized light. The units of $[\theta]$ are degrees per centimeters squared per decimole.

Results

Fluorescence Emission Spectra and Quantum Yields. Oxidized thioredoxin shows a corrected emission maximum at 342 mμ (Figure 1), indicative of fluores-

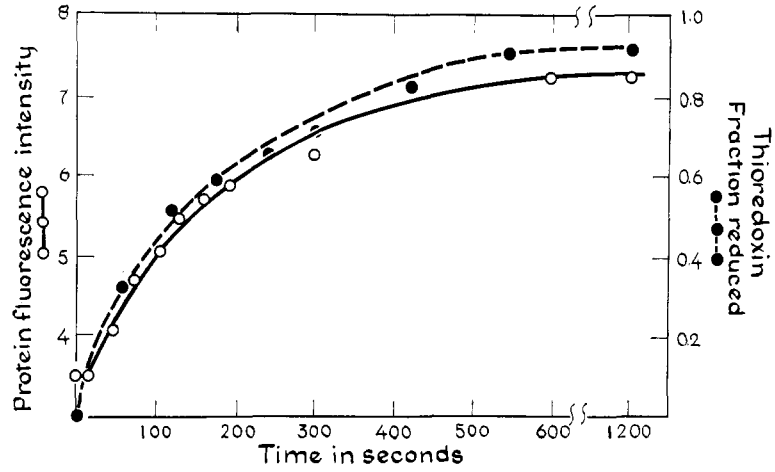

FIGURE 2: Kinetics of the enzymatic reduction of thioredoxin (- - - -) and of the increase in quantum yield of fluorescence (———).

cence from the tryptophan residues of the protein. Reduction of the disulfide bond leads to a striking change in the quantum yield of fluorescence, while the wavelength of the emission maximum remains the same (Figure 1). The quantum yield of emission of oxidized thioredoxin is 0.04, while that of reduced thioredoxin is 0.10, on excitation at 280 mμ. The ratio of the fluorescence intensities of the oxidized and reduced forms is constant for excitation wavelengths between 270 and 300 mμ. Reduction of thioredoxin by excess DTE gave the same increase in quantum yield as reduction by TPNH in the presence of thioredoxin reductase. The effectiveness of DTE in completely reducing thioredoxin was confirmed by the observation that the chemically reduced material no longer reacted with TPNH in the presence of thioredoxin reductase.

The time course of increase in quantum yield closely paralleled the kinetics of reduction (Figure 2). The extent of reduction of thioredoxin by the enzymatic system was determined fluorimetrically by following the conversion of TPNH to TPN^+.

Fluorescence Polarization. The fluorescence polarization of the dansyl chromophore was measured for the oxidized and reduced forms of thioredoxin which contained an average of two covalently attached dansyl groups per protein molecule, based on the absorbance at 280 and 340 mμ. Dansylation of oxidized thioredoxin did not interfere with its capacity to be reduced either by DTE or by thioredoxin reductase. For the fluorescence polarization measurements, the dansylated enzyme was reduced by DTE to avoid a significant background fluorescence from excess TPNH.

The fluorescence polarization of the oxidized form of dansylated thioredoxin was appreciably lower than the limiting polarization that would be observed in the absence of molecular rotation (Table I). Thus, the fluorescence polarization of the attached dansyl group should be sensitive to either an increase or decrease in the rotational relaxation time of thioredoxin. The significant finding is that the fluorescence polarization of dansylated thioredoxin was virtually unaltered on reduction of the disulfide bond (Table I).

TABLE I: Fluorescence Polarization of Dansylated Thioredoxin.[a]

	p
Oxidized thioredoxin	0.256 ± 0.003
Reduced thioredoxin	0.253 ± 0.003
Limiting polarization (p_0)	0.364 ± 0.003

[a] The polarization of oxidized and reduced thioredoxin in 0.1 M phosphate buffer (pH 6.8) was measured at 5°. The limiting polarization was the asymptotic value obtained on measuring the fluorescence polarization of reduced thioredoxin in a 2:1 glycerol–water mixture between −5 and 20°.

Optical Rotatory Dispersion and Circular Dichroism. The optical rotatory dispersion curves of the oxidized and reduced forms of thioredoxin are quite similar between 220 and 300 mμ (Figure 3). For these experiments, thioredoxin was reduced with DTE to avoid an optical density and optical rotatory contribution from TPNH and TPN^+. Both forms of thioredoxin showed a trough at 234 ± 2 mμ and a crossover point at 223 ± 2 mμ. The mean residue rotation [M'] of the oxidized form was −2050 ± 100, while [M'] of the reduced form was −2250 ± 100.

The circular dichroism of the oxidized and reduced forms of thioredoxin between 215 and 300 mμ were nearly superimposable (Figure 3). A minimum was observed at 221 ± 2 mμ, where the ellipticity was −5000 ± 1000 deg cm^2/dmole.

Discussion

The large increase in fluorescence quantum yield accompanying reduction of thioredoxin indicates that there is a significant change in the environment of one or both tryptophan residues when the disulfide bond is reduced to give the sulfhydryl form of thioredoxin. This conformational change could either be localized to the region of the protein containing the tryptophan residues or it could be more extensive. *An over-all conformational transition on reduction of thioredoxin seems unlikely since little change was observed in the optical rotatory and fluorescence polarization spectra.* The similarity of the optical rotatory dispersion and circular dichroism spectra of the oxidized and reduced forms of thioredoxin indicates that there is no appreciable change in the α-helical content upon reduction. It is likely that a change in the helicity of five residues would have been detected. The values obtained here for $[M']_{233}$ and for $[\theta]_{222}$ suggest that thioredoxin has a low α-helical content (Carver *et al.*, 1966; Holzwarth and Doty, 1965). The small differences in the optical rotatory and circular dichroism spectra of the oxidized and reduced forms may reflect the rotatory contribution of the disulfide chromophore (Beychok, 1966). A second finding, based on the constancy of the fluorescence polarization of dansylated thioredoxin in the oxidized and reduced forms, is that the rotational relaxation time of the protein is unaffected by reduction of the disulfide bond. This observation suggests that reduction of thioredoxin does not lead to a large change in the shape or flexibility of the molecule. The fluorescence of the dansyl chromophore attached to thioredoxin is only partially polarized, and so fluorescence polarization would have revealed large-scale structural changes if they had occurred. In addition, in a previous experimental study (Laurent *et al.*, 1964), there was no detectable difference in the sedimentation coefficient of the oxidized and reduced forms of thioredoxin. The absence of any change in the translational frictional coefficient also suggests that there is no gross conformational change on reduction of thioredoxin. These findings lead to the conclusion that *there is a localized conformational change on reduction of thioredoxin. Furthermore, one or both tryptophan residues are located near the active site of the protein.*[2]

The 2.5-fold increase in quantum yield on reduction of thioredoxin is one of the largest observed fluorescence changes accompanying a conformational change in a protein. It should be noted that the present data do not reveal whether the increased quantum yield arises from a change in environment of one or both of the tryptophan residues in the enzyme. A study of the nanosecond kinetics of the tryptophan emission might distinguish between these alternatives.

[2] This conclusion is supported by the recent unpublished work of A. Holmgren on the amino acid sequence of thioredoxin. A decapeptide obtained in 40% yield from a partial pepsin digest contains two tryptophans and two half-cystines.

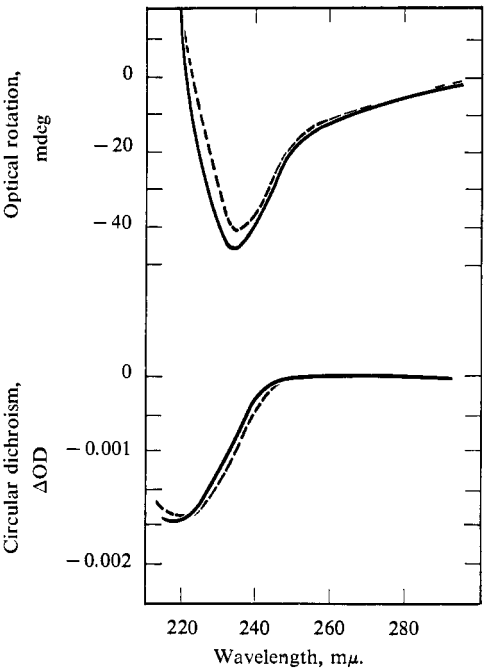

FIGURE 3: Optical rotatory dispersion and circular dichroism of the oxidized (– – – –) and reduced (———) forms of thioredoxin. Spectra were taken at 25°.

The sensitivity of the quantum yield of fluorescence to the structural change accompanying reduction of thioredoxin is related to the low quantum yield, q = 0.04, of the oxidized form. This value should be compared with the quantum yield of L-tryptophan in water, q = 0.20, and the yields of a series of tryptophan containing proteins for which q ranges from 0.038 to 0.152 (Teale, 1960). These values of q are based on over-all quantum yield at 280 mμ. When corrected for the fractional absorption of tryptophan at 280 mμ, q' ranges from 0.05 to 0.475 for these proteins, while for oxidized thioredoxin, q' = 0.052. On reduction, the quantum yield of thioredoxin increases to a value more typical of proteins that contain tryptophan. It is evident that the conformational change accompanying reduction of thioredoxin abolishes a strong quenching interaction present in the oxidized protein.

The fluorescence of tryptophan can be quenched in a number of ways. Proton-transfer reactions may occur during the excited-state lifetime (Weber, 1961). Loss of the ring NH proton probably gives rise to a nonfluorescent species. Alternatively, a proton may be transferred to the excited ring. The recent finding of a deuterium isotope effect on the quantum yields of tryptophan and indole suggests that proton transfer is an important mechanism of quenching (Stryer, 1966). In addition, hydrogen bonding between the imino hydrogen of the indole nucleus and an acceptor such as the carbonyl oxygen of the peptide backbone has been implicated as an interaction that results in quenching (C. H. Suelter and G. Weber, to be published).

Finally, it seems likely that interactions between adjacent tryptophan residues tend to lower the quantum yield, since a block copolymer of lysine and tryptophan has a lower quantum yield than the randomly sequenced copolymer (Fasman *et al.*, 1966). It is not yet possible to determine which of these interactions is altered when thioredoxin is reduced.

In contrast to the quantum yield, the wavelength of the emission maximum is the same for the oxidized and reduced forms of thioredoxin. The emission maximum of tryptophan is known to be sensitive to the polarity of its environment. In native proteins, the emission maximum ranges from 328 mμ, indicative of a nonpolar environment, to 342 mμ, for a highly polar environment (Teale, 1960; Green, 1964). The emission maximum of thioredoxin, 342 mμ, reveals that its tryptophan residues are in a distinctly polar environment. The constancy of the emission maximum on reduction indicates that the polarity of the environment of the tryptophan residues is unaffected by the conformational change.

This study emphasizes the usefulness of fluorescence techniques in investigating certain types of conformational changes in proteins. The recent observation of fluorescence changes accompanying the binding of inhibitors to lysozyme shows that the intrinsic fluorescence of proteins is sensitive to small conformational changes at the active site if tryptophan residues are located in that region (Lehrer and Fasman, 1966). In lysozyme, it is known from X-ray studies that the binding of inhibitors does not lead to gross structural changes (Johnson and Phillips, 1965).

The conformational change accompanying the conversion of oxidized to reduced thioredoxin may affect the binding affinity of thioredoxin to other proteins. Oxidized thioredoxin must be bound by thioredoxin reductase in order to be converted to the reduced form. In turn, reduced thioredoxin must interact with the B1 and B2 enzymes. The binding sites of oxidized and reduced thioredoxin necessarily overlap to an appreciable extent, since the disulfide or sulfhydryl group is common to both. Preferential binding of the oxidized form of thioredoxin to thioredoxin reductase would be favored by a conformational difference between the oxidized and reduced forms of thioredoxin apart from the state of the disulfide bond. Similarly, such a conformational change might favor preferential binding of the reduced form to the B proteins. Studies on the binding affinities of these proteins are needed to test this hypothesis.

Acknowledgment

Peter Reichard wishes to thank Dr. Arthur Kornberg and other members of the Department of Biochemistry at Stanford Medical School for the hospitality extended to him during his sabbatical visit.

References

Beychok, S. (1966), *Science 154*, 1288.

Carver, J. P., Schechter, E., and Blout, E. R. (1966), *J. Am. Chem. Soc. 88*, 2562.

Fasman, G. D., Bodenheimer, E., and Pesce, A. (1966), *J. Biol. Chem. 241*, 916.

Green, N. M. (1964), *Biochem. J. 90*, 564.

Holmgren, A., Reichard, P., and Thelander, L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 830.

Holzwarth, G., and Doty, P. (1965), *J. Am. Chem. Soc. 87*, 218.

Johnson, L. N., and Phillips, D. C. (1965), *Nature 206*, 761.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9*, 351.

Laurent, T. C., Moore, E. C., and Reichard, P. (1964), *J. Biol. Chem. 239*, 3436.

Lehrer, S. S., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 133.

Moore, E. C., Reichard, P., and Thelander, L. (1964), *J. Biol. Chem. 239*, 3445.

Stryer, L. (1965), *J. Mol. Biol. 13*, 482.

Stryer, L. (1966), *J. Am. Chem. Soc. 88*, 5708.

Teale, F. W. J. (1960), *Biochem. J. 76*, 381.

Weber, G. (1961), *in* Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, pp 81–107.