Spectroscopic Characterization of Thioredoxin Covalently Modified with Monofunctional Organoarsenical Reagents†

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ABSTRACT: Thioredoxin upon reduction with mercaptoethylamine was subjected to covalent modification by the monofunctional organoarsenical reagents $H_2NPhAsO$ and $HO(CH_2)_4AsCl_2$. The degree of modification was monitored by the percentage loss in free thiol content as measured by the reaction with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid). The modification results in the formation of a stable 15-membered cyclic dithioarsenite ring that readily extrudes the arsenic moiety upon the addition of 2,3-dimercaptopropanol. The conformational effects of this modification were monitored by steady-state fluorescence and circular dichroism. On the basis of circular dichroic spectra, it appeared that the protein experiences no significant backbone conformational change from this modification. The degree of conformational change was found to be within the range observed upon reduction of the oxidized thioredoxin. Steady-state fluorescence revealed that the arsenicals caused strong quenching of the tryptophan fluorescence. Stern–Volmer titrations revealed that the quenching was a function of both the nature of the organic group and its covalent attachment to the "spatially close" thiols. The analysis of the spectroscopic results obtained with the arsenical reagents provides further insight into the nature of the conformational change that has been observed upon reduction of thioredoxin.

Thioredoxin is a small ubiquitous protein with a M_r of 11 700 (Lim et al., 1985), containing a redox-active cystine on an exposed β-turn (Holmgren et al., 1975). Thioredoxin can occur in the oxidized (thioredoxin-S_2) or reduced [thioredoxin-$(SH)_2$] state and functions as an electron transport protein between NADPH[1] and the reduction of ribonucleotides to deoxyribonucleotides via ribonucleotide reductase and thioredoxin reductase (Thelander & Reichard, 1979), as well as other reductive processes (Holmgren, 1985). Its primary sequence of 108 amino acid residues as well as its X-ray structure to 2.8-Å resolution are known (Holmgren, 1968; Holmgren et al., 1975). Thioredoxin contains two tryptophan residues, Trp-28 and Trp-31, which are found in the region of the active-site disulfide residues, Cys-32 and Cys-35, and provide an ideal intrinsic probe to study the active site by fluorescence. Upon reduction of the disulfide bond the quantum yield of tryptophan fluorescence increases markedly (Stryer et al., 1967; Holmgren, 1972), which is indicative of a localized change in the microenvironment of the tryptophan residues. Thioredoxin isolated from *Saccharomyces cerevisiae* has a single tryptophan in a position homologous to Trp-31 in *Escherichia coli* and shows only a 20% increase in quantum yield upon the reduction of the disulfide bond (Holmgren, 1972). The author concluded that the reduction of thioredoxin-S_2 in *E. coli* has its main effect on the fluorescence emission of Trp-28.

Reduction of a disulfide bond may yield spatially close thiols that are readily modified covalently by trivalent organoarsenical reagents of the form R—As=O (or R–AsX_2), where R is an organic group and X is a halide ion (Stocken & Thompson, 1949; Stevenson et al., 1978). These reagents have been applied to enzyme systems such as 2-ketoacid dehydrogenase multienzyme complexes (Stevenson et al., 1978; Adamson & Stevenson, 1981; Adamson et al., 1984, 1986; Holmes & Stevenson, 1986), lipoamide dehydrogenase (Danson et al., 1986), mitochondrial coupling factors (Sanaoi, 1982), transport systems (Frost & Lane, 1985), pyridine nucleotide transhydrogenase (Voordouw et al., 1981), and adenylate cyclase (Drummond, 1981). These reagents form stable cyclic dithioarsenites that are readily reversed on the addition of small molecular weight dithiol reagents such as 2,3-dimercaptopropanol (Whittaker, 1947; Stocken & Thompson, 1946).

Our main objective was to establish a means by which we could monitor the reduction of thioredoxin-S_2 and its subsequent reversible covalent modification, and hence inactivation, using monofunctional organoarsenicals. We also wanted to determine whether or not, upon modification, there would be a perturbation of the secondary and tertiary structure of thioredoxin. As evaluation of the effect of chemical modification on the structure of thioredoxin is a necessary prerequisite for studies involving thioredoxin chemically modified with a bifunctional reagent (e.g., $BrCH_2CONHPhAsO$) such that this form of thioredoxin (thioredoxin $\begin{matrix}\ulcorner S \urcorner \\ \llcorner S \lrcorner\end{matrix}$ As-$PhNHCOCH_2Br$) could function as an active-site-directed inactivator for thioredoxin reductase and ribonucleotide reductase in a manner similar to pyruvate dehydrogenase multienzyme complex (Adamson et al., 1984, 1986; Holmes & Stevenson,86).5 μ In this study we have monitored the inhibition of thioredoxin by measuring the loss of activity and

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[1] Abbreviations: Å, angstrom; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; M_r, molecular weight; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; Tris, tris(hydroxymethyl)aminomethane; $H_2NPhAsO$, *p*-aminophenyl arsenoxide; $HO(CH_2)_4AsCl_2$, (4-hydroxybutyl)dichloroarsine.

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also by observing the changes in the spectroscopic properties of the protein. Fluorescence, fluorescence quenching, and circular dichroism in the near and far ultraviolet have provided useful insights into the nature of the conformational change observed upon reduction of thioredoxin.

Experimental Procedures

Materials

2,3-Dimercaptopropanol was obtained from Sigma Chemical Co. Mercaptoethylamine and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Pierce Chemical Co. L-Tryptophan and L-tyrosine were from Calbiochem. 1,4-Dibromobutane and arsenic trioxide were obtained from Aldrich Chemical. *E. coli* K-12, $^3/_4$ log phase, enriched medium was obtained as a frozen paste from Grain Processing Co., Muscatine, IA. Thioredoxin-S_2 (salt free) was purchased from MICO (U.S.A.) Inc., New York (agent for IMCO, Stockholm, Sweden). All other chemicals were obtained from Fisher Chemicals unless otherwise noted. All aqueous solvents were prepared with deionized H_2O by using Fisher ion-exchange cartridges with the house distilled water. All experiments were performed at 20 °C unless otherwise noted.

Methods

Syntheses. (*A*) *Dihydrolipoamide.* The synthesis of dihydrolipoamide was as described by Reed et al. (1958).

(*B*) *p-Aminophenyl Arsenoxide.* The synthesis of *p*-aminophenyl arsenoxide was as described by Stevenson et al. (1978).

(*C*) *(4-Hydroxybutyl)dichloroarsine.* The procedure followed was similar to that of Banks et al. (1947). 1,4-Dibromobutane (200 mmol) in 5 mL of 95% ethanol was added slowly at room temperature to a stirred solution of arsenic trioxide (40 mmol) in 30 mL of 10 M NaOH. The mixture was refluxed until titration of aliquots with standard iodine solution indicated that the reaction was complete. Volatile organic residues were removed by vacuum distillation (water aspirator), and the residual solution was adjusted to pH 9.0 with concentrated HCl. A precipitate was removed, and the solution was further acidified to pH 3.0 with concentrated HCl. When the solution was cooled to 4 °C, a precipitate of (4-hydroxybutyl)arsonic acid was obtained. The infrared spectrum of (4-hydroxybutyl)arsonic acid agreed well with the published data for butylarsonic acid (McBrearty et al., 1968). Reduction of the arsonic acid to the dichloroarsine was obtained by dissolving the arsonic acid in concentrated HCl, containing a trace of potassium iodide, and bubbling the solution with SO_2 for 4 h. A yellow oil layer was removed and extracted with diethyl ether. The ether was evaporated, and the yellow oil was extracted and later recovered from dichloromethane. The product $HO(CH_2)_4AsCl_2$ was characterized by infrared spectroscopy (McBrearty et al., 1968; Petit & Turner, 1967) (CH_2, 2952, 2924, 2868, 1454, and 805 cm^{-1}; C–OH, 1062 cm^{-1}; C–As, 603 cm^{-1}; and AsCl, 395 and 365 cm^{-1}) and mass spectroscopy (m_1 201, m/e 183, 166, 131, 127, 117, 101, 89, and 75). Mass spectroscopy revealed that the dichloroarsine was susceptible to hydrolysis with the formation of a chlorohydroxyarsine. The chemical reactivities of these two arsine derivatives are very similar (Doak & Freedman, 1970).

Concentration Determination of $HO(CH_2)_4AsCl_2$. A stock solution of this reagent was prepared in 95% ethanol. The concentration was determined by preparing a series of 10-fold serial dilutions in 95% ethanol and analyzing an aliquot (20 μL) of each dilution. Dihydrolipoamide (20 nmol in 10 μL of 95% ethanol) was added to 960 μL of 20 mM potassium phosphate, pH 7.0, and incubated with 20 μL of arsenical taken from the serial dilutions. The trivalent arsine readily forms a six-membered cyclic dithioarsenite complex with reduced lipoamide (Whittaker, 1947). Excess (uncomplexed) dihydrolipoamide, which was present in some dilutions, was assayed by using DTNB and monitoring the production of the mercaptonitrobenzoate anion at 412 nm. In this case, 1 mol of DTNB reacted with 1 mol of dihydrolipoamide to yield 1 mol of oxidized lipoamide and 2 mol of mercaptonitrobenzoate (Brown & Perham, 1976). The concentration of the arsenical in the stock solution was calculated via this indirect but accurate method.

Protein Preparation Thioredoxin was isolated from *E. coli* BH2012/pCJF4 (Lim et al., 1985), a superproducing strain containing the thioredoxin gene cloned in the *lac* promoter, pUC13, and possessing about 300–500 copies per cell. This strain was kindly donated by J. A. Fuchs of the University of Minnesota. Cells were grown in 100 L of rich medium (Luria Broth) using a New Brunswick Gen II fermenter for 10 h with moderate aeration (50 L/min) at 33 °C, pH 7.0. Cells were harvested at 10 °C by tangential flow filtration, followed by centrifugation at 10000*g*, to yield approximately 1700 g of wet paste which was stored at −20 °C. Frozen cell paste (500 g) was soaked overnight in 1 L of 50 mM Tris, pH 8.0, and was homogenized for 2 min in a Waring blender prior to being disintegrated at 600 kg/cm^2 for 5 min with a Manton-Gaulin homogenizer. All steps were carried out at 2–4 °C. Purification of thioredoxin from this cell extract was processed by method 2 of Holmgren and Reichard (1967).

Thioredoxin reductase was prepared from commercially available *E. coli* K-12 by the "large-scale" procedure of Pigiet and Conley (1977).

Protein Assay. Thioredoxin was assayed for free thiol groups in a coupled reaction by measuring the reduction of DTNB in the presence of NADPH and thioredoxin reductase (Laurent et al., 1964). In this assay thioredoxin-S_2 was transformed to thioredoxin-$(SH)_2$ in which the new thiol moieties were measured by DTNB. Thioredoxin possessing a cyclic dithioarsenite complex would remain unaltered under these conditions, and hence no reaction would occur with DTNB. Each assay contains 50 μmol of potassium phosphate (pH 7.0), 0.10 μmol of NADPH, and ~3 μg of thioredoxin reductase incubated for 10 min with less than 30 nmol of thioredoxin at 20 °C in a final volume of 990 μL. To this was added 0.10 μmol of DTNB (0.4% w/v in 10% ethanol) to make up a final volume of 1 mL.

Thioredoxin reductase activity was determined by using DTNB according to the method of Williams et al. (1967). Each assay contains 50 μmol of potassium phosphate (pH 7.6), 1.0 μmol of EDTA, 0.1 μmol of NADPH, 0.2 μmol of DTNB, and 2 nmol of thioredoxin (MICO, U.S.A.) in a final volume of 1 mL. One unit is the amount of enzyme required to give rise to an absorbance change of 1 per minute at 412 nm.

Protein Purity. Thioredoxin had a specific activity of 72–73 nmol per A_{280} unit[2] and was shown to migrate as a single band on 7.5% SDS–polyacrylamide gel electrophoresis stained with Coomassie blue. It also eluted as a single peak on SEC–HPLC using Toya Soda Micropak TSK-G2000SW and TSK-G3000SW columns in series, monitoring at 260 nm with a band-pass of 16 nm. (Corbett & Roche, 1984).

Thioredoxin reductase had a specific activity of 530 units/mg, and the spectral ratio of A_{271}:A_{456} was 4.0.

[2] An A_{280} unit is defined as the absorbance of a solution giving a value of 1.00 for the difference in absorbance at 280 and 310 nm.

Circular Dichroism. All spectra were acquired by using a Jasco J-500A spectropolarimeter equipped with a DP-500N data processor and calibrated with 0.1% *d*-10-camphorsulfonic acid (w/v in water) according to the procedure of Chen and Yang (1977). Protein solutions at a final concentration of 23.3 μM were placed in cylindrical cells with path lengths of 1.0 mm in the far-UV region and 10.0 mm in the aromatic region. The sample was typically scanned 8 times from 270 to 195 nm or from 310 to 240 nm, at 10 nm/min, using a time constant of 4 s and using the automatic bandwidth selector. Spectra were electronically digitized, averaged, and smoothed once before plotting.

Mean residue molar ellipticity ($[\theta]_\lambda$), at a given wavelength λ, was calculated by using the formula:

$$[\theta]_\lambda \text{ (deg cm}^2 \text{ dmol}^{-1}) = (11\,700/108)\theta_\lambda/100[\text{P}]l$$

where θ_λ is the observed ellipticity in degrees, l is the path length in cm, [P] is protein concentration in mol dm^{-3}, and 11 700 is the molecular weight of thioredoxin containing 108 amino acids. The percent α-helix and β-structure were calculated by using the procedure developed by Siegel et al. (1980). A program based on this procedure was written in BASIC 3.0 and run on a Hewlett-Packard HP 9000 Series 200 computer.

Fluorescence. All spectra were measured on an Aminco SPF-500 spectrofluorometer equipped with a calculator interface accessory operated with a Hewlett-Packard HP 9815A computer. Data were recorded by use of a Hewlett-Packard HP 7225B plotter. For a typical experiment, a protein sample with an A_{280} between 0.1 and 0.3 for a 1-cm path length was used. Emission spectra were obtained by scanning from 300 to 540 nm at 50 nm/min with the spectrometer in the ratio mode and an emission band-pass of 8 nm and excitation band-pass of 2 nm. Excitation spectra were collected with the emission wavelengths set at 320 and 355 nm, by scanning from 220 to 460 nm at 20 nm/min with all other parameters the same. When the spectrometer is in the ratio mode, emission intensity is automatically corrected for variations in the light source intensity as a function of wavelength. Where necessary, spectra were electronically digitized and smoothed 3 times (five-point smoothing over a 2.5-nm range) by use of the calculator accessory.

Chemical Reduction of Thioredoxin. Thioredoxin (269 μg, 23.3 nmol) was dissolved at room temperature in 1 mL of degassed 50 mM potassium phosphate buffer, pH 7.0, in a 3-mL QS1000 fluorescence cell. The solution was purged with N_2(g) for 5 min. Mercaptoethylamine (100 μL) from a stock solution (28.3 mg of mercaptoethylamine hydrochloride/mL of degassed H_2O, and pH adjusted to 7.0 with 1 M NH_3) was added to the cell. All reductive experiments were carried out in the spectroscopic cells.

Covalent Modification of Thioredoxin-$(SH)_2$ by $H_2NPhAsO$ and $HO(CH_2)_4AsCl_2$. $H_2NPhAsO$ (5 mg) was partially dissolved in 2 mL of 95% ethanol and filtered to remove insoluble material. By use of an extinction coefficient of 1.33×10^4 M^{-1} cm^{-1} in 95% ethanol at 262 nm (Robinson, 1980) and exact molar concentration for $H_2NPhAsO$ was determined.

Thioredoxin-$(SH)_2$ was incubated with a 100-fold molar excess of $H_2NPhAsO$ at 20 °C under a nitrogen atmosphere for 5 min. The fluorescent properties were monitored (Figure 2), and the sample was then dialyzed against 50 mM potassium phosphate, pH 7.0, or purified directly by SEC–HPLC. Purification by SEC–HPLC involves concentration by speed vacuum to 0.1 mL before loading onto the HPLC column. The pooled protein fraction was taken to dryness on a speed vacuum and redissolved into 1 mL of 50 mM potassium phosphate buffer, pH 7.0.

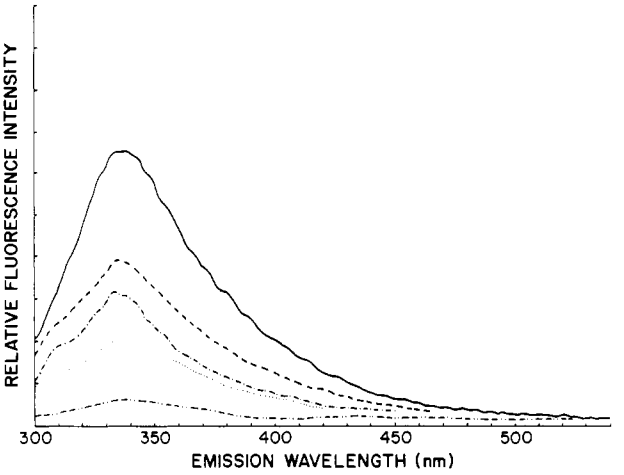

FIGURE 1: Fluorescence emission spectra for thioredoxin-$(SH)_2$ (—) excited at 280 nm; spectra for thioredoxin-S_2 before (- - -) and after (- - -) O_2 is removed; spectra upon modification of thioredoxin-$(SH)_2$ with $HO(CH_2)_4AsCl_2$ (···) and with $H_2NPhAsO$ (- - ··· - -).

A 0.48 mM solution of $HO(CH_2)_4AsCl_2$ in 95% ethanol was made as described above. Thioredoxin-$(SH)_2$ was incubated with a 100-fold molar excess of $HO(CH_2)_4AsCl_2$ (as for $H_2NPhAsO$), and the fluorescent properties were monitored. The sample was then concentrated 10-fold by rotary evaporation at 35 °C and modified thioredoxin isolated by SEC–HPLC.

Fluorescence Quenching Studies with Arsenicals. Reduced thioredoxin (10 nmol) in 1.0 mL of 50 mM potassium phosphate, pH 7.0, was titrated at 20 °C with 0.94 nmol of $H_2NPhAsO$ and 0.96 nmol of $HO(CH_2)_4AsCl_2$. Oxygen was removed from solutions by purging with N_2 and capping the cell before measurement of fluorescence.

Oxidized thioredoxin (5 and 10 nmol) in 1.0 mL of buffer was titrated at 20 °C with 4.8 nmol of $HO(CH_2)_4AsCl_2$ and 9.4 nmol of $H_2NPhAsO$, respectively.

To analyze the quenching, Stern–Volmer titration curves were plotted by using the equation:

$$F_0/F = 1 + K_D[\text{Q}]$$

where F_0 is the initial fluorescence and F is the resulting fluorescence due to the presence of a certain concentration of quencher Q. If this curve is linear, then a single quenching mechanism is operative, which can be either dynamic (collisional) or static (complex formation). In both cases quenching requires that there is molecular contact between the fluorophore and the quencher and that there is a single class of fluorophore all equally accessible to the quencher (Lakowicz, 1983). From a linear Stern–Volmer plot a value for the quenching constant K_D can be obtained.

RESULTS

Chemical Reduction of Thioredoxin. The reduction of thioredoxin was monitored by the increase in quantum yield observed by steady-state fluorescence (Stryer et al., 1967) (Figure 1). Our initial studies of the effect of reduction of the disulfide bond upon fluorescence showed that fluorescence increased a maximum of 52% (or 1.5-fold) as opposed to 2.0–2.5-fold previously reported for pH 6.8 (Stryer et al., 1967). When the fluorescence of oxidized thioredoxin was recorded in a nondegassed buffer open to air, the fluorescence was observed to be decreased by 25% relative to that observed in the absence of O_2. It appears that previously reported results (Holmgren, 1972; Stryer et al., 1967) were for non-

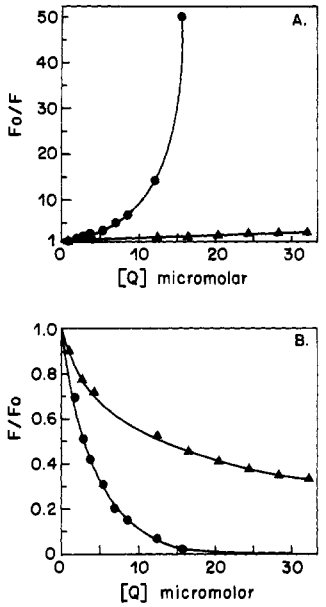

FIGURE 2: Stern–Volmer (A) and inverse Stern–Volmer (B) titration plots of 10 μM thioredoxin-$(SH)_2$ in 50 mM potassium phosphate buffer, pH 7.0, quenched with $H_2NPhAsO$ (●) and $HO(CH_2)_4AsCl_2$ (▲). Fluorescence emission was collected at 340 nm upon excitation at 280 nm.

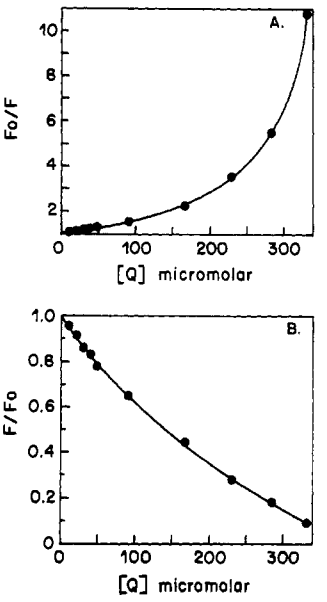

FIGURE 3: Stern–Volmer (A) and inverse Stern–Volmer (B) titration plots of 10 μM thioredoxin-S_2 in 50 mM potassium phosphate buffer, pH 7.0, quenched with $H_2NPhAsO$. No quenching was observed with $HO(CH_2)_4AsCl_2$.

deoxygenated buffer systems and that the effect observed here has not been reported before.

Covalent Modification of Thioredoxin-$(SH)_2$ by p-Aminophenyl Arsenoxide. The chemically modified thioredoxin (dialyzed 16 h, 4 °C, or SEC–HPLC) was shown to have 5% of the fluorescence (Figure 1) and 9 ± 1% free thiol content of that observed for unmodified thioredoxin-$(SH)_2$. 2,3-Dimercaptopropanol was added to the protein in a 10-fold molar excess over thioredoxin, and the reduced thioredoxin so formed was concentrated, subjected to SEC–HPLC, dried, and reconstituted as indicated earlier. Following these procedures, the reduced thioredoxin was reoxidized as indicated by the fluorescence level of 49% relative to reduced thioredoxin. This value is comparable to that observed for oxidized thioredoxin. The arsenical was removed from thioredoxin as indicated by the 102% (±2%) recovery for thiols in the assay as outlined under Methods. SEC–HPLC was used to remove excess 2,3-dimercaptopropanol and mercaptoethylamine to prevent their reaction with DTNB and to also remove the 2,3-dimercaptopropanol–$AsPhNH_2$ complex since it interfered with the fluorescence of native thioredoxin (spectrum not shown).

These results suggest that 95% of thioredoxin-$(SH)_2$ binds arsenical and upon addition of 2,3-dimercaptopropanol almost 100% reduced thioredoxin is regenerated. The reduced thioredoxin slowly oxidizes in aerated solution (Laurent et al., 1964).

Covalent Modification of Thioredoxin-$(SH)_2$ by HO-$(CH_2)_4AsCl_2$. Thioredoxin chemically modified with HO-$(CH_2)_4AsCl_2$ and purified by SEC–HPLC contained 7% (±1%) free thiol and 33% of the fluorescence of that of native reduced thioredoxin (Figure 1). Treatment of this preparation with a 5-fold molar excess of 2,3-dimercaptopropanol over thioredoxin (to remove the arsenical), followed by SEC–HPLC, yielded purified thioredoxin that contained 100% (±2%) free thiols and 47% of the fluorescence compared with unmodified thioredoxin-$(SH)_2$.

Fluorescence Quenching Studies with Arsenicals. The Stern–Volmer plots for the quenching of thioredoxin-$(SH)_2$ by the two organoarsenicals are shown in Figure 2A. The quenching by $HO(CH_2)_4AsCl_2$ demonstrates a single mechanism with a quenching constant, K_D, of 0.061 μM^{-1} with a correlation coefficient of 0.9952. However, the quenching by $H_2NPhAsO$ shows a dual (complex) quenching mechanism. Stern–Volmer plots with an upward curvature, concave toward the *y* axis, show that the fluorophore is being quenched both collisionally and by complex formation between the quencher and the fluorophore (Lakowicz, 1983). From Figure 2B, it can be seen that when thioredoxin-$(SH)_2$ and arsenical are in a 1:1 molar ratio, 90% of the fluorescence is lost with $H_2NPhAsO$ as opposed to 45% with $HO(CH_2)_4AsCl_2$. We have shown by monitoring the protein fraction eluted from the SEC–HPLC with DTNB that 95% of thioredoxin-$(SH)_2$ is covalently modified by arsenical. The corresponding loss in fluorescence for this modified thioredoxin was 95% with $H_2NPhAsO$ and 67% with $HO(CH_2)_4AsCl_2$. From the inverse Stern–Volmer plot (Figure 2B) we observe that a molar ratio of $H_2NPhAsO$ to thioredoxin-$(SH)_2$ of 2:1 and $HO(CH_2)_4AsCl_2$ to thioredoxin-$(SH)_2$ of 4:1 is required to bring about the same degree of fluorescence quenching.

Quenching of thioredoxin-S_2 by the $H_2NPhAsO$ is not as efficient; however, it still shows a dual quenching mechanism (Figure 4). $H_2NPhAsO$ in the same molar ratio as used for thioredoxin-$(SH)_2$ gives less than 5% loss in fluorescence as seen by comparing Figure 2B to Figure 3B. There was no significant loss in fluorescence when thioredoxin-S_2 was titrated with the $HO(CH_2)_4AsCl_2$. Therefore, quenching with HO-$(CH_2)_4AsCl_2$ requires covalent attachment. Considering these results, we suggest that a molar ratio of 2:1 for $H_2NPhAsO$ and 4:1 for $HO(CH_2)_4AsCl_2$ to thioredoxin-$(SH)_2$, respectively, is required to effect 95% chemical modification.

To further investigate the quenching of thioredoxin by organoarsenicals, a concentration of tryptophan equivalent to the amount found in thioredoxin was titrated with the two arsenicals in 50 mM phosphate buffer, pH 7.0. Both arsenicals demonstrated a single quenching mechanism. The K_D value obtained for $H_2NPhAsO$ was 0.013 μM^{-1}, and that for HO-$(CH_2)_4AsCl_2$ was 0.029 μM^{-1}, with correlation coefficients of 0.9952 and 0.9764, respectively. In the same manner, the K_D value for L-tyrosine, obtained with $H_2NPhAsO$, was 0.010 μM^{-1}, and that obtained with $HO(CH_2)_4AsCl_2$ was 0.040

Table I: Fluorescence Values of Thioredoxin and Modified Thioredoxin with Organoarsenicals

system	emission λ_{max}[a] (nm)	emission λ (nm)	excitation λ_{max} (nm)
thioredoxin-S_2	334	320	289
		355	286
thioredoxin-$(SH)_2$	338	320	289
		355	288
thioredoxin-$(SH)_2$ + $HO(CH_2)_4AsCl_2$	336	320	291
		355	294
thioredoxin-$(SH)_2$ + $H_2NPhAsO$[b]	338		

[a] Excitation wavelength was 280 nm. [b] Excitation spectra were not obtainable due to the signal to noise ratio becoming too large to decisively determine the excitation λ_{max}.

μM^{-1}, with correlation coefficients of 0.9839 and 0.9937, respectively. This shows that $H_2NPhAsO$ is a poor quencher compared to $HO(CH_2)_4AsCl_2$ of tryptophan and tyrosine fluorescence in solution.

Fluorescence Excitation Studies. Oxidized thioredoxin showed excitation wavelengths of 286 and 289 nm when the emission wavelength was held at 355 and 320 nm, respectively. Upon reduction, the excitation maximum for the 355-nm emission is red-shifted to 288 nm. Modification of thioredoxin-$(SH)_2$ with $HO(CH_2)_4AsCl_2$ led to a red shift of the excitation maxima of both the 320- and 355-nm emissions. It was observed (Table I) that the excitation of the 355-nm emission experienced the larger shift. These observations (Table I) reflect the changing microenvironment of the tryptophan residues.

Circular Dichroism. Ellipticity spectra were recorded for thioredoxin in the oxidized, reduced, and chemically modified states. In the far-ultraviolet region scans were obtained where $H_2NPhAsO$ was added in a 2:1 molar ratio and $HO(CH_2)_4AsCl_2$ was added in a 4:1 molar ratio. The ellipticity spectra under these conditions were identical, and thus only one spectrum is reported (Figure 4). As a control, thioredoxin-S_2 (23.3 nmol/mL) was titrated with both $H_2NPhAsO$ and $HO(CH_2)_4AsCl_2$ in a 1:1 and 1:10 molar ratio (Figure 5). With $HO(CH_2)_4AsCl_2$ there was no change in the ellipticity spectra compared to that for thioredoxin-S_2; however, with $H_2NPhAsO$ changes are evident. In Figure 4, the effect of reduction and chemical modification is clearly seen in the region 190–205 nm, which is dominated by α-helical secondary structure, whereas the region between 205 and 245 nm has contributions from all secondary structures (Cantor & Schimmel, 1980). From X-ray crystallographic studies of thioredoxin-S_2 the α-helix content has been determined to be 38% (Reutimann et al., 1981). Therefore, a small perturbation of the α-helical content of thioredoxin upon reduction will have its major effect in the region from 190 to 205 nm, where the contribution of α-helical structures dominates, rather than the region from 205 to 240 nm.

In the aromatic region reduced (Figure 6A) and oxidized (Figure 6B) thioredoxin were titrated with a 2-fold and 5-fold molar ratio of $H_2NPhAsO$ to protein, respectively. In both cases, we observed that the aromatic CD spectrum is increasingly distorted by $H_2NPhAsO$.

Ellipticity spectra for reduced thioredoxin in the presence of a 5-fold molar excess of $HO(CH_2)_4AsCl_2$ are presented in the difference spectra in Figure 7A. We observed minimal distortion relative to the reduced state in the presence of the arsenical.

Discussion

Reduction of thioredoxin was achieved by use of the monothiol reagent mercaptoethylamine as opposed to the use of dithiol reagents such as dithioerythritol. The use of a monothiol reagent allowed investigation of the properties of modified thioredoxin without the reagent reacting (or complexing) with the organoarsenicals (Whittaker, 1947). Dithiol reagents will not only complex free arsenical but also reverse the arsenical modification of thioredoxin whereas the monothiol at low concentration will not.

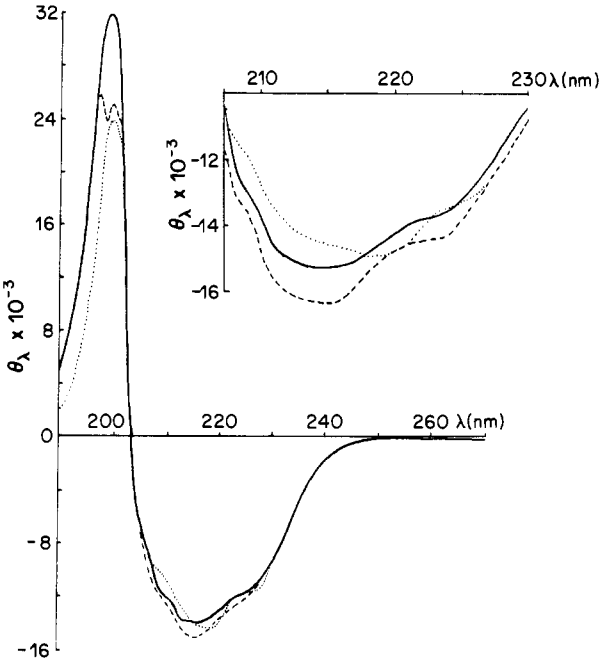

FIGURE 4: Far-ultraviolet circular dichroism spectra of thioredoxin-S_2 (—) which was subsequently reduced to thioredoxin-$(SH)_2$ (- - -) with mercaptoethylamine. The modified thioredoxin-$(SH)_2$ with organoarsenicals is shown (···). Protein solution was 23.3 μM in 50 mM potassium phosphate buffer, pH 7.0, and was placed in a cylindrical cell of 1.0-mm path length. θ_λ is the observed ellipticity in degrees.

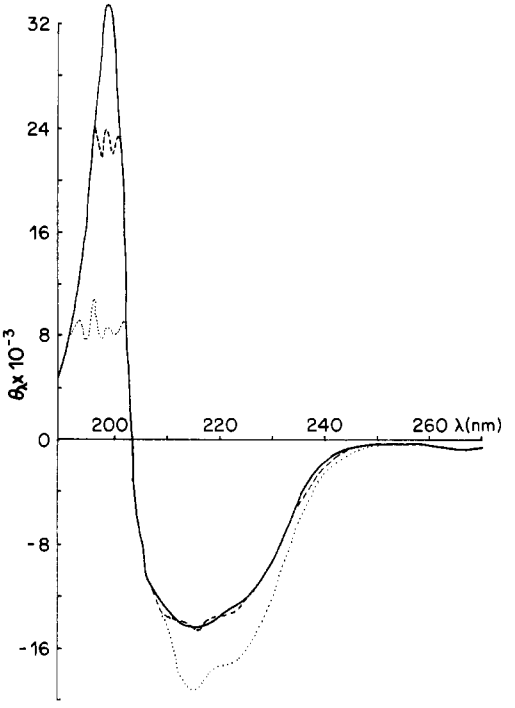

FIGURE 5: Far-ultraviolet circular dichroism spectra of thioredoxin-S_2 in which $H_2NPhAsO$ was added in a 1:1 ratio (- - -) and a 10:1 ratio (···) to the thioredoxin-S_2 (—). No significant change in the spectra was observed upon addition of $HO(CH_2)_4AsCl_2$. Experimental conditions are the same as in the legend of Figure 4.

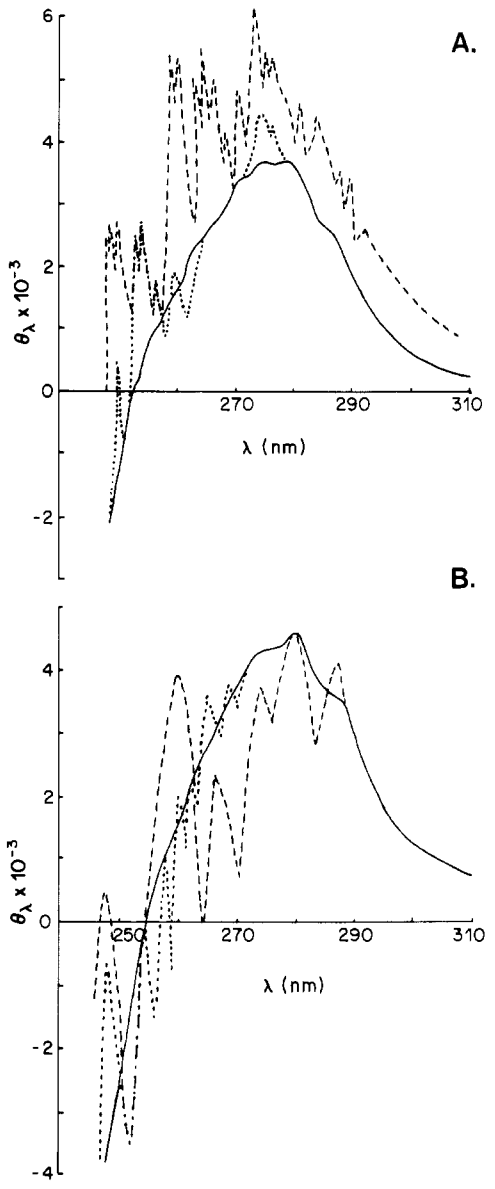

FIGURE 6: (A) Aromatic circular dichroism spectra of thioredoxin-$(SH)_2$ (—) in which $H_2NPhAsO$ was added in a 2:1 ratio (···) and a 5:1 ratio (---). (B) Aromatic circular dichroism spectra of thioredoxin-S_2 (—) in which $H_2NPhAsO$ was added in a 2:1 ratio (···) and 5:1 ratio (---). Protein solution was 23.3 μM in 50 mM potassium phosphate buffer, pH 7.0, and was placed in a cylindrical cell of 10.0-mm path length. θ_λ is the observed ellipticity in degrees.

We have available to us a selection of trivalent organoarsenicals in which the arsenical terminal is attached to an aromatic or alkyl moiety (Robinson, 1980; Brown and Stevenson, unpublished data). The unique interaction of the organic group with reduced thioredoxin attached via the arsenical moiety to two spatially close cysteinyl residues has provided a method to elucidate the differences observed in the fluorescence and the CD of thioredoxin associated with the reduction of the disulfide bond.

A question that is frequently asked in discussions of the chemical modifications of a protein is the following: Does the modification affect the secondary and/or the tertiary structure in such a manner that one is no longer observing the native protein? In the present study of the modification of thioredoxin with organoarsenicals, the spectroscopic changes associated with the formation of the protein–arsenical complex suggest that the effect on the native conformation of the protein is minimal.

Reduced thioredoxin covalently modified by a 100-fold molar excess of $H_2HPhAsO$ exhibited a complete loss of both tyrosine and tryptophan fluorescence when excited at 280 nm, suggesting that $H_2NPhAsO$ was a strong quencher. When a 100-fold molar excess of $H_2NPhAsO$ was added to oxidized thioredoxin, the effect was the same. A UV absorption scan, equivalent to a 1000-fold molar excess, showed that there was no absorption at 340 nm (results not shown), indicating that fluorescence was not quenched by energy transfer to the arsenical.

Reduced thioredoxin when covalently modified by a 100-fold molar excess of $HO(CH_2)_4AsCl_2$ showed a 67% loss in fluorescence. The oxidized thioredoxin, in contrast, showed no loss of fluorescence upon addition of a 100-fold molar excess of $HO(CH_2)_4AsCl_2$. Addition of 2,3-dimercaptopropanol to the modified thioredoxin removed the arsenical, reversed the modification, and reestablished (after SEC–HPLC) the fluorescence found in the native oxidized thioredoxin.

Stern–Volmer titrations were used to study the quenching of fluorescence in thioredoxin (Figures 2 and 3). These plots are nonlinear and indicate that complex formation and collisional quenching occur when $H_2NPhAsO$ binds or associates with reduced or oxidized thioredoxin. Oxidized thioredoxin is not quenched by $HO(CH_2)_4AsCl_2$, and thus the fluorophores are not accessible to the quencher. In contrast, reduced thioredoxin is quenched by $HO(CH_2)_4AsCl_2$, most probably due to the fact that the arsenical is covalently attached to the thiol groups. The linear Stern–Volmer plot observed in this case (Figure 2A) suggests a single quenching mechanism. Thus, covalent modification by the arsenicals not only increases the efficiency of quenching but also makes the fluorophores more accessible to the arsenicals. Quenching studies using each arsenical in solution with concentrations of L-tryptophan and L-tyrosine equivalent to their content in thioredoxin in solution revealed that the arsenicals quench the free fluorophores by a single mechanism. $H_2NPhAsO$ is several times more efficient at quenching the tryptophan and tyrosine fluorescence in the protein than in free solution. By comparison, $HO(CH_2)_4AsCl_2$ is only twice as efficient at quenching, as suggested by the observed K_D values. The quenching by the organoarsenicals used in this study appears to be a function of the organic group. Published reports of As(III) as a quencher of fluorescence are not known to us.

Analysis of Figure 1 shows that modification of thioredoxin-$(SH)_2$ with $HO(CH_2)_4AsCl_2$ causes very little loss in tyrosine fluorescence at 305 nm. However, not only does modification by $H_2NPhAsO$ cause almost a complete loss in tryptophan fluorescence but also there is an enhanced energy transfer from tyrosine to tryptophan giving rise to a complete loss in tyrosine fluorescence at 305 nm. The parameters for energy transfer between two chromophores are distance and orientation. Conceivably, the binding of $H_2NPhAsO$ causes a change in the orientation of tryptophan relative to tyrosine and hence in the efficiency of energy transfer from tyrosine to tryptophan. This effect is also observed with oxidized thioredoxin but at much higher concentrations of $H_2NPhAsO$.

Far-ultraviolet circular dichroism was used to calculate the change in the α-helical and β-structural content following reduction and arsenical modification of oxidized thioredoxin. We observed a 6% increase in α-helix and a 3% decrease in β-structure for the transition thioredoxin-S_2 to thioredoxin-$(SH)_2$. Modification with either of the organoarsenicals led to a 1% increase in α-helix and a 2% decrease in β-structure compared to that of oxidized thioredoxin. This degree of change is within the limit observed for reduction; hence the

modified protein most probably has a conformation somewhere between that of thioredoxin-S_2 and thioredoxin-$(SH)_2$.

The CD in the aromatic region is reflective of aromatic chromophores in asymmetric environments. We see from panels A and B of Figure 6 that $H_2NPhAsO$ binds to thioredoxin in both the oxidized and reduced states and perturbs the aromatic CD. Examination of the difference CD spectra for thioredoxin-$(SH)_2$ relative to thioredoxin-S_2 (Figure 7A) shows that a positive ellipticity is gained in the phenylalanine region and ellipticity is lost in the tyrosine and tryptophan region. Therefore, there is a loss of asymmetry in the microenvironment of one or more aromatic residues upon reduction consistent with the change in tertiary structure suggested by the fluorescence data.

Modification of reduced thioredoxin with $H_2NPhAsO$ results in the formation of two positive bands at 250 and 255 nm that are due to the absorption of the H_2NPh- chromophore. Modification with $HO(CH_2)_4AsCl_2$ however yields little change from the reduced state and consequently negligible perturbation of the intrinsic chromophores of the protein. This result suggest that the bands at 250 and 255 nm for $H_2NPhAsO$ covalently bound to reduced thioredoxin are due to induced CD in the H_2NPh- chromophore. Further evidence is afforded by subtracting the CD spectrum of thioredoxin-S_2 from thioredoxin-(SH_2), both titrated with $H_2NPhAsO$ (Figure 7B), thus allowing us to observe only that due to covalent modification. $H_2NPhAsO$ at a molar ratio of 2:1 to oxidized and reduced thioredoxin [the ratio required for 95% modification of thioredoxin-$(SH)_2$] revealed a strong positive circular dichroic band at 253 nm. This suggests that the H_2NPh- chromophore is buried in a relatively nonpolar environment, the asymmetry of which induces the observed CD in its absorption band at 253 nm. Increasing the ratio of reagent to thioredoxin to 5:1 leaves the 253-nm band relatively unchanged. The appearance of a number of positive bands between 260 and 290 nm is indicative of a perturbation in the environment of the aromatic chromophores of the protein and a change in its tertiary structure. The reduction and modification of thioredoxin are also accompanied by noncovalent binding of $H_2NPhAsO$ at ratios greater than 2:1. In contrast with covalent cyclic dithioarsenite complexes, the noncovalent binding of $H_2NPhAsO$ is of low affinity and most probably involves the hydrophobic surface of thioredoxin (Eklund et al., 1984) via hydrophobic interaction.

If we consider the reduction of the disulfide bond as the opening up (relaxation) of the 14-membered polypeptide ring, comprising -Cys^{32}-Gly^{33}-Pro^{34}-Cys^{35}-, then the cyclic dithioarsenite 15-membered rings formed on covalent modification by the arsenicals are "relaxed" compared to the 14-membered rings of the oxidized state but are not as relaxed as the reduced state of thioredoxin.

The reduction of the disulfide bond within the reverse turn opens the 14-membered ring, allowing the β-turn to "unwind". This unwinding appears to add to the α-helical content at the expense of the β-structure (Table II). Kishore and Balaram (1986) have studied synthetic peptide models containing a disulfide bond and have shown that reduction is indeed accompanied by a backbone conformational change. We propose that the disulfide bond "locks" the α_2-helix, residues 35–49 (Holmgren, 1968), into a specific conformation, one that partially exposes Trp-28 to the solvent (Holmgren & Roberts, 1976). Upon reduction and the consequent relaxation of the β-turn, the α_2-helix is no longer "locked" and is now free to "swing" over the surface of the β pleated sheets, particularly that of β_2 (residues 22–29 inclusive), which contains Trp-28. This dynamically increases the area of the accessible hydrophobic surface.

Tryptophan fluorescence can increase as a result of the removal of a quenching group (e.g., a disulfide bond) or as a consequence of being in a more rigid or hydrophobic environment (Lakowicz, 1983). This latter condition could be satisfied by the "swinging helix" model.

The quenching of the fluorescence of thioredoxin-$(SH)_2$ appears to be complex, and more than one mechanism operates. If the arsenical simply "restresses" the β-turn, then we would expect the fluorescence to be approximately identical with that of the oxidized state for both arsenicals. The fact that it is not and that each arsenical shows different characteristics points to the function of the organic group in the reagents.

In the case of thioredoxin-S_2, only $H_2NPhAsO$ quenches, suggesting that it is the phenyl ring that is quenching as opposed to the –AsO moiety; otherwise $HO(CH_2)_4AsCl_2$ should also quench. The CD studies show that $H_2NPhAsO$ does associate noncovalently with thioredoxin-S_2 (Figure 6B). A solution of $H_2NPhAsO$ showed no CD properties on its own, and we conclude that in order to elicit a CD contribution, the phenyl ring of $H_2NPhAsO$ associates with the hydrophobic surface of thioredoxin (Eklund et al., 1984). $HO(CH_2)_4AsCl_2$ simply does not possess sufficient hydrophobicity to bring about efficient quenching.

We suggest that the mechanism of fluorescence quenching in thioredoxin-$(SH)_2$ by covalently bound arsenical (i) involves the formation of the –S–As–S– bridge that could act as a quencher or play a role in "locking" the α_2-helix, (ii) is collisional in that the organo groups are now juxtaposed to Trp-28, and (iii) also involves a $\pi-\pi$ interaction of the H_2NPh- moiety with Trp-28. The latter interaction is most probably responsible for the spectral differences observed between the two arsenicals. However, we cannot rule out the possibility that if –S–As–S is a quenching group then the organo group may also affect its efficiency.

Excitation spectra for thioredoxin in the oxidized, reduced, and modified states were obtained when the emission wavelengths were held at 320 and 355 nm. Kronman (1976) has suggested that the different areas of the emission spectral peak may reflect fluorescent contributions from different tryptophan residues in the protein. Further, Reutimann et al. (1981) have suggested that the larger than normal observed spectral half-bandwidth could be due to the superposition of two slightly displaced emission bands of the two tryptophan residues. We make the assumption that, at each of these selected emission wavelengths, one tryptophan is predominantly fluorescent. Reduction of thioredoxin-S_2 followed by modification with an organoarsenical mainly causes a red shift in the excitation of one of the tryptophans (Table I). This observation is not inconsistent with the "swinging helix" model and with the findings of Holmgren and Roberts (1976) where reduction of thioredoxin-S_2 affects the microenvironments of one tryptophan more than the other.

The present results provide convincing evidence that the monofunctional organoarsenicals modify, and hence inactivate, reduced thioredoxin but cause negligible changes in the secondary structure of the protein. The extent of modification was monitored by the percentage loss in free thiol content as measured by the reaction with DTNB. This modification results in the formation of a stable 15-membered cyclic dithioarsenite ring. The fluorescence and circular dichroism data are consistent with minor changes in the tertiary structure.

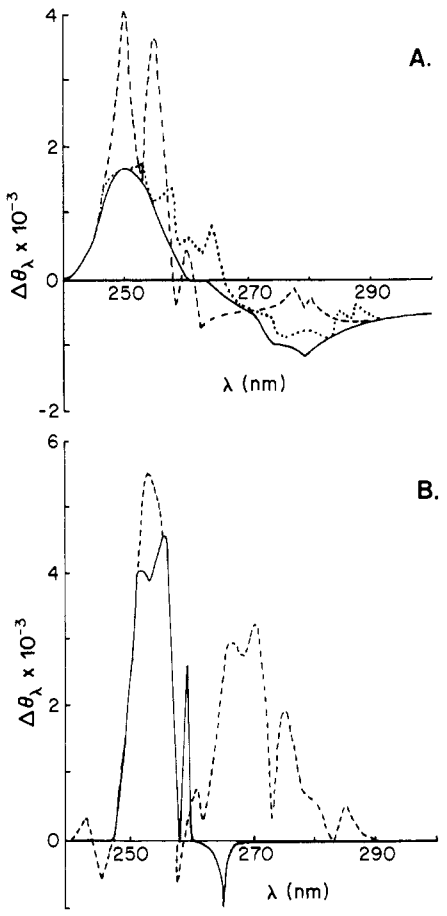

FIGURE 7: (A) Aromatic circular dichroism difference spectra of thioredoxin-$(SH)_2$ relative to thioredoxin-S_2 in the presence of no arsenical (—), $H_2NPhAsO$ added in a 2:1 ratio (---), and $HO(CH_2)_4AsCl_2$ added in a 5:1 ratio (···). (B) Aromatic circular dichroism difference spectra, with subtraction of thioredoxin-S_2 + $H_2NPhAsO$ from thioredoxin-$(SH)_2$ + $H_2NPhAsO$ with a molar ratio of $H_2NPhAsO$ to thioredoxin of 2:1 (—) and 5:1 (--). Difference spectra were generated from Figure 6.

Table II: Percentages of α- and β-Structure As Calculated from Circular Dichroism[a]

system	α-helix	β-structure[b]
X-ray values[c]	38	43
thioredoxin-S_2	26.1	39.3
thioredoxin-$(SH)_2$	27.6	38.2
thioredoxin $\begin{matrix}\text{—S—}\\ \text{—S—}\end{matrix}$ $AsPhNH_2$	26.4	38.4
thioredoxin-S_2 + $H_2NPhAsO$ (1:1)	28.1	34.0
thioredoxin-$(SH)_2$ + $H_2NPhAsO$ (10:1)	40.0	29.1

[a] Errors in the calculated percent α-helix and percent β-structure are ±1.6 and ±3.2, respectively. [b] Includes β-sheet + β-turn. [c] Data of Reutimann et al. (1981).

Hence, these reagents have potential for the study of the interactions of thioredoxin with other enzymes.

Registry No. $HO(CH_2)_4AsCl_2$, 28440-95-1; $H_2NPhAsO$, 1122-90-3; 1,4-dibromobutane, 110-52-1; (4-hydroxybutyl)arsonic acid, 105858-36-4.

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Active-Site Cobalt(II)-Substituted Horse Liver Alcohol Dehydrogenase: Characterization of Intermediates in the Oxidation and Reduction Processes as a Function of pH†

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ABSTRACT: Substitution of Co(II) for the catalytic site Zn(II) of horse liver alcohol dehydrogenase (LADH) yields an active enzyme derivative, $Co^{II}E$, with characteristic Co(II) charge-transfer and d–d electronic transitions that are sensitive to the events which take place during catalysis [Koerber, S. C., MacGibbon, A. K. H., Dietrich, H., Zeppezauer, M., & Dunn, M. F. (1983) *Biochemistry 22*, 3424–3431]. In this study, UV–visible spectroscopy and rapid-scanning stopped-flow (RSSF) kinetic methods are used to detect and identify intermediates in the LADH catalytic mechanism. In the presence of the inhibitor isobutyramide, the pre-steady-state phase of alcohol (RCH_2OH) oxidation at pH above 7 is characterized by the formation and decay of an intermediate with λ_{max} = 570, 640, and 672 nm for both aromatic and aliphatic alcohols (benzyl alcohol, *p*-nitrobenzyl alcohol, anisyl alcohol, ethanol, and methanol). By comparison with the spectrum of the stable ternary complex formed with oxidized nicotinamide adenine dinucleotide (NAD^+) and 2,2′,2″-trifluoroethoxide ion (TFE^-), $Co^{II}E(NAD^+,TFE^-)$, the intermediate which forms is proposed to be the alkoxide ion (RCH_2O^-) complex, $Co^{II}E(NAD^+,RCH_2O^-)$. The timing of reduced nicotinamide adenine dinucleotide (NADH) formation indicates that intermediate decay is limited by the interconversion of ternary complexes, i.e., $Co^{II}E(NAD^+,RCH_2O^-) \rightleftharpoons Co^{II}E(NADH,RCHO)$. From competition experiments, we infer that, at pH values below 5, NAD^+ and alcohol form a $Co^{II}E(NAD^+,RCH_2OH)$ ternary complex. RSSF studies carried out as a function of pH indicate that the apparent pK_a values for the ionization of alcohol within the ternary complex, i.e., $Co^{II}E(NAD^+,RCH_2OH) \rightleftharpoons Co^{II}E(NAD^+,RCH_2O^-) + H^+$, fall in the range 5–7.5. Using pyrazole as the dead-end inhibitor, we find that the single-turnover time courses for the reduction of benzaldehyde, *p*-nitrobenzaldehyde, anisaldehyde, and acetaldehyde at pH above 7 all show evidence for the formation and decay of an intermediate. Via spectral comparisons with $Co^{II}E(NAD^+,TFE^-)$ and with the intermediate formed during alcohol oxidation, we identify the intermediate as the same $Co^{II}E(NAD^+,RCH_2O^-)$ ternary complex detected during alcohol oxidation.

In recent years, both our understanding of the equine liver alcohol dehydrogenase (LADH,[1] EC 1.1.1.1) catalytic mechanism and our understanding of enzyme catalysis in general have been greatly enriched by the findings of chemists and enzymologists working on LADH [see reviews by Brändén et al. (1975), Dunn (1975, 1984), Klinman (1981), Brändén & Eklund (1980), and Zeppezauer (1983)]. Nevertheless, several aspects of the LADH catalytic mechanism are incompletely resolved: (1) the roles played by protein conformation change are poorly understood; (2) there is no general agreement about the coordination state of the active-site metal during catalysis; (3) there is no agreement about the identities and roles of ionizable groups at the site; and (4) our understanding of elelectrostatic field effects at the site is incomplete.

The most direct evidence indicating that LADH undergoes changes in protein conformation during catalysis is provided

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[1] Abbreviations: E or LADH, horse liver alcohol dehydrogenase; $Co^{II}E$, specifically active-site Co(II)-substituted alcohol dehydrogenase; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide, respectively; RCH_2OH and RCH_2O^-, alcohol and alkoxide ion, respectively; RCHO, aldehyde; NBZA and NBZOH, *p*-nitrobenzaldehyde and *p*-nitrobenzyl alcohol, respectively; pyr, pyrazole; IBA, isobutyramide; TFA and TFE^-, 2,2′,2″-trifluoroethanol and 2,2′,2″-trifluoroethoxide ion, respectively; MPD, 2-methyl-2,4-pentanediol; H_2NADH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide; RSSF, rapid-scanning stopped flow; CME, Cys-46-carboxymethylated LADH; DACA, 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldehyde; LMCT, ligand to metal charge transfer; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Alc, alcohol; Me_2SO, dimethyl sulfoxide; ADPR, adenine diphosphoribosyl; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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