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Reduction of thioredoxin significantly decreases its partial specific volume and adiabatic compressibility

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Abstract

The partial specific volume and adiabatic compressibility were determined at several temperatures for oxidized and reduced *Escherichia coli* thioredoxin. Oxidized thioredoxin had a partial specific volume of 0.785–0.809 mL/g at the observed upper limit for all proteins whereas the partial specific volume of reduced thioredoxin was 0.745–0.755 mL/g, a value in the range found for a majority of proteins. The adiabatic compressibility of oxidized thioredoxin was also much larger ($9.8–18 \times 10^{-12}$ cm^2 dyne^{-1}) than that of the reduced protein ($3.8–7.3 \times 10^{-12}$). Apart from the region immediately around the small disulfide loop, the structures of the oxidized (X-ray, crystal) and reduced protein (nuclear magnetic resonance, solution) are reported to be very similar. It would appear that alterations in the solvent layer in contact with the protein surface must play a major role in producing these large changes in the apparent specific volumes and compressibilities in this system. Some activities of thioredoxin require the reduced structure but are not electron transfer reactions. The large changes in physical parameters reported here suggest the possibility of a reversible metabolic control function for the SS bond.

Keywords: adiabatic compressibility; partial specific volume; reduction; thioredoxin

A brief overview of thioredoxins, and in particular the most studied example from *Escherichia coli*, has been provided in Kaminsky and Richards (1992). For general reviews of the properties and functions of the thioredoxins one should consult Holmgren (1985, 1989).

The protein from *E. coli* contains two cysteine residues in positions 32 and 35. These exist either in the reduced form or as a disulfide bond in the four-residue loop. The redox cycle and electron transfer are central to some but not all functions. The structure of the reduced form, but not the specific SH groups, is required for other functions. The structure of the oxidized form in crystals has been determined by X-ray diffraction (Söderberg et al., 1974; Katti et al., 1990) whereas the structure of the reduced form in solution has been determined by nuclear magnetic resonance (NMR) (Dyson et al., 1990). Except for the disulfide loop region, these two structures are very similar. If this latter statement is correct, what is the difference between the two structures that accounts for the functional differences observed and the large change in thermal stability?

In NMR experiments Hiraoki et al. (1988) and Dyson et al. (1990) found redox-sensitive changes, including hydrogen exchange properties, in the region of the SS bond. In the previous paper Kaminsky and Richards (1992) have corroborated that there is a small group of amide protons whose exchange behavior is dramatically altered by the redox state. These appear to occur in two small nonadjacent regions, the disulfide loop and the first β-strand. In the same experiments many of the other protons appear to be unaffected, attesting to the general similarity of the two structures.

If the equilibrium structures are so similar, then the relevant difference may lie in the dynamics of the two forms. Hydrogen exchange is one measure of the dynamic behavior of the molecules and reflects a certain class of conformational fluctuations in a protein. The structural changes involved in providing solvent access to protected protons may be large, intrinsically rare events and probably involve the lowest frequency modes of motion. These modes may or may not be related to biolog-

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ical function. A more general measurement of the mean fluctuations in a system is provided by compressibility. We decided to check this property of the two states of thioredoxin.

Compressibility is the change in volume in response to variations in pressure. In solutions the velocity of sound is directly related to the adiabatic compressibility (see Sarvazyan, 1991, for a recent review). Compressibility data have been used to correlate internal motions with secondary structural features (Gavish et al., 1983), thermal stability, and hydrophobicity (Gekko & Hasegawa, 1986). Such measurements have demonstrated that an increase in volume fluctuations occurs upon oxidation of cytochrome c, despite small differences, if any, between the crystal structures of its two redox forms (Eden et al., 1982; see, however, the quantitative revisions provided by Kharakoz & Mkhitaryan, 1986).

To obtain the apparent specific compressibility of the protein component, the apparent specific volume of the protein is required in addition to the sound velocity data. In the course of collecting these data, a large change with redox state in the apparent specific volume was observed. This was not expected for two very similar structures. The atoms themselves are essentially incompressible at modest pressures. The observed compressibility in either liquids or molecular solids depends on voids in the structures and their collapse or rearrangement on compression. In similar structures the atomic positions are essentially the same, and thus the voids are similar. The dense packing observed in most structures and the small volume change normally observed on denaturation make mysterious the observed change in apparent volume. The source of this change has not yet been identified, but we are inclined to attribute it to some concerted change in the first hydration layer surrounding the protein.

Results

Density of thioredoxin solutions

Direct calculation of the partial specific volume of thioredoxin solutions was made from the density of thioredoxin at several concentrations. The apparent volume fraction of the solvent X_1^{app} is given by the following equation (Shiio, 1958):

$$X_1^{app} = (\rho - c)/\rho_0, \tag{1}$$

where ρ is the density of the solution, ρ_0 is the density of the pure solvent, and c is the protein concentration in grams per milliliter. The apparent specific volume of the protein, $\bar{v}_2^{app}$, was determined by dividing the apparent volume fraction of the solute $(1 - X_1^{app})$ by the concentration of protein in grams per milliliter:

$$\bar{v}_2^{app} = (1 - X_1^{app})/c. \tag{2}$$

The partial specific volume ($\bar{v}_2$) is a measure of protein volume including the net contribution, if any, due to the hydration layer. Because $\bar{v}_2^{app}$ is essentially independent of concentration, it is numerically equal to $\bar{v}_2$, and protein–protein interactions are absent in the concentration range used.

The relationship between density of the solution (ρ_s), concentration, and apparent specific volume is derived from Equations 1 and 2, with the result:

$$\rho_s = \rho_0 + c(1 - \rho_0 \bar{v}_2^{app}), \tag{3}$$

where ρ_0 is the density of the buffer. The density of the solutions of oxidized and reduced thioredoxin showed a linear dependence on protein concentration at the temperatures studied: 5 °C, 15 °C, and 30 °C. Least-squares analysis indicates that the data are adequately fit by a straight line; no higher order concentration-dependent phenomena were indicated. From these data the apparent specific volume of the protein was calculated for each solution using Equation 3. The results are plotted in Figure 1. The extrapolation of the apparent specific volume to zero protein concentration provided the partial specific volumes ($\bar{v}_2$) listed in Table 1. The experimental errors were calculated from Equation 2 using the following error estimates, $\sigma\rho = 6 \times 10^{-6}$ g/mL, $\sigma c = 1 \times 10^{-4}$ g/mL.

To verify the proper performance of the density meter and the accuracy of the procedure, the partial specific volume of lysozyme was determined. The measured value in water at 20 °C of 0.720 mL/g is within the range of published values (0.702–0.733 mL/g; Durchschlag, 1986).

The temperature variation of $\bar{v}_2$ for the reduced protein corresponds to a coefficient of thermal expansion of $5.4\ (\pm 0.6) \times 10^{-4}\ °C^{-1}$. This value falls within the range found for most proteins ($2.5\text{–}10 \times 10^{-4}\ °C^{-1}$). The temperature behavior of the expansion coefficient for the oxidized form is unusual with evidence for a maximum around 15 °C. Three points are just not enough to define these changes properly or to attempt an interpretation. It is clear that the values are much higher than those for the reduced protein.

Sound velocity in thioredoxin solutions

The sound velocity in thioredoxin solutions was calculated from the acoustical resonant frequency of the probe containing the solution. Because signal attenuation increases with the viscosity of the solution, the maximum protein concentration was kept below 1.5% (w/v). The results in Figure 2 show that the velocity difference between oxidized and reduced thioredoxin increases with temperature. The sound velocity is greater in solutions of the reduced protein. The error bars in Figure 2 represent the root-mean square error from the least-squares fitted

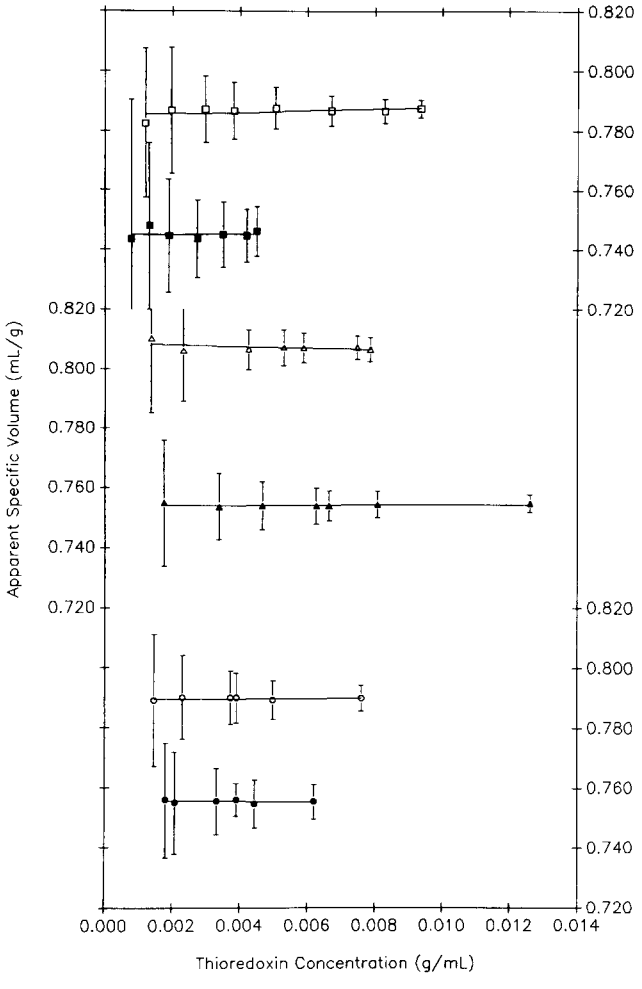

Fig. 1. The apparent specific volume of thioredoxin as a function of concentration. All solutions were dialyzed against 50 mM sodium phosphate, pH 7.0. Buffer for solutions of the reduced protein contained, in addition, 2 mM dithiothreitol. For the 30 °C reduced samples, 2 mM EDTA was added with the dithiothreitol. The filled symbols are data for the reduced protein; the open symbols are for the oxidized form: 5 °C ■, □; 15 °C ▲, △; 30 °C ●, ○.

Table 1. *Partial specific volume*

Temperature: °C	Oxidized thioredoxin: mL/g	Reduced thioredoxin: mL/g
5	0.785 ± 0.007	0.745 ± 0.006
15	0.809 ± 0.008	0.748 ± 0.007
30	0.790 ± 0.006	0.755 ± 0.001

line. If the error is estimated from the precision of the individual measurements (assuming temperature stability of ±0.0001 °C and electronic stability 0.01%) then the error bars would be ±0.05% of the determined sound velocity or double the error indicated.

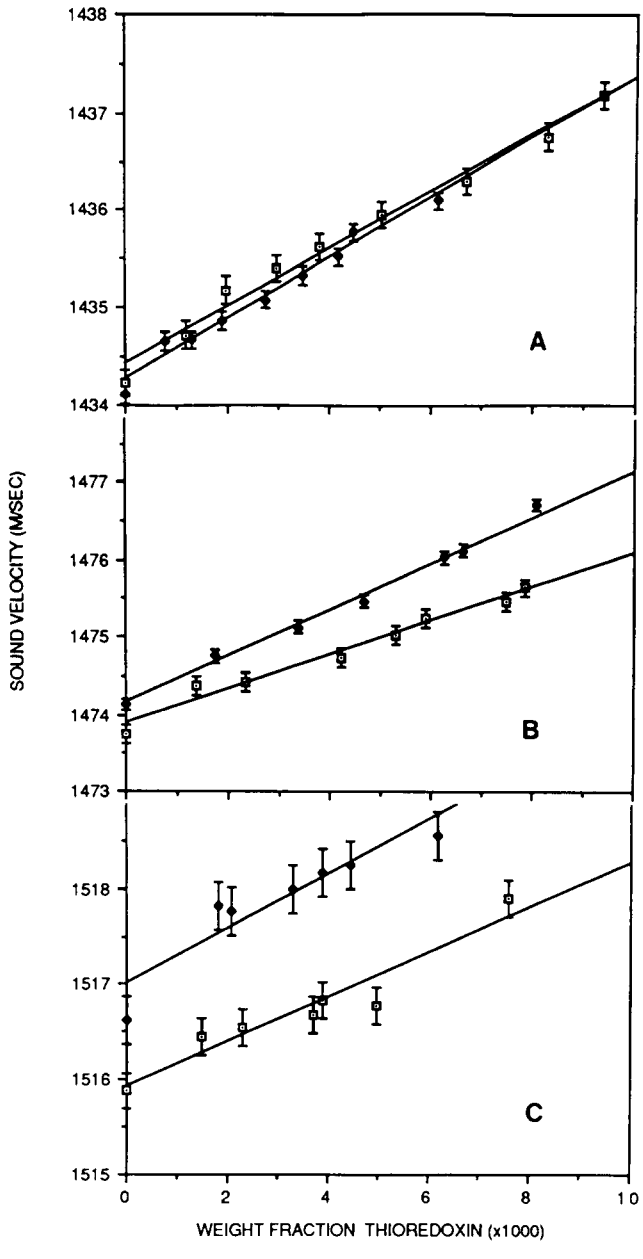

Fig. 2. Sound velocity in oxidized (open squares) and reduced (filled diamonds) thioredoxin solutions vs. the weight fraction of the protein. Panels **A**, **B**, and **C** represent experiments performed at 5 °C, 15 °C, and 30 °C, respectively.

Compressibility calculation

The adiabatic compressibility (β_s) of the protein solution was calculated directly from the density and sound velocity data using the Laplace equation (Gekko & Hasegawa, 1986):

$$\beta_s = (\rho U^2)^{-1}, \quad (4)$$

where U is the sound velocity in the solution. The calculation of partial specific adiabatic compressibility is analogous to that of partial specific volume described above. Compressibility, as given in Equation 4 is used to derive a suitable expression for β_s that allows extrapolation to zero protein concentration. The definition of compressibility is

$$\beta = (1/\bar{v}_2)\delta\bar{v}_2/\delta P, \quad (5)$$

where P is pressure. Combining Equations 2 and 5 and substituting for X_1^{app} from Equation 1, the partial specific compressibility is then given by the expression (Gekko & Noguchi, 1979)

$$\beta = \beta_0 + (\bar{\beta}_s - \beta_0)\bar{v}_2 c, \quad (6)$$

where β is the adiabatic compressibility of the solution, β_0 is the adiabatic compressibility of the solvent, and $\bar{\beta}_s$ is the partial specific adiabatic compressibility of the protein. The low concentration of protein contributes such a small effect on the solution properties of density and compressibility that to obtain reasonable estimates of these protein parameters measured values of these bulk properties must be determined with a precision of at least one part in 10^6.

The adiabatic compressibility of thioredoxin solutions was calculated from Equation 4. The values of β for oxidized and reduced thioredoxin solutions are plotted against weight fraction of the protein at three different temperatures in Figure 3. The partial specific adiabatic compressibility is also a function of the solution density, and this varied substantially with redox states. Therefore, a significant change in β_s was observed. The results are listed in Table 2. Standard deviations of $\bar{\beta}_s$ were calculated from the error estimates used in partial specific volume calculations and the standard deviations of the intercept and slope of the least-squares fit line to the compressibility data. $\bar{\beta}_s$ for oxidized thioredoxin is larger than for the reduced protein. In addition, $\bar{\beta}_s$ increased with temperature for reduced thioredoxin, whereas it decreased above 15 °C for the oxidized protein. Because the thermal denaturation point of the oxidized form is another 50 °C above this temperature, it seems unlikely that this effect is due to a thermal transition in protein structure.

Discussion

In the normal thermodynamic analysis of a binary solution the properties of the solution are divided up among two components. By definition the apparent specific volume of the component designated as the solute is derived on the assumption that the specific volume of all of the solvent is identical to that of the pure solvent. The apparent specific volume of the solute may or may not bear any simple relation to the geometrical volume occupied by its atoms. In sufficiently dilute solutions each solute molecule is completely surrounded with solvent, and solute–solute interactions can be neglected.

At the molecular level in a two-component solution there is a minimum of three regions to be considered: the bulk solvent, the solute molecules, and the interface region between these two. The bulk solvent is sufficiently removed from the solute to be unperturbed, and its properties are identical with the pure solvent. The solute is defined by its geometrical parameters, which, to a first approximation, are unaffected by its environment. The difference in measured volume from that of an ideal mixture may be attributable to the interface region and altered packing of the solvent molecules that it contains.

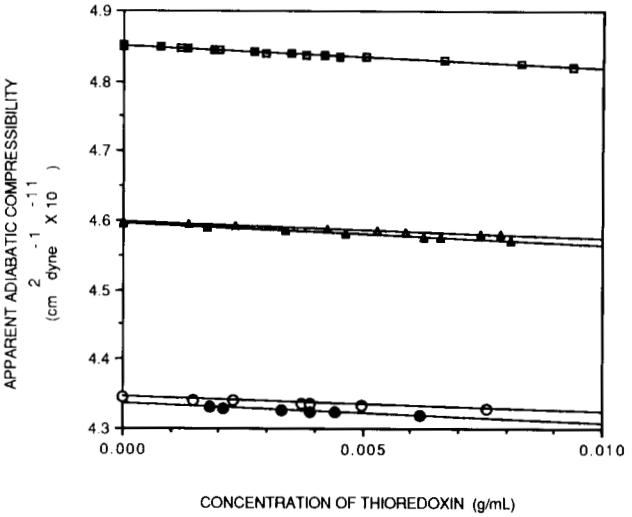

Fig. 3. The apparent adiabatic compressibility ($cm^2\ dyne^{-1} \times 10^{-11}$) of thioredoxin vs. concentration of the protein. The filled symbols are data for the reduced proteins; the open symbols are for the oxidized form: 5 °C ■, □; 15 °C ▲, △; 30 °C ●, ○.

Table 2. *Partial specific adiabatic compressibility*

Experiment	Slope: $cm^4\ sec^{-2}$ ($\times 10^{11}$)	Intercept: $cm^2\ dyne^{-1}$ ($\times 10^{11}$)	Partial specific adiabatic compressibility: $cm^2\ dyne^{-1}$ ($\times 10^{12}$)
Oxidized 5 °C	−3.037 ± 0.002	4.850 ± 0.001	9.81 ± 0.37
Oxidized 15 °C	−2.247 ± 0.004	4.597 ± 0.002	18.16 ± 0.32
Oxidized 30 °C	−2.154 ± 0.003	4.345 ± 0.001	16.18 ± 0.25
Reduced 5 °C	−3.324 ± 0.001	4.851 ± 0.0003	3.89 ± 0.37
Reduced 15 °C	−2.941 ± 0.010	4.595 ± 0.007	6.63 ± 0.50
Reduced 30 °C	−2.721 ± 0.008	4.336 ± 0.0003	7.32 ± 0.15

The present evidence on the structure of thioredoxin from crystallography for the oxidized form (Katti et al., 1990) and from NMR for the reduced form (Dyson et al., 1990) shows that the two structures are remarkably similar, if not identical, except, of course, in the region close to the SS redox center. It appears very likely that the geometrical volume of the protein in the two redox states differs by less than 1%. The actual changes in the active site region cannot be reliably estimated from the available data, but only a small group of atoms are involved.

In the experiments reported here two almost identical solutes appear to have markedly different apparent specific volumes. The value of about 0.75 mL/g for reduced thioredoxin is typical of those published for over a hundred proteins (Durchschlag, 1986). However, the measured $\bar{v}_2$ values for oxidized thioredoxin, 0.785–0.809, are in the upper extreme range of those reported. For example, $\bar{v}_2$ for glyceraldehyde-3-phosphate dehydrogenase (in 2 M potassium phosphate, pH 7) is 0.812 (Aune & Timasheff, 1970), and that for bovine serum albumin (in 1 M sodium sulfate, pH 5.6) is 0.788 (Arakawa & Timasheff, 1984). (Both of these proteins are much larger than thioredoxin, but in a survey of the literature Gekko & Noguchi [1979] found no correlation between partial specific volume or adiabatic compressibility and molecular weight.) Because $\bar{v}_2$ is sensitive to the buffer composition and pH of the solution, there is a range of values for any particular protein. Hence, in the present experiments, the solvent conditions for the comparison of reduced and oxidized thioredoxin were made as similar as possible. Durchschlag and Jaenicki (1982) have noted that phosphate buffer tends to increase $\bar{v}_2$ as determined from density measurements. This effect may have contributed 0.01 mL/g to each redox form of thioredoxin. This contribution to $\bar{v}_2$ should not affect the magnitude of the difference between the redox forms.

The volume change on reduction is on the order of 400–700 mL/mol. Because of the structural similarity of the protein in the two redox forms, we are inclined to attribute this difference to changes in the solute-solvent interface. The origin of such a large change is obscure at this time. Conversion of neutral groups to ones having a formal charge would produce an electrostrictive contraction that may amount to 20 mL per charge pair. The appearance or disappearance of polar but uncharged groups or nonpolar groups are likely to produce smaller effects. No alterations in the protein large enough to produce volume changes of hundreds of milliliters per mole are obvious in the structural data currently available.

Local accessible surface areas can be significantly changed both in total area and/or in the polar/nonpolar ratio by relatively small movements of the side chains on the surface. This can be seen in the changes produced by thermal motion as modeled in molecular dynamics simulations. The influence of such effects on electrostatic calculations has been provided by the study of Northrup et al. (1990). Although the local changes can be quite large, the fluctuation in values averaged over the whole protein is much smaller. The volume and compressibility measurements reported here reflect only the properties of the mean values of the molecular structural parameters.

The interatomic distance between the oxygen atom of water and the carbon atom of a nonpolar group decreases by about 1 Å if the carbon atom is changed to a nitrogen or oxygen due to the formation of a hydrogen bond (Thanki et al., 1988). This corresponds to the interpenetration of the van der Waals spheres of the two atoms and thus a volume change. From simple sphere geometry, such a chemical alteration would have to involve about 15% of the total surface area to produce the observed effect on volume. Although this kind of alchemical change can be modeled in a computer, it could only happen in a protein of fixed covalent structure by significant rearrangement of a number of residues. Side-chain movement can expose or cover polar groups such as main-chain peptide bonds in addition to polar atoms in the side chains themselves. Water molecules can bond to, or be excluded from, the carbonyl oxygen atoms without breaking the peptide bonds. However, as the side chains move, covering and exposing will both occur and the net effect averaged over the whole molecule would be expected to be small. In the present system major structural changes apparently do not occur and thus significant alteration in the total surface polar/nonpolar ratio is hard to picture.

The only region known to undergo a structural change is the disulfide bond and its immediate neighborhood. Using the detailed X-ray structure of the oxidized protein, one can calculate the free energy of solvation of the surface of the protein using the solvent accessibilities of the individual atoms and the procedure described and parameterized by Eisenberg and McLachlan (1986). The results of such a calculation are shown in Figure 4. Strikingly, there is a large positive free energy contributed by the residues at the active site turn. Cleavage of the SS bond may not only produce the more polar SH groups but, by inducing other shifts, significantly change the polar/nonpolar area ratio in this region. Because the first β-strand also has a large positive free energy of solvation and it is only 10 Å from the active site, it is possible that cooperative changes occur at this site as well. These two regions correspond closely to the peptides that undergo a change in amide proton exchange rates upon reduction of thioredoxin (Kaminsky & Richards, 1992).

The adiabatic compressibility of reduced thioredoxin is in the middle of the published range of -1.0 to $+12.0 \times 10^{-12}$ cm^2 dyne^{-1} for proteins (Gekko & Hasegawa, 1986). Oxidized thioredoxin has a very large compressibility ($9.8–18.2 \times 10^{-12}$ cm^2 dyne^{-1}), above the largest published value. In this system something has

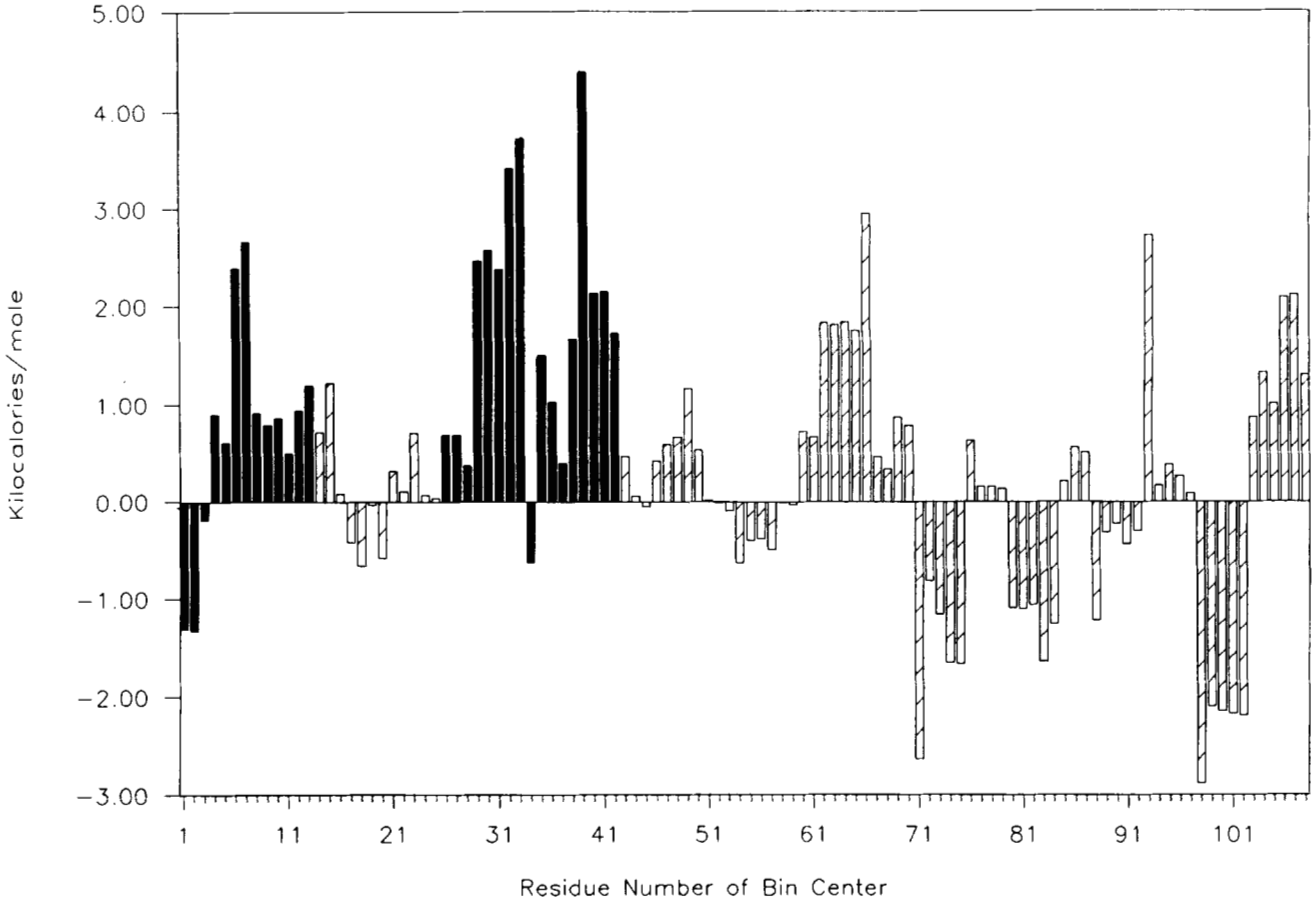

Fig. 4. A plot of the free energy of solvation of oxidized thioredoxin. The energy per residue is calculated as the sum of the products of the solvent accessible area of each component atom and the atomic solvation parameter appropriate for that atom type. The accessible area for each atom was determined from the high resolution X-ray structure of thioredoxin (Katti et al., 1990) using the computer program ACCESS (Richards, 1977). The atomic solvation parameter is a measure of the sign and magnitude of the solvent interaction energy with units of cal $Å^{-2}$ mol^{-1}. The values of this parameter for each of the protein component atom types were taken from Eisenberg and McLachlan (1986). For this figure the free energy was summed over five residue bins centered at the residue indicated on the abscissa. The regions that have significant redox-sensitive hydrogen exchange rates are shown as solid bars.

increased in volume. Because the atomic volumes cannot have changed, the increase must reflect an increase in void volume in the system. Under these circumstances the compressibility would also be expected to increase as observed. If the structures of the two redox forms are essentially the same, the total internal void volume of the proteins will also be the same. Thus the compressibility results also point to the solvent interface layer as the principal location of the altered packing.

Although the values of compressibility and partial specific volume for oxidized thioredoxin are both very large, the relationship between the two are consistent with other proteins. Correlations between adiabatic compressibility and partial specific volume or thermal stability have been reported (Gekko & Hasegawa, 1986). The data in Figure 5 are taken from their paper with the values for oxidized and reduced thioredoxin added for comparison. The values of adiabatic compressibility and partial specific volume for both forms of thioredoxin are consistent with the correlation between these two parameters for the other proteins tested. Adiabatic compressibility also shows a weak positive correlation with thermal stability. Using the best fit line from the data of Gekko and Hasegawa (1986), thioredoxin, with a thermal denaturation point of about 85 °C, is predicted to have an adiabatic compressibility of approximately 16×10^{-12} cm^2 $dyne^{-1}$ at 25 °C, close to that found in this study.

Although the large changes in volume, in thermal denaturation temperature (Hiraoke et al., 1988), and in fluorescence quantum yield (Holmgren, 1972), all correlate with redox changes, it is not at all clear what structural or dynamic alterations mediate these effects. One is left with the assumption that some cooperative effect is occurring in the solvent interface layer. It has been sug-

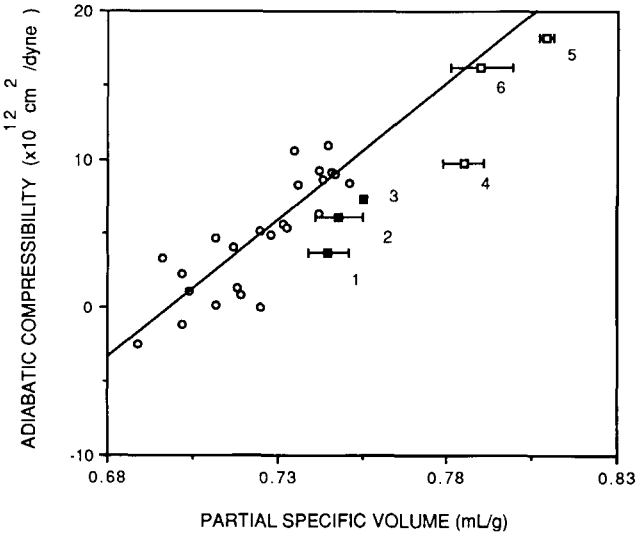

Fig. 5. The adiabatic compressibility, $\bar{\beta}_s$, versus the partial specific volume, $\bar{v}_2$, of various proteins. The line represents the linear least-squares best fit to all the data presented except that of thioredoxin. This figure is derived from the publication of Gekko and Hasegawa (1986). The symbols for proteins discussed in the text are as follows: 1, Reduced thioredoxin at 5 °C; 2, reduced thioredoxin at 15 °C; 3, reduced thioredoxin at 30 °C; 4, oxidized thioredoxin at 5 °C; 5, oxidized thioredoxin at 15 °C; 6, oxidized thioredoxin at 30 °C.

gested that the solvent interface layer could, in fact, affect thermal stability (Gekko & Hasegawa, 1986) and hydrogen exchange (Scarpa et al., 1967). This could conceivably be related to a subtle change in the net distribution of the polar and nonpolar patches between the two redox states. However, without a detailed model it is hard to picture a mechanism. At the moment it remains a mystery.

Whatever the source of the volume change, the effect may produce alterations in the interactions of this multifunctional protein with other ligands. For example, in its role in the maturation of the phage M13, thioredoxin must have its reduced structure even though the redox properties are not directly involved in that function. The C32S-C35S mutant missing the disulfide bond does just as well (Russel & Model, 1986). This suggests that in the appropriate systems the redox reaction can be used as a biological control process through changes in the physical properties of the protein rather than as a chemical mechanism to pass electrons back and forth.

Methods and materials

Thioredoxin purification and sample preparation

Thioredoxin was purified to homogeneity by the method of LeMaster and Richards (1988). The protein was lyophilized and resuspended in 50 mM sodium phosphate buffer (pH 7.0) and extensively dialyzed against 50 mM sodium phosphate (pH 7.0) with or without 2 mM dithiothreitol (DTT; Pierce Chemical) to produce reduced or oxidized thioredoxin, respectively. The protein solution was degassed and particulates were removed by filtration through a 0.45-μm filter.

Density measurements

Solution densities were determined by the vibrating U-shaped capillary method of Kratky et al. (1969) on a Mettler-Paar density meter (model DMA60) with two jacketed measuring cells (model DMA602). A distilled water reference solution was used to minimize the effects of temperature or pressure fluctuations during the measurement. The density of each sample was determined at the same temperature (±0.1 °C) used in the sound velocity measurements for that same sample. Temperature was monitored via thermocouples in each of the cells. The density, ρ, of the solution was calculated from the following equation:

$$\rho = (Q^2 - A)/B, \tag{7}$$

where A and B are cell constants determined from calibration with water and air, and Q is a number that is indicated by the density meter and is related to the period of oscillation. Calibration measurements for A and B were performed after every two or three protein samples. The actual values for A and B were then interpolated from the calibration points to the time of the actual Q value measurement. The maximum variation in A or B was less than 0.016% during an 8-h period. T values were recorded only after achieving equilibration in the last of six decimal places. After each measurement, the sample cell was rinsed with distilled water and dried with acetone.

The errors associated with the determination of solution density were calculated from the variation in the measured values of the parameters A, B, and Q used in Equation 7. The following estimates were used: $\sigma Q/Q = 2 \times 10^{-6}$, $\sigma A/A = 2\text{-}5 \times 10^{-6}$, $\sigma B/B = 0.2\text{-}1.5 \times 10^{-5}$.

Protein analysis

Thioredoxin concentrations were determined by absorbance at 280 nm using an extinction coefficient of 13.7 mM^{-1} cm^{-1} for both redox states (Reutimann et al., 1981). The redox state of thioredoxin was determined by fluorescence spectroscopy using an excitation wavelength of 280 nm and scanning the emission from 300 to 400 nm. Reduced thioredoxin exhibits a six- to sevenfold greater quantum yield than the oxidized form as well as a shift

in the wavelength of maximum emission. To confirm the redox state, DTT was added to the solution and a second scan performed. No spectral changes were observed for any sample of the reduced thioredoxin upon addition of DTT.

Sound velocity measurements

Sound velocity measurements were made by the method first developed by Shiio et al. (1955), later refined to the "sing-around" technique (Greenspan & Tschiegg, 1957), which measures the acoustical resonance of a chamber filled with a solution. Experiments were performed with a Mapco Nusonics concentration analyzer (model 6080) with a sound transmitter operating at 2 MHz. The device emitted a pulse of sound that traversed the chamber filled with the protein solution. A receiver detected the pulse, and a positive feedback loop then triggered the next pulse. The pulse generation frequency was measured to six digits with a frequency meter (Heath/Zenith model SM-2420). The sound velocity through liquids is extremely temperature dependent; therefore, the sample chamber was placed in a large, precisely controlled temperature bath.

The probe was rinsed with alcohol, several changes of sterile water, and finally dialysate before loading the protein solution in a manner to minimize bubbles. Trapped air was monitored by the attenuation of the sound signal: data were not collected if the attenuation was above 20%. Typical attenuation values were 10–12%. The probe was submerged in a temperature bath set to the indicated temperature, estimated with an accuracy of ±0.01 °C, but maintained at the preset temperature with a precision of ±0.0001 °C by a temperature controller (Tronac Inc., model PTC-41). Temperature calibration was accomplished by comparing the measured sound velocity of distilled, degassed, filtered water with standard charts provided by Mapco. The sound velocity, U, was obtained by the following equation:

$$U = \frac{fA(1 + \alpha T)}{7 - fB}, \tag{8}$$

where f is the measured frequency, T is the temperature, α is the coefficient of thermal expansion of stainless steel (the vessel material), and A and B are the sonic path length and electronic time delay, fixed parameters of the individual vessel and transducer. The long-term (24 h) reproducibility of the sound velocity measurement was ±0.08 m/s at 5 °C and 30 °C and ±0.02 m/s at 15 °C. The reduced stability at 5 °C and 30 °C was probably due to less effective temperature regulation when further away from room temperature.

Freshly dialyzed protein was used as the highest protein concentration sample, and the final dialysate was used as the zero protein concentration sample. The two solutions were mixed to obtain intermediate concentrations. After each sound velocity measurement, an aliquot was removed directly from the probe for immediate analysis of protein concentration and then stored at −20 °C for subsequent redox state and density determinations.

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