to Tyr-36 in α-lactalbumin, may have a pK value close to 11.6 (Latovitzki et al., 1971).

One or two tyrosines in α-lactalbumin were known to be unreactive with cyanuric fluoride or with N-acetylimidazole, which may be consistent with the present results (Gorbunoff, 1967; Kronman et al., 1971). Prieels et al. (1975) have suggested that Tyr-103 in human α-lactalbumin, in which all the tyrosines occupy the same positions as in bovine α-lactalbumin, is more easily nitrated than Tyr-18 and that these two residues are more exposed than Tyr-36 and -50. The results are also consistent with the assignment in Table I.

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References

Allerhand, A., Norton, R. S., & Childers, R. F. (1977) *J. Biol. Chem. 252*, 1786.
Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, Ser. B 167*, 365.
Brew, K., Castellino, F. J., Vanaman, T. C., & Hill, R. L. (1970) *J. Biol. Chem. 245*, 4570.
Browne, W. J., North, A. C. T., Phillips, D. C., Brew, K., Vanaman, T. C., & Hill, R. L. (1969) *J. Mol. Biol. 42*, 65.
Donovan, J. W. (1973) *Methods Enzymol. 27*, 525.
Eigen, M. (1964) *Angew. Chem., Int. Ed. Engl. 3*, 1.
Garel, J., & Baldwin, R. L. (1975) *J. Mol. Biol. 94*, 621.
Gorbunoff, M. J. (1967) *Biochemistry 6*, 1606.
Hill, R. L., Steinman, H. M., & Brew, K. (1974) in *Lysozyme* (Osserman, E. F., Canfield, R. E., & Beychok, S., Eds.) p 55, Academic Press, New York.
Hiromi, K., Ohnishi, M., Kanaya, K., & Matsumoto, T. (1975) *J. Biochem. (Tokyo) 77*, 957.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd Ed. 7*, 665.
Inada, Y. (1961) *J. Biochem. (Tokyo) 49*, 217.
Kato, K., & Murachi, T. (1971) *J. Biochem. (Tokyo) 69*, 725.
Kita, N., Kuwajima, K., Nitta, K., & Sugai, S. (1976) *Biochim. Biophys. Acta 427*, 350.
Kronman, M. J., & Robbins, F. M. (1970) in *Fine Structure of Proteins and Nucleic Acids* (Fasman, G. D., & Timasheff, S. N., Eds.) p 271, Marcel Dekker, New York.
Kronman, M. J., Holmes, L. G., & Robbins, F. M. (1967) *Biochim. Biophys. Acta 133*, 46.
Kronman, M. J., Holmes, L. G., & Robbins, F. M. (1971) *J. Biol. Chem. 246*, 1909.
Kurz, L. C., & Holtzer, A. (1977) *Arch. Biochem. Biophys. 178*, 546.
Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359.
Latovitzki, N., Halper, J. P., & Beychok, S. (1971) *J. Biol. Chem. 246*, 1457.
Nitta, K., Kita, N., Kuwajima, K., & Sugai, S. (1977) *Biochim. Biophys. Acta 490*, 200.
Prieels, J., Dolmans, M., Leonis, J., & Brew, K. (1975) *Eur. J. Biochem. 60*, 533.
Robbins, F. M., & Holmes, L. G. (1970) *Biochim. Biophys. Acta 221*, 234.
Robbins, F. M., Andreotti, R. E., Holmes, L. G., & Kronman, M. J. (1967) *Biochim. Biophys. Acta 133*, 33.
Steinhardt, J., & Stocker, N. (1973a) *Biochemistry 12*, 1789.
Steinhardt, J., & Stocker, N. (1973b) *Biochemistry 12*, 2798.
Strosberg, A. D., von Hoeck, B., & Kanarek, L. (1971) *Eur. J. Biochem. 19*, 36.
Sugai, S., Yashiro, H., & Nitta, K. (1973) *Biochim. Biophys. Acta 328*, 35.
Tojo, T., Hamaguchi, K., Imanishi, M., & Amano, T. (1966) *J. Biochem. (Tokyo) 60*, 538.
Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J., & Hiromi, K. (1978) *Anal. Biochem. 84*, 370.
Warme, P. K., Momany, F. A., Rumball, S. V., Tuttle, R. W., & Scheraga, H. A. (1974) *Biochemistry 13*, 768.

Conformations of Denatured and Renatured Ovotransferrin†

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ABSTRACT: Conformational properties of native, denatured, and renatured ovotransferrin were studied. The samples were denatured either in 7.2 M urea or in acidic (pH 3.0) conditions for periods up to a few hours. Combined data from quasi-elastic light scattering and transient electric birefringence were used to estimate the molecular dimensions under the various conditions. The native ovotransferrin is best described as a prolate ellipsoid with a major axis a = 68 Å and a minor axis b = 21 Å. Such an ellipsoidal shape is consistent with a globular particle where the solvation factor is ~0.28 mg/mg of solute. The urea-denatured sample was more expanded and more globular than the native sample. This observation was supported by a decrease in helical content, which was shown using circular dichroism data. Complete recovery of conformation and capacity to form a colored complex with Fe^{3+} seemed to occur with the simple dilution of urea or by adjustment of the low pH sample to pH 7.3.

Ovotransferrin (OT)[1] is an iron (Fe^{3+}) binding egg-white protein which is homologous with serum transferrin (Feeney & Komatsu, 1966; Greene & Feeney, 1968; Feeney & Allison, 1969; Osuga & Feeney, 1977; Thibodeau et al., 1978). The amino acid content of OT indicates there are 22 CySH residues (Osuga & Feeney, 1977), all of which exist as 11 disulfide bonds. These bonds are not easily destroyed even in the

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[1] Abbreviations used: OT, ovotransferrin; CD, circular dichroism; QELS, quasi-elastic light scattering; TEB, transient electric birefringence; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; NTA, nitrilotriacetate.

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environment of 8 M urea (Azari & Feeney, 1961). Consequently, the conformational changes of OT that occur upon denaturation by urea or other denaturing agents, which do not break disulfide bonds, are constrained by these bonds.

The reversibility of the denatured OT has been reported by many previous workers. Phelps & Cann (1956) reported that the denaturation by acid is reversible if the exposure in an acidic medium at room temperature is not too long. They found that at low pH (<pH 4.2) OT denatures with a concurrent decrease in sedimentation coefficient and an increase in viscosity. Azari & Feeney (1961) found that the iron-free OT can also be reversibly denatured in 8.0 M urea at pH 9.5 and 37 °C, while Fe–OT was completely stable under similar conditions. OT and Fe–OT have also been extensively studied by Glazer & McKenzie (1963), who noted "partial recovery" of iron-binding capacity, as well as recovery of other physical properties, when the denaturing agent, either 5 M urea at pH 4.5 or 6 M urea at pH 5.9–6.1, was diluted to half the original concentration at 30 °C. CD spectra of native OT and Fe–OT have also been reported (Tan, 1971; Tomimatsu & Vickery, 1972). Although studies of specific functional sites of OT have been conducted using spin labels (Zweier & Aisen, 1977) and the depth and location of these label-binding sites have been extensively investigated for many systems (cf., Cornell & Kaplan, 1978), the overall hydrodynamic conformation upon denaturation of OT still remained unclear. There existed the question of whether the changes occurring upon denaturation were due to a total unfolding or a swelling of the molecule.

In contrast to OT, diferric OT is extremely resistant to denaturation (Azari & Feeney, 1961). No denaturation of diferric OT was observed in 8 M urea, even after 5 h at 37 °C, and diferric OT was much more stable than OT to denaturation by solvents or by heat. Interest in the reversible denaturation of OT is also stimulated by the relatively high stabilities of its metal complexes.

To provide further characterization of the denatured and renatured states of OT, a series of studies was conducted exploiting several of the newer techniques for protein characterization. QELS (Chu, 1974) and TEB (O'Konski, 1976) methods were used to characterize the size and shape of the OT molecules in their native, denatured, and renatured states. CD spectra of the peptide backbone region (200–250 nm) were examined to determine the α-helical/random coil contents. The binding capacity of the renatured states was compared with the control system as a test for chemical reversibility. The bulk of the work was performed using a 7.2 M urea denaturation/renaturation sequence. Acid denaturation at pH 3 was also examined by QELS and iron-binding techniques.

Our results indicate that the urea-denatured state is highly expanded toward a more spherical prolate ellipsoid than is the native system or the renatured system. Acid denaturation does not expand the molecule as much as does the urea denaturation. α-Helical content of the denatured species was considerably lower than either the native or renatured species. Both denaturation processes were essentially 100% reversible under certain controlled conditions.

Materials and Methods

Materials

OT was isolated from chicken egg white as previously reported (Feeney & Komatsu, 1966; Rogers et al., 1977). UltraPure grade urea (Schwarz/Mann) was recrystallized once from 95% ethanol just before use. Tris (Eastman Kodak Co.) was reagent grade and glycerol (Mallinckrodt, Inc.) was SpectrAR grade, and both were used without further purification. The Millipore filter (pore size 0.22 μm) and Chelex 100 (100–200 mesh) were purchased from Millipore Corporation and Bio-Rad Laboratories, respectively.

All the solutions used were treated with Chelex in order to eliminate trace amounts of metal contaminants according to the method reported by Willard et al. (1969).

Urea has been known to undergo decomposition, especially at high temperature, with the formation of a cyanate ion (Stark et al., 1960). To avoid such a side reaction, the urea solution was carefully prepared just before use.

Methods

Preparation of Apotransferrin. Iron-free OT was prepared by dialysis against 0.1 M acetate buffer–0.01 M EDTA (pH 4.5), deionized water, 0.1 M sodium perchlorate, and deionized water, in that order, and then it was lyophilized. The purity was checked by gel electrophoresis and iron-binding activity. Pure iron-free OT was used throughout the experiments.

Denaturation and Renaturation. OT in 0.1 M Tris-HCl buffer (pH 7.8) was used as the native sample. The urea-denatured sample was prepared by adding a ninefold volume of 8 M urea/0.1 M Tris-HCl buffer (pH 7.8) into the native protein solution. The renatured sample was made by adding a ninefold volume of 0.1 M Tris-HCl buffer (pH 7.8) into the denatured protein solution, which was then kept up to 2 h at room temperature.

Phelps & Cann (1956) reported that OT was denatured if the protein were exposed to a pH below 4.2; however, the effect of pH was reversible under certain experimental conditions (e.g., ionic strength of 0.1; pH changing from 5.3 to 3.0). OT in pH 3 solution (1 mM HCl + 99 mM NaCl) was used as the acid-denatured sample in our experiment. The pH of the sample was adjusted by HCl and NaOH when necessary, but the ionic strength was kept constantly at 0.1 M throughout the experiment. The renatured sample was prepared by the addition of 0.1 M Tris-HCl buffer (1.5 times the volume of the denatured sample at pH 7.8) into the denatured sample.

Protein Concentration Determinations. The protein concentrations of the final samples of each set of experiments were roughly standardized to 0.8 mg/mL for QELS and CD, 2 mg/mL for TEB, and 10 mg/mL for assay of iron-binding activity.

The measured urea-denatured and renatured samples were dialyzed against distilled water. Then the protein concentrations were measured by using $E_{280}^{1\%} = 11.6$ (Osuga & Feeney, 1977) and the Lowry method (Lowry et al., 1951).

QELS for Translational Diffusion Coefficients. Translational diffusion coefficients (D_t) were measured by the QELS method (Chu, 1974). Since this light scattering method gives a z-averaged diffusion coefficient (Berne & Pecora, 1976), solution heterogeneity can cause serious interpretational difficulties. Therefore, the sample solution was made as homogeneous and dust-free as possible.

In order to accomplish this task, solutions were carefully centrifuged and filtered before measurement to remove small amounts of contaminants. Sample cells for these studies were cleaned by chromic acid washes and distilled water rinses, followed by an ethanol reflux bath. These cells were then sealed with paraffin wrappers, and they were not opened to the atmosphere until introduction of the sample. All sample loadings were conducted under a laminar flow clean bench. The native OT solution was centrifuged at 15 000 rpm for 20 min. The urea-denatured sample was prepared by adding urea solution into the supernatant of the centrifuged native protein sample. For renaturation, the urea-denatured sample was centrifuged at 8000 rpm for 20 min, and then Tris buffer was

added. The acid-denatured sample was centrifuged at 8000 rpm for 20 min. The supernatants of these samples were filtered through a Millipore filter (pore size 0.22 μm) into a dust-free light scattering cell.

The light source for the QELS experiments was a krypton ion laser (Spectra Physics Model 165) operating at 647.1 nm. As determined by optical density spectra of the native OT samples at this wavelength, the optical absorption of the residual tracer metallic ions is essentially negligible. The scattering spectrometer has been previously described (Selser et al., 1976). An improved modification of the past apparatus was the use of a thermoelectrically cooled GaAs photomultiplier tube, RCA C31034A, which has a cathode quantum efficiency at the operating wavelength of ~30%. The autocorrelation spectra were obtained using a Malvern K7023 24-channel correlator. Signals were sampled, processed, and accepted or rejected according to the techniques described by Yeh et al. (1978).

TEB for Rotational Diffusion Coefficients. The rotational diffusion coefficient, D_R, of these globular proteins can, in principle, be measured by the method of depolarized QELS. However, the requirements of high sensitivity and time resolution made the situation somewhat difficult to handle using our present apparatus. Consequently, TEB decay studies were conducted to determine D_R.

Samples for the TEB experiments were dissolved in 0.01 M Tris-HCl buffer (pH 7.8) in the presence of glycerol instead of 0.1 M Tris buffer. Glycerol was used so that the rotational diffusion coefficient would be measurable with the apparatus. OT in a buffer composed of 60% glycerol and 0.01 M Tris was used for the native sample. Glycerol, 35%, in 7.2 M urea/0.01 M Tris buffer was used for the denatured sample. The renatured sample, lyophilized after dialysis against distilled and deionized water, was dissolved in 60% glycerol/0.01 M Tris buffer.

The details of the TEB method have been amply reviewed (Fredericq & Houssier, 1973; O'Konski, 1976). Briefly stated, when a pulse of strong electric field is applied onto a molecular system, the permanent and induced dipoles align preferentially with respect to the field. Such molecular alignments lead to optical birefringence for the duration of the alignment pulse. When the pulse is turned off, the oriented molecules relax toward a random distribution. Since the rerandomization of dipoles is via a rotational diffusion process, the observed decay time of the birefringent signal in the field-free situation is a good measure of D_R. The method of the experiment and the interpretations of data were similar to those discussed by O'Konski & Krause (1976). OT samples in 35 and 60% glycerol experience approximately a three- and tenfold increase in the solvent shear viscosity, respectively. The ionic strength was decreased from the 0.1 to 0.01 M Tris buffer to prevent the possibility of conduction heating. Since pH 7.8 is not far from the isoelectric point of the OT system (Feeney & Komatsu, 1966), desalting by this factor of 10 was not considered a problem. The native sample was checked and shown to have the same D_t as the sample in the higher ionic strength environment.

CD Measurement. The CD spectra were measured using a Cary 60 recording spectropolarimeter fitted with a Model 6002 CD attachment in a constant temperature room (23 °C). The OT samples were prepared in the same manner as those for light scattering measurements, except that the centrifugation process was not used for CD measurement. The optical path lengths of the cells were 1.0, 0.5, and 0.1 mm. CD results are reported in terms of $[\theta]$, the mean residue ellipticity, in units of deg·cm^2/dmol. The mean residue weight was taken as 112 (Tomimatsu & Vickery, 1972).

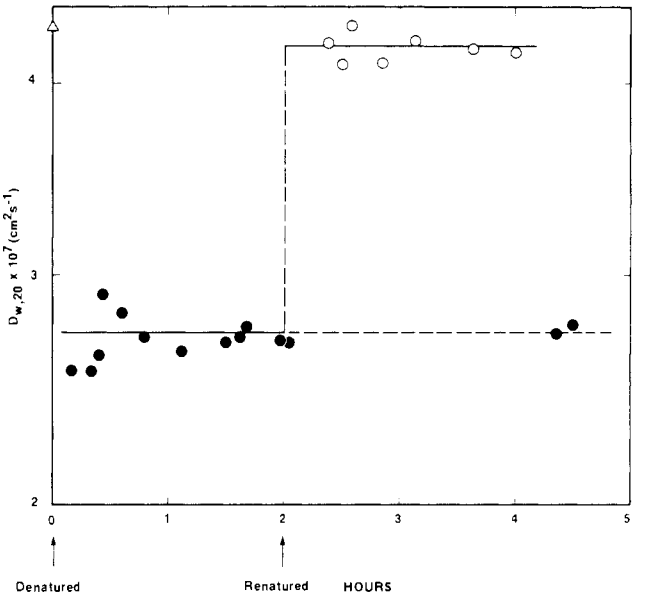

FIGURE 1: Time development of denatured and renatured OT. Native OT (△). Denaturation (●) was achieved by the use of 8.0 M urea. Renaturation (○) was conducted with 0.7 M urea concentration. Note the control denatured sample was reexamined after 4 h.

Iron-Binding Activity Measurement. The iron-binding activity was determined by a spectrophotometric titration at 470 nm (Harris & Aisen, 1975; Rogers et al., 1977). All samples except the urea-denatured sample were standardized at 10 mg/mL of protein concentration. The urea-renatured sample was dialyzed against distilled and deionized water and then lyophilized before dissolution into 0.1 M NH_4HCO_3 in the presence of 0.7 M urea (10 mg/mL). The samples in 0.1 M NH_4HCO_3 were titrated with 3.5 mM Fe–NTA solution. A plot of the absorbance at 470 nm vs. the amount of added iron gave the equivalence point. The equivalence point divided by protein concentration gave the amount of iron bound by OT. An iron-binding activity measurement was taken after each of the series of other physical studies.

Results and Discussion

Translational Diffusion Coefficient. The extrapolated zero concentration value of $(D_t)^0_{w,20}$ was found to be 4.47 ± 0.1 × 10^{-7} cm^2 s^{-1} by the present technique. This value is somewhat lower than those previously obtained by Fuller & Briggs (1956), where $(D_t)_{w,20} = 5.30 \times 10^{-7}$ cm^2 s^{-1}, using a sedimentation technique. One of the possible reasons for such dissimilarity in D_t is the difference in the averaging considered between the two techniques for a heterogeneous sample system, the z-average being more susceptible to the presence of higher molecular weight aggregates. In an analysis of the different averaging techniques, it was shown (see Appendix) that a 30% difference in D_t may be possible between these two experiments just on this basis alone.

Time Course of D_t upon Denaturation and Renaturation. All these studies were conducted at a concentration of ~1 mg/mL using a value of $(D_t)_{w,20} \sim 4.31 \pm 0.30 \times 10^{-7}$ cm^2 s^{-1} for the native sample. Figure 1 shows the change of $(D_t)_{w,20}$ to a value of 2.70 ± 0.15 × 10^{-7} cm^2 s^{-1} upon 8.0 M urea addition. This steady-state value was measured 15 min after the urea addition, and it remained constant for at least 4 h.

When a portion of the sample was returned to a urea concentration of 0.7 M, recovery of $(D_t)_{w,20}$ to the value of 4.21 ± 0.30 × 10^{-7} cm^2 s^{-1} was complete in approximately 20 min.

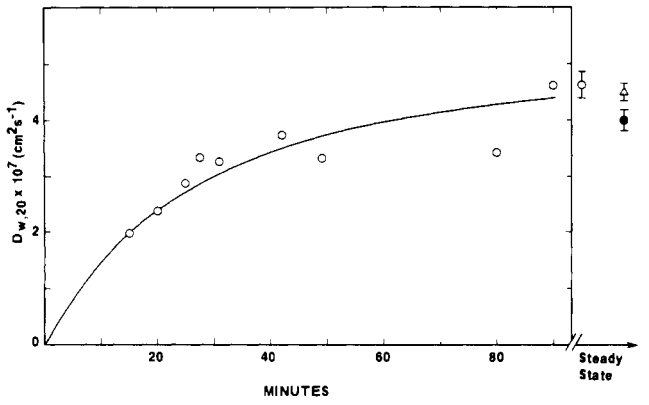

FIGURE 2: Time development of the renaturation process of acid denatured OT. Concentration of OT in the denatured state (pH 3) was ~10 mg/mL. The sample was diluted 10:1 in Tris buffer at pH 7.8. Note comparison values of D_t for steady-state native (△), denatured (●), and renatured (○) samples.

Again, no excessive aggregation was observed even after 2 h.

In order to show that the effect seen was due to denaturation and not simply to an aggregation process, the scattered intensities for all experiments were compared. It can be shown that the ratio of the intensity of scattered light to the sample concentration (I/c) is proportional to the molecular weight of the sample (Fabelinskii, 1968). Present data indicate that all observed values of I/c are constant to about 20%. Consequently, high molecular weight aggregation is an unlikely explanation for the observed changes in D_t.

The $(D_t)_{w,20}$ values were also obtained from acid-denaturation studies. Here, two features are prominent. The denatured sample has a value of $(D_t)_{w,20}$ which is only about 10% less than that of the corresponding native sample.

Evidence for a still further difference between the two denaturing agents was obtained when renaturation of the acid-denatured sample was initiated by a 10:1 dilution of the pH 3 solution. A precipitate immediately developed upon the introduction of the high pH buffer. After 2 h this precipitate essentially disappeared (Figure 2). The concept of different pathways for the recovery from denaturation is an interesting one for further exploration.

TEB Measurements of D_R. It is well-known that, even though the rise of the TEB signal toward its steady-state value is the result of dynamic equilibrium between the alignment forces and the randomizing forces, the decay in the field-free case is completely described by rotational diffusion motion. Consequently, TEB decay is often used to measure the rotational diffusion coefficient. For globular proteins, the field-denaturation effect is considered to be at a minimum.

In these studies, the introduction of glycerol was checked for possible adverse interactions with the sample system. It was determined that, after correcting for the viscosity, the value for OT was not affected by glycerol. Furthermore, glycerol does not give rise to any TEB signals on its own (Krause & O'Konski, 1963).

The decay signals of the native and renatured samples (urea free in the latter case) were fitted to the best single exponential decay. The denatured sample, due to the presence of ~7.2 M urea, had to be fitted to a two-component exponential. The faster instrument-limited component was attributed to the decay of urea, while the slower one was ascribed to the OT sample.

Table I summarizes the TEB decay results. It is evident that the denatured sample experienced more than a threefold decrease in D_R, while simultaneously suffering a drop of ~40% in D_t.

Table I: Denaturation and Renaturation of Ovotransferrin; Urea Denaturation

sample	QELS, $\langle D_t \rangle_z \times 10^7$ (cm^2 s^{-1})	conversion by eq 9,[a] $\langle D_t \rangle_w \times 10^7$ (cm^2 s^{-1})	TEB relaxation, $\langle \tau \rangle$ (μs)	viscosity corr to w, 20°C, $\langle D_R \rangle_w \times 10^{-6}$ (s^{-1})[b]	sense of biref. δ_B	Perrin eq[c] prolate ellipsoid A a	b	ρ	solvation factor,[d] δ_1 (mg/mg of sol.)	CD[f] α%	β%	RC%	Fe^{3+} binding[g] (%)
native	4.31 ± 0.30	6.14 ± 0.43	0.82 ± 0.14	2.17 ± 0.32	−	68	21	0.31	0.28	11 (11)	32	57	86
denatured 7.2 M urea	2.70 ± 0.15	3.86 ± 0.21	0.81 ± 0.11	0.72 ± 0.08	+	84	42	0.50	4.16[e]	6 (0)	19	75	<5 (0)
renatured	4.21 ± 0.30	6.00 ± 0.43	0.93 ± 0.07	1.91 ± 0.13	−	67	22	0.33	0.36	12 (10)	29	59	82

[a] Dispersion factor, $\delta = 0.1$, used in eq 9 (see text). [b] Weight averaging is directly obtained by TEB. [c] Oblate ellipsoid values were disk-like, incompatible with other reported results. [d] $\bar{\nu} = 0.73$, molecular weight 77 000 values used in determining δ_1. [e] See text for a discussion of this high value. [f] Values in parentheses calculated from method of Chen & Yang (1971); other values by method of Greenfield & Fasman (1969). [g] Values are calculated from direct color determinations on solutions. True values should be higher.

From the column labeled δ_B in Table I, it can be seen that the native and renatured OT both have negative birefringence factors, δ_B, while the denatured OT has a positive δ_B value. The presence of such a sign switch usually signifies extensive structural arrangement during denaturation, so that the strong electrical polarizability axis and the strong optical polarizability axes develop mutually perpendicular orientations. The details of this phenomenon are highly significant in our attempts to characterize more fully the OT denaturation–renaturation process. More extensive studies are being conducted.

The use of D_t and D_R with Perrin's equations (Perrin, 1934, 1936) for ellipsoids of revolution allows determination of the actual changes in sizes and shapes. In Table I, the D_t and D_R data are systemized, and the corresponding axial ratios were computed using an inversion method for the Perrin equations. The validity of our inversion technique was first verified by comparisons of the lysozyme data from Dubin et al. (1971) and an inversion technique developed recently by Wright & Baxter (1976). In Table I the first column data on $\langle D_t \rangle_z$ has been converted to $\langle D_t \rangle_w$ according to eq 9 of the Appendix. Even though the saturation field was not actually reached in our TEB experiments due to instrumental difficulties, the fields were strong enough to suggest the onset of saturation in many cases. Under saturation conditions, O'Konski et al. (1959) showed that the decay time, and therefore $\langle D_R \rangle$, is also the weight-averaged value. A more recent analysis of polydispersed systems by Kobayasi (1968) indicated that, at very low field strengths, the $\langle D_R \rangle$ may be weighted even more than the z-averaging weights, depending on whether the contribution to the birefringence signal was from the permanent or the induced dipole moment. This problem is currently under investigation.

The axial dimensions obtained in this study for native apo-ovotransferrin (a = 68 Å; b = 21 Å) compare favorably with those determined by Rosseneu-Motreff et al. (1971) for the homologous protein, apo-serum transferrin (a = 62 Å; b = 24.6 Å). It is of interest to note that these dimensions indicate that the apo form of transferrin is less spherical than the Fe^{3+} forms. Rosseneu-Motreff et al. (1971) determined that Fe^{3+}–serum transferrin has a = 55 Å and b = 27.6 Å, while Wright & Baxter (1976) reported a = 49 Å and b = 31 Å for the same system.

The results in Table I show that the axial ratios of the prolate ellipsoids ranged from ~0.3 for the native and renatured species to ~0.5 for the denatured sample, which fitted the weight-averaged data well. The ~0.5 ratio probably indicates an overall swelling and accounts for the increase in hydrodynamic size. The axes' dimensions were used to determine the hydrodynamic volume, using $v_h = (4\pi/3)ab^2$. From this hydrodynamic volume, a solvation factor, δ_1, can be determined using the equation (Tanford, 1961)

$$v_h = (M/N)(\bar{v} + \delta_1 v_1^\circ)$$

where M is the OT molecular weight (~77 000), v_1° is the specific volume of the solvent taken as 1.0 cm^3/g, and N is Avogadro's number. $\bar{v}$, the specific volume for the dry solute, is taken as 0.73 cm^3/g (Fuller & Briggs, 1956). The value of δ_1 for the native sample determined with this equation is ~0.28 g/g of solute, and the renatured species has nearly the native δ_1 value.

The calculated solution factor for the 7.2 M urea-denatured ovotransferrin is much larger than that for the native species (Table I). The reason for this difference is not known at this time. There are, however, two different models which might explain the difference. First, there may be some urea bound to the protein, but, since the affinity of urea to the protein is not known, the additional term in the solvation equation ($\delta_{urea} v^\circ_{urea}$) was not incorporated in the calculation. Secondly, and perhaps more importantly, the expanded denatured protein could surround much solvent, even though only a small portion of the solvent might actually be bound to the protein. Since the techniques used to determine sizes are only sensitive to the location of the proteins, extensive protein voids which are filled by the solvent not necessarily bound to the protein could contribute to exaggeration of the calculated solvation factor.

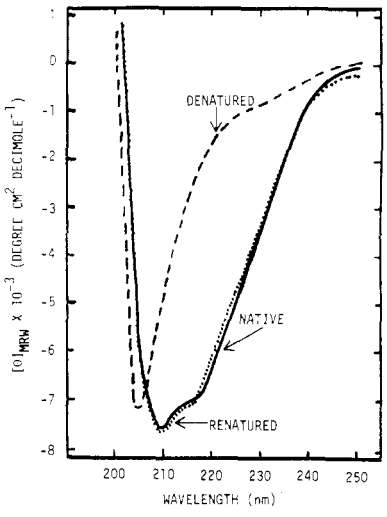

FIGURE 3: CD spectra from 200 to 250 nm for OT. Mean residue weight of 112 is used (see text). Native, c = 1.04 mg/mL (—); 7.2 M urea denatured sample, c = 1.04 mg/mL (---); and renatured sample, c = 0.83 mg/mL (···).

CD. The CD spectrum of OT in the 200–250-nm region is shown in Figure 3. The spectrum in this region depends primarily on the secondary structure of the protein. Disulfide bonds and side-chain chromophores are also expected to contribute to the absorption in this range, but their contributions are generally small compared with that of the backbone chromophore.

Native OT has been reported to exhibit a negative maximum around 210 nm and a shoulder around 213–220 nm (Tan, 1971; Tomimatsu & Vickery, 1972), and our data confirm their existence. However, due to the signal-to-noise ratio in our experiment, we could not observe the shoulder around 230 nm reported by Tomimatsu & Vickery (1972). The denatured OT gave a CD pattern that was observed within 15 min after exposure in 7.2 M urea solution (pH 7.8). There were no significant changes in the CD spectrum of the denatured OT when the sample was allowed to stand for more than 2 h at room temperature under these denaturing conditions. When the Tris-HCl buffer (pH 7.8) was added into the denatured protein solution, complete recovery of the secondary structure was observed in less than 10 min.

Based on the method of Greenfield & Fasman (1969) for calculating percentages of various structures, the native OT at pH 7.8 has the following structure: 11% α helix, 32% β structure, and 57% random coil (Table I). This estimation is subject to the error limits assessed by those authors. Their discussion states that, when a protein is highly ordered, with either α helix or β structure predominating, the results are within 5% of the X-ray data. However, when the proteins lack an extensive range of structural regularity, the deviations from X-ray data are much larger. These results do, however, give a good approximate idea of the secondary structure of the proteins (Greenfield & Fasman, 1969). Tan (1971) applied the same method on his sample of OT and obtained 28% α helix, 32% β structure, and 40% random coil; but he used a

Table II: Denaturation and Renaturation of Ovotransferrin; Acid Denaturation

sample	pH	$(D_t)_{20,w} \times 10^7$ ($cm^2\ s^{-1}$)	$E_{280}^{1\%}$ [a]	concn[b] (mg/mL)	% Fe^{3+} binding[b]
native	7.8	4.6 ± 0.20	0.80 ± 0.02	0.97 ± 0.02	86
denatured	3.0	4.0 ± 0.15	0.66 ± 0.03	0.72 ± 0.02	<5 (0)
renatured	7.3	4.5 ± 0.15	0.79 ± 0.05	0.95 ± 0.02	77

[a] $E_{280}^{1\%}$ is a concentration measurement. This should be compared with the Lowry method (see text). [b] Values are calculated from direct color determinations and were uncorrected. True values should be higher.

mean residue weight of 131 which does not seem to be justifiable (Williams, 1962). Tomimatsu & Vickery (1972) recalculated Tan's data using a mean residue weight of 112 and reported 19% α helix. On the other hand, these authors reported 16% α helix based on the method of Chen & Yang (1971), while our data gave 11% α helix. All these calculations are, at least, consistent in showing that OT is a low-helix-containing protein.

The decreases of α helix and β-pleated sheet, and a concomitant increase of random coil structure, were observed for the urea-denatured OT. However, the denatured protein still retained some amount of ordered structure. When Chen & Yang's method is used for the computation of helical content (1971), present data indicate that the denatured state has no helical structure, but our CD spectrum is significantly different from that of 100% random coil (Greenfield & Fasman, 1969; Chen & Yang, 1971).

There was no significant difference between the native and the renatured OT. Greenfield & Fasman's method (1969) agreed well with Chen & Yang's method (1971), and both supported the conclusion that complete recovery of protein from the denatured state occurred.

Recovery of Iron-Binding Activity. Iron-binding tests were done to determine the chemical reversibility of the renatured OT samples. In both the urea-denatured case and the acid-denatured cases, when the renaturation was accomplished at pH $\gtrsim$7.0, iron binding was very nearly 100% (Tables I and II). Consequently, chemical recovery is also assumed to be total for the renatured protein.

Inferred Hyperchromicity in $E_{280}^{1\%}$ Measurements. It is well-known that $E_{280}^{1\%}$ is a measure of the content of aromatic chromophores in a protein system. Knowing the percentage of aromatic residues in a protein molecule, $E_{280}^{1\%}$ is an approximate measure of the concentration of proteins.

Such a calculation, however, does not take into account partial shielding of the aromatic chromophores, leading to reduced absorption signals. Upon certain denaturation processes, an enhancement of $E_{280}^{1\%}$ may be an indication of more aromatic residue exposure. This is commonly called hyperchromicity.

For the acid-denaturation studies, the noncorrespondence of the two concentration measurements reflects such hyperchromicity (Table II). Thus, the acid-denaturation process seems to affect the exposure of aromatic residues in OT. On the other hand, urea-denaturation processes do not seem to provide a conclusive result in this respect. The presence of urea, which also absorbs in this spectral region, may also cause ambiguous readings.

Conclusions

Urea-Denaturation Studies. It is well-known that even 8.0 M urea does not usually cause cleavage of covalent bonds in proteins. In particular, the 11 disulfide bonds (Osuga & Feeney, 1977) provide a basis for some preservation of structure, and renaturation is virtually complete. Examination of the hydrodynamic data of the denaturated system showed that the overall shape was not extensively altered. This could indicate that the disulfide bridges are distributed throughout the protein.

However, the regions between the disulfide bonds seem to have been effectively denatured. Since a major role of urea is believed to involve a hydrophobic mechanism which favors exposure to the solvent of nonpolar groups in the protein interior, such extensive rearrangements may be seen as the opening of many of the helical regions and other previously shielded regions. The CD spectra of the denatured samples are consistent with these conclusions.

The unfolding of protein in urea is primarily driven by a large and favorable entropy change which overrides an unfavorable enthalpic change. Consequently, when the urea concentration is lowered by dilution or dialysis, the return to a situation which becomes enthalpically and also entropically favorable is usually very likely.

Acid-Denaturation Studies. The isoelectric point of OT is slightly below pH 7.0. Consequently, most of our studies of urea denaturation, whether at 0.1 M Tris or 0.01 M Tris, should have made little difference to the native protein conformation. On the other hand, extreme acidic or basic conditions will lead to a different charge relationship on the OT system and, consequently, to denaturation. Phelps & Cann (1956) have shown that, at low pH, OT undergoes denaturation as shown by a decrease in the sedimentation coefficient and an increase in the viscosity coefficient.

QELS data indicated that the nature of acid denaturation is unlike that of urea denaturation. The low pH (pH 3) sample gave only a 10% increase in the hydrodynamic size, compared with a nearly 40% change in size for the urea-denatured sample.

Renaturation of the acid-denatured sample gave us much more difficulty. Recovery at pH 5 was incomplete, and, generally, the QELS data indicated the presence of aggregates. When a pH 7 $\rightarrow$ 3 $\rightarrow$ 7 transition was effected, the return to native size was nearly 100%, as was binding activity. However, when the renaturation was effected by diluting a high concentration of denatured sample with a pH 7 buffer, the recovery to native form was very slow (Figure 2). Perhaps most striking was the onset of high molecular weight aggregates which, in time, dissipated. Such an effect was suggested as plausible by Haschemeyer & Haschemeyer (1973); however, the mechanism is unclear. The interesting kinetics of this process are being further investigated.

The results which we have reported here, indicating the difference in the two denaturation processes, are conducive to further theoretical studies, as well as to experimental confirmation. Recently, a comparison of lysozyme denaturation by thermal means and by 6 M guanidine hydrochloride was made by Nicoli & Benedek (1976).

Several of the unresolved observations mentioned in the previous section should be further pursued. These include the change in the sign of the birefringence signal, δ_B, upon urea denaturation (Table I) and the temporary aggregation of the acid-renatured species (Figure 2). A fuller understanding of

these interactions is clearly necessary in any attempt to develop theoretical models for these proteins.

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Appendix: Relationship between z-Averaged and Weight-Averaged Diffusion Coefficients

QELS from a polydispersed system of macromolecules typically provides a measurement of the diffusion coefficient which is different from a sedimentation measurement. QELS provides a z-averaged diffusion coefficient

$$\langle D\rangle_z = \sum_i N_i M_i^2 D_i / \sum_i N_i M_i^2 \quad (1)$$

where N_i and M_i are the number and the molecular weight of the ith species, respectively.

On the other hand, a sedimentation measurement of diffusion is a weight-averaged value

$$\langle D\rangle_w = \sum_i N_i M_i D_i / \sum_i N_i M_i \quad (2)$$

The difference in the factors of the two averages usually causes the QELS measurement to predict larger sized particles than those sizes predicted by sedimentation methods for a polydispersed system. If the polydispersity is not too excessive (such excessiveness will be quantified later), then one can express $\langle D\rangle_z$ in terms of $\langle D\rangle_w$. We shall derive this relationship in a manner analogous to the discussion previously reported for vesicle samples (Selser & Yeh, 1976).

The translational diffusion coefficient for a sphere of radius R_i of species i is given by $D_i = A/R_i$, where $A = kT/6\pi\eta$, η being the solvent viscosity. If one further assumes that the density of the heterogeneous species is a constant

$$\rho = M_i/(4\pi/3)R_i^3 \quad (3)$$

then eq 1 and 2 may be written as

$$\langle D\rangle_w = A\langle R^2\rangle_N/\langle R^3\rangle_N \quad (4a)$$

$$\langle D\rangle_z = A\langle R^5\rangle_N/\langle R^6\rangle_N \quad (4b)$$

where

$$\langle R^n\rangle_N \equiv \sum_i N_i R_i^n / \sum_i N_i \quad (5)$$

and n is an integer.

In general, $\langle R^n\rangle_N$ is a difficult quantity to obtain experimentally; consequently, one expands R^n about $\langle R\rangle_N$ and evaluates $\langle R^n\rangle_N$. Keeping only terms through second-order smallness, one finds that

$$\langle R^n\rangle_N = \langle R\rangle_N^n \left[1 - \frac{n(n-1)}{2}(\delta_R)_N\right] \quad (6)$$

where

$$(\delta_A)_\alpha \equiv (\langle A^2\rangle_\alpha - \langle A\rangle_\alpha^2)/\langle A\rangle_\alpha^2 \quad (7)$$

Here, $(\delta_A)_\alpha$ is the variance of the quantity A in the αth averaging technique. So, $\alpha = N, w, z$ for number, weight, and z-averaging techniques, respectively.

Even though $(\delta_R)_N$ is also not accessible by the QELS technique, the quantity $(\delta_D)_z$ is; so is $(\delta_D)_w$ by the sedimentation diffusion technique. It is easy to show that

$$(\delta_D)_w + 1 = \langle R\rangle_N\langle R^3\rangle_N/\langle R^2\rangle_N^2 \quad (8a)$$

$$(\delta_D)_z + 1 = \langle R^4\rangle_N\langle R^6\rangle_N/\langle R^5\rangle_N^2 \quad (8b)$$

Combining eq 8 with eq 6 and 7, and keeping only terms of second order smallness, one obtains $(\delta_D)_z \simeq (\delta_D)_w \simeq (\delta_R)_N \equiv \delta$. Under these conditions

$$\langle D\rangle_w \simeq D_z/(1 - 3\delta) \quad (9)$$

Since the acceptance criterion for QELS data is set at $\delta \simeq 0.1$, such a value has been used in the analysis of the translational diffusion data.

References

Azari, P. R., & Feeney, R. E. (1961) *Arch. Biochem. Biophys. 92*, 44.

Berne, B. J., & Pecora, R. (1976) *Dynamic Light Scattering*, Wiley, New York.

Chen, Y.-H., & Yang, J. T. (1971) *Biochem. Biophys. Res. Commun. 44*, 1285.

Chu, B. (1974) *Laser Light Scattering*, Academic Press, New York.

Cornell, C. N., & Kaplan, L. J. (1978) *Biochemistry 17*, 1750.

Dubin, S. B., Clark, N. A., & Benedek, G. B. (1971) *J. Chem. Phys. 54*, 5158.

Fabelinskii, I. L. (1968) *Molecular Scattering of Light*, Plenum Press, New York.

Feeney, R. E., & Komatsu, S. K. (1966) *Struct. Bonding (Berlin) 1*, 149–206.

Feeney, R. E., & Allison, R. G. (1969) *Evolutionary Biochemistry of Proteins, Homologous and Analogous Proteins from Avian Egg Whites, Blood Sera, Milk, and Other Substances*, Wiley, New York.

Fredericq, E., & Houssier, C. (1973) *Electric Dichroism and Electric Birefringence*, Clarendon Press, Oxford.

Fuller, R. A., & Briggs, D. R. (1956) *J. Am. Chem. Soc. 78*, 5253.

Glazer, A. N., & McKenzie, H. A. (1963) *Biochim. Biophys. Acta 71*, 109.

Greene, F. C., & Feeney, R. E. (1968) *Biochemistry 7*, 1366.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108.

Harris, D. C., & Aisen, P. (1975) *Biochemistry 14*, 262.

Haschemeyer, R. H., & Haschemeyer, A. E. V. (1973) *Proteins: A Guide to Study by Physical and Chemical Methods*, pp 352–385, Wiley, New York.

Kobayasi, S. (1968) *Biopolymers 6*, 1491.

Krause, S., & O'Konski, C. T. (1963) *Biopolymers 1*, 503.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Nicoli, D. F., & Benedek, G. B. (1976) *Biopolymers 15*, 2421.

O'Konski, C. T. (1976) *Molecular Electro-Optics, Part I, Theory and Methods*, Marcel Dekker, New York.

O'Konski, C. T., & Krause, S. (1976) in *Molecular Electro-Optics, Part I, Theory and Methods* (O'Konski, C. T., Ed.) pp 63–120, Marcel Dekker, New York.

O'Konski, C. T., Yoshioka, K., & Orttung, W. H. (1959) *J. Phys. Chem. 63*, 1558.

Osuga, D. T., & Feeney, R. E. (1977) in *Food Proteins* (Whitaker, J. R., & Tannenbaum, S. R., Eds.) pp 209–266, Avi Publishing, Westport, Conn.

Perrin, P. F. (1934) *J. Phys. Rad., Ser. 7 5*, 497.

Perrin, P. F. (1936) *J. Phys. Rad., Ser. 7 7*, 1.

Phelps, R. A., & Cann, J. R. (1956) *Arch. Biochem. Biophys. 61*, 51.

Rogers, T. B., Gold, R. A., & Feeney, R. E. (1977) *Biochemistry 16*, 2299.

Rosseneu-Motreff, M. Y., Soetewey, F., Lamote, R., &

Peeters, H. (1971) *Biopolymers 10*, 1039.
Selser, J. C., & Yeh, Y. (1976) *Biophys. J. 16*, 847.
Selser, J. C., Yeh, Y., & Baskin, R. J. (1976) *Biophys. J. 16*, 337.
Stark, G. R., Stein, W. H., & Moore, S. (1960) *J. Biol. Chem. 235*, 3177.
Tan, A.-T. (1971) *Can. J. Biochem. 49*, 1071.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, p 356, Wiley, New York.
Thibodeau, S. N., Lee, D. C., & Palmiter, R. D. (1978) *J. Biol. Chem. 253*, 3771.
Tomimatsu, Y., & Vickery, L. E. (1972) *Biochim. Biophys. Acta 285*, 72.
Willard, J. M., Davis, J. J., & Wood, H. G. (1969) *Biochemistry 8*, 3137.
Williams, J. (1962) *Biochem. J. 83*, 355.
Wright, A. K., & Baxter, J. E. (1976) *Biophys. J. 16*, 931.
Yeh, Y., Selser, J. C., & Baskin, R. J. (1978) *Biochim. Biophys. Acta 509*, 78.
Zweier, J. L., & Aisen, P. (1977) *J. Biol. Chem. 252*, 6090.

Reduction of Methemerythrin by Deoxymyoglobin: a Protein–Protein Redox Reaction Not Involving Electron-Transfer Proteins†

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ABSTRACT: The stoichiometry and kinetics of reaction of methemerythrin with the deoxy forms of myoglobin and hemoglobin have been examined at $I = 0.2$ M and 25 °C. One mole of methemerythrin (on the basis of the monomer unit containing two irons) reacts with 2 mol of deoxymyoglobin and with 0.5 mol of deoxyhemoglobin. All reactions are second order. Rate constants for reaction with deoxymyoglobin are 0.25 M^{-1} s^{-1} (*Phascolopsis gouldii*) and 5.6 M^{-1} s^{-1} (*Themiste pyroides*) at pH 6.3. There is little effect of raising the ionic strength to 1.35 M and only a small decrease in rate when the pH is adjusted to 8.2. The rate constant for reaction of deoxyhemoglobin with *P. gouldii* methemerythrin is ~0.1 M^{-1} s^{-1} at pH 6.3. Metmyohemerythrin from *T. pyroides* reacts slightly slower than the octamer form ($k = 2.0$ M^{-1} s^{-1} at pH 6.3 and 7.0). Oxymyoglobin is converted to metmyoglobin by methemerythrin. The electron-transfer path is discussed and a self-exchange rate constant for hemerythrin assessed as 10^{-3} M^{-1} s^{-1} on the basis of Marcus's theory.

The kinetics of redox reactions involving metalloproteins are being increasingly studied (for recent reviews, see Bennett, 1973; Moore & Williams, 1976; Sutin, 1977; Wherland & Gray, 1977). Most of the work so far involves inorganic reactants, and there are few reported investigations of electron transfer between metalloproteins (Chien et al., 1978; Wherland & Pecht, 1978, and cited references). In even these at least one of the partners appears to be a protein for which electron transfer is an important biological function.

We have been studying a number of redox reactions of hemerythrin and decided to include its reactivity toward myoglobin. Both are well-characterized metalloproteins, whose structures, particularly that of myoglobin, are well understood (Kendrew et al., 1960; Phillips, 1978; Kurtz et al., 1978). Both exist in well-defined iron(II) and iron(III) states and hemerythrin has the added interest of being available both as a monomer and as an octamer, containing two linked irons in each monomeric unit. A noncomplementary redox reaction between two- and one-electron redox reagents is therefore involved. Both proteins in their reduced state function as O_2-storage or O_2-carrying proteins. However, in the oxy form, both autoxidize to the iron(III) species (Brown & Mebine, 1969; Bradić et al., 1977) and redox processes are therefore important. The only reported redox kinetics involving myoglobin and oxymyoglobin with another metalloprotein is that with ferricytochrome *c* (Wu et al., 1972; Ataullakhanov et al., 1976; Atanasov et al., 1977). In this paper we examine the stoichiometry and kinetics of reaction of horse heart and sperm whale deoxymyoglobin with octameric methemerythrin from the coelomic fluid of *Phascolopsis gouldii* and *Themiste pyroides* in the pH 6.3–8.2 range. Some data were also obtained for monomeric metmyohemerythrin from the retractor muscle of *T. pyroides* and for replacing myoglobin by hemoglobin.

Experimental Section

Materials. The marine worms *P. gouldii* and *T. pyroides* were obtained, respectively, from Marine Biological Laboratory, Woods Hole, MA, and Pacific Biomarine Supply, Venice, CA. Oxyhemerythrin was obtained from the coelomic fluid of these worms by the methods of Klotz et al. (1957) and Klippenstein et al. (1972). Metmyohemerythrin was obtained directly from the retractor muscles of *T. pyroides* by a modification of the procedure of Klippenstein et al. (1972), omitting the addition of azide. Methemerythrin was prepared from oxyhemerythrin by adding 2–3 molar excess of $Fe(CN)_6^{3-}$ or by dialyzing against 1 mM $Fe(CN)_6^{3-}$. The product was then dialyzed several times against the appropriate buffer system and absence of iron(II)- or iron(III)–cyano complexes in the final dialysate checked by absorption measurement at 320 or 420 nm. The spectral properties of the various forms agreed with literature descriptions (Klotz, 1971; Klippenstein et al., 1972; Dunn et al., 1977). Sperm whale myoglobin (Sigma) was reduced to the deoxy form by dialyzing overnight at 4 °C against deaerated buffer containing a slight excess of dithionite. The deoxy protein was then dialyzed against buffer to remove excess of dithionite. In one run, oxymyoglobin prepared from the muscle of sperm whale was purified by the method of Keyes et al. (1971) and was then converted into

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