Atassi, M. Z., & Habeeb, F. S. A. (1972) *Methods Enzymol. 25*, 546–553.
Coggins, J. R., Hooper, E. A., & Perham, R. N. (1976) *Biochemistry 15*, 2527–2533.
Dottavio-Martin, D., & Ravel, J. M. (1978) *Anal. Biochem. 87*, 562–565.
Fields, R., & Dixon, H. B. F. (1968) *Biochem. J. 108*, 883–887.
Geoghegan, K. F., Ybarra, D. M., & Feeney, R. E. (1979) *Biochemistry 18*, 5392–5399.
Gidley, M. J., & Sanders, K. M. (1982) *Biochem. J. 203*, 331–334.
Goldberger, R. F., & Anfinsen, C. B. (1962) *Biochemistry 1*, 401–405.
Hartly, B. S. C. (1980) *Biochem. J. 119*, 805–822.
Hirs, C. H. W. (1956) *J. Biol. Chem. 219*, 611–621.
Hughes, R. C., & Jeanloz, R. W. (1966) *Biochemistry 5*, 253–258.
Hunter, M. J., & Ludwig, M. L. (1972) *Methods Enzymol. 25*, 585.
Inman, J. K., Perham, R. N., Dubois, G. C., & Appella, E. (1980) *Methods Enzymol. 91*, 559–569.
Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.
Jentoft, N., & Dearborn, D. G. (1983) *Methods Enzymol. 91*, 570–579.
Klee, W. A., & Richards, F. M. (1957) *J. Biol. Chem. 229*, 489–504.
Lundblad, L. R., & Noyes, M. C. (1984) in *Chemical Reagents for Protein Modification*, Vol. 1, CRC Press, Boca Raton, FL.
Manjula, B. N., Acharya, A. S., Mische, S. M., Fairwell, T., & Fischetti, V. A. (1984) *J. Biol. Chem. 259*, 3686–3693.
Manjula, B. N., Acharya, A. S., Fairwell, T., & Fischetti, V. A. (1986) *J. Exp. Med. 163*, 129–138.
Manning, J. M., & Acharya, A. S. (1984) *Am. J. Pediatr. Hematol./Oncol. 6*, 51–54.
Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192–2201.
Powers, J. R., & Whitaker, J. R. (1977) *J. Food Biochem. 1*, 239–260.
Saroff, H. A., & Carroll, W. R. (1962) *J. Biol. Chem. 237*, 3384–3387.
Smith, R. J., Capaldi, R. A., Muchmore, D., & Dahlquist, F. (1978) *Biochemistry 17*, 3719–3723.
Wong, W. S. D., Dristjansson, M. M., Osuga, D. T., & Feeney, R. E. (1985) *Int. J. Pept. Protein Res. 26*, 55–62.
Yamasaki, R. B., Osuga, D. T., & Feeney, R. E. (1982) *Anal. Biochem. 126*, 183–189.
Yasuda, Y., Takahashi, N., & Murachi, T. (1971) *Biochemistry 10*, 2624–2630.

Oxidation of Tryptophans in an Interhelical Hydrophobic Cluster of Myoglobin Alters the Thermodynamics of the Denaturation Transition†

Jeffrey A. Radding‡
Department of Chemistry, Indiana University, Bloomington, Indiana 47405
Received September 26, 1986; Revised Manuscript Received January 23, 1987

ABSTRACT: Model folding studies of sperm whale myoglobin have illustrated the presence of hydrophobic interfacial regions between elements of secondary structure. The specific oxidation of two tryptophan residues, in the A-H helix contact of sperm whale myoglobin, to the less hydrophobic oxindolylalanine residues is utilized to probe the contribution of hydrophobic packing density in this contact region. The acid denaturation of the modified protein is no longer a simple two-state process exhibiting the presence of stable intermediates. The relative stability of the intermediate is shown to be +5.3 kcal/mol less stable than native myoglobin. This value is consistent with the predicted relative stability, based upon electrostatic model calculations, of the docking of the A helix with a des-A helix myoglobin. The presence of stable intermediate structures in the denaturation pathway of the modified protein is consistent with the proposed role of hydrophobic interactions in damping structural fluctuations and statistical mechanical models of noncooperative protein unfolding. These results demonstrate the relationship between large-scale fluctuations and the frictional forces governing small-scale motions within the protein core.

In their description of the packing of α-helices in myoglobin, Richmond and Richards (1978) examined the loss of solvent contact area upon the association of secondary structures. Defining the site of interhelix contact regions in the protein by a perpendicular line segment to the axes of the two compared helices (the contact normal), calculation of the loss of solvent accessibility upon association of the isolated helices revealed six major contact pairs. The large decreases in accessibility imply strong hydrophobic interactions in these interhelical contacts (Chothia, 1974).

One of these interhelical contacts is between the A helix and the H helix and is centered about the valine at position 10 in the myoglobin sequence. Two of the six surrounding residues in this complex are tryptophan residues at positions 7 and 14 (Figure 1A). Tryptophan residues are relatively large, and their contribution to the hydrophobic cluster may be only partial as they are not completely buried, yet tryptophan residues have potentially one of the largest decreases in contact area upon folding (Richmond & Richards, 1978).

Comparing the locations of these interhelical contact region as defined by Richmond and Richards (1978) with the $\langle\chi^2\rangle$ values (root mean square deviations in atomic coordinates) of the backbone atoms of myoglobin obtained by X-ray dif-

† This work was supported by U.S. Public Health Service Research Grants HL-14680 and AI-08614.
‡ Present address: Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06510.

0006-2960/87/0426-3530$01.50/0

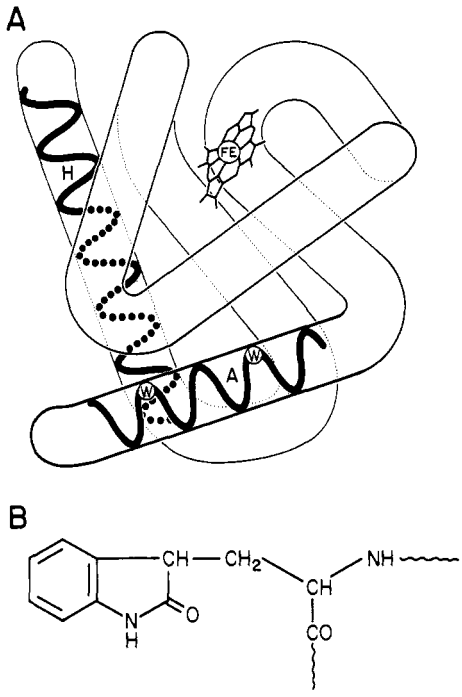

FIGURE 1: (A) Schematic representation of the three-dimensional structure of sperm whale myoglobin showing the relative orientation of the A and H helices. The approximate positions of the α-carbon of tryptophans-7 and -14 are marked with a W. For greater detail of the residues involved in the A-H interhelical contact, see Richmond and Richards (1978). (B) Structural formula of oxindolylalanine.

fraction at 80 and 300 K (Frauenfelder et al., 1979; Hartmann et al., 1982), it is apparent that most of these interhelical contacts represent minima in the flexibility of the protein structure. More importantly, however, is the fact that in one contact, that between the A and H helices at Val-10, there is little temperature dependence in $\langle\chi^2\rangle$. The temperature dependence and absolute values of $\langle\chi^2\rangle$ are near those found in solids, indicating that this interhelical contact is very closely packed, i.e., solidlike (Hartmann et al., 1982).

The interhelical complex between the A and H helices in myoglobin provides a unique opportunity to disrupt a densely packed region of hydrophobic contacts and to observe the effects of an artificially induced defect on the stability and dynamics of a protein molecule. To accomplish this, a modification of the tryptophan residues in the hydrophobic cluster between the A and H helices is performed by the specific oxidation of the indole rings of the tryptophans to form oxindolylalanine (Oia) residues (Savige & Fontana, 1977a, 1980). This alters the geometry of the C_γ carbon from planar to tetrahedral, causing a tilt in the plane of the ring relative to the C_β–C_γ bond (Figure 1B). This altered geometry disrupts the closely packed nature of the interface. In addition, oxindolylalanine is less hydrophobic than tryptophan. Oia elutes earlier than tryptophan in reversed-phase high-performance liquid chromatography (HPLC), and phase-transfer equilibrium studies indicate that Oia is approximately 0.5 kcal/mol less hydrophobic than tryptophan (Nozaki & Tanford, 1971; Radding, 1983).

Experimental Procedures

Materials. The major component of sperm whale myoglobin was isolated and purified as previously described (Hapner et al., 1968). The heme prosthetic group was removed and the apomyoglobin frozen and lyophilized (Yonetani, 1967).

Oxidation of tryptophan to oxindolylalanine in sperm whale myoglobin was accomplished by modification of the method of Savage and Fontana (1977a, 1980). Lyophilized sperm whale apomyoglobin was dissolved in 33% 12 N HCl/glacial acetic acid (v/v). Protein concentration was 3.2 mM. To this viscous solution, a 100 molar excess over protein of crystalline phenol was added. The solution was swirled in the dark for several minutes. A 60 molar excess over protein of dimethyl sulfoxide was added and the mixture swirled in the dark at room temperature for 30 min. The reaction was then placed in an ice bath and the reaction stopped by addition of a large volume of ice-cold water. The protein solution was then dialyzed against cold water for several changes in the dark. The protein solution was then frozen and lyophilized.

The reduction of methionine sulfoxide was accomplished by the method of Savage and Fontana (1977b). The modified apoprotein was then dialyzed against several changes of water, followed by dialysis against 8 M urea at 4 °C.

Reconstitution of the apoprotein with hemin was accomplished by dissolving the appropriate amount of hemin in 20 mM KCN/8 M urea. This solution was then added to the apoprotein solution and allowed to stir briefly at room temperature. The protein solution was then exhaustively dialyzed against water, followed by dialysis against phosphate buffer, pH 6.5, I = 0.1 M, and 1 mM KCN.

Purification of the [$Oia^{7,14}$]*myoglobin* was accomplished by chromatography of the reconstituted holoprotein on Sephadex CM-C50 (2.5 × 45 cm) equilibrated with phosphate buffer, pH 6.5, I = 0.1 M, and 1 mM KCN. The major band eluted by this protocol was collected and utilized for further studies. The protein was stored in the cold with cyanide as ligand. The yield was 15%. Conversion of the cyanoferri protein to the aquoferri ligand was accomplished as described by DiMarchi et al. (1980) and Garner et al. (1975).

Amino acid analysis was performed by the method of Liu and Chang (1971) for analysis of tryptophan and oxindolylalanine content (Savage & Fontana, 1977a). Routine analysis was performed by the method of Spackman et al. (1958). Methionine sulfoxide content was determined by direct hydrolysis in 4 N methanesulfonic acid and by reaction of the sample with CNBr followed by hydrolysis in 6 N HCl (determined as methionine).

Cellulose acetate electrophoresis was performed by using a Beckman "Microzone" electrophoresis apparatus. Buffer systems utilized were 0.05 M sodium phosphate, pH 6.5, and 0.1 M tris(hydroxymethyl)aminomethane/ethylenediaminetetraacetic acid/borate (Tris/EDTA/borate), pH 9.2, with and without additional 1 mM KCN.

Gel filtration of the product was performed on Sephadex G-200 (1.5 × 100 cm) in [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris)/HCl buffer, pH 6.0, I = 0.2 M. The column was calibrated by using standard proteins (Mol-Ranger, Pierce Chemical Co.). High-pressure gel permeation chromatography was performed with a Varian LC 5000 liquid chromatograph equipped with a SynChropak GPC-100 gel permeation column (4 × 250 mm) in phosphate buffer, pH 6.5, I = 0.1 M, and 1 mM KCN.

Absorption spectra were determined by using a Perkin-Elmer 552 UV–vis scanning spectrophotometer.

Circular dichroism (CD) spectra were obtained on a Jasco optical rotary dispersion recorder equipped with a Sproul Scientific SS-10 CD modification.

Acid stability measurements were performed by the method of Flanagan et al. (1983) using constant ionic strength sodium acetate buffers. The reversibility of the acid denaturation of the modified protein was determined by reversal of pH with observation of the recovery of the Soret band. As the modified

Table I: Amino Acid Composition of [Oia^{7,14}]Myoglobin and Sperm Whale Myoglobin[a]

amino acid	modified protein	sperm whale myoglobin
Asp	8.28 (8)[b]	8.96 (8)
Thr	4.17[c] (5)	4.49[c] (5)
Ser	3.91[c] (6)	4.20[c] (6)
Glu	19.30 (19)	19.91 (19)
Gly	11.25 (11)	10.73 (11)
Ala	16.51 (17)	14.80 (17)
Val	8.16 (8)	8.80 (8)
Met	2.33 (2)	1.90 (2)
Ile	8.72 (9)	8.63 (9)
Leu	18.59 (18)	18.01 (18)
Tyr	2.87 (3)	2.93 (3)
Phe	6.40 (6)	6.20 (6)
Lys	19.63 (19)	20.17 (19)
His	11.98 (12)	12.16 (12)
Arg	4.71 (4)	5.11 (4)
Trp	0.00 (0)	ND[d]
Oia[e]	2.15 (2)	ND[d]

[a] Hydrolysis performed in 4 N methanesulfonic acid, 110 °C, 24 h. [b] Expected values based on the sequence of sperm whale myoglobin. [c] Values uncorrected for partial destruction during hydrolysis. [d] ND = not determined. [e] Oia, oxindolylalanine (Savige & Fontana, 1977a).

protein demonstrated reduced stability compared to native sperm whale myoglobin, the pH limit for the fully native structure was extended to pH 6.3. Protein concentrations were 1 μM. Data for sperm whale myoglobin acid denaturation are taken from Flanagan et al. (1983).

Potentiometric hydrogen ion titrations of the modified and native proteins were performed as previously described (Shire et al., 1974a; DiMarchi et al., 1978). Protein concentrations were 100 μM. Tenth normal acid or base was delivered with a Gilmont Ultrahigh Precision micrometer, capable of additions of 1×10^{-4} mL.

Simultaneous multiparameter acid denaturation curves were constructed by observation of the Soret absorbance of the aquoferri derivative of the modified protein, by the far-UV CD spectrum as described above, and by UV difference spectroscopy of the tryptophans in native sperm whale myoglobin and the oxindolylalanines in the modified protein using the method of Herskovits and Laskowski (1962; Herskovits, 1969; Donovan, 1973). The base line of the UV difference spectra was normalized for the contribution of the heme absorbance (Herskovits, 1969). Protein concentrations were 10 μM. All samples were temperature controlled at 25 °C.

Results

Purification and Characterization of [Oia^{7,14}]Myoglobin. The major band eluted from the cation-exchange column was subjected to cellulose acetate electrophoresis at two different pH values. Both demonstrated a single band comigrating with native sperm whale myoglobin. In addition, gel permeation chromatography of the major band demonstrated the product was not aggregated.

Amino acid analyses of the modified and native proteins are shown in Table I. The absence of tryptophan and the quantitative appearance of oxindolylalanine were observed. Methionine residues appeared to be unoxidized by direct determination of methionine and methionine sulfoxide content. Reaction of the modified molecule with cyanogen bromide followed by hydrolysis in 6 N HCl demontrated no methionine sulfoxide present in the protein (determined in the analysis as methionine).

The UV–visible spectra of three ligand complexes of the modified protein, the cyanoferri (Figure 2A), aquoferri (Figure 2B), and (carbonmonoxy)ferro derivatives (Figure 2C), gave strong Soret band absorbances at the correct wavelengths. The

Table II: Far-UV Circular Dichroism of [Oia^{7,14}]Myoglobin and Sperm Whale Myoglobin[a]

wavelength (nm)	modified protein	sperm whale myoglobin[b]
209	−20 170[c]	−24 400[c]
222	−21 460	−25 280

[a] Determined in phosphate buffer, pH 6.5, I = 0.1 M, and 1 mM KCN as the cyanoferri derivatives. [b] Rothgeb and Gurd (1978). [c] Values are mean residue ellipticities (in degrees centimeter squared per decimole).

Table III: Recoveries of Sperm Whale Myoglobin and [Oia^{7,14}]Myoglobin from Acid Denaturation

pH	time (h)	volume (mL)	A_{409}[a]	% recovery
		Sperm Whale Myoglobin		
6.574		3.010	0.1650	
3.498		3.130	0.0693	
6.570	0.17	3.315	0.1292	62.6
6.570	3.00	3.315	0.1402	74.1
6.570	19.00	3.315	0.1451	79.2
		[Oia^{7,14}]Myoglobin		
6.494		3.050	0.1487	
3.848		3.140	0.0735	
6.460	0.17	3.255	0.1153	55.6
6.505	4.00	3.255	0.1298	74.8

[a] Absorbance of Soret band of aquoferri derivative.

UV spectra demonstrated a shift in the wavelength maxima from 280 to 250 nm, consistent with the oxidation of tryptophan to oxindolylalanine. Quantitative determination of absorbance coefficients for the cyanoferri derivative of the modified protein demonstrated a slight loss in the Soret coefficient when compared to native sperm whale myoglobin but was within the range of variation found in other myoglobin species (Rothgeb & Gurd, 1978). The spectroscopic data are indicative of the integrity of the heme–protein association.

The far-UV CD spectrum of the modified protein showed significant α-helical content (Table II) but demonstrated a 15% decrease in the mean residue ellipticity compared to native protein. As CD spectra for proteins are based upon empirical data bases and aromatic side chains contribute to the far-UV CD spectrum of proteins, it is unclear if the loss in mean residue ellipticity correlates to changes in secondary structure of the modified protein. The substitution of Oia into a protein or peptide of known structure and its affect on CD spectra are not known. The presence of characteristic α-helical CD bands in the modified protein indicates that gross changes in secondary structure have not occurred. Moreover, any changes in secondary structure are unlikely to be localized in a particular region of the protein, as this would be energetically unfavorable, but may represent small changes in structure which have been globally distributed to reduce strain.

It has been demonstrated previously that myoglobin molecules which have been truncated experience a significant loss in helical content and are unable to properly bind the heme prosthetic group (Wang, 1977; Wang et al., 1978). The ability to derive a stable heme–protein complex, in conjunction with the physical and analytical data described above, demonstrates that the fraction of purified modified protein used in the following studies consists of an intact sequence, despite the rather harsh oxidation conditions.

Acid Stability Measurements. In the preliminary characterization of the modified protein, the reversibility of the acid denaturation of the protein was determined. Table III shows the percent recovery of the Soret absorbance of the aquoferri derivatives of both native and modified proteins. Both molecules demonstrate a recovery in excess of 70% within 3 h of

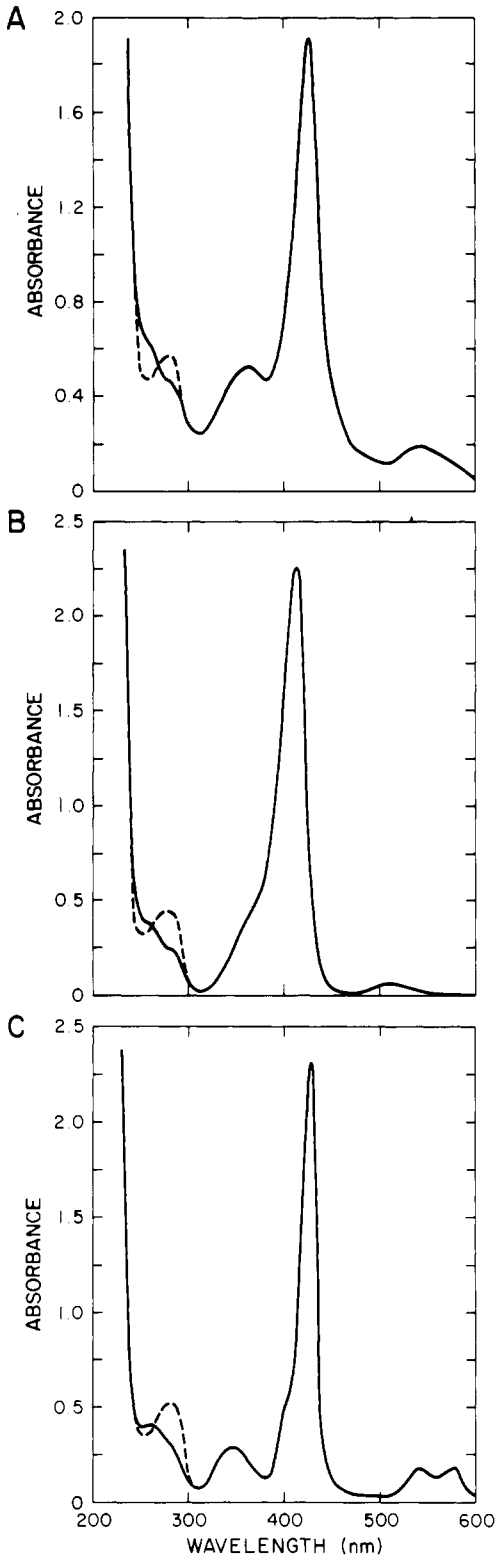

FIGURE 2: Absorbance spectra of [Oia^{7,14}]myoglobin with three different ligands in 0.1 M phosphate buffer, pH 6.5: (A) cyanoferri derivative; (B) aquoferri derivative; (C) (carbonmonoxy)ferro derivative. Dashed lines represent the UV spectrum of native sperm whale myoglobin.

returning to conditions favorable for renaturation. The loss of the native protein to irreversible denaturation has been shown to be the product of a slow-forming irreversible denatured state, probably due to aggregation of protein and released heme (Shen & Hermans, 1972).

The acid denaturation profile of the modified protein is shown in Figure 3A. The modified protein (curve A) shows

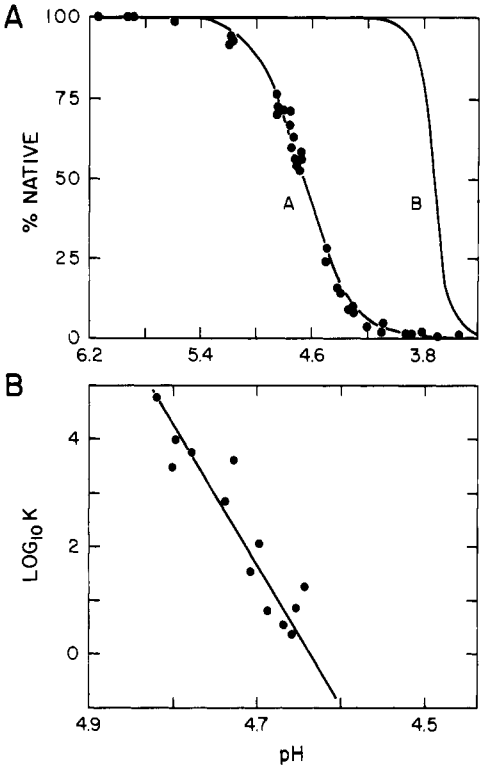

FIGURE 3: (A) Acid denaturation profile of the aquoferri derivatives of [Oia^{7,14}]myoglobin (curve A) and native sperm whale myoglobin (curve B) as monitored by the change in the heme absorbance at 409 nm and I = 0.01 M. Data for sperm whale myoglobin are taken from Flanagan et al. (1983). (B) Dependence of log K on pH at I = 0.01 M for points near the midpoint of denaturation of [Oia^{7,14}]myoglobin. The slope of the line, $\Delta\bar{\nu}_{H^+}$, is 2.5.

a marked decrease in stability relative to native myoglobin (curve B). The midpoint of denaturation has increased from pH 3.70 for native sperm whale myoglobin (Flanagan et al., 1983) to pH 4.63. Figure 3B illustrates the correlation of the equilibrium constant with pH obtained from points near the midpoint of the denaturation curve. Following the method of Flanagan et al. (1983), extrapolation of the calculated line to pH 4.0 allows comparison of the free energy of denaturation to that of native myoglobin. The relative stability of [Oia^{7,14}]myoglobin to native myoglobin is calculated to be +5.3 kcal/mol at pH 4.0. As will be demonstrated subsequently, it turns out that this value represents the relative stability of an intermediate(s) structure in the unfolding pathway relative to native myoglobin.

Another feature of the acid denaturation curve of the modified protein is the decreased slope of the transition. The theory of linked functions (Wyman, 1964) establishes that for an equilibrium between native and denatured states:

$$\delta \ln K_{app}/\delta \ln a_{H^+} = \Delta\bar{\nu}_{H^+}$$

thereby relating the equilibrium constant for denaturation as a function of pH to the change in the number of protons bound upon denaturation (Tanford, 1968; Hermans & Acampora, 1967). A large part of this effect is due to the pH at which the midpoint of denaturation occurs (pH_{mid}). Recently, a linear correlation between the slopes of the acid denaturation curve and pH_{mid} at I = 0.01 M has been established for several species of cetacean myoglobins (Flanagan et al., 1983). Extrapolation of this correlation to the midpoint of denaturation for the modified protein at pH 4.63 gives a theoretical value for $\Delta\bar{\nu}_{H^+}$ = 3.8. The observed value of 2.5 is just within 2 standard deviations of the correlated line. If this difference is real, the apparent decrease in protons bound upon denatu-

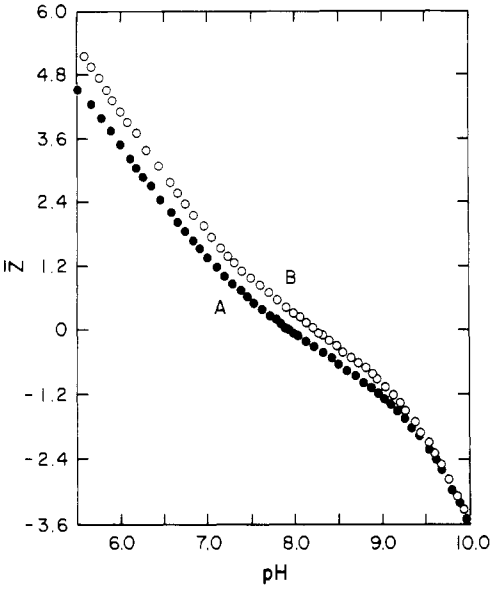

FIGURE 4: Potentiometric hydrogen ion titration of the aquoferri derivatives of [Oia^{7,14}]myoglobin (curve A) and sperm whale myoglobin (curve B) at I = 0.01 M.

ration relative to that predicted by theory is indicative of the presence of stable intermediates in the denaturation of the modified protein (Tanford, 1968).

Potentiometric Titration. The potentiometric hydrogen ion titration curves (Figure 4) of the aquoferri derivatives of the modified protein (curve A) and native sperm whale myoglobin (curve B) in 0.01 M KCl indicate that although there are no formal charge changes in the modified protein, there are fewer bound protons in the folded structure of the modified protein than in the folded structure of native sperm whale myoglobin. As no significant charge differences could be detected by electrophoresis and no changes in amino acid composition of the modified protein can account for this observed charge difference, it is most likely due to slight differences in conformation of the "native" states of the modified and native proteins. The groups affected have apparent pK values consistent with changes in histidine residues (below pH 6.5). There is a largely buried histidine residue near the site of the modification in the three-dimensional structure of the native protein. Preliminary NMR evidence suggests that alteration of the titration behavior of this residue (His-119) has occurred, most likely due to perturbation of the local environment near this residue (Radding, 1983).

If no formal charge changes have occurred in the primary sequence of the modified protein, differences in charge behavior are not due to charge differences in the denatured states of the modified and native proteins. The observation by titration of fewer bound protons in the folded state of [Oia^{7,14}]myoglobin should therefore lead to a relative increase in the number of protons bound upon acid denaturation of the modified protein, when corrected for pH effects. The increased number of protons bound should lead to a relative increase in the slope of the denaturation curve compared with native sperm whale myoglobin. As previously described, the data from the acid denaturation curve demonstrate that fewer protons are bound upon acid denaturation of the modified protein, and in conjunction with the potentiometric hydrogen ion titration curve, the proton binding data seem inconsistent, if one assumes that acid denaturation in this system is a two-state process.

Comparison of the number of protons bound upon denaturation derived from the relationship $\delta \ln K_{app}/\delta \ln a_{H^+}$ with

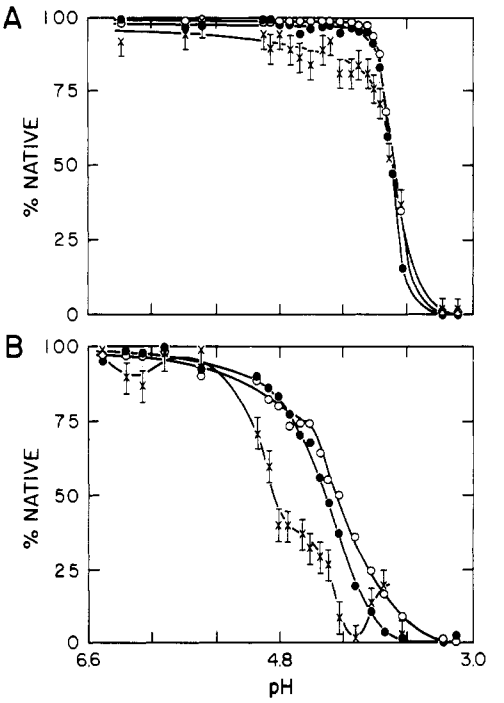

FIGURE 5: (A) Simultaneous multiparameter acid denaturation profile for the aquoferri derivative of sperm whale myoglobin: (●) Soret absorbance at 409 nm; (○) far-UV CD band at 222 nm; (×) UV acid difference spectral band at 291 nm. Error bars are the estimated error due to extrapolation of the heme absorbance to base line. (B) Similar determination as in (A) for [Oia^{7,14}]myoglobin. The UV acid difference spectral band for oxindolylalanine is at 264 nm.

the observed values obtained by direct titration has been used as a criteria for establishment of the two-state model for protein denaturation (Tanford, 1968; Hermans & Acampora, 1967). The inconsistencies observed by using this type of comparison in this study could arise due to differences in the denatured states of the modified and native sperm whale myoglobins, but this seems unlikely. Alternatively, it would appear that the modification of the tryptophan residues in sperm whale myoglobin has caused the protein to no longer fit the two-state model of protein denaturation.

Simultaneous Multiparameter Denaturation Studies. The acid denaturation of native sperm whale myoglobin and the modified protein was observed by utilizing three conformational probes. For native sperm whale myoglobin (Figure 5A), the conformational probes were the Soret absorbance at 409 nm, the far-UV CD band at 222 nm, and the acid difference spectral band of the tryptophan residues at 291 nm. The error bars in the acid difference probe of the tryptophan absorbances represent the estimated error due to extrapolation to base line of the heme absorbance (Herskovits, 1969). The midpoints of all three probes of the protein structure are similar, indicating the denaturation of native sperm whale myoglobin may be a two-state process. This is consistent with previous observations (Hermans & Acampora, 1967; Acampora & Hermans, 1967; Privalov & Khechinashvili, 1974; Privalov, 1979). Interestingly, the UV difference spectral probe at 291 nm indicates changes in the environments of the tryptophan residues which precede denaturation as monitored by the heme absorbance and secondary structure of the protein. This confirms results obtained previously using measurements of the near-UV CD spectrum of the protein at 295 nm as a function of pH (Gurd et al., 1980).

The acid denaturation of [Oia^{7,14}]myoglobin was determined in similar fashion (Figure 5B), except that the observed UV difference spectral band of the oxindolylalanine residues was

an intense negative absorbance at 264 nm (Radding, 1983). Strikingly, the environment of the oxindolylalanine residues undergoes a multiphasic transition prior to denaturation of the modified protein as monitored by the heme absorbance. This multiphasic transition is mirrored by changes in the secondary structure as monitored by the CD probe. The midpoints of the three probes are also seen to be quite different. These results strongly evidence the departure of the modified protein from the two-state denaturation process, previously established for native sperm whale myoglobin, to a process which involves stable intermediates, therefore confirming the inequality in the number of protons bound upon denaturation as observed by direct titration and calculated from the slope of the denaturation curve.

Discussion

The mechanism of the loosening of the A helix in native myoglobin has been described previously (Gurd et al., 1980). The near-UV CD bands of the tryptophan residues at 295 nm were utilized as a conformational probe of the A helix. This predenaturational change in native myoglobin is also observed in this study utilizing perturbation difference UV spectroscopy. There is no associated change in the far-UV CD spectrum of the native molecule, indicating that a major conformational change has not occurred.

The data from the simultaneous multiparameter denaturation curves suggest that in the modified protein, the A helix as monitored by the oxindolylalanine environments and mirrored by the far-UV CD has undergone a localized transition. The midpoint of the first phase of the denaturation transition of the modified helix correlates to the transition pH of 4.9 (from stabilizing to destabilizing) of the summed electrostatic free energy between Lys-16, Arg-118 His-119, and Asp-122 (Gurd et al., 1980). This suggests that the mechanism of the first phase of the denaturation transition is similar to that which induces the predenaturational changes observed in native myoglobin.

The relative stability of the modified protein to native myoglobin of +5.3 kcal/mol is open to uncertainty as determination of this parameter from the equilibrium studies presented is dependent upon the observed transition being two state in nature. The two-state denaturation of sperm whale myoglobin is well established. The noncooperative unfolding of the modified myoglobin was first suggested by observation of the inequality in the proton binding of the molecule as determined from the slope of the denaturation curve and by inference through direct titration. In addition, a necessary, but insufficient, criteria for demonstrating a transition to be two state is the correspondence of observed changes in all conformational probes. The modified myoglobin obviously does not meet these simple criteria.

The change in free energy of +5.3 kcal/mol for the modified protein, calculated from the heme transition subsequent to the A helix transition, thus represents the free energy of a stable intermediate structure(s), if a two-state transition for the second phase is assumed. Electrostatic model calculations of the assembly formation of the A helix and the des-A helix myoglobin predict the des-A helix protein to be +5 kcal/mol less stable than native myoglobin at pH 4.0 (March, 1983; Garcia-Moreno et al., 1985). This agrees well with the observed free energy difference between the stable intermediate and native myoglobin as monitored by the heme absorbance, indicating the loss of important A helix electrostatic interactions upon localized unfolding. The total randomization of the A helix is unlikely, however, as the loss of stabilizing electrostatic interactions and hydrophobic burial would exceed the net stability of the entire protein (Richmond & Richards, 1978; Finney et al., 1980).

The alteration of a small globular protein from a single thermodynamic domain to one demonstrating at least two distinct, but linked, domains is the result of introducing packing defects through oxidation of the tryptophan rings in the cluster between the A-H helix contacts. In this respect, it is necessary to distinguish between the burial of surface area of the helix and the integrity of the interhelical contact in terms of the concentration of nonpolar groups. The introduction of defects in packing would not greatly affect the level of stability acheived by hydrophobic surface burial of the helix through the exclusion of solvent but would affect the frictional forces within the cluster due to nonpolar contacts in the A-H helix interface (Lesk & Chothia, 1980).

As introduction of packing defects in the A-H helix contact results in a decrease in the concentration of nonpolar contacts between the helices, the modified helix may thus be viewed as undergoing fluctuations, induced by electrostatic interactions as a function of pH, which are transmitted throughout the helix and lead to localized unfolding. This is consistent with previous observation of the relationship between the concentration of nonpolar contacts within a protein and the motility of protein structures as demonstrated by scanning microcalorimetry and hydrogen isotope exchange studies (Privalov, 1979).

The microstability of small globular proteins is found not to correlate with the macrostability of the molecules but correlates with their denaturational change in specific heat capacity, a function of the denaturational enthalpy and the denaturational entropy (Privalov, 1979). Through these latter values, the specific heat capacity of a protein is related to the concentration of nonpolar contacts and reflects the nonuniform distribution of these clusters (Privalov & Khechinashvili, 1974; Privalov, 1979). As motility appears to be mainly a function of the entropy term, microunfolding would appear to require localized disruption of hydrogen bonding (Nakanishi et al., 1973). Clusters of nonpolar contacts in proteins therefore obstruct migrating fluctuations in the proteins' structure (Privalov, 1979; Wutrich et al., 1980). Disruption of the close-packed A-H helix contact in the modified myoglobin leads to seperation of the helix and protein into distinct, yet linked, thermodynamic domains. In native myoglobin, the close contact provided by the tryptophan residues dampens the effect of the stress induced by electrostatic destabilization at low pH. The result is a smaller amplitude fluctuation, retaining important electrostatic and hydrophobic interactions. The influence of nonpolar interactions thus dynamically extends beyond that of strictly solvent sheilding of electrostatic interactions (Garcia-Moreno et al., 1985; Busch et al., 1985) and hydrophobic burial (Chothia, 1974) by influencing the motility of structures through longer range interactions.

The detection of the stable intermediate structure in the denaturation pathway is a direct consequence of unfolding blocks of hydrophobic residues prior to overall protein denaturation and is consistent with the statistical mechanical models for noncooperative denaturation of globular proteins. The noncooperativity of the unfolding reaction is therefore a function of the dependence between entropy gain and enthalpy loss accompanying denaturation (Gō, 1975, 1976). The modification of the tryptophan residues in sperm whale myoglobin therefore demonstrates that even in a single polypeptide chain structures which may not obviously consist of seperate domains can be dynamically discrete structures (Privalov, 1979, 1982; Ringe & Petsko, 1986; Rogero et al., 1986) and supports the concept that large-scale fluctuational

events are governed by the frictional effects of more local small-amplitude fluctuations within the protein core (Karplus, 1986).

ACKNOWLEDGMENTS

I thank Dr. M. A. Flanagan and Dr. K. L. March for useful discussions. In addition, the advice of Dr. T. M. Lalor, Dr. F. F. Richards, and Dr. C. M. Radding was invaluable.

Registry No. Oia, 32999-55-6; L-Trp, 73-22-3.

REFERENCES

Acampora, G., & Hermans, J., Jr. (1967) *J. Am. Chem. Soc. 89*, 1543–1547.

Busch, M. R., Maskalick, D. G., Neireiter, G. W., Harris, D. E., & Gurd, F. R. N. (1985) *Biochemistry 24*, 6707–6716.

Chothia, C. (1974) *Nature (London) 248*, 338–339.

DiMarchi, R. D., Garner, W. H., Wang, C. C., Hanania, G. I. H., & Gurd, F. R. N. (1978) *Biochemistry 17*, 2822–2829.

DiMarchi, R. D., Neireiter, G. W., Heath, W. F., & Gurd, F. R. N. (1980) *Biochemistry 19*, 2454–2465.

Donovan, J. W. (1973) *Methods Enzymol. 27*, 497–525.

Finney, J. L., Gellatly, B. J., Golton, I. C., & Goodfellow, J. (1980) *Biophys. J. 32*, 17–33.

Flanagan, M. A., Garcia-Moreno, E. B., Friend, S. H., Feldmann, R. J., Scouloudi, H., & Gurd, F. R. N. (1983) *Biochemistry 22*, 6027–6037.

Frauenfelder, H., Petsko, G. A., & Tsernoglou, D. (1979) *Nature (London) 280*, 558–563.

Friend, S. H., & Gurd, F. R. N. (1979a) *Biochemistry 18*, 4612–4619.

Friend, S. H., & Gurd, F. R. N. (1979b) *Biochemistry 18*, 4620–4630.

Garcia-Moreno, E. B., Chen, L. X., March, K. L., Gurd, R. S., & Gurd, F. R. N. (1985) *J. Biol. Chem. 260*, 14070–14082.

Garner, M. H., Bogardt, R. A., & Gurd, F. R. N. (1975) *J. Biol. Chem. 250*, 4398–4403.

Gō, N. (1975) *Int. J. Pept. Protein Res. 7*, 313–323.

Gō, N. (1976) *Adv. Biophys. 9*, 65–113.

Gurd, F. R. N., Friend, S. H., Rothgeb, T. M., Gurd, R. S., & Scouloudi, H. (1980) *Biophys. J. 32*, 65–75.

Hapner, K. D., Bradshaw, R. A., Hartzell, C. R., & Gurd, F. R. N. (1968) *J. Biol. Chem. 243*, 683–689.

Hartmann, H., Parak, F., Steigmann, W., Petsko, G. A., Ringe Ponzi, D., & Frauenfelder, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4967–4971.

Hermans, J., Jr., & Acampora, G. (1967) *J. Am. Chem. Soc. 89*, 1547–1552.

Herskovits, T. T. (1969) *Arch. Biochem. Biophys. 130*, 19–29.

Herskovits, T. T., & Laskowski, M., Jr. (1962) *J. Biol. Chem. 237*, 2481–2492.

Karplus, M. (1986) *Methods Enzymol. 131*, 283–307.

Lesk, A. M., & Chothia, C. (1980) *Biophys. J. 32*, 35–46.

Liu, K. L., & Chang, Y. J. (1971) *J. Biol. Chem. 246*, 2842–2848.

March, K. L. (1983) Ph.D. Thesis, Indiana University, Bloomington, IN.

Matthew, J. B., Gurd, F. R. N., Flanagan, M. A., March, K. L., & Shire, S. J. (1985) *CRC Crit. Rev. Biochem. 18*, 91–197.

Nakanishi, M., Kasamichi, T., & Ikegami, A. (1973) *J. Mol. Biol. 75*, 673–682.

Nozaki, Y., & Tanford, C. (1971) *J. Biol. Chem. 246*, 2211–2217.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.

Radding, J. A. (1983) Ph.D. Thesis, Indiana University, Bloomington, IN.

Richmond, T. J., & Richards, F. M. (1978) *J. Mol. Biol. 119*, 537–555.

Ringe, D., & Petsko, G. (1986) *Methods Enzymol. 131*, 389–432.

Rogero, J. R., Englander, J. J., & Englander, S. W. (1986) *Methods Enzymol. 131*, 508–517.

Rothgeb, T. M., & Gurd, F. R. N. (1978) *Methods Enzymol. 52C*, 473–486.

Savige, W. E., & Fontana, A. (1977a) *Methods Enzymol. 47*, 442–453.

Savige, W. E., & Fontana, A. (1977b) *Methods Enzymol. 47*, 453–459.

Savige, W. E., & Fontana, A. (1980) *Int. J. Pept. Protein Res. 15*, 285–297.

Shen, L. L., & Hermans, J., Jr. (1972) *Biochemistry 11*, 1842–1844.

Shire, S. J., Hanania, G. I. H., & Gurd, F. R. N. (1974) *Biochemistry 13*, 2967–2974.

Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1205.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.

Wang, C. C. (1977) Ph.D. Thesis, Indiana University, Bloomington, IN.

Wang, C. C., DiMarchi, R. D., & Gurd, F. R. N. (1978) in *Semisynthetic Peptides and Proteins* (Offord, R. E., & DiBello, C., Eds.) pp 59–69, Academic Press, London.

Wutrich, K., Wagner, G., Richarz, R., & Braun, W. (1980) *Biophys. J. 32*, 549–560.

Wyman, J., Jr. (1964) *Adv. Protein Chem. 19*, 223–286.

Yonetani, T. (1967) *J. Biol. Chem. 242*, 5008–5013.