Conformational Studies on Modified Proteins and Peptides. Artificial Myoglobins Prepared with Modified and Metalloporphyrins*

S. F. Andres† and M. Z. Atassi‡

ABSTRACT: Artificial myoglobins have been prepared with Cu- or Zn-metalloporphyrins or with a heme derivative nitrated at the vinyl side chains and their conformations investigated by optical rotatory dispersion and circular dichroism and by immunochemical methods. Native myoglobin (Mb), Fe-Mb, and Cu-Mb exhibit identical rotations at the negative minima at 233 mμ and at the positive maxima at 199 mμ. Measurements of the reduced molar ellipticities of these derivatives at the negative circular dichroism bands at 221 and 208 mμ were in agreement with optical rotation measurements. These results suggest that the three derivatives possess identical conformations. On the other hand, Nheme-Mb shows a small conformational change while Zn-Mb shows an appreciable degree of unfolding. A lower contribution of helical structure was, therefore, present in Zn-Mb and Nheme-Mb than that found in native myoglobin, Fe-Mb, or Cu-Mb. Furthermore, studies of the changes in the rotatory dispersion behaviors of these derivatives and of apomyoglobin with decreasing pH have revealed that native myoglobin and Cu-Mb appear to possess structures of comparable stabilities. Nheme-Mb was next in the order of stability, while the acid denaturation of Zn-Mb was complete at a relatively higher pH than the other derivatives. The conformational differences were in agreement with previous studies of the immunochemical behaviors of native myoglobin, Fe-Mb, ApoMb, Cu-Mb, and Zn-Mb as well as with results presented here regarding the antigenic reactivity of the Nheme-Mb derivative. Such results confirm that, under appropriate conditions, immunochemical methods may be employed as a powerful tool to monitor conformational changes in proteins. Derivatives of the type studied here are useful in understanding the role played by some selected specific interactions in their contributions to the mode of folding of a given protein in solution.

In a previous report from this laboratory (Atassi, 1967b), the preparation and characterization of some artificial myoglobins prepared with various modified and metalloporphyrins have been described in detail. Immunochemical investigations on these derivatives have revealed that Fe-Mb[1] (prepared by recombination of ApoMb with resynthesized ferriheme) was immunochemically indistinguishable from native Mb. In addition, the antigenic reactivities of Cu-Mb and Fe-Mb were identical, while Zn-Mb reacted poorly with antisera to Mb. With the exception of Cu-Hb which has not been prepared, the corresponding derivatives of human adult hemoglobin exhibit similar trends in antigenic reactivity relative to the homologous antigen (Atassi and Skalski, 1969). These changes in antigenic reactivities were attributed to conformational reorganizations caused by the different coordination tendencies of the various metals (Atassi, 1967b; Atassi and Skalski, 1969).

In the present studies, the conformation of these Mb derivatives is investigated by optical rotatory dispersion and circular dichroism measurements. Also, a new derivative of Mb has been prepared by recombination of ApoMb with ferriheme previously nitrated at the vinyl groups (Atassi, 1969). Such a derivative is useful in studying the role of the vinyl side chains in the heme–protein interaction. The immunochemical behavior of this derivative is reported here together with its optical rotatory dispersion and circular dichroism properties. Finally, changes in the rotatory behavior of each of these derivatives with decreasing pH have been monitored in order to determine their relative stabilities in solution.

Materials and Methods

Metmyoglobin and Apomyoglobin. Sperm whale Mb used in these studies was the major chromatographic component 10 (MbX) obtained by CM-cellulose chromatography of the crystalline protein (Atassi, 1964). The apoprotein was prepared from MbX by a procedure similar to that used for the preparation of apohemoglobin (Atassi and Skalski, 1969).

Preparation of Metalloporphyrins and Nitration of Ferriheme. Removal of the iron from ferriheme was done by a pro-

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‡ Established Investigator of the American Heart Association, to whom requests for reprints should be addressed.

[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin; MbX, the major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964); Fe-Mb, control prepared from ApoMb and resynthesized heme (from ferric iron and protoporphyrin IX); Cu-Mb, Zn-Mb, derivatives prepared by recombination of ApoMb with the respective metalloporphyrins; Nheme-Mb, derivative prepared by recombination of ApoMb with ferriheme previously nitrated at the vinyl side chains.

cedure previously reported in detail (Atassi, 1967b). Zinc and copper metalloporphyrins were prepared and exhaustively characterized by the procedure already described (Atassi, 1967b). Nitration of the vinyl side chains of ferriheme and purification and characterization of the derivative were performed according to the procedures recently described in detail elsewhere (Atassi, 1969).

Reconstitution of the Artificial Myoglobins. The procedures for reconstitution of ApoMb with various porphyrin derivatives and for removal of excess ferriheme or derivative on CM-cellulose have been described elsewhere (Atassi and Caruso, 1968).

Antisera. These were prepared in goats and in rabbits against MbX, and the procedure has already been described in detail (Atassi, 1967a). Antisera from individual animals were kept separate and stored in 8-ml portions at −40°. Goat antisera G3 and G4 and rabbit antiserum 77 were used in the present studies.

Analytical Methods. All metalloporphyrin preparations were examined for purity by thin-layer chromatography (silica gel, Eastman chromatogram sheets). The metal content of zinc, copper, and other metalloporphyrin preparations has already been reported (Atassi, 1967b) and analyses were performed on a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Spectral measurements were carried out with a Zeiss PMQII spectrophotometer. Continuous spectra were performed with a Perkin-Elmer Model 124 recording spectrophotometer. Immunochemical methods employed here (*i.e.*, agar double diffusion and quantitative precipitin analyses) have already been described in detail (Atassi and Saplin, 1968). Starch gel electrophoresis and determination of Stokes radius, a, and molar frictional coefficient, f/f_0, by gel filtration on a precalibrated Sephadex G-75 column (2.2 × 55 cm) were done under conditions previously described in detail (Atassi and Caruso, 1968).

Preparation of Protein Solutions and Determination of Their Concentrations. After removal of excess heme (or modified heme) on CM-cellulose by elution with 0.01 M NaH_2PO_4 containing 0.01% KCN (pH 6.2), the derivatives were eluted from the columns with 0.01 M phosphate buffer at pH 7.6, containing 0.01% KCN (Atassi and Caruso, 1968). Following elution from the column, samples were dialyzed extensively against water (glass double distilled), centrifuged (5600 rpm, 60 min, 0°) to remove any insoluble protein, and then used for optical rotatory dispersion and circular dichroism studies at appropriate dilutions.

Concentrations of protein solutions were determined from their nitrogen contents using a micro-Kjeldahl procedure similar to that described by Markham (1942), by using Nessler's reagent standardized with ammonium sulfate, and by using the Folin–Lowry color reagent (Lowry *et al.*, 1951), (Fischer Scientific), calibrated with accurately prepared MbX solutions. Also, the absorption at 280 mμ was always determined. Three or four replicate analyses were done on each solution by at least two of the foregoing methods, in addition to determination of the absorption at 280 mμ, and they varied by ±0.5%. The nitrogen contents for Cu-Mb, Zn-Mb, and Nheme-Mb were considered identical with the nitrogen content of MbX. The nitrogen contents of MbX and of ApoMb have already been calculated (Atassi and Saplin, 1968) from their amino acid compositions and are 17.36 and 17.66%, respectively.

Optical Rotatory Dispersion and Circular Dichroism Measurements. All experiments were carried out at 25° on solutions of the proteins in water. Solutions contained 0.08–0.12 mg/ml. Measurements were made with a Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism accessory.

Measurements on each protein were at several concentrations employing cells with light paths of 0.5, 1, 5, and 10 mm. For measurements below 220 mμ only 0.5- and 1-mm cells were used with maximum damping (pen period 30), very low scan speeds (30 sec/mμ) and a 0.1 deg full range. Solvent base-line scans were performed before and after each protein sample. Optical rotatory dispersion results are reported in reduced mean residue rotations, $[m']_\lambda$, corrected for the refractive index dispersion of water, n_λ

$$[m']_\lambda = \left(\frac{3}{n^2 + 2}\right)\frac{M_R}{100}[\alpha]_\lambda$$

where M_R is the mean residue molecular weight (taken as 116.4 for Mb and its derivatives and as 112.4 for ApoMb), and $[\alpha]_\lambda$ is the specific rotation at various wavelengths, λ. Also, optical rotatory dispersion data were analyzed quantitatively by means of the Moffitt equation (Moffitt and Yang, 1956)

$$[m']_\lambda = a_0 \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} + b_0 \frac{\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

The parameter b_0 was determined from the slope of the plot $[m']_\lambda((\lambda^2 - \lambda_0^2)/\lambda_0^2)$ against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ with $\lambda_0 = 216$ mμ. The plots were linear in the range 240–270 mμ. In the present work a b_0 value of −630° was assumed to represent a 100% helical conformation (Urnes and Doty, 1961).

The circular dichroism accessory records data directly in terms of ellipticity, θ, in degrees. In analogy to $[m']_\lambda$, circular dichroism data are reported here as reduced molar ellipticities, $[\theta']_\lambda$, by correcting for the refractive index dispersion of water. Units of $[\theta']$ are in deg cm² per dmole.

Results

Characterization of the Porphyrin Derivatives and Their Corresponding Artificial Myoglobins. Spectral properties and metal analyses for iron, copper, and zinc metalloporphyrins have already been reported (Atassi, 1967b). The ultraviolet, visible, and infrared spectral properties of nitrated heme have been reported together with its elemental analysis (Atassi, 1969). The purity of all these derivatives was confirmed by thin-layer chromatography utilizing the solvent systems reported elsewhere (Atassi, 1969).

Purity of the artificial Mb derivatives was confirmed by starch gel electrophoresis. Electrophoretic mobilities of the derivatives (relative to MbX = 1) were: Fe-Mb, 1.00; Cu-Mb, 0.97; Zn-Mb, 0.91; and Nheme-Mb, 0.96. Complete complex formation of modified or metalloporphyrin with ApoMb was confirmed by metal analysis (Atassi, 1967b). Determinations showed that all metalloporphyrin–apomyoglobin complexes had metal contents similar to those expected (±6%) from a complex formation of 1 mole of metalloporphyrin/mole of ApoMb.

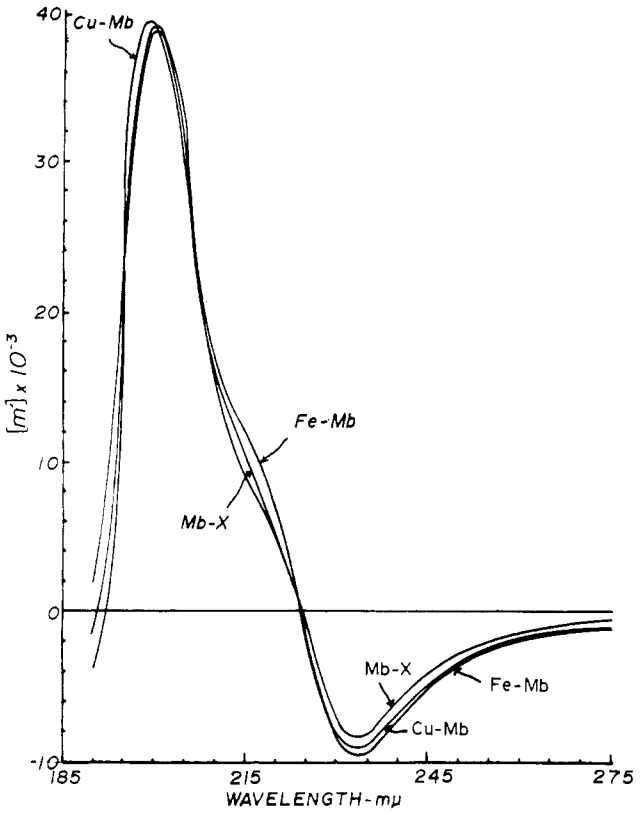

FIGURE 1: Ultraviolet optical rotatory dispersion curves of MbX, Fe-Mb, and Cu-Mb in water. Values were obtained from single scans. For averages of four or more scans, see Table II.

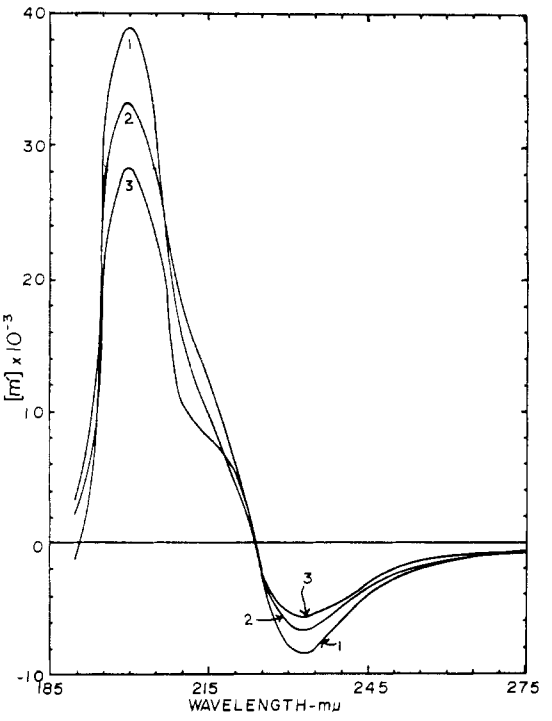

FIGURE 2: Ultraviolet optical rotatory dispersion curves of artificial Mb derivatives in water. Values were obtained from single scans. For averages of four or more scans, see Table II. MbX (1), ApoMb (2), and Zn-Mb (3).

The spectral properties of Nheme-Mb have not been reported and these are summarized in Table I. It can be seen that the Soret band is shifted from 424 (for cyan-MbX) to 417 mμ for the cyanmet form of Nheme-Mb. Also, the absorption in the Soret region is suppressed appreciably (Table I).

Gel filtration was carried out on a Sephadex G-75 column which had been calibrated with human serum albumin, hen ovalbumin, bovine ribonuclease A, and horse heart cytochrome *c*. Calculation of the molecular parameters of the artificial myoglobins was done by the procedure described elsewhere in detail (Atassi and Caruso, 1968). It was found that MbX, Fe-Mb, Cu-Mb, and Zn-Mb possessed identical values for stokes radius (18.5 Å) and f/f_0 (1.06). Comparison with optical rotatory dispersion, circular dichroism, and immunochemical data suggests that this technique might not always be sensitive to conformational changes.

TABLE I: Absorption Maxima and Ratios of Optical Densities at These Maxima of MbX and Nheme-Mb.[a]

	Absorption Max. (mμ)				Ratios of Optical Densities at Absorption Max.		
	A	B	C	D	A:D	B:D	C:D
MbX	279	360	424	542	3.05	2.61	9.08
Nheme-Mb	275	364[b]	417	542[b]	4.19	3.66	4.55

[a] Solutions were in 0.01 M phosphate buffer, pH 7.6, containing KCN (0.01%). [b] Absorption maximum very weak, mostly a small arrest in the curve.

Optical Rotatory Dispersion of Artificial Myoglobins. All the present Mb derivatives showed a negative rotation minimum at 233 mμ and a positive extremum at 199 mμ. The optical rotatory dispersion spectra of MbX, Fe-Mb, and Cu-Mb were identical, both in the values of rotation at the negative minimum and positive maximum and in the value of b_0 (Figure 1). The behavior of Zn-Mb was different. Zn-Mb exhibited a lower rotatory power at the negative minimum at 233 mμ and at the positive maximum at 199 mμ (Figure 2). The rotatory power of Zn-Mb was even lower than that of ApoMb. The b_0 value for Zn-Mb (−299) was also lower than that of Fe-Mb (−414) or even ApoMb (−321).

Nitration of the vinyl side chains of ferriheme appeared to exert some influence on the conformation of the artificial myoglobin prepared from it. The optical rotatory dispersion spectrum of Nheme-Mb is shown in Figure 3 together with that of MbX. Some differences are apparent in the values of $[m']_{233}$ and $[m']_{199}$ for Nheme-Mb which were −7,680 and +35,700, respectively (Table II). The corresponding values for MbX were −9,240 and +39,000, respectively. The b_0 value of Nheme-Mb was −328. The results of the optical rotatory dispersion measurements are summarized in Table II. From the b_0 values the per cent helical content is calculated for the present proteins. Although, of course, many complications already outlined by various workers (Beychok, 1968;

TABLE II: Optical Rotatory Dispersion Parameters for the Artificial Myoglobin Derivatives.[a]

Derivative	pH	$[m']_{233}$	$[m']_{199}$	b_0	% Helix
MbX	6.54	−9,240	+38,900	−410	65
Fe-Mb	7.44	−9,640	+41,700	−414	66
ApoMb	5.79	−7,366	+33,100	−321	51
Cu-Mb	6.84	−9,170	+39,400	−415	66
Zn-Mb	7.25	−5,650	+28,800	−299	48
Nheme-Mb	7.10	−7,680	+35,700	−328	52

[a] Values represent the average of four or more determinations with varying concentrations and light paths. $[m']_{233}$ values varied ±3% or less, and $[m']_{199}$ values varied ±8% or less. Values of per cent helix are obtained directly from the b_0 values with the assumption that a b_0 value of −630 represents a helical content of 100% (Urnes and Doty, 1961).

Schellman and Lowe, 1968; Woody and Tinoco, 1967; Greenfield *et al.*, 1967; Epand and Scheraga, 1968) make it difficult to correlate transitions responsible for optical rotatory dispersion and circular dichroism behavior with helical content, the present calculated values are useful as an indication of the *relative* conformations of these proteins.

Circular Dichroism Measurements. Circular dichroism studies were carried out in the range 260–205 mμ. All the present proteins showed two negative ellipticity bands at 221 and at 208 mμ. Both ellipticity bands in Zn-Mb were lower in magnitude than the corresponding $[\theta']$ values for MbX, Cu-Mb, and ApoMb. The circular dichroism spectra of these four proteins are shown in Figure 4. It can be seen that the ellipticity of Zn-Mb at the two minima was suppressed to such an extent that it was even lower than that of ApoMb. On the other hand, Cu-Mb and MbX had identical circular dichroism spectra. These results are in agreement with the foregoing optical rotatory dispersion data.

Circular dichroism spectra of Nheme-Mb and of MbX were quite similar (Figure 5). They differ only by 4% from each other which is just within the normal experimental deviation. Circular dichroism spectra of free nitrated heme in solution showed that this has no contribution to the circular dichroism spectrum in the ultraviolet region. Therefore, it appeared from the circular dichroism data that MbX and Nheme-Mb (Figure 5 and Table III) possess closely similar conformations.

TABLE III: Circular Dichroism Parameters for the Artificial Myoglobin Derivatives.[a]

Derivative	pH	$[\theta']_{221}$	$[\theta']_{208}$	$[\theta']_{221}$: $[\theta']_{208}$
MbX	6.54	−20,200	−19,500	1.04
ApoMb	5.79	−15,800	−15,300	1.03
Cu-Mb	6.84	−19,600	−18,000	1.08
Zn-Mb	7.25	−10,500	−11,000	0.96
Nheme-Mb	7.10	−21,400	−21,300	1.00

[a] Values represent the average of four or more determinations. Both $[\theta']_{221}$ and $[\theta']_{208}$ values varied ±8% or less. Values are corrected for the refractive index dispersion of water which was the solvent in all of these studies.

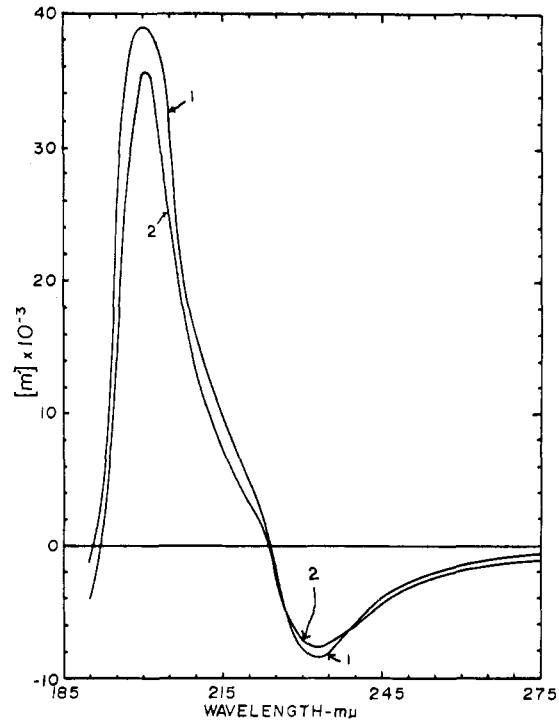

FIGURE 3: Ultraviolet optical rotatory dispersion curves of artificial Mb derivatives in water. Values were obtained from single scans. For averages of four or more scans, see Table II. MbX (1) and Nheme-Mb (2).

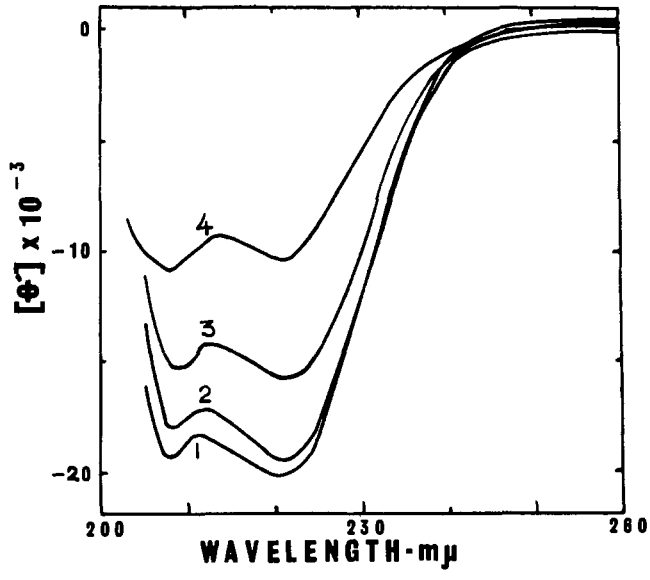

FIGURE 4: Circular dichroism spectra of artificial Mb derivatives in water. MbX (1), Cu-Mb (2), ApoMb (3), and Zn-Mb (4).

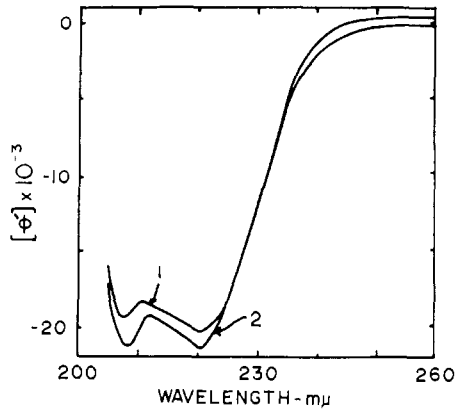

FIGURE 5: Circular dichroism spectra of artificial Mb derivatives in water. MbX (1) and Nheme-Mb (2).

On the other hand, optical rotatory dispersion measurements suggested different conformations. This is especially true with the b_0 value. Due to these differences between optical rotatory dispersion and circular dichroism data, it was decided to investigate the immunochemistry of Nheme-Mb. The antigenic reactivity of proteins is highly sensitive to conformational changes (Atassi, 1967b; Sela *et al.*, 1967; Atassi and Skalski, 1969; Atassi *et al.*, 1969).

Immunochemistry of Artificial Myoglobins. The antigenic reactivities of Fe-Mb, Cu-Mb, and Zn-Mb have been studied in detail with several antisera to MbX and compared with the reaction of the homologous antigen (Atassi, 1967b). In quantitative precipitin experiments, it was reported that Fe-Mb, MbX, and Cu-Mb were immunochemically identical, possessing equal antigenic reactivities with antisera to MbX. With the same antisera, Zn-Mb exhibited lower antigenic reactivity than MbX. In fact, its reactivity was even lower than that of ApoMb (*cf.* the present optical rotatory dispersion and circular dichroism data).

The immunochemical behavior of Nheme-Mb has not been reported and was studied in the present work. In agar double diffusion, Nheme-Mb gave single precipitin lines which fused completely with the single line given by MbX, showing no spurs or intersections. The antigenic reactivity was also examined quantitatively by precipitin analysis. Figure 6 shows the reaction of antiserum G4 with MbX and Nheme-Mb. Nheme-Mb shows an increase in antigenic reactivity relative to MbX. Identical results were obtained with goat antiserum G3 and rabbit antiserum 77. The increased antigenic reactivity of Nheme-Mb confirms the presence of a conformational change relative to MbX.

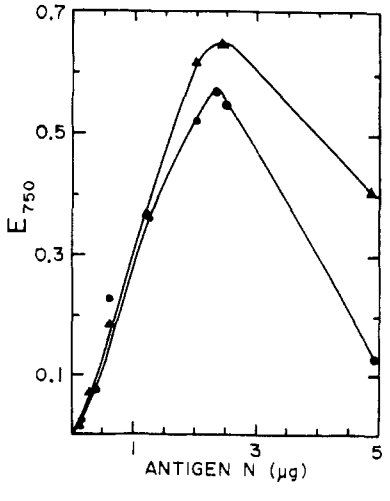

FIGURE 6: Precipitin analyses with MbX (●) and Nheme-Mb (▲) and goat antiserum G4 which had been diluted with 0.15 M NaCl (1:1). E_{750} indicates the amount of protein in the immune precipitate as determined by the Folin–Lowry method (Lowry *et al.*, 1951).

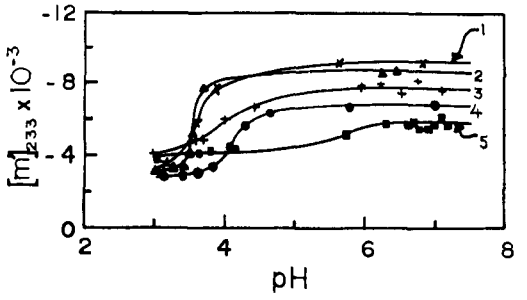

FIGURE 7: pH-stability profiles of artificial Mb derivatives. Reduced mean residue rotations at 233 mμ, $[m']_{233}$, are plotted against pH. Cu-Mb (1), MbX (2), Nheme-Mb (3), ApoMb (4), and Zn-Mb (5).

pH Denaturation Curves of the Artificial Myoglobins. Although some artificial Mb derivatives possess conformations that are identical with MbX at neutral pH conditions, it is of significance to determine the relative stabilities of the derivatives under changing conditions of pH. Denaturation curves of the artificial myoglobins were obtained by following changes in the rotatory dispersion behaviors of these derivatives with decreasing pH. The results of this study are summarized in Figure 7. It is evident that Zn-Mb is the most unstable of all of these artificial myoglobins since its acid denaturation is complete at a relatively higher pH than the other derivatives. However, the final value of $[m']_{233}$ at pH 3.0 is slightly higher than that of Cu-Mb, MbX, and Nheme-Mb. ApoMb appears to be slightly more unstable than Nheme-Mb. However, it was highly interesting that Cu-Mb and MbX seem to possess structures of comparable stabilities. Identical trends were observed when b_0 values were determined. Table IV summarizes b_0 values obtained from the

TABLE IV: Variation of b_0 Values with pH for the Artificial Myoglobin Derivatives.[a]

MbX		Cu-Mb		Zn-Mb		Nheme-Mb	
pH	b_0	pH	b_0	pH	b_0	pH	b_0
6.54	−410	6.84	−415	7.25	−299	7.10	−328
3.70	−314	3.86	−439	4.13	−199	3.69	−170
3.53	−149	3.59	−241	3.79	−201	3.60	−163
3.49	−148					3.38	−169

[a] Values are obtained from the slopes of least-square lines which result upon plotting $[m']_\lambda$ $((\lambda^2 - \lambda_0^2)/\lambda_0^2)$ as a function of $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ with $\lambda_0 = 216$ mμ.

optical rotatory dispersion spectra of the artificial myoglobins at selected values of pH. It was not possible to carry out the reverse titrations on these derivatives due to their excessive precipitation when this was attempted.

Discussion

Information on the antigenic structure of sperm whale Mb (see Atassi and Thomas, 1969) represents the most advanced knowledge for such a globular protein. Therefore, this protein could serve as a good model for investigating the effect of some selected modifications, in parts that are known to be outside the antigenic sites, on the immunochemical behavior of the protein and its relationship to conformation. Since the heme group in myoglobin and hemoglobin is not part of an antigenic site (Reichlin *et al.*, 1963), modifications of this structure should provide an excellent means for examining the effect of purely conformational factors on the antigen–antibody reaction. Previous studies from this laboratory have, indeed, revealed that the antigenic reactivity of myoglobin (Atassi, 1967b) and hemoglobin (Atassi and Skalski, 1969) is influenced by the nature of the porphyrin modification. Investigations were carried out on artificial myoglobin and hemoglobin derivatives which were prepared with various modified and metalloporphyrins, and the observed changes in antigenic reactivity were attributed entirely to conformational reorganization caused by the different coordination tendencies of the various metals or by the modification of the carboxyl side chains of the heme (Atassi, 1967b). Such derivatives, where the modification is clearly not in an antigenic region, provided (Atassi, 1967b) an unequivocal demonstration that the antigenic reactivity of proteins is highly influenced by changes in the conformation of the antigen. Also, conversely, immunochemical methods may be employed, under appropriate conditions, as a method for investigating conformational changes (Atassi, 1967b). Binding of ligands to proteins has been followed immunochemically (Von Fellenberg *et al.*, 1968; Von Fellenberg and Levine, 1967). However, caution should be exercised in such studies since binding of a foreign molecule to a protein may sterically cover one or more of its antigenic sites. Changes in immunochemical reactivity of a protein obtained with such binding studies could be due either to conformational changes or to masking of antigenic site(s) and, therefore, the interpretation is not so straightforward. In the present work, conformational studies have been carried out on the aforementioned artificial myoglobin derivatives and the results correlated with changes in immunochemical behavior.

Optical rotatory dispersion and circular dichroism provide a valuable approach for monitoring conformational changes of Mb derivatives, particularly since from these measurements estimates of the α-helical content of the native protein are in good agreement with those obtained by X-ray crystallography (Urnes *et al.*, 1961; Kendrew *et al.*, 1961). Excellent reviews of this technique have appeared recently (Urnes and Doty, 1961; Todd, 1960; Schellman and Schellman, 1964; Beychok, 1968), and several applications of the technique to myoglobin and hemoglobin have been reported (Urnes *et al.*, 1961; Breslow *et al.*, 1965; Harrison and Blout, 1965; Beychok and Blout, 1961; Li and Johnson, 1969a,b; Nagai *et al.*, 1969; Javaherian and Beychok, 1968; Willick *et al.*, 1969; Geraci and Li, 1969; Simon and Cantor, 1969; Epand and Scheraga, 1968). Methods for obtaining quantitative estimations of α-helical contents of proteins from their optical rotatory dispersion curves (Shechter and Blout, 1964; Moffitt and Yang, 1956; Moffitt *et al.*, 1957; Yang, 1967) have been employed and indicate that ApoMb is more unfolded than the native protein in solution (Harrison and Blout, 1965; Breslow *et al.*, 1965; Urnes *et al.*, 1961). While it has been questioned whether such treatment of optical rotatory dispersion data does in fact yield absolute helical contents of proteins (Urnes and Doty, 1961; Tanford, 1961), the use of the parameter b_0 from the Moffitt–Yang equation in calculating values of the helical contents of the artificial Mb derivatives in the present study will serve as a valid measure of their *relative* conformations in solution. It is relevant that calculation of the helical contents from present circular dichroism spectra using the procedure of Greenfield and Fasman (1969) gives values for the per cent α helix in MbX and ApoMb of 63 and 48%, respectively, which are in excellent agreement with our results obtained from the b_0 values (Table II). The use of circular dichroism in the study of protein conformation has been recently reviewed (Beychok, 1968). Since the observed rotations and ellipticities of proteins in the ultraviolet region arise from the n–π^* peptide transition at 223 mμ and are essentially uninfluenced by heme Cotton effects (*e.g.*, Breslow *et al.*, 1965), measurements in this wavelength region should provide a valid indication of the conformational reorganizations of the artificial Mb derivatives prepared for the present investigation.

Previous results from this laboratory have shown that Fe-Mb that had been prepared by recombination of apoprotein with resynthesized heme (from ferric iron and protoporphyrin IX) is immunochemically indistinguishable from Mb by all immunochemical tests. Furthermore, Cu-Mb possessed equal reactivity to the homologous antigen when allowed to react with antisera to MbX (Atassi, 1967b). The cupric complex, which is of the d^9 type, possesses a considerable Jahn–Teller distortion and its complexes are mostly square-planar. These complexes often have two ligands in the axial direction at a greater distance. Since the lone electron in Cu^{2+} is involved in bonding to the porphyrin nitrogen atoms (Falk and Nyholm, 1958; Havemann *et al.*, 1961), the attainment of the octahedral coordination is probably due to weak bonding to the two extra donor atoms. This suggests that Cu-Mb complex formation may take place especially with the contribution of apomyoglobin–porphyrin side-chain interactions (Atassi, 1967b). The present optical rotatory dispersion and circular dichroism studies suggest that the final complex has the same configuration as the ferric (Fe-Mb) complex, and this has been confirmed by our previous immunochemical data.

On the other hand, Zn-Mb reacted poorly with the antisera to Mb and, in fact, precipitated less antibody nitrogen than the apoprotein when allowed to react with antisera to MbX (Atassi, 1967b). The zinc complex possesses the d^{10} configuration which is a completely filled system. The coordination number of Zn^{2+} is four, and it prefers tetrahedral ligand distribution. Thus, some weakening of the bonding orbitals might be anticipated on coordination with the square-planar porphyrin. These Zn^{2+}-porphyrin chelates are diamagnetic (Falk and Nyholm, 1958). The Zn^{2+} ion has enough

electronegativity to allow the addition of a fifth but not a sixth ligand (Falk, 1964). It has been suggested that on addition of one extra ligand the bond to one of the porphyrin nitrogen atoms is released, and the metal ion is able to approximate the tetrahedral sp^3 hybridization that it prefers (Falk and Phillips, 1964). In terms of the structure of Mb this will imply that linkage is only to one of the histidine residues. With nothing to attach the other histidine residue to the neighborhood of the porphyrin, an appreciable configurational change should be expected to result (Atassi, 1967b). Such a change could account for the decrease in the rotatory power observed with Zn-Mb, and is in good agreement with the immunochemical properties of Zn-Mb.

The vinyl side chains of ferriheme have been implicated in the heme protein linkage in heme proteins through hydrophobic bonds (Rossi-Fanelli *et al.*, 1964). Therefore, the preparation of a Mb derivative with ferriheme nitrated at the vinyl side chains by the procedure recently described (Atassi, 1969) is valuable for studying the heme–apoprotein interaction. It is significant that optical rotatory dispersion measurements of Nheme-Mb indicate the presence of appreciable conformational change and, in fact, this derivative showed optical rotatory dispersion parameters similar to those of the apoprotein. The circular dichroism spectra of Nheme-Mb and MbX differed only by 4% from each other which is just within the normal experimental deviation. While this might suggest that the two proteins possess closely similar conformations, the results of optical rotatory dispersion measurements suggested an appreciable conformational alteration due to nitration of the vinyl side chains of ferriheme. This may be due to substitution of chromophore and, although the latter does not absorb in the peptide region, its influence of asymmetry on binding may be observed in the optical rotatory dispersion but not in the circular dichroism behavior of the protein derivative. The immunochemical behavior of Nheme-Mb confirms the presence of conformational changes. It is relevant in this connection that the carboxyl side chains of ferriheme have also been shown to be important for the attainment of native conformation and, hence, the immunochemical reactivity of Mb (Atassi, 1967b) and hemoglobin (Atassi and Skalski, 1969). It appears that the conformational change in Nheme-Mb results in increased antigenic reactivity relative to the homologous antigen. Although this result was not expected, it, nevertheless, is not unusual. Similar results have been obtained in immunochemical studies on human hemoglobins prepared from protoporphyrin IX and apohemoglobin and from ferriheme esterified with 4-pyridinepropanol and apohemoglobin (Atassi and Skalski, 1969). While no satisfactory explanation can be offered for these observations, it is possible that soluble antigen–antibody complexes are obtained with the native protein and that with these artificial Mb derivatives most or all of the soluble complexes become insoluble. This might well be facilitated by the conformational reorganization obtained in these derivatives. This phenomenon has been discussed in detail elsewhere (Atassi and Skalski, 1969; Atassi *et al.*, 1965).

While some artificial Mb derivatives possess conformations that are identical with MbX at neutral pH conditions, a determination of the relative stabilities of such derivatives with decreasing pH is of significance. The effects of urea and ethanol on the denaturation of sperm whale Mb with decreasing pH has recently been reported (Hermans *et al.*, 1969). In the present work on the acid denaturation of the various derivatives, the stability of the Cu-Mb structure resembled closely that of Fe-Mb or MbX, while Zn-Mb was the most unstable of these artificial myoglobins. These trends in stabilities of the artificial myoglobins from optical rotatory dispersion or circular dichroism measurements are identical with those revealed by our investigations of the immunochemical behaviors of these derivatives.

In conclusion, these results indicate that Cu-Mb possesses a conformation identical with that of MbX. The structures also possess comparable stabilities. On the other hand, Zn-Mb exhibits a loss of helicity compared with MbX which is even more drastic than that observed in the apoprotein. Also, the vinyl side chains in ferriheme play some role in the heme–apoprotein interaction. The excellent agreement between the measurements reported here and previous studies of the antigenic reactivities of these artificial Mb derivatives strongly indicates that conformational changes in a protein can influence its antigenic reactivity (Atassi, 1967b). Conversely, Crumpton (1966) has neatly demonstrated that upon binding with antibody, conformational changes may be induced in the antigen. Furthermore, the results with artificial Mb derivatives demonstrate that, under appropriate conditions, immunochemical methods may be employed as a powerful tool to monitor conformational changes in proteins (Atassi, 1967b). Very recently, Sela (1969) has reviewed this subject. Derivatives of the type investigated here are useful in understanding the role played by some selected specific interactions in their contributions to the mode of folding of a given protein in solution.

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Haptoglobin–Hemoglobin Interaction. Stoichiometry*

A. C. Peacock, J. V. Pastewka, R. A. Reed, and A. T. Ness

ABSTRACT: The fully saturated and the partially saturated intermediate products of the reaction between haptoglobin type 1-1 and cyanmethemoglobin were identified by polyacrylamide gel electrophoresis. Quantitative estimates of the protein constituents of each product were made by scanning gels in the ultraviolet for total protein and in the Soret band for hemoglobin. These measurements indicated that the fully saturated complex consisted of one molecule each of haptoglobin and hemoglobin; the intermediate complex consisted of one molecule of haptoglobin and one-half molecule of hemoglobin. A scheme involving sequential addition of half-molecules of hemoglobin fits the observations.

The reaction of haptoglobin 1-1 (Hp 1-1)[1] with excess hemoglobin yields a stable complex of one molecule of haptoglobin with one molecule of hemoglobin. An additional intermediate complex appears when less than an equivalent amount of hemoglobin is added (Allison and Rees, 1957). The intermediate compound is probably a complex of one molecule of haptoglobin and one-half molecule of hemoglobin (Laurell, 1959; Shim *et al.*, 1965; Hamaguchi, 1967; Ogawa *et al.*, 1968). The reaction between haptoglobin and hemoglobin probably involves one-half molecules of hemoglobin, *i.e.*, hemoglobin dissociated into $\alpha\beta$ subunits of molecular weight 32,250 (Bunn, 1967, 1969; Chiancone *et al.*, 1968; Giblett, 1968; Nagel and Gibson, 1967). The existence of the intermediate compound is thus well documented, but there

* From the Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received December 17, 1969.*

[1] Abbreviations used are: Hp, haptoglobin; CNMetHb, cyanmethemoglobin.