Conformational Studies on Modified Proteins and Peptides

V. CONFORMATION OF MYOGLOBIN DERIVATIVES MODIFIED AT TWO CARBOXYL GROUPS*

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SUMMARY

Conformational studies have been carried out on two myoglobin (Mb) derivatives in which glutamic acid residues 83 and 85 were modified by coupling, after activation with carbodiimide, with glycine methyl ester (i.e., di(glycylmethyl ester)-myoglobin, (GlyMe)₂-Mb) or with histidine methyl ester, (HisMe)₂-Mb. Determination of the molecular parameters by gel filtration showed that Mb and (HisMe)₂-Mb had identical molecular parameters, while in (GlyMe)₂-Mb a large degree of unfolding and increase in the asymmetry of the molecule was observed. These studies also showed that even in the corresponding apoproteins, apo-myoglobin (ApoMb) and (HisMe)₂-ApoMb appeared to have similar conformational parameters, whereas (GlyMe)₂-ApoMb was more unfolded. In optical rotatory dispersion measurements, (HisMe)₂-Mb was only slightly less rotatory than Mb at the negative minimum at 233 nm and at the positive 199 nm extremum. Also the b₀ value of (HisMe)₂-Mb (-382) was slightly lower than that of Mb (-419). On the other hand, (GlyMe)₂-Mb showed a large degree of unfolding as evidenced by a sharp decrease of all of its optical rotatory dispersion parameters. Measurement of rotatory behaviors with decreasing pH showed that large unfolding of (HisMe)₂-Mb takes place in a somewhat slightly higher pH range (pH 4.75 to 3.5) than the corresponding range for MbX (pH 4.0 to 3.0). (GlyMe)₂-Mb, on the other hand, exhibited only a small decrease in rotatory power as the pH is lowered from 4.0 to 3.0. In circular dichroism measurements, the reduced molar ellipticity values at the negative bands at 208 nm and at 221 nm for Mb and (HisMe)₂-Mb were equal in magnitude both around neutrality and at acid pH. The ellipticity values for (GlyMe)₂-Mb were greatly suppressed relative to Mb. Circular dichroism measurements at acid pH showed little or no change in the [ø] values for (GlyMe)₂-Mb, indicating that little, if any, further unfolding of the molecule had taken place. The results are discussed in terms of the three-dimensional structure of Mb in the crystalline state and suggest that the two carboxyl groups at positions 83 and 85 do not participate in hydrogen bonding in the native protein.

Factors and interactions responsible for protein folding have stimulated a great deal of interest. To study interactions responsible for protein folding in solutions, many workers have investigated the conformation of peptides from a variety of proteins. Another approach has been to study the conformation of intact protein under conditions that will lead to unfolding, in order to derive information concerning interactions which are being broken in the process of unfolding. Conformational changes on binding of small molecules with proteins have also been studied. These approaches will yield, in most cases, an over-all type of information that will not specifically describe the contribution of a defined amino acid location to the folding of a given protein, as can be obtained from x-ray diffraction on the crystalline protein. Correlation of the three-dimensional structure in solution with that in the crystalline state is difficult, especially when the folding of selected regions or the orientations of specific amino acid side chains are being considered. A good way to approach this is to study the conformation of protein derivatives modified, in more than one way, if possible, at specific amino acid residues. If the interactions in which these residues participate in the crystalline state are known, then solution studies will help in the crystal-solution conformational correlations. Thus valuable results have been obtained concerning the contribution of the heme metal and various heme side-chains to the metmyoglobin structure by studying derivatives prepared with various metalloporphyrins and modified hemes (1). Myoglobin has been chosen as the protein model for the present studies since its three-dimensional structure is well known (2) and the helical content of the protein in solution, derived from optical rotatory dispersion and circular dichroism measurements (3–8), has been determined and found to be in good agreement with the crystal structure.

In previous communications we have reported the conformation of peptides obtained by cleavage of apomyoglobin at arginine peptide bonds (8) and at proline peptide bonds (9). The latter peptides are of particular interest since they represented intact helices due to the fact that the point of cleavage was always at the corner between helices. These studies showed that a helical segment in the protein becomes less helical when isolated...
intact in the free state. Short range interactions have an appreciable contribution and long range interactions play a major role in stabilizing protein folding (8, 9). This paper reports on a detailed study of the contribution of interactions by two specific amino acid side chains to the conformation of Mb in solution. The conformations of two derivatives modified at glutamic acid residues 83 and 85 by the coupling of two different substituents (i.e. a non-polar and a hydrophylic substituent) have been investigated by ORD and CD measurements. Also their hydrodynamic parameters have been determined.

EXPERIMENTAL PROCEDURES

Materials—The preparation of crystalline sperm whale myoglobin from skeletal muscle of the sperm whale was carried out as described in detail elsewhere (7). The Mb used in the present studies was the major chromatographic component No. 10 (MbX) obtained by chromatography on CM-cellulose (10). The ApoMb was prepared from MbX according to the procedure described by Atassi and Skalski (11).

Coupling of Glycine Methyl Ester or Histidine Methyl Ester to Aporhoglobin—Coupling of glycine methyl ester or histidine methyl ester to carboxyl groups of ApoMb was accomplished by activation of these groups with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The reaction was studied (12) in detail under various conditions in order to find those most suitable for the preparation of derivatives modified at the minimum number of carboxyl groups. The preparation, characterization, and determination of the locations of the modification have been described in detail (12), together with their immunological properties. The present work provides a detailed conformational analysis of di(glycyl methyl ester)-myoglobin, (GlyMe)2-Mb, and of di(histidyl methyl ester)-myoglobin, (HisMe)2-Mb. In these derivatives, glutamic acids 83 and 85 are linked through an amide bond to the ε-amino group of glycine methyl ester or histidine methyl ester.

Reconstitution of Metmyoglobin—The procedures for reconstitution of ApoMb with hemin chloride (Eastman Organic Chemicals) and for removal of excess ferriheme on a CM-cellulose column have been described elsewhere (13).

Analytical Methods—Concentrations of protein solutions were based on their nitrogen contents which were calculated from their amino acid compositions (see Table I). Nitrogen contents of protein solutions were determined by a micro Kjeldahl procedure (14) and by using Nessler’s reagent standardized with ammonium sulfate. Three or more replicate analyses were performed and they varied ±0.5% or less. Optical densities were read in a Zeiss PMQ II spectrophotometer. Continuous spectra were done with a Perkin-Elmer model 110 recording spectrophotometer. Procedures for starch gel electrophoresis were carried out as described elsewhere (15).

Determination of Molecular Parameters by Gel Filtration—Protein samples (1 to 2 mg) were dissolved in cold (0 °C) 0.01 m phosphate buffer (0.5 ml) containing 0.01% KCN at pH 6.08 and applied onto a column (2.5 × 59 cm) of Sephadex G-75. Elution was effected with the same phosphate buffer. The column was calibrated with horse heart cytochrome c, bovine ribonuclease A, α-lactalbumin, ovalbumin, and human serum albumin. The effluent was continuously monitored with a double beam flow analyzer equipped with an automatic scale expansion. Determination of Stokes radius (a) and frictional ratios (f/f0) were done as described elsewhere in detail (15).

Optical Rotary Dispersion and Circular Dichroism Measurements—ORD and CD studies were carried out on solutions of the proteins in water (glass double distilled) at 25 °C. Solutions contained 0.08 to 0.25 mg of protein per ml. Measurements were made with a Cary model 60 spectropolarimeter, equipped with a model 6001 circular dichroism accessory. Measurements on each protein were made at several concentrations using cells with light paths of 1, 5, and 10 mm. For measurements below 220 nm, only 1-mm cells were employed with maximum damping (pen period 30), a full range of 0.1 degree, and very low scan speeds (30 s per nm). Scans of solvent base-line were made before and after each sample, and protein samples were scanned at least five times at each concentration. ORD and CD were the average of these scans.

ORD data are reported in reduced mean residue rotation [m ' 0 ] corrected for the refractive index dispersion of water n0. Experimental procedure and quantitative treatment of data were done as described elsewhere in detail (8). The mean residue molecular weights employed in the present work for myoglobin and derivatives are shown in Table I. The Moffitt-Yang parameter, 00, was calculated from their equation (16) with λε = 216 nm.

Ellipticity, [θ], is recorded directly in degrees by the circular dichroism accessory which was calibrated as described elsewhere (7). CD data are given here as reduced molar ellipticities [θ], by correcting for the refractive index dispersion of water (i.e. [θ] = [θ] 3/(n0 2 + 2)). Units of [θ] are in deg cm2 per decimole.

Change of rotatory behavior of MbX and derivatives with pH was studied by the addition of increasing amounts of acid (2 N HCl) or alkali (2 N NaOH) to a solution of the protein in water. Solutions were centrifuged (4000 rpm, 1 hour, 0 °C), after each change in pH, before ORD or CD measurements were carried out. Protein concentration at each pH was determined directly by removing aliquots for triplicate nitrogen analyses.

RESULTS

Preparation and Characterization of Derivatives—Preparation of (GlyMe)2-Mb and (HisMe)2-Mb was accomplished by recombination of (GlyMe)2-ApoMb and (HisMe)2-ApoMb with ferriheme. This was necessary in order to avoid modification of the propionyl side chains of hemin during the carbodiimide coupling reaction. The two Mb derivatives were shown to be homogene-

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Nitrogen content %</th>
<th>Mean residue weight</th>
</tr>
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<tbody>
<tr>
<td>MbX</td>
<td>17,816</td>
<td>17.36</td>
<td>116.4</td>
</tr>
<tr>
<td>ApoMb</td>
<td>17,200</td>
<td>17.66</td>
<td>112.4</td>
</tr>
<tr>
<td>(GlyMe)2-Mb</td>
<td>17,858</td>
<td>17.39</td>
<td>117.4</td>
</tr>
<tr>
<td>(HisMe)2-Mb</td>
<td>18,118</td>
<td>17.54</td>
<td>118.4</td>
</tr>
</tbody>
</table>

* Values were calculated from the amino acid composition of each protein.
ous by starch gel and acrylamide gel electrophoresis. The locations of the modification were determined by peptide mapping of tryptic hydrolysates of ApoMb, (GlyMe)₂-ApoMb, and (HisMe)₂-ApoMb, elution of the modified peptides, and their amino acid analysis. In each derivative, modifications were found to be at glutamic acids 83 and 85 (12).

**Spectral Studies**—Continuous spectra were done on solutions of MbX, (GlyMe)₂-Mb, and (HisMe)₂-Mb in 0.01 M phosphate buffer at pH 7.2, containing 0.01% KCN. The spectra of MbX and (HisMe)₂-Mb were identical throughout the range 600 to 230 nm, each giving absorption maxima at 540, 421, 359, and 280 nm (Fig. 1). The spectra were also quantitatively similar (Ε₂₈₀/Ε₅₄₀: MbX, 0.322; (HisMe)₂-Mb, 0.326; Ε₂₈₀/Ε₅₄₀: MbX, 3.27; (HisMe)₂-Mb, 3.33). Differences were observed in the spectrum of (GlyMe)₂-Mb which showed absorption maxima at 530, 415, 360, (shoulder) and 274 nm. Also there was an appreciable increase in the ultraviolet absorption of (GlyMe)₂-Mb (ε₂₈₀ = 4.37 × 10⁴; cf. MbX, 3.02 × 10⁴; (HisMe)₂-Mb, 3.01 × 10⁴) and a decrease in the absorption at the Soret band (415 nm).

**Determination of Molecular Parameters by Gel Filtration**—Calculation of molecular parameters by gel filtration on a Sephadex G-75 column was done by the procedure described earlier in detail (13). The results are summarized in Table II. It can be seen that MbX and (HisMe)₂-Mb possessed identical values for Stokes radius (19.0 and 19.1 Å, respectively) and for ʃʃ₀ (1.09), whereas these values were greatly increased in (GlyMe)₂-Mb. Table II also shows that even in the corresponding apomyoglobin, ApoMb and (HisMe)₂-ApoMb had identical molecular parameters and that these were appreciably increased in (GlyMe)₂-ApoMb. The sensitivity of this technique to the conformational changes can also be seen from Table II, which clearly shows that ApoMb is less folded or more asymmetrical than MbX. Combination of ApoMb with heme yielded the more folded structure of MbX and this increase in folding was identical with the increase in folding obtained on combination of (HisMe)₂-ApoMb with heme. No decrease in unfolding was observed on recombination of (GlyMe)₂-ApoMb with heme (Table II).

**Optical Rotatory Dispersion Measurements**—In ORD studies, MbX and the two derivatives each gave a negative rotation minimum at 233 nm and positive extremum at 199 nm. Fig. 2 gives the ORD spectra of MbX, (GlyMe)₂-Mb, and (HisMe)₂-Mb. The rotatory behavior of MbX and (HisMe)₂-Mb showed some differences in that (HisMe)₂-Mb was slightly less rotatory both at 233 nm and at 199 nm. On the other hand, in (GlyMe)₂-Mb the rotatory power was appreciably suppressed at these two bands. In addition, the Ə₀ value of (GlyMe)₂-Mb was lowe

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**Fig. 1.** Continuous spectra of: 1, MbX, 154.6 μg per ml; 2, (HisMe)₂-Mb, 175.3 μg per ml; and 3, (GlyMe)₂-Mb, 113.0 μg per ml. Solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%).
TABLE II
Gel filtration data and molecular parameters of myoglobin, apomyoglobin and their derivatives

Gel filtration values were obtained from three replicate determinations and varied ±1.1% or less. $K_D$ is the distribution coefficient given by $K_D = (V_e - V_o)/V_i$, where $V_e$ is the effluent volume at the apex of a protein peak, $V_o$ is the void volume, and $V_i$ is the volume of unbound solvent within the gel phase (28).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol wt</th>
<th>Elution volume</th>
<th>$K_D$</th>
<th>$a$</th>
<th>$b/a$</th>
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<tbody>
<tr>
<td>MbX</td>
<td>17,816</td>
<td>147.90</td>
<td>0.3922</td>
<td>19.0</td>
<td>1.09</td>
</tr>
<tr>
<td>(HisMe)$_2$-Mb</td>
<td>18,118</td>
<td>146.10</td>
<td>0.3877</td>
<td>19.1</td>
<td>1.09</td>
</tr>
<tr>
<td>(GlyMe)$_2$-Mb</td>
<td>17,958</td>
<td>63.55$^a$</td>
<td>0.0049</td>
<td>52.0</td>
<td>2.98</td>
</tr>
<tr>
<td>ApoMb</td>
<td>17,200</td>
<td>118.15</td>
<td>0.2581</td>
<td>24.8</td>
<td>1.44</td>
</tr>
<tr>
<td>(HisMe)$_2$-ApoMb</td>
<td>17,502</td>
<td>116.30</td>
<td>0.2105</td>
<td>35.1</td>
<td>1.45</td>
</tr>
<tr>
<td>(GlyMe)$_2$-ApoMb</td>
<td>17,342</td>
<td>62.90$^a$</td>
<td>0.0019</td>
<td>54.7</td>
<td>3.17</td>
</tr>
</tbody>
</table>

* The elution volumes for (GlyMe)$_2$-Mb and (GlyMe)$_2$-ApoMb are too close to the void volume (62.50 ml). Therefore the value of $a$ for these two derivatives might even be higher than those given.

Fig. 2. ORD spectra of Mb and derivatives. $Mb_A$ is the ORD spectrum of native MbX; $Mb_B$ is the ORD spectrum of a control Mb prepared from ApoMb, which had been subjected to the same reaction conditions as those used in the preparation of the Mb derivatives, followed by recombination with unmodified ferriheme. GME$_2$-Mb and HME$_2$-Mb denote (GlyMe)$_2$-Mb and (HisMe)$_2$-Mb, respectively. Measurements were carried out on the protein solutions in water.

TABLE III
ORD parameters of myoglobin and derivatives

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>$[m']_{233}$</th>
<th>$[m']_{300}$</th>
<th>$[m']_{430}$</th>
<th>$b/a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MbX</td>
<td>6.70</td>
<td>-9,320</td>
<td>+46,400</td>
<td>-419</td>
<td></td>
</tr>
<tr>
<td>Control Mb$^*$</td>
<td>6.70</td>
<td>-9,280</td>
<td>+47,200</td>
<td>-417</td>
<td></td>
</tr>
<tr>
<td>(HisMe)$_2$-Mb</td>
<td>6.78</td>
<td>-8,200</td>
<td>+41,000</td>
<td>-382</td>
<td></td>
</tr>
<tr>
<td>(GlyMe)$_2$-Mb</td>
<td>6.52</td>
<td>-3,480</td>
<td>+17,200</td>
<td>-115</td>
<td></td>
</tr>
<tr>
<td>ApoMb</td>
<td>5.90</td>
<td>-7,280</td>
<td>+33,000</td>
<td>-315</td>
<td></td>
</tr>
</tbody>
</table>

* Control Mb represents a preparation obtained by recombination of unmodified ferriheme with ApoMb, which had been subjected to the same reaction conditions as those used in the preparation of (GlyMe)$_2$-Mb and (HisMe)$_2$-Mb.

Fig. 3. pH-stability profiles of Mb and derivatives. ○, MbX; ●, control Mb prepared as described under Fig. 2; ▲, (HisMe)$_2$-Mb; Δ, (GlyMe)$_2$-Mb; □, ApoMb.

The effect of carboxyl group modification on the relative stabilities of the present proteins was studied by determining the change in their rotatory behaviors in the pH range 2 to 7. The results of this work are summarized in Table III. The conformation of MbX suffers a large degree of unfolding in the pH range 4 to 3. The unfolding of (HisMe)$_2$-Mb takes place in a slightly higher pH range (pH 4.75 to 3.5) suggesting that this derivative is slightly less stable than MbX. (GlyMe)$_2$-Mb, which is greatly unfolded to start with, undergoes only a small decrease in $[m']_{233}$ from -3400 to -3000 as the pH is lowered between 4 and 3. For comparison, Fig. 3 gives the pH denaturation curve than that of MbX, (HisMe)$_2$-Mb, or even ApoMb. The ORD parameters for these proteins are summarized in Table III.
Fig. 4. CD spectra of: 1, MbX in water at pH 6.0; 2, (HisMe)$_2$-Mb in water at pH 6.3; 3, MbX in water at pH 3.2; 4, (HisMe)$_2$-Mb in water at pH 3.2.

of ApoMb, and it can be seen that (GlyMe)$_2$-Mb is even more unfolded than ApoMb.

Circular Dichroism Measurements—Conformational changes were also monitored by CD studies. The CD spectra of MbX and (HisMe)$_2$-Mb are given in Fig. 4 and the spectrum of (GlyMe)$_2$-Mb is shown in Fig. 5. Also, Figs. 4 and 5 show the CD spectra of these three proteins at acid pH. Each of the present proteins gave, around neutrality, negative ellipticity bands at 208 and 220 nm, the latter band being only a shoulder in (GlyMe)$_2$-Mb. The ellipticity bands for MbX and (HisMe)$_2$-Mb were equal in magnitude both around neutrality and at the acid pH. On the other hand, the ellipticity values in water for (GlyMe)$_2$-Mb were greatly suppressed relative to MbX. CD measurements at acid pH showed little or no change in the [θ] values of (GlyMe)$_2$-Mb, indicating that little, if any, further unfolding of the molecule had taken place. The results suggest that conformational alterations in (HisMe)$_2$-Mb, if present, were too small to be detectable by CD measurements and that (GlyMe)$_2$-Mb is as much unfolded at neutral pH conditions as is possible for it to unfold. In fact, from CD measurements also, (GlyMe)$_2$-Mb is even more unfolded than is ApoMb (Fig. 5).

DISCUSSION

Reaction of carbodiimides with proteins displays excellent specificity for carboxyl groups. First attempts to modify carboxyl groups with these reagents used carbodiimides alone but characterization of the derivatives obtained was difficult (17-20). However, when the carbodiimide-activated carboxyl groups were made to couple with the ε-amino group of an amino acid or peptide ester (21, 22), the derivatives could then be characterized more easily. On coupling of activated carboxyl groups with an amino acid ester, the latter is linked by a peptide bond between its ε-amino group and the modified carboxyl group. For the preparation of the present derivatives, the modification reaction was first carried out with ApoMb which was subsequently recombined with unmodified ferriceme to yield the corresponding Mb derivatives. This was necessary in order to avoid modification of the carboxyl groups on the propionyl side chains of the heme. Modification of the propionyl side chains of heme has been shown to give rise to change in conformation and immunochernistry of myoglobin and hemoglobin (11, 23). Since the purpose of the present work was to study the role of selected carboxyl groups on the polypeptide side chain, the carboxyl groups on the heme must not be modified.

One of the major disadvantages of carboxyl group modification through activation reactions (17-22, 24) is the possibility that intramolecular and intermolecular cross-links may take place between activated carboxyl groups and amino groups on the same or on another protein molecule. Intermolecular cross-links could be greatly minimized by carrying out the reaction on a relatively dilute mixture of reactants. Indeed, the homogeneity of the present derivatives by starch gel and acrylamide gel electrophoresis and the results of sedimentation studies ruled out the presence of any significant amounts of intermolecular condensation products. The existence of intramolecular cross-links is more difficult to investigate. Such derivatives should be de-
fectable on starch gel or acrylamide gel electrophoresis. Even if these derivatives were not resolved by gel electrophoresis, the newly formed link should be well resolved by peptide mapping. No such cross-links were detected in the peptide maps of (GlyMe)₂-ApoMb and (HisMe)₂-ApoMb (12). It may, therefore, be stated that within the limits of detection of the available techniques no intramolecular or intermolecular cross-links were found in the present derivatives.

Spectral measurements showed no differences between MbX and (HisLe)₂-Mb, reflecting, therefore, absence of change in the environment of the heme group. Charge effects on the heme group play a major role on the visible spectrum, since absorption maxima in this part of the spectrum arise from electron displacement to the periphery of the porphyrin nucleus. When changes lead to a decrease of π-electron density at the periphery, absorption maxima will occur at a lower wavelength (25, 26). Therefore, the visible spectrum of (GlyLe)₂-Mb suggests, most likely, that linkage of the iron atom in the heme is only to the proximal histidine. The sixth coordination position of the iron is occupied by cysteine which in this case does not effect a bridge with the distal histidine residue (histidine 64) in this derivative as a result of unfolding. The unfolding can also be inferred from the ultraviolet part of the spectrum.

Elution volumes of proteins on calibrated Sephadex columns can be related to their Stokes radii (13, 27–31). This provides a simple tool to monitor shape changes of proteins and has been employed to evaluate conformational changes associated with chemical modification of proteins (32, 33). The technique has also already been employed successfully to monitor conformational changes in specifically modified hemoglobin and myoglobin derivatives (11, 13) and to study conformation of Mb from various species (7). In (GlyLe)₂-Mb a large degree of unfolding or “swelling” and increase in the asymmetry of the molecule was detected by gel filtration studies. On the other hand, no changes in the molecular parameters were observed in (HisLe)₂-Mb. It is noteworthy that the present work shows that gel filtration is quite capable of detecting conformational differences between myoglobin and apomyoglobin. The “tightening” of ApoMb upon recombination with heme had previously been concluded from studies on infrared spectra (34), from ORD and CD measurements (1, 4, 5, 8), and from immunochemical studies (26, 35).

The foregoing conclusions derived from gel filtration concerning conformational changes were well confirmed by results of ORD and CD measurements. These studies indicated that (GlyLe)₂-Mb was much less helical than native Mb. In fact this derivative seemed more unfolded than ApoMb. Despite this great degree of unfolding in (GlyLe)₂-Mb, satisfactory combination of the heme group is still achieved at 1:1 molar ratio of (GlyLe)₂-ApoMb and heme. A similar greatly unfolded Mb derivative has been reported (26) and was prepared by recombination of ApoMb with an equimolar amount of zinc metalloporphyrin. With (HisLe)₂-Mb, the ORD results may suggest small conformational changes relative to native Mb. However, the CD spectra were almost superimposable. The immunochemistry of these derivatives has been investigated and it has been found (12) that (HisLe)₂-Mb and MbX were immunochemically identical, while (GlyLe)₂-Mb showed lower antigenic reactivities with antisera to Mb or to (HisLe)₂-Mb. These findings all suggest that little or no conformational differences exist between Mb and (HisLe)₂-Mb, while (GlyLe)₂-Mb is greatly unfolded.

The carboxyl groups in myoglobin are directed to the surface of the molecule for optimum contact with the water medium (2).

Coupling with glycine methyl ester would eliminate the hydrophilic character of the carboxyl group and the new non-polar substituent will exert a directive force for refolding of that region so that the non-polar side chain will be surrounded by a more favorable non-polar environment within the protein molecule. This directive force should be expected to give rise to a conformational change. The magnitude of the conformational change will depend on the location of the modified carboxyl groups. Glutamic acid residue 83 is in the bend between helices F and F while glutamic acid residue 85 represents the first residue in helix F (2). Modification of glutamic acid 83 should not exert a detrimental effect on the conformation as would the modification of glutamic acid 85. However, the possibility of a cooperative effect of the two modifications must be considered. Coupling of the two carboxyl groups with histidine methyl ester would retain the hydrophilic nature of the side chain and would obviate the directive effect for refolding of that region. Indeed conformational studies confirmed this line of reasoning. There is some doubt from the x-ray diffraction of crystalline Mb (36) that the carbonyl groups of glutamic acids 83 and 85 may participate in hydrogen bonds to undefined peptide bond carboxyl groups. These two hydrogen bonds, if they were present in native Mb, would be completely eliminated in both (GlyLe)₂-Mb and (HisLe)₂-Mb. It is, therefore, significant that the absence of conformational changes in (HisLe)₂-Mb would indicate that either the two hydrogen bonds do not exist in Mb or that if they do exist in Mb and are broken in (HisLe)₂-Mb then their presence is not essential for the integrity of the native conformation. The reasoning for their absence in Mb is more acceptable.

In conclusion two Mb derivatives, in which glutamic acid residues 83 and 85 were modified by coupling with glycine methyl ester or with histidine methyl ester, have been studied in detail by ORD and CD measurements and their molecular parameters determined. The results show that the protein suffers gross conformational changes when the two carboxyl groups were coupled with glycine methyl ester. On the other hand, no conformational changes were present when the same two side chains were coupled with histidine methyl ester. The results are discussed in terms of the three-dimensional structure of Mb in the crystalline state and contribution of these interactions to the three-dimensional structure.

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