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Immunochemistry of Sperm-Whale Myoglobin. Conformation and Immunochemistry of Derivative Reduced at Some Carboxyl Groups by Diborane†

M. Z. Atassi* and M. T. Perlstein

ABSTRACT: A homogeneous derivative was prepared by reduction of apomyoglobin (ApoMb) with diborane at $-10°$ for 4 hr. In the derivative, glutamic acid residues at positions 83 and 85 were reduced to their corresponding hydroxy acid (*i.e.*, 2-amino-5-hydroxyvaleric acid). Recombination of the reduced ApoMb with unmodified ferriheme yielded the corresponding reduced myoglobin (R-Mb) derivative. In starch gel electrophoresis, R-Mb migrated as a single band with a mobility of 0.90 relative to native myoglobin (Mb). Absorption spectra in the ultraviolet–visible range, optical rotatory dispersion and circular dichroism parameters of R-Mb and Mb were quantitatively identical. Immunochemical studies showed that R-Mb and Mb had equal reactivity both with antisera to Mb and with antisera to R-Mb. The results show that no conformational changes take place upon reduction of the γ-carboxyl groups of glutamic acid residues 83 and 85. Also these two amino acids are not located in an antigenic reactive region of Mb. From these findings and other already published results, it was possible to narrow down further the size of a previously located antigenic reactive region in Mb so that it will now fall within, but may not include all of, the sequence 86–102.

Information concerning the antigenic structure of sperm-whale myoglobin has been reported in several previous publications from this laboratory. Discrete antigenic reactive regions have been exhaustively delineated by studying the specific immunochemical interactions of a variety of overlapping peptides obtained by chemical and enzymic cleavage procedures and by specific chemical modification of various amino acids in protein and immunochemically reactive peptides. The reactive regions occupy four corners between helices on the surface of the molecule. Interactions between adjacent *reactive regions* and their incorporation into *reactive sites* are difficult to investigate, but are important for understanding the three-dimensional nature of the reactive site. This may be approached by studying the conformation and immunochemistry of specifically modified derivatives which will also be helpful in further narrowing down of antigenic reactive regions. Previously, valuable information has been obtained concerning the contribution to antigenic structure of the methionines (Atassi, 1967a, 1969), the tryptophans (Atassi and Caruso, 1968), the tyrosines (Atassi, 1968), and the arginines (Atassi and Thomas, 1969). We wish now to report on the role of two glutamic acid residues.

Carboxyl groups can be modified by esterification but these reactions lack specificity. Reaction with carbodiimides yields derivatives that are not easy to characterize (Sheehan and Hlavka, 1956; Franzblau *et al.*, 1963; Goodfriend *et al.*, 1964; Riehm and Scheraga, 1966) and this complication is avoided when activation with carbodiimides is followed by coupling with amino acid or peptide esters (Hoare and Koshland, 1966; Wilchek *et al.*, 1967). Carboxyl groups can also be modified by reaction with isoxalium salts (Bodlaender *et al.*, 1969), giving an enol ester. The reactive enol ester may be coupled with nucleophiles. In these procedures, where the carboxyl group is activated, reaction may give rise to intramolecular (and intermolecular) cross-linking with favorably placed amino groups. Carbodiimides have been shown to react with sulfhydryl groups in proteins (Carraway and Triplett, 1970). Also, the modification is not permanent and will be removed by acid or alkaline hydrolysis and cannot be used to identify the residue modified (*i.e.*, aspartic, glutamic, or C terminus). Recently, it was reported that car-

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boxyl groups in proteins can be reduced to their corresponding alcohols (Rosenthal and Atassi, 1967; Atassi and Rosenthal, 1969). The applicability of this reaction was demonstrated on 20 different model peptides and six different proteins. No conformational or biological studies have yet been done on reduced proteins. The present paper describes the preparation and characterization of a myoglobin derivative reduced by diborane at γ-glutamyl carboxyl groups. The immunochemistry and conformation of the derivative are reported in detail.

Experimental Section

Materials. Diborane was purchased as 1 M solution in tetrahydrofuran from Ventron Metal Hydrides Division. Hemin chloride was from Eastman Organic Chemicals. Metmyoglobin used for these studies was the major chromatographic component no. 10 (MbX) obtained by CM-cellulose chromatography (Atassi, 1964), and was homogeneous by starch gel, acrylamide gel, and disc electrophoresis. The preparation of ApoMb[1] from MbX is described below.

Preparation of Apomyoglobin. A portion of MbX (200 mg) was dissolved in cold (0°) water (8–9 ml), and the solution was added dropwise to a magnetically stirred acetone–HCl mixture (500 ml containing 0.025 ml of 4 N HCl), at 0°. When addition was complete, the mixture was allowed to stir overnight at −10°, after which it was centrifuged (−10°, 2000 rpm, 20 min) and the supernatant was kept. The precipitate, which was slightly yellowish at this stage, was redissolved in cold (0°) water (8–9 ml) and added dropwise to the acetone–HCl supernatant. The mixture was again left stirring overnight at −10°, and then centrifuged (−10°, 2000 rpm, 20 min). The precipitate was washed twice on the centrifuge (at −10°) with acetone–HCl (20 ml each), and the combined supernatant and washings were recentrifuged (−10°, 3000 rpm, 30 min) to recover any small amounts of precipitate that might have escaped into the supernatant and washings. The ApoMb precipitate was dissolved in water (50 ml) at 0°. The solution, which was completely colorless and had no absorption at 420 nm, was dialyzed extensively against distilled water and freeze-dried. Recovery of ApoMb was 173 mg (*i.e.*, 94% of the theoretical yield).

Reduction of Apomyoglobin with Diborane. The carboxyl groups must first be protonated since nonprotonated carboxyl groups cannot be reduced (Atassi and Rosenthal, 1969). Apomyoglobin (311 mg) was dissolved in water at 0° (50 ml) in a 1000-ml, round-bottom flask. Precooled (0°) trifluoroacetic acid (3 ml) was added to the ApoMb solution, and the the mixture was swirled vigorously for 20 sec, after which it was immediately frozen and then freeze-dried. The glassy residue was finely powdered in the flask with a spatula, and a solution (60 ml) of 1 M diborane in tetrahydrofuran (at −10°) was added. The mixture was stirred magnetically for 4 hr at −10° and then dioxane (10 ml) was added. The reaction mixture (at −10°) was evaporated down to about 25 ml with a stream of nitrogen. To this was added a second aliquot (10 ml) of dioxane and evaporation (down to about 20 ml) on a rotary evaporator followed at −10°. Three more additions of dioxane were made, with the mixture being evaporated down to about 20 ml after each addition. Following the last dioxane addition, the mixture was brought down to about 5–10 ml, and the residue was dissolved in water (50 ml at 0°). To this was added a 0.02 M phosphate buffer at pH 7.5 containing 10% glycerine (50 ml at 0°), and the solution was dialyzed (at 0°) against two changes (4 l. each) of 0.01 M phosphate buffer at pH 7.5 containing 5% glycerine, and then extensively against distilled water. The solution was cleared by centrifugation (0°, 2000 rpm, 30 min) and then freeze-dried.

To account for the conditions of the preparation, which may influence the immunochemistry and conformation of the protein, a control ApoMb was prepared which was subjected to all the manipulation employed in the diborane reduction, except that in this case tetrahydrofuran without diborane was used.

By replacing tetrahydrofuran with dioxane, at the end of the reduction before drying the residue, the derivative was almost completely soluble (more than 95%) in water. If the reduced protein was dried directly from tetrahydrofuran before the addition of water, it was found to be very slightly (not more than 10%) soluble in water.

Reconstitution of Metmyoglobin. Recombination of ApoMb or derivatives with unmodified ferriheme and removal of excess heme on CM-cellulose columns were carried out by the procedure previously described in detail (Atassi and Caruso, 1968).

Antisera. The preparation of rabbit and goat antisera to MbX has already been described in detail (Atassi, 1967a). Antisera against R-Mb were raised in rabbits by a similar procedure. Antisera G3 and G4 were goat antisera against MbX, and antisera S11 and S12 were rabbit antisera against R-Mb. All antisera were kept and studied separatedly and they were stored frozen at −40° in 5- to 8-ml portions.

Analytical Methods. Procedures employed here for immunochemical studies (*i.e.*, agar double diffusion, precipitin, and absorption experiments) have been described elsewhere in detail (Atassi and Saplin, 1968). Optical density measurements were done in a Zeiss PMQII spectrophotometer. Continuous spectra in the range 700–230 nm were performed with a Perkin-Elmer Model 124 recording spectrophotometer. The procedure for starch gel electrophoresis has been described (Atassi and Caruso, 1968). Amino acid analyses of protein hydrolysates were carried out on a BioCal BC-200 amino acid analyzer. Acid hydrolysis was in constant boiling HCl at 110° for 22, 48, or 72 hr in nitrogen-flushed, evacuated sealed tubes. For tryptophan determination hydrolysis was in 3 N-*p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole in nitrogen-flushed, evacuated sealed tubes at 110° for 22 or 48 hr (Liu and Chang, 1971). Tryptic digestion and peptide mapping were done as described elsewhere (Atassi and Saplin, 1968). Peptide maps were stained with ninhydrin or with specific stains for various amino acids (Easley, 1965).

Concentrations of protein solutions were based on their nitrogen contents. Analyses for nitrogen were done by a micro-Kjeldahl procedure (Markham, 1942) and by using Nessler's reagent standardized with ammonium sulfate. At least three replicate analyses were done on each solution and they varied ±0.5% or less. The nitrogen content for MbX was taken as 17.36%, which is the value calculated from the amino acid composition (Atassi and Saplin, 1968). The nitrogen content for the derivative was calculated here as 17.40%.

Optical Rotatory Dispersion and Circular Dichroism Measurements. Optical rotatory dispersion and circular dichroism

[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin; MbX, the major chromatographic component no. 10 obtained by CM-cellulose chromatography (Atassi, 1964); R-ApoMb, ApoMb that had been reduced with diborane; R-Mb, metmyoglobin prepared by recombination of R-ApoMb with unmodified ferriheme.

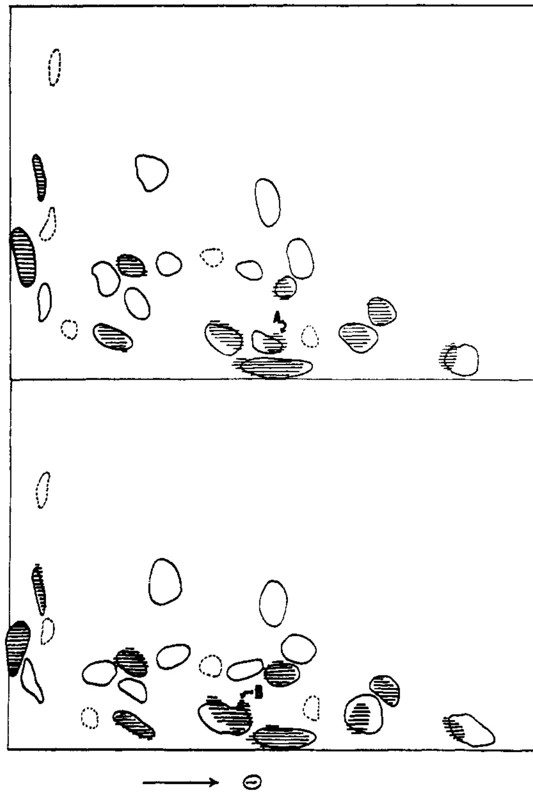

FIGURE 1: A tracing of the peptide maps of the soluble tryptic peptides of (a) R-ApoMb, (b) ApoMb. Maps show all the ninhydrin-positive spots. Shaded areas represent spots that are positive with Pauly stain. For details see text.

TABLE I: Amino Acid Composition of ApoMb and Its Diborane-Reduced Derivative.[a]

Amino Acid	ApoMb[b]	R-ApoMb[c]
Asp	7.86	7.81
Thr	4.95	4.76
Ser	5.88	5.94
Glu[e]	*19.1*	*17.1*
Pro	4.10	3.74
Gly	10.5	10.6
Ala	16.8	16.8
Val	7.78	7.71
Met	2.02	1.98
Ile	8.69	8.59
Leu	17.8	18.1
Tyr	2.98	3.00
Phe	6.19	6.00
Trp[d]	1.66	1.69
Lys	19.0	19.1
His	11.83	11.7
Arg	4.00	4.00

[a] The compositions are expressed in moles of amino acid per mole of protein. Values of serine and threonine have been obtained by extrapolation to zero hydrolysis time. [b] Values represent the average of four analyses (two 22-hr and two 72-hr hydrolyses). [c] Values represent the average of eight analyses (five 22-hr and three 72-hr hydrolyses). [d] Tryptophan was determined from duplicate hydrolyses with *p*-toluenesulfonic acid. [e] Glutamic acid is reduced to 2-amino-5-hydroxyvaleric acid; for recovery and details see text.

studies were carried out on solutions of the proteins (0.1–0.2 mg/ml) in water (glass double distilled) at 25°. 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 in cells of light paths 1, 5, and 10 mm. For measurements below 200 nm, only 1-mm cells were employed with maximum damping (pen period 30), a full range of 0.1°, and very low scan speeds (30 sec/nm). Solvent base-line scans were made before and after each sample, and protein samples were scanned at least five times at each concentration. Optical rotatory dispersion and circular dichroism parameters were the average of these scans.

Optical rotatory dispersion data are reported in reduced mean residue rotation $[m']_\lambda$ corrected for the refractive index dispersion of water, n_λ. Experimental procedure and quantitative treatment of data were done as described elsewhere in detail (Atassi and Singhal, 1970a). The mean residue molecular weight employed for myoglobin and derivative was 116.4. The Moffitt–Yang parameter, b_0, was calculated from their equation (Moffitt and Yang, 1956) with $\lambda_0 = 216$ nm.

Ellipticity, θ, is recorded directly in degrees by the circular dichroism accessory. Circular dichroism data are given here as reduced molar ellipticities $[\theta']$, by correcting for the refractive index dispersion of water (*i.e.*, $[\theta'] = [\theta]3/(n^2 + 2)$). Units of θ' are in deg cm^2 per decimole.

Results

Reduction of ApoMb with Diborane and Determination of the Modified Residues. Amino acid composition of R-ApoMb is shown in Table I. The results showed that two glutamic acid residues were reduced in the derivative to their corresponding alcohol (*i.e.*, 2-amino-5-hydroxyvaleric acid) and δ-lactone. The color yield constant of 2-amino-5-hydroxyvaleric acid has been shown to be 0.971 ± 0.036 of that of aspartic acid and it appears on the analyzer 2–3 min before glutamic acid (Atassi and Rosenthal, 1969). The formation of 2-amino-δ-valerolactone from the hydroxy acid can take place during hydrolysis and drying of the hydrolysates. The decrease in the amount of glutamic acid was entirely acconted for by the total of the recovery of the hydroxy acid (1.30 moles/mole of Mb) and the lactone (0.51 mole/mole of Mb). The amino acid composition of R-Mb also suggests no reduction of the C-terminal glycine residue in this derivative. Also, no reduction of aspartic acid was observed.

Tryptic hydrolysis was carried out on ApoMb and R-ApoMb. Amino acid analysis was carried out on acid hydrolysates of the soluble and insoluble tryptic peptides. Amino acid compositions of the insoluble tryptic peptides of ApoMb and of R-ApoMb were identical. The insoluble tryptic peptides of ApoMb correspond to sequences 1–16, 64–78, and 103–118 (Edmundson, 1963). The reduced glutamic acid residues were, therefore, not within these portions of the molecule. Amino acid compositions of the soluble tryptic peptides showed that those of R-ApoMb contained two reduced glutamic acid residues that were accounted for entirely by 2-amino-5-hydroxyvaleric acid and its δ-lactone. Peptide mapping was carried out on the soluble tryptic peptides of R-ApoMb and ApoMb. Figure 1 shows a tracing of the two maps. The only

TABLE II: Positions of Absorption Maxima and Ratios of Optical Densities at the Maxima for MbX and R-Mb.[a]

	Absorption Max (nm)				Ratios of Optical Densities at Abs Max		
	A	B	C	D	B/A	C/A	D/A
MbX	279	360	422	540	0.940	3.43	0.340
R-Mb	279	358	421	540	0.980	3.45	0.347

[a] Solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%).

difference between the two maps is that peptide B in ApoMb moves to position A in R-ApoMb. Peptides A and B were each cut from ten maps that were lightly stained with ninhydrin (0.05% ninhydrin in ethanol), eluted with 0.1 N acetic acid, cleared by centrifugation, and then freeze-dried. Amino acid analysis of acid hydrolysate of the residues gave: (B) Glu, 1.82; Gly, 0.91; Ala, 1.00; Leu, 0.93, Lys, 2.10; His, 1.84; (A) Glu, 0.10; Gly, 0.96; Ala, 1.00, Leu, 1.02; Lys, 1.85; His, 1.82; 2-amino-5-hydroxyvaleric acid, 1.22; 2-amino-δ-valerolactone, 0.49. By comparison with the known sequence of myoglobin (Edmundson, 1965), these compositions corresponded well to: B, sequence 79–87; and A, sequence 79–87, in which the two glutamic acid residues (at positions 83 and 85) were reduced to 2-amino-5-hydroxyvaleric acid. The results, therefore, indicate that in R-Mb glutamic acid residues 83 and 85 were reduced to the corresponding alcohol.

Properties of the Modified Myoglobin. Recombination of R-ApoMb with ferriheme was carried out to yield the corresponding R-Mb derivative. Starch gel electrophoresis at pH 9.2 of R-Mb showed that the derivative was completely homogeneous and its electrophoretic mobility was 0.896 (relative to MbX = 1.00). Assuming that the charge of Mb at pH 9.2 was only due to the 21 free carboxyl groups, and that a linear relationship between mobility and charge exists, then the number of carboxyl groups reduced in R-Mb will be 2.19 groups per mole. This is in good agreement with the results obtained by amino acid analysis. Spectral studies on the cyanmet forms of MbX and of R-Mb were carried out in the range 230–700 nm. Table II shows the positions of the absorption maxima given by the two proteins as well as the ratios of the optical densities at these maxima. The results indicated that the two proteins had identical spectral properties. Control Mb, prepared from ApoMb which had been subjected to tetrahydrofuran treatment, behaved exactly like MbX in electrophoresis, spectral, optical rotatory dispersion, circular dichroism, and immunochemical properties.

Conformation of the Derivatives. OPTICAL ROTATORY DISPERSION STUDIES. In these studies, MbX and R-Mb each gave a negative rotation minimum at 233 nm and a positive rotation extremum at 199 nm. Figure 2 shows the optical rotatory dispersion spectra of MbX and R-Mb in the range 260–190 nm. The two proteins had equal rotatory powers both at the negative minimum ($[m']_{233}$: R-Mb, −9850; MbX, −9860) and at the positive maximum ($[m']_{199}$: R-Mb, +45,200; MbX, +46,100). Also the b_0 values were identical (R-Mb, −420; MbX, −417).

CIRCULAR DICHROISM STUDIES. Circular dichroism measurements were carried out on MbX and R-Mb in the range 260–205 nm. Each of the two proteins gave negative ellipticity

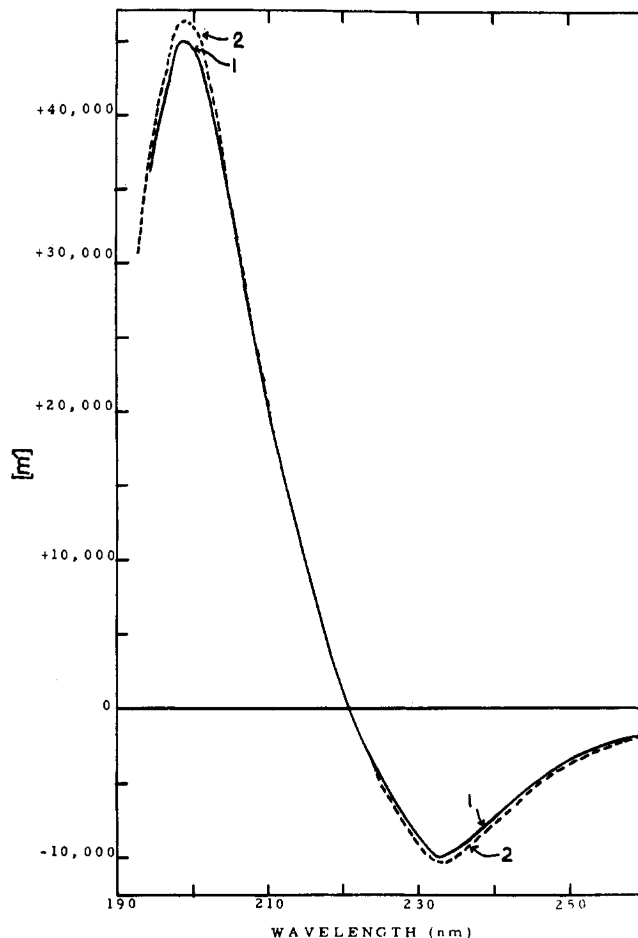

FIGURE 2: Optical rotatory dispersion spectra of R-Mb (1) and MbX (2). Measurements were carried out on solutions in water.

bands at 221 and 208 nm. The $[\theta']$ values at these two bands were very comparable ($[\theta']_{220}$: R-Mb, −21,400; MbX, −20,900; $[\theta']_{208}$: R-Mb, −19,300; MbX, −18,400). Figure 3 shows the circular dichroism spectra for MbX and R-Mb. The results of optical rotatory dispersion and circular dichroism measurements suggest that the two proteins possessed similar conformations.

Immunochemistry of the Derivative. REACTION WITH ANTISERA TO MYOGLOBIN. In agar double diffusion with these antisera, R-Mb gave a single sharp precipitin line which fused completely with the line given by MbX, showing no spurs or intersections. In quantitative precipitin analysis with these antisera, R-Mb possessed equal antigenic reactivity to the homologous antigen (98.0 ± 1.0% with antiserum G3 and 99.7 ± 0.5% with antiserum G4 relative to MbX as 100%). In addition, absorption of these sera with R-Mb removed completely the reactivity of the serum supernatants with MbX. Figure 4 shows an example of the precipitin reaction with goat antiserum G3.

REACTION WITH ANTISERA TO R-Mb. With these antisera, MbX and R-Mb showed precipitin lines of complete identity (*i.e.*, no spurs or intersections were observed). Figure 5 shows the quantitative precipitin reaction or rabbit antisera S11 and S12 with MbX and R-Mb. With these antisera, MbX and R-Mb reacted in almost equal efficiency although MbX exhibited a slightly lower reactivity (98.4 ± 1.0% with antiserum S11, and 95.8 ± 1.0% with antiserum S12 relative to RMb as 100%). However, absorption of the antiserum with MbX

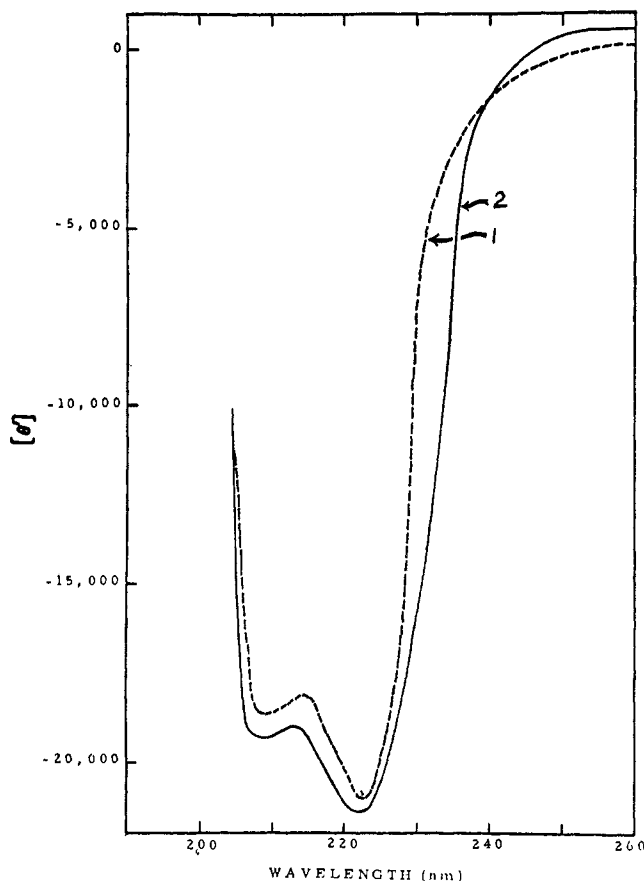

FIGURE 3: Circular dichroism spectra of MbX (1) and R-Mb (2). Measurements were carried out on solutions in water.

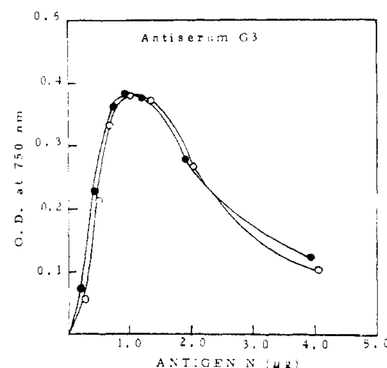

FIGURE 4: Quantitative precipitin reaction of (●) MbX and (○) R-Mb with goat antiserum G3 to MbX. For assay, precipitates were dissolved in 0.5 ml of 0.5 N NaOH and then determined with the Folin–Lowry method (Lowry *et al.*, 1951).

removed completely the reactivity of these sera with the homologous antigen.

Discussion

Specificity of reaction with diborane to reduction of carboxyl groups was well demonstrated by the results of amino acid analysis. The amino acids that sometimes suffer modification when relatively vigorous conditions are used for reduction of proteins (Atassi and Rosenthal, 1969) are histidine, arginine, and proline. In the present work, none of these amino acids exhibited a change in content. Previous studies (Atassi and Rosenthal, 1969) have shown that specificity was influenced by temperature of the reaction. When modification of a limited number of carboxyl groups is desired, in order to study the role of the modified residues in protein function, then reaction can be carried out under very mild conditions (0° or lower) and nonspecificity is then avoided. In fact, it has been shown (Atassi and Rosenthal, 1969) that reaction at $-10°$ was absolutely specific for carboxyl groups and may be employed routinely for carboxyl group reduction in proteins. Specificity of the reaction to reduction of carboxyl group was well demonstrated (Rosenthal and Atassi, 1967; Atassi and Rosenthal, 1969) on 20 model peptides and six different proteins. Peptide bond cleavage was not encountered on reduction of any of the proteins which also included ApoMb reduced with diborane at $-10°$ (Atassi and Rosenthal, 1969). In proteins containing disulfide bonds, reduction of the disulfide bond takes place (Atassi and Rosenthal, 1969). Myoglobin, however, contains no disulfide bonds and this complication does not have to be taken into account. In order to avoid the possible reduction of the propionyl side chains of the heme group, reaction was carried out on ApoMb which was then recombined with unmodified ferriheme. The carboxyl side chains of heme play an active part in the linkage of heme to apoprotein (Lemberg and Legge, 1949; O'Hagan, 1961; Atassi, 1967b). Modification of the propionyl side chains of heme has been shown to influence the immunochemical behavior of myoglobin and hemoglobin (Atassi, 1967b; Atassi and Skalski, 1969).

Due to their hydrophilic character the carboxyl groups in Mb are virtually all exposed to be in complete contact with the surrounding water (Kendrew *et al.*, 1961) and it is likely that a similar situation exists in ApoMb. It is, therefore, noteworthy to point out the higher accessibility of the two glutamic acid residues at positions 83 and 85. Recent work carried out in our laboratory on coupling of carboxyl groups in ApoMb with glycine methyl ester or histidine methyl ester by activation with carbodiimides showed that glutamic-83 and glutamic-85 were also most accessible in ApoMb (Atassi and Singhal, 1972a). Similarly remarkable differences in accessibility were also observed in the reaction of the arginine residues in ApoMb with cyclohexanedione (Atassi and Thomas, 1969) and in the reaction of the lysine residues in Mb with 3,3-tetramethyleneglutaric anhydride (Atassi, 1967c). At any rate, it is not really unusual that not all the carboxyl groups of ApoMb were reducible under the mild conditions of the reaction, since it has been well demonstrated (Beychok and Warner, 1959; Donovan *et al.*, 1960; Hoare and Koshland, 1966; Hornishi *et al.*, 1968) that carboxyl groups in proteins differ in their accessibility.

In the reduction of the carboxyl groups, the charge of the carboxylate ion is lost. However, the change in the size of the side chain upon conversion from $-COOH$ to $-CH_2OH$ is very minimal, and therefore rearrangement of that region due to change in size of the carboxyl group may be ignored. On the other hand, if the reduced carboxyl group had been involved in an intramolecular ionic interaction, then loss of its charge may result in a conformational alteration. The two carboxyl groups in Mb, reduced here, seem to be free and not involved in ion pairs. Coupling of glutamic acids 83 and 85 to glycine methyl ester by activation with carbodiimide yielded a myoglobin derivative which exhibited gross conformational changes (Atassi and Singhal, 1972b), most likely due to loss of the hydrophilic character of the two carboxyl groups. Since loss of charge, on reduction of these two carboxyl groups with diborane, is accompanied by retention of their hydro-

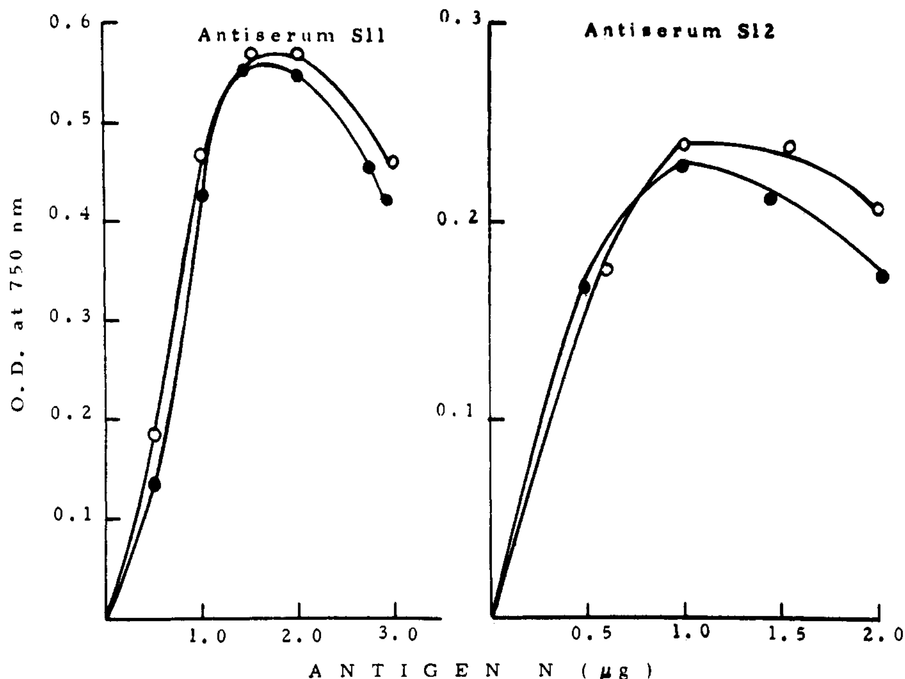

FIGURE 5: Precipitin reactions of (●) MbX and (○) R-Mb with antisera to R-Mb. Antisera S11 and S12 were raised in rabbits against R-Mb. E_{750} indicates the amount of protein in immune precipitate as determined by the Folin method (Lowry *et al.*, 1951).

philic character, little or no driving force will be exerted to change the orientation of the side chain and thus modify the configuration of that region. The absence of detectable conformational changes in R-Mb is, therefore, not unexpected.

It is significant that no change in immunochemical properties was observed in R-Mb relative to MbX. If carboxyl groups were present in an antigenic reactive site of a protein, then their participation in the binding with the corresponding antibody combining site, at neutral pH, will be effected through ionic interaction. Therefore loss of charge by reduction will be sufficient to detect the involvement, or otherwise, of the modified carboxyl group in the antigen–antibody reaction. The reaction of this region accounts for 16–20% of the total antigenic reactivity of Mb (Atassi and Saplin, 1968; Atassi and Singhal, 1970b). The small difference (3–4%) observed between the precipitin reaction of R-Mb and Mb with only one of the four antisera tested (antiserum S12) cannot be taken to indicate presence of the reduced carboxyl groups within a reactive region. In addition, each protein quantitatively absorbed the reaction of this antiserum with the other protein. The immunochemical results and the absence of conformational changes which could influence immunochemical behavior (Atassi, 1967b; Habeeb, 1967; Atassi and Skalski, 1969; Atassi, 1970; Andres and Atassi, 1970) suggest that glutamic acids 83 and 85 are not part of an antigenic reactive region in Mb.

Previous studies have shown that an antigenic reactive region (or regions) exists wihin the sequence 79–96 (Atassi and Saplin, 1968) and terminates before tyrosine-103 (Atassi, 1968). The present findings help in narrowing down further the antigenic reactive region in that location. Since the space intervals between residues 80, 83, and 85 are far too short for antigenic reactive regions to be present and be completed between the nonreactive residues 83 and 85, it must be concluded that the reactive region commences after glutamic acid-85 (*i.e.*, it is located within, but may not include all of, the sequence 86–102). Figure 6 shows a schematic diagram of the mode of folding of this region within the intact Mb molecule. It can be seen that this region is located on the corner between helices F and G (Kendrew *et al.*, 1961). Antibodies directed specifically against various parts of this reactive region, as well as other single reactive regions (all

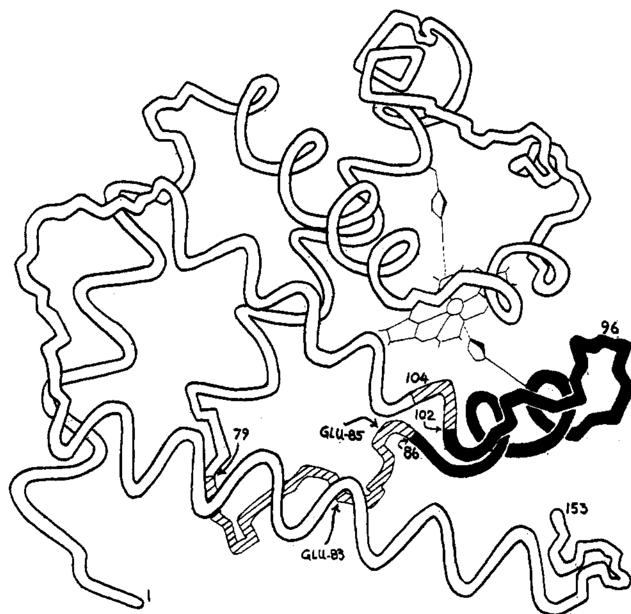

FIGURE 6: Location and mode of folding within the native protein of the antigenic reactive region present in the sequence 79–104. The hatched portions 79–86 and 103–104 have been shown to be *outside* the antigenic reactive region. The diagram is *not* intended to imply that the whole sequence 86–102 constitutes a reactive region but rather that the reactive region is located within this sequence. Work for further narrowing down of this reactive region is in progress. For details see text.

obtained by organic synthesis), are being prepared and the results will be reported elsewhere in detail.

In conclusion, by reaction of ApoMb with diborane, it has been possible to prepare a derivative reduced only at glutamic acid residues 83 and 85. Reduction of the carboxyl groups at these locations resulted in no detectable change in conformation. Immunochemical results showed that the modified residues were not present within antigenic reactive sites of Mb.

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