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# Immunochemistry of Sperm-Whale Myoglobin. Conformation and Immunochemistry of Derivatives Prepared by Reaction with Diazonium-1*H*-tetrazole. Evaluation of the Specificity of the Reagent†

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ABSTRACT: Sperm-whale myoglobin (Mb) was reacted with diazonium-1*H*-tetrazole at two different pH values. The specificity of each reaction was carefully determined, and the conformational and immunochemical properties of the derivatives were investigated. Studies of the electrophoretically homogeneous derivatives obtained from reaction of Mb with the reagent at pH 6.9 and 8.8 revealed that diazonium-1*H*-tetrazole possessed a marked lack of specificity for histidine or tyrosine even under carefully controlled conditions where, from spectral measurements, one histidine residue was expected to have reacted. Several other amino acids were modified to significant extents as well. Spectral, optical rotatory dispersion, and circular dichroism measurements performed on the derivatives revealed that reaction with the reagent resulted in severe conformational changes relative to Mb. Furthermore, neither derivative exhibited significant antigenic reactivity, relative to the native protein, with antisera directed against MbX. Reaction of human serum albumin with diazonium-1*H*-tetrazole under identical conditions resulted in similar severe modifications of the primary structure and spectral properties of the native protein. The antigenic reactivities of all of these derivatives were further examined employing rabbit antisera prepared against a diazotized derivative of Mb. The results of these investigations revealed that treatment of Mb or human serum albumin with the reagent led to the creation, immunochemically speaking, of new proteins for which the antibody response was directed against new antigenic determinants with the added groups acting as haptens and the nature of the carrier backbones bearing little consequence. Also, diazonium-1*H*-tetrazole is completely nonspecific and totally unsatisfactory as a reagent for selective modification of proteins.

Information concerning the antigenic structure of sperm-whale myoglobin (Mb),[1] reported in several previous communications from this laboratory, represents the most advanced such knowledge for a globular protein (for recent review, see Atassi, 1972). The interaction between *regions* that are close in three-dimensional structure but distant in sequence, to form reactive *sites*[2] has been difficult to investigate. Disruption of such interactions by chemical modification has been useful in yielding some information on this question and most instrumental in the location and delineation of the antigenic reactive region of Mb. This paper reports the detailed immunochemistry as well as the conformations of Mb derivatives prepared by reaction with diazonium-1*H*-tetrazole.

Diazonium-1*H*-tetrazole appeared when introduced (Horinishi *et al.*, 1964) as a very promising reagent that apparently minimized the undesirable nonselective reaction characteristics of common diazonium compounds (Cohen, 1968). This reagent has been applied to differentiate free and bound histidine residues in proteins (Horinishi *et al.*, 1964) and to determine the molar contents of free and iron-linked histidine residues in horse heart and baker's yeast cytochrome *c* (Horinishi *et al.*, 1965). DHT has also been utilized to clarify the role of tyrosine residues in the biological activity of myosin (Shimada, 1970). Several other proteins have been

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received August 25, 1972.* This paper is the 13th article in the series: Immunochemistry of Sperm-Whale Myoglobin. This work was supported by Grant AM-13389 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin: MbX, the major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964); apoMb, apomyoglobin; DHT, diazonium-1*H*-tetrazole; D-Mb$_7$ and D-Mb$_9$, derivatives prepared by reaction of MbX with DHT at pH 6.9 and 8.8, respectively; HSA, human serum albumin; D-HSA$_7$ and D-HSA$_9$, derivatives prepared by reaction of HSA with DHT at pH 6.9 and 8.8, respectively.

[2] The terms antigenic reactive *regions* and antigenic reactive *sites* are used according to the definitions previously given (Atassi and Saplin, 1968).

coupled with DHT as well (Cohen, 1968; Ohtuski *et al.*, 1970). Although diazonium coupling has proven useful in the above investigations of histidine and tyrosine in proteins using visible absorption spectrophotometry, it remained highly probable that several other functional groups reacted equally well, without producing spectral changes (Howard and Wild, 1957; Cohen, 1968). Also, no studies have been undertaken regarding the effect of such a reaction on the immunochemistry and conformation of proteins.

We have, therefore, in the present work studied in detail the specificity of diazotization with DHT using Mb as the protein model. Derivatives of Mb have been prepared under various conditions. The immunochemistry of the derivatives has been investigated, and this was accompanied by detailed analyses of their conformations.

## Materials and Methods

*Materials.* Sperm-whale Mb used for these studies was the major chromatographic component 10 (MbX) obtained by CM-cellulose chromatography of the crystalline protein (Atassi, 1964). 5-Aminotetrazole monohydrate was obtained from Aldrich Chemical Co. and hemin chloride from Eastman Organic Chemicals. Preparation of apoMb has been described (Atassi and Perlstein, 1972).

*Preparation of DHT and Its Reaction with Metmyoglobin.* DHT was prepared from 5-aminotetrazole monohydrate and sodium nitrite according to previously reported procedures (Horinishi *et al.*, 1964; Takenaka *et al.*, 1969). For reaction with MbX, 50 ml (17.80 mM) of freshly made DHT solution (adjusted to pH 5.0 with KOH) were added to 100-ml solutions of 300 mg of MbX (0.017 mM) buffered in 0.1 M phosphate (pH 6.90) or in 0.67 M bicarbonate (pH 8.8). Each reaction was allowed to proceed with constant stirring at room temperature for 2 hr and was terminated by exhaustive dialysis against several changes of cold distilled $H_2O$ (0°). Following dialysis, the resulting D-Mb solutions were centrifuged (0°; 1000 rpm; 1 hr) and freeze-dried.

*Column Chromatography.* In order to remove all traces of unreacted Mb and to resolve reaction product(s), the D-Mb preparations (370 mg each) were dissolved in 0.01 M sodium dihydrogen phosphate containing 0.01% KCN, pH 5.9 (20 ml), and, following adjustment of the pH to 5.4 with 4 N HCl, were applied onto columns of CM-cellulose (2.5 × 37 cm) which had been preequilibrated with the same initial phosphate solution. The effluent was monitored by reading absorbance of the fractions (3.3 ml for D-$Mb_7$ and 2.2 ml for D-$Mb_9$) at 600 nm. Finally, those fractions containing protein were pooled, dialyzed extensively against distilled water, centrifuged (3000 rpm, 60 min, 0°) to remove any insoluble protein, and freeze-dried for use in further studies.

*Antisera.* These were prepared in goats and in rabbits against MbX and D-$Mb_9$ by the procedure already described in detail (Atassi, 1967a). Antisera from individual animals were kept separate and stored at −40° in 8-ml portions. Rabbit antiserum S-10 against D-$Mb_9$ and goat antisera G-3 and G-4 against MbX were employed in the present studies.

*Immunochemical Methods.* Double diffusion in 1% agar was performed by the method of Ouchterlony (1949). Quantitative precipitin analyses were performed according to the procedure described in detail elsewhere (Atassi and Saplin, 1968).

*Analytical Methods.* Spectral measurements were carried out with a Zeiss PMQII spectrophotometer. Continuous spectra were performed with a Perkin-Elmer Model 124 double-beam recording spectrophotometer.

TABLE I: Molecular Weights, Nitrogen Contents, and Mean Residue Molecular Weights of MbX, HSA, and Their Diazotized Derivatives.[a]

| Derivative | Mol Wt | % N Content[b] | Mean Residue Mol Wt |
|---|---|---|---|
| MbX | 17,816 | 17.36 | 116.4 |
| ApoMbX | 17,200 | 17.66 | 112.4 |
| D-$Mb_7$ | 22,904 | 32.94 | 149.7 |
| D-$Mb_9$ | 22,904 | 32.94 | 149.7 |
| HSA | 67,836 | 15.64 | 113.2 |
| D-$HSA_7$ | 80,412 | 26.88 | 134.2 |
| D-$HSA_9$ | 80,508 | 26.95 | 134.4 |

[a] Values are obtained from corresponding amino acid compositions. For the diazotized derivatives, molecular weights and nitrogen contents were calculated assuming bisazo coupling for histidine and tyrosine and monoazo coupling for the other modified amino acid residues. [b] Actual values may be slightly higher for HSA and its derivatives due to the release of nitrogen from Asn and Gln upon acid hydrolysis.

Concentrations of protein solutions were determined from their nitrogen contents using a micro-Kjeldahl procedure, similar to that described by Markham (1942), and by using Nessler's reagent standardized with ammonium sulfate. Also, the absorption at 280 nm was always determined. Three or four replicate analyses were done on each solution, in addition to determination of the concentration from the extinction at 280 nm, and they varied by ±0.5%. The nitrogen contents for the D-Mb derivatives were determined from their amino acid compositions. These data as well as those obtained for HSA and for D-HSA derivatives are summarized in Table I.

Electrophoresis was performed on gels consisting of a 6% solution of acrylamide containing 0.05% KCN at an applied voltage of 250 V for 1 hr. Amino acid analysis of acid (110°, 22 or 72 hr in double-distilled, constant-boiling HCl, three-times nitrogen-flushed evacuated sealed tubes) and alkaline (saturated $Ba(OH)_2$; Ray and Koshland, 1962) hydrolysates was carried out on a Bio-Cal BC-200 amino acid analyzer. Thin-layer chromatography (silica gel, Eastman chromatogram sheets) was performed in a solvent system consisting of water–pyridine–1-propanol (5.5: 2.0: 0.1, v/v).

*Optical Rotatory Dispersion and Circular Dichroism.* All experiments were carried out at 25° on solutions of the proteins in water. Solutions contained 0.08–0.23 mg/ml. Measurements were conducted at several concentrations employing cells with light paths of 1 and 5 mm. Solvent base-line scans were performed before and after each protein sample.

Optical rotatory dispersion results are reported in terms of reduced mean residue rotations, $[m']_\lambda$, corrected for the refractive index dispersion of water, and are analyzed quantitatively by means of the Moffitt–Yang equation (Moffitt and Yang, 1956) taking $\lambda_0 = 216$ nm. A value of 116.4 is taken as the mean residue molecular weight for MbX while a value of 149.7 has been calculated for its diazotized derivatives (Table I). The circular dichroism accessory records data directly in

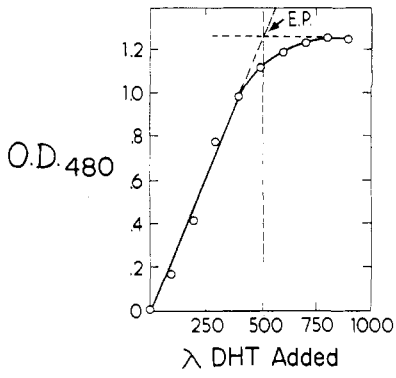


FIGURE 1: Reaction of MbX ($1.87 \times 10^{-5}$ mM) in 0.67 M bicarbonate (pH 8.8) with varied concentrations of DHT conducted according to the procedure of Horinishi *et al.* (1964). $OD_{480}$ represents the absorption maximum for histidinebis(azo-1*H*-tetrazole). Data are corrected for dilution of the protein solution by addition of the reagent. For details, see text.

terms of ellipticity, $\theta$, in degrees. In analogy to $[m']_\lambda$, circular dichroism data are given here as reduced molar ellipticities, $[\theta']_\lambda$, by correcting for the refractive index dispersion of water. Units of $[\theta']_\lambda$ are in (deg cm²)/dmol. Such treatment of the experimental data has been previously discussed in detail (Andres and Atassi, 1970; Singhal and Atassi, 1970).

## Results

*Reaction of Myoglobin and Human Serum Albumin with Diazonium-1H-tetrazole and Characterization of the Derivatives.* In a preliminary experiment to determine the most suitable relative concentration of reagent to protein, varied concentrations of DHT were reacted with a fixed concentration of MbX at the optimum pH of 8.8, and the degree of reaction was estimated from the absorbancy value at the absorption maximum for histidinebis(azo-1*H*-tetrazole) (480 nm) recorded after 20 min at room temperature according to the procedure of Horinishi *et al.* (1964). The resultant reaction curve (Figure 1) indicates that maximum coupling of eight histidine residues is achieved upon the addition of a $10^4$ molar excess of DHT relative to protein. Therefore, in order to limit the extent of coupling to preferably one histidine residue, the necessary molar excess of reagent to protein was derived from Figure 1. This was tenfold less than that required for the modification of eight histidine residues. Subsequent large-scale reactions, therefore, employed a $10^3$ molar excess of DHT relative to protein.

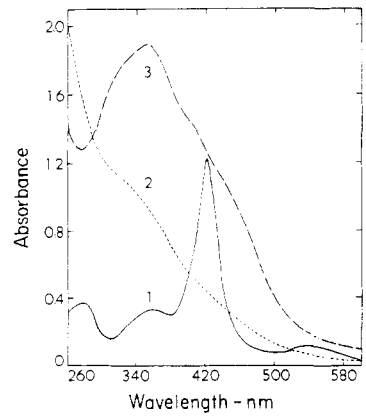


FIGURE 2: Continuous spectra of MbX and its D-Mb derivatives recorded from 260 to 600 nm. Samples were in 0.01 M phosphate buffer containing 0.01% KCN (pH 7.1) and had the following concentrations: (1) MbX, 222 μg/ml; $\epsilon_{280} = 3.02 \times 10^4$; (2) D-$Mb_7$, 123 μg/ml; $\epsilon_{280} = 2.81 \times 10^5$; (3) D-$Mb_9$, 109 μg/ml; $\epsilon_{280} = 2.69 \times 10^5$.

TABLE II: Amino Acid Composition of MbX and Its DHT Derivatives.[a]

| Amino Acid | MbX | DHT Derivatives D-$Mb_7$ | D-$Mb_9$ |
|---|---|---|---|
| Trp | 1.93 | 0 | 0 |
| Lys | 19.1 | 1.29 | 0.28 |
| His | 12.0 | 0.84 | 0.09 |
| Arg | 4.16 | 4.00 | 4.00 |
| Asp | 8.04 | 10.8 | 8.51 |
| Thr | 5.16 | 5.17 | 5.10 |
| Ser | 5.98 | 6.10 | 6.04 |
| Glu | 19.0 | 18.9 | 18.9 |
| Pro | 4.27 | 2.98 | 3.92 |
| Gly | 10.7 | 12.2 | 12.2 |
| Ala | 17.3 | 16.7 | 16.7 |
| Val | 7.88 | 6.87 | 6.69 |
| Met | 1.86 | 0.18 | 1.01 |
| Ile | 9.08 | 9.33 | 9.33 |
| Leu | 18.1 | 17.7 | 18.1 |
| Tyr | 2.90 | 0.90 | 0.22 |
| Phe | 6.11 | 2.16 | 4.68 |

[a] The results represent the average of five acid hydrolyses (three 22- and two 72-hr hydrolyses). Tryptophan was determined from alkaline hydrolysis. Values for serine and threonine were obtained by extrapolation to zero hydrolysis time. The amino acid composition is in residues per mole. In addition, nine new peaks appeared on the analyzer in the D-Mb analyses due to reaction products. Two reaction products appeared as a shoulder under aspartic acid and under glycine explaining the high values obtained for these amino acids.

A second preliminary experiment was carried out to determine whether the heme group in MbX would react with DHT. Modification of the side chains of heme affects the conformation and immunochemistry of Mb (Atassi, 1967b; Andres and Atassi, 1970). Hemin chloride was reacted with DHT at pH 6.9 and 8.8 under the conditions described previously. The reaction products were examined at intervals by thin-layer chromatography over a 2-week period taking care to minimize their exposure to direct light. No differences in the relative mobilities of the reaction products to their unreacted controls could be detected over the entire period. Consequently, the decision was made to perform the reaction on native MbX directly and not on its apoprotein.

Both D-Mb preparations were eluted from CM-cellulose columns of identical size at pH 5.9. No traces of unreacted MbX, which would not emerge from the columns with gradient elution until a pH value of 7.2 had been reached (Atassi, 1964), could be detected. Purity of both preparations was indicated by the symmetry of the $A_{600}$ elution profiles and was confirmed by acrylamide gel electrophoresis. Electro-

TABLE III: Amino Acid Composition of HSA and Its DHT Derivatives.

| Amino Acid | HSA[a] | DHT Derivatives | |
|---|---|---|---|
| | | D-HSA$_7$[b] | D-HSA$_9$[b] |
| Lys | 58.5 | 5.46 | 0.90 |
| His | 15.9 | 4.40 | 0.98 |
| Arg | 23.2 | 23.0 | 23.0 |
| Asp | 53.3 | 53.0 | 54.8 |
| Thr | 29.4 | 25.8 | 25.4 |
| Ser | 24.5 | 22.1 | 23.5 |
| Glu | 90.0 | 85.6 | 90.9 |
| Pro | 29.0 | 18.9 | 28.5 |
| Gly | 13.1 | 18.7 | 20.7 |
| Ala | 65.2 | 64.3 | 63.8 |
| $^1/_2$-Cys | 31.8 | 18.4 | 27.8 |
| Val | 40.0 | 40.4 | 38.2 |
| Met | 5.77 | 1.85 | 2.02 |
| Ile | 8.90 | 8.93 | 6.20 |
| Leu | 62.8 | 63.0 | 61.6 |
| Tyr | 17.6 | 7.69 | 0.94 |
| Phe | 31.0 | 25.1 | 28.1 |

[a] The results represent the average of eleven acid hydrolyses. Tryptophan was not determined. Values for serine and threonine were obtained by extrapolation to zero hydrolysis time. The amino acid composition is in residues per mole. [b] The results represent the average of two 22-hr acid hydrolyses. Values for serine and threonine were not extrapolated to zero hydrolysis time. In addition, at least seven new peaks appeared on the analyzer due to reaction products. Two reaction products appeared under aspartic acid and under glycine explaining the high values obtained for these amino acids.

phoretic mobilities of the derivatives (relative to MbX = 1) were: D-Mb$_7$, 12.24; and D-Mb$_9$, 10.36.

Spectra of the D-Mb derivatives in 0.01 M phosphate buffer containing 0.01% KCN (pH 7.1) were recorded over the wavelength range 260–600 nm (Figure 2). Both showed radical departures from the spectrum obtained for MbX with the apparent blue shifts of the 280-nm absorption maximum. Furthermore, D-Mb$_9$ exhibited a rather broad area of absorption centered at 354 nm which merely appears as a shoulder in the D-Mb$_7$ spectrum. Absorption in the Soret region appears to be abolished for both derivatives although it may be obscured by the shoulder appearing at approximately 400 nm in the D-Mb$_9$ spectrum. In addition, the D-Mb derivatives exhibited an extinction coefficient at 280 nm which was more than eight times that observed for MbX (Figure 2). An even greater increase was obtained for diazotized derivatives of HSA (D-HSA$_7$, $7.06 \times 10^5$; D-HSA$_9$, $5.49 \times 10^5$; HSA, $3.48 \times 10^4$).

Amino acid analyses of acid and alkaline hydrolysates of MbX, HSA and their corresponding derivatives obtained from reaction with DHT are shown in Tables II and III. At least seven new peaks also appeared on the analyzer due to reaction products which, in some instances, nearly coincided with the elution positions of amino acids, making resolution and quantitation impossible to achieve. This accounts for the high values obtained for Asp in D-Mb$_7$ and D-HSA$_9$ as well as for Gly in all of the derivatives. Although from the spectral

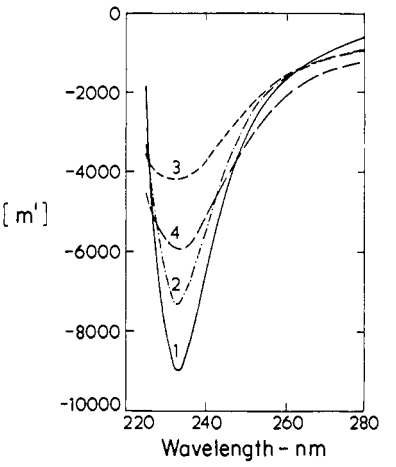


FIGURE 3: Ultraviolet ORD curves of MbX and its diazotized derivatives in water. Values represent the average of four or more scans. ApoMb is also included for comparison: (1) MbX, (2) apoMb, (3) D-Mb$_7$, (4) D-Mb$_9$.

data, one histidine residue should have been modified under the conditions employed, Table II shows that, in fact 42 amino acid residues were modified in D-Mb$_7$ and included 11 (out of 12) histidine residues and 2 (out of 3) tyrosine residues. In D-Mb$_9$, in addition to modification of the twelve histidine and the three tyrosine residues, 24 other amino acid residues were modified. Drastic modifications were also observed with D-HSA$_7$ and D-HSA$_9$ (Table III). Accordingly, the molecular weights and nitrogen contents of the derivatives would change appreciably (Table I). In these calculations, bisazo coupling for histidine and tyrosine while monoazo coupling for all the other modified amino acid residues were assumed. In addition to histidine and tyrosine, amino acids that suffered modification at both pH values were: tryptophan, lysine, proline, cystine (HSA), methionine, phenylalanine, and the N-terminal amino acid (in Mb) at pH 6.9; and tryptophan, lysine, cystine (HSA), methionine, phenylalanine, and the N-terminal valine (in Mb) at pH 8.8. While it has been reported that DHT-modified histidine and tyrosine residues are destroyed on acid hydrolysis (Spande *et al.*, 1970), our results indicate that reversion to the original amino acid residues does not occur.

*Evaluation of Conformational Changes.* Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements, performed on the D-Mb derivatives in water, both reveal that treatment of MbX with DHT at pH 6.9 and 8.8 results in disruption of its three dimensional structure to an extent even greater than that observed for its apoprotein. Quantitative analyses of the optical rotatory dispersion spectra over the wavelength range 280–225 nm (Figure 3) indicate that, while MbX had an $[m']_{233}$ value of $-9080$ and a $b_0$ value of $-405$, its diazotized derivatives exhibited $[m']_{233}$ values of $-4200$ and $-6040$, and $b_0$ values of $-240$ and $-243$ for D-Mb$_7$ and D-Mb$_9$, respectively. These results are consistent with values obtained for the reduced molar ellipticities at 221 nm (Figure 4) which were $-22{,}640$, $-6690$, and $-9300$, for MbX, D-Mb$_7$, and D-Mb$_9$, respectively. For apoMb, the values of $[m']_{233}$, $b_0$, and $[\theta']_{221}$ were $-7360$, $-321$, and $-14{,}100$ respectively.

*Immunochemistry of the Derivatives.* Double diffusion experiments carried out with antiserum G-3 to MbX suggested that little or no cross-reaction could be expected between

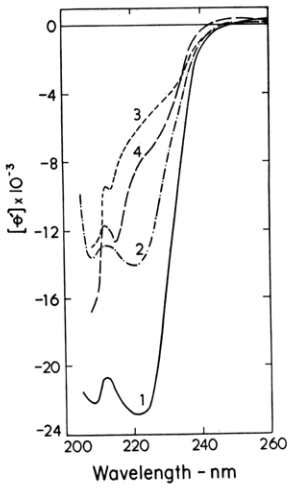


FIGURE 4: CD spectra of MbX and its diazotized derivatives in water. ApoMb is also included for comparison: (1) MbX, (2) apoMb, (3) D-$Mb_7$, (4) D-$Mb_9$.

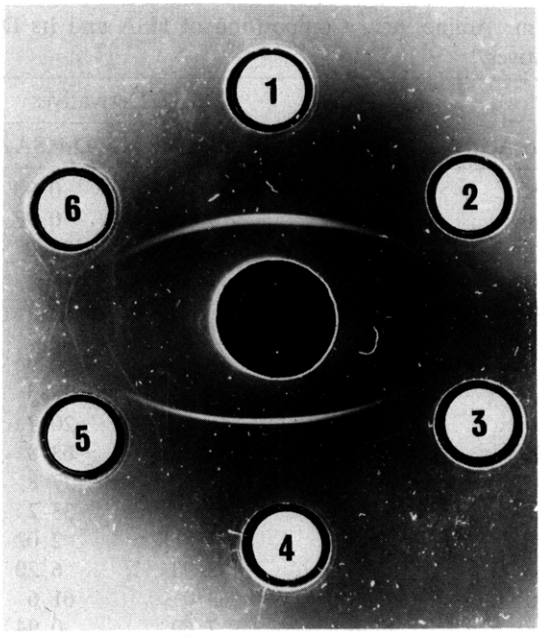


FIGURE 5: Double diffusion in agar; the central well contained goat antiserum G-3 directed against MbX. Moving clockwise: wells 1 and 4, MbX; 2 and 5, D-$Mb_7$; 3 and 6, D-$Mb_9$.

the native protein and its D-Mb derivatives (Figure 5). Identical results were obtained with antiserum G-4. This lack of antigenic reactivity of the diazotized derivatives was borne out in quantitative precipitin analyses with the same antisera (Figure 6). D-$Mb_7$ and D-$Mb_9$ reacted only 3–4% with these antisera relative to the homologous antigen. Neither HSA nor any of its derivatives, of course, showed any reaction with antisera to Mb.

Antibodies were also raised in rabbits against D-$Mb_9$. In agar double diffusion with antiserum S-10 to D-$Mb_9$, single precipitin lines were visible only with wells containing the homologous antigen and D-$HSA_9$. No lines could be observed when MbX, D-$Mb_7$, D-$HSA_7$, and HSA control were tested.

In the quantitative precipitin reaction carried out with antiserum S-10 (Figure 7), D-$HSA_9$ exhibited an antigenic reactivity of 82% relative to that of the homologous antigen, D-$Mb_9$. D-$Mb_7$ showed the next highest extent of cross-reaction (22%), followed by D-$HSA_7$ (13%). No reaction was observed for the HSA control. It is highly significant that MbX also failed to exhibit any cross-reaction with S-10.

## Discussion

The specificity of DHT should first be considered. This has been reported to be specific for histidine and tyrosine (Horinishi *et al.*, 1964). Although histidine residues in several proteins have been modified specifically with affinity labels, it has in fact been difficult to find selective reagents for the modification of this residue (Stark, 1970). Since diazonium salts couple with histidine or tyrosine to produce colored products which can be monitored spectrophotometrically, attempts have been made to exploit the technique as a method for assaying histidine and tyrosine in proteins. However, diazonium coupling has been found to be generally unsuitable for use in selective modification (Cohen, 1968). One of the most promising reagents which has been explored to minimize undesirable characteristics of common diazonium compounds is diazonium-1*H*-tetrazole. This reagent has been applied successfully to distinguish differences in the reactivity of histidine residues in native proteins by visible absorption spectrophotometry (Horinishi *et al.*, 1964). Recently, it has been used to examine the reactivities of histidine and tyrosine residues in native horse ferrihemoglobin and ferrimyoglobin as well as those in cyano derivatives and apoproteins of these hemoproteins (Takenaka *et al.*, 1970). In these reports, however, the amino acid composition, conformation, and immunochemistry of the derivatives were not investigated. While the effect of the formation of colorless derivatives of various amino acid residues in suppressing the formation of the colored bisazo derivatives can be largely overcome in the above studies (Horinishi *et al.*, 1964), such is not the case in this series of investigations. Therefore, we were forced to undertake a thorough study of the specificity of the reagent toward the various amino acid residues of Mb.

Previous investigations have established an optimum pH of 8.8 for the reaction of DHT with proteins (Horinishi *et al.*, 1964; Takenaka *et al.*, 1969). We hoped to minimize reaction of the amino groups and, hence, increase the specificity of the reagent for histidine by conducting the reaction also at a pH of 6.9. However, our results obtained from amino acid analyses of both derivatives demonstrate a marked lack of specificity of the reagent for histidine and/or tyrosine in either case. It is pertinent to mention here that from the spectral data (Figure 1) only one histidine residue should have been modified. Clearly, amino acid analysis indicates that reliance on spectral data may lead to erroneous conclusions. In addition to histidine and tyrosine, DHT also reacted with lysine, tryptophan, methionine, proline, cystine, and phenylalanine (Tables II and III). Also the SH groups of myosin have been reported to be modified by DHT (Shimada, 1970) These findings, therefore, clearly indicate the unacceptability of the reagent for selective modification of histidine and/or tyrosine residues in proteins.

Conformational studies reported here indicate that modification of Mb with DHT results in a large extent of unfolding. The disappearance of absorption in the Soret region in the continuous spectra of the D-Mb derivatives taken together with previous observations indicating the possible release of

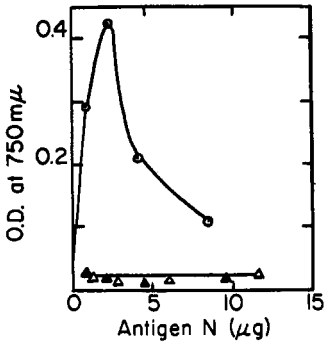


FIGURE 6: Quantitative precipitin curves of MbX and its diazotized derivatives with MbX antiserum G-3 which had been diluted with 0.15 M NaCl (1:1). $OD_{750}$ indicates the amount of protein in the immune precipitate as determined by the Folin–Lowry method (Lowry *et al.*, 1951). Identical results were obtained with MbX antiserum G-4. Antigens are: (○) MbX, (△) D-$Mb_7$, (▲) D-$Mb_9$.

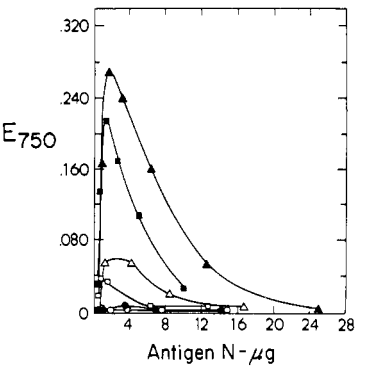


FIGURE 7: Quantitative precipitin curves of MbX, HSA, and their diazotized derivatives with antiserum S-10 to D-$Mb_9$. $E_{750}$ indicates the amount of protein in the immune precipitate as determined by the Folin–Lowry method (Lowry *et al.*, 1951). Antigens are: (○) MbX; (●) HSA, (△) D-$Mb_7$, (□) D-$HSA_7$, (▲) D-$Mb_9$, (■) D-$HSA_9$.

the heme group from horse ferrimyoglobin at low DHT concentrations (Takenaka *et al.*, 1970) suggests, at least in part, one likely explanation for such unfolding. However, previous results obtained from ORD and CD measurements indicate that apoMb retains approximately 78% of the helical content of the native protein (Andres and Atassi, 1970). The present derivatives show a much larger degree of unfolding (see Figures 3 and 4). Therefore, the conformational changes cannot be accounted for entirely by the release of the heme group.

The immunochemical data obtained with the antiserum S-10 prepared against D-$Mb_9$ were highly interesting. The fact that native Mb failed to exhibit any cross-reaction whatsoever indicates that, immunochemically speaking, reaction with DHT had resulted in the creation of a new protein. The high degree of cross-reaction exhibited by D-$HSA_9$ confirms that the antibody response is directed against the modified amino acid residues while the nature of the carrier backbone becomes virtually irrelevant. As expected, D-$Mb_7$ and D-$HSA_7$ also exhibited significant degrees of cross-reaction, although not nearly as great as those observed for the derivatives prepared at pH 8.8. It may be pointed out here that conformational changes in the derivatives may contribute, in part, for the lack of cross-reaction of the derivatives with antisera to Mb or of Mb with antisera to D-$Mb_9$. Purely conformational changes (*i.e.*, imposed without modification of any antigenic reactive regions) have been shown to influence antigenic reactivity (Atassi, 1967a; Atassi and Skalski, 1969; Andres and Atassi, 1970).

In conclusion, while the formation of colorless derivatives of various amino acid residues obtained by reaction of native proteins with DHT may not interfere in experiments employing visible absorption spectrophotometry, it can be expected to exert marked influences on the conformational organization and immunochemical reactivity of the protein under investigation. Our results with sperm-whale myoglobin and human serum albumin indicate that DHT is completely nonspecific and totally unsatisfactory as a reagent for selective modification of histidine, tyrosine or, for that matter, any other amino acids in proteins. Thus, great caution must be exercised in the formulation of conclusions regarding changes in biological function of proteins modified with DHT. Reaction of proteins with DHT yields derivatives with new antigenic determinants which are virtually independent of the carrier backbone.

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