Immunochemistry of Sperm Whale Myoglobin. III. Modification of the Three Tyrosine Residues and Their Role in the Conformation and Differentiation of Their Roles in the Antigenic Reactivity*

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ABSTRACT: Apomyoglobin has been nitrated completely and specifically at the three tyrosine residues. Nitromyoglobin prepared from nitrated apomyoglobin and unmodified ferriheme exhibited electrophoretic and spectral differences but had conformational parameters identical with those of metmyoglobin X. Nitrated apomyoglobin and nitrated metmyoglobin possessed lower antigenic reactivities relative to apomyoglobin and to metmyoglobin X. Fragments 56–131 and 132–153, obtained by cleavage of apomyoglobin at methionines 56 and 131, were also nitrated. Peptide 56–131 and its derivative nitrated at tyrosine 103 showed identical reactivities with antisera to metmyoglobin X. It was therefore concluded that tyrosine 103 is not located in an antigenic reactive region in metmyoglobin. On nitration of tyrosines 146 and 151 the inhibitory activity of peptide 132–153 was completely abolished. The inhibitory activity lost compared well with the decrease in antigenic reactivity observed with nitrated apomyoglobin and nitrated metmyoglobin. Also, nitrated metmyoglobin did not react with antisera to fragment 132–153 (Atassi, M. Z., and Saplin, B. J. (1968), *Biochemistry 7*, 688).

These findings suggest that one or both of tyrosines 146 and 151 are present in a reactive region in metmyoglobin.

Recent work from this laboratory on the antigenic structure of Mb[1] has provided valuable information concerning the nature and location of some reactive regions[2] on the molecule. Studies on fragments obtained by enzymic and chemical cleavage procedures were useful in determining the approximate location of some reactive regions in Mb (Atassi and Saplin, 1968). But further delineation of reactive regions in a peptide based solely on shortening the peptide might lead to erroneous conclusions due to the inherent limitations of the technique. The ability of the peptide to attain a favorable orientation for reactivity with the antibody combining site could very well be impaired upon shortening, even though the segment removed may not be part of a reactive region in the peptide. Similarly for the same considerations of configuration, a nonreactive fragment may indeed still be a part of a reactive region in the intact protein. Evidence utilizing an independent approach must be presented. Specific chemical modification of selected amino acid residues in Mb has been particularly useful in mapping out amino acids in reactive and nonreactive regions. Thus it has been demonstrated that the methionine residues at positions 55 and 131 (Atassi, 1967a) and tryptophan at position 7 (Atassi and Caruso, 1968) were not essential parts of the antigenic sites of Mb. I wish now to report on the role of the three tyrosine residues.

It has recently been demonstrated that tetranitromethane which nitrates phenols (Schmidt and Fischer, 1920) can be successfully employed for the specific nitration of tyrosine residues in proteins especially in the absence of SH groups (Riordan *et al.*, 1966, 1967; Sokolovsky *et al.*, 1966). This paper therefore describes the preparation and chemical and physicochemical characterization of a myoglobin derivative modified specifically at the three tyrosine residues. The immunochemical reactivity of the derivative is reported and the role of tyrosine 103 is differentiated from the role(s) of tyrosines 146 and 151.

Materials and Methods

Metmyoglobin and Apomyoglobin. Sperm whale myoglobin used in these studies was the major chromatographic component 10 (Mb X), obtained by CM-cellu-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin; Mb X, the major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964); TNM, tetranitromethane; nitro-Mb, metmyoglobin nitrated at all three tyrosine residues; nitro-ApoMb, apomyoglobin nitrated at all three tyrosine residues.

[2] The distinction between reactive region and reactive site has already been defined (Atassi and Saplin, 1968).

lose chromatography (Atassi, 1964). The apoprotein was prepared from Mb X by the procedure of Theorell and Åkeson (1955).

Reaction of Apomyoglobin with Tetranitromethane. To Apomyoglobin (3 μmol) in 0.05 M Tris–1 M NaCl buffer (pH 8.0) (8 ml) was added 30 μmol of TNM (Aldrich Chemical Co.) as a 10% solution in 95% ethanol and the mixture was allowed to stir magnetically at room temperature (22°) for 2 hr. After reaction, the mixture was dialyzed extensively against distilled water, centrifuged (0°, 0.5 hr at 5600 rpm), and the supernatant was filtered on a column (2.2 × 15 cm) of Sephadex G-10 in order to remove excess reagent. The column was eluted with distilled water. The tubes containing the protein fraction were pooled and freeze dried. Extent of nitration was determined spectrally from the extinction at 381 mμ (Sokolovsky *et al.*, 1966) and by amino acid analysis of acid and alkaline hydrolysates.

Cleavage at the Methionine Sites and Nitration of Peptides 56–131 and 132–153. Reaction of ApoMb with cyanogen bromide was according to the procedure of Gross and Witkop (1962). Separation of the resultant fragments was done by gel filtration on appropriate grades of Sephadex (Edmundson, 1963). Purity and immunochemical reactivities of these peptides has recently been reported in detail (Atassi and Saplin, 1968). The peptides will be designated according to their location in the primary structure of ApoMb as reported by Edmundson (1965).

For nitration, 40 μl of TNM solution (10% TNM in 95% ethanol) was added to the peptide (7–9 mg) in 0.05 M Tris–1 M NaCl buffer (pH 8.0) (0.5 ml). The mixture was stirred magnetically for 3 hr at room temperature after which the yellow solution was filtered on a column (1.2 × 10 cm) of Sephadex G-10 which was eluted with water. The tubes containing the nitrated peptide were pooled and freeze dried. Extent of nitration was determined by amino acid analysis.

Antisera. These were prepared against MbX by the procedure previously described (Atassi, 1967a). Antisera from individual animals were kept separate and stored in 8–10-ml portions at −40°. Rabbit antisera 80 and 100 and goat antiserum G1 were used in the present studies.

Analytical Methods. The purity of each peptide was confirmed by peptide mapping by the procedure recently described (Atassi and Saplin, 1968). Concentrations of protein and peptide solutions were based on their nitrogen contents determined by a micro-Kjeldahl procedure (Markham, 1942). Spectral measurements, starch gel electrophoresis, and sedimentation experiments were done under the conditions previously described (Atassi and Caruso, 1968). Immunochemical studies with agar double diffusion, quantitative precipitin, and inhibition experiments were performed by the procedures and under conditions already described in detail (Atassi and Saplin, 1968). Results of inhibition experiments were expressed in per cent inhibition, *i.e.*, 100 × (total precipitate with antigen − total precipitate in the presence of peptide)/total precipitate with antigen. Calculation of Stoke's radius, a, and molar frictional coefficient, f/f_0, by gel filtration on a column (2.2 × 55 cm) of Sephadex G-75 which had been calibrated with four standard proteins had already been described (Atassi and Caruso, 1968). Alkaline hydrolysis of proteins was in saturated $Ba(OH)_2$ by the procedure of Ray and Koshland (1962). Acid hydrolysis was at 110° for 22 or 72 hr in constant-boiling HCl (double distilled) in nitrogen-flushed evacuated, sealed tubes. Amino acid analyses were done on Spinco Model 120C amino acid analyzer.

Reconstitution of Metmyoglobin. Reconstitution of ApoMb or nitro-ApoMb with hemin chloride (Eastman Organic Chemicals) and removal of excess ferriheme were done by the procedure recently described in detail (Atassi and Caruso, 1968).

Results

Characterization of the Modified Protein. The direct reaction of TNM with MbX was considered since it would simplify the whole approach and improve yield. However, the possibility of reaction of TNM with heme had to be taken into account. Modification of the side chains of heme has already been shown to change the antigenic reactivity of Mb (Atassi, 1967b). In a preliminary experiment, ferriheme was treated with 15 molar excess of TNM under conditions similar to those described in the Experimental Section. After 24 hr the reaction mixture was examined by thin-layer chro-

TABLE I: Amino Acid Compositions of ApoMb and Its Nitrated Derivative.[a]

Amino Acid	Amino Acid Composition (residues/mol)	
	ApoMb	Nitro-ApoMb
Trp	1.91	1.88
Lys	19.0	18.9
His	12.0	12.2
Arg	4.03	4.06
Asp	7.92	7.83
Thr	4.78	4.83
Ser	5.82	5.66
Glu	19.2	19.0
Pro	4.18	3.83
Gly	10.8	11.0
Ala	16.7	17.0
Val	7.85	7.92
Met	2.14	1.95
Ile	8.66	9.05
Leu	18.0	17.8
Tyr	2.95	0
Phe	6.11	5.91
3-NO_2Tyr	0	3.04

[a] The results represent the average of four acid hydrolyses (two 22 hr and two 72 hr). Tryptophan was determined by duplicate analyses of alkaline hydrolysates. Values for serine and threonine were obtained by extrapolation to zero hydrolysis time.

TABLE II: Absorption Maxima and Ratios of Optical Densities at These Maxima of Metmyoglobin and Its Nitrated Derivative.[a]

	Max (mμ)					Ratios of OD's at Absorption Max			
	A	B	C	D	E	A/E	B/E	C/E	D/E
MbX		279	360	424	542		3.20	2.83	9.83
Nitro-Mb	268	280	360	417	540	4.92	4.27	4.71	11.76

[a] Solutions were in 0.01 M phosphate buffer (pH 7.0), containing KcN (0.01%).

matography (silica gel, Eastman chromatogram sheets). A new reaction product appeared which, in the following solvents, had R_F values: water–1-butanol–pyridine (220:4:16, v/v), ferriheme, 0.20; reaction product, 0.33; benzene–methanol–*N*-ethylmorpholine (10:5:3, v/v), ferriheme, 0; reaction product, 0.10; and 1-butanol–acetic acid–water (4:1:5, v/v), ferriheme, 0.89; reaction product, 0. After 5-days reaction, ferriheme was almost completely converted into the new reaction product. The reaction product of ferriheme with TNM is being characterized and the results will be reported elsewhere. For the present problem, therefore (*i.e.*, investigating the antigenic reactive regions of Mb), it was advisable to treat ApoMb with TNM and then combine the derivative with unmodified ferriheme.

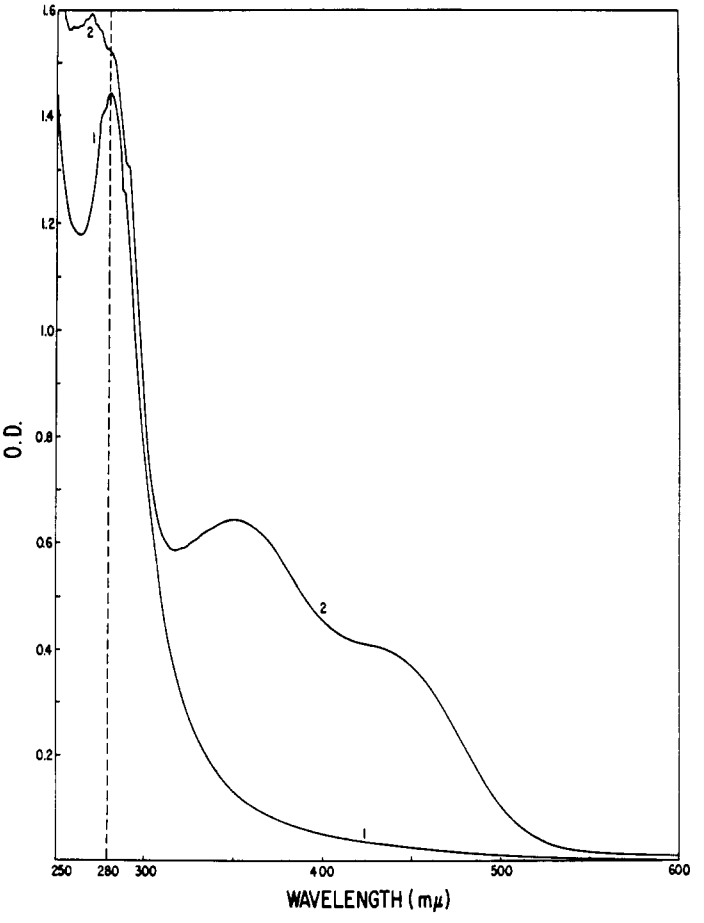

FIGURE 1: Absorption spectra of (1) ApoMb and (2) nitro-ApoMb. Proteins were dissolved in distilled water.

Amino acid analyses of acid and alkaline hydrolysates of ApoMb and the derivative obtained from its reaction with TNM are shown in Table I. The results demonstrate that reaction of ApoMb with 10 molar excess of TNM for 2 hr resulted in the complete modification of all three tyrosine residues in ApoMb. The disappearance of tyrosine on the analyzer was accounted for entirely by the appearance of a new derivative which eluted at the same position as authentic 3-nitrotyrosine. No other amino acids were modified. The results of amino acid analysis agreed quite well with the extent of nitration, determined spectrally from the extinction at 381 mμ (Sokolovsky *et al.*, 1966). These determinations suggested the presence of 2.98 nitrotyrosine residues in nitrated ApoMb. The absorption spectra of ApoMb and nitro-Apo Mb are shown in Figure 1. Nitration of the tyrosine residues resulted in the appearance of a new peak in the visible region of the spectrum at 350 mμ and also a shoulder between 420 and 440 mμ. In the ultraviolet region, the arrest in the curve at 290 mμ, given by tryptophan residues, was still pronounced. However, the major absorption maximum appeared at 268 mμ while the maximum at 280 mμ was only a shoulder in the curve.

Nitroapomyoglobin was then recombined with unmodified ferriheme and the spectral behavior of the resultant nitro-Mb was investigated. Table II summarizes the spectral properties of the cyanmet forms of Mb X and nitro-Mb. The spectrum of nitro-Mb in its cyanmet form, showed peaks at 268 (sh), 280 (sh), 290 (sh), 360, 417, and 540 mμ (Figure 2). The absorption at 268 mμ was higher than that at 280 mμ. The extinction at 360 was almost doubled (relative to Mb X) and that at the Soret peak was also slightly increased. The spectral behavior of nitro-ApoMb suggests that the observed increase in extinction of nitro-Mb in the visible region was most likely due to the contribution of the nitrotyrosine residues rather than to any conformational changes in the protein.

On starch gel electrophoresis nitro-Mb gave one band which appeared to be more negatively charged than Mb X with a mobility (relative to Mb X = 1) of 4.70. When gels were heavily loaded, a trace of another band with a mobility of 5.32 was obtained. Results of the amino acid analyses (Table I) have already confirmed that the increase in the apparent negative charge of nitro-Mb was not due to the modification of any basic amino acid

TABLE III: Amino Acid Compositions of Fragments 56–131 and 132–153 and Their Nitro Derivatives.[a]

Amino Acid	Peptide 56–131		Peptide 132–153	
	Before Nitration	After Nitration	Before Nitration	After Nitration
Lys	9.86	10.0	3.99	3.89
His	7.95	7.92	0.01	0
Arg	1.00	0.983	1.01	0.970
Asp	3.01	3.06	1.93	2.11
Thr	2.84	2.95	0.03	0.02
Ser	3.99	4.00	0.01	
Glu	7.03	7.00	3.26	3.00
Pro	3.01	2.99		
Gly	6.10	6.20	2.04	2.01
Ala	9.97	10.1	3.16	3.09
Val	2.88	2.97		
Ile	5.82	5.77	1.17	1.09
Leu	8.04	8.13	3.27	3.22
Tyr	1.01	0.007	2.03	0.012
Phe	1.97	2.08	1.02	1.00
3-NO_2Tyr		0.992		2.09

[a] Values are expressed in moles of amino acid per mole of peptide. Results represent the average of three acid hydrolyses (two at 22 hr and one at 72 hr). Values for serine and threonine were obtained by extrapolation to zero hydrolysis time.

residues. The increase in the over-all negative charge of the protein may be partly accounted for by the ionization of the phenolic hydroxyl group in 3-nitrotyrosine whose pK_a now has decreased to 7.2 (Sokolovsky *et al.*, 1967) as compared with that of the same group in tyrosine (10.1; Edelhoch, 1962). In myoglobin the pK_a values of the three tyrosines are 10.3, 11.5, and 1 with $pK_a = >12.8$ (Hermans, 1962). In sedimentation studies nitro-Mb sedimented at the same rate as Mb X when solutions had the same concentration. 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 nitro-Mb was done by the method already described in detail (Atassi and Caruso, 1968). By this procedure Mb X and nitro-Mb possessed identical values for Stoke's radius (18.5 Å) and f/f_0 (1.06).

Preparation of the Nitrated Peptides. Preparation of highly purified ApoMb peptides obtained by cleavage at the methionine sites together with the immunochemical properties of these peptides has recently been described (Atassi and Saplin, 1968). Table III shows the amino acid composition of peptides 56–131 and 132–153 and their corresponding nitrated derivatives. The compositions of the peptides before nitration agree well with those reported by Edmundson (1963). After nitration the tyrosine residues were converted quantitatively into 3-nitrotyrosine. No other amino acids were modified.

Immunochemical Reactivity of Nitro-Mb. In agar double-diffusion experiments nitro-Mb gave a single precipitin line with rabbit and goat antisera to Mb X. No spurs or intersections were obtained with the line given by Mb X. In quantitative precipitin analysis nitro-ApoMb and nitro-Mb precipitated less antibody nitrogen at maximum than ApoMb or Mb X with the three sera tested. However, the amounts of antigen required by the native proteins or their nitrated derivatives to achieve maximum precipitation were similar. Figure 3 shows an example of the precipitin reactions of recombined Mb and of nitro-Mb with antisera 80 and Gl. Table IV summarizes the results of several precipitin experiments of antisera Gl, 80, and 100 with ApoMb, nitro-ApoMb, and their corresponding metmyoglobins. It is relevant to point out here that Mb X and Mb obtained by recombination of ApoMb with ferri-

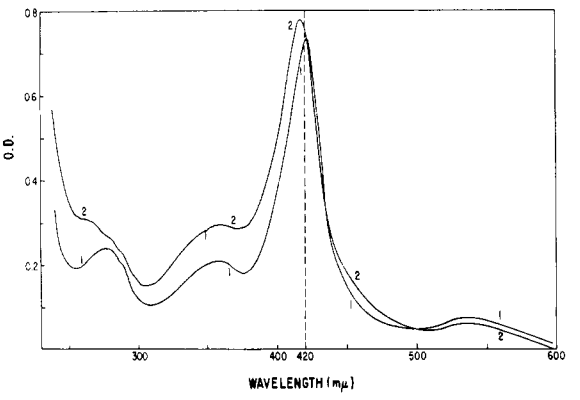

FIGURE 2: Absorption spectra of (1) Mb X (20.6 μg of nitrogen/ml) and (2) nitro-Mb (17.4 μg of nitrogen/ml). Solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%).

TABLE IV: Relative Amounts of Precipitation Formed by Nitro-ApoMb and Nitro-Mb.[a]

	Nitro-ApoMb			Nitro-Mb		
	% Ppt Rel to Reaction of Antiserum with ApoMb			% Ppt Rel to Reaction of Antiserum with Mb X		
Antiserum	Expt	Av	Range	Expt	Av	Range
G1	3	85.0	84.2–86.4	5	85.5	83.5–86.8
80	2	87.7	86.5–88.9	4	88.1	86.8–89.4
100				2	86.2	85.4–87.0

[a] The precipitation reaction was carried out with one goat antiserum and two rabbit antisera against MbX. The percentage of precipitation relative to ApoMb or MbX was based on the total nitrogen values in the precipitates at the point of maximum precipitation.

heme have been demonstrated to be physicochemically and immunochemically identical (Atassi, 1967b).

In order to determine the factors responsible for the decrease in reactivity of Mb upon nitration of its three tyrosyl residues, further studies were necessary so that the role played by the various tyrosyl residues in the antigen–antibody reaction may be differentiated. The three tyrosine residues in myoglobin are located at positions 103, 146, and 151 (Edmundson, 1965). Reactivity of nitro-Mb with antisera to fragment 132–153 was examined. These sera were prepared by absorption of antisera 80 and 100 with fragments 1–55 and 56–131. It was shown (Atassi and Saplin, 1968) that out of six sera absorbed by this procedure, thus leaving in the supernatant only antisera to fragment 132–153, two sera (*i.e.*, 80 and 100) gave specific immune precipitates with the homologous antigen. Therefore, reactivity of nitro-Mb with antisera to fragment 132–153 should provide valuable information concerning the role of tyrosines 146 and 151. It was, therefore, extremely significant to find that nitro-Mb did not react with either antisera to fragment 132–153 prepared from antisera 80 and 100.

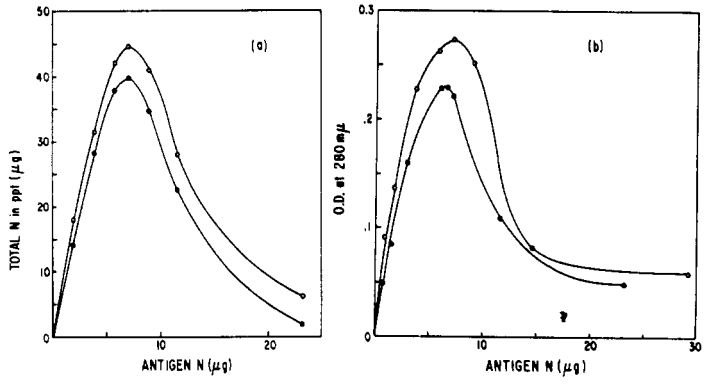

FIGURE 3: Precipitin studies on recombined Mb (○) and nitro-Mb (●). (a) Reactions with rabbit antiserum 80. (b) Reactions with goat antiserum G1. The differences in the methods employed for measuring the total amounts of immune precipitates should be noted. The precipitates in b were dissolved in 0.5 ml of 0.5 N NaOH and the volume was made up to 3 ml with 0.15 M NaCl.

Immunochemical Reactivities of the Nitrated Peptides. A. FRAGMENT 56–131 NITRATED AT TYROSINE 103. The reactivity of fragment 56–131 with several rabbit and goat antisera to Mb X and to ApoMb has already been reported (Atassi and Saplin, 1968). It was shown that this peptide formed immune precipitates with all the antisera tested and that, of the three peptides obtained by cleavage at the methionine sites, it was the immunodominant region of the molecule. Fragment 56–131 and its nitrated derivative gave similar amounts of precipitates with antisera G1 and 80 to Mb X. Upon absorption of each of these sera with the minimum amounts of fragments 56–131 or nitrated 56–131 necessary to achieve maximum precipitation, the reactivities remaining in each supernatant toward the homologous antigen were also identical. Figure 4 shows an example of an absorption experiment with antiserum G1. This suggested equal reactivities for peptides 56–131 and nitrated 56–131 with antisera to Mb X.

B. Peptide 132–153 Nitrated at Tyrosines 146 and 151. The reactivity of fragment 132–153 with six different goat and rabbit antisera to Mb X and ApoMb has been reported in detail (Atassi and Saplin, 1968). It was shown that with all the sera tested this was an inhibitory peptide. Its maximum inhibitory activity against the antisera to Mb X ranged between 11 and 21% with Mb X and between 14 and 21% with ApoMb. Table V summarizes the results of inhibition experiments with peptide 132–153 and its nitrated derivative. It can be seen that upon nitration of the tyrosine residues at positions 146 and 151, the inhibitory activity of fragment 132–153 disappeared completely.

Discussion

In attempts to correlate protein structure with function utilizing chemical modification studies of a given amino acid, it is not sufficient to ensure the specificity of the chemical reaction and the absence of conformational alterations. It is, in addition, necessary to determine if the modification will alter the properties of the residue in question sufficiently so that its participation in the biological role in which it is normally involved

TABLE V: Maximum Inhibitory Activities of Fragment 132–153 and Nitrated 132–153.[a]

Antiserum	Peptide 132–153[b]	Nitrated Peptide 132–153[c]
G1	20.8 ± 1.1 (4.1)	0 (20.0)
80	11.2 ± 0.9 (4.1)	0 (20.3)
100	10.8 ± 1.0 (9.5)	0 (35.3)

[a] Results are expressed in maximum per cent inhibition by the peptide of the precipitin reaction of Mb X with various antisera. [b] Values in parentheses represent peptide/antigen molar ratio at 50% of maximum inhibition. [c] Values in parentheses represent maximum molar excess of peptide used in the inhibition experiment.

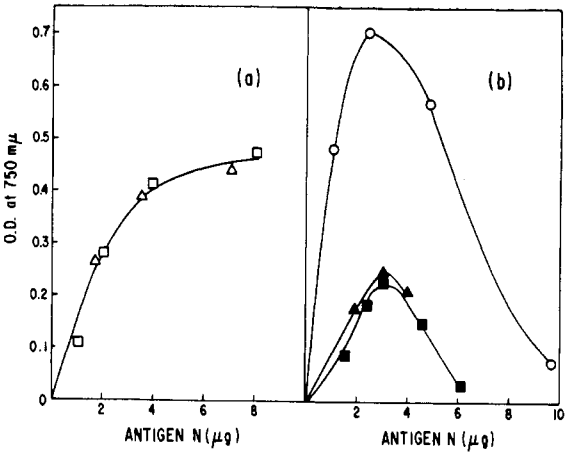

FIGURE 4: Absorption experiments with goat antiserum G1. Part a shows the reactions of peptides 56–131 (△) and nitrated 56–131 (□) with antiserum G1 which had been diluted 1:1 with 0.15 M NaCl. Part b gives the reaction of serum which had been absorbed with peptide 56–131 (▲), and nitrated 56–131 (■) toward Mb X; the reaction of unabsorbed serum (which had been diluted with an equal volume of 0.15 M NaCl to account for the volume of the peptide solution used in the absorption experiment) is also shown for comparison (○). 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). Notice that the amounts of maximum precipitate obtained with peptide 56–131 and nitrated 56–131 were almost identical. In part b it can be seen that absorption with peptide 56–131 removed 35.4% and with nitrated 56–131 removed 32.6% of the antibody to Mb X.

might be influenced. On nitration of tyrosyl residues *ortho* to the phenolic hydroxyl group the inductive effect of the nitro group on the aromatic nucleus will increase the acidity of the phenolic hydroxyl group whose ionization will be promoted since the electron-withdrawing mesomeric effect leads to stabilization of the resultant anion. The increase in acidity upon nitration of tyrosine at the *ortho* position is shown by a decrease of the value of pK_a from 10.1 for tyrosine (Edelhoch, 1962) to 7.2 for 3-nitrotyrosine (Sokolovsky *et al.*, 1967). This difference in pK_a is similar to that observed between phenol (pK_a = 10.0) and *o*- and *p*-nitrophenols (pK_a = 7.21 and 7.16, respectively). For comparison, halogenation results in a very small decrease in the pK_a value (*e.g.*, pK_a for *p*-chlorophenol is 9.39; Fieser and Fieser, 1961). Thus, it is very likely that iodination of tyrosyl residues might not yield conclusive results regarding the biological role of these residues. Similarly, for these reasons it would be expected that the presence or absence of changes in biological activity upon nitration of tyrosine residues in a protein should reflect a true picture regarding the involvement of these residues in a reactive region. Since the reaction of tetranitromethane with ferriheme may take place in Mb, it was decided to nitrate the tyrosines in ApoMb, then recombine nitro-ApoMb with unmodified ferriheme. This precaution will avoid complications arising from changes in antigenic reactivity of Mb upon modification of the heme group (Atassi, 1967b).

The decrease in antigenic reactivity upon nitration of the three tyrosine residues in ApoMb and Mb could reflect a true involvement of some (or all) of these residues in reactive region(s) of the molecule or could indeed be the result of drastic conformational changes. Although ApoMb, which is more unfolded in any case than Mb (Breslow *et al.*, 1965; Atassi and Cacciotti, 1966), showed a decrease in reactivity upon nitration comparable with that obtained with nitro-Mb, the presence of some conformational changes common to Mb and ApoMb will have to be taken into account. Since nitro-Mb and Mb X possessed identical conformational parameters (*i.e.*, Stoke's radius and frictional ratio values), appreciable conformational changes may not be present. It is noteworthy that tyrosines 103 and 146 are present in helices G and H, respectively (Kendrew *et al.*, 1961), and it is therefore significant that their nitration does not disrupt the integrity of these helices. The present data do not rule out, however, the presence of small changes in conformation that may not be detectable by the methods employed. A more detailed study of the conformation of this derivative is in progress and will be reported later. In the crystal structure tyrosines 103 and 151 are exposed while tyrosine 146 is buried. It appears, therefore, that in ApoMb, under the conditions of the reaction, these tyrosine residues are equally accessible for nitration. In a recent report (Atassi, 1967a) it was shown that the tyrosine residues in Mb were equally accessible to oxidation with sodium metaperiodate at pH 6.8 or 5.0 for 5–6 hr at 22°. Upon oxidation of ApoMb at pH 5.0 and 0° for 7 hr, only one tyrosine residue was modified (Atassi, 1967c). The modified tyrosine residue was located at position 151 in the peptide chain (Atassi, 1967a). By the use of iodination followed by performic acid oxidation, Hermans and Wan Lu (1967) reported that tyrosine 151 was the most reactive tyrosine residue in Mb.

Peptide 56–131 and its derivative nitrated at tyrosine 103 removed equivalent amounts of antibody from antisera to Mb X. It was apparent from this finding that the decrease in the antigenic reactivity of nitro-Mb does not result from the nitration of tyrosine 103. Therefore,

tyrosine 103 is not located in an antigenic reactive region.

The absence of reaction of nitro-Mb with antisera to fragment 132–153 gave the first indication that the decrease in reactivity of nitro-Mb with antisera to Mb X might be mainly the result of nitration of tyrosines 146 and 151. This conclusion was confirmed by the finding that nitration of tyrosines 146 and 151 in peptide 132–153 resulted in the complete loss of its inhibitory activity with all the antisera tested. Conformational factors are not too important here since it is very unlikely that the isolated peptide will be able to assume in an appreciable way a configuration similar to that it has in the native protein. Therefore, nitration of tyrosines 146 and 151 will not be expected to exert a meaningful influence upon the configuration of the isolated peptide. It can therefore be safely concluded that one or both of tyrosines 146 and 151 are present in antigenically reactive regions of Mb. It is not possible from the present data to differentiate between the independent roles of these two tyrosine residues. Several attempts at partially nitrating the peptide resulted in heterogeneous preparations which were not too useful for immunochemical studies. It is relevant to point out here that peptide 148–153 isolated from tryptic digest of ApoMb showed an appreciable inhibitory activity with antisera to Mb X and to ApoMb (Atassi and Saplin, 1968; also see Crumpton, 1967). However, with a given serum, the inhibitory activity exhibited by peptide 148–153 was always lower than that given by the larger peptide 132–153. The smaller inhibitory activity obtained with peptide 148–153 may arise from the possibility that peptide 132–153 contains more than one reactive region and hence its inhibitory activity would be expected to be higher than that of peptide 148–153. Alternatively, peptide 148–153 may carry most or all of the reactive regions in the larger peptide but the inhibitory activity of the smaller peptide is enhanced by the rest of peptide 132–153 which might not be part of a reactive region. With the larger peptide, the reactive region will be able to achieve the appropriate orientation necessary for a more favorable fit on the antibody combining site. Such an example has already been reported for the role of sequence 1–7 in the reactivity of fragment 1–55 (Atassi and Saplin, 1968). Therefore, information derived from the modification of an active peptide will most likely be of a more conclusive nature than that obtained from shortening the peptide. The appropriate orientation of a reactive region is more likely to be retained upon limited and specific modification of a peptide than upon removal of, what what might well be, a critical orientating influence of a segment in that peptide. The complete loss of inhibitory activity by fragment 132–153 upon nitration of tyrosines 146 and 151 could suggest at first glance that one or both of these tyrosine residues are present in the only reactive region in that peptide. Nevertheless, the results do not exclude the existence of another reactive region in peptide 132–153 which requires, for proper function, an intact reactive region around the tyrosyl residues.

The present results can be safely correlated with previous information concerning the role of the methionine residues (Atassi, 1967a), the iron of ferriheme and the heme side chains (Atassi, 1967b), the tryptophan residues (Atassi and Caruso, 1968), and the reactivity of tryptic peptides and of peptides obtained by cleavage at the methionine and tryptophan sites (Atassi and Saplin, 1968). This is due to the fact that serum Gl was used throughout these studies and appreciable overlaps were always ensured between sera G1, G4, 11, 80, 93, and 100. Therefore, complications due to differences in specificities and affinities of sera, have been minimized. With antisera 11 and 17 the C-terminal dipeptide was not an essential part of an antigenic site in Mb X (Atassi, 1966).

In conclusion, the results show that the tyrosine residues of Mb can be quantitatively nitrated. The conformational parameters of Mb X and nitro-Mb were identical suggesting the absence of any detectable conformational alterations in nitro-Mb. Tyrosine 103 is not part of a reactive region in Mb whereas one, or both, of tyrosines 146 and 151 are present in a reactive region of the molecule.

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The Conversion of [3β-^{3}H]5β-Cholestan-3α-ol into [3β-^{3}H]Deoxycholic Acid in Man*

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ABSTRACT: Since 5β-cholestan-3α-ol possesses the structural features of bile acids at C-3 and at the A/B ring juncture, its conversion into bile acids was investigated. [3β-^{3}H]5β-Cholestan-3α-ol was prepared and administered to a patient with a bile fistula. Over 15% of the radioactivity in the biliary steroids was associated with the dihydroxycholanic acids of which labeled deoxycholic acid comprised the major portion. Preparation of suitable derivatives showed that ^{3}H was retained at C-3β through the transformation.

Two pathways have been proposed for the formation of C_{24} bile acids. The first (Danielsson, 1963) involves changes in the sterol nucleus prior to production of the carboxyl group. While the sequence is not fully established, it is probable that 7α hydroxylation of cholesterol is an early step (Bergstrom and Lindstedt, 1956; Bergstrom *et al.*, 1958) followed by 12α hydroxylation (Danielsson, 1962) which occurs either before or after the saturation of the double bond and inversion at C-3 (Danielsson, 1961a,b). Loss of three carbons leads to chenodeoxycholic[1] and cholic acid. In support of this pathway, Staple and coworkers have isolated radioactive 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid and 5β-cholestane-3α,7α-diol from human bile after the administration of [26-^{14}C]cholesterol (Staple and Rabinowitz, 1962; Rabinowitz *et al.*, 1966). The second pathway, reported by Mitropoulos and Myant (1967) in rats and rat liver preparations, involves first the production of 3β-hydroxy-Δ^5-cholenic acid followed by hydroxylation steps to afford bile acids oxygenated at C-3, -6, and -7. Whether such a pathway exists in man is not known. Bongiovanni (1965) was unable to find 3β-hydroxy-Δ^5-cholenic acid in human gall bladder bile. Both of these pathways exclude 3,12-dihydroxy steroids from the biosynthetic sequence either as C_{27} intermediates or as primary bile acids. Deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid), the principal bile acid of human feces and a major constituent of the bile of man and animals, is formed by bacterial reduction of cholic acid (Lindstedt, 1957; Ekdahl and Sjövall, 1957).

The metabolism of 5β-cholestan-3α-ol, structurally identical with naturally occurring bile acids at C-3 and at the A/B ring juncture, was studied in man. [3β-^{3}H]5β-Cholestan-3α-ol was prepared and administered by vein to a subject with a bile fistula. The major portion of the radioactivity recovered from bile was found in neutral sterols. About 15% of the total, however, was associated with deoxycholic acid and the tritium was solely at the 3β position.

Materials and Methods

Radiochemical Purity of the Administered Sterol. [3β-^{3}H]5β-Cholestan-3α-ol was prepared by the reduction of 5β-cholestan-3-one with [^{3}H]lithium aluminum hydride (Rosenfeld *et al.*, 1967). To 100.4 mg of nonradioactive 5β-cholestan-3α-ol was added 1.0×10^7 cpm of the labeled compound (specific activity 99,600 cpm/mg). The material, in 2 ml of hot ethanol, was mixed with 5 ml of a solution of 1 g of digitonin in 80% ethanol. The nonprecipitable fraction was dissolved in 15 ml of dimethyl sulfoxide (Issidorides *et al.*, 1962) and extracted with petroleum ether (bp 30–60°). The petroleum ether was washed three times with 50% ethanol and afforded 99 mg of crystalline material. The digitonin separation was repeated to yield 97 mg in the nonprecipitable fraction. This was dissolved in 1 ml of pyridine and converted into the tosylate with 250 mg of *p*-toluenesulfonyl chloride in 1 ml of pyridine at room temperature overnight. After the usual extraction and washing procedures, 128 mg of 5β-cholestan-3α-yl tosylate was obtained and recrystallized twice from acetone. Radioactivity was measured at each

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[1] Trivial names used: chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanic acid.