Immunochemistry of Sperm Whale Myoglobin. IV. The Role of the Arginine Residues in the Conformation and Differentiation of Their Roles in the Antigenic Reactivity*

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ABSTRACT: Reaction of apomyoglobin with cyclohexanedione has been investigated. Modified apomyoglobin was recombined with ferriheme and the metmyoglobin obtained was subjected to chromatography on CM-cellulose. A homogeneous fraction was isolated which was modified at three arginine residues. The modified arginines were located at positions 31, 118, and 139. The derivative possessed spectral and hydrodynamic parameters identical with those of metmyoglobin X. With antisera to metymyoglobin X it reacted less than the homologous antigen. Apomyoglobin prepared from this metmyoglobin derivative also reacted less than unmodified apomyoglobin. Fragments obtained by cleavage of apomyoglobin at methionines-55 and -131 were also modified with cyclohexanedione. The immunochemical reaction of peptide 1–55 was unaffected upon modification of arginines 31 and 45. Similarly the inhibitory activity of peptide 132–153 did not change after modification of arginine-139. On the other hand, modification of arginine-118 in peptide 56–131 resulted in an appreciable decrease of the immunochemical reactivity of this peptide.

This decrease in the reaction of the modified peptide accounted for the entire loss of immune reaction obtained with modified apomyoglobin and almost all that obtained with modified metmyoglobin. These findings suggest that of the four arginine residues in metmyoglobin, only arginine-118 is located in an antigenic reactive region. The results also show that the environment of an amino acid type determines its involvement in the antigenic structure of a globular protein. The present data have been integrated with previous results concerning the antigenic structure of metmyoglobin.

The environment of a given amino acid side chain determines its contribution to the antigenic structure of a globular protein. The mode in which reactive regions[1] are incorporated into a given reactive site is difficult to investigate. Useful clues may be obtained from the X-ray crystallographic structure of the protein, since it has been demonstrated (Atassi and Saplin, 1968) that reactive regions in Mb[2] occupy corners between helices on the surface of the molecule. Conversely, assuming that this finding holds for other globular proteins, the molecular interaction of a protein with its antibody may be used as a conformation probe for investigating correspondence between solution and crystal structures. On the other hand, when the crystal structure is not available, determination of antigenic-reactive regions will locate some of the surface segments of the molecule. The incorporation of reactive regions into reactive sites may be studied by disruption of interactions between these regions by the use of denaturing agents or best by specific chemical modification of selected amino acid sites in the protein. Thus valuable information has been obtained concerning contribution, to antigenic structure, of the methionines (Atassi, 1967a, 1969), the tryptophans (Atassi and Caruso, 1968), and the tyrosines (Atassi, 1968). We wish now to report on the role of the four arginine residues.

Specific modification of the guanido group of arginine can be accomplished by condensation with 1,2-cyclohexanedione (Toi *et al.*, 1967). This paper describes the preparation and chemical and physicochemical characterization of myoglobin derivatives modified at three or less arginine residues. The immunochemical reactivities of the derivatives are reported and the contribution of each of the four arginine residues to the antigenic structure is differentiated.

Materials and Methods

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

Reaction of Apomyoglobin with 1,2-Cyclohexanedione. To a solution (50 ml) of ApoMb (23.25 μmol) in 0.1 N NaOH (50 ml) was added 930 μmol of CHD (Baker Chemical Co., Phillipsburg, N. J.) as a 5% solution in 0.1 N NaOH. The

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[1] The distinction between antigenic reactive *regions* and antigenic reactive *sites* has already been defined (Atassi and Saplin, 1968).

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Mb, metmyoglobin; ApoMb, apomyoglobin; MbX, the major chromatographic component 10 obtained by CM-cellulose chromatography (Atassi, 1964); CHD, 1,2-cyclohexanedione; CHD_3-Mb, metmyoglobin modified at three arginine residues with CHD; CHD_3-ApoMb, the apoprotein prepared from CHD_3-Mb; Cont-ApoMb, alkali-pretreated (0.1 N NaOH, room temperature, 2 hr) apomyoglobin; Cont-Mb, metmyoglobin prepared from recombination of Cont-ApoMb and unmodified ferriheme.

mixture was allowed to stir magnetically at room temperature for 2 hr, after which it was neutralized with 1 N HCl, dialyzed extensively against distilled water, cloudiness was removed by centrifugation, and supernatant was freeze dried. Extent of modification was determined by amino acid analysis of acid and alkaline hydrolysates. Since ApoMb might suffer alkaline denaturation that could influence its immunochemical behavior, a control sample was prepared in which ApoMb was subjected to the conditions used in the foregoing preparation but in this case CHD was not added.

Reconstitution of Metmyoglobin and Chromatography of the Derivative. The procedures for reconstitution of ApoMb with hemin chloride (Eastman Organic Chemicals) and for removal of excess ferriheme on CM-cellulose have been described elsewhere (Atassi and Caruso, 1968). However, in the present work, a longer column (2.5 × 60 cm) was used for the CHD derivative. After elution of excess ferriheme with 0.01 M NaH_2PO_4 containing 0.01% KCN (pH 6.2), the column was subjected to a gradient elution from pH 6.2 to 7.6 using 0.01 M phosphate buffers containing 0.01% KCN. The procedure for this chromatography has already been described in detail (Atassi, 1964).

Cleavage at the Methionine Sites and Modification of Peptides 1–55, 56–131, and 132–153 with CHD. Cleavage at the methionine sites 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 reaction with CHD, aliquots of 5% CHD solution in 0.1 N NaOH (calculated to contain 20 molar excess of CHD per arginine residue) were added to the peptide (8–9 mg) in 0.1 M NaOH. The mixture was allowed to stir magnetically at room temperature for 2 hr. Each reaction mixture was then filtered on a column (1 × 15 cm) of Sephadex G-10 which was eluted with water. The tubes containing the modified peptide were combined and freeze dried. Extent of arginine modification was determined by amino acid analysis. Since alkali treatment might influence the immunochemical reactivity of these peptides, control preparations (1–2 mg) were carried out in which each peptide was subjected to the conditions used in the reaction with CHD except that in this case, CHD was not added.

Antisera. These were prepared in goats and in rabbits against MbX and the procedure has been described in detail (Atassi, 1967a). Antisera from individual animals were kept separate and stored in 8–10-ml portions at −40°. Goat antisera G1, G3, and G4 and rabbit antiserum 77 were used in the present studies.

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

Tryptic Digestion and Peptide Mapping. Digestion with trypsin was done on ApoMb or CHD_3-ApoMb. For hydrolysis, the protein (9–10 mg) was in 2 ml of water at 37° and the pH was maintained at 8.0 on the pH-Stat by the addition of 0.1 N NaOH. Hydrolysis was started by the addition of 10 μl of 0.5% trypsin (DFP, twice crystallized, Worthington Biochem Corp.) in 10^{-3} N HCl. A second 10-μl aliquot of enzyme solution was added 2 hr after the first addition. Hydrolysis was allowed to continue for 16 hr after which the pH was lowered to 5.0 with 2 N acetic acid. The insoluble tryptic peptides were removed by centrifugation (5600 rpm, 30 min), washed on the centrifuge six times with distilled water, and the residue was freeze dried. This was subjected to hydrolysis and amino acid analysis. The supernatant, containing the soluble tryptic peptides was freeze dried, redissolved in 200 μl of water, and subjected to peptide mapping as described elsewhere (Atassi and Saplin, 1968). The peptide maps were stained with ninhydrin and/or with specific stains for various amino acids (Easley, 1965).

Results

Reaction of Apomyoglobin with 1,2-Cyclohexanedione. Reaction of CHD with proteins takes place under alkaline conditions (Toi *et al.*, 1967) and alkaline treatment might lead to some irreversible changes that could influence the reactivity of Mb with antibody. Trial preparations were therefore carried out in order to determine suitable alkali and CHD concentrations. Four portions of ApoMb (0.5 μmol) were dissolved in 0.1 N NaOH (1 ml each). To these, aliquots of CHD solution (50 mg in 1 ml of 0.1 N NaOH), calculated to contain 20, 30, 40, and 120 molar excess of CHD, respectively, were added. Mixtures were stirred magnetically at room temperature for 2 hr after which they were neutralized with 1 N HCl, dialyzed extensively against distilled water, and freeze dried. A similar set of four reactions was carried out in 0.2 N NaOH. The foregoing derivatives were then recombined with unmodified ferriheme and each examined by electrophoresis on starch gel. Each of the above preparations was heterogeneous. Bands observed had mobilities (relative to MbX = 1): 2.42, 3.98, 5.32, and 6.26. The relative distribution of intensities depended upon the preparation. Reactions with 20 or 30 molar excess CHD gave products containing negligible amounts of the faster moving components. Products from reaction with 40 or 120 molar excess of CHD contained more of the faster moving components, and only traces of unmodified MbX. Reaction with 40 molar excess was therefore selected for the large-scale preparation. Since no differences were observed when reactions were carried out in 0.1 or 0.2 N NaOH, the former was employed in the present preparation.

Determination of the Modified Residues. Chromatography of the recombined CHD-Mb (obtained from ApoMb that had been reacted with 40 molar excess of CHD) was carried

TABLE I: Amino Acid Composition of MbX and Its CHD Derivatives.[a]

Amino Acid	Controls		CHD Derivatives[b]				
	MbX	Cont-Mb	Component 1	Component 2	Component 3	Component 4	Component 5
Trp	1.93	1.90	1.89	1.83	1.91	1.94	1.79
Lys	19.0	19.1	19.0	18.9	18.8	19.0	18.9
His	11.9	12.0	11.8	12.0	11.9	11.9	12.0
Arg	4.02	4.03	1.03	2.32	3.26	3.18	3.99
Asp	7.96	8.10	8.13	8.05	7.97	8.20	7.99
Thr	5.03	4.89	4.95	5.12	4.88	4.81	5.10
Ser	5.86	5.81	5.82	5.91	5.93	5.79	5.67
Glu	19.1	19.0	18.7	18.9	18.9	19.2	18.8
Pro	4.11	4.06	3.96	3.99	4.01	4.18	4.29
Gly	11.1	10.9	10.8	11.0	11.2	10.9	11.0
Ala	16.8	16.9	17.2	17.0	17.0	16.6	17.2
Val	7.88	7.94	8.08	7.83	7.62	7.75	7.77
Met	2.02	2.11	1.95	2.03	2.12	2.06	1.83
Ile	8.71	8.69	8.76	8.72	9.01	8.54	8.75
Leu	17.9	18.0	18.2	18.1	18.0	17.9	18.3
Tyr	2.97	3.05	2.89	3.03	3.01	3.00	2.95
Phe	5.89	6.01	6.26	6.22	5.88	5.86	5.97

[a] The results represent the average of four acid hydrolyses (two 22 and two 72 hr). Tryptophan was determined from duplicate alkaline hydrolyses. Values for serine and threonine were obtained by extrapolation to zero hydrolysis time. The amino acid composition is in residues per mole. [b] These represent the chromatographic components obtained from the recombined CHD reaction product (see Figure 1).

out on CM-cellulose. Five components were obtained (Figure 1). Each component was subjected to rechromatography on a similar column and purity of each preparation was monitored by electrophoresis. After two rechromatography experiments, peak 1 was obtained in an electrophoretically homogeneous form and corresponded to the band with mobility 5.32 (relative to MbX = 1). Peaks 2, 3, and 4 could not be obtained in an electrophoretically homogeneous form even after four rechromatography experiments. However, 65–75% enrichment in certain electrophoretic bands was obtained and chromatographic peaks corresponded to the following relative electrophoretic mobilities: peak 2, 3.98; peak 3, 2.42; peak 4, 2.42; and peak 5, 1. The splitting of each of these myoglobin derivatives on electrophoresis might be related to the existence of Mb in various molecular species with identical chemical and immunochemical properties but differing only in charge (Atassi, 1964).

Increase in electrophoretic mobilities of the various components is accounted for entirely by modification of the arginine residues. Table I shows the amino acid composition of the various chromatographic components. It can be seen that in peak 1, three arginine residues were modified. Component 2 had 2.32 modified arginine residues, but it was contaminated with components 3 and 4 to the extent of 30%. Therefore completely pure component 2 will contain 2.02 modified arginine residues. Component 3 whose arginine content was 3.26

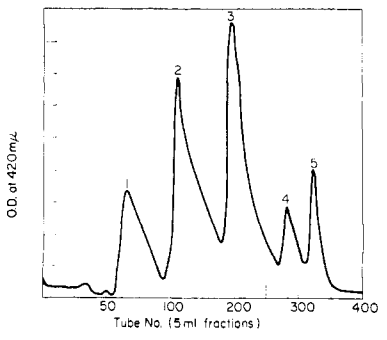


FIGURE 1: Chromatogram of recombined CHD-Mb on CM-cellulose. CHD-ApoMb (400 mg) was recombined with ferriheme and applied on CM-cellulose column (2.5 × 60 cm). For details, see text.

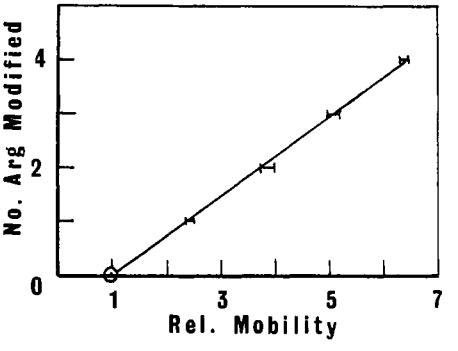


FIGURE 2: A plot showing change in relative electrophoretic mobility for number of arginine residues modified with CHD. Mobilities are expressed relative to the mobility of MbX = 1.

TABLE II: Absorption Maxima and Ratios of Optical Densities at These Maxima of MbX and CHD_3-Mb.[a]

	Absorption Max (mμ)					Ratios of Optical Densities at Absorption Max			
	A	B	C	D	E	A/E	B/E	C/E	D/E
MbX		280	359	424	542		3.21	2.86	10.06
Cont-Mb		280	359	424	542		3.17	2.89	10.02
CHD_3-Mb	270	280 (sh)	357	422	538	3.40	3.32	2.96	10.67[b]

[a] Solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%). [b] When the absorption of this derivative at 422 mμ is corrected for the contribution of CHD_3-ApoMb this ratio becomes 9.95.

residues was contaminated with component 4 (30%) and with component 2 (20%). This component therefore has one modified arginine residue. Similarly component 4, with 3.18 unmodified arginines, was contaminated with 3 (30%), 2 (15%), and 5 (10%). Figure 2 shows that electrophoretic mobility increases linearly with the number of arginine residues modified. A derivative in which all four arginine residues are modified could not be prepared even when reaction was carried out in the presence of 1200 molar excess of CHD for 4 hr. Only traces (0.4 mg) of a component migrating on electrophoresis with a relative mobility of 6.26 could be isolated in the products (400 mg) of reaction with 40 molar excess of CHD. This component was modified at all four arginine residues. However, since only slight traces were available, a single analysis was performed on the short column of the analyzer.

In order to determine the location of the modified arginine residues, tryptic digests of components 1, 2, 3, and 4 were examined. The insoluble portions of the tryptic digests of each of components 2, 3, and 4 had one unmodified arginine residue. Peptide mapping on the soluble portions of the digests of components 2, 3, and 4 gave ambiguous results. This is not surprising since none of these components could be isolated in an electrophoretically homogeneous form and appreciable cross-contaminations were present. However, component 1 was homogeneous and it was possible to locate the positions of the modified arginine residues. It has been shown (Edmundson, 1963) that arginine-118 falls in an insoluble tryptic peptide (*i.e.*, peptide 103–118). Amino acid analysis showed that the insoluble tryptic peptides of component 1 contained no unmodified arginine and that the soluble tryptic peptides had one unmodified arginine residue present. The peptide maps of the soluble tryptic peptides of ApoMb and of component 1 are shown in Figure 3. ApoMb gives three spots that stain positively with Sakaguchi stain for arginine. These are designated A_I, A_{II}, and A_{III}. In the maps of component I only one peptide, B_I, gave a positive stain for arginine. The position of peptide B_I superimposed well with the position of peptide A_I. To confirm this and determine the identity of these two peptides, spots A_I and B_I were cut out from lightly stained maps (0.05% ninhydrin in ethanol), eluted with 0.2 N acetic acid, cleared by centrifugation, and evaporated to dryness on a rotary evaporator. The residue was dissolved in 0.2 N acetic acid (1 ml) and filtered on a column (1 × 35 cm) of Sephadex G-10 in order to remove any contaminating larger peptides. Ninhydrin-positive material in the fractions (0.5 ml) was revealed by applying small spots from alternate tubes on a filter paper, drying the latter, and staining it with ninhydrin. Only traces of contaminating larger peptides were present and

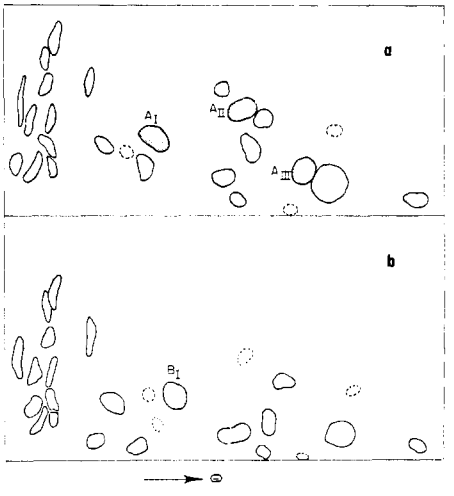


FIGURE 3: A tracing of the peptide maps of the soluble tryptic peptides of (a) MbX and (b) CHD_3-Mb. Shaded spots represent the only peptides that gave a positive reaction with Sakaguchi stain for arginine. Peptides A_I and B_I corresponded to sequence 43–45, A_{II} corresponded to sequence 134–139, and peptide A_{III} corresponded to sequence 17–31. For details, see text.

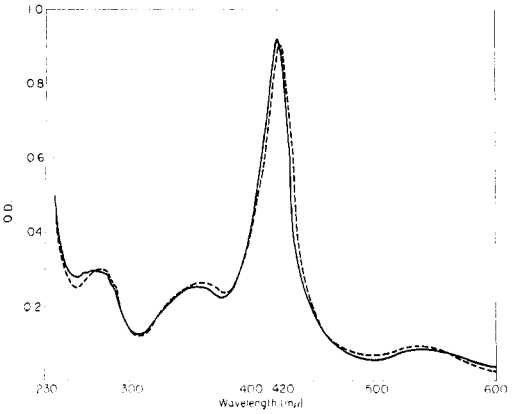


FIGURE 4: Absorption spectra of CHD_3-Mb (solid line) and of MbX (dashed line). Solutions were in 0.01 M phosphate buffer (pH 7.2) containing KCN (0.01%).

TABLE III: Amino Acid Compositions of Peptides 1–55, 56–131, and 132–153 and Their CHD Derivatives.[a]

Amino Acid	Peptide 1–55[b]		Peptide 56–131		Peptide 132–153	
	Unmodified	CHD Derivative	Unmodified	CHD Derivative	Unmodified	CHD Derivative
Trp	1.91	1.83				
Lys	4.91	4.86	9.86	9.87	3.99	4.00
His	3.71	4.06	7.95	8.09	0.01	0
Arg	2.03	0.100	1.00	0.086	1.01	0.110
Asp	3.11	2.94	3.01	2.98	1.93	1.87
Thr	1.90	1.79	2.84	2.91	0.03	0
Ser	2.05	2.22	3.99	3.95	0.01	0
Glu	8.96	8.47	7.03	7.01	3.26	3.15
Pro	1.21	1.14	3.01	3.00		
Gly	2.98	2.86	6.10	5.93	2.04	2.00
Ala	4.18	3.84	9.97	10.0	3.16	3.05
Val	4.25	4.29	2.88	2.96		
Ile	1.90	1.84	5.82	5.78	1.17	1.11
Leu	7.14	6.99	8.04	8.11	3.27	3.20
Tyr	0	0	1.01	0.990	2.03	1.99
Phe	2.77	2.78	1.97	2.02	1.02	1.01

[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 threonine and serine have been extrapolated to zero hydrolysis time. [b] Values for tryptophan in this peptide were obtained by duplicate alkaline hydrolyses.

moved out in the void volume. The fractions belonging to the bulk of the ninhydrin-positive material, which was retarded, were combined, evaporated to dryness, and subjected to hydrolysis and amino acid analysis. Peptide A_I had the following composition: Asp, 0.91; Phe, 1.00; and Arg, 0.96. Peptide B_I had the following composition: Asp, 0.88; Phe, 1.00: and Arg, 0.94. It is therefore clear that peptides A_I and B_I are identical. From the known sequence of Mb and the positions of tryptic attack it can be concluded that these two peptides corresponded to sequence 43–45. Therefore, arginine-45 was not modified in component I.

Peptides A_{II} and A_{III} were also extracted, centrifuged, dried, hydrolyzed, and subjected to amino acid analysis. It suffices to mention here that peptide A_{II} corresponded to sequence 134–139 and peptide A_{III} corresponded to sequence 17–31. In conclusion arginines-31, -118, and -139 were modified in component I. This component will therefore be designated CHD_3-Mb.

Properties of the Modified Protein. The electrophoretic behavior of the CHD derivatives has been described. Table II summarizes the spectral properties of the cyanmet forms of MbX and of CHD_3-Mb. The spectrum of CHD_3-Mb in its cyanmet form (Figure 4) showed peaks at 270, 280 (sh), 290 (sh), 357, 422, and 538 mμ. There was an appreciable increase in the extinction at the Soret peak. However, ApoMb that had been reacted with CHD was yellow before recombination with ferriheme. In an attempt to account for the increase in absorption at the Soret band, the apoprotein of CHD_3-Mb was prepared and its spectral behavior was investigated. Figure 5 shows the spectra of ApoMb and CHD_3-ApoMb. CHD_3-ApoMb has an absorption maximum in the visible region at 407 mμ. When the absorption at 422 mμ in CHD_3-Mb is corrected for the contribution of CHD_3-ApoMb in that region, then the absorption of CHD_3-Mb becomes identical with that of MbX (see Table II).

Gel filtration was carried out on a Sephadex G-75 column which had been calibrated with human serum albumin, hen ovalbumin, bovine ribonuclease A, and horse heart cytochrome *c*. Calculation of the molecular parameters of the derivatives was done by the procedure recently described in detail (Atassi and Caruso, 1968). By this procedure, MbX and all CHD-Mb chromatographic components possessed identical values for Stokes radius (18.5 Å) and f/f_0 (1.06). In sedimentation studies, MbX and all components from the reaction with CHD had identical $s^0_{20,w}$ values (2.05 ± 0.04 S).

Modification of the Peptides Obtained by Cleavage at the Methionine Sites. 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 compositions of peptides 1–55, 56–131, and 132–153 together with their corresponding CHD derivatives. Reaction with CHD leads to the complete modification of the arginine residue(s) in each peptide. No other amino acids were modified.

Immunochemical Reactivity of the Myoglobin Derivatives. All CHD-myoglobin components and alkaline-pretreated controls gave single lines in agar double-diffusion experiments with each of the sera tested. No spurs or intersections were formed with the line given by MbX. In quantitative precipitin analyses, the reaction curves of MbX and Cont-Mb superimposed well with all the sera examined here. Similarly ApoMb and Cont-ApoMb were immunochemically identical. Also components 2, 3, and 4 possessed antigenic reactivities

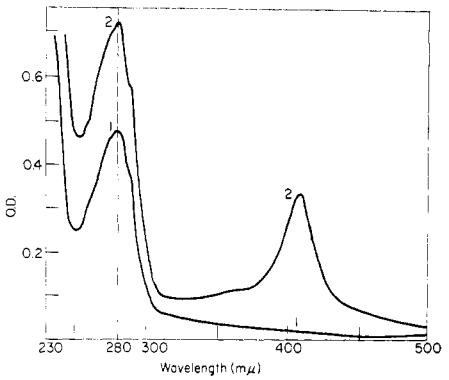


FIGURE 5: Absorption spectra of (1) ApoMb (59.2 μg of N/ml) and (2) CHD_3-ApoMb (82.5 μg of N/ml) solutions were in 0.15 M NaCl.

identical with that given by the homologous antigen. Table IV summarizes the precipitin reactions of these components with antisera 77, G1, G3, and G4. Since all the present studies were performed on myoglobins obtained by recombination of ApoMb or modified ApoMb with ferriheme, it is relevant to mention here that MbX and recombined Mb have already been shown to be physicochemically and immunochemically identical (Atassi, 1967b).

The antigenic reactivity of CHD_3-Mb was appreciably lower, with each serum, than the homologous reaction. The reactions of CHD_3-Mb with antisera 77 and G4 are shown in Figure 6 and Table V summarizes the results of several precipitin analyses with antisera G1, G3, G4, and 77. In Table V also, the immunochemical behavior of CHD_3-ApoMb is summarized. The reaction of CHD_3-ApoMb (relative to reaction of ApoMb or Cont-ApoMb) was lower than the reaction of CHD_3-Mb (relative to reaction of MbX or Cont-Mb).

Due to the difficulty in obtaining homogeneous preparations of partially modified components 2, 3, and 4, it was decided to study the immunochemistry of arginine-modified peptides obtained by cleavage of ApoMb at the methionine sites. Information obtained from these studies should aid in the differentiation of the roles played by the various arginine residues in the antigenic structure of Mb.

Immunochemistry of the Modified Peptides. A. PEPTIDE 1–55. The specific interaction of this peptide with six different rabbit and goat antisera to MbX and ApoMb has been reported in detail (Atassi and Saplin, 1968). It was shown that this peptide gave a specific immune precipitate with all the sera tested. Fragment 1–55 and its CHD derivative precipitated equal amounts of antibody with each of antisera G1, G4, and 77. In addition, each antiserum was absorbed with the minimum amounts of fragments 1–55 or its CHD derivative necessary to achieve maximum precipitation and the reactions of the supernatants with MbX were determined. With each antiserum, the reactivities remaining in the supernatants, from absorption with the fragment or its derivative, were identical. The results of these experiments are summarized in Table VI. The results clearly show that peptide 1–55 and its CHD derivative are equally reactive immuno-

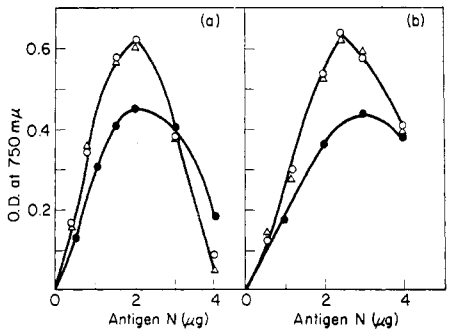


FIGURE 6: Precipitin reactions on MbX (○), Cont-Mb (△), and CHD_3-Mb (●). (a) Reactions with goat antiserum G4. (b) Reactions with rabbit antiserum 77. For assay, precipitates were dissolved in 0.5 ml of 0.5 N NaOH and then determined with the Folin–Lowry method (Lowry *et al.*, 1951).

TABLE IV: Precipitin Reactions with CHD Derivatives of Metmyoglobin.[a]

	% Precipitate Relative to Reaction of Antiserum with MbX[b]			
Antiserum	Component 1	Component 2	Component 3	Component 4
G1		98.3	99.3	97.9
G3	72.5	98.7	99.6	99.8
G4	72.4	99.1	99.5	100.0
77	68.9	100.1	99.3	98.8

[a] Reaction was carried out (with three goat antisera and one rabbit antiserum to MbX) on the chromatographic components obtained from the recombined CHD reaction product (see Figure 1). [b] Results are expressed as per cent precipitation by each component, at equivalence relative to the homologous reaction. Each value (except those of component 1) represents the average of two independent precipitin analyses. Values for component 1 are obtained from Table V.

TABLE V: Relative Amounts of Precipitation Formed by CHD_3-ApoMb and CHD_3-Mb.[a]

Antiserum	CHD_3-ApoMb, % Ppt Rel to Reaction of Antiserum with ApoMb	CHD_3-Mb, % Ppt Rel to Reaction of Antiserum with MbX
G1	60.2 ± 1.3	
G3	61.7 ± 1.1	72.5 ± 0.96
G4	63.52 ± 1.3	72.4 ± 1.2
77	65.8 ± 1.0	68.9 ± 1.1

[a] Reaction was carried out with three goat antisera and one rabbit antiserum to MbX. Results are expressed in per cent precipitation, at equivalence, relative to reaction of serum with ApoMb or MbX. Values were obtained from three independent determinations.

TABLE VI: Relative Amounts of Precipitation Formed by Peptides 1–55, 56–131, and Their CHD Derivatives.

	% Ppt Relative to Reaction of Antiserum with MbX[a]					
	Peptide 1–55			Peptide 56–131		
Antiserum	Untreated[b]	Control[c]	CHD Derivative	Untreated[b]	Control[c]	CHD Derivative
G1	27.6	27.4	27.3	56.9	55.4	14.9
G3				51.7	51.2	13.10
G4	8.20	8.17	8.04	81.9	75.1	36.7
77	25.6	25.1	24.9	49.0	48.3	13.3

[a] The percentage of precipitation relative to MbX was derived from maximum precipitation by peptide and precipitation at equivalence by MbX. Values represent the average of three analyses each and ranged ±1.3% or better. [b] Values obtained from untreated peptide. [c] Values obtained with alkali-pretreated peptide control preparations. Notice that alkali treatment did not influence the immunochemical reactivity of the peptide.

chemically. It is relevant to mention here that peptide 1–55 and alkaline-pretreated control were immunochemically similar (Table VI).

B. FRAGMENT 56–131 MODIFIED AT ARGININE-118. The reactivity of fragment 56–131 with several rabbit and goat antisera to MbX and to ApoMb has already been reported (Atassi and Saplin, 1968). With all the antisera tested, fragment 56–131 formed an immune precipitate. In addition, it was shown that, of the three fragments obtained by cleavage at the two methionines, peptide 56–131 was the immunodominant region of the molecule. Figure 7 shows the precipitin reactions of peptide 56–131 and its CHD derivative with rabbit antiserum 77. It can be seen that the modified peptide precipitated appreciably less antibody than peptide 56–131. Similar results were obtained with antisera G1, G3, and G4. Upon absorption of each serum with the minimum amount of peptide or derivative, necessary to effect maximum precipitation, the reactivities remaining in the supernatants toward MbX were different. The supernatant from absorption with peptide 56–131 reacted less efficiently than the supernatant from the CHD derivative. In each case, the immune precipitate given by the peptide accounted completely for the loss of supernatant reactivity with MbX (Figure 7). The results with various antisera are summarized in Table VI. It may be pointed out here that peptide 56–131 exhibited virtually no immunochemical changes as a result of alkaline treatment.

C. PEPTIDE 132–153 MODIFIED AT ARGININE-139. Peptide 132–153 has been previously shown to be an inhibitory peptide with six different rabbit and goat antisera to MbX and ApoMb (Atassi and Saplin, 1968). Its maximum inhibitory activity ranged between 11 and 21% with MbX and between 14 and 21% with ApoMb. Figure 8 gives the inhibition curves obtained with peptide 132–153 and its CHD derivative with antiserum G4 and it shows that the inhibitory activities of the two peptides were identical with this serum. Similar re-

TABLE VII: Maximum Inhibitory Activities of Peptide 132–153 and Its CHD Derivative[a]

Antiserum	Peptide 132–153	CHD Derivative
G3	18.3 ± 1.1 (5.3)	18.0 ± 1.2 (5.1)
G4	13.5 ± 1.3 (13.4)	13.4 ± 0.9 (13.7)
77	21.0 ± 1.0 (6.8)	20.5 ± 1.4 (7.0)

[a] Results are expressed in maximum per cent inhibition by the peptide of the precipitin reaction of MbX with various antisera. Values in parentheses represent peptide/antigen molar ratio at 50% maximum inhibition. Untreated peptide and alkali-pretreated control also possessed identical inhibitory activities.

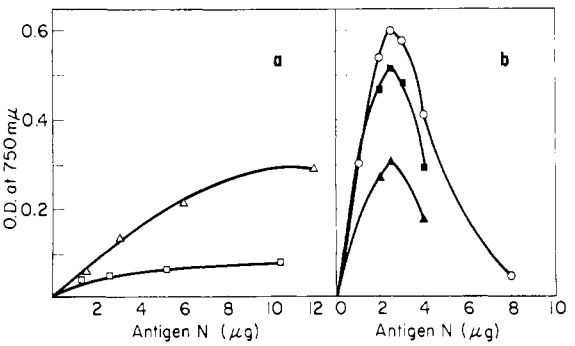


FIGURE 7: Precipitin reactions and absorption experiments on peptides 56–131 and its CHD derivative with rabbit antiserum 77. Part a shows the reaction of peptide 56–131 (△) and its CHD derivative (□) with antiserum which had been diluted 1:1 with 0.15 M NaCl. In part b reactions with MbX are shown with serum which had been absorbed with peptide 56–131 (▲) and CHD derivative of peptide 56–131 (■). For comparison, the reaction of antiserum 77 which had been diluted 1:1 with 0.15 M NaCl (to account for the volume of peptide solutions used in absorption experiments) is shown. Notice that peptide 56–131 precipitated at maximum 48.3% relative to MbX and absorption of the serum with this peptide removed 49.2% of the serum reactivity toward MbX. On the other hand, the CHD derivative of peptide 56–131 precipitated at maximum 13.3% relative to MbX and absorption of the serum with this peptide removed 14.8% of serum reactivity toward MbX. Therefore as a result of modification of arginine-118, decrease in reaction with serum 77 of the peptide was 35.0%, by direct precipitin reactions on the peptide, and 34.4% from absorption experiments.

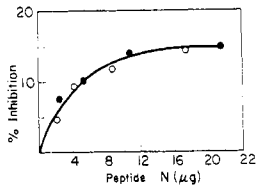


FIGURE 8: Inhibition experiments with antiserum G4 and peptide 132–153 (○) and its CHD derivative (●).

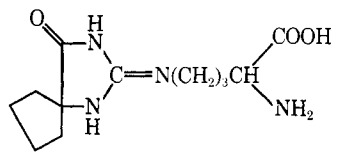
sults were obtained with all the sera tested in the present work and these findings are summarized in Table VII. It may be mentioned here that alkali pretreatment of peptide 132–153 did not affect its immunochemical reactivity.

Discussion

It is necessary at the outset to consider the modification of the arginine residues with CHD. In this particular case, three factors must be considered: (a) conditions of the reaction are rather drastic; (b) specificity of the reaction; and (c) whether the modification of the arginine residues is sufficient to influence involvement of these residues in the biological function in which they normally participate.

In modification of the arginine residues of ApoMb, the alkaline condition necessary for reaction was of major concern. Treatment with alkali might result in denaturation that could influence the antigenic reactivity of the protein. Such irreversible changes with immunochemical effects have already been shown to take place upon treatment with 8 M urea at pH 3.0 (Atassi and Caruso, 1968). Therefore treatment with 0.1 N NaOH may even have a more pronounced effect. Preliminary investigations were carried out in which ApoMb was subjected to 0.1 or to 0.2 N NaOH for 2 hr at room temperature. Upon neutralization and dialysis, appreciable precipitation (up to 50%) was obtained. Nevertheless, the portion remaining in solution showed immunochemical behavior completely identical with that of ApoMb. Also, after recombination with ferriheme, Mb from alkali-pretreated ApoMb was immunochemically identical with MbX. These controls strongly suggested that the reaction may be utilized for the modification of arginine residues in Mb. The present results do not exclude that, with other proteins, alkaline denaturation might be a limiting factor, and therefore each protein will have to be investigated independently with appropriate controls.

Reaction of CHD with ApoMb was completely specific for arginine. No modification of other amino acids was detected in acid and alkaline hydrolysates. CHD condenses with the guanido group of arginine to give N^5-(4-oxo-1,3-diazaspiro-[4.4]non-2-ylidene)-L-ornithine (Toi *et al.*, 1967) or CHD-arginine (I). Recently, Atassi and Habeeb (1969) showed that changes in biological activity of a modified protein are dependent upon nature of modification. It can be seen that, in addition to the loss of the positive charge of the side chain of arginine, the modified residues will exert a considerable steric influence. If participation of an arginine residue in antigenic reaction is due to charge interaction with the antibody combining site, then its modification with CHD should completely eliminate such interaction. The modification used here will therefore be sufficient to reveal any contribution of the arginine residues to the antigenic structure of Mb.



(1)

The arginine residues in ApoMb showed remarkable differences in their accessibility to the reaction with CHD. It is also significant that arginine-45 did not react when a large molar excess (1200) of CHD was employed even though the protein is expected to be completely unfolded under the alkaline conditions of the reaction. Arginine-118 followed in the order of reactivity. It is perhaps relevant to mention here that differences in the availabilities of the two tryptophan residues to reaction with 2-hydroxy-5-nitrobenzyl bromide were also observed for ApoMb in 8 M urea at pH 3.0 (Atassi and Caruso, 1968). Under similar conditions, α-chymotrypsin also exhibited unequal accessibility of the tryptophans to reaction with 2-hydroxy-5-nitrobenzyl bromide (Koshland *et al.*, 1964). Aune *et al.* (1967) reported that ribonuclease, lysozyme, and chymotrypsinogen retained regions of ordered structure on acid and heat denaturation but these regions were susceptible to destruction by guanidine hydrochloride. It was recently suggested (Miller and Goebel, 1968) that in 6 M guanidine hydrochloride, proteins possess appreciable regions that are highly associated but not regularly ordered. These and many other examples in the literature indicate that, under some dissociating conditions, a protein is not unraveled in such a way that equal exposure of all residues is obtained.

The decrease in antigenic reactivity observed with CHD_3-Mb could suggest involvement of one (or more) arginine residue(s) in the reaction of Mb with its antibody. On the other hand, the presence of conformational changes as a result of modification might influence the immunochemical properties of the protein. Whatever caused the immunochemical change, clearly it was not due to alkaline denaturation. That MbX, Cont-Mb, and CHD_3-Mb possessed identical conformational parameters (*i.e.*, Stokes radius and frictional ratio values) suggests that appreciable conformational changes may not be present. It is noteworthy that arginines-31 and -139 are present in the long helices B and H, respectively (Kendrew *et al.*, 1961), and it is therefore significant that their modification does not destroy the integrity of these helices. The conformation of CHD_3-Mb is therefore highly interesting and the present data do not rule out the presence of small conformational changes that may not be detectable by the methods employed here. A more detailed study of the conformation of this derivative is in progress and will be reported later.

It is significant that CHD_3-ApoMb reacted less efficiently than ApoMb with antisera to MbX. Since ApoMb is more unfolded in any case than Mb (Harrison and Blout, 1965; Breslow *et al.*, 1965; Atassi and Cacciotti, 1966; Epand and Scheraga, 1968), the decrease in the reactivity is more likely to be due to the modification rather than to conformational changes. However, the possibility of conformational changes common to both CHD_3-Mb and CHD_3-ApoMb should not be overlooked. The chromatographic components 2, 3, and 4 each possessed antigenic reactivity equal to that of the homologous antigen. Accordingly, it was tempting to conclude that an arginine residue, which was modified in CHD_3-Mb but not in the other three

derivatives, was responsible for the decrease of immunochemical reaction obtained with CHD_3-Mb. In each of components 2, 3, and 4 arginine-118 appeared to be unmodified. The three derivatives also possessed conformational parameters identical with those of MbX or Cont-Mb. The present data appeared to imply that arginine-118 is located in an antigenic reactive region in Mb. However, due to the heterogeneity of components 2, 3, and 4, the results must be treated with caution and may be considered at best, suggestive but not conclusive. Some stronger independent evidence was obviously required and it was derived from the immunochemical behavior of the modified peptides.

Peptide 1–55 and its derivative modified at both arginines-31 and -45 precipitated equivalent amounts of antibody from antisera to MbX. It is relevant to mention here that peptide 17–31, which was isolated from tryptic hydrolysates of ApoMb, possessed significant immunochemical inhibitory activity (Atassi and Saplin, 1968). The present results suggest that arginine-31 is not involved in the specific interaction of peptide 17–31. Another tryptic peptide (peptide 35–42) was shown to possess almost no inhibitory activity (Atassi and Saplin, 1968), and this inactive region may extend to, and include, arginine-45. Also, it may be concluded that the loss of some antigenic reactivity obtained with CHD_3-Mb is not caused by the modification of arginine-31.

The ability of peptide 56–131 to form immune precipitates was appreciably impaired upon modification of arginine-118. This decrease in the activity of the peptide would account for the entire loss of immune reaction obtained with CHD_3-ApoMb and almost all that obtained with CHD_3-Mb (Table VIII). An explanation for this is that the free peptide will be able to approximate more frequently the configuration it has in ApoMb than that it possesses in Mb. The loss of precipitating power in the modified peptide was not due to alkali treatment. Conformational changes in the peptide, as a result of modification, are not too significant here. Recently Atassi and Saplin (1968) concluded, from the immunochemical behavior of peptides obtained by cleavage at the methionine sites and that of other ApoMb peptides, that these peptides are not able to assume, in an appreciable way, the configuration they have in the native protein. These conclusions have been confirmed by the recent results of Epand and Scheraga (1968) from optical rotatory dispersion and circular dichroism measurements on the cyanogen bromide fragments of Mb. Therefore modification of arginine-118 will not be expected to have a meaningful effect on the configuration of isolated peptide 56–131. Accordingly differences in immunochemical behavior will not arise from configurational changes. The only satisfactory explanation for the present data is that arginine-118 is located in an antigenic reactive region in Mb.

The inhibitory activity of peptide 132–153 was unchanged on modification of arginine-139. This leads to the conclusion that arginine-139 is not part of a reactive region in that peptide. Confirmation of this conclusion was derived from the finding that CHD_3-Mb reacted equally as well as MbX with antisera to fragment 132–153 (Atassi and Saplin, 1968).

It may be necessary at this stage to offer briefly an integration of the present data with all previous findings concerning the antigenic structure of Mb. A systematic movement along the polypeptide chain will be employed. By modification at tryptophan-7 (Atassi and Caruso, 1968) and by cleavage at tryptophan-7 with periodate (Atassi, 1967c; Atassi and Saplin, 1968), the N-terminal heptapeptide in helix A is not located in an active region in Mb. The N-terminal heptapeptide was also isolated from a chymotryptic digest of ApoMb (Crumpton and Wilkinson, 1965) and shown to be noninhibitory. The region between tryptophan-7 and tryptophan-14 is inconclusive since modification of both tryptophans-7 and -14 resulted in appreciable unfolding in Mb (Atassi and Caruso, 1968). Moving along the polypeptide chain, the bend AB and most of helix B (peptide 17–31) comprises an active region (Atassi and Saplin, 1968) which does not include arginine-31 (the present work). Between the antigenically nonreactive arginines-31 and -45, tryptic peptide 35–42 was found inactive. Therefore, this inactive region quite likely extends all the length between residues 31–45 (part of helix B, all of helix C and a portion of the bend CD) and includes arginines-31 and -45. Not much is known concerning the region 46–55 (most of bend CD and part of helix D), but methionine-55 is not located in an antigenic reactive region (Atassi, 1967a). Information concerning any reactive regions on helix E (sequence 58–77) is inconclusive and further investigations are now being carried out. In the bend EF, helix F and part of bend FG (sequence 79–96) an active region (or regions) is located (Atassi and Saplin, 1968). This reactive region is terminated before tyrosine-103 (in helix G) which is not part of a reactive region (Atassi, 1968). Between tyrosine-103 in helix G and arginine-118 in the same helix a reactive region has already started with arginine-118 forming a part of that region (the present paper). This same reactive region extends into sequence 119–133 (an inhibitory tryptic peptide, Atassi and Saplin, 1968) which occupies all of bend GH and part of helix H. It is likely, however, that this reactive region does not extend to include lysine-133 but terminates before methionine-131 which is not located in a reactive region (Atassi, 1967a). The region between lysine-133 and arginine-139 in helix H is probably inactive since the latter does not contribute to the antigenic structure (the present paper). The C-terminal hexapeptide carries a reactive region (Crumpton, 1967; Atassi and Saplin, 1968). The segment around the tyrosyl residues at 146 and 151 (at the end of helix H and the beginning of the

TABLE VIII: Decrease in Relative Amount of Immune Precipitate Obtained with Derivative of Peptide 56–131, CHD_3-ApoMb, and CHD_3-Mb.

	Decrease in % Relative Precipitation Due to Modification		
Antiserum	CHD Derivative of Peptide 56–131 Rel to Control Peptide 56–131[a]	CHD_3-ApoMb Rel to ApoMb[b]	CHD_3-Mb Rel to MbX[b]
G1	40.5	39.8	
G3	38.1	38.3	27.5
G4	38.4	36.5	27.6
77	35.0	34.2	31.1

[a] Obtained from Table VI. [b] Obtained from Table V.

randomly folded C-terminal pentapeptide) probably represents the only such reactive region in segment 132–153. Nitration of the tyrosines completely abolishes the activity of peptide 132–153 (Atassi, 1968). This reactive region terminates at (but includes) tyrosine-151 because the C-terminal dipeptide is not part of a reactive region in MbX (Atassi, 1966). Finally the heme group in myoglobin and hemoglobin is not part of an antigenic site (Reichlin *et al.*, 1963). The antigenic reactivity is sensitive to conformational changes of myoglobin (Atassi, 1967b). Artificial myoglobin derivatives, prepared with various modified and metalloporphyrins (Atassi, 1967b), afforded good models in which the location of the modification clearly did not involve an antigenic reactive region. The conformational reorganization, which was caused by the different coordination tendencies of the various metals or by the modification of the side chains of the heme, gave rise to appreciable changes in antigenic reactivity (Atassi, 1967b).

This has been only a translational scanning along the direction of the polypeptide chain to give locations of reactive regions along the chain. The information represents the most advanced knowledge concerning the antigenic structure of a globular protein. Nevertheless the mode into which reactive regions, from distant parts in the sequence come together in the three-dimensional structure to be incorporated into reactive sites, is still not too clear. It may be partially understood by relying heavily on the X-ray structure and consequently being trapped into excessive speculation.

The finding that conformational changes in myoglobin (Atassi, 1967b) and hemoglobin (Atassi and Skalski, 1969) will influence reactivity with antibodies to the native antigen supports the hypothesis that the primary antibody response is directed against the native three-dimensional structure of a globular protein. The presence of the reactive regions at discreet surface locations of the molecule and the failure to uncover more reactive regions (Atassi and Saplin, 1968) in the three fragments obtained by cleavage at the two antigenically inactive methionines (Atassi, 1967a) suggest that transcription of a protein antigen does not proceed through proteolytic fragmentation of the protein. However, this might be relatively more significant in late-course sera.

In conclusion, the present data show that of the four arginine residues in myoglobin only arginine-118 is located in an antigenic-reactive region. This confirms previous observations with the tyrosine residues (Atassi, 1968) that the environment determines the involvement of an amino acid type in the antigenic structure of a globular protein. The environment will be the immediate sequence around the residue in question as well as the approaching folds of the polypeptide chain from otherwise distant regions in the sequence. The results concerning the arginine residues have been integrated with previous data concerning the antigenic structure of Mb.

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