Reaction of Myoglobin with 3,3-Tetramethyleneglutaric Anhydride

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(Received 11 July 1966)

1. The extent of reaction of the protein with 3,3-tetramethyleneglutaric anhydride was measured by determining the loss of lysine and the equivalent appearance of 3,3-tetramethyleneglutarimidolysine in the hydrolysate at various time-intervals. Of the 19 lysine residues in sperm-whale myoglobin, three did not react with 3,3-tetramethyleneglutaric anhydride. Also, the *N*-terminal valine did not react. 2. Slight spectral changes were observed on modification of myoglobin. 3. Electrophoretic changes were great since the derivative was negative throughout the range pH 6–8.8. 4. The derivative did not react with antisera to native sperm-whale myoglobin.

There is now a variety of chemical reagents available for modification of lysine residues in proteins and peptides. These reagents usually react with other amino acids. In addition, determination of the extent of substitution at the ϵ -amino groups is not easy and does not achieve a high degree of accuracy. When acetylation is used the extent of modification can be determined by titration of the acetic acid obtained on steam-distillation (Johansen, Marshall & Neuberger, 1960; Augustyniak & Martin, 1965), by hydrazinolysis (Narita, 1958) and dinitrophenylation of the acetyl hydrazide (Phillips, 1963) or by gas-liquid chromatography (Schroeder, Cua, Matsuda & Fenninger, 1962; Ward & Coffey, 1964). The extent of substitution at the amine and imidazole groups can be determined by adaptations of the ninhydrin reaction (Cobbett, Gibbs & Leach, 1964; Leach, 1966), fluorodinitrobenzene or by the use of 2,4,6-trinitrobenzenesulphonic acid (Okuyama & Satake, 1960; Habeeb, 1966a).

The present paper describes the use of TGA* for the specific modification of the ϵ -amino groups of lysine in sperm-whale Mb. The chemical and immunochemical characterization of the derivative was carried out. Owing to its stability in acid, the lysine-TGA reaction product could be determined by amino acid analysis of acid hydrolysates of the protein derivative and was equivalent to the decrease in the lysine content.

MATERIALS AND METHODS

Reaction of metmyoglobin with 3,3-tetramethyleneglutaric anhydride. Sperm-whale Mb used in these studies was the

major chromatographic component, MbX, obtained by chromatography on CM-cellulose (Atassi, 1964) of the twice-crystallized protein. The TGA was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

For reaction, metmyoglobin (100 mg.) was dissolved in saturated sodium acetate (5 ml.). It was then mixed, at 0°, with a 55 molar excess of TGA (52 mg. in 15 ml.). TGA was only slightly soluble in water, and therefore a few drops of acetone were added to bring it into solution before mixing with Mb. The mixture was magnetically stirred in the ice bath for 5 hr. It was then dialysed extensively against distilled water and centrifuged at 6000 rev./min. for 30 min. The supernatant was divided into two portions. One portion (5 ml.) was freeze-dried and used for amino acid and *N*-terminal analysis, and the remainder was dialysed against 10 mM-phosphate buffer, pH 7.2, containing KCN (0.01%), and kept in solution for immunochemical and other studies.

Analytical methods. Electrophoresis was done at room temperature in starch gel by using the discontinuous buffer system of Poulik (1957) in gels and buffer containing KCN (0.05%). The gels were stained with Amido Black (Smithies, 1959). Spectral analyses in the range 230–700 m μ were carried out in a Cary model 14 spectrophotometer. Precipitin reactions were done according to the procedure of Heidelberger & Kendall (1935). Double diffusion in 1% agar was by the method of Ouchterlony (1949). Nitrogen determinations were carried out in a micro-Kjeldahl apparatus similar to that described by Markham (1942).

For amino acid analysis, portions (2–3 mg.) of the salt-free protein were dissolved in 2 ml. each of constant-boiling HCl (double-distilled), flushed with N₂, evacuated (the procedure repeated twice), sealed and heated at 110° for 18, 22, 30, 40, 50 or 72 hr. The hydrolysates were freed from excess of HCl on a rotary evaporator and assayed on a Spinco model 120C amino acid analyser. Quantitative *N*-terminal analyses were done by dinitrophenylation as described by Atassi & Saplin (1966).

Immunization. The preparation of rabbit antibodies to sperm-whale Mb has been described in detail by Atassi (1967). Rabbit antisera nos. 11 and 100 were used in the present work.

* Abbreviations: TGA, 3,3-tetramethyleneglutaric anhydride; Mb, metmyoglobin; TG-Mb, 3,3-tetramethyleneglutaryl-metmyoglobin.

RESULTS

Electrophoretic and spectral behaviour. Electrophoresis of TG-Mb showed that the derivative was homogeneous and very negatively charged at all pH values between 6.0 and 8.8. No trace of unchanged myoglobin was observed.

Spectral investigations were on solutions containing 0.15–0.2 mg. of protein/ml. of 10 mM-phosphate buffer, pH 7.2, containing potassium cyanide (0.01%). Fig. 1 shows the spectra of Mb and TG-Mb in their cyanmet forms, the latter having peaks at 275, 370, 415 and 540 m μ . In addition to a shift observed with the Soret peak, the spectra showed slight differences in the relative intensities of the peaks (Table 1).

Fig. 1. Spectra of Mb (—) and of TG-Mb (····) in their cyanmet forms. Solutions were in 10 mM-phosphate buffer, pH 7.2, containing KCN (0.01%), and adjusted so that they had identical extinctions at 280 m μ .

Table 1. Ratios of extinctions at the absorption maxima of Mb and TG-Mb

Spectra were determined on the cyanmet forms at pH 7.2, in 10 mM-phosphate buffer containing KCN (0.01%).

Sample	E_{280}/E_{360}	E_{280}/E_{Soret}	E_{Soret}/E_{540}
Mb	1.12	0.327*	9.62
TG-Mb	0.960	0.385†	9.89

* Absorption maximum in the Soret region is at 422.7 m μ .

† Absorption maximum in the Soret region is at 415 m μ .

Determination of the modified amino acid residues. Amino acid analysis of TG-Mb revealed that there was a decrease in the number of lysine residues in myoglobin. The decrease in the lysine content was accompanied by the appearance of a new peak in the analyser 33 min. after phenylalanine (Fig. 2). The constant for this compound was assumed to be equal to the average of the constants of all the amino acids on the long column except half-cystine and proline. By doing this, the decrease in the lysine content was quantitatively accounted for by the appearance of the new peak. The amount of lysine in the hydrolysate, however, increased as the duration of hydrolysis was prolonged. This was accompanied by the disappearance of an equivalent amount of the new product. Determination of the number of lysine residues actually modified was possible by carrying out hydrolysis for 18, 22, 30, 40, 50 and 72 hr. The results of these analyses are shown in Table 2. By extrapolation of the yield of the product to zero hydrolysis time 15.97 mol./mol. of Mb was obtained. Similarly, when the yield of lysine was extrapolated to zero hydrolysis time, 3.15 residues of lysine/mol. were obtained (Fig. 3). These results indicate that three lysine residues did not undergo modification with TGA.

Amino acid analysis of the water extract from the 25 hr. and 50 hr. acid hydrolysates of the DNP-protein revealed that only three lysine residues reacted with fluorodinitrobenzene. Also, valine decreased from 8 to 7 residues/mol. Amino acid analysis of the ether-soluble fraction, after hydrolysis with barium hydroxide, revealed the presence of valine (0.71 mol./mol. of Mb). These results indicated that the *N*-terminal valine residue had not reacted with TGA, since it was still available for reaction with 1-fluoro-2,4-dinitrobenzene.

Immunochemical studies. On double diffusion in agar TG-Mb failed to react with antisera to sperm-whale Mb. Two different rabbit antisera were

Fig. 2. Relative positions in the amino acid analyser of tyrosine (A), phenylalanine (B) and the reaction product of lysine with TGA (C). The rate of buffer flow through column was 68 ml./hr. and the temperature was 55°.

Table 2. *Amino acid composition of TG-Mb*

Mb that had been treated with TGA was subjected to acid hydrolysis for 18, 22, 30, 40, 50 and 72 hr. Results were calculated by assuming the molar proportions aspartic acid:glycine:alanine:leucine:histidine:arginine to be 8:11:17:18:12:4. The results represent the average of two analyses at each hydrolysis period.

Amino acid Duration of hydrolysis.....	Amino acid composition (residues/mol.)					
	18hr.	22hr.	30hr.	40hr.	50hr.	72hr.
Lys*	6.78	7.75	9.28	10.53	13.40	17.69
His	12.07	12.00	11.73	12.18	12.02	12.31
Arg	3.98	3.40	4.10	3.94	3.90	4.01
Asp	7.40	8.10	7.75	7.68	7.87	8.11
Thr	4.60	4.76	4.76	4.79	4.56	4.41
Ser	5.54	5.73	5.63	5.43	5.20	4.69
Glu	18.76	18.90	19.03	19.43	19.05	19.08
Pro	4.28	3.84	4.25	4.18	3.98	4.29
Gly	11.01	10.81	10.96	11.21	11.10	10.88
Ala	16.42	17.30	17.19	17.01	17.03	16.62
Val	6.96	6.55	7.58	8.09	7.88	7.52
Met	2.19	1.95	2.02	2.02	1.97	1.84
Ile	6.64	7.19	7.75	8.34	8.73	9.06
Leu	17.43	16.77	17.99	18.26	18.10	18.30
Tyr	2.77	2.68	3.01	3.04	2.89	2.96
Phe	6.05	5.89	6.26	6.41	5.86	5.93
Product†	12.17	11.32	10.03	8.01	5.51	0.91
Product+Lys	18.95	19.07	19.31	18.53	18.91	18.60

* Lysine extrapolated to zero hydrolysis time = 3.15.

† Product extrapolated to zero hydrolysis time = 15.97.

Fig. 3. Rate of hydrolysis of lysine in TG-Mb. O, Lysine content of Mb (mol./mol. of Mb); ●, content of the reaction product (3,3-tetramethyleneglutarimido lysine) in the hydrolysate (mol./mol. of Mb).

tested. These results were confirmed by quantitative precipitin analysis, since no precipitation of antibody nitrogen was obtained with TG-Mb over a wide range of antigen concentrations.

DISCUSSION

The spectral shift in the visible region observed with the cyanmet form of TG-Mb reflects a change in the haem-group environment that could be more negatively charged than in Mb. Conformational changes will also take place on blockage of the lysine residues with a rather bulky side group that, in addition, carries a charge of opposite sign to that of lysine. In this connexion, Mb that has been completely acetylated at the amino groups only has a spectrum identical with that of native protein when the cyanmet forms are investigated (Atassi, 1966). Nevertheless, drastic conformational reorganization apparently takes place on complete acetylation of proteins (Habeeb, 1966b). This suggests that the spectral behaviour of Mb in the visible range may not always be sensitive to conformational changes and that charge effects on the haem group play a major role, especially since all visible peaks arise from displacement of electrons towards the periphery. Thus when changes lead to a decrease of π -electron density at the periphery of the porphyrin nucleus (i.e. increase of porphyrin basicity) absorption will occur at a lower wavelength (Falk, 1964).

The antigenic reactivity of the protein does not survive complete modification of its amino groups, probably owing to the combined effect of con-

formational disturbance and of charge reversal (or elimination as in acetylation; Atassi, 1966) of the amino groups.

Reaction of the lysine residues in Mb with TGA will probably give rise to 3,3-tetramethyleneglutaryl-lysine (I). This will cyclize in acid to 3,3-tetramethyleneglutarimido-lysine (II). The cyclization would account for the stability of the derivative to acid hydrolysis and for its appearance on the analyser after phenylalanine. Mb has 19 lysine residues, three of which form a basic centre at positions 77, 78 and 79 in the peptide chain (Edmundson, 1965). Structures as shown in (II) cannot sterically be fitted on three adjacent lysine residues in the peptide chain. Since three lysine residues did not react with TGA, it is possible that these are the same residues that form the above basic centre. Similarly, the failure of TGA to react with the *N*-terminal end can be caused by steric hindrance exerted by the isopropyl side group of valine. It is proposed that the cyclization of compound (I) into compound (II) takes place only on mixing TG-Mb with constant-boiling hydrochloric acid before hydrolysis and will not take place when the reaction is carried out under the alkaline conditions of the sodium acetate solution. This might explain the high solubility of TG-Mb in water. TGA should prove useful for modification of lysine residues in other proteins, especially since it would be easy to determine the degree of substitution with accuracy. The *N*-terminal end will very likely take part in this reaction in the absence of steric hindrance.

The author thanks Mr R. Schmidt for his technical assistance. This work was supported by the Office of Naval Research, Biochemistry Branch, Contract no. Nonr-

4564(00), and by a grant (no. AM-08804-02) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service. The author is an Established Investigator of the American Heart Association.

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