Biochem. J. (1967) **102**, 478

Periodate Oxidation of Sperm-Whale Myoglobin and the Role of the Methionine Residues in the Antigen–Antibody Reaction

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(*Received* 12 *May* 1966)

1. Oxidation of sperm-whale metmyoglobin and its apoprotein with periodate has been investigated under various conditions of pH and temperature to find those under which the reagent acted with specificity. 2. At pH 6·8 and 22° consumption of periodate ceased in 3½hr. at 43 moles of periodate/mole of myoglobin. The two methionine residues, the two tryptophan residues, the three tyrosine residues and two histidine residues were oxidized; serine increased in the hydrolysates from 6 to 9 residues/mol. 3. At pH 5·0 and 22°, consumption levelled off in 4½hr. at 26 moles of periodate/mole of myoglobin and resulted in the modification of the two methionine residues, the two tryptophan residues, the three tyrosine residues and two histidine residues; serine increased from 6 to 7 residues/mol. and, also, ferrihaem suffered considerable oxidation. 4. Oxidation at pH 5·0 and 0° resulted at completion (4hr.) in the consumption of 22 moles of periodate/mole of myoglobin and in the modification of the methionine, tyrosine and tryptophan residues. Spectral studies indicated oxidation of the haem group. This derivative reacted very poorly with rabbit antisera to MbX (the major component no. 10 obtained by CM-cellulose chromatography; Atassi, 1964). 5. Oxidation of apomyoglobin at pH 5·0 and 0° was complete in 4hr. with the consumption of 7·23 moles of periodate/mole of apoprotein. The rate of oxidation in decreasing order was: methionine; tryptophan; tyrosine; and after 7hr. of reaction the following residues/mol. were oxidized: methionine, 2·0; tryptophan, 1·6; tyrosine, 0·99. No peptide bonds were cleaved. Metmyoglobin prepared from the 7hr.-oxidized apoprotein showed that the reactivity with antisera to MbX had diminished considerably. 6. Milder oxidation of apoprotein (2 molar excess of periodate, pH 5·0, 0°, 2hr.) resulted in the modification of 1·66 residues of methionine/mol. Metmyoglobin prepared from this apoprotein was identical with native MbX spectrally, electrophoretically and immunochemically. It was concluded that the methionine residues at positions 55 and 131 were not essential parts of the antigenic sites of metmyoglobin.

Oxidation with periodate is an important tool in the structural studies of carbohydrates. Its application to proteins, however, has not been so well investigated. Oxidation of some amino acids with periodate was first reported by Nicolet & Shinn (1939). Recently Clamp & Hough (1965) showed that all α-amino acids are oxidizable with periodate, but at differing rates. They also found that, in general, oxidation was more rapid at high pH. The reaction has also been applied by various investigators to proteins and loss of biological activity was often reported, but the oxidized protein was usually not well characterized. Dixon (1962) was successful in oxidizing the *N*-terminal serine residue of corticotrophin with periodate. Knowles (1965) showed that periodate-oxidized α-chymotrypsin was a homogeneous partially active preparation in which one of the two methionine residues in the protein had been converted into its sulphone. Reaction of Mb* with periodate and other oxidizing agents was investigated spectrally by George & Irvine (1954). They showed that reaction with potassium periodate was extremely slow at pH 8·6, resulting in an intermediate that, when reduced with potassium ferrocyanide followed by addition of azide, yielded 80–85% of the original Mb. When oxidation was

* Abbreviations: Mb, metmyoglobin; MbX, the major component no. 10 obtained by CM-cellulose chromatography (Atassi, 1964).

at pH 8·2 only 60% of the original Mb was recovered. The intermediate formed with periodate had the same spectral characteristics as those of the intermediate compounds produced by hydrogen peroxide, methyl hydroperoxide or ethyl hydroperoxide in which the iron was considered to have an effective oxidation number of +4 (George & Irvine, 1952, 1953).

The present paper reports a detailed investigation of the oxidation of Mb and apomyoglobin with sodium metaperiodate at various conditions of temperature and pH. The physicochemical properties and amino acid compositions of the protein derivatives have been studied together with their immunochemistry.

MATERIALS AND METHODS

Preparation of myoglobin and apomyoglobin. Myoglobin used in these studies was the major component MbX obtained by CM-cellulose chromatography (Atassi, 1964). The present work was done on the met form. The apoprotein was prepared by a method similar to that described by Theorell & Åkeson (1955).

Periodate oxidation. Oxidation was carried out with sodium metaperiodate (A.R.; Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.) in the dark. The protein was dissolved in the appropriate buffer solution (4–5mg./ml.) and mixed with a 60–70 molar excess of 0·1M-sodium metaperiodate solution in water. A blank solution containing the same amount of periodate but no protein was prepared. Portions (1ml. each) were withdrawn at suitable intervals for the determination of periodate consumption, which was carried out by the sodium arsenite–iodine titration procedure (Fleury & Lange, 1933*a*,*b*). In the present work the sample was added to 2ml. of 0·02M-sodium arsenite containing 0·1g. of $NaHCO_3$ and 0·1g. of KI and the mixture was titrated with 0·01N-iodine solution (obtained as 0·1N-iodine solution from Fisher Scientific Co., Pittsburgh, Pa., U.S.A.). To eliminate the possibility of interaction of the protein with arsenite or iodine during the titration, controls were run that contained the protein and all the reagents except periodate. These were identical with blanks that contained no protein. In the samples taken for chemical characterization, oxidation was terminated by the addition of an excess of ethylene glycol (about 0·1ml./ml. of sample). The sample was then divided into two portions. One portion was dialysed extensively against distilled water, freeze-dried and used for amino acid analysis and structural investigations. The second portion was dialysed against 10mM-phosphate buffer, pH 7·2, containing KCN (0·01%) and used for immunochemical and other physical studies that have to be performed in solution. This was done because freeze-dried oxidized myoglobin was insoluble.

Oxidations were done in 0·1M-acetate buffers at pH 6·8 and 5·0 at 22° or 0°. All reagents were checked periodically by oxidation of mannitol (periodate consumption, 5·0 moles/mole).

For the oxidation of amino acids and ferrihaem, 10mg. samples were dissolved in 0·1M-acetate buffer, pH 5·0, mixed with 3ml. of 0·1M-$NaIO_4$ each and the volumes each made up to 10ml. Periodate consumption was determined with the arsenite–iodine titration procedure as above.

Analytical methods. Electrophoresis was on starch gel, at room temperature, in the discontinuous buffer system of Poulik (1957); the buffers contained KCN (0·05%) and the gels were stained with Amido Black (Smithies, 1959). Spectral analyses were done in a Cary model 14 spectrophotometer. Double diffusion in 1% agar was by the method of Ouchterlony (1949); the lines were sharper when the agar contained KCN (0·05%). Precipitin experiments were done according to the procedure of Heidelberger & Kendall (1935), with 0·2ml. of antiserum and various amounts of antigen. Nitrogen determinations were done in a micro-Kjeldahl apparatus similar to that described by Markham (1942). The concentrations of protein solutions were determined from their nitrogen content, which was assumed to be 16% for both Mb and γ-globulin.

Acid hydrolysis of salt-free protein samples (2–3mg.) was in 2ml. of constant-boiling HCl (double-distilled) in sealed tubes under vacuum (after flushing with N_2) at 110° for 22 or 72hr. Alkaline hydrolysis in saturated $Ba(OH)_2$ was by the procedure of Ray & Koshland (1962). The hydrolysates were assayed on a Spinco model 120C amino acid analyser.

Tryptic hydrolysis of heat-denatured apomyoglobin and periodate-oxidized apomyoglobin was at 40° and pH 8·0 and was continued until consumption of 0·1N-NaOH ceased. The procedure employed was similar to that described by Edmundson & Hirs (1962). Peptide 'mapping' was on Whatman no. 3MM papers (63cm. × 25cm.). Ascending chromatography in butanol–acetic–water (4:1:5, by vol.) was followed by electrophoresis at pH 3·75 in a Savant electrophoresis apparatus with pyridine–acetic acid–water buffer (1:10:289, by vol.) and a potential gradient of 24·5v/cm. for 50min. A control hydrolysate of unoxidized apoprotein was run simultaneously, on a separate paper, with the hydrolysate from the periodate-oxidized apoprotein. The peptide spots were revealed at room temperature with ninhydrin (0·2% solution in acetone) and tyrosine peptides were detected by exposure to nitrogen oxides (Block, Durrum & Zweig, 1958). For correspondence between peptide spots and their sequence, the assignments of Banaszak, Andrews, Burgner, Eylar & Gurd (1963), for the above solvent systems, were used.

Reconstitution of metmyoglobin. For reconstitution with apomyoglobin crystalline haemin chloride (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was dissolved in 0·02M-Na_2HPO_4 and added slowly to the magnetically stirred apomyoglobin solution in 0·02M-phosphate buffer, pH 7·2, containing KCN (0·01%). The ferrihaem added was in about 1·5–2 molar excess (and one-fifth the volume) of the apomyoglobin solution and the operation was carried out away from direct light. When the addition of ferrihaem to apomyoglobin was complete the mixture was magnetically stirred at 0° for a further 2hr. After dialysis against several changes of 10mM-phosphate buffer, pH 7·2, containing KCN (0·01%), it was centrifuged before use in subsequent studies. With this combination procedure, only a small amount (10–15%) of the protein was precipitated.

Immunization. Antibodies to MbX were raised in rabbits weighing 2–2·5kg. by injecting a mixture of equal volumes of the cyanmetmyoglobin derivative [16–22mg. in 2·7ml. of 0·01M-phosphate, pH 7·2, containing KCN (0·01%)] and adjuvant. In the first injection the adjuvant was a 4:1

mixture of Adjuvant Complete Freund and Adjuvant Complete H37 Ra (both from Difco Laboratories, Detroit, Mich., U.S.A.). The antigen–adjuvant mixture was split between three rabbits. Each rabbit was injected into the hind toepads (0·6ml. each) and subcutaneously into the neck (0·5ml.). A week later the rabbits were injected at the same sites with a mixture of equal volumes of protein solution and adjuvant (2·7ml. each) in which the adjuvant was a 3:2 mixture of Complete Freund and Complete H37Ra. In the third injection, which was given a week after the second injection at the above sites, the Complete Freund and Complete H37 Ra were in the proportions 2:3. Equal volumes of protein solution and adjuvant (2·7ml. each) were used. The animals were bled a week after the third injection.

Antibodies were raised in goats by injecting mixtures of equal volumes of the cyanmetmyoglobin derivative (7mg./ml.) in the above phosphate buffer and adjuvant. Three injections were administered and were spaced a week apart and the adjuvant compositions were similar to those given to the rabbit. In each injection 2·5ml. of protein–adjuvant mixture was administered at several sites in each of the goat's front flanks. Final bleeding was performed a week after the third injection. Only antisera that gave single lines in agar double-diffusion tests were used for the present studies. Antisera from individual animals were kept separate and stored in 8–10ml. portions at −40°. Rabbit antisera nos. 11 and 100 and goat antiserum G1 were employed in these studies.

RESULTS

Periodate oxidation of metmyoglobin. The rates of periodate consumptions of Mb are shown in Fig. 1. At 22° and pH 6·8 periodate consumption levelled off in about 3½hr. at 43 moles of periodate/mole of Mb. On the other hand, when the pH was lower (pH 5·0 and 22°) oxidation was complete in 4½hr. and consumption ceased at 26·1 moles of periodate/mole of Mb. At about 10hr. over-oxidation began and periodate consumption rose very sharply (e.g. consumption, in moles of periodate/mole of Mb, was: 20hr., 29·5; 32hr., 39·4). The amino acid compositions of oxidized metmyoglobins are shown in Table 1. Since under conditions of acid hydrolysis any methionine sulphoxide that had been formed as a result of oxidation might revert to methionine (Ray & Koshland, 1962; Knowles, 1965), it was necessary to carry out alkaline hydrolyses on the oxidized derivative.

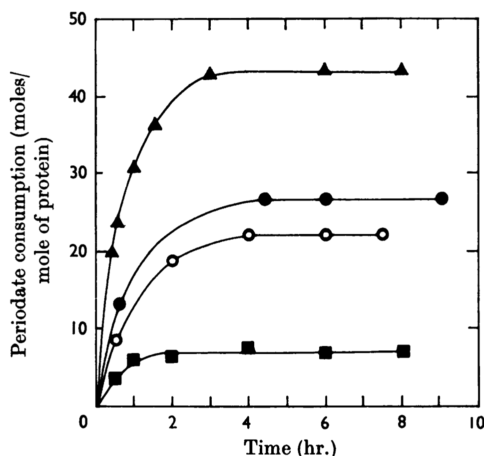

Fig. 1. Rate of periodate consumption of MbX and its apoprotein under various conditions. ▲, MbX, pH 6·8 and 22°; ●, MbX, pH 5·0 and 22°; ○, MbX, pH 5·0 and 0°; ■, apomyglobin, pH 5·0 and 0°. Oxidation was with a 60–70 molar excess. Experimental details are given in the text.

The results show that at pH 6·8 and 22° the two methionine residues were oxidized to methionine sulphoxide. Also, the two tryptophan residues, the three tyrosine residues and two histidine residues suffered oxidation. Serine in the hydrolysate increased from 6 to 9 residues/mol. The only explanation I can offer for this unexpected finding is that the excess of serine may have arisen from the oxidation product of tyrosine, which might well be serine or simply behave like serine on the analyser. The oxidized derivative was almost completely insoluble and therefore could not be subjected to any further investigations. It should be pointed out that Mb controls that had been treated with ethylene glycol (i.e. no periodate) had the same amino acid composition as the native protein.

At pH 5·0 and 22°, the two methionine residues, the two tryptophan residues, the three tyrosine residues and two histidine residues were oxidized. In addition, serine increased from 6 to 7 residues/mol. Visually, the oxidized protein suffered an almost total loss of its red colour. Spectral investigations showed that the Soret band and other absorption peaks in the visible region had disappeared completely. This suggested the possibility that the haem group might have undergone oxidation. Also, under these oxidation conditions, Mb suffered appreciable precipitation and, in view of the extensiveness and non-specificity of the oxidation, no further studies were carried out on this derivative.

To account for the periodate consumption of Mb and the high ammonia values in the acid hydrolysates, histidine, methionine, serine, threonine, tryptophan and tyrosine were subjected to periodate oxidation under the same conditions (0·1M-acetate buffer, pH 5·0, 22°) and their consumptions determined. These were similar to the values reported by Clamp & Hough (1965). Consumptions of asparagine, glutamine and haemin chloride, after oxidation for 16hr., were (in moles of periodate/mole): 0·32, 0·29 and 5·11 respectively. When

Table 1. *Amino acid compositions of periodate-oxidized metmyoglobin and apomyoglobin*

Sperm-whale Mb was oxidized with a 60–70 molar excess of $NaIO_4$ under various conditions. Apomyoglobin was oxidized with a 2·29 molar excess of $NaIO_4$. The values for acid hydrolysates were calculated by assuming the molar proportions aspartic acid:glycine:alanine:leucine:lysine:arginine to be 8:11:17:18:19:4. For alkaline hydrolysates the proline:phenylalanine molar ratio was assumed to be 4:6. The results represent the average of three acid hydrolyses (two 22hr. and one 72hr.) and two alkaline hydrolyses. Values for threonine and serine were obtained by extrapolation to zero hydrolysis time.

	Amino acid composition (residues/mol.)									
	Mb		Mb oxidized at pH 6·8 and 22°, 6hr.		Mb oxidized at pH 5·0 and 22°, 5hr.		Mb oxidized at pH 5·0 and 0°, 5hr.		Apomyoglobin oxidized at pH 5·0 and 0°, 2hr.	
Amino acid	Acid hydrolysis	Alkaline hydrolysis	Acid hydrolysis	Alkaline hydrolysis	Acid hydrolysis	Alkaline hydrolysis	Acid hydrolysis	Alkaline hydrolysis	Acid hydrolysis	Alkaline hydrolysis
Met sulphoxide	—	—	—	2·08	—	1·96	—	2·01	—	1·66
Asp	7·93	—	8·49	—	8·41	—	8·13	—	8·06	—
Met sulphone	—	—	—	—	0·23	0·12	0·08	—	—	—
Thr	5·01	—	4·60	—	4·85	—	5·09	—	4·82	—
Ser	5·65	—	8·84	—	7·13	—	6·06	—	5·62	—
Glu	19·0	—	19·2	—	19·3	—	19·1	—	19·3	—
Pro	3·91	4·02	3·69	3·84	3·85	3·99	3·72	4·00	3·67	4·00
Gly	10·9	—	11·2	—	11·2	—	11·3	—	10·8	—
Ala	17·1	—	17·3	—	17·0	—	17·1	—	16·8	—
Val	7·53	—	7·69	—	7·85	—	7·87	—	7·81	—
Met	1·86	1·97	1·69	0	1·76	0	2·03	0	1·84	0·38
Ile	8·71	—	8·62	—	8·80	—	8·79	—	8·63	—
Leu	18·1	—	18·1	—	17·9	—	18·1	—	17·8	—
Tyr	2·89	3·01	0	0·02	0·27	0·08	0·37	0·26	2·89	2·69
Phe	6·23	5·99	5·64	6·01	5·83	5·98	5·87	5·92	5·83	6·01
Trp	—	1·87	—	0	—	0	—	0	—	1·88
Lys	18·7	—	18·7	—	19·0	—	19·1	—	19·1	—
His	12·2	—	10·4	—	10·3	—	12·1	—	12·3	—
Arg	4·01	—	4·11	—	3·90	—	3·95	—	3·96	—

periodate consumption was calculated for the number of amino acid residues of Mb that had been oxidized, it was not possible to account for the total periodate consumption of the protein. However, the 16hr.-oxidation values for tyrosine (0·98 mole of periodate/mole) and ferrihaem (given above) were not the final ones at which oxidation of these molecules ceased. The consumptions of ferrihaem and tyrosine increased steadily with time, so that at 349hr. the consumptions (in moles of periodate/mole) were: ferrihaem, 27·7; tyrosine, 7·66. Since the extent of oxidation of ferrihaem and tyrosine in the intact protein is not known, it was not possible to correlate the periodate uptake of Mb with its amino acid composition.

In an attempt to render the reaction less drastic, the protein and periodate solutions were precooled to 0–2° before mixing and the mixture was maintained at that temperature during the reaction. Fig. 1 shows the rate of periodate uptake at 0° and at pH 5·0. Periodate uptake ceased after 4hr. at 22 moles of periodate/mole of Mb. Table 1 shows the amino acid composition of a derivative previously oxidized for 5hr. at 0°. Periodate uptake and amino acid data indicated that the oxidation of Mb was less drastic at pH 5·0 and 0°. Only the tyrosine, tryptophan and methionine residues were modified. However, spectral investigations suggested that the ferrihaem had suffered some oxidation. This was indicated from spectral studies, which showed a great decrease in the extinction at 415mμ (Fig. 2).

To avoid the complications resulting from ferrihaem oxidation, it was considered advisable to modify the apoprotein first with periodate and then form the complex between it and unaltered ferrihaem, and study its chemistry and immunochemistry.

Periodate oxidation of apomyoglobin. The rate of periodate uptake by apomyoglobin is shown in Fig. 1. Periodate consumption ceased in about 4hr. at 7·23 moles of periodate/mole of apoprotein. Portions (2ml.) of the oxidation mixture were withdrawn at intervals and oxidation was stopped immediately by the addition of 0·2ml. of ethylene glycol. The samples were dialysed extensively against distilled water and freeze-dried, and their amino acid compositions were determined after acid and alkaline hydrolysis. Only three amino acid species suffered oxidation, i.e. methionine, tryptophan and tyrosine. The rate of oxidation of the three amino acids in apomyoglobin is shown in Fig. 3. The oxidation of the two methionine residues and one tryptophan residue was most rapid. One tyrosine residue was oxidized at a relatively lower rate.

The two methionine residues in myoglobin are located at positions 55 and 131, the two tryptophan residues are at positions 7 and 14, and the three tyrosine residues are at positions 103, 146 and 151 (Edmundson, 1965). To determine the location of the modified tyrosine residue in periodate-oxidized apomyoglobin, tryptic hydrolysis of a 7hr.-oxidation product was carried out at pH 8·0 and 40°. In myoglobin, tyrosine 103 falls in one of the insoluble tryptic peptides from the 'core'

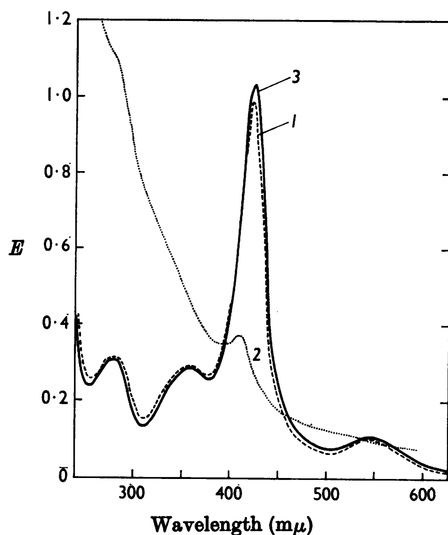

Fig. 2. Spectra of the cyanmet forms of MbX and various oxidized derivatives. Curve *1* (----), MbX; curve *2* (····), MbX, previously oxidized with a 60–70 molar excess of $NaIO_4$ in 0·1M-acetate, pH 5·0, at 0° for 7hr.; curve *3* (——), Mb obtained by forming a complex between previously oxidized apomyoglobin (2 molar excess of $NaIO_4$, pH 5·0, 0°, 2hr.) and unaltered ferrihaem. Solutions contained 120–140μg. of Mb/ml. except for solution *2*, which contained about five times as much protein. Samples were all in 10mM-phosphate buffer, pH 7·2, containing KCN (0·01%).

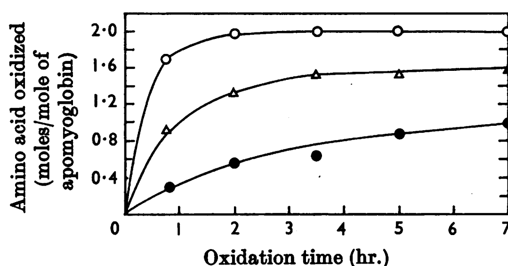

Fig. 3. Rate of oxidation of various amino acid residues on oxidation of apomyoglobin with periodate. ○, Methionine; △, tryptophan; ●, tyrosine. Oxidation was with a 60–70 molar excess of $NaIO_4$ at pH 5·0 and 0°. Experimental details are given in the text.

Table 2. *Amino acid composition of the insoluble and soluble portions of the tryptic hydrolysates of apomyoglobin and periodate-oxidized apomyoglobin*

Apomyoglobin was oxidized with a 60–70 molar excess of $NaIO_4$ for 7hr. in 0·1M-acetate buffer, pH5·0, at 0°. Tryptic hydrolysis was carried out at pH8·0 and 40°. Details are given in the text. Results were calculated on the basis that in the insoluble fraction the molar proportions phenylalanine:lysine:glycine:leucine are 1:3:4:7. The corresponding proportions in the soluble fraction were assumed to be 5:16:8:10 (Edmundson & Hirs, 1962). Contents are expressed as molar proportions of the constituent amino acids. Values represent the average of two 22hr. acid hydrolysates each. No alkaline hydrolysis was carried out.

	Amino acid composition (residues/mol.)			
	Insoluble fraction		Soluble fraction	
Amino acid	Apomyoglobin	Oxidized apomyoglobin	Apomyoglobin	Oxidized apomyoglobin
Asp	2·08	2·33	7·66	8·00
Thr	1·34	1·25	3·49	3·46
Ser	2·59	2·94	2·87	3·04
Glu	6·16	6·09	13·4	13·5
Pro	0·750	0·681	3·51	3·43
Gly	3·68	3·75	7·23	7·66
Ala	4·08	4·13	13·6	13·3
Val	5·38	5·83	2·34	2·59
Met	0·238	0·140	1·97	1·71
Ile	3·61	3·52	4·25	4·34
Leu	6·65	6·83	9·66	9·85
Tyr	0·710	0·760	2·03	0·89
Phe	0·997	1·04	4·28	4·62
Lys	2·85	3·13	15·6	15·8
His	4·22	4·31	7·22	6·93
Arg	0·981	0·850	2·86	3·02

(Edmundson, 1963, 1965). Amino acid analysis of the tryptic precipitate and the supernatant showed that the modified tyrosine residue was in the supernatant fraction (Table 2). Peptide-'mapping' experiments on the tryptic supernatant suggested that the modified tyrosine residue is located at position 151 in the peptide chain.

Since, as mentioned above, the rate of oxidation of the two methionine residues in apomyoglobin is greater than those of tryptophan and tyrosine (Fig. 3), it seemed possible that a derivative modified only at the methionine sites could be made under appropriate conditions. Also, since periodate oxidation of aromatic systems can be dependent on the total concentration of periodate (Kaiser & Weidman, 1964), it seemed that oxidation with a small amount of periodate could produce the desired derivative. Apomyoglobin (60mg.) was dissolved in cold (0°) 0·1M-acetate buffer, pH5·0 (15ml.). To this solution was added a 2·29 molar excess of periodate (1ml. of 8mM-sodium metaperiodate, at 0°), the mixture was magnetically stirred in the dark and the reaction terminated after 2hr. with 0·4ml. of ethylene glycol. A portion (2ml.) was dialysed exhaustively against distilled water and freeze-dried for amino acid analysis. The remainder of the solution was dialysed against several changes of 20mM-phosphate buffer, pH7·2, containing potassium cyanide (0·01%) and then formed into a complex with unmodified ferrihaem. Amino acid analysis of acid and alkaline hydrolysates of the derivative showed oxidation of the two methionine residues only (Table 1).

Properties of metmyoglobins reconstituted from oxidized apomyoglobins. A portion of the apomyoglobin reaction mixture (with 60–70 molar excess of periodate), which was withdrawn at 7hr., and the reaction terminated with excess of ethylene glycol, was dialysed against distilled water, followed by dialysis against several changes of 20mM-phosphate buffer, pH7·2, containing potassium cyanide (0·01%). The apoprotein fraction was then formed into a complex with unmodified ferrihaem. This derivative was used for physicochemical and immunochemical investigations.

The complex formed from oxidized apomyoglobin and ferrihaem, in its cyanmet form, gave peaks at 540, 420, 358 and 275mμ. The positions of the absorption maxima of the oxidized derivative were similar to those shown by native cyanmetmyoglobin. The relative intensities of these maxima were different in the complex formed from oxidized apoprotein and ferrihaem and in the native protein. By starch-gel electrophoresis

the oxidized derivative had an electrophoretic mobility similar to that of the native protein. The band given by the oxidized derivative was always wider than the band given by the native protein. The possibility of cleavage of some peptide bonds during oxidation was investigated by ultracentrifugal studies of the oxidation product. The oxidized derivative sedimented at the same rate as the native protein when the solutions had the same concentration. Molecular-weight measurements on a Sephadex G-100 column were carried out on the 7hr.-oxidation derivative by a method similar to that described by Andrews (1964). In the present work, however, 10mM-phosphate buffer, pH 7·2, containing potassium cyanide (0·01%) was used and the column was calibrated as described by Atassi & Saplin (1966). In two experiments, the oxidized protein emerged at elution volumes corresponding to molecular weights of 17600 and 18000. It was therefore concluded that no cleavage of peptide bonds had taken place.

The Mb derivative that was reconstituted from mildly oxidized apomyoglobin (2 molar excess of periodate, pH 5·0, 0°) was identical with MbX in their cyanmet forms spectrally (see Fig. 2) and electrophoretically.

Immunochemistry of the oxidized derivatives. The derivative of Mb oxidation at pH 5·0 and 0° gave, in agar double-diffusion experiments, very faint lines with rabbit antibodies to the native protein. On quantitative precipitin analysis increasing amounts of the derivative precipitated increasing amounts of antibody nitrogen (Fig. 4).

Mb that was reconstituted from oxidized apomyoglobin (60–70 molar excess of periodate) and unaltered ferrihaem gave, in agar double diffusion, one precipitin line, which was weaker than the line of the native protein, but no spurs or intersections were formed. On quantitative precipitin analysis the oxidized derivative precipitated less antibody nitrogen than the native protein and a greater amount of the derivative was required for maximal precipitation (Fig. 4). Table 3 shows antibody/antigen molar ratios at equivalence. It should be pointed out that native and reconstituted sperm-whale Mb are immunochemically identical (Fig. 4). This has also been reported for horse myoglobin by Reichlin, Hay & Levine (1963).

Finally, immunochemical tests in agar double-diffusion plates on reconstituted Mb, from the mildly oxidized apoprotein (2 molar excess of periodate), showed that the derivative in which only the two methionine residues were modified was identical with native MbX (no spurs or intersections were formed) when examined with the rabbit and goat antisera to the native protein. Fig. 5 shows the results of precipitin reactions of the derivative and of reconstituted Mb, with rabbit antiserum no. 100 and goat antiserum G1. Table 4 summarizes the results of all the precipitin reaction experiments with antisera no. 11, no. 100 and G1. It can be seen that the derivative in which only

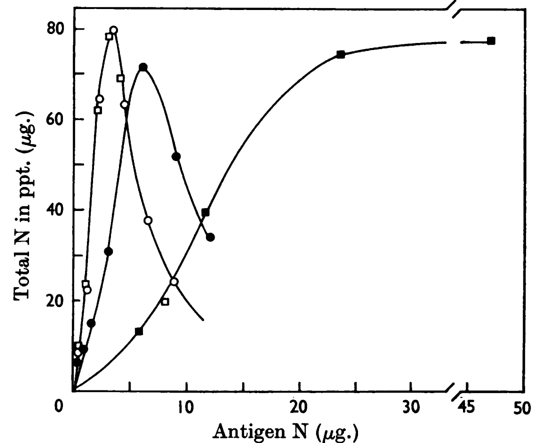

Fig. 4. Precipitin analyses with antiserum to MbX (rabbit antiserum no. 11). □, Native MbX; ○, Mb obtained from recombination of apomyoglobin with ferrihaem; ■, MbX previously oxidized with periodate at pH 5·0, at 0° for 5hr.; ●, a derivative obtained by forming a complex between previously oxidized apoprotein (60–70 molar excess of $NaIO_4$, pH 5·0, 0°, 7hr.) and ferrihaem.

Table 3. *Relative amounts and compositions of immune precipitates formed by metmyoglobin prepared from 7hr.-oxidized apomyoglobin with a 60–70 molar excess of periodate*

Values were from single experiments and are based on total nitrogen values in the precipitates at the point of equivalence.

Antiserum to MbX	Percentage of ppt. relative to homologous reaction	Antibody/antigen molar ratio at equivalence	
		Re-formed Mb	Re-formed derivative
No. 11	90·7	2·62	1·32
No. 100	84·0	2·10	1·10

two methionine residues were modified precipitated, at equivalence, amounts of antibody nitrogen that were 93·8, 94·4 and 98·3% of the homologous reaction. The molar ratio of antibody to antigen at the maximum is given for each experiment. It is apparent that the modification of the methionine residues did not disturb any antigenic reactive sites. The slight decrease in the antigenic efficiency of the derivative could be the result of oxidation of some small amounts of tyrosine and tryptophan.

DISCUSSION

If myoglobin or, better still, its apoprotein can be considered as a well-characterized model protein, then the present study should give valuable information about the mode of attack of proteins by periodate under various conditions. The information reported above should not be taken as a general rule applicable to all proteins. The order of susceptibility to periodate oxidation of various amino acid residues in proteins, and the extent of this oxidation, will depend largely on conditions of the reaction and on the structure of the protein. It is nevertheless possible to point out that protein oxidation will be more extensive as temperature and pH are raised and also that methionine and tryptophan are probably the most rapidly oxidized residues in proteins. Since myoglobin contains no cysteine or cystine, no information can be obtained here about the susceptibility of these residues to periodate oxidation. It might be mentioned that, on dealing with periodate-oxidized glycoproteins and their biological activity, it cannot be assumed that the carbohydrate groups undergo oxidation preferentially or more rapidly than the protein moiety. Each case will have to be investigated separately.

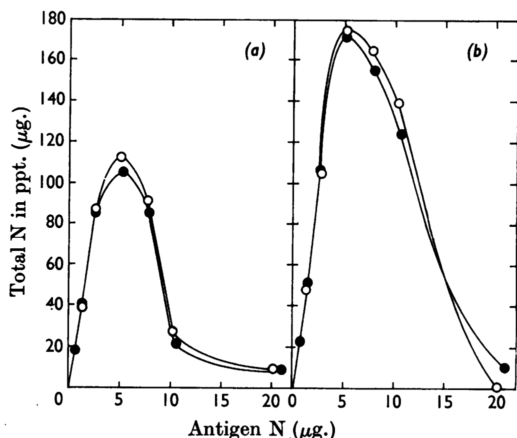

Fig. 5. Precipitin studies on the derivative of mild oxidation. ○, Mb obtained by recombination of apomyoglobin with ferrihaem; ●, derivative obtained by forming a complex between previously oxidized apoprotein (pH 5·0, 0°, 2 molar excess of $NaIO_4$, 2hr.) and ferrihaem. (a) Reactions with rabbit antiserum no. 100 (average of three independent determinations of each point); (b) reactions with goat antiserum G1 (average of five independent determinations of each point).

It is apparent from the present studies that the oxidation of Mb with periodate cannot be attributed solely to the contribution of the haem group. The products of oxidation of ferrihaem were not identified here. It has been reported, however, that porphyrins undergo degradation to monopyrroles when subjected to oxidation with chromic oxide (Fischer & Wenderoth, 1939; Muir & Neuberger, 1949) or with potassium permanganate (Nicolaus, Mangoni & Caglioti, 1956; Nicolaus, 1960). It is likely that extensive periodate oxidation of haem might lead to its degradation to monopyrroles, which would account for the observed loss of red colour.

The finding that apomyoglobin suffered less oxidation than Mb under identical conditions suggested a possible catalytic role played by the haem iron in myoglobin. At least ferrihaem is

Table 4. *Relative amounts and compositions of immune precipitates formed by metmyoglobin reconstituted from apomyoglobin oxidized with a 2 molar excess of periodate*

The precipitation reaction was carried out with two rabbit antisera and a goat antiserum against MbX. The percentage of precipitation relative to the homologous reaction was based on the total nitrogen values in the precipitates at the point of maximum precipitation. Both myoglobin and its oxidized derivative showed maximum antibody precipitation at the same antigen concentration.

		Percentage of ppt. relative to homologous reaction		Antibody/antigen molar ratio at equivalence			
				Re-formed Mb		Re-formed derivative	
Antiserum to MbX	No. of experiments	Average	Range	Average	Range	Average	Range
No. 100	3	93·8	91·5–98·3	2·39	2·23–2·65	2·09	2·03–2·12
No. 11	2	94·4	93·6–95·2	2·61	2·50–2·71	2·58	2·53–2·62
G1	5	98·3	95·5–100	3·58	3·18–3·77	3·41	3·18–3·82

known to catalyse the decomposition of hydrogen peroxide (Haldane, 1931; Kremer, 1965). Oxidation might have taken place by a free-radical mechanism. Myoglobin free radicals are apparently formed from amino acids close to the iron atom when hydrogen peroxide attacks Mb (King, Looney & Winfield, 1964). These authors also found, by electron-spin resonance, that the principal free radical present shortly after adding hydrogen peroxide at 0° was that of tyrosine.

Some information on the correlation of myoglobin structure with antigenic function can be obtained from the present work. Derivatives obtained by oxidation of Mb itself under the conditions described above give very little information about the role of the modified residues in the antigen–antibody reaction. Owing to the extensive nature of the modification, even under the mildest conditions, it is unlikely that the protein would retain its native conformation on oxidation. Conformational changes could influence the immunochemistry of the protein. In addition, it is also not possible, on extensive modification, to isolate the role played by the various modified groups in the antigenic function.

Oxidation of apomyoglobin, on the other hand, because it was less drastic, provided derivatives that gave some valuable information about the reactive sites. The results obtained with Mb formed as a complex between unaltered ferrihaem and the apoprotein that had been oxidized with a 2 molar excess of periodate were significant. In this derivative, 1·66 residues of methionine were oxidized and only negligible amounts of tyrosine and tryptophan.

It is very likely that the conformation of the protein is not altered much on the oxidation of the two methionine residues to methionine sulphoxide, since the derivative resembled native myoglobin spectrally, electrophoretically and immunochemically. The present immunochemical studies showed that this derivative was almost equally efficient as antigen, precipitating 94–98% of antibody nitrogen relative to the native protein. This suggests at once that one methionine residue is definitely not a part of a reactive site. It is, in fact, clear that neither methionine residue can be involved in reactive sites since at least 65% modification of the second residue was achieved and therefore reactivity would diminish by more than 2–6% if the second methionine residue were a part of an antigenic site. The two methionine residues of myoglobin are at positions 55 and 131 (Edmundson, 1965). Methionine 55 is in a seven-residue helical segment (helix D; Kendrew *et al.* 1961), whereas methionine 131 is in the longer helix H. Preliminary studies on cleavage of the oxidized protein with cyanogen bromide suggest that the partially oxidized methionine residue is at position 131, since partial (about 10%) cleavage was obtained in that location whereas no cleavage has been obtained at position 55. Cyanogen bromide cleaves at methionine sites (Gross & Witkop, 1961), but no cleavage occurs when the methionine residues are oxidized (Gross & Witkop, 1962; Koshland, Strumeyer & Ray, 1962).

Further oxidation of the protein resulting in the modification of one tyrosine and one or two tryptophan residues leads to an appreciable decrease of the reactivity of Mb with its antibody. To determine whether this is due to the modification of one tyrosine residue or the two tryptophan residues, or both, further investigations will be necessary. It has been found that the *C*-terminal amino acid (and possibly the *C*-terminal dipeptide) is not part of an antigenic site (Atassi, 1966). On the other hand, Crumpton & Wilkinson (1965) have shown that the *C*-terminal heptapeptide has a significant inhibiting activity to the extent of 15% with one antiserum pool when tested with the precipitation of the apoprotein. There is therefore a strong possibility that the decrease in the reactivity of the derivative is due, at least in part, to the modification of tyrosine 151. No conformational factors enter here since this tyrosine is in the non-helical *C*-terminal pentapeptide segment of the protein. There is increasing evidence that the structure of Mb in solution is mostly similar to that of the crystalline state (Urnes, 1965).

The role played by the haem group in giving the haemoprotein a certain conformation necessary for its antigenic specificity has already been reported for both myoglobin (Reichlin *et al.* 1963) and haemoglobin (Reichlin *et al.* 1963; Atassi, Brown & McEwan, 1965). This, indeed, was the reason why in all the present derivatives an unmodified ferrihaem was incorporated into the protein, when possible, before immunochemical studies were carried out.

The author thanks Miss Barbara J. Saplin for her technical assistance. This research was supported by the U.S. Public Health Service (grant no. AM-08804-02) and by the Office of Naval Research, Biochemistry Branch, contract no. Nonr-4564(00).

REFERENCES

Andrews, P. (1964). *Biochem. J.* **91**, 222.
Atassi, M. Z. (1964). *Nature, Lond.*, **202**, 496.
Atassi, M. Z. (1966). *Nature, Lond.*, **209**, 1209.
Atassi, M. Z., Brown, R. K. & McEwan, M. (1965). *Immunochemistry*, **2**, 379.
Atassi, M. Z. & Saplin, B. J. (1966). *Biochem. J.* **98**, 82.
Banaszak, L. J., Andrews, P. A., Burgner, J. W., Eylar, E. H. & Gurd, F. R. N. (1963). *J. biol. Chem.* **238**, 3307.

Block, R. J., Durrum, E. L. & Zweig, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 139. New York: Academic Press Inc.
Clamp, J. R. & Hough, L. (1965). *Biochem. J.* **94**, 17.
Crumpton, M. J. & Wilkinson, J. M. (1965). *Biochem. J.* **94**, 545.
Dixon, H. B. F. (1962). *Biochem. J.* **83**, 91.
Edmundson, A. B. (1963). *Nature, Lond.*, **198**, 354.
Edmundson, A. B. (1965). *Nature, Lond.*, **205**, 883.
Edmundson, A. B. & Hirs, C. H. W. (1962). *J. molec. Biol.* **5**, 683.
Fischer, H. & Wenderoth, H. (1939). *Liebigs Ann.* **537**, 170.
Fleury, P. & Lange, J. (1933*a*). *J. Pharm. Chim., Paris*, **17**, 107.
Fleury, P. & Lange, J. (1933*b*). *J. Pharm. Chim., Paris*, **17**, 196.
George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
George, P. & Irvine, D. H. (1953). *Biochem. J.* **55**, 230.
George, P. & Irvine, D. H. (1954). *Biochem. J.* **58**, 188.
Gross, E. & Witkop, B. (1961). *J. Amer. chem. Soc.* **83**, 1509.
Gross, E. & Witkop, B. (1962). *J. biol. Chem.* **237**, 1856.
Haldane, J. B. S. (1931). *Proc. Roy. Soc.* B, **108**, 559.
Heidelberger, M. & Kendall, F. E. (1935). *J. exp. Med.* **62**, 6971.
Kaiser, E. T. & Weidman, S. W. (1964). *J. Amer. chem. Soc.*, **86**, 4354.
Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C. & Shore, V. C. (1961). *Nature, Lond.*, **190**, 666.
King, N. K., Looney, F. D. & Winfield, M. E. (1964). *Biochim. biophys. Acta*, **88**, 235.
Knowles, J. R. (1965). *Biochem. J.* **95**, 180.
Koshland, D. E., Strumeyer, D. H. & Ray, W. J. (1962). *Brookhaven Symp. Biol.* **15**, 101.
Kremer, M. L. (1965). *Nature, Lond.*, **205**, 384.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Muir, H. & Neuberger, A. (1949). *Biochem. J.* **45**, 163.
Nicolaus, R. A. (1960). *Rass. Med. sper.* **7**, Suppl. 2, 1.
Nicolaus, R. A., Mangoni, L. & Caglioti, L. (1956). *Ann. Chim., Roma*, **46**, 793.
Nicolet, B. H. & Shinn, L. A. (1939). *J. Amer. chem. Soc.* **61**, 1615.
Ouchterlony, Ö. (1949). *Acta path. microbiol. scand.* **26**, 507.
Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.
Ray, W. J. & Koshland, D. E. (1962). *J. biol. Chem.* **237**, 2493.
Reichlin, M., Hay, M. & Levine, L. (1963). *Biochemistry*, **2**, 971.
Smithies, O. (1959). *Advanc. Protein Chem.* **14**, 141.
Theorell, H. & Åkeson, A. (1955). *Ann. Acad. Sci. fenn.* 2A, **60**, 303.
Urnes, P. (1965). *J. gen. Physiol.* **49**, 75.