PRODUCTS OF METMYOGLOBIN OXIDATION AT ACID pH

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Summary

When the oxidation of metmyoglobin or methaemoglobin by H_2O_2 is carried out at pH 5 or less the principal product absorbs at 525 mμ. It is taken to be the conjugate acid of the ferrylmyoglobin or ferrylhaemoglobin which is the main oxidation product at neutral pH and which absorbs at 545 mμ. It appears to be susceptible to further attack by H_2O_2, yielding an inert ferric complex which absorbs at 586 mμ, and which cannot be reduced to an oxygen carrier. Formation of the 586 mμ complex could be a minor factor in the aging of erythrocytes. Whale metmyoglobin differs from that of horse in not being oxidized to the ferryl state by chloroiridate ion if the pH is less than 6·5.

I. INTRODUCTION

In earlier papers we have described in detail the oxidation of metmyoglobin by H_2O_2 at pH 6–8 to give ferrylmyoglobin and a free radical (King and Winfield 1963), and in brief the nature of the free radical (King, Looney, and Winfield 1964). The present paper concerns the products which appear at lower pH, while a subsequent communication will deal with the identity of the free radical and the oxidation of the globin moiety.

II. METHODS AND MATERIALS

A description of the experimental techniques and the preparation of horse heart metmyoglobin (Mb^{III}) have been given elsewhere (King and Winfield 1963).

Horse methaemoglobin (Hb^{III}) is prepared at 0°C by washing horse blood cells in 0·1M phosphate buffer at pH 7·4, haemolysis in distilled water, precipitation with ammonium sulphate (314 g/l), dialysis against distilled water, centrifugation, a second ammonium sulphate precipitation and dialysis, followed by oxidation to the ferric state by ferricyanide ion, and a final dialysis against the buffer appropriate to the oxidation experiment in which the Hb^{III} is to be used.

Sperm whale myoglobin (WMb^{III}) from Seravac Laboratories, Capetown, South Africa, is dialysed against 0·05M Tris buffer at pH 8·6, then chromatographed on diethylaminoethylcellulose, following the method of Brown (1961). The purity of the fraction selected for experiment is 99% as determined by the method of de Duve (1948).

Hydrogen peroxide (British Drug Houses, Analar grade), potassium chloroiridate (Johnson, Matthey, and Co.), and potassium ferricyanide (Hopkin and Williams Ltd., Analar grade) are used without further purification.

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III. Results

(a) *Product Absorbing at 525 mμ*

At pH 5 or lower the principal product of the reaction between H_2O_2 and Mb^{III} (or WMb^{III} or Hb^{III}), determined spectrophotometrically, has an absorption band at 525 mμ (Fig. 1). Its second peak in the visible region is at 548 mμ and therefore is obscured by the 545 mμ band of "alkaline" ferrylmyoglobin, Mb^{IV} (Keilin and Hartree 1935; George and Irvine 1952; King and Winfield 1963). Figure 1 is the result of correcting for the presence in the mixture of Mb^{III} and Mb^{IV} using a method based on that of Hardy and Young (1948).

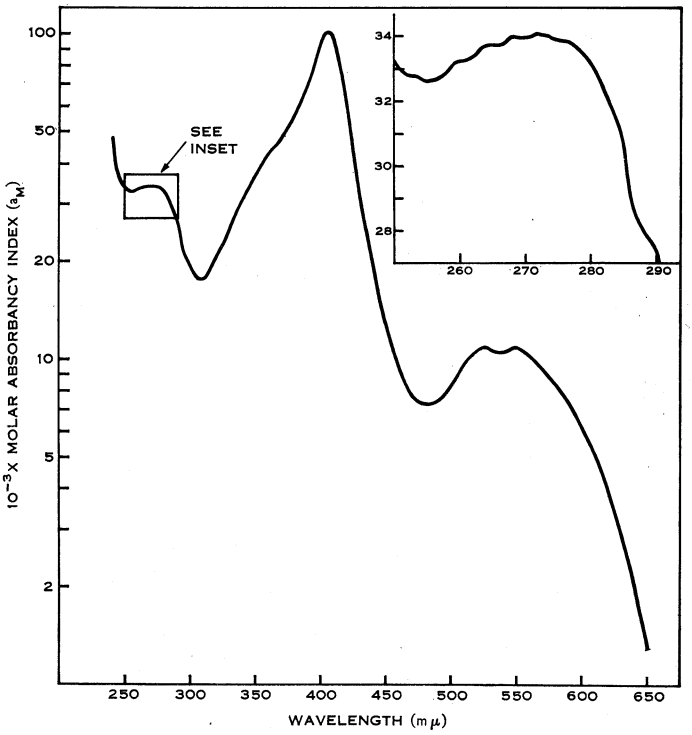


Fig. 1.—Estimated absorption spectrum of the complex obtained by oxidizing horse metmyoglobin (Mb^{III}) at low pH, derived from the observed spectrum by correction for the presence of other oxidation products and of residual Mb^{III}. Temperature 0°C; pH 4·5; initial Mb^{III} concentration $1·2\times10^{-4}$M for visible and Soret regions, $3·85\times10^{-5}$M for the ultraviolet region; mole ratio of H_2O_2 to Mb^{III} = 1·39; time 1–10 min after adding H_2O_2.

At pH 3·5 little except the 525 mμ complex and Mb^{III} can be detected spectrophotometrically during the first minute after adding H_2O_2 at 0°C. But at this pH precipitation of the protein takes place too quickly to permit detailed investigation. At pH 4·5 the loss of protein amounts to only 3% per hour. The relative proportions of Mb^{IV} and the 525 mμ complex are then found to be about 1 : 3·5 after 4·8 min (at a haemoprotein concentration of $0·8\times10^{-4}$M, H_2O_2 concentration of 4×10^{-4}M, and temperature of 0·2°C). Precise experimentation is

still difficult because the 525 mμ complex is converted fairly rapidly to one which absorbs at 586 mμ.

Increasing the initial concentration of Mb^{III} leads to a higher proportion of Mb^{IV} in the oxidation products. Modification of the globin by several cycles of oxidation and reduction (King and Winfield 1963) increases the ease of formation of the 525 mμ compound.

If the pH is raised from 4·5 to 8·0 after forming the 525 mμ complex, the latter is converted to Mb^{IV} (Table 1). In a reverse experiment, Mb^{IV} formed at pH 8·0 is largely converted to the 525 mμ complex on addition of sufficient acid to lower the pH to 4·5. In both directions the time required for conversion is less than the time required for scanning of the absorption bands (15 sec).

TABLE 1

INTERCONVERSION OF Mb^{IV} AND THE 525 mμ COMPLEX BY CHANGE OF pH

Initial pH	Complex	Percentage of Each Complex in Reaction Mixture	
		pH 4·5	pH 8·0
4·5*	Mb^{IV}	6	57
	525 mμ	53	−2
	Mb^{III}	18	18
	586 mμ	24	28
8·0†	Mb^{IV}	89	6
	525 mμ	0	83
	Mb^{III}	10	10

* Aqueous KOH added 4 min after mixing H_2O_2, acetic acid, and Mb^{III} at pH 4·5 Haemoprotein concentration $0{\cdot}75\times10^{-4}$M; initial H_2O_2 concentration $1{\cdot}7\times10^{-4}$M; phosphate buffer $0{\cdot}7\times10^{-1}$M, temperature 0°C.

† Aqueous acetic acid added 4 min after mixing H_2O_2, KOH, and Mb^{III} at pH 8·0. Other conditions as in first experiment.

It can be shown that the complex is not a phosphate or acetate by working in the absence of these ions, e.g. in citrate buffer.

Reduction of the 525 mμ complex by ferrocyanide ion or by ascorbate is not noticeably faster than reduction of Mb^{IV} at the same pH. As with Mb^{IV} (George and Irvine 1952; King and Winfield 1963), one equivalent of ferrocyanide is required for reduction of one mole to the ferric state. Reduction by ethanol is slow.

When Mb^{III} is oxidized at pH 4·5 and 0·2°C by chloroiridate ion, a one-electron oxidant, the product is again the 525 mμ complex, but precipitation of the protein is too fast to permit quantitative experiments. WMb^{III} behaves differently; there is negligible oxidation of the haem group when the pH is less than 6·5. Since the chloroiridate is consumed, an amino acid residue must be oxidized in preference to the metal.

(*b*) *Product Absorbing at 586 mμ*

A nearly pure solution of the 586 mμ complex can be prepared as follows: 2·4 ml of 0·1M aqueous H_2O_2 is added to 10 ml of 0·2M acetate buffer (pH 4·5) containing 0·5 g of Mb^{III}, at 0°C. After 2 and 4 hr, 1·2 and 0·6 ml respectively of H_2O_2 solution are added. After standing a further 2 hr the mixture is dialysed against running distilled water. It is then chromatographed on a diethylaminoethylcellulose column; with water as eluant the 586 mμ complex moves behind the other pigments. The absorption spectrum of the selected fraction is shown in Figure 2.

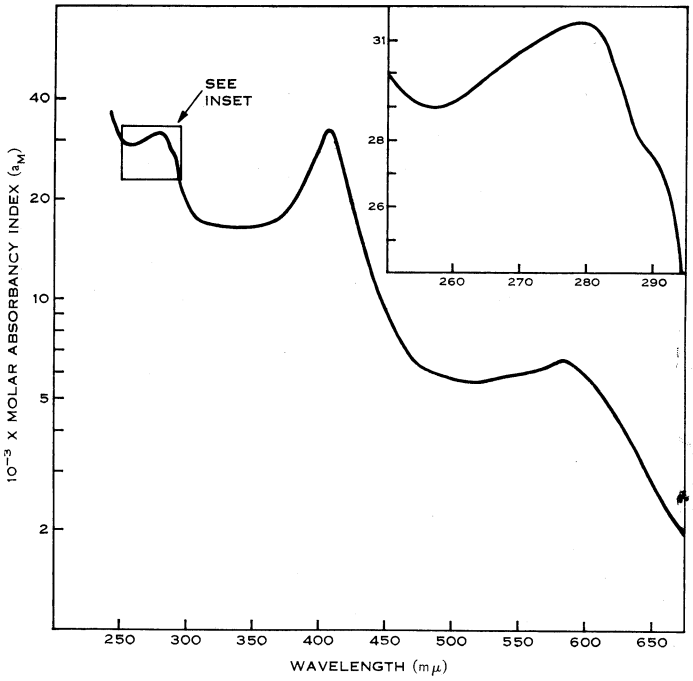


Fig. 2.—Absorption spectrum of the irreversibly formed product obtained by oxidizing whale metmyoglobin at low pH. Temperature 0°C; pH 4·5; product purified by chromatography (see text).

Negligible amounts of the complex are found during oxidation of Mb^{III} at pH 8. But once formed (at low pH), its absorption spectrum is insensitive to pH, or to the addition of ferrocyanide or more H_2O_2. Addition of dithionite causes extensive reaction, with no products which we can recognize. Sodium borohydride reacts only slowly, with a general loss of absorption in the visible region.

Although no immediate change in absorption spectrum is detected on addition of alkali to pH 8, the rate of denaturation (precipitation) is greatly increased, suggesting that the protein moiety is different in structure from that of Mb^{III}. Comparison of the ultraviolet absorption of the two haemoproteins leads us to believe that not more than three phenylalanine residues per molecule have been

oxidized (King, Looney, and Winfield, unpublished data). At pH 8 an extensive oxidation of the globin of Mb^{III} fails to produce the 586 mμ complex.

Although approximate, the values for the molar absorbancies given in Figure 2 for the principal bands of the 586 mμ compound are sufficient to demonstrate the unusually weak absorption in the Soret region (about 20% of that for Mb^{III}). The ultraviolet absorption is suggestive of a low-spin complex (George, Beetlestone, and Griffith 1961); there is no electron-spin resonance absorption (measured at $-102°C$ and at $-30°C$).

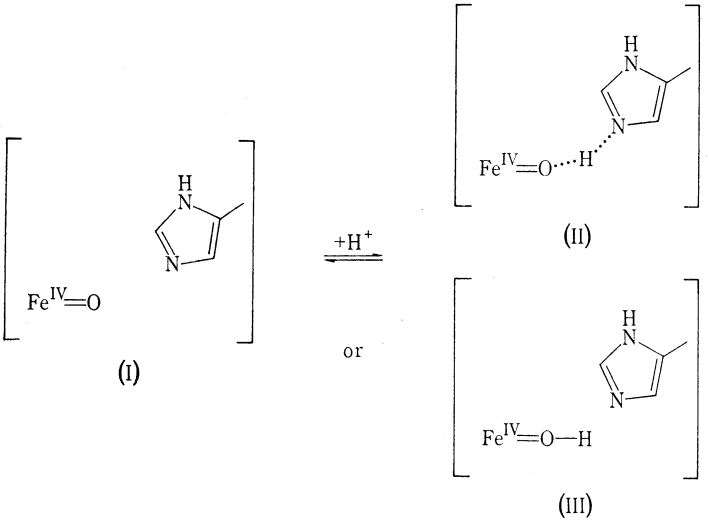
Hb^{III} yields a 586 mμ complex at about the same rate at pH 4·5 as does Mb^{III} or WMb^{III}.

IV. Discussion

(*a*) *525 mμ Complex*

From the experiments described it is concluded that the 525 mμ complex is probably H^+Mb^{IV} (the conjugate acid of ferrylmyoglobin). It is the hypothetical complex assumed by George and Irvine (1959) to contribute to the oxidation of ferrocyanide by Mb^{IV}, in their explanation of the kinetics of ferrocyanide oxidation by H_2O_2 when it is catalysed by Mb^{III}.

In both Mb^{IV} and H^+Mb^{IV} the metal must be in the formal oxidation state Fe^{IV}, in order to satisfy the experimental evidence (Keilin and Hartree 1935; King and Winfield 1963). But there is no evidence regarding the location of the added proton, except the inference that it is H^+Mb^{IV} rather than Mb^{IV} which degenerates to the inactive 586 mμ complex. From the structure of Mb^{III} and its azide (Stryer, Kendrew, and Watson 1964) we may guess that the most likely structure for Mb^{IV} contains the ferryl ion and the distal histidine arranged as in (I), while the most attractive alternatives for H^+Mb^{IV} are (II) and (III) or a mixture of the two.



The failure of chloroiridate to oxidize WMb^{III} to the ferryl form may be due to the presence in WMb^{III} of a readily accessible tyrosine residue which is absent from Mb^{III} (Holleman and Biserte 1959; Braunitzer *et al.* 1964). Differences between the ultraviolet absorption spectra of Mb^{III} and WMb^{III} used in our oxidation experiments are consistent with the presence of one more tyrosine residue in the material from sperm whale. The six ultraviolet absorption bands ascribed to the phenylalanine residues (Lavin and Northrup 1935) are detectable in both spectra, but are much less prominent for WMb^{III}.

(*b*) *586 mμ Complex*

From the effect of pH upon its rate of formation, and the need for excess H_2O_2, it is tentatively concluded that the complex has its origin in a reaction of H^+Mb^{IV} with H_2O_2. The resistance of the 586 mμ complex to oxidation, the irreversibility of its formation, and the occurrence of a haemoglobin analogue, together with the indication that it is produced by oxidation of a ferryl complex, suggest that the porphyrin has been oxidized.

When myoglobin is subjected to prolonged oxidation, the aromatic groups are progressively oxidized. Although the capacity to combine reversibly with O_2 is modified, it is not destroyed by eight two-electron oxidations of the globin (King and Winfield 1963). From the number of aromatic residues known to be present we can estimate* that 8–18 such oxidations occur before the haemoprotein becomes non-functional. Oxidation to the 586 mμ complex, on the other hand, can take place quickly, without the need for a long series of reactions before the haemoprotein becomes inactive. To be fast it requires an acid pH, but so does the autoxidation of haemoglobin, which is nevertheless known to proceed rapidly enough at physiological pH to maintain an easily detectable concentration of Hb^{III} in the erythrocyte (Lemberg and Legge 1949). Whether the 586 mμ complex can also be formed under physiological conditions will depend upon the concentration of those peroxides small enough to penetrate to the haem group within a haemoglobin molecule (Winfield 1965). The complex might appear in detectable amounts in blood cells deficient in catalase, or glutathione peroxidase, when Hb^{III} attacked by H_2O_2 quickly encounters a second molecule of the peroxide.†

Because the iron atom of the 586 mμ complex of haemoglobin will not initiate peroxide decomposition, further oxidation of the complex within the erythrocyte is unlikely, except in so far as aromatic groups of its globin can be attacked by free radicals of other haemoprotein molecules (King and Winfield 1963; Winfield 1965). In this sense it may have a very limited usefulness as a supplementary source of the reducing power which is needed to "repair" functional haemoglobin which has suffered oxidation to a free-radical state.

* The number cannot be determined accurately by experiment, because once the capacity to decompose H_2O_2 rapidly is lost, there is no method available for continuing to oxidize the aromatic residues selectively.

† Peroxide for the oxidation to the 586 mμ complex cannot be generated within the molecule of H^+Hb^{IV}. While passing from its source to the molecule which is to be oxidized the peroxide is susceptible to enzyme action (Cohen and Hochstein 1963).

V. References

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