Metmyoglobin Oxidation during Electron Transport Reactions in Mitochondria

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ABSTRACT Studies of the intracellular role of myoglobin were carried out by recording spectrophotometric changes in acid metmyoglobin and oxymyoglobin during electron transport reactions with mitochondria prepared from pigeon heart muscle by the method of Chance and Hagihara. The absorption peak of metmyoglobin at 409 mμ disappeared when substrate was added to normal or antimycin-inhibited preparations, and was replaced by a new maximum at 423 to 424 mμ, identified as due to the oxidation to ferrylmyoglobin. Further investigation revealed that the oxidation of metmyoglobin took place with the simultaneous oxidation of reduced flavoprotein. Hydrogen peroxide, formed by the reaction of reduced flavoprotein with oxygen, was considered to be the probable intermediate for the oxidation of metmyoglobin in experiments in which catalase was added as a competitor for the oxidant. When DPNH was added to the reaction mixture, the reductant acted to resynthesize the ferri-derivative by reaction with ferrylmyoglobin. Oxymyoglobin could not be used in place of metmyoglobin in these systems. Under the experimental conditions, oxymyoglobin dissociated when dissolved oxygen was depleted from the medium by enzyme oxidations; the resultant ferromyoglobin underwent oxidation to metmyoglobin.

INTRODUCTION

The observations presented in this paper are the result of studies which were concerned with the intracellular role of myoglobin. This muscle hemoprotein is regarded primarily as an agent which facilitates the intracellular diffusion of oxygen and its storage. However, even before myoglobin was clearly identified as a protein separate from hemoglobin, studies were undertaken to explore the possibility of an additional catalytic role for it in red muscle. These investigations were encouraged by the differences between red and white muscle found in early histological, physiological, and biochemical studies.

The results through 1948 have been summarized in reviews by Needham

(1), Millikan (2), and Biorck (3). Those most significant in relationship to the present paper were the decreased lactic acid production, elevated cholesterol content, and elevated oxygen uptake in red muscle. The more recent results of Feinberg and his associates (4) with respect to oxygen uptake reinforce earlier findings. A consistent correlation has been found between oxygen uptake by cardiac muscle and the work output of the muscle as determined by the product of the blood pressure and heart rate under a variety of experimental conditions (*e.g.*, epinephrine, hypoxia, hypothermia) (5–7). A large excess of oxygen is taken up, however, in relation to the measurable work output. The problem of calculating the intrinsic work of the muscle makes it difficult to correlate the oxygen uptake with the chemical requirements for energy production. However, this large oxygen requirement is of interest in relationship to the results of Bing (8) who found that fatty acid was used for over 60 per cent of the energy production in cardiac muscle.

In studying the oxygen utilization of red muscle, *in vivo*, Millikan (2) measured the fluctuations in oxymyoglobin during muscle contraction and concluded that the deoxygenation of the hemoprotein served to maintain the oxygen tension in the tissue during periods of contraction when requirements rose tenfold. Although this served to maintain a steady supply of oxygen for enzyme requirements, it did not rule out an additional role for the protein in the catalytic activity of the cell. At the same time, Sacks (9) studied the aerobic recovery of red muscle after aerobic and anaerobic contraction and concluded that anaerobic contraction, characterized by the outpouring of lactic acid, represented an abnormal experimental situation in red muscle. Both Millikan and Sacks considered the possibility of a further role of myoglobin, although this idea was presented on the basis of intuition rather than experimental evidence. Indeed, Millikan pointed out those characteristics of myoglobin which placed it between hemoglobin and catalase: its ease of oxygen dissociation and its rapid oxidation to metmyoglobin.

The oxidation of metmyoglobin by peroxide was first reported by Keilin and Hartree in 1950 (10). They observed the formation of an oxidation product which they considered to be ferrylmyoglobin. The mechanism of this reaction was subsequently studied by George and Irvine (11) with myoglobin from horse heart. On the basis of further experimental evidence, these authors concluded that the reaction product represented a one-electron oxidation of the iron in the hemoprotein to Fe^{IV}. In turn, ferrylmyoglobin could be reduced to metmyoglobin by a one-electron reductant, potassium ferrocyanide (12). The reaction product was more stable above pH 8; α-, β-, and γ-bands were recorded at 580, 549, and 423 mμ, respectively (13). Changes in the spectrum of ferrylmyoglobin were observed as the pH approached the acid range due to destruction of the product.

In this paper, the spectrum of the oxidation product of metmyoglobin was

recorded when metmyoglobin was added to mitochondria in the presence of substrate. This spectrum corresponded to that which has been attributed to ferrylmyoglobin (10, 11, 13). The reduction of ferrylmyoglobin to metmyoglobin by DPNH was also shown to take place. These results support the earlier concept of an additional catalytic role for myoglobin in red muscle.

METHODS

Preparation of Myoglobin

Myoglobin was purified from horse heart essentially according to the method of Theorell (14). At two steps in purification, the phosphate precipitation procedure of Morgen (15) was used: (1) 3 M phosphate precipitation was introduced after the lead acetate step, and (2) 3 M phosphate precipitation followed by 4 M phosphate precipitation was added after the hemoprotein solution was concentrated to one-tenth of the original volume. The hemoprotein, isolated as metmyoglobin, was stored at −20° as an ammonium sulfate precipitate. The purity of the final product, as determined by the method of de Duve (16), was greater than 99 per cent and represented a yield of 37 per cent.

For use in enzyme studies, an aqueous suspension of metmyoglobin was saturated with carbon monoxide, reduced with sodium dithionite, $Na_2S_2O_4$, and dialyzed rapidly against a constantly flowing solution of 0.01 M EDTA (pH 7) saturated with carbon monoxide. Oxymyoglobin was prepared by passing a fine stream of oxygen (saturated with water vapor) over the surface of a solution of carboxymyoglobin at room temperature. The replacement of carbon monoxide by oxygen was monitored by the shift in the absorption peak in the Soret region from 424 mμ to 418 mμ, and by the change in spectrum in the region of the α- and β-bands according to the values reported by Theorell and Åkeson (17). Equilibration with oxygen was continued until there were no further changes in the spectrum. The solution was centrifuged to remove any denatured protein and the concentration determined before use in enzyme studies (17). Metmyoglobin was obtained by autoxidation of oxymyoglobin during storage at −20°C.

Preparation of Mitochondria

Mitochondria were prepared from pigeon heart muscle by the method of Chance and Hagihara (18). The homogenizing medium, consisting of 0.225 M mannitol, 0.075 M sucrose, 1.0 mM tris, 0.05 mM tetrasodium EDTA, was adjusted to pH 7.4 with HCl (19). Suspensions of mitochondria were assayed for protein by the biuret reaction according to the method of Gornall, Bardawill, and David (20) and were diluted to 12 to 18 mg protein/ml. Fresh preparations of mitochondria did not consume oxygen in the absence of ADP when assayed manometrically by the method of Slater and Cleland (21), nor could they oxidize DPNH in the absence of ADP and substrate.

These preparations were freed of any traces of red blood cells by refrigeration overnight. The cells sedimented and the suspended mitochondria could be decanted.

Ageing for 24 to 48 hours resulted in increased activity with respect to both oxygen uptake and DPNH oxidation in the absence of ADP.

Electron micrographs showed that the fresh preparation was uncontaminated by other cellular particles and that the individual mitochondria were intact (22). These micrographs were made with samples taken from the top of the pellet.

Reaction Mixture for Spectrophotometric Measurements

Homogenizing medium (1 ml) was mixed in a cuvet with an aqueous solution (0.85 ml) containing 20 μmoles potassium phosphate (pH 7.4), 1 μmole ADP, and 80 to 200 Kunitz-McDonald units of hexokinase (Sigma, type II) with 50 μmoles glucose (21). Mitochondria in homogenizing medium (equivalent to 1.8 to 2.6 mg mitochondrial protein) were added and the volume adjusted to 2 ml. All solutions were in equilibrium with air.

Other biochemical preparations used included: (*a*) β-DPNH (Sigma Chemical Co., synthesized enzymatically), dissolved in phosphate buffer at pH 7.4 such that 0.75 μmole could be added to the reaction mixture described above in a volume of 5 to 10 μl. (DPNH prepared by chemical reduction could not be used in these experiments because the residual dithionite present in it reduced metmyoglobin to ferromyoglobin. Frozen DPNH solutions were also inhibitory (23).) The oxidation of DPNH was recorded by changes in optical density at 340 mμ. Calculations were based on the extinction coefficient of DPNH reported by Horecker and Kornberg (24). (*b*) Catalase was a lyophilized preparation with a minimum of 300 units/mg from the Worthington Biochemical Corporation.

A Cary model 11 recording spectrophotometer was used in these studies. An opal glass plate was placed in the light path according to the method of Shibata (reference 25, procedure A). This makes it possible to record spectra in suspensions which are strongly light-scattering. Optical density determined in this manner cannot be related quantitatively to extinction coefficients measured in clear solutions.

RESULTS

Oxidation of Metmyoglobin to Ferrylmyoglobin

Spectrophotometric changes in the visible spectrum of myoglobin were recorded in reaction mixtures containing mitochondria, substrate, and cofactors. The reaction and reference cuvets were equivalent in all respects except for the addition of myoglobin in the reaction cuvet. In experiments with oxymyoglobin, the absorption maximum at 418 mμ (17) decreased as the oxygen supply was exhausted from the medium and was replaced by a peak at 409 mμ, characteristic of metmyoglobin. Addition of metmyoglobin in place of oxymyoglobin resulted in an unexpected reduction of the peak at 409 mμ and the appearance of a new maximum at 423 to 424 mμ. This change in spectrum required substrate, such as succinate or fumarate, mitochondria, and oxygen. The reaction was not inhibited by antimycin, but was actually enhanced in its presence.

The reaction of mitochondria with metmyoglobin in the presence of fumarate, oxygen, and antimycin can be seen in the spectra shown in Fig. 1. The difference spectrum in Fig. 1 *b* is that of the reduced-minus-oxidized flavoprotein and cytochromes in the mitochondrial preparation. This can be compared with Fig. 1 *c* in which a difference spectrum was recorded 2 minutes after antimycin was added to the reaction cuvet. Absorption bands

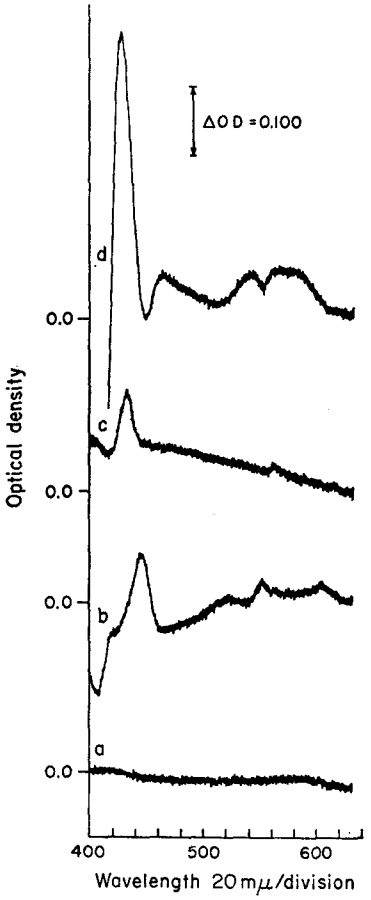

FIGURE 1. The oxidation of metmyoglobin in antimycin-inhibited systems. Difference spectra taken with complete reaction mixture in both cuvets. Fumarate used as substrate. Further additions as indicated. (*a*) Base line, aerobic + substrate minus aerobic + substrate. (*b*) Anaerobic + substrate minus aerobic without substrate. (*c*) Spectrum recorded 2 minutes after the addition of antimycin (1 μg) to complete reaction mixture with substrate. Aerobic-inhibited minus aerobic. (*d*) Spectrum recorded 2 minutes after adding 4.4×10^{-6} M metmyoglobin to (*c*). Aerobic-inhibited + metmyoglobin minus aerobic.

which appeared at 430 and 562 mμ were attributed to the increased reduction of cytochrome *b* (26). The spectrum in Fig. 1 *d* was recorded within 2 minutes after 4.4×10^{-6} M metmyoglobin was added to the antimycin-blocked mitochondria in Fig. 1 *c* and shows the contribution from myoglobin as well as the difference in the state of oxidation of the mitochondria in the two cuvets. There was a shift in the absorption maximum of myoglobin in the Soret region from 409 mμ to 424 mμ. In addition, a broad band with a maximum at 465 mμ in the region of the absorption of oxidized flavoprotein (26) appeared after the addition of metmyoglobin to these preparations. Further, two max-

ima at 542 mμ and 580 mμ were superimposed upon the spectrum of reduced cytochrome *b*.

The new absorption bands which appeared at 424, 542, and 580 mμ corresponded with those which Keilin and Hartree (10) and George and Irvine (11) reported for ferrylmyoglobin. In order to identify the product absorbing at 424 mμ, potassium ferrocyanide (1.5×10^{-3} M) was added after the enzymatic reaction with metmyoglobin (4.4×10^{-6} M) had resulted in a definite maximum at 424 mμ. Addition of the reductant resulted in the reappearance of the absorption peak at 409 mμ, corresponding to the formation of ferrimyoglobin. Subsequent addition of a slight excess of hydrogen peroxide reversed the reaction and the product had a maximum at 423 to 424 mμ, similar to the absorption peak which appeared during the enzyme-catalyzed reactions.

For a clearer comparison of the spectra which appeared in the mitochondrial reactions with those reported previously, a slight excess of hydrogen peroxide was added to 4.4×10^{-6} M metmyoglobin in 2.0 ml of 0.3 M homogenizing medium (pH 7.4). Under these conditions, without mitochondria, the same absorption peaks were observed as in the present enzymatic experiments and as were reported previously for ferrylmyoglobin (13) in this pH range.

The ratio of the absorption maxima of ferrylmyoglobin and metmyoglobin, calculated on the basis of their extinction coefficients at 423 mμ (13) and 409 mμ (17), respectively, is 0.63. This ratio was found to be 0.68 in the present experiments (see Fig. 2). The spectrum of ferrylmyoglobin in Fig. 2 cannot be compared precisely with those recorded at pH 6 in a more recent paper by King and Winfield (27) since the oxidation product is affected by the pH of the reaction medium (13).

On the basis of these experimental results, the absorption maxima which appeared in the reactions with metmyoglobin were identified with the compound which George and Irvine (13) have considered to be ferrylmyoglobin.

Inhibition of Metmyoglobin Oxidation by Catalase

George and Irvine (28) have demonstrated that the reaction with metmyoglobin takes place with several oxidants other than hydrogen peroxide; *e.g.*, potassium chloriridate, potassium molybdocyanide, alkyl peroxides (28, 29). Hydrogen peroxide, however, can be present in cells as the product of the reaction of reduced flavoprotein with oxygen (30) and is, therefore, the most obvious candidate for the oxidation of metmyoglobin in these enzyme systems.

In order to confirm this, catalase was added to the reaction mixture as a means of removing hydrogen peroxide. The results are illustrated in Fig. 2. These spectra were recorded 5 minutes after all the reactants were mixed. In both cases the reaction and reference cuvets differed only in the myo-

globin content of the former. The addition of metmyoglobin to antimycin-inhibited systems resulted in the formation of ferrylmyoglobin (Fig. 2 *a*). However, when catalase (10^{-9} M) was present, there was no change in the spectrum of metmyoglobin (Fig. 2 *b*). The spectrum of ferrylmyoglobin (in

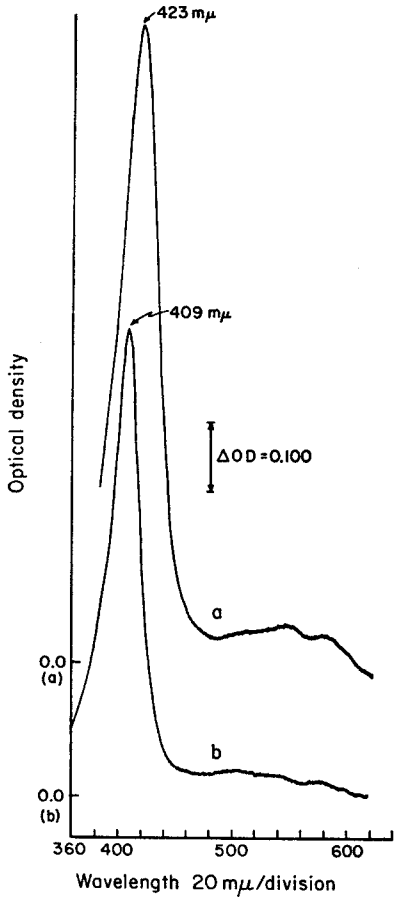

FIGURE 2. Effect of catalase on the oxidation of metmyoglobin in the presence of antimycin. Difference spectra taken with complete reaction mixture and antimycin (1 μg) in both cuvets. (*a*) Spectrum recorded 5 minutes after the addition of mitochondria. Myoglobin 8.8 × 10^{-6} M. Aerobic-inhibited + metmyoglobin minus aerobic-inhibited. (*b*) Catalase (approximately 10^{-9} M by weight) in both cuvets. Spectrum recorded 5 minutes after the addition of mitochondria. Metmyoglobin, 4.4 × 10^{-6} M. Aerobic-inhibited with catalase + metmyoglobin minus aerobic-inhibited with catalase.

Fig. 2 *a*) tends to obscure the 450 to 480 mμ region where any differences in the oxidation-reduction state of flavoprotein in these longer incubation periods would be seen for comparison with Fig. 1 *d*.

Reduction of Ferrylmyoglobin by DPNH

The oxygenase reaction which metmyoglobin exhibited in the formation of ferrylmyoglobin is of potential use in accounting for the increased oxygen uptake measured in red muscle (1) and cardiac muscle (4). The absence of lactic acid in heart muscle is also a striking characteristic. Since the lactic acid formed by white muscle as the oxygen tension decreases is related to an

accumulation of extramitochondrial DPNH, it was of interest to measure changes in the rate of oxidation of DPNH in the reactions with mitochondria and myoglobin. DPNH was added to give a final concentration of 2.5×10^{-4} M so that it was present in excess of the dissolved oxygen in these reactions. With fresh, coupled mitochondria, succinate, and ADP, the change in optical density at 340 mμ was −0.10 per minute per mg mitochondrial protein. With 6×10^{-6} M metmyoglobin present, the rate increased to −0.13. Although these rather low rates were determined in mixtures with high total absorption, the increased oxidation of DPNH with metmyoglobin was con-

TABLE I

THE EFFECT OF MYOGLOBIN ON THE OXIDATION OF DPNH

Complete reaction mixture used as described under Methods; mitochondria aged 24 hours. DPNH (0.75 μmole) added at zero time.

Additions	DPNH oxidized per min. per mg mitochondrial protein
	mμmoles
None	50
Oxymyoglobin, 1.2×10^{-5} M	50
None	48
Metmyoglobin, 2.3×10^{-5} M	43
None	40
Antimycin, 1 μg	<1
Antimycin, 1 μg, oxymyoglobin, 1.8×10^{-5} M	<1
Antimycin, 1 μg, metmyoglobin, 2.3×10^{-5} M	6
Mitochondria omitted, metmyoglobin, 8.8×10^{-6} M	<1

firmed by a comparable decrease of 25 per cent in the time during which the oxidation took place. These results suggested that there might be some relationship between the oxidation of metmyoglobin and DPNH. This was investigated further with aged preparations in experiments comparable to those illustrated in Figs. 1 and 2. With these preparations, the control rates of DPNH oxidation were much higher than in fresh preparations. The results are presented in Table I. Here there was essentially no change in the rate of oxidation of DPNH with mitochondria and substrate when either form of hemoprotein was added to the reaction mixture in the absence of antimycin. In experiments with oxymyoglobin, the additional oxygen added in bound form allowed for an increase in the total amount of DPNH oxidized, but did not influence the rate of this oxidation. When no further changes were recorded at 340 mμ in experiments with both oxy- and metmyoglobin, measurements in the Soret region revealed only a peak at 409 mμ, the absorption maximum for metmyoglobin.

The oxidation of DPNH in the presence of mitochondria and substrate was sensitive to inhibitors such as antimycin and cyanide. Cyanide, however, was not a suitable inhibitor in these studies due to its reaction with metmyoglobin (31) as well as with DPN and flavoproteins (32). The effect of metmyoglobin in partially reversing the inhibition of DPNH oxidation due to antimycin can be seen in Table I. In measurements with several mitochondria preparations, metmyoglobin consistently reversed the inhibition produced by antimycin by 12 to 25 per cent. Under the same conditions, oxymyoglobin did not stimulate DPNH oxidation.

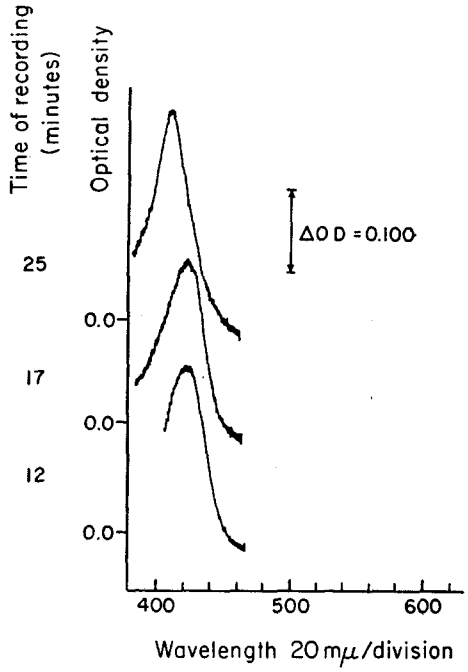

FIGURE 3. The reaction of metmyoglobin with fumarate, antimycin and DPNH. The reaction mixture was prepared as described under Methods with mitochondria aged 24 hours. Antimycin, 1 μg, DPNH, 3.7×10^{-4} M, and metmyoglobin, 1.3×10^{-5} M were used. Mitochondria were added at zero time.

Since metmyoglobin stimulated DPNH oxidation in the presence of antimycin, it was of interest to see whether the oxidation of metmyoglobin was affected by DPNH. The results can be seen in Fig. 3. It took 12 minutes for the maximum at 409 mμ to be replaced by that of ferrylmyoglobin at 424 mμ. This may be compared with Figs. 1 *d* and 2 *a*, in which ferrylmyoglobin was formed in less than 2 and 5 minutes respectively, with antimycin but without DPNH.

If mitochondria were omitted from the reaction mixture, metmyoglobin did not stimulate the oxidation of DPNH. However, when ferrylmyoglobin was synthesized by direct chemical reaction of metmyoglobin with hydrogen peroxide, the subsequent addition of DPNH brought about reduction of the oxidation product of metmyoglobin. This reaction has been suggested by

George and Irvine (33) and probably accounts for the oxidation of DPNH which was observed in the present experiments with antimycin.

The possibility of a reaction between DPNH and ferrylmyoglobin in the enzyme system was investigated further by making use of the competition of catalase for hydrogen peroxide (as illustrated in Fig. 2) to measure its effect on the rate of oxidation of DPNH in the presence of metmyoglobin. As shown in Fig. 4, the inhibition of the oxidation of DPNH which resulted when antimycin was added to the reaction mixture was reversed to the extent of 12 per cent upon the addition of metmyoglobin. This partially restored rate of oxidation was again suppressed when catalase was added. The oxidation of DPNH, stimulated by the addition of metmyoglobin (Fig. 4), explains the

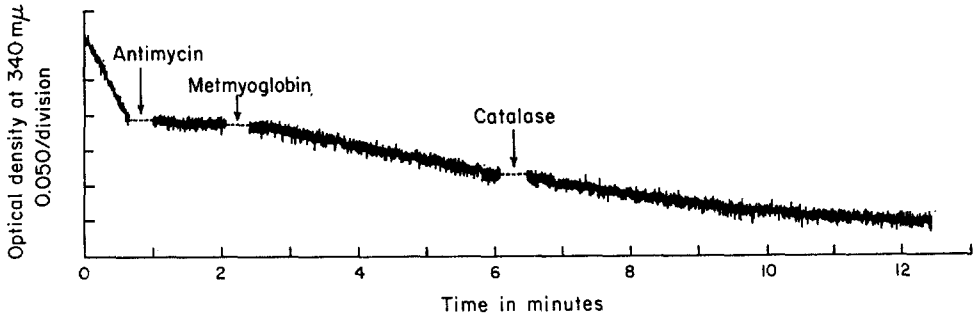

FIGURE 4. Effect of antimycin, metmyoglobin, and catalase on the oxidation of DPNH. The complete reaction mixture with fumarate was used; 10^{-4} M DPNH was added at zero time and then 1 μg antimycin, 4.4 × 10^{-6} M metmyoglobin, and 10^{-9} M catalase as indicated in the figure. The reference cuvet contained the complete reaction mixture with fumarate.

slow appearance of the ferrylmyoglobin peak in Fig. 3 as compared to Figs. 1 *d* and 2 *a*, for it reflects the continuous resynthesis of metmyoglobin in the presence of DPNH.

The results with catalase shown in Fig. 4 confirm those of Fig. 2 which demonstrated that catalase was more effective than metmyoglobin in removing the oxidant formed during mitochondrial activity. The oxidation shown in Fig. 4 did not come to a halt because of the depletion of oxygen from the medium because only 10^{-4} M DPNH was used in order to avoid anaerobiosis. The final portion of Fig. 4 reflects the removal of peroxide by reaction with catalase rather than by oxidation of metmyoglobin and DPNH.

DISCUSSION

The results demonstrate that the disappearance of metmyoglobin which was observed in the enzyme-catalyzed reactions is due to an oxidation in which ferrylmyoglobin is formed; this, in turn, can be reduced directly by DPNH. The reaction of metmyoglobin is independent of the cytochrome chain for it

is antimycin-insensitive. However, spectrophotometric evidence indicates that there is simultaneous alteration in the absorption of flavoprotein with the oxidation of metmyoglobin. The flavine enzymes which participate in the oxygenase reaction are believed to be associated with the mitochondria and not with other intracellular particles such as microsomes. The evidence which supports this includes the lack of any direct oxidation of DPNH in the preparations, the inhibition of the oxidation of DPNH by antimycin (without metmyoglobin), and the absence of other particles in these preparations (22). The various reactions which have been referred to in this paper are summarized in Fig. 5. The figure shows that ferrylmyoglobin can react further with hydrogen peroxide to yield oxymyoglobin (10) with an absorption peak

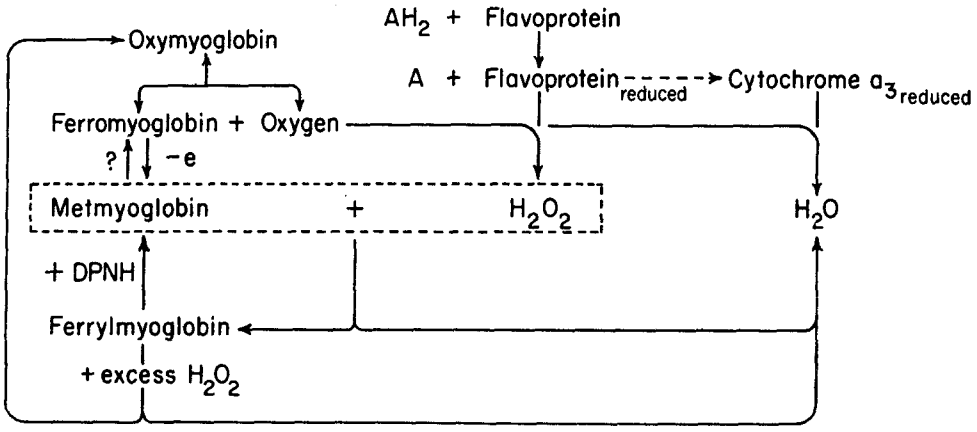

FIGURE 5. Summary of reactions.

at 418 mμ. This reaction then competes with the reduction by DPNH to prevent the accumulation of ferrylmyoglobin.

The turnover rate cannot be determined solely on the basis of spectrophotometric evidence, but also requires measurement of oxygen uptake. This can be seen from a comparison of Figs. 1 *d* and 2 *a*. Under the conditions present in Fig. 1 *d*, accumulated peroxide favored the further reaction of ferrylmyoglobin to form oxymyoglobin. The ratio of the peak heights of the α- and β- and the γ-bands is 1 to 6 in Fig. 1 *d* and 1 to 10 in Fig. 2 *a*. This indicates that oxymyoglobin has contributed to the α- and β-bands in Fig. 1 *d*. This additive contribution is not present in the Soret region where the γ-bands of oxy- and ferrylmyoglobin do not coincide (418 *vs.* 423 mμ). This means that the oxidation-reduction state of myoglobin cannot be determined quantitatively from the recorded spectra.

The rate of oxidation of DPNH in fresh preparations of mitochondria increased by 30 per cent with the addition of metmyoglobin. With antimycin, 12 to 25 per cent of the rate of this oxidation persisted with metmyoglobin present.

However, this cannot be translated directly into reaction rates with respect to ferrylmyoglobin formation either, because of the secondary reaction of the latter with hydrogen peroxide.

Reduced flavine enzymes are known to react with oxygen to produce hydrogen peroxide (30). In 1936, Theorell (34) reported that the autoxidation of reduced flavine mononucleotide was directly dependent upon the partial pressure of oxygen. According to his results, oxygen pressure of 150 mm Hg, present in reactions equilibrated with air, resulted in half of the reduced flavine undergoing autoxidation with the formation of hydrogen peroxide. More recent work of Massey and Gibson (35) has been concerned with the detailed mechanism of the reactions of flavoproteins. On the basis of their work with D-amino acid oxidase, they have proposed that the formation of hydrogen peroxide takes place by simultaneous interaction of the semiquinoid flavine enzyme with oxygen and substrate. Their results also favor this form of the flavine enzyme as the one absorbing at 465 mμ. In the experiments described in the preceding section, all solutions were in equilibrium with atmospheric gases; the addition of metmyoglobin to the reaction mixture resulted in the formation of ferrylmyoglobin and in the appearance of the broad absorption band of oxidized flavoprotein with a maximum at 462 to 465 mμ. In the experiments with myoglobin, reduced flavoproteins did not undergo marked oxidation in the presence of oxygen until metmyoglobin was added. Therefore, the mechanism proposed by Massey and Gibson may be operating in this reaction. The contribution of non-heme iron to the absorption at 465 mμ could not be determined from the spectra because the 550 mμ absorption was obscured by cytochrome C (36).

Although the location of myoglobin in the cell has not been determined, traces of the hemoprotein were shown to be present in preparations from heart muscle by Colpa-Boonstra and Minnaert (37). Whether this was an accidental inclusion due to the precipitation of metmyoglobin during the fractionation procedure is not known. However, the preparations used in the present studies appeared to be free of myoglobin as judged from the absence of absorption at 409 mμ (γ-band of metmyoglobin) and 580 mμ (α-band of oxymyoglobin). The metmyoglobin concentration used in this paper corresponds to a few per cent of the total myoglobin in heart muscle, 3×10^{-4} M (38).

The oxidation of metmyoglobin reported in the present paper is consistent with the earlier observation of the enhanced oxygen uptake in red muscle as compared with white muscle which was mentioned in the Introduction. The reaction also provides an additional pathway for the oxidation of extramitochondrial DPNH. The contribution of the oxygenase reaction to the metabolism of the muscle cell cannot be evaluated from the experiments with mitochondria preparations. The formation of ferrylmyoglobin will easily be

obscured in intact cells by the more strongly absorbing oxymyoglobin, both compounds having similar positions for their α-, β-, and γ-bands. However, the results presented in this paper encourage a further evaluation of the steady-state concentrations of oxy-, met-, and ferrylmyoglobin in muscle in order to determine the contribution of the oxidase and oxygenase pathways to the over-all oxygen uptake of the tissue.

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