Interaction of Myoglobin and Cytochrome c*

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SUMMARY

Spectrophotometric studies of various mixtures of reduced and oxidized forms of myoglobin and cytochrome c revealed that oxymyoglobin is capable of reducing ferricytochrome c at a molar ratio of 1. Other combinations, i.e., oxymyoglobin and ferricytochrome c; metmyoglobin and ferricytochrome c; or metmyoglobin and ferrocytochrome c showed no spectral change during the time course studied.

The pH optimum of the reaction was found to be 7.2. The Q10 was about 5 and the activation energy was about 29 kcal per mole. Aging of oxymyoglobin tended to increase the rate of the reaction, but metmyoglobin added to the reaction mixture had no effect on the system. Antimycin A, amytal, and rotenone were without effect on the reaction. The partial carbamylation of histidyl residues of myoglobin increased the reaction rate to twice that of the control. The formation of a complex between oxymyoglobin and cytochrome c was not detected by various physical methods.

Oxygen had an inhibitory effect on the reaction of the oxymyoglobin-ferricytochrome c system. This suggested that deoxymyoglobin rather than oxymyoglobin is the reacting species and that the rate-limiting step is the dissociation of oxygen from oxymyoglobin. This was confirmed by measurements showing that deoxymyoglobin reacted with ferricytochrome c at rates significantly greater than those of oxymyoglobin-cytochrome c systems. This reaction was shown to proceed in stoichiometric fashion under equilibrium conditions. Carbon monoxide myoglobin was unreactive.

It is well known that myoglobin and cytochrome c have structural similarities. Both are hemoproteins with one polypeptide chain and both are capable of transforming reversibly to two oxidation-reduction forms. The primary structures of both proteins from many species are fairly well established. The role of cytochrome c in mitochondrial electron transport has been extensively studied and elucidated, but interaction of cytochrome c and myoglobin has received little attention.

Because of the capability of myoglobin to bind oxygen reversibly it has been thought that this protein is responsible for the transport of oxygen in muscle (1-3). However, one essential feature of this theory is that myoglobin must be able to undergo translational movement. Recently, James (4), on the basis of histochecmical studies, suggested that myoglobin is unable to undergo such movement. Forster (5) has concluded that the role of myoglobin in facilitating oxygen diffusion is not of major importance. There is disagreement on this point; a recent review by Wittenberg (6) discusses these possibilities in detail. Other evidence suggests that myoglobin may have a more direct role in mitochondrial respiration. This evidence includes the observation that the concentration of myoglobin in muscle is directly proportional to the respiratory activity of that muscle (7), and the finding that oxymyoglobin is associated with mitochondria and changes its spectrum to the reduced form on the addition of succinate to the mitochondria, suggesting that oxymyoglobin donates its oxygen directly to the electron transport system (8). It has also been suggested that myoglobin forms a complex with mitochondrial structural protein (9); however recent work has shown that such structural protein preparations may be heterogeneous and nonmembrane protein (10).

In the present study, we report a reaction between ferrous myoglobins and ferricytochrome c that appears to be a simple electron exchange reaction similar to those between cytochromes.

EXPERIMENTAL PROCEDURE

Materials

Sperm whale myoglobin was obtained from Calbiochem and purified by DEAE-cellulose column chromatography (11). Oxymyoglobin was prepared by the addition of about 2 mg of sodium hydrosulfite to a metmyoglobin solution containing about 100 mg; the decomposition and oxidation products of the hydrosulfite were removed by a column of mixed bed ion exchange resin (Bio-Rad AG 501-X8) (12). The carbon monoxide derivative was obtained by blowing a stream of carbon monoxide gas over a solution of oxymyoglobin which was placed as a thin layer on the bottom of a flask sitting on ice. Deoxymyoglobin was prepared by passing for 2 to 3 hours a stream of nitrogen gas (washed in alkaline pyrogallol and water) over a solution of oxymyoglobin layered as just indicated. In this case the flask containing the myoglobin was connected to a second flask containing cytochrome c, and the latter was connected to a Thunberg cuvette. The reaction of deoxymyoglobin with cytochrome c the solutions were mixed under nitrogen and poured into the Thunberg cuvette which was then sealed and placed in a cell compartment of a Cary model 15 spectrophotometer. The wavelength of measurement was 500 nm and the solution was scanned at 100 nm intervals.

The same general procedure was followed in those experiments...
in which relative amounts of deoxymyoglobin and cytochrome c were varied. In this case the first flask contained buffer and the cytochrome c solution, with deoxymyoglobin in the second.

The flasks were allowed to warm to room temperature (15 min) before mixing. Suitable controls were done to establish amounts of solutions remaining in the flasks after transfer to the cuvette.

Horse heart cytochrome c, type VI, antimycin A, smytal (amobarbital), and rotenone were obtained from Sigma and used without further purification. A small amount (approximately 10%) of the reduced form was present in the cytochrome c.

Methods

Spectrophotometry—The spectrophotometric studies were carried out with either a Cary model 11 or 15 recording spectrophotometer. For the measurement of difference spectra cylindrical tandem cells with stoppers were used. Reference solutions of myoglobin and cytochrome c were placed in separate compartments of one cell; one compartment of another cell was filled with the mixture of the two; while the other compartment was filled with buffer solution. Single compartment cylindrical cells or Thunberg cuvettes were used for mixtures in other experiments. When reduced oxygen levels were required, the reactant solutions were saturated with nitrogen gas and then mixed.

The concentrations of oxymyoglobin, metmyoglobin, ferri- and ferrocyanochrome c were measured spectrophotometrically using

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\epsilon_{1}^m = 14.6, \epsilon_{1}^{15n} = 157 (13), \epsilon_{1}^{15m} = 106, \epsilon_{1}^{15m} = 27.7
\]

(18), respectively. Corrections for the autoxidation of oxymyoglobin were made by noting the absorbance at 700 nm and using the method of Goldblum and Brown (15). This was generally not a problem.

Kinetic Analysis—The rate of the reaction was followed by measuring the increase in optical density at 550 nm of ferrocyanochrome c. With systems containing oxymyoglobin the change in concentration due to autoxidation was corrected by measuring \(A_{581}\) of the blank solution just prior to each recording of the difference spectrum. The extinction coefficients of systems of myoglobin and cytochrome c derivitives at various pHs were determined in 0.05 M Tris-maleate buffer (Table I). The molar absorption of cytochrome c at 581 nm was low when compared with myoglobin and did not change appreciably with pH. Therefore in the calculations \(\epsilon_{1}^{m}\) (ferri- or ferrocyanochrome c minus ferrocyanochrome c) was assumed to be a constant equal to 2.8.

Equilibrium Analysis—The titration of 30 mM ferrocyanochrome c with increasing amounts of deoxymyoglobin, and vice versa, was done under equilibrium conditions. In these cases, deoxy-

myoglobin and ferrocyanochrome c solutions were mixed and the absorbance of the 550-nm peak of ferrocyanochrome c was determined for several minutes until it had reached a maximum, usually about 15 min. The spectrum over the visible region was recorded to determine if any oxymyoglobin was present. Generally there was none; when there were indications of the presence of small amounts of oxymyoglobin the reaction was allowed to proceed for a longer time period, up to 30 min.

Carboxymethylation of Myoglobin—The method of Banaszak et al. (16) was employed to carboxymethylate the histidine residues of myoglobin. A small amount of potassium ferricyanide was added to the sperm whale myoglobin to oxidize residual oxymyoglobin in the solution. The solution was passed through a mixed bed ion exchange resin (Bio-Rad AG 501-X8) to remove excess ferricyanide, and then dialyzed overnight against a large volume of 0.1 M Tris-maleate, pH 7.0. Recrystallized bromosaccharin was dissolved in the same buffer, the pH of the solution was adjusted to near neutrality, and it was made up to a final concentration of 1.6 M. Seven milliliters of myoglobin solution (about 10 mg per ml) was mixed with 1 ml of bromosaccharin, and incubated in the dark at room temperature for 9 days. A control myoglobin solution, without the addition of bromosaccharin but containing 1 ml of buffer, was similarly treated. One drop of chloroform was added to each solution to prevent bacterial growth. After the incubation, the solutions were dialyzed exhaustively against water. Oxymyoglobin was prepared from these samples just prior to experimentation.

Measurement of Oxygen Evolution—Oxygen evolution during the reaction of oxymyoglobin and ferricyanochrome c was measured by an oxygen electrode attached to a monitor (Yellow Springs Instrument, models 5331 and 53, respectively), using a reaction chamber described previously (12). The electrode was standardized with water saturated with oxygen at 25°, and the oxygen level recorded for about 1 hour in order to estimate the rate of oxygen consumption by the electrode. The water in the chamber was then replaced by cold buffer treated the same way as the reaction mixture, and the base-line was recorded after the temperature became equilibrated with that of the water bath. The chamber was dried thoroughly and filled with portions of reaction mixture which had just been mixed and were held on ice. The reaction was started by incubating the reaction vessel in a 25° water bath. The oxygen level was recorded continuously. The optical density of the reaction mixture was measured in a round, stoppered cuvette. Thereafter the spectrum was recorded around 581 nm at timed intervals in order to compare the rate of oxygen evolution and that of disappearance of oxymyoglobin. Corrections for the autoxidation of oxymyoglobin were made by measuring the change of \(A_{581}\) of an oxymyoglobin solution held at the same temperature and having the same concentration as that in the reaction mixture. The reaction mixture and the oxymyoglobin solution in cuvettes were also incubated at 25°. At the end of the reaction (about 2 hours) the reaction mixture was taken from the chamber and the spectrum was measured in order to estimate unreacted oxymyoglobin that remained in the solution.

Other Analytical Methods—Sedimentation velocity studies were carried out in a Spinco model E analytical ultracentrifuge. The pictures were taken on Spectroscopic type 1-N plates with the aid of a red filter on the light source.

Gel filtration chromatography was performed on a column

<table>
<thead>
<tr>
<th>pH</th>
<th>Myoglobin &amp; Ferricyanochrome c</th>
<th>Myoglobin &amp; Metmyoglobin</th>
<th>Difference Spectrum</th>
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<td>5.0</td>
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(0.9 × 55 cm) of Sephadex G-75 (superfine) using 0.05 M Tris-HCl, pH 8.0, as the buffer.

RESULTS

Spectral Changes  The changes in spectra with time of a mixture of oxymyoglobin and ferricytochrome c in the Soret region and between 500 to 640 nm are shown in Fig. 1. As the peak at 581 nm decreased, the maxima at 521 and 550 nm increased with time. This clearly indicated that as oxymyoglobin was oxidized, ferricytochrome c became reduced. The peak at 414 nm increased, and shifted toward shorter wavelengths. This also corresponded to a change from a mixture of oxymyoglobin and ferricytochrome c to that of metmyoglobin and ferrocytochrome c. The rates of change at 581 and 550 nm were equivalent, indicating that an equimolar reaction was taking place.

Combinations of metmyoglobin and ferrocytochrome c or oxymyoglobin and ferrocytochrome c showed no spectral change during the time course studied.

The difference spectra of the oxymyoglobin-ferricytochrome c system against the same solutions, unmixed, are shown in Fig. 2. The changes in absorption at various wave lengths are assigned as follows:

The decrease at 237 and 280 nm was attributed to the conformational changes as a result of transformation from oxy- to metmyoglobin, and from ferri- to ferrocytochrome c, resulting in alteration of the environment of tyrosine and tryptophan residues of both proteins.

The increase at 313 nm was attributed to the formation of ferrocytochrome c; the decrease at 352 nm to the disappearance of ferricytochrome c; the increase at 408 nm and the decrease at 428 nm to the concurrent formation of metmyoglobin and ferrocytochrome c; the increase at 521 and 550 nm to the formation of ferrocytochrome c; the decrease at 538 and 581 nm to the disappearance of oxymyoglobin.

Effect of pH—The reaction between oxymyoglobin and cytochrome c depended on the pH of the medium. In 0.05 M Tris-maleate, the optimum pH for the system was about 7.2. This optimum pH was very close to that for the oxidation of cytochrome c in the presence of cytochromes c and a (17).

Effect of Temperature—The temperature dependence of the reaction of oxymyoglobin and ferricytochrome c was determined in 0.05 M Tris-maleate buffer, pH 8.0. The Q10 was approximately 5, and the energy of activation was calculated to be about 29 kcal per mole.

Effect of Metmyoglobin—During the course of the study, it was
noticed that the rate of reaction of oxymyoglobin and ferricytochrome c increased if aged oxymyoglobin were used. It was thought that the metmyoglobin produced by autoxidation of oxymyoglobin during storage might act as a catalyst for the reaction. In order to test this assumption, metmyoglobin was added to freshly prepared oxymyoglobin and the reaction with ferricytochrome c was carried out at room temperature. In another experiment, metmyoglobin was added to a fresh oxymyoglobin solution at concentrations up to 70% of the total myoglobin concentration. Similarly, no increase in reaction rate was observed.

Effect of Antimycin A, Amytal, and Rolenone—The oxidation-reduction potential of myoglobin is very close to that of cytochrome b (+0.046 and 0.0, respectively). It was thought that the inhibitors for the cytochrome b-cytochrome c system might also have some effect on the oxymyoglobin-cytochrome c system. However, under the conditions studied, none of these inhibitors had any effect. Estabrook (18) reported that for maximum inhibition in a pigeon heart mitochondrial system the molar quantity of antimycin A should approximate that of cytochrome b. The concentrations of antimycin A used in present study were 1 to 2 moles of inhibitor per mole of myoglobin or cytochrome c. The concentrations of amytal and rotenone (5 and 10 mM each) were those used by Estabrook et al. (19).

Effect of Carboxymethylation of Myoglobin—The fact that the pH optimum of the oxymyoglobin-ferricytochrome c system was at 7.2 suggested that some of the amino acid residues ionizing near neutral pH might be participating in the reaction. The effect of carboxymethylation of histidyl residues of myoglobin on its interaction with cytochrome c was examined. It had been shown previously that at pH 7.0 and 25°C carboxymethylation is limited to 8 of the 12 histidyl residues of sperm whale oxymyoglobin, and that many of the properties of the native protein are not altered (16). The carboxymethylation of myoglobin did not inhibit the reaction of oxymyoglobin and ferricytochrome c. Instead, the reaction rate increased to about twice that of the control. It was also noticed that the oxymyoglobin prepared from modified protein underwent autoxidation faster than that prepared from an unmodified one. The appearance of metmyoglobin was detectable by visual observation immediately after the preparation and all the oxymyoglobin became autoxidized after 1 week of storage in a 4°C refrigerator. Only approximately 10% of unmodified oxymyoglobin was autoxidized during the same period of time.

Absence of Myoglobin-Cytochrome c Complex—Limited studies showed no evidence for formation of a complex between myoglobin and cytochrome c. Sedimentation velocity studies in an analytical ultracentrifuge revealed two boundaries, corresponding to those of myoglobin and cytochrome c. Furthermore, gel filtration on a polyacrylamide gel did not reveal the presence of a complex.

Absence of Small Molecular Weight Carrier—Two lines of evidence support the conclusion that the electron exchange between oxymyoglobin and cytochrome c was not mediated by a contaminating small molecular weight component. First, both proteins were quite highly purified and the preparation of oxymyoglobin routinely included a final passage through a mixed bed ion exchange column. The eluting material had a conductivity of only about 2 or 3 microemhos cm⁻¹. Cytochrome c solutions subjected to similar chromatography and showing similar conductivities were not altered in their reactivity with oxymyoglobin.

The second line of evidence was obtained through experiments in which oxymyoglobin was placed inside a dialysis sack and dialyzed against a solution of cytochrome c. Aliquots of cytochrome c were removed over long time intervals (up to 24 hours) and no evidence of reduction was obtained. Similarly oxymyoglobin removed at the termination of the experiment remained in the ferrous state as determined spectrophotometrically.

Oxygen Evolution during Reaction—Because of the difficulties in measuring the concentration in the reaction mixture and the exact volume of solutions mixed in the reaction chamber, oxymyo-
globin and ferricytochrome $c$ were previously mixed with known concentrations of solutions on ice. In this way the reaction was kept to a minimum before the reaction vessel was filled. However, this procedure gave an uncertainty to the base-line since cold solutions absorb more oxygen than those at higher temperatures. A correction was made by measuring the oxygen concentration of cold buffer solution treated the same way as the reaction mixture. A correction for autoxidation of myoglobin was necessary, although this reaction was slow under the experimental conditions. Since the electrode consumes oxygen, the autooxidation rate inside the reaction chamber may not be the same as that in the stopped cuvette. However, we assumed that the difference was too small to affect the calculations. With these considerations, the amount of oxygen evolved per mole of oxymyoglobin reacted was found to be 0.96 mole as an average of five determinations. The rate of disappearance of oxymyoglobin measured spectrophotometrically paralleled the rate of evolution of oxygen.

Effect of Oxygen Concentration on Rate of Reaction—It was noted that oxygen had an inhibitory effect on the rate of reaction of oxymyoglobin-ferricytochrome $c$ systems. Yamazaki et al. (20) reported similar results and suggested that this reaction with ferricytochrome $c$ is responsible for the oxidation of oxymyoglobin when the latter is extracted from muscle. When the reaction was carried out under air, the rate of increase of $A_{590}$ was approximately the same as that of the decrease at $A_{581}$. However, at a very low concentration of oxygen ($6 \mu M$, or $4 \text{mm} \text{Hg}$), the increase at $550 \text{nm}$ was much faster than the decrease at $581 \text{nm}$ at the beginning of the reaction. This suggested that the reacting species was deoxymyoglobin, rather than oxymyoglobin, since under low oxygen tension some of the oxymyoglobin would be dissociated into the deoxy form and thus be able to react immediately when ferricytochrome $c$ was added.

Reactivity of Other Ferrous Myoglobin Derivatives—As just mentioned, the influence of oxygen level on the reaction suggested that deoxymyoglobin might be the reacting species. Consequently, this material was prepared and reacted in an oxygen-free environment with cytochrome $c$. The reaction rate was markedly enhanced over that with oxymyoglobin as indicated in Fig. 3. The carbon monoxide derivative of myoglobin was unreactive provided care was taken to remove traces of oxygen from solutions used by means of bubbling carbon monoxide gas. Furthermore, as shown in Fig. 4 titrations of ferricytochrome $c$ with deoxymyoglobin or titrations of deoxymyoglobin with ferrocyanochrome $c$ under equilibrium conditions provide strong evidence for equimolar reaction of the two proteins.

**DISCUSSION**

The reaction between myoglobin and cytochrome $c$ is feasible from a thermodynamic point of view because the oxidation-reduction potentials for these proteins are $+0.046$ and $+0.255$ volts, respectively. In this respect, this system is very similar to the members of mitochondrial electron transport system, cytochrome $b$ or cytochrome $c_1$ and cytochrome $c$. Nevertheless, the interaction between myoglobin and cytochrome $c$ apparently has not been reported except for a brief mention by Yamazaki et al. (20).

Our results show clearly that deoxymyoglobin is the reacting species. Oxymyoglobin reacted at a significantly slower rate, and carbon monoxide myoglobin did not react at all, at least during the time interval utilized. These results presumably reflect differences in dissociation behavior of the latter two liganded species. The difficulty in removing an electron from liganded forms of hemoglobin has long been recognized (21); this phenomenon has been subjected to detailed analysis with both hemoglobin and myoglobin derivatives (22, 23).

In spite of a great deal of research, little is understood about how an electron is transferred from one hemoprotein to the other. Chance and coworkers (24, 25) have postulated a rotational mechanism between cytochromes, whereas Winfield (26) suggested free-radical involvement in the reaction. The physiological significance, if any, of the reaction between myoglobin and cytochrome $c$ is not known; in any case, the interaction may serve as a model system for exploring the mechanism of electron transport between hemoprotein molecules.

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