Competitive Inhibition by Myoglobin of the Reduction of Cytochrome c by Xanthine Oxidase*

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During a study of the reduction of cytochrome c by milk xanthine oxidase, it was observed that relatively impure preparations of cytochrome c were reduced more slowly than highly purified preparations. This difference was found to relate to the presence, in impure preparations, of a potent inhibitor of the reduction of cytochrome c by xanthine oxidase. This inhibitor was of interest because its action was formally competitive with respect to ferrixytochrome c, whereas it had no effect on the reduction of oxygen by this enzyme. Identification of the inhibitor as myoglobin and characterization of the inhibition form the subject of this report.

EXPERIMENTAL PROCEDURE

Material and Methods—A unit of xanthine oxidase is defined as that amount which catalyzes an increase in absorbancy at 265 mμ of 1.0 per minute at 25° when acting aerobically in a medium (1.0 ml final volume, pH 7.8) containing 5 × 10⁻⁴ m xanthine, 0.050 m potassium phosphate, and 1 × 10⁻⁴ m EDTA. Specific activity is defined as units per milliliter divided by A₄₅₃.

Milk xanthine oxidase (450 mg, 2200 units), obtained from the Worthington Biochemical Corporation, was dialyzed against several changes of 0.010 m potassium phosphate, pH 6.8, and then adsorbed onto a 2.2 × 20 cm column of hydroxyapatite prepared according to the method of Tiselius, Hjerten, and Levin (1). Inactive protein (250 mg) was eluted with 300 ml of 0.10 m potassium phosphate, pH 6.8. The phosphate concentration of the eluting fluid was raised to 0.20 m, and the elution was continued until the specific activity of the eluted enzyme reached 10.0. At this point, 0.50 m potassium phosphate, pH 6.8, was applied to complete the elution. The active eluates were combined and brought to 0.75 saturation with solid ammonium sulfate, and the enzyme which precipitated was stored under saturated ammonium sulfate at -20°.

Identity of Inhibitor

Presence of Inhibitor in Impure Preparations of Cytochrome c—One unit of xanthine oxidase, with xanthine as substrate, catalyzed a Δ₆₈₀ per minute of 0.82 when purified cytochrome c was present. When absorbancy is translated into the corresponding molar concentration, it is apparent that xanthine oxidase transmits electrons to cytochrome c at 0.2 the rate at which electrons are transmitted to oxygen. In contrast, impure cytochrome c was not reduced at a measurable rate by the small amounts of enzyme used in these assays. Indeed, preparations of impure cytochrome c appeared to inhibit, competitively, the reduction of purified cytochrome c.

Identity of Inhibitor in Crude Preparation of Cytochrome c—The inhibitor in impure cytochrome c was found to be destroyed by both dilute acid and alkali. It was not readily adsorbed by activated charcoal or by Amberlite XE-64. It was nondialy-
able and was destroyed by brief boiling. Since the inhibitor was eliminated when the impure cytochrome c was purified according to the method of Margoliash (12), who identified the globin of myoglobin as a contaminant of horse heart cytochrome c preparations, it appeared possible that the inhibitor in crude preparations of cytochrome c is myoglobin or the corresponding globin.

Myoglobin and the corresponding globin were then prepared from pig heart, and both were found to inhibit the reduction of cytochrome c by xanthine oxidase. The inhibition was competitive with respect to cytochrome c and noncompetitive with respect to xanthine. The inhibitor constant, \( K_i \), was found to be \( 6 \times 10^{-7} \) M for pig heart myoglobin and \( 4 \times 10^{-7} \) M for the corresponding globin.

From these data for \( K_i \) of pig heart myoglobin, and from the observed \( K_i \) for impure horse heart cytochrome c of 10 \( \mu \)g per ml, it could readily be calculated, assuming that \( K_i \) for horse heart myoglobin is identical with that for pig heart myoglobin, that the commercial "type 11" cytochrome c would have to be almost pure myoglobin. As determined by the spectrophotometric assay, the purity of "type 11" cytochrome c is about 70% cytochrome c. Hence, it appeared likely that horse heart myoglobin would prove to be a more potent inhibitor of cytochrome c reduction than is pig heart myoglobin. As shown in Fig. 1, the globin of horse heart myoglobin inhibits xanthine oxidase competitively with cytochrome c and has a \( K_i \) of \( 2 \times 10^{-7} \) M. Horse heart myoglobin was similarly inhibitory with a \( K_i \) of \( 3.5 \times 10^{-8} \) M.

Effects on Cytochrome c Reduction by Other Enzymes—The effect of the myoglobins and corresponding globins of horse and pig heart on the rate of cytochrome c reduction by several other enzyme systems was investigated. The reduction of cytochrome c by 1.5 \( \times 10^{-3} \) M N-methylnicotinamide, catalyzed by rabbit liver aldehyde oxidase, was strongly inhibited by these myoglobins and globins, in each case competitively with respect to ferrocyanochrome c. The inhibitory constants obtained with this enzyme were: pig heart myoglobin, \( 5 \times 10^{-4} \) M; pig heart globin, \( 2.7 \times 10^{-2} \) M; and horse heart myoglobin, \( 1.1 \times 10^{-4} \) M. The reduction of cytochrome c by neither DPNH cytochrome c reductase nor sulfite-cytochrome c reductase was subject to inhibition by myoglobin. As reported by Margoliash (13), cytochrome c oxidase was not inhibited by horse heart myoglobin.

Effects of Ferrocyanochrome c—In the studies described above, the rate of reduction of cytochrome c by xanthine oxidase plus xanthine remained linear until the concentration of ferrocyanochrome c became limiting, thus indicating that the ferrocyanochrome c formed was not inhibitory, and, presumably, was not significantly bound to the enzyme. It appeared of interest to explore this point further. With ferrocyanochrome c at \( 4 \times 10^{-4} \) M, addition of ferrocyanochrome c up to \( 16 \times 10^{-4} \) M had only a trivial effect on the rate of reduction of ferrocyanochrome c and no effect on the degree of inhibition caused by 2 \( \mu \)g per ml of horse heart globin. Thus, ferrocyanochrome c competes with neither ferrocyanochrome c nor with horse heart globin for xanthine oxidase.

Effects of Ionic Strength—If the binding of cytochrome c and of myoglobin to xanthine oxidase were largely dependent on electrostatic interactions, then variation of the ionic strength of the medium might be expected to alter the measured affinity constants. To test this point, \( K_a \) for cytochrome c and \( K_i \) for horse heart globin were determined in 0.0025 M potassium phosphate buffer, pH 7.8, and found to be \( 1.3 \times 10^{-4} \) M and \( 2.4 \times 10^{-4} \) M, respectively. Thus, a 20-fold decrease in the concentration of phosphate buffer had a negligible effect on the affinity constant for cytochrome c and for horse heart globin. It is noteworthy that, at these low concentrations of buffer, xanthine oxidase was rapidly and irreversibly inhibited in the reaction mixtures, and it was necessary to add 0.4 mg per ml of crystalline bovine serum albumin to prevent this loss of activity.

DISCUSSION

The globin of horse heart myoglobin, demonstrated to be a contaminant of partially purified preparations of cytochrome c by Margoliash (13), is here shown to be a potent competitive inhibitor of cytochrome c reduction by milk xanthine oxidase and by rabbit liver aldehyde oxidase. The heme of myoglobin is not involved in this inhibition, since its removal with acid-acetone actually enhances slightly the inhibitory action of the globin. Heat denaturation of the globin completely destroys its inhibitory action. The myoglobins of pig and horse heart, although similar in physical properties, nevertheless exhibit markedly different affinity constants. Human hemoglobin, the globin of human hemoglobin, Veroeno (the didecyld salt of ethylenediaminetetraacetic acid), protamine sulfate, ribonucleic acid, and bovine serum albumin were without effect on the rate of cytochrome c reduction. Ferrocyanochrome c, but not ferrocyanochrome c, competes with myoglobin. The inability of ferrocyanochrome c to compete with either ferrocyanochrome c or with myoglobin indicates that ferrocyanochrome c has negligible affinity for the enzyme. Since the binding of myoglobin to the enzyme is independent of its heme group and, since the heme iron of cytochrome c is unable readily to form bonds to exogenous ligands (14), it appears likely that changes in the protein structure of cytochrome c which occur upon reduction of the heme iron are responsible for the observed change in affinity. The report that ferrocyanochrome c is more resistant to proteolytic digestion than is ferrocyanochrome c (15) is in accord with this suggestion, as is the occurrence of an oxidation-linked ionization of cytochrome c about pH 7.0 (16). Large variations in ionic strength had no significant effect on the \( K_a \) for cytochrome c or on the \( K_i \) for horse heart globin. These observations indicate that the interaction of myoglobin with xanthine oxidase is a highly specific protein-protein interaction which occurs at the cytochrome c-binding site of this enzyme. The oxygen-binding site of this enzyme would appear to be distinct from the cytochrome c-bind-
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ing site, since myoglobin does not inhibit the reduction of oxygen. Reported studies of the reduction of cytochrome c by milk xanthine oxidase may require re-evaluation in terms of these results. Thus, the observation that cytochrome c reduction by this enzyme was very slow in comparison with oxygen reduction (17, 18) may have been due to the use of incompletely purified cytochrome c which retained myoglobin or the corresponding globin. Indeed, the $K_m$ here reported for purified cytochrome c may yet be high because of persistent contamination by small amounts of myoglobin or its globin moiety. As indicated by the striking differences in the inhibitory powers of pig and horse heart myoglobins, inhibition of cytochrome c reduction is highly specific and might provide a sensitive assay for probing the structure of myoglobin and for comparing genetic differences in that structure.

**SUMMARY**

Partially purified preparations of cytochrome c were found to contain a potent competitive inhibitor of the reduction of cytochrome c, but not of oxygen, by milk xanthine oxidase and by rabbit liver aldehyde oxidase. The inhibitor was identified as the protein moiety of myoglobin. The reduction of cytochrome c by several other enzymes is not affected by myoglobin. Several aspects of this inhibition of cytochrome c reduction are described.

**REFERENCES**

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