The Reduction of Cytochrome c by Milk Xanthine Oxidase*

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SUMMARY

The reduction of cytochrome c by xanthine oxidase and the competitive inhibition of this process by carbonic anhydrase and by myoglobin have been studied by kinetic and by equilibrium binding methods. Carbonic anhydrases isolated from bovine and from human erythrocytes differed strikingly in their ability to inhibit competitively the reduction of cytochrome c. The $K_i$ for cytochrome c was a function of the concentration of xanthine oxidase, as were $K_i$ for carbonic anhydrase and $K_i$ for myoglobin, whereas $K_i$ for xanthine was invariant under the same conditions. Binding studies performed by a variety of methods indicated that carbonic anhydrase does not bind to xanthine oxidase. Carbonic anhydrase was found to be a potent inhibitor of the sulfite-oxygen chain reaction initiated either by the reduction of oxygen at an electrode or by xanthine oxidase plus xanthine. The data are consistent with the conclusion that xanthine oxidase, when catalyzing the aerobic oxidation of xanthine, generates an unstable reduced form of oxygen, presumably the superoxide anion, and that this radical is the agent which directly reduces cytochrome c and initiates the sulfite-oxygen chain reaction. Carbonic anhydrase and myoglobin appear to inhibit the reduction of cytochrome c and the initiation of sulfite oxidation by reducing the steady state concentration of the superoxide anion. It is proposed that they accomplish this end by catalyzing the following dismutation reaction:

$$O_2^− + O_2^− + 2H^+ → O_2 + H_2O_2$$

The mechanism by which oxygen mediates the transfer of electrons from xanthine oxidase to cytochrome c has been the subject of considerable discussion. It has been suggested that hydrogen peroxide plays a key role in this process, either by serving as the direct reductant of cytochrome c (2) or by peroxidatively generating substrate radicals, which then reduce cytochrome c (3). There are, however, several observations which effectively eliminate hydrogen peroxide as a possible intermediate in the oxygen-dependent reduction of cytochrome c by xanthine oxidase. Thus, the $K_i$ for oxygen in the oxygen-dependent reduction of cytochrome c was found to be 30-fold greater than the $K_i$ for oxygen in the enzymic reduction of oxygen to hydrogen peroxide (3). Tiron was seen to be a potent inhibitor of the oxygen-dependent reduction of cytochrome c, competitive with respect to cytochrome c, whereas it was without effect on the aerobic oxidation of xanthine to urate (3). Tiron was similarly seen to exert a discriminatory effect. The addition of a hydrogen peroxide-generating system, such as the snake venom L-amino acid oxidase plus L-leucine, to milk xanthine oxidase, in the presence of oxygen, did not confer upon it the ability to reduce cytochrome c (3). Adding a peroxide-trapping system, such as catalase plus ethanol, did not interfere with the oxygen-mediated reduction of cytochrome c (3). Since several lines of chemical evidence (6-8) had indicated that xanthine oxidase was capable of the univalent reduction of oxygen to the superoxide anion, it was proposed (3) that this radical of oxygen, bound to the enzyme, was the actual reductant of cytochrome c in this enzyme system.

Certain proteins were found to mimic the action of Tiron in that they inhibited the oxygen-mediated reduction of cytochrome c, competitively with respect to cytochrome c, without interfering with the aerobic oxidation of xanthine (9, 10). The effects of these proteins were intriguing from several points of view. Thus, their inhibitory constants ($K_i$) were unusually low, being of the order of $10^{-3}$ M for horse heart myoglobin (9) and $10^{-2}$ M for bovine erythrocyte carbonic anhydrase (10), whereas a large number of other proteins, chosen at random, were entirely without effect. In both cases the inhibition of cytochrome c reduction was dependent upon the native conformation of the protein, yet the heme-free apomyoglobin and the zinc-free apocarbonic anhydrase were as effective inhibitors as the corresponding holo-proteins. The ability of myoglobin to inhibit the reduction of cytochrome c exhibited a remarkable species specificity, $K_i$ varying by orders of magnitude among myoglobins from rather...
closely related species. As shown in the present report, this species specificity is also a property of the inhibition of cytochrome c reduction by carbonic anhydrase.

It has been assumed (9, 10) that the inhibitions of cytochrome c reduction by myoglobin and by carbonic anhydrase were based upon highly specific protein-protein interactions: that these proteins have a great affinity for the cytochrome c-binding site of xanthine oxidase and thus directly compete with cytochrome c for binding to that site. This hypothesis was based, in turn, upon the assumption that the oxygen-dependent reduction of cytochrome c by xanthine oxidase involves the binding of the cytochrome c to the enzyme. It is the purpose of the present report to present evidence which supports a contrary view: that cytochrome c is reduced, by the aerobic xanthine oxidase system, without binding to the xanthine oxidase, and that myoglobin and carbonic anhydrase compete with cytochrome c in free solution for the reducing species generated by the aerobic xanthine oxidase system. It will further be suggested that the reducing species in question is univalently reduced oxygen, and that myoglobin and carbonic anhydrase eliminate this radical from the field of action, possibly by catalyzing a dismutation reaction.

EXPERIMENTAL PROCEDURE

Milk xanthine oxidase was purchased from Worthington and was purified to a specific activity of 14 by a published procedure (11). The concentration of xanthine oxidase solutions was determined by use of a molar extinction coefficient of 70,000 at 450 μM (12). Horse heart cytochrome c, type III, was obtained from Sigma; its quantitative determination was based on the molar extinction coefficients given by Massey (13). The mixed bovine erythrocyte carbonic anhydrases were purified and assayed by the procedures described by Rickli et al. (14) for carbonic anhydrases from human erythrocytes, except that lyophilization as a means of concentration was replaced by dialysis against neutral saturated ammonium sulfate solutions. The final product had a specific activity of 3 × 10^10. A fluorometric assay for carbonic anhydrase, which was precise, convenient, and sensitive, was also used. It was based upon the observation by Chen and Kernohan (15) that carbonic anhydrase specifically forms a highly fluorescent 1:1 complex with dansylamide.1 When solutions containing carbonic anhydrase were added to 10^-4 M dansylamide in 0.05 M phosphate buffer at pH 7.0 and 25°C, excitation at 290 nm resulted in a fluorescence emission at 470 nm which was strictly proportional to the concentration of carbonic anhydrase and the range 5 × 10^-5 to 1 × 10^-4 M. It appeared likely that the assay could have been used at even lower concentrations of carbonic anhydrase. It should be noted that this assay, which was used in the binding studies, measures the concentration of dansylamide-binding sites rather than carbonic anhydrase activity. Horse heart myoglobin was obtained from Pentex as a paste under ammonium sulfate and as a lyophilized powder. The concentrations of solutions of myoglobin were determined by using a molar extinction of 1.6 × 10^6 at 409 μM (10). Human erythrocyte carbonic anhydrase B and C and the variant of horse erythrocyte carbonic anhydrase were generously provided by Dr. J. T. Edsall. Dansylamide was prepared from dansyl chloride as described by Weber (17). All kinetic measurements were made in cells having a light path of 10.0 cm, in a Cary Model 15 spectrophotometer equipped with a 0 → 0.10 slide wire and a thermostated cell block. Other spectrophotometric assays were performed with a Gilford model 2000 instrument. Fluorescence measurements were made with an Amino-Bowman spectrophotofluorometer in cells with a 1.0-cm light path. All measurements were made at 25°C. The aerobic oxidation of sulfite was measured in terms of oxygen consumption in Warburg microrespirometers at 37°C. The experiments involving the initiation of sulfite oxidation at an electrode were performed with a Warburg vessel which was fitted with sealed-in platinum electrodes (7), across which a potential of 1.0 volt was applied. Thermobarometric corrections were applied, but no correction was made for flask constants, since all of the experiments reported were performed in the same Warburg flask. For this reason, manometric results are reported directly in terms of pressure changes in units of millimeters of Brodie's solution. The constant of the flask used would certainly have been within the range 1.2 to 1.4.

RESULTS

Inhibition of Cytochrome c Reduction by Carbonic Anhydrases—Enzymes from the erythrocytes of several species of mammals were tested for their abilities to compete with cytochrome c in the oxygen-dependent reduction of cytochrome c by xanthine oxidase. These measurements were made at 25°C and at pH 10.0 in 0.05 M carbonate buffer containing 1 × 10^-4 M EDTA, as previously described (10). The results, which are summarized in Table I, indicated striking differences in inhibitory potencies among the several species of carbonic anhydrases tested. All were strictly competitive with respect to cytochrome c.

Effect of Xanthine Oxidase Concentration on K₅ for Cytochrome c—K₅ should be independent of the concentration of enzyme, provided that the interaction of substrate and enzyme does involve the formation of a transient complex. This statement may also be made with respect to K₅ for an electron acceptor and to Kₛ for an inhibitor. All of this, of course, assumes that the enzyme concentration is small compared to the concentrations of substrate, electron acceptor, and inhibitor. The constancy of Kₛ for the substrate xanthine was investigated over a 10-fold range in concentration of xanthine oxidase. This was performed at 25°C in 0.05 M phosphate buffer at pH 7.8 containing 1 × 10^-4 M EDTA and 2 to 12 × 10^-4 M xanthine. The concentration of xanthine oxidase was varied from 1.13 × 10^-10 to 1.13 × 10^-8 M, and Kₛ for xanthine was found to be 2.2 × 10^-4 M, in agreement with published values (6). Moreover, Kₛ for xanthine was entirely independent of the concentration of xanthine oxidase. A similar experiment was performed in which saturation curves for cytochrome c were determined at a fixed concentration of xanthine and at a variety of concentrations of xanthine oxidase. As shown in Fig. 1, Kₛ for cytochrome c was strikingly dependent upon the concentration of xanthine oxidase. The competitive

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1 The abbreviation used is: dansylamide, 5-dimethylaminonaphthalene-1-sulfonylamine.
inhibition of the reduction of cytochrome c by bovine carbonic anhydrase was also investigated as a function of the concentration of xanthine oxidase. As shown in Fig. 2, the $K_i$ for carbonic anhydrase was markedly dependent upon the concentration of xanthine oxidase. Myoglobin from horse heart, like carbonic anhydrase, is a competitive inhibitor of cytochrome c reduction (9, 10). Its $K_i$ was also found to be a function of the concentration of xanthine oxidase. These results indicated that the reduction of cytochrome c by the aerobic xanthine oxidase system and the competitive inhibition of this reduction by carbonic anhydrase and by myoglobin did not involve the binding of xanthine oxidase to cytochrome c, carbonic anhydrase, or myoglobin. Direct binding studies were undertaken to probe this conclusion.

**Binding Studies**—The existence of direct affinities between xanthine oxidase and bovine carbonic anhydrase was explored by three techniques. The first was a modified equilibrium dialysis in which particles of a cross-linked dextran gel replaced dialysis sags. This method has been described in a study of the binding of testosterone to serum proteins (18). The second method utilized columns of dextran gels which were equilibrated with a stream of the small molecule and then challenged with an aliquot of the large molecule. This method has also been thoroughly described (19, 20). In the equilibrium method Sephadex G-100 was used, while in the column method Sephadex G-75 was used. Both of these gels have the ability to admit carbonic anhydrase (mol wt $\approx 30,000$) to the interior of the gel particles while excluding xanthine oxidase (mol wt $\approx 300,000$). The third method was based upon the expectation that the association of these two proteins might be accompanied by some change in their intrinsic fluorescence. There are precedents for this expectation. Thus, the association of haptoglobin with hemoglobin is accompanied by a quenching of the fluorescence of the haptoglobin (21).

The equilibration experiments were performed at 4° in Pyrex tubes fitted with Teflon-lined, screw-capped closures. Each tube contained 150 mg of dry Sephadex G-100 and graded amounts of carbonic anhydrase with and without a fixed concentration of xanthine oxidase, in a total fluid volume of 4.0 ml, which was buffered at pH 7.0 with 0.05 M phosphate containing $1 \times 10^{-4}$ M EDTA. The tubes were continuously agitated for 18 hours in a manner which effected slow, end-over-end tumbling of the tube contents. After equilibration, the excluded volumes were separated by filtration and were assayed for carbonic anhydrase by the fluorometric procedure. Xanthine oxidase at 3.75 $\times 10^{-7}$ M was found to cause 15% masking of the fluorescence upon which this method was based. Corrections were applied for this masking effect. The assay was further validated by adding known amounts of carbonic anhydrase to the separated excluded volumes, as internal standards. The rate of equilibration of carbonic anhydrase between the excluded and included volumes was investigated. Equilibration was a first order process with a half-life of less than 1 hour under the conditions of these experiments. Sephadex G-100 was observed to bind carbonic anhydrase. Thus, the equilibrium concentration of carbonic anhydrase in the excluded volume was less than was expected on the basis of equal distribution between the included and excluded volumes. As expected, xanthine oxidase was confined to the excluded volume. Other proteins diminished the binding of carbonic anhydrase to the Sephadex particles, and in their presence the concentration of carbonic anhydrase in the

**Fig. 1.** Effect of enzyme concentration on $K_i$ for cytochrome c. Initial rates of cytochrome c reduction were measured as a function of the concentration of ferricytochrome c at the indicated concentrations of xanthine oxidase. Plots of the data on reciprocal coordinates defined straight lines, from which the values of $K_i$ were derived. All reactions were performed at 25° in $10^{-4}$ M EDTA with the pH buffered at 7.8 with 0.05 M phosphate. Cells with a 10.0-cm light path were used.

**Fig. 2.** Effect of enzyme concentration on $K_i$ for carbonic anhydrase. Initial rates of cytochrome c reduction were measured as in Fig. 1 in the absence and in the presence of bovine carbonic anhydrase. $K_i$ for carbonic anhydrase was calculated from the slopes of the reciprocal plots of the kinetic data. In all cases, carbonic anhydrase was strictly competitive with respect to cytochrome c. $K_i$ for carbonic anhydrase is graphed here as a function of the concentration of xanthine oxidase.

excluded phase approached that to be anticipated for the case of complete equilibration between the phases. Myoglobin, cytochrome c, and xanthine oxidase were equally effective in this regard. There were no indications, from these studies, of any affinity between carbonic anhydrase and xanthine oxidase.

Binding studies by the column method (19) were performed under anaerobic conditions. A stream of nitrogen, rendered oxygen-free by passage through a column of heated colloidal copper (22), was used to sweep dissolved oxygen out of the eluting buffer. Tubing connections were metal or glass to avoid the introduction of oxygen by diffusion through rubber or plastic.
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with a stream of anaerobic 0.05 M potassium phosphate and 1 X containing 1 X 1OP through a column (1 X 30 cm) of Sephadex G-75 in a stream of dize rapidly on the column, but promptly reoxidized to the blue dye upon emerging from the column. It failed to reoxidize rapidly on the column, but promptly reoxidized to the blue dye upon emerging from the column. These precautions were taken because of the possibility that only the reduced form of xanthine oxidase might exhibit affinity for carbonic anhydrase. A column (1 X 30 cm) of Sephadex G-75 was equilibrated at 49 with a stream of anaerobic 0.05 M potassium phosphate and 1 X 10-4 M EDTA at pH 8.8, containing 1 X 10-3 M bovine carbonic anhydrase. A 1.0-ml aliquot of 4.7 X 10-4 M xanthine oxidase containing 9 X 10-4 M acetaldehyde and 5% ethylene glycol, to increase its density, was then placed on the column. Fractions were assayed for xanthine oxidase activity and for carbonic anhydrase activity. Fig. 3 illustrates the results obtained. It is clear that the peak of xanthine oxidase was not associated with a peak of carbonic anhydrase, nor was it followed by a trough in the level of carbonic anhydrase. There was therefore no evidence for the binding of carbonic anhydrase to xanthine oxidase reduced by an aldehyde substrate under anaerobic conditions. This experiment was repeated with the modification that the influent stream and the sample of xanthine oxidase contained 9 X 10-4 M acetaldehyde and 1 X 10-2 M sodium sulfite. This would allow for the efficient scavenging of any residual oxygen by the sulfite-oxygen chain reaction, initiated by xanthine oxidase plus its substrate (7), and would provide additional assurance of anaerobic conditions. The results were again devoid of any indications of affinity between carbonic anhydrase and reduced xanthine oxidase.

Carbonic anhydrase exhibited an excitation band at 290 mp with a shoulder at 250 mp and an emission band at 360 mp. Xanthine oxidase behaved similarly, but showed no shoulder in its excitation spectrum. When xanthine oxidase and carbonic anhydrase, each at 3.5 X 10-4 M in 0.05 M potassium phosphate and 1 X 10-4 M EDTA at pH 7.8 and at 25°, were mixed, the fluorescence spectrum of the mixture was that to be anticipated on the basis of simple additivity of the spectra of the separate components. This was true in the absence as well as in the presence of 9 X 10-4 M acetaldehyde. There was therefore no fluorometric indication of any association reaction between xanthine oxidase and carbonic anhydrase.

Effect of Carbonic Anhydrase on Initiation of Sulfite-Oxygen Chain Reaction—The existence of a free radical chain mechanism for the aerobic oxidation of sulfite has been well documented (23-25). This chain reaction has been used as a chemical amplifier to detect the production of reactive radicals. Free radicals generated by enzymic (6, 7, 26-28), photochemical (29), and electrode (7) processes have been detected in this way. Experiments performed with platinum electrodes sealed through the walls of a Warburg microrespirometer showed (7) that radicals of oxygen generated by reduction at the electrode surface could be detected by the sulfite-oxygen chain reaction. Because the results already in hand could be explained on the basis of the generation of oxygen radicals by xanthine oxidase and the competition between cytochrome c and carbonic anhydrase for these radicals, it seemed reasonable to anticipate that carbonic anhydrase might interfere with the initiation of the sulfite-oxygen chain reaction at the platinum electrode. When 1.0 volt d.c. was impressed across platinum electrodes immersed in 2.2 ml of 0.05 M phosphate buffer which contained 5 X 10-4 M EDTA and 0.018 M Na2S2O4 at pH 7.8 and 37°, a rapid uptake of oxygen was observed. This consumption of oxygen could be started and stopped at will by applying or discontinuing the voltage. Bovine carbonic anhydrase at 2.3 X 10-7 M caused a 28% inhibition of this oxygen consumption. Human erythrocyte carbonic anhydrase B, which was not an effective inhibitor of the reduction of cytochrome c by xanthine oxidase (Table I), was used as a control. At 2.3 X 10-7 M it was without effect on electrode-initiated oxidation of sulfite, thus establishing the specificity of the effect of the bovine carbonic anhydrase. Surprisingly, horse heart myoglobin at 2.3 X 10-7 M was found not to inhibit the electrode-initiated oxidation of sulfite. It appeared possible that myoglobin, which is prone to surface denaturation, had coated the platinum electrode with a layer of unfolded protein and had thus denied the native myoglobin access to the immediate vicinity of the electrode surface. Since a highly reactive radical such as O2- is not likely to survive long enough to diffuse far from its point of origin, such a coating of the electrode would prevent interaction of the radical with native proteins in the bulk solution. These considerations predicted that myoglobin should prevent the inhibitory action of bovine carbonic anhydrase on the electrode-initiated process. Myoglobin at 2.3 X 10-7 M did completely eliminate the inhibitory effect of 2.3 X 10-7 M bovine carbonic anhydrase.

Milk xanthine oxidase, when catalyzing the aerobic oxidation of xanthine, can initiate the sulfite-oxygen chain reaction (6, 7). It presumably does so by virtue of generating oxygen radicals, which in turn initiate the chain reaction. In this case, bovine carbonic anhydrase and horse heart myoglobin should inhibit the initiation of sulfite oxidation. Fig. 4 indicates that this was the case. Reaction flasks contained 8 X 10-8 M xanthine oxidase, 9 X 10-5 M xanthine, 1 X 10-4 M EDTA, 0.036 M sodium sulfite, and 1.3 X 10-7 M bovine serum albumin in a volume of 2.2 ml buffered at pH 7.8 with 0.05 M potassium phosphate. The data of Line 1 were obtained in the absence of additional components. The data of Line 2 were obtained in the presence of 1.8 X 10-7 M human carbonic anhydrase B. Its failure to inhibit was in accord with its high Ki in the cytochrome c reduc-
Fig. 4. Effects of carbonic anhydrase and of myoglobin on sulfite oxidation initiated by xanthine oxidase. Reaction mixtures contained 0.036 M sodium sulfite, 9 \times 10^{-4} M xanthine, 8 \times 10^{-11} M xanthine oxidase, 1 \times 10^{-4} M EDTA, 1.3 \times 10^{-7} M bovine serum albumin, and 0.05 M potassium phosphate in a final volume of 2.2 ml. The pH was 7.8, and the temperature was 37\degree C. Reactions were started at the arrow by tipping the xanthine oxidase into the reaction mixture. Additional components were: Line 1, none; Line 2, 1.8 \times 10^{-7} M human carbonic anhydrase B; Line 3, 3.6 \times 10^{-7} M lyophilized horse heart myoglobin; Line 4, 2.3 \times 10^{-8} M bovine carbonic anhydrase; Line 5, 4.6 \times 10^{-8} M bovine carbonic anhydrase; Line 6, 9.2 \times 10^{-8} M bovine carbonic anhydrase; Line 7, 1.07 \times 10^{-7} M horse heart myoglobin.

The data of Line 3 were obtained in the presence of 3.6 \times 10^{-7} M horse heart myoglobin which had been lyophilized. Its failure to inhibit may be contrasted with the inhibition caused by 1.05 \times 10^{-5} M horse heart myoglobin which had not been lyophilized (Line 7). This result agrees with previous observations that lyophilization of horse heart myoglobin destroys its ability to inhibit the aerobic reduction of cytochrome c by xanthine oxidase. The data of Lines 4, 5, and 6 were obtained in the presence of 2.3, 4.6, and 9.2 \times 10^{-8} M bovine carbonic anhydrase, respectively. We may conclude that the oxygen-dependent reduction of cytochrome c by xanthine oxidase plus xanthine share a common intermediate, and that horse heart myoglobin or bovine carbonic anhydrase can act to eliminate that intermediate, whereas a variety of other proteins, including human carbonic anhydrase B and lyophilized horse heart myoglobin, cannot do so.

If we propose that this common intermediate is univalently reduced oxygen, then we may ask whether, in the present case, this radical acts only as the initiator of the sulfite-oxygen chain reaction or whether it also acts as one of the chain-propagating radicals. If one could find a means of initiating the sulfite-oxygen chain reaction which was not susceptible to inhibition by bovine carbonic anhydrase, one would have to conclude that the oxygen radical common to the reduction of cytochrome c and to the oxidation of sulfite by xanthine oxidase was not also involved in the propagation steps of the sulfite-oxygen chain reaction. The sulfite-oxygen chain reaction can be initiated by light in the presence of a suitable photosensitizing dye (29). As shown in Fig. 5, the oxidation of sulfite by light and methylene blue was not inhibited by 4.5 \times 10^{-7} M bovine carbonic anhydrase. These reaction flasks contained 0.036 M sodium sulfite, 1 \times 10^{-4} M EDTA, and 6.4 \mu g of methylene blue adsorbed onto 2.0 mg of Celite, all in a total volume of 2.2 ml buffered at pH 7.8 with 0.05 M potassium phosphate. Reactions were initiated at the arrows by tipping the adsorbed dye into the reaction mixture. Additional components were: Line 1, none; Line 2, 4.5 \times 10^{-7} M bovine carbonic anhydrase.

Fig. 5. Effect of carbonic anhydrase on the photosensitized oxidation of sulfite. Reaction mixtures contained 0.036 M sodium sulfite, 1 \times 10^{-4} M EDTA, 0.05 M potassium phosphate, and 6.4 \mu g of methylene blue adsorbed onto 2.0 mg of Celite in a total volume of 2.2 ml. Other conditions were as described in Fig. 4. Reactions were initiated at the arrows by tipping the adsorbed dye into the reaction mixtures. Additional components were: Line 1, none; Line 2, 4.5 \times 10^{-7} M bovine carbonic anhydrase.
methylene blue adsorbed on Celite on the initiation of sulfite oxidation by xanthine oxidase was investigated. This was performed in the dark under the conditions described for Fig. 4, except that 6.4 μg of methylene blue adsorbed on 2.0 mg of Celite were also present. Methylene blue on Celite did not prevent 2.3 x 10^-3 m bovine carbonic anhydrase from causing 70% inhibition of the initiation of sulfite oxidation in this case.

**DISCUSSION**

Let us assume that xanthine oxidase, when acting on its substrates in the presence of oxygen, is capable of the univalent reduction of oxygen to the perhydroxyl radical or to its conjugate base, the superoxide anion. Thus

\[ EH_2 + O_2 \rightarrow EH \cdot + HO_2^- \]  \hspace{1cm} (1)

The univalently reduced enzyme could then give rise to another perhydroxyl radical by reacting with a second molecule of oxygen; but one or the other of these modes of generation of the oxygen radical would be rate-limiting so that we can, for the purposes of the present discussion, limit ourselves to considering either one. The perhydroxyl radicals thus generated could spontaneously dismutate as follows:

\[ HO_2^- + HO_2^- \rightarrow H_2O_2 + O_2 \]  \hspace{1cm} (2)

The perhydroxyl radical could alternately act to reduce ferricytochrome c. Thus

\[ HO_2^- + cytochrome c^{+++} \rightarrow O_2 + H_2O_2 + cytochrome c^{++} \]  \hspace{1cm} (3)

In the absence of cytochrome c, the steady state level of HO_2^- would depend upon a balance between its rate of generation and its rate of dismutation. Thus:

\[ \frac{d(HO_2^-)}{dt} = k_1 \langle EH_2 \rangle \langle O_2 \rangle - k_2 \langle HO_2^- \rangle^2 = 0 \]

During the period of observation, the concentration of oxygen would be essentially constant, as would the proportion of the total enzyme in the reduced state. Hence

\[ K(E_0) = k_2[HO_2^-]^2 \]

and

\[ [HO_2^-] = \sqrt{\frac{K(E_0)}{k_2}} \]

The steady state level of HO_2^- should therefore vary as the square root of the total concentration of enzyme. This prediction cannot be tested directly, because of the difficulty of measuring minuscule steady state levels of HO_2^- However, an indirect test is possible. Thus, if HO_2^- is the reductant of cytochrome c, the initial rate of reduction of cytochrome c will directly reflect the level of HO_2^- and should in turn be related to the square root of the concentration of total xanthine oxidase. This should hold true provided that the concentration of cytochrome c used is too low to perturb the steady state level of HO_2^- significantly. Fig. 6 illustrates the affirmation of these expectations. The initial rates of cytochrome c reduction, which are graphed in Fig. 6 as a function of the square root of the enzyme concentration, are the rates which would have been observed in the presence of 5 x 10^-8 m cytochrome c. Rates were not actually measured at such low levels of cytochrome c. At each enzyme concentration, initial rates were measured as a function of cytochrome c concentration. These data defined straight lines when graphed on reciprocal coordinates, and these lines were extrapolated to give the rates which would have been observed at 5 x 10^-8 m cytochrome c. The slopes and intercepts of these lines defined the values of K, for cytochrome c presented in Fig. 1.

As the concentration of cytochrome c is raised, at any fixed level of xanthine oxidase, the proportion of the oxygen radicals generated, which are utilized for the reduction of cytochrome c, will also be raised. At saturating levels of cytochrome c, all of the HO_2^- generated in Reaction 1 will be used in Reaction 3. Thus, at saturating levels of cytochrome c, the initial rates of reduction of cytochrome c (V_max) should be directly proportional to the concentration of xanthine oxidase. That this was the case is shown by the data illustrated in Fig. 7.

If carbonic anhydrase or myoglobin acts to catalyze a dismutation of oxygen radicals as in Reaction 2, then at any given concentration of xanthine oxidase and of cytochrome c it will act to lower the steady state level of HO_2^- and will thus inhibit the rate of reduction of cytochrome c without inhibiting the rate of oxidation of xanthine. However, the dismutation reaction, whether spontaneous or catalyzed, is in competition with cytochrome c for the HO_2^- available; hence carbonic anhydrase and myoglobin must inhibit competitively with respect to cytochrome c.

At any given concentration of cytochrome c, the higher the concentration of xanthine oxidase, the greater the rate of generation of HO_2^- and the greater the proportion of HO_2^- being spontaneously dismutated. If carbonic anhydrase or myoglobin provides a catalyzed pathway for the dismutation reaction, then this catalyzed pathway will be in competition for HO_2^-, with the spontaneous dismutation as well as with the reduction of cytochrome c. Hence, the larger the relative flux of HO_2^- through the spontaneous dismutation pathway, the greater the amount of carbonic anhydrase or of myoglobin which will be required to effect a given decrease in the steady state level of HO_2^-.
therefore expect that $K_i$ for carbonic anhydrase and for myoglobin will increase with increases in the concentration of xanthine oxidase. Fig. 2 illustrates this particular aspect of the behavior of this system.

It is clear that the observed dependencies on the concentration of xanthine oxidase of $K_v$ for cytochrome $c$, $K_i$ for myoglobin and for carbonic anhydrase, and the initial rate of cytochrome $c$ reduction at low levels of cytochrome $c$ can all be explained on the basis of the production of $\text{HO}_2^-$ by the xanthine oxidase system, and of the catalysis of a dismutation reaction by carbonic anhydrase and by myoglobin. It is equally clear that this proposal precludes the necessity for any direct association reactions between xanthine oxidase and cytochrome $c$, myoglobin, or carbonic anhydrase. Binding studies performed by three techniques did indeed fail to detect any such association under conditions in which the kinetic constants would ordinarily have led to the expectation of quantitative association. The results of these binding studies therefore support the hypothesis presented above. More direct support was provided by means of the electrode-initiated sulfite-oxygen chain reaction. It has been shown (7) that the cathode-initiated oxidation of sulfite responds to the impressed voltage in such a way as to define a polarographic reduction wave of oxygen. Hence, the initiation of the sulfite-oxygen chain reaction at a platinum cathode is a consequence of the reduction of oxygen at that electrode. Since $\text{H}_2\text{O}_2$ does not initiate the sulfite-oxygen chain reaction (7), and since there is independent evidence for the univalent reduction of oxygen at electrodes (30), it was concluded (7) that the cathodic initiation of the sulfite-oxygen chain reaction was a direct consequence of the generation of univalently reduced oxygen at the electrode. The ability of bovine carbonic anhydrase to inhibit the cathodic initiation of the sulfite-oxygen chain reaction is therefore in accord with the hypothesis under discussion. The failure of myoglobin to do so was inexplicable until it was observed to prevent the action of carbonic anhydrase in the electrode-initiated system. This indicated the possibility that myoglobin was unfolding over the surface of the cathode. The relative ease with which myoglobin is denatured at interfaces, and the resistance of carbonic anhydrase to this type of unfolding, is in accord with this explanation, as is the observation that lyophilization destroyed the inhibitory potency of myoglobin but had no effect on that of carbonic anhydrase. Xanthine oxidase plus xanthine is an effective initiator of the sulfite-oxygen chain reaction (6, 7). This process is also presumably a consequence of the generation of univalently reduced oxygen. Since in this case the oxygen radicals are generated throughout the solution rather than at the surface of an electrode, both carbonic anhydrase and myoglobin should act as effective inhibitors. Fig. 4 indicates that they do so.

The inability of bovine carbonic anhydrase to inhibit the sulfite-oxygen chain reaction initiated by light in the presence of methylene blue has important implications with regard to the mechanism of this chain reaction. A variety of chain-propagating and chain-terminating steps can be and have been proposed (29–25) to fit kinetic data, but direct evidence for or against the radical species proposed is not available. The present observations indicate that univalently reduced oxygen cannot be involved in the chain propagating steps of this reaction.

The suggestion that carbonic anhydrase and myoglobin specifically catalyze a dismutation of univalently reduced oxygen requires additional comment. A direct demonstration of this activity is as desirable as it is difficult to attain because of the great chemical reactivity of the proposed substrate. This particular dismutase activity could be of importance to living cells. Thus, $\text{HO}_2^-$ could be generated by a variety of aerobic univalent oxidations, such as the oxidation of hemoglobin to methemoglobin. Once generated, this radical could be very damaging to the components of the cell by virtue of its high reactivity. It is tempting to speculate that a herefore unsuspected function of carbonic anhydrase and of myoglobin is to protect the cell against such damage by catalyzing a rapid dismutation of oxygen radicals. Attempts to gain evidence for or against this proposal are under way.

Note Added in Proof—It has recently proven possible to separate the carbonic anhydrase activity from the dismutase activity of the preparations of bovine erythrocyte carbonic anhydrase used in the above studies. This was accomplished by column chromatography on Whatman DE-32 and establishes that the bovine erythrocyte dismutase is distinct from carbonic anhydrase and was present as an impurity in the carbonic anhydrase preparations used in these studies. This finding modifies neither the results nor the conclusions of the various kinetic studies described above. It does, however, cast doubt upon the binding studies whose negative results were based upon the presumed identity of the dismutase and the carbonic anhydrase. It was therefore necessary to validate the binding studies by measuring dismutase activities rather than carbonic anhydrase. The experiment illustrated in Fig. 3 was therefore repeated with the modification that the column effluent was assayed for dismutase. This was done by observing the degree of inhibition of the rate of reduction of cytochrome $c$ by aldehyde oxidase (31) acting on $N$-ethylquinolinium chloride at pH 8.8, caused by fixed aliquots of the column effluent. This assay was unaffected by the presence of xanthine oxidase. The results of this experiment were virtually identical to those shown in Fig. 3. The method which succeeded in separating dismutase activity from bovine erythrocyte carbonic anhydrase was applied to horse

![Fig. 7. Relationship between the maximal rate of reduction of cytochrome c and the concentration of xanthine oxidase. Reaction conditions were as described in the legend to Fig. 1. Maximum velocities were obtained by extrapolation of data obtained at finite concentrations of cytochrome c.](http://www.jbc.org/content/148/3/5759/F1.large.jpg)
heart myoglobin. Myoglobin and dismutase failed to separate to any degree. It therefore appears likely that myoglobin, per se, possesses dismutase activity. This conclusion is in accord with the results of Quinn and Pearson (32). The results shown in Table I may reflect varying contents of dismutase rather than species differences in the carbonic anhydrases tested.

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