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INVOLVEMENT OF SUPEROXIDE IN THE REACTIONS OF THE CATECHOL DIOXYGENASES

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SUMMARY

The effect of copper salicylate on the rates of reaction of protocatechuate 3,4-dioxygenase (intradiol cleavage) and 3,4-dihydroxyphenylacetate 2,3-dioxygenase (extradiol cleavage) was monitored. The data obtained is consistent with the dismutation of superoxide by the copper complex resulting in the uncoupling of the oxygen reduction step from the product formation step. Mechanistic interpretations are presented.

INTRODUCTION

The catechol dioxygenases catalyze the cleavage of catechols into aliphatic products with the incorporation of dioxygen.[1] Some of these enzymes effect ring cleavage at the carbon-carbon bond between the hydroxyl groups (intradiol cleavage) while others break the C-C bond adjacent to one of the hydroxyl groups (extradiol cleavage). Pyrocatechase and protocatechuate 3,4-dioxygenase are examples of the intradiol cleaving enzymes; they are pink in color and contain high-spin ferric iron at the active site.[1] The color has been assigned to tyrosinate-to-iron charge transfer in both cases by resonance Raman spectroscopy.[2,3] The extradiol cleaving enzymes, on the other hand, are colorless and are believed to have ferrous iron in the active site, though this has yet to be unequivocally demonstrated; examples of this class are metapyrocatechase and 3,4-dihydroxyphenylacetate 2,3-dioxygenase.[1]

Mechanisms for the reaction of the catechols with oxygen through the intermediacy of the active site iron have been proposed and superoxide is usually

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suggested as an intermediate.[4,5] We have investigated the involvement of superoxide in these reactions with superoxide dismutase as well as with copper complexes. These copper complexes have been shown to act as superoxide dismutases with catalytic efficiencies similar to that of the bovine erythrocyte enzyme.[6,7] Indeed, the involvement of superoxide has recently been demonstrated in the reaction mechanisms of diamine oxidase,[8] prolyl hydroxylase,[9] and lysyl hydroxylase[9] with the use of the copper complexes. In these reactions, the superoxide formed is thought to be inaccessible to the bovine erythrocyte enzyme.

MATERIALS AND METHODS

Protocatechuate 3,4-dioxygenase from Pseudomonas aeruginosa was prepared as previously described.[10,11] 3,4-Dihydroxyphenylacetate 2,3-dioxygenase was prepared from Bacillus brevis as will be reported in a forthcoming paper. Copper salicylate was synthesized from copper sulfate and sodium salicylate in aqueous solution.[12] All other reagents were obtained commercially.

All absorbance measurements were performed on a Cary 219 Spectrophotometer while oxygen uptake rates were obtained on a Gilson K-1C oxygraph.

RESULTS AND DISCUSSION

Protocatechuate 3,4-dioxygenase (EC 1.13.1.3) catalyzes the incorporation of the elements of molecular oxygen into catechols to yield cis,cis-muconic acids.[1] Superoxide has been proposed as an intermediate in the reaction.[4,5] In our experiments, bovine erythrocyte superoxide dismutase shows no inhibitory effect on the enzyme reaction, while copper salicylate ($Cu(sal)_2$) does. Figure 1 shows the time course of the cleavage of protocatechuate by the dioxygenase as monitored by the oxygraph (A) and the spectrophotometer at 260 nm (C). The former measures oxygen uptake while the latter monitors product accumulation. Under steady state conditions, the rates measured by these two methods must be identical. B and D show the effect of added $Cu(sal)_2$ on the rates of O_2 uptake and product accumulation, respectively. Two observations are made: 1) the rate of product

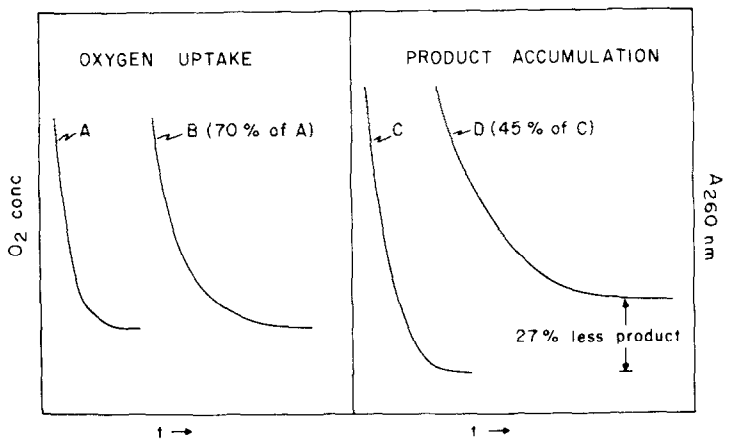


Figure 1. A comparison of the inhibitory effect of $Cu(sal)_2$ on the rates of oxygen uptake (A and B) and product formation (C and D) for the reaction catalyzed by protocatechuate 3,4-dioxygenase. Reaction conditions: 50 μM 3,4-dihydroxybenzoate, 83 μM $Cu(sal)_2$ in 50 mM Tris-HCl buffer, pH 7.5 at 25°C. Note that oxygen concentration decreases with time in A and B, while A_{260nm} increases with time in C and D.

accumulation is significantly smaller than the rate of oxygen uptake in identical experiments and 2) a stoichiometric amount of O_2 is consumed during the inhibited reaction, while a less than stoichiometric amount of product is formed. In fact, the percentage of product missing in the inhibited reaction corresponds to the difference in the rates monitored in B and D. Similar observations are made with different amounts of substrate and inhibitor.

Further steady state kinetic studies on the inhibitory effect of $Cu(sal)_2$ confirm the apparent dichotomy in the two assay methods. Oxygen uptake studies show the inhibitor to be competitive with substrate (Figure 2A), while product accumulation data indicate noncompetitive inhibition (Figure 2B). This means that $Cu(sal)_2$ does not alter V_{max} for oxygen uptake while it decreases the V_{max} for product formation. Experiments with oxygen as variable substrate indicate noncompetitive inhibition for both assay methods and do not provide more information.

The observation of two types of inhibition dependent on assay method suggests that two mechanisms of inhibition are operative. Hayaishi and his co-workers

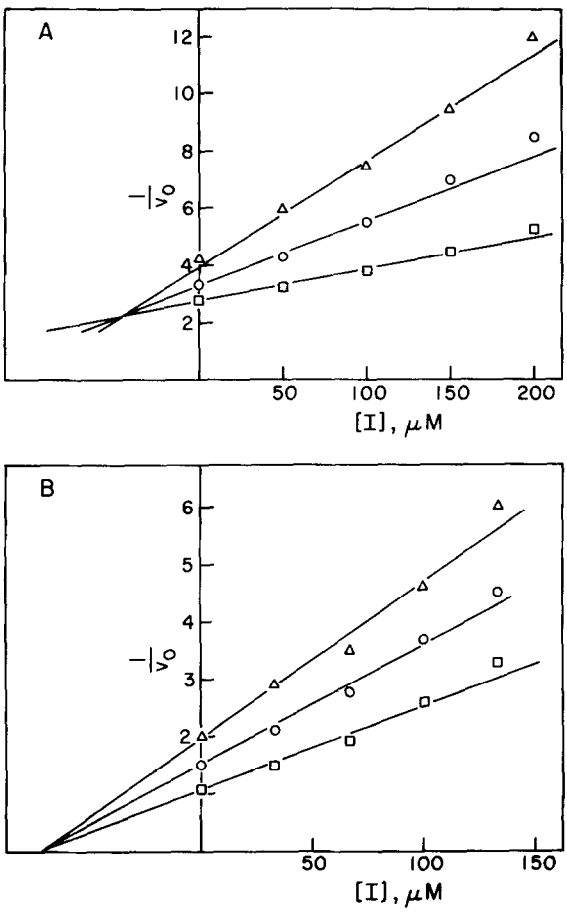
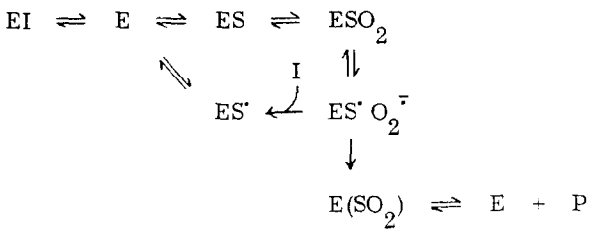


Figure 2. A) Kinetics of inhibition of $Cu(sal)_2$ with respect to 3,4-dihydroxybenzoate, measured as a function of the rate of oxygen uptake. Substrate concentrations were 33 μM (Δ), 50 μM (O), and 92 μM (□).

B) Kinetics of inhibition of $Cu(sal)_2$ with respect to 3,4-dihydroxybenzoate, measured as a function of the rate of product formation. Substrate concentrations as in A).

have demonstrated that catalysis by protocatechuate 3,4-dioxygenase proceeds by an ordered mechanism wherein the catechol binds first, followed by oxygen to form a ternary complex, which then breaks down to product in the rate-determining step.[13] $Cu(sal)_2$ first inhibits by competing with substrate — thus the competitive inhibition diagnostic found in the Dixon plot of oxygen uptake (Figure 2A). Our data indicates that the second mechanism of inhibition operates after oxygen binding and prevents product formation. We suggest that this mode of inhibition is the dismutation by

$Cu(sal)_2$ of superoxide formed as an intermediate in the reaction. This process diverts the ternary complex from breaking down into product and at the same time maintains the oxygen flux. Our conclusions are summarized in the following scheme:



$Cu(sal)_2$, in addition to acting as a competitive inhibitor, uncouples the oxygen consumption step from the product formation step. Such uncoupled reactions are not uncommon for oxygenases (e. g. salicylate hydroxylase[14] and prolyl hydroxylase[15])

Similar results were obtained in studies of the effect of $Cu(sal)_2$ on an extradiol cleaving enzyme, 3,4-dihydroxyphenylacetate 2,3-dioxygenase. In a typical experiment, the rate of oxygen uptake was decreased by 38% in the presence of 166 μM $Cu(sal)_2$, while the rate of product formation was diminished by 58%. 17% less product was formed, in agreement with the difference between the two rates measured.

We have thus demonstrated the involvement of superoxide in the mechanisms of both intradiol and extradiol non-heme iron dioxygenases. One more mechanistic insight can be extracted from our data. In our experiments, we observe stoichiometric consumption of oxygen and less than equivalent formation of product. This implies that the substrate is transformed concomitantly with the formation of superoxide. If the substrate were not involved with the formation of superoxide, then one should observe greater than stoichiometric oxygen consumption and stoichiometric product formation since our experiments were run under limiting

catechol, but not limiting oxygen, conditions. We are currently attempting to elucidate the fate of the transformed substrate.

Such arguments lend credence to the mechanism proposed by Que, et. al.[5] for protocatechuate 3,4-dioxygenase which involves a one-electron transfer from catechol to oxygen in the first step of oxygen reduction. A similar process may be proposed for the extradiol cleavage mechanism with the iron mediating electron transfer. The participation of superoxide is, however, just one of the many steps involved in the mechanism of dioxygenases and much needs yet to be understood.

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