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INVOLVEMENT OF SUPEROXIDE IN THE PROLYL AND LYSYL HYDROXYLASE REACTIONS

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SUMMARY

Four superoxide dismutase active copper chelates, Cu(acetylsalicylate)$_2$, Cu(salicylate)$_2$, Cu(lysine)$_2$ and Cu(tyrosine)$_2$, proved to be inhibitors of prolyl and lysyl hydroxylase. The kinetics of the inhibition are consistent with the proposal that these compounds dismutated $\cdot O_2^-$ at the active site of the enzymes. The data strongly suggest that $\cdot O_2^-$ is the active form of O_2 in the prolyl and lysyl hydroxylase reactions.

Prolyl 4-hydroxylase (E.C.1.14.11.2, termed here prolyl hydroxylase) and lysyl hydroxylase (E.C.1.14.11.4) catalyze the hydroxylation of certain prolyl and lysyl residues in peptide linkages. Both enzymes require Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate, and both reactions involve a stoichiometric decarboxylation of 2-oxoglutarate (for reviews, see 1-3). One atom of the O_2 molecule is incorporated into the hydroxyproline or hydroxylysine, while the other is incorporated into the succinate (4). Kinetic studies (5-8) and other data (9-12) suggest that both reactions involve an ordered binding of Fe^{2+}, 2-oxoglutarate, O_2 and the peptide substrate to the enzymes in this order before the hydroxylation takes place. The O_2 must evidently be activated by some mechanism, the formation of superoxide being one possibility (1-3). Nitroblue tetrazolium, which is capable of scavenging superoxide, inhibits the two enzymes (5,8,13) competitively with respect to O_2 (5,8), but three laboratories have reported that superoxide dismutase does not inhibit the prolyl hydroxylase reaction (1,5,13). It is not known, however, whether the latter finding is due to the inability of the enzyme to come into contact with the active site of the hydroxylase.

Several low molecular weight copper chelates have recently been shown to exhibit the same superoxide dismutase activity as the native enzyme itself

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(14-17), and these compounds have been used to detect the production of $^{\cdot}O_2^-$ at active sites which could not be reached by superoxide dismutase (e.g. 17-19). We report here an inhibition of prolyl and lysyl hydroxylase in the case of four such copper chelates and present kinetic data which strongly suggest that this inhibition is due to dismutation of $^{\cdot}O_2^-$, which thus appears to be the active form of O_2 in these reactions.

MATERIALS AND METHODS

The copper chelates were synthesized as described previously (14,17). Pure prolyl hydroxylase and highly purified lysyl hydroxylase were prepared from ammonium sulphate fractions of chick embryo extract by affinity chromatography procedures, using poly(L-proline) in the case of the former enzyme (20), and concanavalin A-agarose in the latter (21). The enzyme activities were assayed under standard conditions in a final volume of 1.0 ml which contained 0.1-0.5 µg enzyme; 0.1 mg $(Pro\text{-}Pro\text{-}Gly)_{10}\cdot 9H_2O$ for prolyl hydroxylase or 0.5 mg peptide substrate L-I (Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly, purchased from the Protein Research Foundation, Minoh, Osaka, Japan) for lysyl hydroxylase; 0.05 µmol $FeSO_4$; 0.1 µmol 2-oxo[1-^{14}C]glutarate (40 000 dpm); 1 µmol ascorbate; 0.1 mg catalase; 0.5 mg bovine serum albumin; 0.1 µmol dithiothreitol and 50 µmol Tris-HCl buffer, adjusted to pH 7.8 at 25°C. The samples were incubated at 37°C for 30 min and the $^{14}CO_2$ trapped and counted (see 5,22,23). The hydroxylation systems were modified for various experiments as indicated in the legends to the figures.

RESULTS AND DISCUSSION

Inhibition of the prolyl and lysyl hydroxylase reactions by superoxide dismutase active copper chelates. Four superoxide dismutase active copper chelates, Cu(acetylsalicylate)$_2$, Cu(salicylate)$_2$, Cu(lysine)$_2$ and Cu(tyrosine)$_2$, were tested as inhibitors of the two hydroxylases. The enzyme reactions were assayed by measuring the formation of $^{14}CO_2$ during the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate (22,23). All four compounds inhibited both prolyl hydroxylase (Fig. 1A) and lysyl hydroxylase (Fig. 1B), their effectiveness being in decreasing order: Cu(acetylsalicylate)$_2$, Cu(salicylate)$_2$, Cu(lysine)$_2$ and Cu(tyrosine)$_2$. The concentrations required for a 50 % inhibition of both hydroxylases were in the range of about 30-50 µM. The inhibition could not be reversed by the addition of a large excess of Fe^{2+} (up to 250 µM), indicating that the inhibition was not due to chelation of the Fe^{2+} required as a co-substrate. It may also be noted that the concentrations of the copper chelates required for 50 % inhibition of the two hydroxylases are similar to those giving 50 % inhibition in other systems involving $^{\cdot}O_2^-$ (17-19).

The free ligands, acetylsalicylate, salicylate, lysine and tyrosine gave either no inhibition or less than 10 % inhibition when tested at a concentration of 100 µM (not shown). $CuSO_4$, which in itself has a high $^{\cdot}O_2^-$ dismutating activity (14,17), but which also inhibits these hydroxylases by the

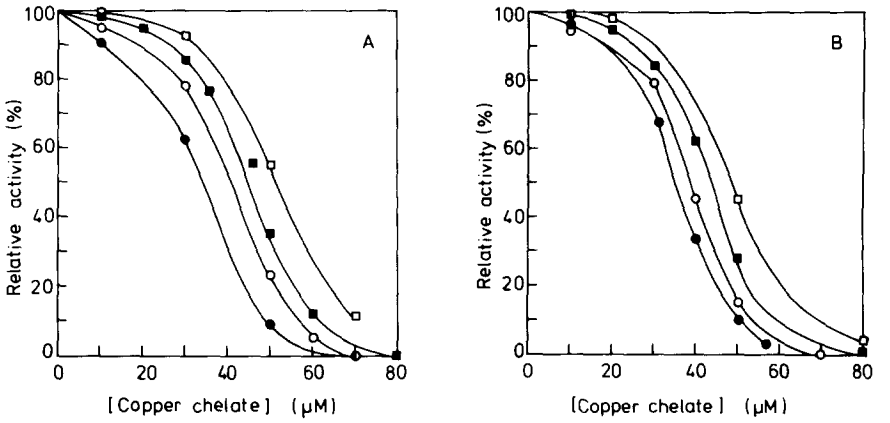

Fig. 1. Inhibition of prolyl hydroxylase (A) and lysyl hydroxylase (B) by Cu-(acetylsalicylate)$_2$ (●), Cu(salicylate)$_2$ (○), Cu(lysine)$_2$ (■) and Cu(tyrosine)$_2$ (□). The reactions were carried out under standard conditions, except that the concentrations of the inhibitors were varied. The reaction rates are expressed as relative velocities, the values obtained in the absence of the inhibitor being taken as 100 %.

competition of Cu^{2+} with Fe^{2+} at the active site (24), inhibited the reaction by about 5 % at a concentration of 30 μM and 50 % at 60 μM. The inhibition by the copper chelates, however, cannot be due to Cu^{2+} dissociated from these compounds, as they inhibited the reaction in lower concentrations than did $CuSO_4$, and are themselves very stable, with stability constants varying from about 10^{14} to 10^{19} (25), so that the concentration of free Cu^{2+} in solutions of 50 μM copper chelates remained below 1 μM.

Kinetics of the inhibition. The inhibition patterns of the copper chelates with respect to the peptide substrate and all the co-substrates were determined for prolyl hydroxylase. All four compounds gave similar patterns. The inhibition was competitive with respect to O_2 (Fig. 2A) and non-competitive with respect to Fe^{2+} (Fig. 2B), 2-oxoglutarate (not shown), the peptide substrate (Fig. 2C) and ascorbate (not shown). In all cases the secondary plots of the slopes and intercepts of the primary plots (26) were parabolic (not shown). These results agree with those expected if the copper chelates dismute $^{\cdot}O_2^-$ at the active site of the enzyme.

Conclusions. The present data indicate that the four superoxide dismutase active copper chelates inhibit prolyl and lysyl hydroxylase in concentrations similar to those required to inhibit other enzymic reactions involving $^{\cdot}O_2^-$ (17-19). The kinetics of the inhibition are consistent with the proposal that these compounds dismutated $^{\cdot}O_2^-$ at the active site, and argue against non-specific side-effects such as chelation of Fe^{2+} or inhibition by reacting

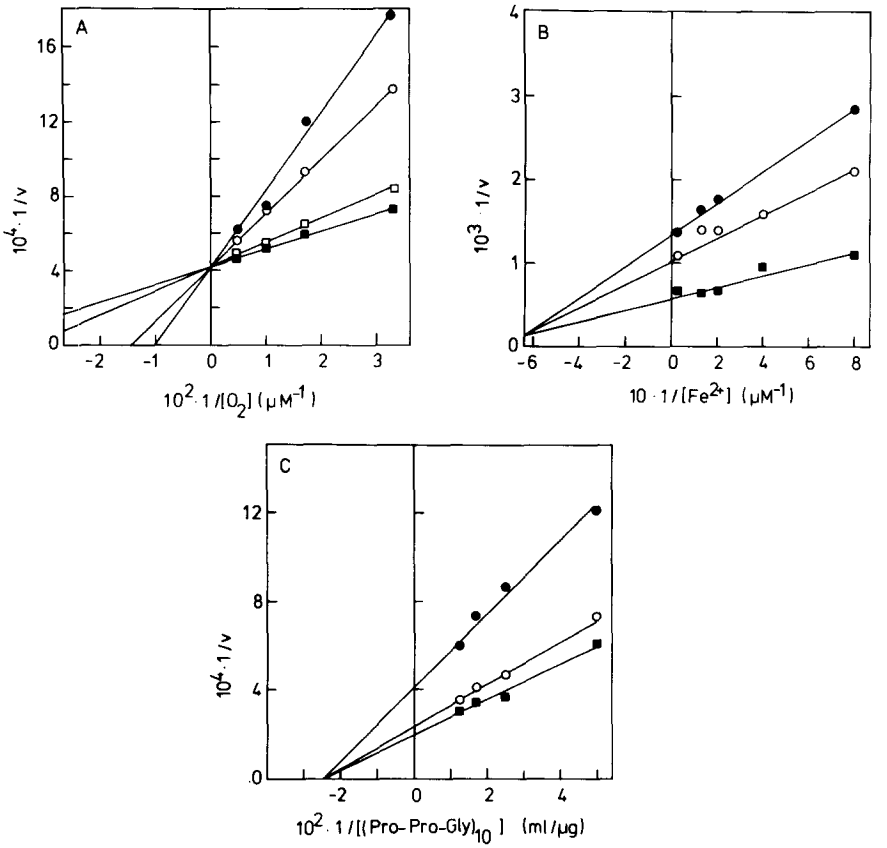

Fig. 2. Inhibition of the prolyl hydroxylase reaction by $Cu(lysine)_2$ with respect to O_2 (A), by $Cu(tyrosine)_2$ with respect to the bivalent iron (B) and by $Cu(lysine)_2$ with respect to the peptide substrate (C). The concentrations of the copper chelates were A: (●) 36 μM, (o) 30 μM, (□) 24 μM, (■) none, B: (●) 60 μM, (o) 45 μM, (■) none, C: (●) 45 μM, (o) 35 μM, (■) none. The reaction velocity (v) was measured in dpm.

with the Fe^{2+} binding site. These results thus strongly suggest that $\cdot O_2^-$ is involved in the prolyl and lysyl hydroxylase reactions.

These two hydroxylases belong to the group of 2-oxoglutarate dioxygenases, other members of which include prolyl 3-hydroxylase, 4-butyrobetaine hydroxylase, thymine 7-hydroxylase, pyrimidine deoxyribonucleoside 2'-hydroxylase, ε-N-trimethyllysine hydroxylase and p-hydroxyphenylpyruvate hydroxylase (1-3). It seems likely that all these enzymes have basically similar reaction mechanisms, and hence it seems probable that $\cdot O_2^-$ is the active form of O_2 in all the reactions catalyzed by 2-oxoglutarate dioxygenases.

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