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Investigations in Serum Copper

III. Coeruloplasmin as an Enzyme

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Coeruloplasmin, the dark blue copper containing protein present in mammalian plasma has been described by us in earlier papers [1, 2]. We have also shown that this copper proteide promotes the oxidation of *para*phenylene diamine (ppd).

In this paper we intend to show that coeruloplasmin must be regarded as an enzyme with an active group containing copper.

METHODS

Coeruloplasmin has been prepared according to a method described in an earlier paper [2]. The preparations used had a copper content of about 0.35 per cent. Copper has been determined with sodium diethyl dithiocarbamate after wet ashing and protein with the biuret method.

*Para*phenylene diamine (ppd) c. p. Coleman and Cell Co. has been used.

The enzymatic activity has been determined with the Warburg technique at 37° C. The consumption of oxygen per unit time, during the period of the most rapid oxidation, has been used as a measure for the velocity of the oxidation.

pH was measured with glass electrodes.

ENZYMATIC CHARACTER OF THE OXIDATION OF PPD BY COERULOPLASMIN

As ppd is a substance which is fairly instable in solutions it is always necessary to compare the action of a proposed enzyme on this substance with its spontaneous oxidation.

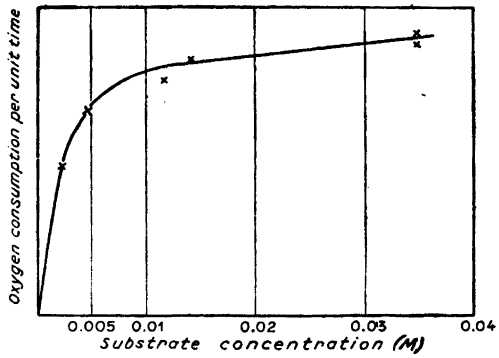

Fig. 1. Influence of substrate concentration on the activity of coeruloplasmin. The Michaelis constant as derived from the curve will be about $2.5 \cdot 10^{-3}$. The experiment has been performed at pH 6.0. The ppd was acidified with HCl. Enzyme concentration was $1.2 \cdot 10^{-6}$ M.

Under optimal conditions * at pH 6.0 the QO_2 at 37° C of the oxidation of ppd in the presence of coeruloplasmin amounts to about 1 500. Under the same conditions and in the presence of an equivalent amount of inorganic copper the O_2 consumption amounts to only about 3 per cent of that found in the presence of coeruloplasmin.

If albumine is added to the system ppd-inorganic copper the oxidation velocity is diminished. Albumin has no such effect on the system ppd-coeruloplasmin.

The relation between oxidation velocity and the concentration of substrate in the system ppd-coeruloplasmin is recorded in Fig. 1.

Variations of the oxygen in the atmosphere between 16 and 100 vol. per cent do not influence the oxidation velocity of ppd by coeruloplasmin.

These experiments seem to furnish conclusive proofs of the enzymatic character of the oxidation of ppd by coeruloplasmin.

There are also clear differences between the oxidation of ppd in the presence of coeruloplasmin on one hand and in the presence of inorganic copper on the other. As is well known the oxidation of ppd in the presence of inorganic copper proceeds much faster in slightly alkaline than in slightly acid solution. The optimal activity of coeruloplasmin, however, lies between pH 5 and 6.

When ppd is oxidized in the presence of copper there is an accumulation of H_2O_2. The amount of oxygen consumed amounts to 3 *M* O_2 per 2 *M* ppd.

By adding catalase we have been able to show that no H_2O_2 is accumulated when ppd is oxidized in the presence of coeruloplasmin. If catalase is present during the oxidation of ppd by coeruloplasmin this does not interfere with the

* Factors influencing the optimal conditions are discussed in paper 4 of this series /.

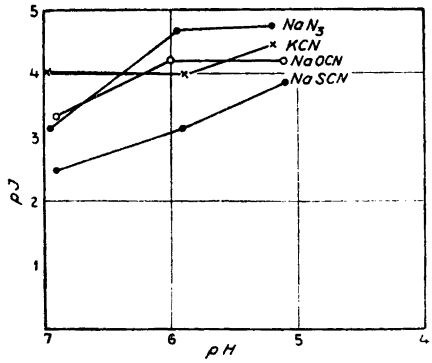

Fig. 2. Concentration of inhibitors which result in 50 per cent inhibition of coeruloplasmin at different pH:s. pI = −log of the molar concentration of the inhibitory substances. Enzyme concentration was kept at $1.9 \cdot 10^{-6}M$ ($7.6 \cdot 10^{-6}M$ Cu) and substrate concentration at $4.6 \cdot 10^{-3}M$. All points are results of a series of determinations performed at each pH with varying concentrations of inhibitor.

velocity of the reaction. The amount of oxygen consumed in this oxidation is 3 *M* O_2 per 4 *M* ppd. The same figures have been obtained by Graubard [4] and by Gregg and Miller [5] when ppd was oxidized by laccase from mushrooms.

DOES THE ACTIVE GROUP IN THE ENZYME CONTAIN COPPER?

If the copper is eliminated from coeruloplasmin by dialysis against KCN at pH 7 during 48 hours the resulting colorless preparation has lost its enzymatic activity. The remaining cyanide was of course eliminated by prolonged dialysis against distilled water before testing the activity. Neither the blue color nor the activity can be restored by adding copper to this preparation.

There is a proportional decrease in blue color and copper content if coeruloplasmin is dialyzed in acetate buffer at a pH below 5.

If a preparation which has lost some of its color by acidification for some hours to pH 5 is tested on its enzymatic activity there is a decrease in this activity proportional to the decrease in blue color.

From all these experiments it can be concluded that coeruloplasmin is an enzyme with copper in the active group and that its blue color is dependent on its copper content.

SUBSTRATE SPECIFICITY OF COERULOPLASMIN

Coeruloplasmin has greater activity against ppd than against other substrates hitherto tested. The activity of coeruloplasmin against other substrates which are generally oxidized by oxidases has been tested. These experiments have been performed in the presence of albumin or gelatin in order to depress the action of traces of heavy metals. The activity of coeruloplasmin against these substrates being considerably less than against ppd. With this precau-

tion we have been able to show that the following substances can act as substrates for coeruloplasmin: hydrochinone, catechol, pyrogallol, dopa, adrenaline and ascorbic acid. For all these substrates the optimum pH lies between 5 and 6. The experiments have also shown that it is doubtful whether monophenols and mono-amines are attacked at all.

INFLUENCE OF SOME INHIBITORS ON THE OXIDATION OF PPD BY COERULOPLASMIN

As early as 1944 [6] it was shown that the oxidation of ppd by a serum fraction containing copper was inhibited by KCN. We have now studied the inhibitory effect of KCN, NaN_3, KSCN and NaOCN * on the oxidation of ppd by pure coeruloplasmin. It is evident from Fig. 2 that all these substances inhibit the oxidation at fairly low concentrations especially on the acid side of pH 7. The activity of coeruloplasmin is also inhibited by sodium diethyl dithiocarbamate.

The effect of CO has also been studied. No inhibitory effect could be shown with a gas mixture containing 50—85 vol. per cent CO not even in complete darkness.

SUMMARY AND CONCLUSIONS

Our investigations have shown that coeruloplasmin is a true oxidase wich contains copper in its active group. For the following reasons we think that it should be classified as a laccase:

a. It is a blue copper proteide.

b. Its best substrate is *para*phenylene diamine (ppd).

It also acts on poly-phenols but probably not on monophenols and mono-amines.

c. It is inhibited by KCN, NaN_3, NaOCN, KSCN and sodium diethyl dithiocarbamate but not by CO.

Coeruloplasmin differs from laccases of plant origin in being less active. We have determined the optimal QO_2 at 37° C with ppd as substrate to 1 500.

* NaOCN was synthetisized according to Dupré and Schütz [7]. This preparation is completley free from cyanide.

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