KINETIC STUDIES OF CERULOPLASMIN-AZIDE INTERACTION

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1. Introduction

Ceruloplasmin is a copper-containing protein in plasma which has oxidase activity towards polyphenols, aromatic polyamines [1] and ferrous ions [2]. It contains 8 copper atoms per molecule, and 4 of them which are paramagnetic are in cupric (Cu^{2+}) state [3]. The intense blue color of the enzyme has been attributed to type 1 Cu^{2+} which shows the characteristic hyperfine structure in the ESR spectrum [4]. The blue type 1 Cu^{2+} is believed to undergo a cyclic change between Cu^{2+} (blue) and Cu^{+} (non-blue) states in its catalytic reactions in the presence of substrate and oxygen [5]. However, the nature and the catalytic role of non-blue cupric ions (type 2 Cu^{2+}) [4] and the diamagnetic copper atoms (which are believed to be Cu^{+}) have not been elucidated.

Azide is known to strongly inhibit the catalytic activity for oxidation of *N,N*-dimethyl-*p*-phenylenediamine (DPD), one azide molecule per enzyme molecule being sufficient for complete inhibition [6]. The binding of azide with ceruloplasmin has been studied spectrophotometrically [7, 8] and by ESR [8] in static experiments, and the appearance of a new absorption band around 380 nm has been shown to be parallel to the azide binding with type 2 Cu^{2+} [8]. However, the kinetics of azide binding and the mechanism of inhibition of the catalytic reactions by azide seem to be open to further investigations.

In this communication, we wish to report on the effect of the binding of azide with ceruloplasmin. The rate of initial reduction of type 1 Cu^{2+} by DPD in the pre-steady state of the enzyme reaction is not affected by azide. Kinetic studies have shown that azide binds with ceruloplasmin with apparent dissociation constant of 2.7×10^{-4} M in the absence of substrate.

2. Materials and methods

Human ceruloplasmin was prepared from Cohn IV-1 fraction of human plasma by the method of Deutsch [9]. The ratio of absorbance at 610 nm to that at 280 nm was used as an index of purity [10]. Samples with this ratio larger than 0.040 were stored at 4°, and used within a few days after preparation. Reagent grade *N,N*-dimethyl-*p*-phenylenediamine sulfate (DPD) and sodium azide were used without further purification.

A stopped-flow apparatus [11] was used for the kinetic measurements of the azide binding with the enzyme observed at 380 nm, the initial reduction of type 1 Cu^{2+} in the pre-steady state of the reaction with DPD at 610 nm, and the steady-state rate of DPD oxidation at 550 nm. The optical path was 10 mm and the dead-time of the apparatus was about 1 msec. The rate constants of azide binding in low concentration of azide (below 2 mM) were estimated from the measurements with 1 cm cells in a Hitachi 139 spectrophotometer coupled to a potentiometric recorder. All the experiments were performed under aerobic condition at 25° and pH 5.5 with acetate buffer. In the study of azide inhibition, the reaction was induced by mixing the enzyme and the substrate solutions containing azide of identical concentration which had been prepared 10 min before mixing the two solutions.

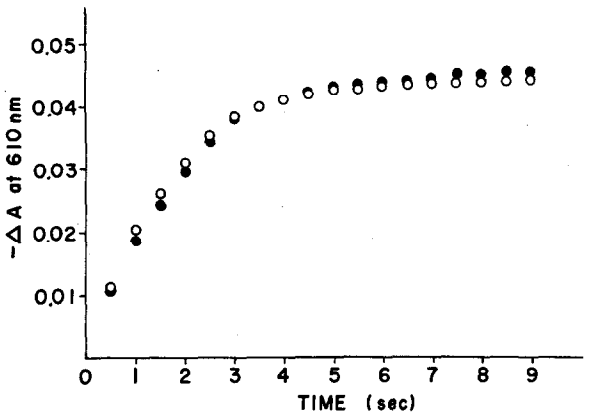

Fig. 1. The time course for the reduction of ceruloplasmin by DPD sulfate in the absence (○) and presence (●) of 15 μM azide. The reactions were followed at 25° in a 0.02 M acetate buffer containing 50 μM EDTA, pH 5.5, with 5.0 μM enzyme and 100 μM DPD.

3. Results and discussion

The process of initial reduction of type 1 Cu^{2+} by DPD was observed in the absence and presence of azide at 610 nm. The result is shown in fig. 1. Unexpectedly, the rate of reduction of type 1 Cu^{2+} was found to be scarcely affected by azide (1.5×10^{-5} M), in spite of the fact that the steady-state rate of product formation observed at 550 nm was reduced to about 60% of the original value, as shown in fig. 2. This result clearly indicates that the elementary step that is inhibited by azide in the overall reaction is not the initial reduction of type 1 Cu^{2+} by the substrate, but some other step(s) in the reaction sequence. This step may be the reoxidation of reduced copper, as was suggested by Curzon in his study of inhibition type [6].

Some examples of the time course of azide binding with ceruloplasmin observed from the absorbance at 380 nm are shown in fig. 3. The curves with azide concentration higher than 2 mM are obviously biphasic in logarithmic plots, indicating that at least two types of azide binding with different rate constants are involved. Below 2 mM, however, only a single phase was observed, which is characterized by apparent first-order rate constant (k). No phase faster than those shown in fig. 3 was observed. Thus the data of static spectrophotometric titration with azide of Kasper [7] at higher azide concentrations may include contribution from the two types of azide binding. The plot of k versus azide concentration [A] (which is sufficiently higher than the enzyme concentration [E]) was linear as shown in fig. 4. This is consistent with a simple bimolecular association equilibrium:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

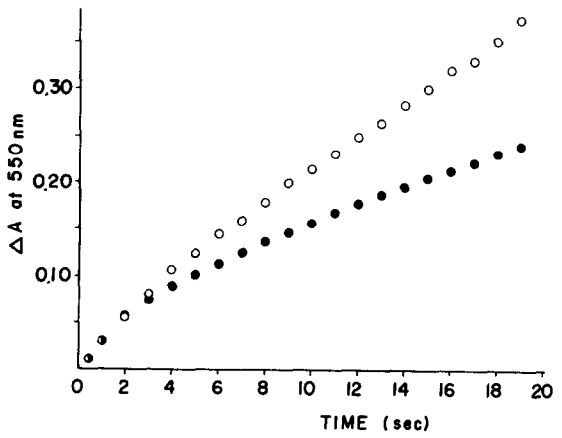

Fig. 2. The time course for the formation of DPD^+ in the absence (○) and presence (●) of 15μM azide. Other conditions are the same as in fig. 1. The slope of the linear part about 4 sec after mixing represents the steady-state of the formation of DPD^+.

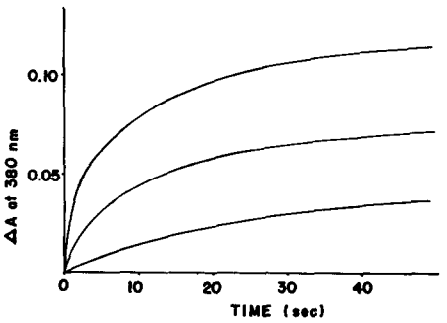

Fig. 3. The time course of absorbance change at 380 nm caused by the azide binding with ceruloplasmin at 25°, pH 5.5., 0.05 M acetate buffer. Ceruloplasmin concentration is 20 μM and azide concentrations are 1, 5, and 25 mM respectively, from the bottom.

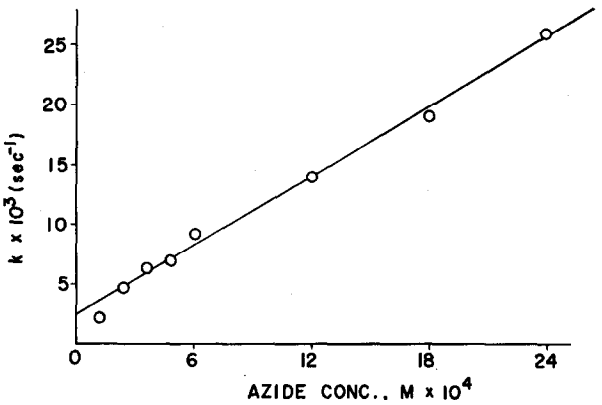

Fig. 4. The apparent first order rate constant k for the binding of azide and ceruloplasmin as a function of total azide concentration at 25°, pH 5.5, in 0.05 M acetate buffer. Ceruloplasmin concentration is 30 μM.

from which the apparent first-order rate constant k is given by:

$$k = k_2 + k_1 [A]$$

The association and dissociation rate constants are obtained from the slope and the intercept of the plot: $k_1 = 9.4\ M^{-1}\ sec^{-1}$ and $k_2 = 2.5 \times 10^{-3}\ sec^{-1}$. The dissociation constant K_a of the complex EA then becomes: $K_a = k_2/k_1 = 2.6 \times 10^{-4}$ M, which is similar in magnitude to that obtained from the static titration of the total absorbance change at 380 nm ($K_a = 3.7 \times 10^{-4}$ M) in a separate experiment. It is of interest that the rate constants are several orders smaller in magnitude than those of the interaction between inorganic cupric ion and azide, which are too rapid to be measured either by the stopped-flow or the temperature-jump method [12].

From the steady-state kinetic study of azide inhibition on DPD oxidation, the inhibition constant K_i of azide was determined to be 3×10^{-6} M, which is close to the value reported by Curzon [6]. It should be noted that this K_i value obtained by the steady-state inhibition by azide is much smaller than the K_a value obtained by the direct observation of azide binding at 380 nm.

The large discrepancy between the K_i and K_a values puts some difficulty in concluding that type 2 Cu^{2+} is responsible for the inhibitory binding of azide with ceruloplasmin.

Further experiments are being undertaken to clarify the effect of azide and fluoride on the steps of ceruloplasmin-catalyzed reactions.

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References

[1] C.G. Holmberg and C.B. Laurell, Acta Chem. Scand. 5 (1951) 476.
[2] S. Osaki, D.A. Johnson and E. Frieden, J. Biol. Chem. 241 (1966) 2746.
[3] P. Aisen, S.H. Koenig and H.R. Lilienthal, J. Mol. Biol. 28 (1967) 225.
[4] A. Ehrenberg, B.G. Malmström, L. Broman and R. Mosbach, J. Mol. Biol. 5 (1962) 450.
[5] L. Broman, B.G. Malmström, R. Aasa and T. Vänngård, Biochem. Biophys. Acta 75 (1963) 365.
[6] G. Curzon, Biochem. J. 100 (1966) 295.
[7] C.B. Kasper, J. Biol. Chem. 243 (1968) 3218.
[8] L.E. Andréasson and T. Vängård, Biochem. Biophys. Acta 200 (1970) 247.
[9] H.F. Deutsch, Arch. Biochem. Biophys. 89 (1960) 225.
[10] C.B. Kasper and H.F. Deutsch, J. Biol. Chem. 238 (1963) 2325.
[11] K. Hiromi, S. Ono, S. Itoh and T. Nagamura, J. Biochem. (Tokyo) 64 (1968) 897.
[12] H. Nakatani, private communication.