A NEW INTERMEDIATE IN THE REOXIDATION OF REDUCED HUMAN CERULOPLASMIN

Takashi MANABE, Nobuko MANABE, Keitaro HIROMI* and Hiroyuki HATANO
Department of Chemistry, Faculty of Science

and

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, Japan*

Received 27 March 1972

1. Introduction

Ceruloplasmin is one of the copper proteins which have a strong blue colour due to an absorption band centering around 600 nm. The blue chromophore of these copper proteins is reduced by various reducing agents whether or not the protein has a catalytic activity. Only the enzymes which show a high rate of aerobic oxidation of the copper atoms have an absorption band at 330 nm [1, 2].

Ceruloplasmin catalyzes the oxidation of Fe^{2+} and certain diamines and polyphenols by oxygen. Recently, it was found that the 330 nm chromophore of ceruloplasmin also participates in the catalytic reaction of this enzyme together with the 610 nm chromophore [3, 4]. However, neither the rate of reduction of the 610 nm chromophore by substrate nor the rates of reoxidation by oxygen observed at 330 nm and 610 nm were consistent with the overall reaction rate of ceruloplasmin [4, 5]. Thus the sequence of the elementary steps, including the rate-limiting steps, in the catalytic reaction of this enzyme still remains to be investigated.

In a recent report [6], we showed that azide, which strongly inhibits the steady-state rate of oxidation of *N, N,* dimethyl-p-phenylenediamine (DPD), does not inhibit the initial reduction of the blue chromophore of ceruloplasmin by DPD. This implies that the rate-limiting step involves a process other than the reduction of 610 nm chromophore, possibly the reoxidation of reduced enzyme by oxygen.

In this communication, we wish to report on a new transient spectral species discovered in the reoxidation step of ceruloplasmin by oxygen. Azide considerably reduced the rate of disappearance of this species, which suggests that it may be one of the important intermediates involved in the reactions catalyzed by this enzyme.

2. Materials and methods

Human ceruloplasmin was purified as previously described [6]. Concentrations of the enzyme were determined spectrophotometrically with $A^{1\%\ 1cm}_{610\ nm} = 0.68$ [7]. Reagent grade chemicals were used in all experiments.

A stopped-flow apparatus with 10 mm optical path and 1 msec dead time was used for all the kinetic measurements reported here.

Ceruloplasmin solutions for reoxidation experiments were made anaerobic in a 100 ml flask by flushing nitrogen gas for 1 hr while gently stirring the solution. A volume of deoxygenated ascorbic acid solution, which contained 8 molar equivalents ascorbate per ceruloplasmin, was added to the flask from a microsyringe for complete reduction of the enzyme. After 2 hr the reduced enzyme was transferred into the reservoir syringe of the stopped-flow apparatus which was flushed with nitrogen gas throughout the

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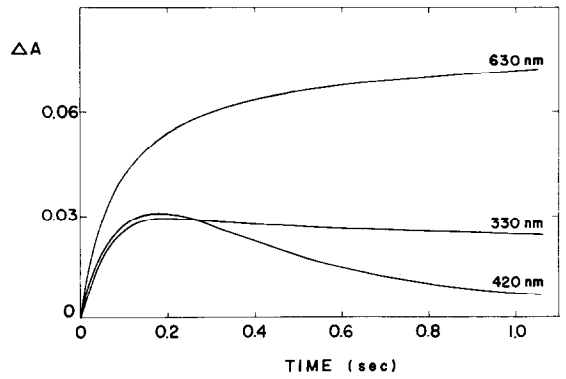

Fig. 1. The time course of absorbance changes at typical wavelengths in the oxidation of completely reduced ceruloplasmin. Solution of reduced ceruloplasmin was mixed with air-saturated buffer solution. The reactions were followed at 25° in a 0.05 M acetate buffer containing 50 μM EDTA, pH 5.5, with 14.7 μM enzyme and 120 μM oxygen.

measurements.

Unless other conditions are indicated, the measurements were conducted at 25° and pH 5.5 in 0.05 M acetate buffer containing 50 μM EDTA.

3. Results and discussion

Ceruloplasmin which had been reduced anaerobically with ascorbate was mixed with air-saturated buffer in a stopped-flow apparatus. Fig. 1 illustrates the time courses of absorbance changes observed at three typical wavelengths. The curve at 420 nm is remarkably different from the curves at 330 nm and 630 nm, in that it reaches a maximum at around 200 msec and then returns to the initial level.

Changes in absorption spectra of the reduced enzyme at 0.2 and 2 sec after initiating the oxidation reaction are shown in fig. 2. An absorption peak around 420 nm is clearly distinguished from the peaks around 330 nm and 610 nm. Different samples of purified ceruloplasmin gave the same results, and EDTA (50 μM to 500 μM) had no effect on the rate of the spectral changes.

Carrico et al. [3] found in their oxidation–reduction titration study of ceruloplasmin that the diamagnetic copper ions can also accept electrons. Three kinds of electron acceptors were distinguished, paramagnetic

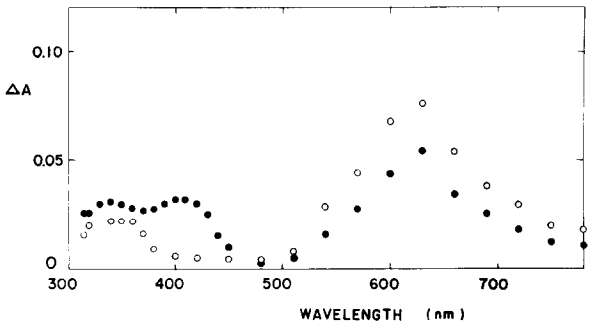

Fig. 2. The spectral changes observed by stopped-flow method at 0.2 (●) and 2 sec (○) after initiating the reoxidation reaction. Conditions are the same as in fig. 1.

copper of Type 1 and Type 2 and a chromophore with an absorption maximum near 330 nm which is associated with the diamagnetic copper. However, no reference was made to the 420 nm chromophore as a transient spectral species.

The effect of azide on the reoxidation process of reduced ceruloplasmin by oxygen was studied at three wavelengths. The results shown in fig. 3A indicate that the rate constants of the initial phases observed at 330 nm and 610 nm are not appreciably reduced by azide. In contrast, the effect of azide on the changes of absorption at 420 nm are evidently different from those observed at 330 nm and 610 nm, as illustrated in fig. 3B. The rate of disappearance of the 420 nm species is reduced by azide to about 19%. In separate experiments, the steady-state rate of the oxidation of DPD (600 μM) was found to be reduced by azide to 19% of the rate obtained in the absence of azide. (The concentrations of ceruloplasmin and azide were the same as in the above reoxidation experiments). It is noteworthy that the rate of the decrease of 420 nm absorption is reduced by azide to the same extent as the steady-state reaction rate.

Although the role of the 420 nm species in the enzyme action is not clear, it is suggested that the species may be an important intermediate in the catalytic reaction of ceruloplasmin. The elementary steps of the reaction of this enzyme are being examined by further kinetic studies on absorbance changes at the relevant wavelengths.

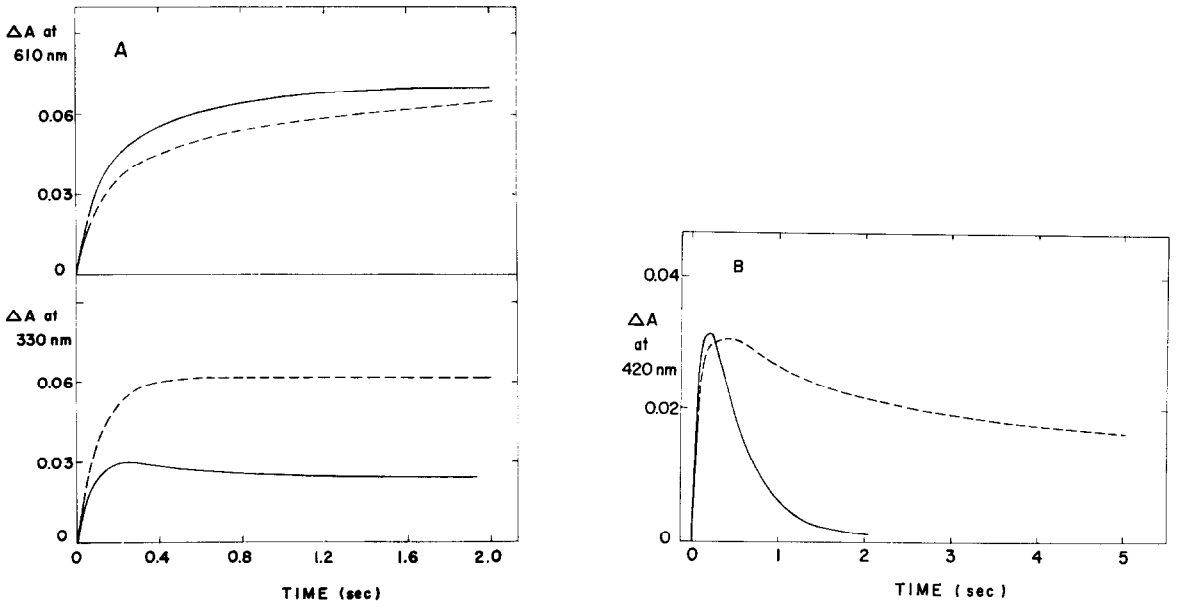

Fig. 3. The time course of the reoxidation of completely reduced ceruloplasmin by oxygen in the presence (------) and absence (——) of 120 μM azide. The enzyme and buffer solutions to be mixed contained the same concentration of azide. Other conditions are the same as in fig. 1. A) Traces at 610 nm and 330 nm; B) Traces at 420 nm. The scales are different from fig. 3A.

Acknowledgements

We wish to thank Dr. O. Nakagawa of the Hematological Laboratory of the Research Institute for Constitutional Medicine for kindly providing us with Cohn IV-1 fraction of human serum plasma.

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