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INHIBITION OF BOVINE CARBONIC ANHYDRASE BY 5-METHYL-1,10-PHENANTHROLINE. DIRECT SPECTROPHOTOMETRIC EVIDENCE FOR TERNARY COMPLEX BETWEEN THE ENZYME AND CHELATING AGENT

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SUMMARY. For the removal of the cobalt ion from cobalt-bovine carbonic anhydrase with 5-methyl-1,10-phenanthroline, it was proved spectrophotometrically that the substitution mechanism proceeded through the ternary complex in which the central metal ion bound with the protein and the chelating agent. The spectrum of the ternary complex had low molar absorption coefficient in visible region, so that it was assumed that the ternary complex had a five- or six-coordination geometry.

INTRODUCTION

For the metal removal reaction of bovine carbonic anhydrase with various chelating agents, it has been assumed that the substitution proceeds through a ternary complex in which the central metal ion binds with protein and chelating agent, and that cleavage of the bound between the metal and protein is the rate-determining step (1).

$$(BCA)M + L \overset{K_{EML}}{\rightleftharpoons} (BCA)ML \xrightarrow{k_2} \text{apo-BCA} + ML \xrightarrow{(n-1)L} \text{apo-BCA} + ML_n \quad (1)$$

where (BCA)M is the metalloenzyme, L is the chelating agent, (BCA)ML, the ternary complex involving the enzyme, chelating agent, and the metal ion, apo-BCA, the apoenzyme, ML, a 1:1 metal-chelating agent complex, and ML_n is the 1:n metal-chelating agent complex. This assumption was derived by the kinetic method for inhibition by chelating agents (1, 2).

In order to detect the ternary complex spectrophotometrically and to provide a direct experimental evidence for the proposed mechanism, we have taken up the cobalt-enzyme and 5-methyl-1,10-phenanthroline as a metal chelating agent. The present paper describes the result on cobalt removal reaction from the cobalt-enzyme by 5-methyl-1,10-phenanthroline.

MATERIALS AND METHOD

Bovine carbonic anhydrase was prepared as described previously (1). The cobalt-enzyme was prepared by the dialysis of apoenzyme against 10^{-3} M Co^{2+} solution in 0.2 M acetate buffer (pH 6.0) (3). Optical spectra were recorded on Shimazu UV-200 spectrophotometer, at 25°. The enzyme activity was measured by using p-nitrophenyl acetate as assay substrate at 25° (4).

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RESULTS

Reaction of 5-Methyl-1,10-phenanthroline with Cobalt-Bovine Carbonic Anhydrase

A series of spectral change occurred on the addition of 5-methyl-1,10-phenanthroline to the protein solution (pH 7.0, 0.1 M phosphate buffer) as shown in Fig. 1A. Within 1 min of the reaction, a scan of the spectrum revealed decrease of the absorbance above 490 nm and the maximum at 550 nm and increase of the absorbance below 490 nm. After 50 min, the absorbance at 550 nm was completely obscured by the increase of that below 490 nm. The absorbance below 490 nm arose from the complex formation of 5-methyl-1,10-phenanthroline with cobalt ion. As this cobalt-enzyme dissociates to yield the complex of 5-methyl-1,10-phenanthroline with cobalt ion, the absorbance of the spectrum of cobalt-enzyme decreased.

The time course of absorbance change at 640 nm is shown in Fig. 1B. Plots of log (A_t/A_0) vs. time were linear but extrapolation to zero reaction time gave a lower absorbance, A_i, than that of the reactants (A is absorbance of the reaction system.). This phenomenon indicates that the fast reaction is followed by a much slower second phase which continues to completion of the reaction. The second phase of the reaction follows first-order kinetics.

Initial Absorbance as Function of 5-Methyl-1,10-phenanthroline

As seen in Fig. 1B, there is an initial decrease of absorbance at 640 nm which can be accurately measured by extrapolating the second phase of the overall kinetics to t_0. The relation of the initial absorbance to increasing concentration of 5-methyl-1,10-phenanthroline is presented in Fig. 2. The sigmoid function was obtained. Thus, observation is interpreted in terms of the equilibrium between (BCA)ML and (BCA)M + L in Eq. (1).

When the equilibrium expressed by Eq. (1) is established, we have the following relationship.

$$E_0 = [(BCA)ML] + [(BCA)M] \quad (2)$$

$$K_{EML} = [(BCA)ML]/([(BCA)M][L]) \quad (3)$$

where E_0 represents the total enzyme concentration and K_{EML} represents the formation constant of ternary complex. The conbination of Eqs. (2) and (3), we obtain

$$\frac{[(BCA)M]}{E_0} = \frac{[(BCA)M]}{[(BCA)M] + [(BCA)ML]} = \frac{1}{1 + K_{EML}[L]} \quad (4)$$

The value of K_{EML} was obtained by the kinetic method which was reported in our previous paper. The value of K_{EML} was 3.8×10^2 M^{-1} and k_2 was 6.8×10^{-2} min^{-1} in 0.1 M phosphate buffer (pH 7.0). [L] was the initial concentration of 5-methyl-1,10-phenanthroline.

In Fig. 2, the dotted line is the curve calculated from Eq. (4) and open circles show A_i/A_0. There is a good agreement between A_i/A_0 and $[(BCA)M]/E_0$. This

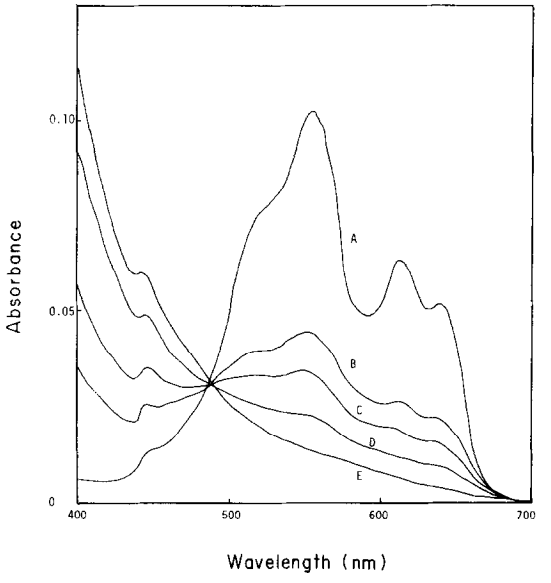

Fig. 1A. Electronic spectra at various periods during transfer of cobalt ion from cobalt-enzyme ((BCA)M) to 5-methyl-1,10-phenanthroline. In the removal of cobalt ion from the cobalt-enzyme, 0.5 ml of cobalt-enzyme solution (6×10^{-4} M) in 0.1 M phosphate buffer (pH 7.0) and 0.5 ml of 5-methyl-1,10-phenanthroline in the same buffer were mixed quickly. At various periods, the spectrum of this solution were measured, and 20 µl of this solution was added to 3 ml of Tris-HCl buffer (pH 7.5, 0.05 M) and esterase activity was measured. All experiments were performed at 25°. A, (BCA)M; B, 45 sec; C, 4min; D, 10 min E, 50 min

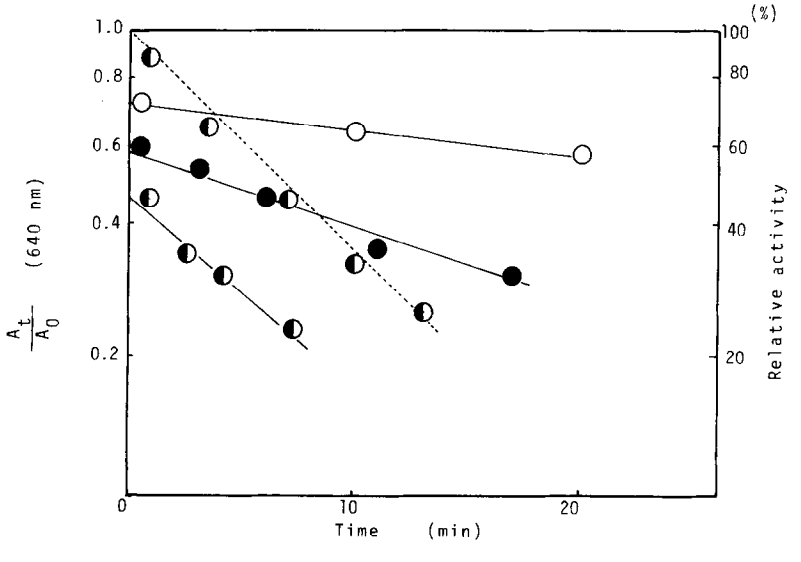

Fig. 1B. Time course of the transfer of cobalt ion from cobalt-enzyme to 5-methyl-1,10-phenanthroline at 640 nm. 5-Methyl-1,10-phenanthroline: —◐— 5×10^{-3} M, —●— 2.5×10^{-3} M, —○— 10^{-3} M. ----◐---- Enzyme activity (5-methyl-1,10-phenanthroline 5×10^{-3} M) In all experiments, the concentration of 5-methyl-1,10-phenanthroline is much larger than that of cobalt-enzyme.

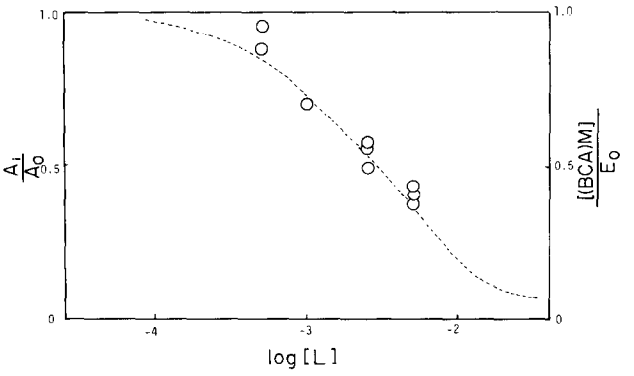

Fig. 2. Initial absorbance as a function of 5-methyl-1,10-phenanthroline concentration. The cobalt-enzyme concentration was held constant at 3×10^{-4} M. The dotted line is the curve calculated from Eq. (4).

obserbation is interpreted that the absorption, A_i, is almost attributed to the absorption of (BCA)M ($\varepsilon_{640} \fallingdotseq 190$). Therefore, (BCA)ML would have a low absorption coefficient or no absorption at 640 nm. There is a good agreement between A_i/A_0 and $[(BCA)M]/E_0$, so that the decrease of initial absorbance would be interpreted in terms of the equilibrium between (BCA)ML and (BCA)M + L.

Kinetic Studies of Second Phase

The second phase of the reaction follows the first order kinetics, as shown in Fig. 1B. Dependency of the second phase of the reaction on the concentration of 5-methyl-1,10-phenanthroline is shown in Fig. 3. A hyperbolic function was obtained. The hyperbolic function between the pseudo-first-order rate constant and 5-methyl-1,10-phenanthroline indicates that the pseudo-first-order rate constant of the second phase depends on the concentration of ternary complex, which fact suggests the validity of Eq. (1).

Competitive Reaction between 5-Methyl-1,10-phenanthroline and Sulfanilamide

In Fig. 1B, the absorbance at 640 nm (5×10^{-3} M, 5-methyl-1,10-phenanthroline) does not agree with the loss of activity. In measurement of the activity, the incubation solution of cobalt-enzyme with 5-methyl-1,10-phenanthroline was diluted with Tris-HCl buffer (3 ml, activity measurement buffer), so that the ternary complex of the enzyme would be dissociated, because of the low formation constant of the ternary complex. Therefore, activity of the incubation solution indicates the total activity of (BCA)M and (BCA)ML, and the difference between the absorbance at 640 nm and the enzyme activity would depend on the formation of a ternary complex. To ascertain this behavior, the following experiments were carried out.

Sulfanilamide is a very strong inhibitor and binds very strongly to this

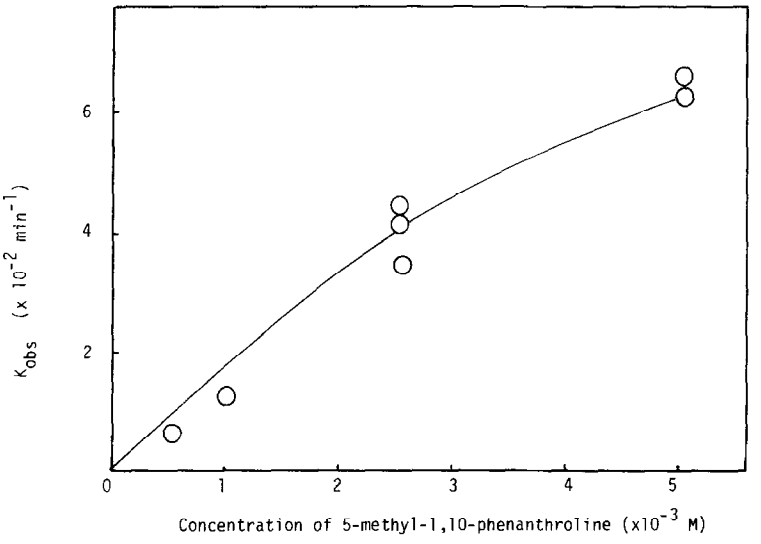

Fig. 3. Pseudo-first-order rate constant (K_{obs}) of the second phase, as a function of 5-methyl-1,10-phenanthroline concentration.

enzyme (5,6). If the formation of a ternary complex occurs in the reaction mixture, sulfanilamide added to the reaction mixture would replace 5-methyl-1,10-phenanthroline in the ternary complex, as shown below:

$$\begin{array}{ccc} (BCA)M + S & \rightarrow & (BCA)MS \\ \uparrow\downarrow +L & & \\ (BCA)ML + S & \rightarrow & \end{array}$$

$$\text{apo-BCA} + LM \xrightarrow{(n-1)L} \text{apo-BCA} + L_nM$$

where S is sulfanilamide and (BCA)MS is the sulfanilamide complex of cobalt-enzyme. The sulfanilamide complex of cobalt-enzyme has a characteristic spectrum in the visible region (7, 8). Spectral changes by the addition of sulfanilamide are shown in Fig. 4A. The absorbance in the characteristic spectrum of sulfanilamide complex decreased with increasing incubation time of cobalt-enzyme and 5-methyl-1,10-phenanthroline. The absorbance at 610 nm and the activity of cobalt-enzyme are plotted against incubation time in Fig. 4B. The decrease of absorbance at 610 nm agreed with the loss of activity. Such a phenomenon indicates that the absorbance of sulfanilamide complex exhibits the concentration of [(BCA)M] + [(BCA)ML]. Therefore, the difference between the absorbance at 640 nm and enzyme activity depends on the formation of a ternary complex.

DISCUSSION

A mechanism such as Eq. (1) has been proposed for the metal removal reaction from bovine carbonic anhydrase. This mechanism was proposed by the kinetic method for inhibition of esterase activity.

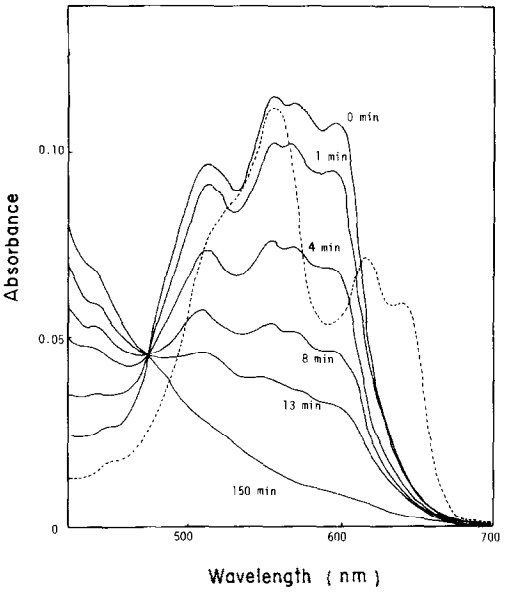

Fig. 4A. Replacement reaction of 5-methyl-1,10-phenanthroline of ternary complex with sulfanilamide. A mixture of the cobalt-enzyme (3×10^{-4} M) and 5-methyl-1,10-phenanthroline (5×10^{-3} M) was incubated at 25° (pH 7.0, 0.1 M phosphate buffer). At various periods, 100 μl of sulfanilamide solution (10^{-2} M) was added to the incubated solution and the spectrum of the complex was measured.

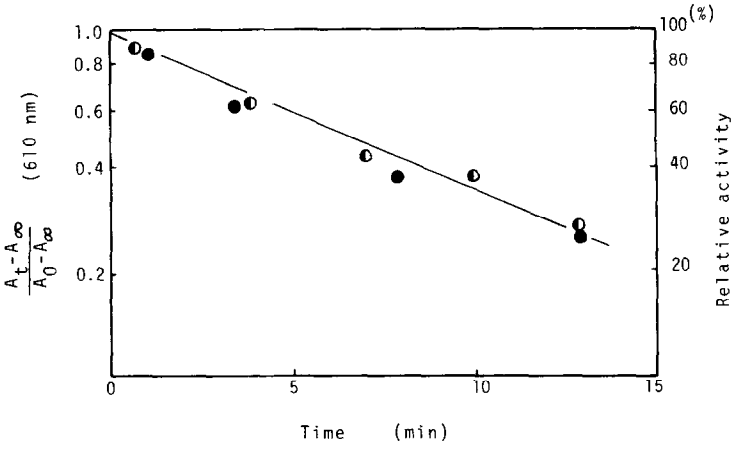

Fig. 4B. Time course of the absorbance at 610 nm (in Fig. 4A) and the enzyme activity. —◐— Enzyme activity (5-methyl-1,10-phenanthroline 5×10^{-3} M), —●— Absorbance at 610 nm

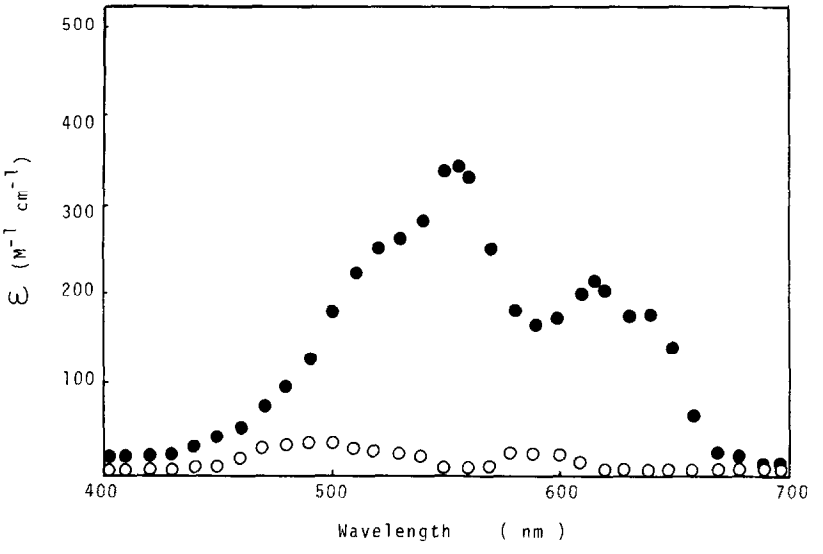

Fig. 5. Spectra calculated from Fig. 1A
The spectrum of (BCA)ML (○) was calculated from Fig. 1A by subtracting the spectrum of 5-methyl-1,10-phenanthroline-cobalt complex and cobalt-enzyme. The concentration of (BCA)M and (BCA)ML was calculated from Eq. (3).
● The spectrum of (BCA)M

In the present experiment, the proposed mechanism was proved directly by the spectroscopic method and it was found that 5-methyl-1,10-phenanthroline bound directly to the cobalt ion in the enzyme. The limit spectrum of the ternary complex ((BCA)ML) was not observed because of poor solubility of 5-methyl-1,10-phenanthroline but the ternary complex has a low absorbance in visible region, as shown in Fig. 1A. The spectrum of (BCA)ML is calculated roughly from the spectrum (within 1 min) of Fig. 1A by subtracting the spectrum of 5-methyl-1,10-phenanthroline-cobalt complex and cobalt-enzyme. The ternary complex has low absorbance in the visible region in Fig. 5.

If it is assumed that 5-methyl-1,10-phenanthroline does not cause detachment of any histidine-nitrogen of the protein, a five or six-coordinated species can be obtained through a bidentate behavior of 5-methyl-1,10-phenanthroline. The spectrum of (BCA)M-acetate shows a low molar absorbance, i.e., about one-third of that of pure cobalt-enzyme system (7), and is unusual for cobalt-enzyme with any other agents. In fact, pseudotetrahedral cobalt complexes have a larger molar absorbance irrespectively of the nature of the donor atom (7). Therefore, Bertini et al. (7,8,9) proposed that the acetate adduct would have a five coordination geometry through three donor atoms of proteic part of the enzyme, the acetate group, and a water molecule. On the other hand, the low intensity of the spectrum of the ternary complex might suggests the posibility of the

existence to some extent of six-coordinated species, whose absoptions would be so low not to be detected. The spectrum of the ternary complex have lower molar absorption coefficient than that of (BCA)M-acetate (7). The low absorbance of the ternary complex in the visible region would be interpreted by five- or six-coordinated species but there is no report on the interaction between cobalt-enzyme and bidentate ligand.

Therefore, further experiments would be necessary for elucidation of the structure of a ternary complex.

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