Coordination Chemical Studies on Metalloenzymes. VIII.\textsuperscript{1)} Reduction of Co(III)-Bovine Carbonic Anhydrase with L-Ascorbic Acid\textsuperscript{2)}

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Co(III)-bovine carbonic anhydrase was reduced by L-ascorbic acid with recovery of the enzyme activity. The rate determined by following the increase in the absorbance at 640 nm (characteristic absorption of Co(II)-bovine carbonic anhydrase) was completely consistent with that determined from the recovery of the esterase activity. This result indicates that the recovery of the enzyme activity with L-ascorbic acid was caused by the reduction of a cobalt ion in the enzyme from Co(III)-bovine carbonic anhydrase to Co(II)-bovine carbonic anhydrase by L-ascorbic acid. The rate of the reduction of Co(III)-bovine carbonic anhydrase has a first-order dependence on the concentrations of both L-ascorbic acid and Co(III)-bovine carbonic anhydrase, and the rate constant is $3.6 - 7.2 \times 10^{-1}$ sec$^{-1}$ M$^{-1}$.

Keywords—Co(III)-bovine carbonic anhydrase; L-ascorbic acid; reduction of Co(III)-enzyme; metalloenzyme; reaction mechanism; coordination chemical study

Bovine carbonic anhydrase is a metalloenzyme containing 1 gram atom of very tightly bound Zn\textsuperscript{2+} per molecule of molecular weight 30000. The zinc atom of the enzyme is essential for its enzyme activity and can be replaced by various divalent metal ions. Co(II)-bovine carbonic anhydrase has a high enzyme activity for CO$_2$ hydration and hydrolysis of p-nitrophenylacetate.\textsuperscript{4)} Recently, Shinar and Navon\textsuperscript{5)} prepared Co(III)-bovine carbonic anhydrase by treating Co(II)-bovine carbonic anhydrase with hydrogen peroxide and investigated in some detail the nature of Co(III)-bovine carbonic anhydrase.\textsuperscript{6)} On the other hand, Van Wart and Vallee tried to prepare Co(III)-carboxypeptidase A by treating Co(II)-carboxypeptidase A with hydrogen peroxide.\textsuperscript{7)} The treatment with hydrogen peroxide affected the spectrum and enzyme activity.\textsuperscript{7)} After the addition of hydrogen peroxide, the enzyme activity decreased and the visible absorption of Co(II)-carboxypeptidase A centered near 555 nm decreased.\textsuperscript{7)} On the other hand, a strong ultraviolet end-absorption gradually developed.\textsuperscript{7)} However, the treatment of Co(II)-carboxypeptidase A with hydrogen peroxide failed to yield a physicochemically homogeneous protein and the decrease in the enzyme activity depended on the modification of amino acid residues of the protein (e.g. tyrosine, tryptophan, etc.), not on the oxidation of the Co(II) ion in the enzyme.\textsuperscript{7)}

Shinar and Navon\textsuperscript{5,6)} observed a yellow color at about 400 nm in the spectrum when cobalt (II)-bovine carbonic anhydrase was oxidized by hydrogen peroxide, and proposed that the yellow color was a result of the oxidation of aromatic amino acid residues.

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\textsuperscript{2)} A part of this work was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April 1978.
\textsuperscript{3)} Location: Tamabe-dori, Mizuko-hu, Nagoya, 467, Japan.
\textsuperscript{7)} H.E. Van Wart and B.L. Vallee, Biochemistry, 17, 3385 (1978).
Therefore, it is not clear whether the loss of the enzyme activity of cobalt(II)-bovine carbonic anhydrase is dependent on the modification of an amino acid residue in its active site or on the oxidation of the cobalt ion.

In order to clarify this point, we measured the rate constant of the reduction of Co(III)-bovine carbonic anhydrase by L-ascorbic acid in terms of both the recovery of the esterase activity, and the increase of the characteristic absorption of Co(II)-bovine carbonic anhydrase at 640 nm. The rate constant, measured in terms of recovery of the esterase activity, was compared with that obtained by measuring the absorbance at 640 nm.

Materials and Methods

Enzyme—Bovine carbonic anhydrase (component B) (BCA) was prepared from bovine erythrocytes by the method of Lindskog and its purity was checked by gel electrophoresis; the zinc content was 1.00 ± 0.05 atom of zinc per molecule. Apocarbonic anhydrase was prepared by successive dialysis of native bovine carbonic anhydrase against 10⁻⁴ M 2,6-pyridinedicarboxylate in 0.2 M acetate buffer, pH 5.0, and then de-ionized water. The Co(II)-bovine carbonic anhydrase (Co(II)-BCA) was prepared by dialysis of the apoenzyme against 10⁻³ M Co(II) ions in 0.2 M acetate buffer, pH 6.0. The cobalt content was 1.00 ± 0.05 atom of cobalt per molecule.

Protein concentration was determined from the absorbance at 280 nm by taking ε = 57000 M⁻¹ cm⁻¹. Concentrations of Co(II)-BCA were determined from the absorbances at 280 nm and 550 nm, with molar absorption coefficients of 57000 and 380 M⁻¹ cm⁻¹ at pH 7.5, respectively. Co(III)-bovine carbonic anhydrase (Co(III)-BCA) was prepared by the oxidation of the corresponding Co(II)-BCA (3 x 10⁻⁴ M in 0.05 M Tris-H₂SO₄ buffer, pH 7.5) with about a 10-fold excess of hydrogen peroxide (3 x 10⁻⁵ M in 0.05 M Tris-H₂SO₄ buffer, pH 7.5). The cobalt content of Co(III)-BCA was 1.00 ± 0.1 atom of cobalt per molecule and the zinc content was smaller than 0.03 atom of zinc per molecule. The esterase activity of Co(III)-BCA was smaller than 3% of that of Co(II)-BCA.

Enzyme Activity—The esterase activity towards p-nitrophenylacetate was measured. About 10 microliters of the enzyme solution was added to 2.7 ml of Tris-HCl buffer (pH 7.5, 0.05 M) and 0.3 ml of the substrate solution (0.005 M p-nitrophenylacetate in acetonitrile) was added to this solution. The absorbance at 400 nm was measured at 25°C.

The hydration activity was measured by the method of Wilbur and Anderson. The time required for saturated CO₂ solution to lower the pH of 0.012 M veronal buffer from 8.3 to 6.5 at 0°C was determined.

Equilibrium Dialysis—The equilibrium dialysis cell (capacity 1.0 ml) was assembled with a sheet of dialysis tubing membrane and filled with 0.8 ml of the solution using a micropipet. Approximately 0.8 ml of 1.2 x 10⁻⁴ M enzyme in 0.1 M acetate buffer solution (pH 5.0) was placed in compartment a, called the enzyme chamber, and the same volume of 2,6-pyridinedicarboxylate solution (pH 5.0, 0.1 M acetate buffer) was placed in compartment b, called the ligand chamber. Dialysis was allowed to proceed for 7 days in a cold room at 4°C. At various intervals, a certain volume of the solution was removed from both the ligand and enzyme chambers. The cobalt content was measured by atomic absorption spectrophotometry. The procedure for equilibrium dialysis was described in the previous paper.

Reagents—2,6-Pyridinedicarboxylate and L-ascorbic acid were purchased from Wako Co., Osaka, Japan. The purity of L-ascorbic acid was found by iodometry to be 98 ± 2%. The concentrations of L-ascorbic acid in various solutions were measured by iodometry.

Kinetics—For the reduction of Co(III)-BCA to Co(II)-BCA, 0.5 ml of Co(III)-BCA stock solution (3 x 10⁻⁴ M) in 0.05 M Tris-H₂SO₄ buffer (pH 7.5) and 0.5 ml of L-ascorbic acid in the same buffer were mixed quickly. A 20 μl portion of this solution was taken at various intervals and was added to 3 ml of Tris-HCl buffer (pH 7.5, 0.05 M), and esterase activities were measured. In order to follow by spectrophotometry the reaction of Co(III)-BCA with L-ascorbic acid, 0.5 ml of Co(III)-BCA stock solution (1.0 x 10⁻⁵ M) in 0.05 M Tris-H₂SO₄ buffer (pH 7.5) and 0.5 ml of L-ascorbic acid (5 x 10⁻⁵—1.0 x 10⁻⁴ M) in the same buffer were mixed quickly and the change of the spectrum was measured with a Shimadzu UV-200 spectrophotometer. Experiments were performed at 20 ± 0.05°C. To prevent the oxidation of L-ascorbic acid by oxygen in air, it was dissolved immediately before each experiment and the experiments were performed under a nitrogen atmosphere.

Results

a) Equilibrium Dialysis of Co(III)-BCA against 2,6-Pyridinedicarboxylate

Figure 1 shows the time courses of the loss of cobalt ions from Co(II)-BCA and Co(III)-BCA with 2,6-pyridinedicarboxylate. In the case of Co(II)-BCA, the cobalt ion was easily removed from the enzyme but that of Co(III)-BCA was not removed even after 7 days. These findings indicate that the rate of dissociation of the cobalt (III) ion from Co(III)-BCA is very slow. The spectrum of Co(III)-BCA is shown in Fig. 2. The shape of the spectrum is consistent with that reported by Shinar and Navon, and is very similar to that of Co(III)-model complexes [(Co(III)-(NH$_4$)$_2$C$_2$O$_4$), etc.].

The slow rate of dissociation of the cobalt ion from Co(III)-BCA and the shape of the spectrum of Co(III)-BCA indicate that the cobalt ion of Co(III)-BCA prepared by the oxidation of Co(II)-BCA with hydrogen peroxide is trivalent.

b) Reduction of Co(III)-BCA with L-Ascorbic Acid

Co(III)-BCA was easily reduced by L-ascorbic acid and its esterase and hydration activities were largely recovered, as shown in Fig. 3. When a hydrogen peroxide solution was added to the solution of Co(II)-BCA, its esterase and hydration activities decreased with time and were completely lost after 10 minutes. At 85 minutes after the addition of hydrogen peroxide to the enzyme solution (indicated by the arrow in Fig. 3), a large excess of L-ascorbic acid was added to this reaction mixture. The esterase and hydration activities slowly recovered up to about 90% of the initial enzyme activities. The recovery of the hydration activity was consistent with that of the esterase activity. These results indicate that L-ascorbic acid reduces Co(III)-BCA to Co(II)-BCA.

To confirm this, the spectrum of the reaction mixture of Co(III)-BCA with L-ascorbic acid was measured at 20°, as shown in Fig. 2. In the reaction of Co(III)-BCA with L-ascorbic acid, the absorbances at 640, 618 and 550 nm, which are characteristic absorbances of Co(II)-BCA, increased with time and the absorbance at 400 nm decreased rapidly at first. After 30 minutes, the absorbances at 618 and 640 nm did not further change, but the absorbance at 400 nm still decreased gradually. As shown in Fig. 2, the intensity of the absorbance at 550 nm in the spectrum 136 min after the addition of L-ascorbic acid was slightly influenced by the absorption band at 400 nm, but the shape of the spectrum from 480 nm to 700 nm is very similar to the spectrum of Co(II)-BCA at pH 7.5. The absorption band at 640 nm was little affected by the absorption band at 400 nm, so that the change in the form of the cobalt ion due to conversion from Co(III)-BCA to Co(II)-BCA was followed in terms of the absorbance at 640 nm. The fraction which was reduced to Co(II)-BCA from Co(III)-BCA by L-ascorbic acid was calculated to be about 90%. Further, the enzyme activity of this reaction solution recovered about 90% of the initial specific activity. These results clearly indicate that L-ascorbic acid reduces Co(III)-BCA to Co(II)-BCA.
Figure 4 shows the time courses of the absorbance at 640 nm and of the increase of the esterase activity. Plots of $\log[(A_e - A_i)/(A_e - A_0)]$ vs. time were linear and plots of the logarithm of the fraction activity recovered vs. time were also linear for 70% of the course of the reaction. These results indicate that the reaction of Co(III)-BCA with L-ascorbic acid follows pseudo-first-order kinetics. The pseudo-first-order rate constant ($k_{obs}$) was derived from the slope of the line in Fig. 4. The pseudo-first-order rate constant obtained from the change of the absorbance at 640 nm was in excellent agreement with that determined from the change of esterase activity (Fig. 4). On the other hand, the yellow color at 400 nm (which may be the result of the oxidation of aromatic amino acid residues) also decreased with time (in Fig. 2) but was not completely lost. The plots of $\log (A_e/A_0)$ vs. time were not linear and the rate of change was not consistent with that of the esterase activity. The pseudo-first-order rate constants obtained from the increase of the absorbance at 640 nm and from the enzyme activity increased with increase in the concentration of L-ascorbic acid. Figure 5 shows the

![Graph showing absorbance vs. wavelength](image)

Fig. 2. Electronic Spectra at Various Periods during the Reduction of Co(III)-BCA by L-Ascorbic Acid

For the reduction of Co(III)-BCA, 0.5 ml of Co(III)-BCA (8.0 x 10^-5 M) in 0.05 M Tris-H$_2$SO$_4$ buffer (pH 7.5) and 0.5 ml of L-ascorbic acid (5.0 x 10^-5 M) in the same buffer were mixed quickly. At various times, the spectrum of this solution was measured with maximum scanning rate (480 nm/min). All experiments were performed at 30°C, Co(III)-BCA; b, 4 min; c, 7 min; d, 21 min; e, 70 min; f, 130 min. Inset, the spectrum of Co(II)-BCA (4.0 x 10^-5 M) at pH 7.5.
Fig. 3. Hydrogen Peroxide Inactivation of Co(II)-BCA and Reactivation of Co(III)-BCA by L-Ascorbic Acid

Activities are expressed as percentages of the unmodified controls. Co(II)-BCA, 1.4 × 10^{-4} M in 0.05 M Tris-H$_2$SO$_4$ buffer, pH 7.5, was treated with 2.0 × 10^{-4} M hydrogen peroxide at 20°. Aliquots (20 µl) were removed at the indicated times and their hydration and esterase activities were measured. The arrow indicates the addition of L-ascorbic acid. L-Ascorbic acid solution (4.0 × 10^{-4} M, 0.05 M Tris-H$_2$SO$_4$ buffer, pH 7.5) of the same volume of the reaction mixture was added to the reaction mixture. Aliquots (40 µl) were removed and their hydration and esterase activities were measured.

•, esterase activity; ○, hydration activity.

Fig. 4. Time Course of the Reduction of Co(III)-BCA to Co(II)-BCA by L-Ascorbic Acid

The conditions of spectral determination were the same as in Fig. 2. To follow the recovery of the enzyme activity, 0.5 ml of Co(III)-BCA (8.0 × 10^{-4} M) in 0.05 M Tris-H$_2$SO$_4$ buffer (pH 7.5) and 0.5 ml of L-ascorbic acid (5.0 × 10^{-4} M) in the same buffer were mixed quickly and 10 µl aliquots of this solution were removed at various times and assayed in 0.05 M Tris-HCl buffer.

•, $A_{540} - A_{400}$ at 640 nm; ○, $A_{540} - A_{400}$ at 400 nm; ○, enzyme activity.

relationship between the pseudo-first-order rate constant ($k_{obs}$) and the concentration of l-ascorbic acid, and also shows that there is a linear relation between the concentration of l-ascorbic acid and the pseudo-first-order rate constant ($k_{obs}$). This result indicates that the reaction is first-order with respect to the concentration of l-ascorbic acid. When the concentration of l-ascorbic acid was varied, $k_{obs}$ obtained from the esterase activity was completely consistent with that obtained from the change of the absorbance at 640 nm.

The effect of the concentration of Co(III)-BCA on the rate of the reaction was tested with 2.5 × 10^{-3} M l-ascorbic acid. The pseudo-first-order rate constant ($k_{obs}$) of the reaction was found to be constant at various concentrations of Co(III)-BCA (1.1 × 10^{-4}—4.9 × 10^{-4} M).

Fig. 5. Effect of the Concentration of L-Ascorbic Acid on the Pseudo-first-order Rate Constant ($k_{obs}$)

All experiments were performed in 0.05 M Tris-H$_2$SO$_4$ buffer (pH 7.5) at 20°. $k_{obs}$, the pseudo-first-order rate constant determined from the change of absorbance at 640 nm; ○, the pseudo-first-order rate constant ($k_{obs}$) obtained from the esterase activity.

Thus, the pseudo-first-order rate constant was independent of Co(III)-BCA concentration over approximately a 4-fold range, indicating that the reaction was first-order with respect to protein concentration. These results indicate that the rate of the reduction from Co(III)-BCA to Co(II)-BCA by L-ascorbic acid is an overall second-order reaction, and its rate may be defined as:

$$\frac{d[\text{Co(II)-BCA}]}{dt} = k_{\text{app}}[L_0][\text{Co(III)-BCA}]$$ (1)

where $[L_0]$ is the initial concentration of L-ascorbic acid and $k_{\text{app}}$ is the apparent first-order rate constant.

L-Ascorbic acid has two protons which can dissociate, and their dissociation constants are $9.16 \times 10^{-5} \text{M}$ and $4.6 \times 10^{-12} \text{M}$. This behavior is shown in Chart 1. To clarify the species of L-ascorbic acid responsible for the reduction, the pH dependence of the rate constant was studied. Figure 6A shows the relationship between the apparent first-order rate constant ($k_{\text{app}}$) and pH from 4.5 to 8.0. The rate of the reduction of Co(III)-BCA was found to be independent of pH from 4.5 to 8.0. Figure 6B shows the effect of changes of ionic strength in the presence of neutral salts, such as sodium chloride. This result shows that the apparent first-order rate constant is independent of the addition of neutral salts.

**Discussion**

Co(III)-BCA was reduced by L-ascorbic acid and recovered its enzyme activity. The rate constant obtained by following the increase in the absorbance at 640 nm (characteristic absorption of Co(II)-BCA) was completely consistent with that determined from changes of the esterase activity. This result clearly indicates that the recovery of the enzyme activity with L-ascorbic acid may be caused by reduction of the cobalt ion in the enzyme from Co(III)-BCA to Co(II)-BCA by L-ascorbic acid.

On the other hand, the yellow color at 400 nm (the result of the oxidation of some amino acids) also decreased with time during reduction by L-ascorbic acid. This result implies that some of these amino acid residues are reduced by L-ascorbic acid. If the amino acid residues that are oxidized by hydrogen peroxide in the active site are reduced by L-ascorbic acid, the rate of reduction of the amino acid residues should be much larger than that of the reduction of the cobalt (III) ion in the enzyme. It is possible that the essential amino acid residue for enzyme activity is quickly reduced by L-ascorbic acid and that the recovery of the enzyme activity depends on subsequent slow reduction of the cobalt (III) ion of the enzyme. Therefore, it cannot be definitely concluded that the decrease of the absorbance at 400 nm is independent
of the recovery of the enzyme activity. However, the oxidation of a cobalt ion from Co(II)-BCA to Co(III)-BCA by hydrogen peroxide is very probably the main reaction that leads to the loss of enzyme activity.

The reduction of Co(III)-BCA by L-ascorbic acid has a first-order dependence on the concentrations of both L-ascorbic acid and Co(III)-BCA, as shown in Eq. (1). The rate of the reduction was independent of pH from pH 4.5 to 8.5. The chemical species of L-ascorbic acid are illustrated in Chart 1; the main species from pH 4.5 to 8.0 is BH⁻, because the acid dissociation constants of BH₂ are 9.16×10⁻⁵ and 4.6×10⁻¹⁸ M.¹⁰ Since the reduction rate of Co(III)-BCA was found to be pH-independent, the reaction species of L-ascorbic acid must be BH⁻. If either BH₂ or BH₃⁻ is a reaction species, the rate constant of the reduction would be pH-dependent. Therefore, the following equation is proposed for the reduction mechanism of Co(III)-BCA.

\[ \text{BH}⁻ + \text{Co(III)-BCA} \xrightarrow{k} \text{BH}⁻ + \text{Co(II)-BCA} \]  
\[ \frac{d[\text{Co(II)-BCA}]}{dt} = k[\text{BH}⁻][\text{Co(III)-BCA}] \]  

On the basis of the reaction of L-ascorbic acid with ferricytochrome C¹⁰ of ferriyanide,¹¹ free radical species of L-ascorbic acid would react with Co(III)-BCA at a very high rate. However, the protein moiety of Co(III)-BCA may react with the free radical species. Even so, since the rate-determining step is the reaction of Eq. 2, its rate constant must be 3.6—7.2×10⁻¹ sec⁻¹ m⁻¹. This value is very similar to that obtained when L-ascorbic acid reduces cytochrome C-552.¹⁰

In general, it is known that various anions (CN⁻, OCN⁻, Cl⁻, etc.) and uncharged complexing agents (aniline, 5-methyl-1,10-phenanthroline, etc.) can bind to Zn(II)- or Co(II)-BCA and that the positive charge of the -NH₄⁺ group of L-alanine decreases the binding constant between the -COO⁻ group of L-alanine and the enzyme.¹²⁻¹⁵ On the basis of these observations, it is proposed that Zn(II)- or Co(II)-BCA may have a positively charged active site.¹⁵ In order to determine the charge of the active site, the dependence of the rate constant of the reduction on ionic strength was determined. In the reaction of cytochrome C with various metal complexes,¹⁶⁻¹⁷ the apparent charge of the active site was obtained from the following relationship between log k and μ¹/².

\[ \log k = \log k_0 + 1.04 Z_A Z_B \sqrt{\mu} \]

where Zₐ and Zₛ are the charges of species.¹¹⁻¹⁸ As shown in Fig. 6B, the rate of the reduction of Co(III)-BCA by L-ascorbic acid is independent of ionic strength. Therefore, our result indicates that the apparent charge of the active site of Co(III)-BCA is almost neutral, because the slope of Fig. 6B is nearly zero. The value of log \(k_{app}\) shows some scatter, so that a clear result could not be obtained from the relationship between log \(k_{app}\) and \(\mu^{1/2}\).

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