Coordination Chemical Studies on Metalloenzymes. IV. Kinetic and Thermodynamic Studies of Metal Removal Reaction from Zinc- and Cobalt-Carbonic Anhydrase with Chelating Agents

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The mechanism and rate of cobalt removal reaction from cobalt-enzyme with various chelating agents were investigated, in comparing with that of zinc-enzyme. The mechanism of cobalt removal reaction was the same as that of zinc-enzyme and the selectivity of chelating agents against zinc-enzyme and cobalt-enzyme was almost the same. The second-order rate constant of cobalt removal from cobalt-enzyme with various chelating agents \( k_{app} \) was much larger (13—30 times) than that of zinc-enzyme. The thermodynamic parameter of metal removal reaction was measured by the reaction of 5-methyl-1,10-phenanthroline with cobalt- and zinc-enzyme. Activation enthalpy for breakdown of the ternary complex of cobalt- and zinc-enzyme was almost the same (28—29 kcal mol\(^{-1}\)) but activation entropy (33 cal deg\(^{-1}\) mol\(^{-1}\)) for breakdown of the ternary complex of cobalt-enzyme was much larger than that (22 cal deg\(^{-1}\) mol\(^{-1}\)) of zinc-enzyme. This is the reason why \( k_{app} \) of the cobalt-enzyme is much larger than that of zinc enzyme.

Keywords—bovine carbonic anhydrase; metalloenzyme; kinetics of metal removal reaction; thermodynamics of metal removal reaction; the inhibition mechanism of chelating agents

Bovine carbonic anhydrase (carbonate hydrolyase, EC. 4.2.1.1)(BCA) is a metalloenzyme containing 1 gram atom of very tightly bound Zn(II) per molecule. Zinc ions can be removed from carbonic anhydrase by dialysis against 1,10-phenanthroline at low pH. Enzyme activity can be restored by the addition of zinc ions to the apoenzyme. Various bivalent metal ions added to apoenzyme form metallo derivatives which, apart from the cobalt(II) form, have zero or near zero activity. The inhibition of bovine carbonic anhydrase by various metal chelating agents and the interaction of apo-bovine carbonic anhydrase with various metal complexes were investigated.

In the previous paper, we reported that the mechanism for the removal of zinc ions from bovine carbonic anhydrase with various chelating agents consisted of the following two reaction pathways:

\[
\begin{align*}
(\text{BCA})M & \xrightleftharpoons{k_{EML}} (\text{BCA})ML & k_2 & \rightarrow \text{apo}-\text{BCA} + LM \\
& \xrightarrow{k_d} \text{apo}-\text{BCA} + M & \text{fast} & (n-1)L \\
& & \xrightarrow{+nL} \text{apo}-\text{BCA} + L_n M
\end{align*}
\]

Chart 1

2) Location: Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467, Japan.
where (BCA)M is a metalloenzyme. L is a chelating agent, (BCA)ML, a ternary complex involving an enzyme, a chelating agent, and a metal ion, apo-BCA, the apoenzyme, LₙM, a coordination compound, and $K_{EML}$ is the equilibrium constant between (BCA)M+L and (BCA)ML. The presence of ternary complex in Chart 1 was also determined by the spectrophotometric method. The reaction mechanism of zinc removal with chelating agents from the native enzyme was governed by the structure of chelating agents, and the rate of zinc removal from the enzyme was governed by the steric factor and stability constants of a chelating agent. These phenomena would be interpreted by the steric hindrance protein, because the zinc binding site is located at the bottom of a crevice of the enzyme. Therefore, the reaction mechanism and the rate of metal removal from the enzyme reflect the physical characteristics of the cleft leading to the active site.

When the zinc ion in carbonic anhydrase is replaced by a cobalt ion, the activity of CO₂ hydration of cobalt-enzyme is one-half of zinc-enzyme. However high resolution difference electron density maps show no significant difference in the metal binding sites of the Zn and Co solid forms of human carbonic anhydrase.

In the present work, the reaction mechanism, the rate and the thermodynamic parameters were determined for cobalt removal from cobalt-enzyme with various chelating agents. These results were compared with those of zinc-enzyme, and the similarity and difference of behavior of chelating agents against cobalt- and zinc-enzyme are discussed.

### Experimental

**Enzyme**—Bovine carbonic anhydrase (component B) 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. Enzyme concentration was determined from the absorbance at 280 nm, using a molar absorptivity of $5.7 \times 10^4$ M⁻¹ cm⁻¹.

**Cobalt-Enzyme**—Apoenzyme was prepared by successive dialysis of BCA against $10^{-2}$ M of 2,6-pyridinedicarboxylate (2,6-PA) in 0.2 M acetate buffer (pH 5.0) and then water. The cobalt-enzyme was prepared by dialysis of the apoenzyme against $10^{-3}$ M Co⁺ solution in 0.2 M acetate buffer (pH 6.0). The cobalt content was 1.00 ± 0.05 atom of cobalt per molecule.

**Reagents**—2,6-PA, 1,10-phenanthroline (OP), and 2,2'-bipyridine (Bipy) were purchased from Wako Pure Chemical Ind., Osaka; they were used without further purification. 2,3-, 2,4-Pyridinedicarboxylate (2,3-, 2,4-PA), 2-pyridinedicarboxylate (2-PA), and 5-methyl-1,10-phenanthroline (5-Me-OP) were purchased from Tokyo Kasei Kogyo Co., Tokyo. 2,3-Pyridinedicarboxylate and 5-Me-OP were recrystallized from methanol and cyclohexane, respectively. Other reagents were used without further purification. A buffer solution was passed through an ion-exchange column (Dowex A-I, Na type) in advance to remove traces of contaminating metal ions.

**Chelating Agents**—Chelating agents used were dissolved in a buffer solution and the pH was adjusted to 5.0 with NaOH solution. The concentration of non-protonated species of the chelating agents was calculated according to Eq. (1),

$$[L] = [L_0]/(1+K\cdot[H^{+}]^n)$$

\[ [L] = [L_0]/(1+K\cdot[H^{+}]^n) \]

\[ \begin{array}{ccc}
\text{2,3-Pyridinedicarboxylic acid} & \text{2,4-Pyridinedicarboxylic acid} \\
\log K_1 & \log K_2 & \log K_1 & \log K_2 \\
5.2 & 4.0 & 5.5 & 4.4 \\
\end{array} \]

<table>
<thead>
<tr>
<th></th>
<th>2,3-Pyridinedicarboxylic acid</th>
<th>2,4-Pyridinedicarboxylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log $K_1$</td>
<td>log $K_2$</td>
</tr>
<tr>
<td>Co⁺</td>
<td>5.2</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>4.4</td>
</tr>
</tbody>
</table>

where [L]₀ is the concentration of a metal-free chelating agent and K₅ is the acid dissociation constant.²⁹ The acid pK₅ values of chelating agents were reported in our previous paper.³⁰

Stability constant of 2,3- and 2,4-PA chelates with cobalt ions were determined by the pH titration method of Yasuda and Yamashita.¹⁰ In the presence of 0.1 M KNO₃, pH titration was carried out at 25° in the higher pH region than 3.5, because the dianion of 2,3- and 2,4-PA take part mainly in the complex formation with cobalt ions.¹⁰) The stability constants of 2,3- and 2,4-PA with cobalt are given in Table 1.

**Kinetics**—Equilibrium between chelating agents and apo-BCA for cobalt ion is given by the following equation:

$$\text{(BCA)Co} + nL \rightleftharpoons \text{apo-BCA} + \text{CoL}_n$$ (2)

The equation tends to the right when concentration of the chelating agent is large enough to remove cobalt ions from BCA completely, so that kinetic experiments were carried out under these conditions.³⁰) In the removal of cobalt ions from cobalt-enzyme, rate of the reaction was measured by following the decrease of esterase activity of the cobalt-enzyme. Kinetic experiments were performed in 0.2 M acetate buffer (pH 5.0). A constant ionic strength was maintained at 0.33 with NaCl as a compensation electrolyte and kinetics was studied at 0° except for the experiments on the temperature effect. Detailed procedure was described in a previous paper.³⁰)

The rate of removal of cobalt ions from the cobalt-enzyme with a chelating agent is measured in a sufficient excess of the chelating agent over BCA, so that plots of the logarithm of the fractional residual activity vs. time give a straight line.

$$\log \left[ \frac{[\text{(BCA)Co}]_0 - [\text{apo-BCA}]}{[\text{(BCA)Co}]_0} \right] = -A \cdot t$$ (3)

where $[\text{(BCA)Co}]_0$ is the initial enzyme concentration and $[\text{apo-BCA}]$ is the apoenzyme concentration. The pseudo-first-order rate constant ($A$) was derived from the slope of the line. The change of $A$ with concentration of chelating agent was determined.

**Results**

1. **Kinetic Reaction of Various Chelating Agents with Cobalt Carbonic Anhydrase**

   a) **PA-type Chelating Agents**—Relationship between the pseudo-first-order rate constant ($A$) and concentration of free chelating agents is shown in Fig. 1. In the relation between $A$ and 2,6-PA concentration, the hyperbolic function shown in the case of zinc-enzyme³⁰) was not obtained in cobalt-enzyme. The relationship between $A$ and 2,6-PA concentration was linear and this behavior was the same in the removal of zinc ions from native enzyme by 2,4-, 2,5-, and 2-PA. In the cobalt removal reaction of 2,4-, 2-, and 2,3-PA, the correlation between $A$ and $[L]$ was linear (cf. Fig. 1) and this behavior is the same as that of zinc removal reaction described in a previous paper.³⁰) Therefore, the following mechanism will be given.

$$\text{(BCA)Co} + L \rightleftharpoons \text{(BCA)CoL} \rightarrow \text{apo-BCA} + \text{LCo}$$

$$+(n-1)L \rightarrow \text{apo-BCA} + \text{L}_n\text{Co}$$

$$K_{EML} = \frac{[\text{(BCA)CoL}]}{[\text{(BCA)Co}] [L]}$$ (5)

$K_{EML}$ is very small because the correlation between $A$ and $[L]$ of 2,3-, 2,4-, 2,6-, and 2-PA is linear (Fig. 1). Therefore, the pseudo-first-order rate constant ($A$) is given by

$$A = \frac{k_{\text{app}} [L]}{2.303}$$ (6)

$$k_{\text{app}} = K_{EML}k_3$$ (7)

The apparent second-order rate constant ($k_{\text{app}}$) was determined from the slope of the lines in Fig. 1 and is given in Table II.

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In the zinc removal reaction from zinc-enzyme, the relationship between A and free 2,6-PA concentration (0.6×10^{-3} - 3.3×10^{-3} M) showed a hyperbolic function. The hyperbolic function was not observed in the cobalt removal reaction and the relationship between A and 2,6-PA concentration (0.6×10^{-3} - 3.3×10^{-3} M) was linear function (Fig. 1), so that $K_{EML}$ values of cobalt-enzyme would be much smaller than that (2.3×10^{3} M^{-1}) of zinc-enzyme.

With PA-type chelating agents, $k_{app}$ decreased in the order of 2-PA, 2,6-PA, 2,4-PA, and 2,3-PA.

**b) OP-type Chelating Agents** — The behavior of OP and Bipy in the removal of cobalt ions from the cabalt-enzyme was similar to that of PA-type chelating agents. The relationship between the pseudo-first-order rate constant ($A$) and the concentration of chelating agents is linear in Fig. 2A. Therefore, OP and Bipy removed cobalt ions from cobalt-enzyme by the same mechanism (Eq. 4) as that of PA-type chelating agents. Apparent second-order rate constants ($k_{app}$) were determined from Eq. (6) and these values are given in Table II. In the zinc removal reaction from zinc-enzyme, the relationship between $A$ and free OP concentration (5×10^{-4} - 5×10^{-3} M) showed a hyperbolic function. The hyperbolic function was not observed in the cobalt removal reaction and the relationship between $A$ and OP concentration (5×10^{-4} - 2.5×10^{-3} M) was linear function (Fig. 2A), so that $K_{EML}$ value of cobalt-enzyme would be much smaller than that (3.8×10^{3} M^{-1}) of zinc-enzyme.

**Fig. 1.** Effect of the Concentration of PA-type Chelating Agent on the Pseudo-first-order Rate Constant ($A$), in a Solution initially Containing 1.2×10^{-4} M Cobalt-enzyme at pH 5.0

O, 2-PA; ●, 2,6-PA; ●, 2,4-PA; ○, 2,3-PA.

**Fig. 2.** Effect on the Concentration of OP-type Chelating Agents on the Pseudo-first-order Rate Constant ($A$)

2A: relationship between the pseudo-first-order rate constant ($A$) and the concentration of chelating agent ([L]). The enzyme concentration was 1.2×10^{-4} M. ○, OP; ●, Bipy.

2B: reciprocal of the pseudo-first-order rate constant ($A$) vs. reciprocal of free 5-Me-OP concentration. The enzyme concentration was 1.2×10^{-4} M.
The behavior of 5-Me-OP in the removal of cobalt ions from cobalt-enzyme was the same as that of zinc removal with 5-Me-OP from native enzyme. Figure 2B shows a linear relationship between $1/A$ and $1/[L]$. In this case, concentration of the ternary complex

**Table II. Rate Constants of the Removal of a Metal Ion from Metalloenzyme and Stability Constants of Chelating Agents**

<table>
<thead>
<tr>
<th>Cobalt-enzyme</th>
<th>Zinc-enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{EM\text{a}}$ (m$^{-1}$)</td>
<td>$k_2$ (sec$^{-1}$)</td>
</tr>
<tr>
<td>PA-type</td>
<td></td>
</tr>
<tr>
<td>2,6-PA</td>
<td>$&lt;2.3 \times 10^2$</td>
</tr>
<tr>
<td>2-PA</td>
<td>—</td>
</tr>
<tr>
<td>2,4-PA</td>
<td>—</td>
</tr>
<tr>
<td>2,3-PA</td>
<td>—</td>
</tr>
<tr>
<td>OP-type</td>
<td></td>
</tr>
<tr>
<td>OP</td>
<td>$&lt;3.8 \times 10^2$</td>
</tr>
<tr>
<td>5-Me-OP</td>
<td>3.9 $\times 10^2$</td>
</tr>
<tr>
<td>Bipy</td>
<td>—</td>
</tr>
</tbody>
</table>

Kinetic experiments were performed at 0°C in 0.2 acetate buffer (pH 5.0). A constant ionic strength of 0.3 was maintained with NaCl as a compensating electrolyte.

a) log $K_1$ values are cited from Sillén and Martell, except for stability constants of 2,3- and 2,4-PA complexes with cobalt ion.

b) These values are cited from our previous paper.

($[(\text{BCA})\text{CoL}]$) is considered to be present in an appreciable amount. Then, the relationship between $1/A$ and $1/[L]$ will be given by:

$$
\frac{1}{A} = \frac{2.303}{k_2 K_{EM}[L]} + \frac{2.303}{k_2} \tag{8}
$$

$K_{EM}$ and $k_2$ were determined from the slope and intercept of the line in Fig. 2B and are given in Table II. With OP-type chelating agents, $k_{app}$ decreased in the order of 5-Me-OP, OP, and Bipy.

2. Comparison of the Rate Constant of Cobalt Removal with That of Zinc Removal from Metalloenzymes

Rate constants of the removal of zinc and cobalt from metalloenzymes and stability constants of chelating agents are given in Table II. In Table II, $k_{app(C0)}/k_{app(Z0)}$ ratios are given to compare the rate constant of zinc removal with that of cobalt removal from metallocarboxylic anhydride. These ratios ($k_{app(C0)}/k_{app(Z0)}$) of various chelating agents were within the range of 13 to 30, except for that of 2,6-PA. This is illustrated in Fig. 3 by a plot of logarithm of the second-order rate constant of cobalt-enzyme against logarithm of the rate constant of zinc-enzyme. In Fig. 3, the relationship between log $k_{app(C0)}$ and log $k_{app(Z0)}$ gives a straight line.

with a slope equal to approximately 1, while the value of 2,6-PA deviated from this line. This phenomenon indicated that relative values of apparent second-order rate constant of PA- and OP-type chelating agents in cobalt-enzyme is the same as that of zinc-enzyme except for that of 2,6-PA, and that cobalt-enzyme has the same selectivity as the zinc-enzyme.

$K_{EML}$ values of cobalt-enzyme were smaller than those of zinc-enzyme in 2,6-PA, OP, and 5-Me-OP but the second-order rate constants ($k_{app}$) of cobalt-enzyme were much larger than that of zinc-enzyme. Therefore, $k_2$ of cobalt-enzyme may be larger than that of zinc-enzyme.

As an example, in 5-Me-OP, $K_{EML}$ of cobalt-enzyme was little smaller than that of zinc-enzyme, but $k_2$ of cobalt-enzyme was much larger than that of zinc-enzyme. Therefore, $k_{app(Co)}$ would be larger than $k_{app(Zn)}$. In order to known the reason why $k_2$ of cobalt-enzyme is much larger than $k_2$ of zinc-enzyme, thermodynamic parameter of rate ($k_2$) and $K_{EML}$ was determined with 5-Me-OP.

3. Effect of Temperature

In the reaction of 5-Me-OP against zinc- and cobalt-enzymes, temperature dependence of $K_{EML}$ and $k_2$ was examined.

The logarithm of the equilibrium constant ($K_{EML}$) of ternary complex is plotted against $1/T$ ($^\circ K$) in Fig. 4A and a linear correlation is given for cobalt-enzyme and zinc-enzyme. $\Delta H^\circ$ values are calculated from Van’t Hoff equation and $\Delta S^\circ$ values are calculated from $\Delta H^\circ$ and $K_{EML}$. These values are shown in Table III. $\Delta H^\circ$ and $\Delta S^\circ$ to form a ternary complex have almost similar values in both cobalt-enzyme and zinc-enzyme.

![Diagram](image)

**Fig. 4.** Effect on Temperature

4A) effect on temperature on the stability constant of ternary complex ($K_{EML}$): 0.2 m acetate buffer ($\mu=0.33$), pH 5.0. ○, (BCA)Zn; ●, (BCA)Co.
4B) Arrhenius plots for the dissociation rate of the ternary complex ($k_2$): 0.2 m acetate buffer ($\mu=0.33$), pH 5.0. The enzyme concentration was $1.2 \times 10^{-4}$ m. ○, (BCA)Zn; ●, (BCA)Co.

Arrhenius plots of $k_2$ were linear in Fig. 4B, and activation enthalpy for the break-down of the intermediate complex is calculated from the slope of the line. Then activation entropy ($\Delta S^\circ$) is calculated from the frequency factor. These values are given in Table III with thermodynamic parameter of the model substances.

Table III. Thermodynamic Parameters for Carbonic Anhydrase and Small Chelating Agents at 25°C

<table>
<thead>
<tr>
<th>Ion</th>
<th>Chelating agents</th>
<th>$\Delta H^{e}$</th>
<th>$\Delta S^{e}$</th>
<th>log $K^{e}$</th>
<th>$\Delta H^{d}$</th>
<th>$\Delta S^{d}$</th>
<th>log $k_{app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn$^{2+}$</td>
<td>OP</td>
<td>-7.5$^{(a)}$</td>
<td>4.4$^{(d)}$</td>
<td>6.55$^{(d)}$</td>
<td>11.7$^{(b)}$</td>
<td>-16$^{(b)}$</td>
<td>0.6$^{(b)}$</td>
</tr>
<tr>
<td></td>
<td>Bipy</td>
<td></td>
<td></td>
<td></td>
<td>11.5$^{(b)}$</td>
<td>-14$^{(b)}$</td>
<td>1.2$^{(b)}$</td>
</tr>
<tr>
<td></td>
<td>2,2',2''-Terpyridine</td>
<td></td>
<td></td>
<td></td>
<td>17.7$^{(b)}$</td>
<td>+1$^{(b)}$</td>
<td>0.1$^{(b)}$</td>
</tr>
<tr>
<td>[(BCA)Zn]</td>
<td>5-Me-OP</td>
<td>~-9</td>
<td>~-19</td>
<td>2.5</td>
<td>28.3</td>
<td>+22</td>
<td>-3.0</td>
</tr>
<tr>
<td>Zn(5-Me-OP)$^{2+}$</td>
<td>apo-BCA</td>
<td></td>
<td></td>
<td></td>
<td>18.8$^{(b)}$</td>
<td>-4$^{(b)}$</td>
<td>-1.8$^{(b)}$</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>OP</td>
<td>-9.1$^{(b)}$</td>
<td>2.1$^{(b)}$</td>
<td>7.3$^{(b)}$</td>
<td>19.6$^{(b)}$</td>
<td>-11$^{(b)}$</td>
<td>-4.0$^{(b)}$</td>
</tr>
<tr>
<td></td>
<td>2,2',2''-Terpyridine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[(BCA)Co]</td>
<td>5-Me-OP</td>
<td>~-8</td>
<td>~-17</td>
<td>2.2</td>
<td>29.0</td>
<td>+33</td>
<td>-1.3</td>
</tr>
<tr>
<td>Co(5-Me-OP)$^{2+}$</td>
<td>apo-BCA</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a) Enthalpy of complex formation or ternary complex formation, kcal mol$^{-1}$.
b) Entropy of complex formation or ternary complex formation, cal deg$^{-1}$ mol$^{-1}$.
c) Formation constant complex and ternary complex.
d) Entropy of activation for the ternary complex dissociation or complex dissociation, kcal mol$^{-1}$.
e) Entropy of activation for the ternary complex dissociation or complex dissociation, cal deg$^{-1}$ mol$^{-1}$.
f) First-order rate constant for dissociation of the ternary complex or the complex, sec$^{-1}$.
g) Ref. 19.
h) Ref. 20, 21.

Discussion

In Fig. 5, the logarithm of the second-order rate constants (log $k_{app}$) of zinc-enzyme and cobalt-enzyme is plotted against the logarithm of stability constants of the chelating agent. Among the same type of chelating agents, a linear correlation between log $K_{1}$ and log $k_{app}$ was established and this behavior is the same as that of zinc-enzyme, but zinc-enzyme and cobalt-enzyme gave different lines for the same type of chelating agents. Among the same type chelating agents, except for 2,6-PA, the rate of the removal of cobalt ions is governed by the log $K_{1}$, that is, the value of the affinity for free metal ions, and this observation is the same as that of zinc-enzyme. Therefore, $k_{app}$ is governed both by steric hindrance of protein (physical characteristics of the cleft to the active site) and stability constant of a chelating agent against cobalt ions.

In OP, 5-Me-OP, 2,6-PA, 2-PA, 2,3-PA, 2,4-PA, and Bipy, the reaction path is found to be through the ternary complex (bimolecular reaction). EDTA removed cobalt ions only by unimolecular reaction of spontaneous dissociation, and nitritotriacetate and trans-cyclohexanediaminetetraacetate removed cobalt ions according to uni- and bi-molecular reactions.15 All of the chelating agents used in the present work removed cobalt ions from the enzyme according to the reaction mechanism shown in Eq. 4. This behavior is the same as that of zinc-enzyme.

In OP-type and PA-type chelating agents, except 2,6-PA, log $k_{app}(Zn)$ vs. log $k_{app}(Co)$ shows a straight line with a slope of 1 (Fig. 3), so that the selectivity of chelating agents

against zinc-enzyme and cobalt-enzyme may be almost the same. The same selectivity of enzymes suggested that the physical characteristics of the cleft leading to active site are approximately the same but the environmental structure of metal site would be very slightly different between cobalt-enzyme and zinc-enzyme, because the value of $k_{\text{app(co)}}/k_{\text{app(zn)}}$ of 2,6-PA is much smaller than that of other PA-type chelating agents, and trans-cyclohexanediaminetetraacetate and nitrilotriacetate can form a ternary complex.

In Table III, $K_{\text{EML}}$ values of zinc-enzyme and cobalt-enzyme are $10^{2.5}$ and $10^{2.4}$, respectively. These are much smaller than those of the stability constant (log $K_1$) between metal ions and 1,10-phenanthroline in aqueous system. In order to know this reason, thermodynamic parameters of $K_{\text{EML}}$ were determined. In the formation of ternary complexes of zinc-enzyme and cobalt-enzyme with 5-Me-OP, the values of $\Delta H^\circ$ and $\Delta S^\circ$ were almost the same. In comparison of thermodynamic parameters of ternary complex with those of 1,10-phenanthroline metal complexes in aqueous system, $\Delta H^\circ$ of the ternary complex was almost the same as that of 1,10-phenanthroline, but $\Delta S^\circ$ was a large negative entropy value (positive entropy for 1,10-phenanthroline metal complexes in aqueous system). The large enthalpy ($\Delta H^\circ$) drives the reaction which is opposed by a large negative entropy change. This is the reason why $K_{\text{EML}}$ of zinc-enzyme and cobalt enzyme are much smaller than the stability constants of 1,10-phenanthroline metal complexes. The relatively large net enthalpy ($\Delta H^\circ$) is more in character with a complex stabilized through a dominant ligand-metal bond rather than hydrophobic interactions. However, we could not interpret the large negative entropy value.

The cobalt removal rate ($k_{\text{app(co)}}$) with chelating agents is much larger than that of zinc. The binding constant between zinc ions and apo-BCA is about $10^4$ larger than that of cobalt ions. Therefore, it is to be expected that the activation enthalpy for dissociation of the ternary complex of zinc-enzyme [(BCA)ML $k_a$,apo-BCA+LM] was much larger than that of cobalt-enzyme, so that thermodynamic parameters of the rate constant($k_a$) were determined for 5-Me-OP.

However, activation enthalpy of cobalt-enzyme and zinc-enzyme is almost the same, and activation entropy of cobalt-enzyme is much larger than that of zinc-enzyme. This is the reason why $k_a$ of cobalt-enzyme is much larger than that of the zinc-enzyme. In Table III, chelate dissociation of small ligands (bidentate and terdentate) is characterized by low activation enthalpy and negative activation entropy, whereas the dissociation reaction of ternary complex to apo-BCA and metal complex has a large activation enthalpy (28—29 kcal mol$^{-1}$) which is partially compensated by fairly large positive activation entropy. This behavior would be interpreted that the transition state of dissociation of ternary complex gives a little conformational change of protein, because ultraviolet difference spectra of BCA characteristic of perturbation of the environment of tryptophan and tyrosine residues have been observed between BCA and apo-BCA.

Therefore, the difference of activation entropy between cobalt-enzyme and zinc-enzyme depends on the conformational change of the transition state. This behavior was shown in the reaction of amino acids with alkaline phosphatase and of a zinc ion with apo-BCA, so that these large activation entropy and enthalpy may be common property in the protein reaction.

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