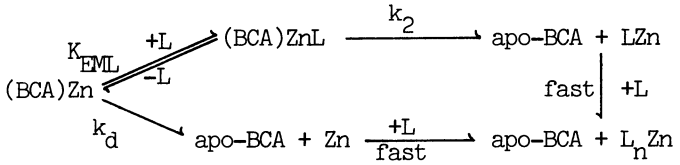
CHEMISTRY LETTERS, pp. 475-478, 1977. Published by the Chemical Society of Japan

DETERMINATION OF THE APPARENT BINDING CONSTANT OF COBALT CARBONIC ANHYDRASE B BY THE KINETIC METHOD[1)]

Yoshinori KIDANI and Junzo HIROSE
Faculty of Pharmaceutical Sciences, Nagoya City University,
Tanabe-dori, Mizuho-ku, Nagoya 467

The second order rate constant (k_f) for the formation of Co(II) complex of bovine carbonic anhydrase and the first order rate constant (k_d) for dissociation of the cobalt-carbonic anhydrase into apoenzyme and Co(II) ion were measured at 0° (pH 5.0) by a catalytic assay method involving p-nitrophenyl acetate as a substrate. The first order rate constant (k_d) has been determined by use of a chelating agent as a scavenging agent for apoenzyme. Combination of k_f with k_d gives apparent binding constant (K_{bind}) for cobalt-carbonic anhydrase. This value ($K_{bind}=k_f/k_d$) was consistent with that obtained by the equilibrium dialysis.

We reported that the mechanism for the removal of zinc ion from bovine carbonic anhydrase[2)] (BCA) with chelating agents consisted of two following reaction pathways.[3)]



Scheme (1)

where (BCA)Zn is the intact native enzyme, L is a chelating agent, (BCA)ZnL, a ternary complex involving enzyme, a chelating agent, and a metal ion, apo-BCA, the apoenzyme, L_nZn, a coordination compound, and K_{EML} is the equilibrium constant between (BCA)Zn + L and (BCA)ZnL. In the reaction of zinc removal from the native enzyme with 1,10-phenanthroline, 5-methyl-1,10-phenanthroline, and 2,6-, 2,3-, 2,4-pyridinedicarboxylic acid, and 2-pyridinecarboxylic acid, the reaction path was found to be through the ternary complex (bimolecular substitution reaction) and 2,2'-bipyridine gave two path ways (unimolecular dissociation and bimolecular substitution reactions).[3)] In the reaction with EDTA, *trans*-1,2-cyclohexanediaminetetraacetic acid and nitrilotriacetic acid, the rate of the removal of zinc ion from native enzyme was governed by the dissociation rate of the zinc-enzyme to a zinc ion and apoenzyme (unimolecular dissociation reaction).[3)]

In the present work, the spontaneous dissociation rate constant (k_d) and the formation rate constant (k_f) of the cobalt-carbonic anhydrase[4)] were determined and the apparent binding constant (K_{bind}) was estimated from the ratio of formation (k_f) and dissociation (k_d) rate constants.[5)] This log K_{bind} value was compared with that of log K_{bind} obtained by the equilibrium dialysis method.

a) Dissociation of the Cobalt-Carbonic Anhydrase In the removal of cobalt ions from the cobalt-enzyme with chelating agents, the rate of reaction was measured by tracing the esterase activity of the enzyme.[6)] Kinetic experiments were carried out at 0° in 0.2 M acetate buffer at pH 5.0 and constant ionic strength of 0.33 was maintained with NaCl.

In the kinetic reaction, sufficient excess of chelating agents over BCA was used so that plots of the logarithm of the fractional residual activity vs. time gave a straight line (Fig. 1A).

$$\log \frac{[(BCA)Co]_0 - [apo\text{-}BCA]}{[(BCA)Co]_0} = -At \quad (1)$$

where $[(BCA)Co]_0$ is the initial enzyme concentration and [apo-BCA] is the apoenzyme concentration. The pseudo-first-order rate constant (A) was derived from the slope of the line.

The relationship between the pseudo-first-order rate constant (A) and the concentration of chelating agents (EDTA, nitrilotriacetic acid, or *trans*-1,2-cyclohexanediaminetetraacetic acid) is shown in Fig. 1B. In the reaction with EDTA, the rate constant (A) was independent of the concentration of chelating agent. This behavior is the same as that observed in zinc-enzyme reaction with EDTA.[3)]

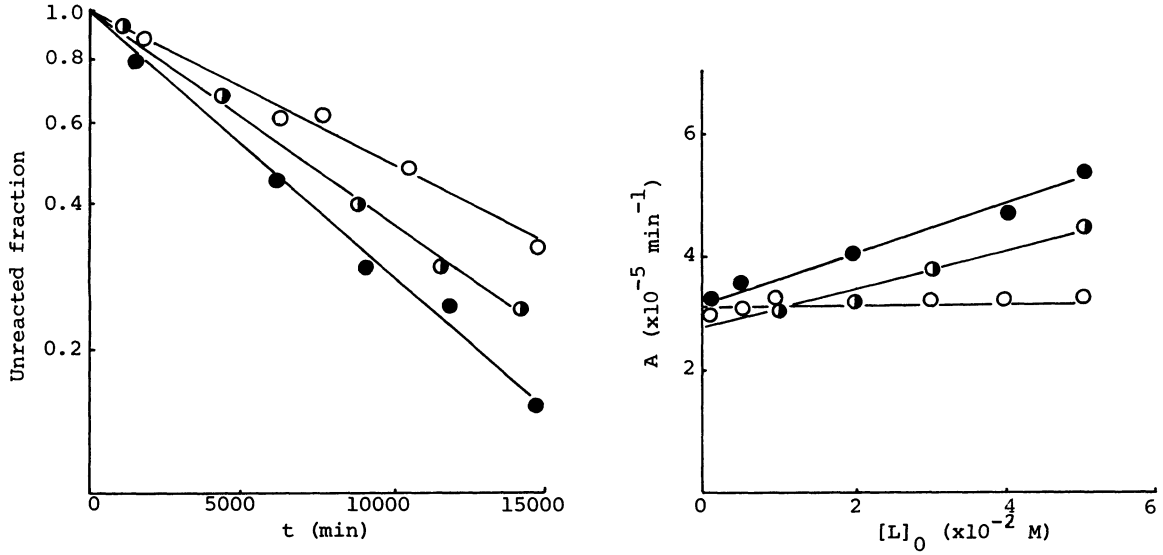


Fig. 1. Dissociaiton reaction of cobalt-carbonic anhydrase

A) Semilogarithmic plots of the transfer of cobalt ions from cobalt-enzyme to chelating agents in 0.2 M acetate buffer, pH 5.0. ○ EDTA 5×10^{-2} M, ● *trans*-1,2-cyclohexanediaminetetraacetic acid 5×10^{-2} M, ◑ nitrilotriacetic acid 5×10^{-2} M

B) Relationship between the pseudo-first-order rate constant (A) and the concentration of chelating agents in 0.2 M acetate buffer, pH 5.0. ○ EDTA, ● *trans*-1,2-cyclohexanediaminetetraacetic acid, ◑ nitrilotriacetic acid. The enzyme concentration was 1.2×10^{-4} M.

Therefore, we assumed that, in Scheme (1), the spontaneous dissociation of the cobalt-enzyme occurs and that a path for formation of a ternary complex is not observed, hence, Eq. (2) is derived.

$$d[apo\text{-}BCA]/dt = k_d[(BCA)Co] \quad (2)$$

$$[(BCA)Co] = [(BCA)Co]_0 - [apo\text{-}BCA] \quad (3)$$

Equation (3) can be inserted into Eq. (2) and integration under the conditions at zero time, [apo-BCA] = 0 gives

$$\log \{([(BCA)Co]_0 - [apo\text{-}BCA])/[(BCA)Co]_0\} = -k_d \cdot t/2.303 \quad (4)$$

The pseudo-first-order rate constant (A) is represented by;

$$A = k_d/2.303 \quad (5)$$

Spontaneous dissociation rate constant (k_d) was 1.2×10^{-6} sec^{-1}.

In the removal of cobalt ions from cobalt-enzyme with *trans*-1,2-cyclohexanediaminetetraacetic acid and nitrilotriacetic acid, plots of the pseudo-first-order rate constant (A) against the concentration of a chelating agent ($[L]_0$) gave a straight line with an intercept (Fig. 1B).

The value of the intercepts in EDTA, *trans*-1,2-cyclohexanediaminetetraacetic acid, and nitrilotriacetic acid was almost the same. Therefore, in the case of cobalt ion removal reaction with nitrilotriacetic acid and *trans*-1,2-cyclohexanediaminetetraacetic acid, both unimolecular dissociation reaction of the spontaneous dissociation of the cobalt-enzyme and bimolecular substitution reaction of the ternary complex formation are considered to take place. This behavior is the same as that observed in the zinc-enzyme reaction with 2,2'-bipyridine.[3]

Therefore, the appearance of apoenzyme is given by

$$d[\text{apo-BCA}]/dt = k_d[(\text{BCA})\text{Co}] + k_t[L]_0[(\text{BCA})\text{Co}] = (k_d + k_t[L]_0)[(\text{BCA})\text{Co}] \quad (6)$$

where k_t is the rate constant of the bimolecular substitution reaction. Hence, the pseudo-first-order rate constant is represented by

$$A = k_d/2.303 + k_t[L]_0/2.303 \quad (7)$$

Extrapolation of the dissociation rate constant (A) to zero concentration of a chelating agent gives an intercept which is considered to be the spontaneous dissociation rate constant (k_d) of the cobalt-enzyme. From the reaction with *trans*-1,2-cyclohexanediaminetetraacetic acid and nitrilotriacetic acid, k_d values were obtained 1.2×10^{-6} sec^{-1} and 1.1×10^{-6} sec^{-1}, respectively.

b) Formation Rate Constant of Cobalt Ion with Apoenzyme The apoenzyme[7] easily recovered its activity when cobalt ion was added.[8] The following experiments were carried out to determine the formation rate constant of cobalt with apo-BCA at pH 5.0 (0°, μ=0.33).[9] The appearance of esterase activity follows apparent second-order kinetics to 70-80% completion under our experimental conditions. The formation reaction of a cobalt ion with apoenzyme is expressed by

$$\text{Apo-BCA} + Co^{2+} \xrightarrow{k_f} [(\text{BCA})\text{Co}] \quad (8)$$

The second-order rate equation is followed by

$$B = \ln\frac{[\text{apo-BCA}]_0([Co^{2+}]_0 - [(\text{BCA})\text{Co}])}{[Co^{2+}]_0([\text{apo-BCA}]_0 - [(\text{BCA})\text{Co}])} = k_f([Co^{2+}]_0 - [\text{apo-BCA}]_0)t \quad (9)$$

where $[\text{apo-BCA}]_0$ and $[Co^{2+}]_0$ are the initial concentrations of apoenzyme and cobalt ions, respectively, and [(BCA)Co] is the concentration of active enzyme.

In Fig. 2, the function B is plotted against t for the experiment at pH 5.0, μ=0.33, with [apo-BCA] = 1.2×10^{-4} M and $[Co^{2+}] = 3\times10^{-4}$ M. It is seen that reasonably good adherence to the second order rate law is observed and the second-order rate constant for various concentrations of cobalt is shown in Table I. Therefore, the formation constant (k_f) between apo-BCA and a cobalt ion was obtained as 7.2×10^{-1} $M^{-1}sec^{-1}$.

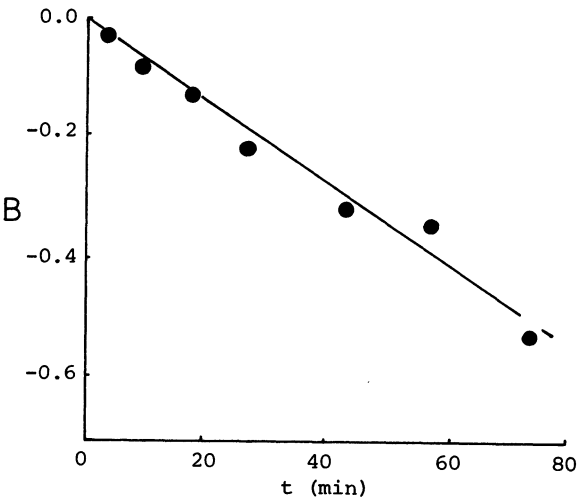


Fig. 2. Representative second order plots for the reaction of Co^{2+} with apocarbonic anhydrase at pH 5.0, 0° in 0.2 M acetate buffer (μ=0.33). Apoenzyme concentration was 1.2×10^{-4} M and Co^{2+} concentration, 3×10^{-4} M.

c) The Apparent Binding Constant between Apo-BCA and Cobalt Ion The binding constant between apo-BCA and cobalt ions will be given by the following equations[10]:

$$\text{Apo-BCA} + Co^{2+} \underset{k_d}{\overset{k_f}{\rightleftharpoons}} [(\text{BCA})\text{Co}] \quad (11)$$

$$K_{bind} = \frac{[(\text{BCA})\text{Co}]}{[\text{apo-BCA}][Co^{2+}]} = \frac{k_f}{k_d} \quad (12)$$

The dissociation rate constant (k_d) of cobalt-enzyme and the formation rate constant (k_f) between apoenzyme and a cobalt ion were determined as shown a) and b) above.

Therefore, the binding constant between apoenzyme and cobalt ion derived from the ratio of the dissociation (k_d) and the formation (k_f) rate constant (Table I) and its binding constant is given in Table II.

In order to prove the validity of the binding constant derived by this kinetic method, equilibrium dialysis was carried out in 0.2 M acetate buffer at pH 5.0. Cobalt-enzyme was dialyzed against 0.2 M acetate buffer containing various concentrations of a chelating agent (2-pyridinecarboxylic acid and 8-hydroxyquinoline-5-sulfonic acid) at 4° in equilibrium dialysis cells. The binding constant was calculated by the method of Lindskog[8] and these values are given in Table II. The values for log K_{bind} obtained by the equilibrium dialysis were almost the same, in spite of the different kind of chelating agents used. Similarly, the value of log K_{bind} obtained by kinetic experiments was consistent with that obtained by the equilibrium dialysis method.

The dissociation rate constant of cobalt-enzyme (1.2×10^{-6} sec^{-1}) was almost the same as that of zinc-enzyme (1.0×10^{-6} sec^{-1}).[3] However, the binding constant of the zinc enzyme is three orders larger than that of cobalt-enzyme. This may be interpreted by the fact that the formation rate constant of zinc-enzyme was much larger than that of cobalt-enzyme.[9]

Table I. Reaction of Co^{2+} with apo-BCA at pH 5.0, 0°, in 0.2 M acetate buffer (μ=0.33)

Concentration of Co^{2+} ($\times10^{-4}$ M)	Formation rate constant (k_f) (sec^{-1} M^{-1})
1.5	0.60
3.0	0.68
5.0	0.78
7.5	0.78
10.0	0.78
	0.72±0.12

Apoenzyme concentration was 1.2×10^{-4} M in all experiments.

Table II. Determination of binding constant for cobalt-carbonic anhydrase by various methods at pH 5.0

Methods	log K_{bind} (M^{-1})
1. Equilibrium dialysis	
2-pyridinecarboxylic acid	6.0±0.3
8-hydroxyquinoline-5-sulfonic acid	6.2±0.4
2. Kinetics	5.8±0.1

All experiments were carried out in 0.2 M acetate buffer at either 0° or 4° and enzyme concentration was 1.2×10^{-4} M.

Reference and Notes

1. Part III in the series of "Coordination Chemical Studies on Metalloenzyme"; the preceding Part II, Ref. 3
2. Bovine carbonic anhydrase (component B) was prepared from bovine erythrocytes.
3. Y. Kidani and J. Hirose, J. Biochem. (Tokyo) in press (1977).
4. 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)
5. R.G. Wilkins and K.R. Williams, J. Amer. Chem. Soc., 96, 2241 (1974).
6. R.W. Henkens, G.D. Watt, and J.M. Sturtevant, Biochemistry, 8, 1874 (1969).
7. Y. Kidani, J. Hirose, and H. Koike, J. Biochem. (Tokyo), 79, 43 (1976).
8. S. Lindskog and B.G. Malmström, J. Biol. Chem., 237, 1129 (1962).
9. R.W. Henkens and J.M. Sturtevant, J. Amer. Chem. Soc., 90, 2669 (1968).
10. K. Gerver, F.T.T. NG, and R.G. Wilkins, Bioinorg. Chem., 4, 153 (1975).

(Received February 5, 1977)