The Kinetics of the Binding of Zinc(II) by Apocarbonic Anhydrase[1]

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Abstract: The reaction of Zn^{2+} with bovine apocarbonic anhydrase to regenerate the active enzyme is accompanied by the liberation of protons and by small changes of absorptivity in the ultraviolet spectrum. The kinetics of the reaction is studied utilizing these two phenomena as well as the appearance of hydrase activity (CO_2 as substrate) and esterase activity (*p*-nitrophenyl acetate as substrate). The rate of the reaction appears to be independent of the method used to follow it. Under all experimental conditions employed the reaction is first order with respect to both Zn^{2+} and apoprotein. Rate constants of the order of 10^4 M^{-1} sec^{-1} are observed; these are two orders of magnitude smaller than those which characterize the combination of Zn^{2+} with small-molecule bi- and tridentate ligands. The rate is generally increased by the addition of neutral salts, although the situation is complicated by specific anion effects. The rate increases with increasing pH in a manner consistent with the view that the rate is enhanced by the dissociation of protons from two groups with pK' values of 5.4 and 7.2. The temperature dependence of the rate corresponds to the activation parameters $\Delta H^{\ddagger}$ = 20.2 kcal $mole^{-1}$ and $\Delta S^{\ddagger}$ = 30 cal deg^{-1} $mole^{-1}$. In contrast, with small bi- and tridentate ligands very small activation enthalpies and small negative activation entropies have been reported. It is evident that the kinetics of the reaction of Zn^{2+} with the apoprotein cannot be understood in terms of the kinetics observed with ligands of known structure. This may result at least in part from a difference in coordination in the two cases, probably tetrahedral in the protein case and octahedral in the small ligand case.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a metalloenzyme containing one atom of very tightly bound Zn(II) per molecule of 30,000 molecular weight. Lindskog and Malmström[3] and Lindskog[4] have shown that under appropriate conditions the zinc atom can be removed, with complete loss by the enzyme of its catalytic properties, and that fully active enzyme, apparently native in all respects, can be regenerated by the addition of 1 equiv of Zn^{2+}. In view of the importance of the zinc atom for the activity of the enzyme, the mode of its attachment to the protein is of great interest.

There is evidence that the binding of metal ions by apocarbonic anhydrase is different in important respects from the binding of the same ions by small ligands. For example, the visible absorption spectrum of the enzyme formed by adding Co^{2+} to the apoenzyme,[3-5] while it indicates that the cobalt is tetrahedrally coordinated,[6] is unlike the spectra of other tetrahedral cobalt complexes and undergoes drastic change when the pH is raised from 6 to 8.

The enthalpy change in the binding of zinc to the bovine apoenzyme has recently been determined.[7] This quantity and the binding constant reported by Lindskog and Nyman[5] lead to a set of thermodynamic parameters for the reaction which are not duplicated by any of the systems involving small ligands which have been reported in the literature. Information on the kinetics of the reaction of Zn^{2+} with small ligands is available.[8] The second-order rate constants for such reactions[9,10] are frequently in the range 10^7–10^8 M^{-1} sec^{-1}. Holyer, *et al.*,[11] have reported lower values, close to 10^6 M^{-1} sec^{-1} at 25°, for the reaction of Zn^{2+} with 1,10-phenanthroline, 2,2′-bipyridine, and 2,2′,2″-terpyridine. A selection of data from the papers by Holyer, *et al.*, is given in Table I.

It has been concluded[8,9] that the rate of formation of many metal complexes is limited by the dissociation of inner-sphere water molecules from the positions to be occupied by the incoming ligands. The second-order rate constant is believed to be the product of an outer-sphere association constant (K_0) and a first-order rate constant (k_1) for exchange of water for ligand, so that the observed rate constant (k_f) is equal to K_0k_1. These views are consistent with the fact that the rates of formation of metal complexes are relatively insensitive to the nature of the ligand.

In view of the knowledge which is available concerning the kinetics of reactions of Zn^{2+} with small ligands which might serve as models for the combining groups in apocarbonic anhydrase, it was decided to investigate the kinetics of the formation of the active enzyme from the apoenzyme.

Experimental Section

Materials. Commercial bovine carbonic anhydrase was chromatographed according to the method of Lindskog.[12] The leading fraction, termed carbonic anhydrase B by Lindskog, was used for

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Table I. The Reaction of Zn^{2+} with Various Small Ligands at 25° (Data of Holyer, *et al.*[11])

Ligand	Temp, °C	k_f,[a] M^{-1} sec^{-1}	k_r,[b] sec^{-1}	E_a,[c] kcal mole^{-1}	$\Delta S^{\pm}$,[d] cal deg^{-1} mole^{-1}
Phenanthroline	25.0	$\geq 3 \times 10^6$	5.0	~8	−4
Bipyridine	30.2–30.5	1.2×10^6	25	6.9	−8
Terpyridine	25.0	1.1×10^6	2.5	8.4	−5

[a] Second-order rate constant for the reaction of Zn^{2+} with indicated ligands. [b] First-order constant for the dissociation of the zinc complexes. [c] Activation energy for the complex formation. [d] Entropy of activation for the complex formation.

the preparation of the apoenzyme by dialysis[8] against 1,10-phenanthroline in 0.1 *M* acetate buffer at pH 5.2 in the cold room. Atomic absorption measurements showed that the enzyme contained 1.00 ± 0.05 atom of zinc per molecule, assuming an absorptivity at 280 mμ of 5.7×10^4 M^{-1} cm^{-1} for the enzyme.[5] Atomic absorption and enzymic activity measurements agreed in showing the zinc removal to be 98% complete, and titration of the apoprotein with Zn^{2+} gave full recovery of activity after the addition of one atom per molecule.[13] Protein concentrations were determined from the absorbance at 280 mμ. The absorptivity of the apoenzyme is actually 400 M^{-1} cm^{-1} less than that of the holoenzyme (*cf.* Figure 1), but this small difference is not significant for the determination of concentrations.

Methods. Absorbance measurements and some reaction rate determinations were made using a Cary 14 spectrophotometer. The rates of rapid reactions were determined with a stopped-flow spectrophotometer[14] which has been found to be capable of following reactions with half-times of 2 msec under favorable conditions.

Carbonic anhydrase activity was routinely determined with *p*-nitrophenyl acetate as substrate.[15] The reaction mixture contained 4.8×10^{-4} *M* *p*-nitrophenyl acetate, 0.05 *M* Tris–HCl buffer (pH 7.5), 5×10^{-5} *M* EDTA, and 5% acetone. The EDTA was added to sequester any inhibiting metal ions which might be present. The reaction was followed at 400 mμ in a 1-cm cell thermostated at 25.0°. Small corrections were made for hydrolysis in the absence of enzyme (10^{-4} *A* unit/sec) and nonspecific catalysis as determined in control experiments using enzyme completely inhibited by acetazolamide (absorbance increase per second is numerically equal to 30 times the enzyme molarity). The absorptivity of *p*-nitrophenol under the assay conditions is 1.39×10^4 M^{-1} cm^{-1}. Specific activities of 0.29–0.36 sec^{-1} were observed for the various enzyme preparations used.

The rate of enzymic hydrolysis of *p*-nitrophenyl acetate varied with pH in the manner to be expected if a single group in the enzyme with a p*K*′ of 7.32 must be in the unprotonated form for the reaction to occur. This is in satisfactory agreement with the p*K* value reported by Pocker and Stone.[15]

Carbon dioxide was also used as substrate for some experiments. CO_2 solutions were prepared by mixing distilled water saturated with CO_2 at 1 atm with an equal volume of boiled, distilled water. The CO_2 solution was mixed in the stopped-flow apparatus at 25.0° with an equal volume of 0.05 *M* imidazole–HCl buffer (pH 7.50) containing 4×10^{-5} *M* *p*-nitrophenol as indicator and 5×10^{-5} *M* EDTA. The rate of liberation of protons was followed at 400 mμ, initial rates being evaluated according to the procedure of Gibbons and Edsall.[16] Nonenzymic hydration of the CO_2 was less than 2% of the catalyzed rate and was neglected. Specific activities of 2.3×10^5 to 3.4×10^5 sec^{-1} were observed for the various enzyme preparations used in this work. Kernohan[17] reported a specific activity of 2.5×10^5 sec^{-1} for the conditions used in our experiments. For six different samples of enzyme, some purified by chromatography and some not, the ratio of specific rate with *p*-nitrophenyl acetate to that with CO_2 was $(1.15 \pm 0.05) \times 10^{-5}$.

Results

Ultraviolet Difference Spectrum. The binding of Zn^{2+} to apocarbonic anhydrase causes small changes in the spectrum of the apoenzyme in the region 230–340 mμ. The ultraviolet difference spectrum shown in Figure 1 (solid curve) has peaks at 235, 287, and 300 mμ. It is interesting that at pH 5.3 the difference spectrum of the zinc–enzyme *vs.* the zinc–enzyme in the presence of 0.05 *M* KCl (dashed curve in Figure 1) is nearly identical with that of the zinc–enzyme *vs.* the apoenzyme. In other words, at this pH KCl appears to abolish the effects of zinc on the spectrum of the apoenzyme. This effect of KCl decreases with increasing pH, approximately in the same manner as the inhibitory effect of Cl^- decreases at higher pH values.[18] Cl^- has no effect on the spectrum of the apoenzyme.

Kinetics of the Reaction of Zn^{2+} with Apocarbonic Anhydrase. The time course of the reaction between apocarbonic anhydrase and Zn^{2+} was followed by four different methods. When the metal ion is bound at pH values up to about 7.3, a proton is liberated, and the number of protons liberated increases to about 2 at pH 9. This proton liberation[4] is conveniently followed by means of an indicator such as *p*-nitrophenol or phenol red. Figure 2 gives curves showing the titration of apoenzyme with Zn^{2+}, followed both by activity measurements and by indicator absorbance changes. In the latter case the continued change in absorbance beyond the equivalence point suggests that nonspecific binding of Zn^{2+} takes place when excess of the metal ion is present.

The small change in the ultraviolet absorption spectrum caused by the binding of Zn^{2+} can also be utilized in following the course of the Zn^{2+} binding. Finally, the rate of recovery of enzymic activity, both with *p*-nitrophenyl acetate and with CO_2 as substrates, has been directly observed.

Rate of Hydrogen Ion Release. The liberation of hydrogen ions, as measured by the change in absorbance of a suitable indicator, follows apparent second-order kinetics to 80–90% completion under all experimental conditions employed. For the reaction

$$E + M \xrightarrow{k_f} EM \quad (1)$$

which is for all practical purposes irreversible, we expect that

$$F(t) \equiv \ln \frac{(E)_0[(M)_0 - (EM)]}{(M)_0[(E)_0 - (EM)]} = k_f t[(M)_0 - (E)_0] \quad (2)$$

where $(E)_0$ and $(M)_0$ are the initial concentrations of apoenzyme and metal ion, (EM) is the concentration of active enzyme, and t is the time. When $(M)_0 > (E)_0$, $(EM) = (A_0 - A)(E)_0/(A_0 - A_\infty)$, where A_0, A, and A_∞ are indicator absorbances at $t = 0$, t, and ∞, respectively. It was ascertained that the indicator absorbance was a linear function of the concentration of added H^+ ions over the range of interest. Representa-

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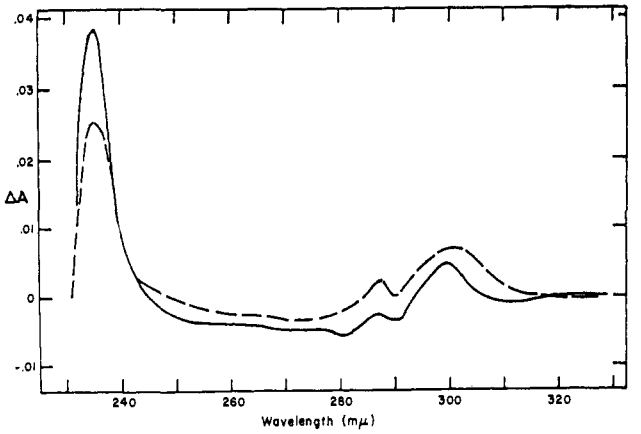


Figure 1. Difference spectra measured at 25°. Protein solutions (1.43×10^{-5} *M*) adjusted to pH 5.3 with HCl, measured in 1-cm cells. Solid curve: reactivated carbonic anhydrase *vs.* apocarbonic anhydrase. Dashed curve: reactivated enzyme *vs.* reactivated enzyme containing 0.05 *M* KCl.

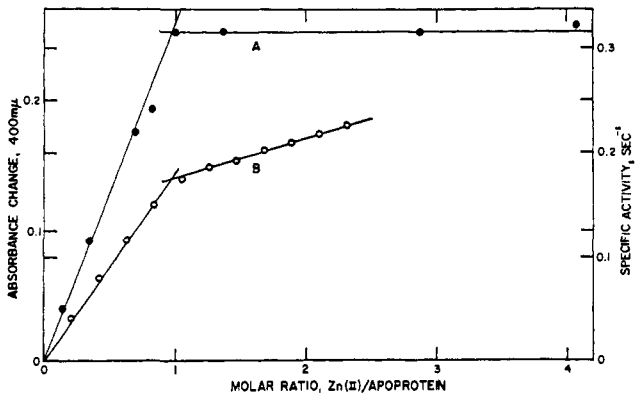


Figure 2. Curve A: titration of 1.45×10^{-4} *M* apocarbonic anhydrase with $ZnCl_2$, followed by measurement of the esterase activity with *p*-nitrophenyl acetate as substrate. The apoenzyme had a residual specific activity less than 0.003 sec^{-1}. See text for details of the assay procedure. Curve B: titration of 7.2×10^{-5} *M* apoenzyme at pH 6.0, 25°, in 0.00125 *M* acetate buffer containing 0.05 *M* KCl, followed by the color change produced in 10^{-4} *M* 2-chloro-4-nitrophenol by the protons liberated.

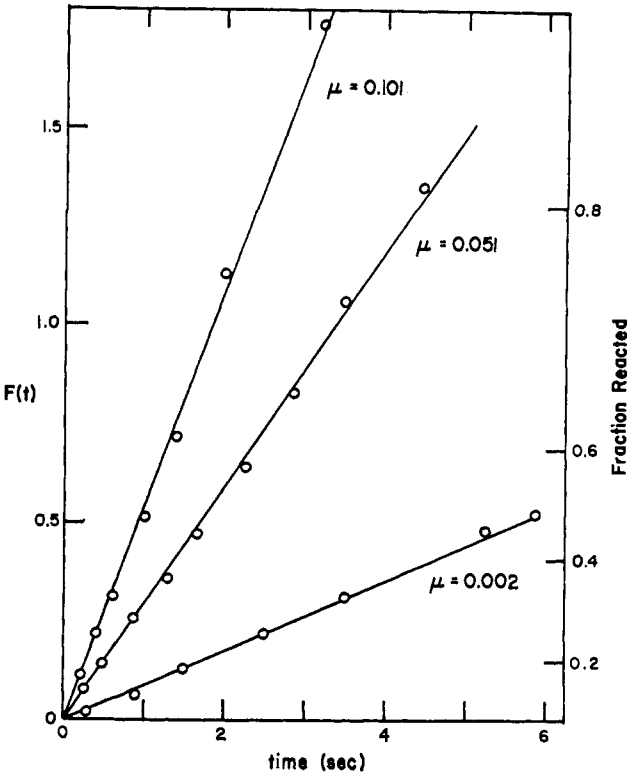


Figure 3. Representative second-order plots for the reaction of Zn^{2+} with apocarbonic anhydrase at pH 5.7, 25°, in 0.00125 *M* acetate buffer containing 10^{-4} *M* 2-chloro-4-nitrophenol as indicator. The ionic strength (represented for convenience by the symbol μ even though concentrations are expressed in molarities) was adjusted with KCl. The function F(*t*) is defined in eq 2 in the text.

tive plots of F(*t*) *vs.* *t* are shown in Figure 3. The second-order behavior is further substantiated by the plots in Figure 4 which show that the reaction is indeed first order in each of the reactants. The slopes of the lines in Figure 4 give for the rate constant the values 1.6×10^4 M^{-1} sec^{-1} with $(M)_0$ held constant, and 1.7×10^4 M^{-1} sec^{-1} with $(E)_0$ constant. The fact that the same rate constant is obtained with both excess metal ion and excess apoenzyme indicates that the nonspecific binding shown in Figure 2 has no effect on the observed kinetics.

The indicators employed were *p*-nitrophenol, 2-chloro-4-nitrophenol, phenol red, and cresol red, the selection of indicator being based on the pH of the reaction. No specific effects due to the indicators were observed.

Rate of Appearance of Ultraviolet Difference Spectrum. In Figure 5 the function F(*t*) is plotted against *t* for an experiment at pH 7.5, ionic strength 0.024 *M*, with $2(E)_0 = (M)_0 = 1.0 \times 10^{-4}$ *M*. For the open circles (EM) = $(A_0 - A)(E)_0/(A_0 - A_\infty)$, where *A* = absorbance at 300 mμ, and for the filled circles, *A* = absorbance at 235 mμ. It is seen that reasonably good adherence to the second-order rate law is observed.

Rate of Recovery of CO_2–Hydrase Activity. If a solution containing apoenzyme is mixed in the stopped-flow apparatus with a solution containing Zn^{2+} to reactivate the enzyme, CO_2 as substrate, and an indicator to make visible the liberation of protons resulting from the reaction $H_2O + CO_2 = HCO_3^- + H^+$, there is a lag of several tenths of a second before decolorization of the indicator proceeds at maximum rate. This phenomenon, which is illustrated in Figure 6, gives another measure of the rate of combination of Zn^{2+} with the apoenzyme. In the figure, the function $\log f = \log (1 - y)$, where *y* is the fraction of the substrate converted to products at time *t*, is plotted against the time for a reactivation experiment (curve A) and for an experiment (curve B) in which the enzyme was reactivated before being mixed with the substrate. Since the enzyme is present in catalytic amount, the H^+ liberation accompanying the binding of Zn^{2+} is completely negligible.

Because of the high turnover number of CO_2 hydration, the apoenzyme in an experiment of this sort has to be present at small concentration; it is therefore practical to use a large enough excess of Zn^{2+} to ensure that the reactivation follows apparent first-order kinetics. If the presence of substrate has no effect on the reactivation, the concentration of active enzyme (EM) is given by

$$(EM) = (E)_0(1 - e^{-k_1(M)_0 t}) \qquad (3)$$

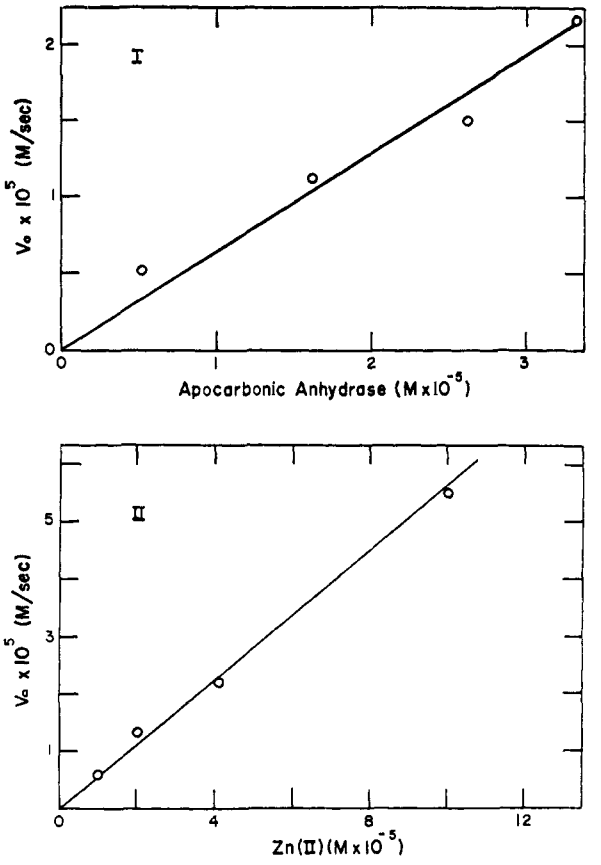


Figure 4. (I): Initial rate of hydrogen ion liberation in the reaction of Zn^{2+} with apocarbonic anhydrase as a function of concentration of apoenzyme at constant Zn^{2+} concentration (4.1×10^{-5} M) (II): Inital rate as a function of Zn^{2+} concentration at constant apoenzyme concentration (3.3×10^{-5} M). pH 6.75, 2.4×10^{-4} M phosphate buffer, 0.09 M KCl, and 1.8×10^{-4} M p-nitrophenol, 25°.

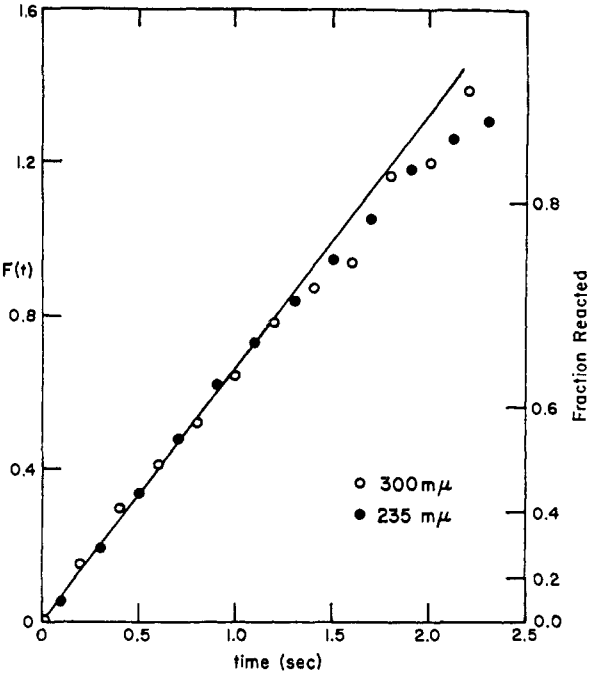


Figure 5. Representative second-order plots for the reaction of Zn^{2+} with apocarbonic anhydrase followed by the change in absorbance at 300 and 235 mμ. Initial concentrations were 4.97×10^{-5} M apoenzyme and 1.00×10^{-4} M $ZnCl_2$ in pH 7.5, 5×10^{-3} M Tris-HCl buffer, containing 0.02 M KCl. The function F(t) is defined in eq 2 in the text.

The initial concentration of substrate, $(S)_0$, was low enough to give apparent second-order kinetics for the hydration reaction

$$EM + S \xrightarrow{k} EM + \text{products} \quad (4)$$

Thus, from eq 3

$$\log (1 - y) = \frac{k(E)_0}{2.3k_f(M)_0}(1 - e^{-k_f(M)_0 t}) - \frac{k(E)_0 t}{2.3} \quad (5)$$

After reactivation is complete, the exponential term becomes zero, so that

$$\log (1 - y_r) = \frac{k(E)_0}{2.3}\left[\frac{1}{k_f(M)_0} - t\right] \quad (6)$$

Setting

$$\Delta \log (1 - y) \equiv \log (1 - y_r) - \log (1 - y) \quad (7)$$

we find that

$$\log [\Delta \log (1 - y)] = \log \frac{k(E)_0}{2.3k_f(M)_0} - \frac{k_f(M)_0 t}{2.3} \quad (8)$$

Thus k_f can be evaluated from the slope of a plot of log [Δ log $(1 - y)$] *vs.* t. The inset in Figure 6 shows such a plot. If the system follows the kinetics assumed in this derivation, the same value of k_f must be obtained

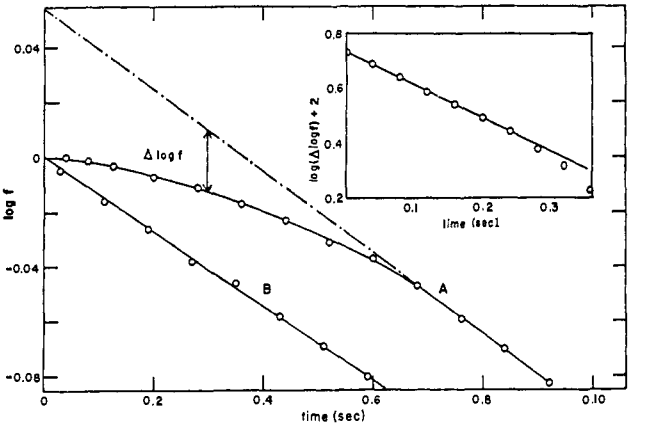


Figure 6. The rate of recovery of CO_2-hydrase activity on adding Zn^{2+} to apocarbonic anhydrase at 25°. Curve A: a solution of Zn^{2+} containing CO_2 was mixed in the stopped-flow apparatus with a solution of apoenzyme. Curve B: a solution containing both Zn^{2+} and apoenzyme was mixed with a solution of CO_2. Initial concentrations (after mixing) were 1.39×10^{-5} M apoenzyme, 2.00×10^{-4} M $ZnCl_2$, and 1.0×10^{-3} M CO_2, in 3.75×10^{-3} M phosphate buffer, pH 7.0, containing 1.00×10^{-4} M p-nitrophenol as indicator, 2.5×10^{-5} M EDTA, and 0.05 M KCl. Both curves have been corrected for the uncatalyzed hydration reaction. The utilization of the plot given in the insert to evaluate the rate constant for the combination of Zn^{2+} and apoprotein is described in the text.

from eq 9, where τ is the time at which $y_r = 0$. In the

$$k_f = \frac{1}{\tau(M)_0} \quad (9)$$

case illustrated in Figure 6 the values of k_f obtained by these two methods are 1.36 and 1.37×10^4 M^{-1} sec^{-1}.

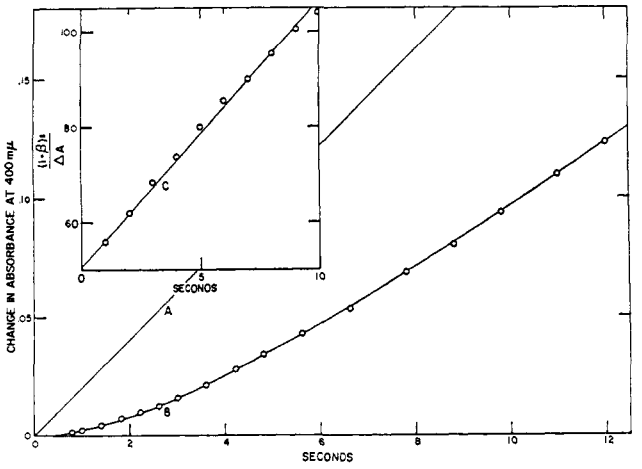


Figure 7. The rate of recovery of esterase activity on adding Zn^{2+} to apocarbonic anhydrase at 25°. Curve A: a solution containing both Zn^{2+} and apoenzyme was mixed in the stopped-flow apparatus with a solution of *p*-nitrophenyl acetate. Curve B: a solution containing Zn^{2+} and substrate was mixed with a solution of apoenzyme. Initial concentrations (after mixing) were 1.14×10^{-5} *M* apoenzyme, 1.14×10^{-5} *M* $ZnCl_2$, and 2.00×10^{-4} *M* substrate in 0.0125 *M* acetate buffer (pH 6.27) containing 0.05 *M* KCl. The utilization of the plot given in the insert to evaluate the rate constant for the combination of Zn^{2+} and apoprotein is described in the text.

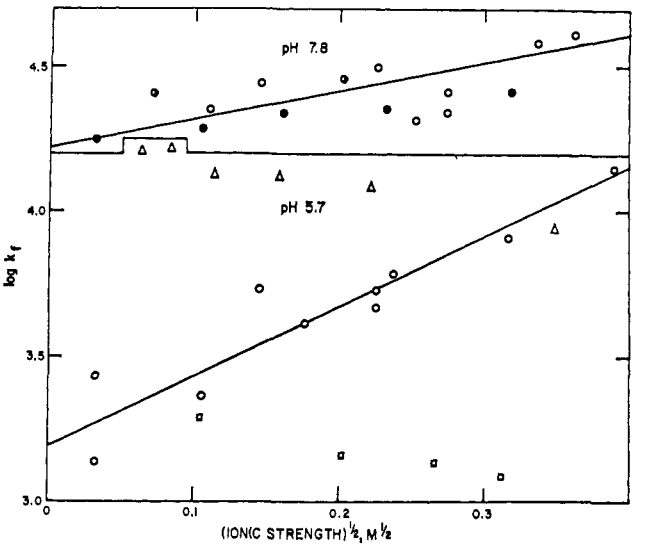


Figure 8. The dependence of the rate constant at 25° for the reaction of Zn^{2+} with apocarbonic anhydrase on the concentration of added neutral salt. The experiments at pH 5.7 were performed in 0.00125 *M* acetate buffer and those at pH 7.8 in 0.00125 *M* Tris–HCl buffer. The data are not corrected for the association of anions with Zn^{2+}. Added salts: (○) KCl, (●), $NaClO_4$, (◐) KNO_3, (□) KF, and (△) K_2SO_4.

Rate of Recovery of Esterase Activity. The method outlined in the preceding section could not be conveniently applied to the measurement of the rate of recovery of esterase activity because of the slowness of the esterase reaction. In this case equal initial concentrations of Zn^{2+} and apoenzyme were used, so that

$$(EM) = \frac{k_f(E)_0^2 t}{1 + k_f(E)_0 t} \tag{10}$$

The change in (S) during the reactivation was no more than a few tenths of 1%, so that we may write

$$\frac{dy}{dt} = k(EM) \tag{11}$$

On integration we obtain

$$y = k(E)_0 t - \frac{k}{k_f} \ln [1 + k_f(E)_0 t] \tag{12}$$

For fully reactivated enzyme under the same conditions

$$y_r = k(E)_0 t \tag{13}$$

The difference $y_r - y$ is unaffected by the nonenzymic hydrolysis of the substrate and is therefore a convenient quantity to use in evaluating k_f. Expansion of the logarithmic term in eq 12 gives

$$y_r - y = \frac{2k(E)_0 t}{2 + k_f(E)_0 t}\left[1 + \frac{1}{3}\frac{[k_f(E)_0 t]^2}{[2 + k_f(E)_0 t]^2} + \cdots\right] \tag{14}$$

Figure 7 summarizes an experiment of this type. Curve A shows the variation with time of absorbance at 400 mμ for fully reactivated enzyme, and curve B the variation observed when apoenzyme was mixed with Zn^{2+} and substrate to initiate the reaction. The difference (ΔA) between the two curves at any time is proportional to $y_r - y$; rearrangement of eq 14 gives

$$\frac{(1 + \beta)t}{\Delta A} = \frac{1}{\alpha k(E)_0} + \frac{k_f}{2\alpha k} t \tag{15}$$

where α is a proportionality constant and $1 + \beta$ represents the quantity in brackets in eq 14 and may be calculated with adequate accuracy using an approximate value for k_f. The quantity $(1 + \beta)t/\Delta A$ is plotted against t in the insert in Figure 7. The ratio of the slope to the intercept at $t = 0$ is $^1/_2 k_f(E)_0$, from which k_f is readily evaluated.

Typical values for the specific rates of recombination of apoenzyme and Zn^{2+} as determined by the four methods outlined above are given in Table II. It appears

Table II. Rate of Recombination of Zn^{2+} with Apocarbonic Anhydrase as Observed by Various Methods at 25°

pH	Ionic strength, *M*[a]	Appt second-order rate constant, M^{-1} sec^{-1} × 10^{-4}: H^+ liberation[b]	Uv difference spectrum	Recov of CO_2 hydrase act.	Recov of esterase act.
5.90	0.001	0.18	0.27		
5.90	0.021	0.42	0.54		
6.27	0.056	0.85			0.81
7.0	0.056	1.5		1.36	
7.40	0.020	1.6	1.33		
7.56	0.056	2.2			2.0

[a] Added salt was KCl. [b] Values interpolated from the data given in Figures 8 and 9.

that the kinetics of recombination is independent of the method of observation. Since the method based on H^+ liberation was the most convenient, it was utilized for obtaining the bulk of the kinetic data reported in this paper.

Effect of Added Salts on the Rate of Recombination. Figure 8 shows the effects of various added salts on the rate of the reaction of Zn^{2+} with the apoenzyme at pH

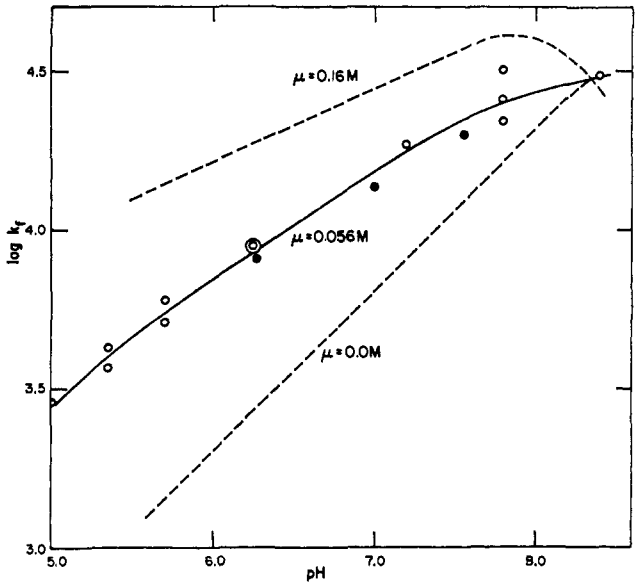


Figure 9. The dependence on pH of the rate constant at 25° for the reaction of Zn^{2+} with apocarbonic anhydrase: O, reaction followed by liberation of protons; ●, reaction followed by appearance of enzymic activity. Added salt was KCl. The dashed curves were estimated by extrapolation of the data in Figure 8 together with some additional data at pH values other than those included in Figure 8.

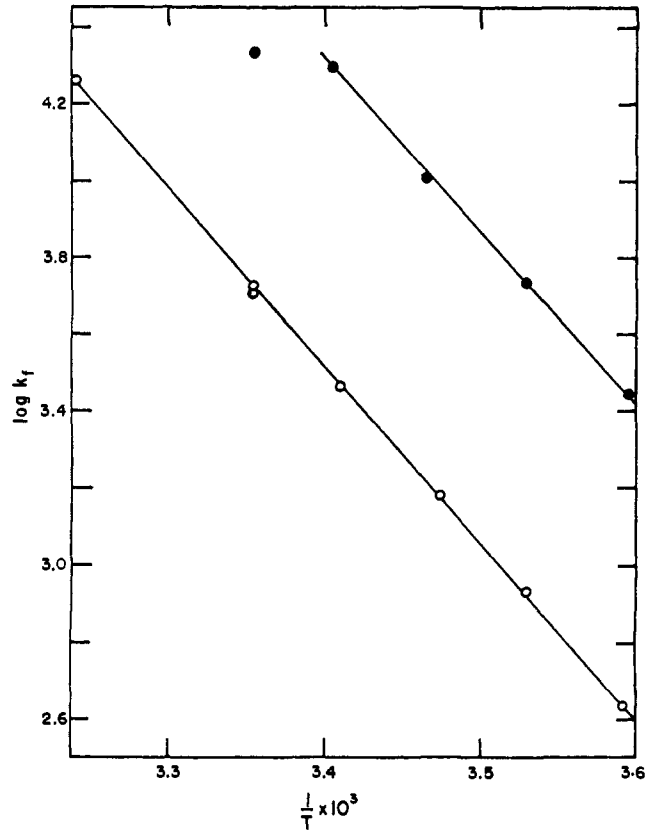


Figure 10. Effect of temperature on the rate constant for the liberation of protons during the binding of Zn^{2+} to apocarbonic anhydrase: (O) 0.00125 *M* acetate buffer, pH 5.5 at 25°; (●) 0.00125 *M* Tris-HCl buffer, pH 7.5 at 25°; added salt, 0.05 *M* KCl.

5.7 and 7.8. There seems to be no consistent explanation for the diversity of the effects observed.

Many Zn(II) salts are only partially dissociated in aqueous solution, and it is to be expected that the rate of the reaction is dependent to some extent on the charge of the Zn(II) species. At pH 5.7 the buffer used was 0.00125 *M* acetate, which at this pH was largely in the ionized form. The association constant[19] for $ZnAc^+$ is 10 M^{-1}, so that a negligible fraction of the Zn^{2+}, usually present at a concentration of 10^{-4} *M*, was bound by acetate. It is very unlikely that any significant interaction took place between Zn^{2+} and the positive ions of the Tris buffer used at pH 7.8.

Of the various added anions, SO_4^{2-} reacts most extensively with Zn^{2+}. Values of the thermodynamic association constant $K^0_{ZnSO_4}$ ranging from 185 to 240 M^{-1}, with a mean of 205 M^{-1}, have been reported.[19] No data are available which permit an accurate estimate of the value of this constant in K_2SO_4 solutions. If we use data given by Harned and Owen,[20] and the simplest possible generalization regarding activity coefficients in mixtures of electrolytes,[21] we obtain as very rough values for the activity coefficients of Zn^{2+} at very low concentration and 0.04 *M* SO_4^{2-} $y(Zn^{2+}) \approx 0.33$ and $y(SO_4^{2-}) \approx 0.30$, so that the effective association constant (K_{ZnSO_4}) is approximately 20 M^{-1}. If we assume that the rate of reaction with $ZnSO_4$ is negligible compared to that with Zn^{2+}, we obtain for k_f in 0.04 *M* K_2SO_4, the highest concentration used, the corrected value of 1.6×10^4 M^{-1} sec^{-1}, which is nearly twice the observed value of 0.87×10^4 M^{-1} sec. On this basis the apparent decrease in k_f with increasing K_2SO_4 concentration is converted into a very small increase.

(19) L. G. Sillen and A. E. Martell, "Stability Constants of Metal-Ion Complexes," The Chemical Society, London, 1964.

(20) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 3rd ed, Reinhold Publishing Corp., New York, N. Y., 1958, pp 553 and 564.

(21) Reference 20, p 602 ff.

The other anion employed in the present work which is bound relative strongly by Zn^{2+} is F^-, with $K^0_{ZnF^+} = 18$ M^{-1}. However, since we have no way of assigning relative reactivities to Zn^{2+} and ZnF^+, it is impossible to correct the observed rates in a manner analogous to the procedure used in the preceding paragraph. For Cl^-, $K^0_{ZnCl^+} = 0.5$ M^{-1}, and no more than 5% of the Zn(II) would be in the form $ZnCl^+$ at the highest Cl^- concentration employed.

Dependence of Recombination Rate on pH. The rate of the reaction of Zn^{2+} with apocarbonic anhydrase increases with increasing pH. The results of a series of experiments with KCl as added salt at ionic strength 0.056 are shown in Figure 9. The dashed curves in the figure, for $\mu = 0$ and 0.16, are estimated curves obtained by extrapolation of the KCl data in Figure 8 together with additional data not included in the figure.

Variation of Recombination Rate with Temperature. Measurements of the rate of reaction of Zn^{2+} with apocarbonic anhydrase over a range of temperatures gave satisfactorily straight-line Arrhenius plots (Figure 10). The energies of activation are $E_a = 21.2$ kcal $mole^{-1}$ at $\mu = 0.051$ (pH 5.5) and 20.8 kcal $mole^{-1}$ at $\mu = 0.051$ (pH 7.5). The corresponding entropies of activation, $\Delta S^{\pm}$, are 27.7 and 30.0 cal deg^{-1} $mole^{-1}$ at 25°.

Discussion

It was shown in the preceding section that the rate of liberation of H^+ ions and the rate of change of absorption in the ultraviolet due to the reaction of Zn^{2+} with the apoprotein are the same, albeit within a fairly wide

uncertainty range, as the rate of recovery of activity. This observation suggests that the coordination site for the Zn^{2+} ion in the enzyme is fully formed before reaction, so that no major rearrangement of atoms or groups is necessary before the zinc atom is ready to start catalyzing a specific reaction.

Chemical relaxation studies[8-10] have shown that the rate constant for the reaction of Zn^{2+} with small ligands is 10^7–10^8 M^{-1} sec^{-1}, with little dependence on the nature of the ligand. More pertinent for present considerations are the data of Holyer, *et al.*,[11] given in Table I, for the reactions of Zn^{2+} with di- and tridentate ligands. It is evident that the reaction of Zn^{2+} with apocarbonic anhydrase is characterized by rates which are two orders of magnitude slower than observed with these polydentate ligands, and by markedly different activation parameters. The very large increase in activation energy seen in the protein case is partially compensated by a large increase in entropy of activation.

According to Dennard and Williams,[6] the Co(II) in the product[3] of the reaction of Co^{2+} with apocarbonic anhydrase is probably tetrahedrally coordinated, at least at low pH. This conclusion is strongly supported by measurements[22] of the magnetic susceptibility and near-infrared absorption of the cobalt–enzyme. Since with small ligands Co(II) shows generally less tendency toward tetrahedral coordination than does Zn(II), it would appear likely that the Zn(II) in carbonic anhydrase is tetrahedrally coordinated. The rate data in Table I refer to the formation of monocomplexes, but probably with octahedral coordination, so that they also may not be directly comparable to those observed with the apoprotein.

Recent experiments[23] suggest the possibility that the transformation of $Zn(H_2O)_6^{2+}$ to $Zn(H_2O)_4^{2+}$ is quite slow. It is therefore pertinent to consider the mechanism

$$Zn(H_2O)_6^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Zn(H_2O)_4^{2+} + 2H_2O \qquad (16)$$

$$Zn(H_2O)_4^{2+} + E \xrightarrow{k_2} EM \qquad (17)$$

Application of the steady-state approximation to the concentration of $Zn(H_2O)_4^{2+}$ gives

$$\frac{d(EM)}{dt} = \frac{k_1(E)(M)}{\frac{k_1 + k_{-1}}{k_2} + (E)} \qquad (18)$$

where (E) is the concentration of apoenzyme and (M) is the total concentration of Zn^{2+} unbound to the protein. Since the octahedral form of Zn(II) in aqueous solution is presumable the predominant form, $k_{-1} > k_1$. Second-order kinetics was observed in all runs so that we may conclude that $k_{-1}/k_2 > 5 \times 10^{-5}$ M, the largest apoenzyme concentration used, and that the forward rate constant is given by $k_f = k_1k_2/k \approx 10^4$ M^{-1} sec^{-1}, or $k_2 > 10^4$ M^{-1} sec^{-1}. Using the value[23] $k_{-1} = 8$ sec^{-1} we see that $k_2 < 1.5 \times 10^5$ M^{-1} sec^{-1}. These two limits on the magnitude of k_2 are far enough apart to make it difficult to rule out this mechanism in the absence of further information.

Effects of salts on the rate of recombination, aside from the complexing of Zn^{2+} with anions discussed in the preceding section, can arise either from binding of ions to the protein or from a primary salt effect. The binding of anions to proteins is usually considered to be more prevalent than that of cations, and although we have not performed experiments designed to show up cationic effects, we shall assume that the binding of monovalent cations such as Na^+ or K^+ can be neglected. It is conceivable that the binding of anions could either speed up or slow down the reaction. Acceleration would be expected to result from increased electrostatic attraction due to the binding of anions in the neighborhood of the binding site for Zn(II). On the other hand, since the Zn(II) appears to be bound in a cavity in the protein,[24] the binding of an anion, for example, to some group at the entrance to the cavity might well interfere with the binding of Zn^{2+} in spite of exerting a favorable electrostatic influence.

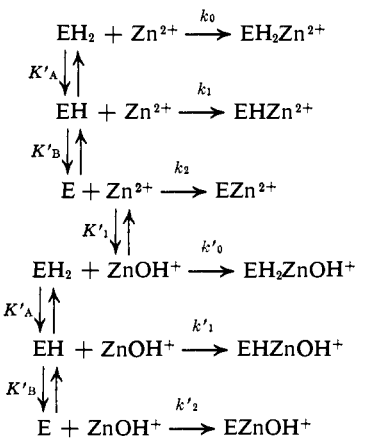
These considerations make it appear that we have such ample leeway for rationalizing all the observed effects that we cannot draw any definite conclusions from the data. However, it is nevertheless pertinent to note that the effects produced by KCl, for which salt more data were obtained than for any other, can be given a consistent interpretation in terms of a primary salt effect. According to the Bronsted–Bjerrum equation,[25] a plot of log k_f against the square root of ionic strength should be a straight line with slope equal to $1.02Z_AZ_B$ at 25°, where Z_A and Z_B are the effective charges on the apoenzyme and the Zn(II) ion. The KCl data in Figure 8 adhere approximately to straight-line plots of this type, as do also data at pH 8.40. The slopes obtained at these three pH values are roughly +2.4, +1.0, and 0, which correspond to charges on the apoenzyme of +1.2, +0.5, and 0.0.

According to Petermann and Hakala,[26] the isoelectric pH of carbonic anhydrase at 0.1 M ionic strength is 5.3, and it is likely that the isoelectric pH of the apoprotein is slightly lower than this since a positive charge is lost on dissociating the Zn^{2+}. Thus the apoprotein carries a net negative charge in the pH range used in the present work. A titration of apocarbonic anhydrase showed that its negative charge increases by six units in the range pH 5.3–8.4. In contrast with these figures, a literal application of the Bronsted–Bjerrum relation indicates an effective positive or zero charge on the apoprotein in this pH range.

The rate of the recombination of Zn^{2+} increases with increasing pH, suggesting that the binding of Zn^{2+} is accelerated by the dissociation of protons from one or more groups at or near the binding site for Zn^{2+}. The dependence on pH appears to decrease with increasing ionic strength, although the data pertaining to this point are fragmentary. It is to be expected that an additional contribution to the pH dependence would stem from the reaction $Zn(H_2O)_6^{2+} \rightleftarrows Zn(H_2O)_5OH^+ + H^+$, for which the p$K$ at 25° and ionic strength 0.056 is 9.2.[27] Analysis of the data at ionic strength 0.056 M with KCl as added salt, using the procedure of Goldsack, *et al.*,[28] leads to the conclusion that at least

(22) S. Lindskog and A. Ehrenberg, *J. Mol. Biol.*, 23, (1967).
(23) T. J. Swift, *Inorg. Chem.*, 3, 56 (1964).
(24) K. Fridborg, K. K. Kannan, A. Liljas, J. Lundin, B. Strandberg, R. Strandberg, B. Tilander, and G. Wirén, *J. Mol. Biol.*, 25, 505 (1967).
(25) See, for example, I. Amdur and G. G. Hammes, "Chemical Kinetics," McGraw-Hill Book Co., Inc., New York, N. Y., 1966, p 119.
(26) M. L. Petermann and N. V. Hakala, *J. Biol. Chem.*, 145, 701 (1942).
(27) D. D. Perrin, *J. Chem. Soc.*, 4500 (1962).

two ionizations of protein groups must be included, as in the mechanism



In this formulation charges on the protein are not indicated, nor is the dissociation of protons from the products. Since in each experiment the rate constant was evaluated using the total proton liberation in that experiment, no explicit attention has to be given to the ionization state of the product. The solid curve in Figure 9 is drawn using the parameters $pK'_1 = 9.2$, $pK'_A = 5.4$, and $pK'_B = 7.2$ and $\log k_0 = 3.02$, $\log k_1 = 3.89$, $\log k_2 = 4.44$, and $\log k'_2 = 4.6$. The value of pK'_A is certainly not well established by the data, nor is the rate constant k_0. It seems unlikely that the $pK'_B = 7.2$ pertains to the same group as the one, with $pK' = 7.3$, which affects the rate of hydrolysis of *p*-nitrophenyl acetate, since one would expect the pK of a group at the active site to be markedly affected by the presence of the Zn(II).

It is well to emphasize the great difference in activation parameters for the reaction of Zn^{2+} with the apoenzyme as compared to the reaction with the ligands studied by Holyer, *et al.*[11] The protein reaction has an unusually large energy of activation, 21 kcal mole^{-1}, which is partially compensated by a fairly large positive entropy of activation amounting to 27–29 cal deg^{-1} mole^{-1}, whereas chelate formation with small ligands is characterized by an unusually low energy of activation of 7–8 kcal mole^{-1} and a small negative entropy of activation of -4 to -8 cal deg^{-1} mole^{-1}. Since the Zn^{2+} is bound to the protein at the bottom of a cleft, the entropy of activation should be made more negative than it is with small ligands by this added orientation requirement. It thus appears that the positive contribution to the entropy must amount to roughly 40 cal deg^{-1} mole^{-1}. If we assume that the transition state in the reaction is similar to the product rather than the reactants, it is reasonable to attribute at least part of this large positive contribution to liberation of bound water molecules from the Zn^{2+} and/or the protein cavity.

Data given by Vallee and Coleman[29] permit an approximate comparison to be made between the rates of reaction of apocarbonic anhydrase and of apocarboxypeptidase A with Zn^{2+}. According to these authors the half-time for the displacement of Zn(II) from carboxypeptidase by Cd^{2+} is 20 hr at pH 8.0, 4°, and 1 *M* ionic strength. Thus the reverse rate constant (k_r) under these conditions is 10^{-6} sec^{-1}. Since at pH 8.0 the rate of reaction of Zn^{2+} with apocarbonic anhydrase is not greatly affected by ionic strength (added KCl), we may estimate from the data in Figure 9 that $k_f = 6.3 \times 10^4$ M^{-1} sec^{-1} at pH 8.0, $\mu = 1$, 25°, and, from the observed temperature coefficient, that $k_f = 10^4$ M^{-1} sec^{-1} at pH 8.0, $\mu = 1$, 4°. According to Lindskog and Nyman,[5] the value of K_{assoc} of bovine carbonic anhydrase at pH 8.0, $\mu = 0.1$, 25°, is 10^{13} M^{-1}. The heat of the association reaction[7] is +3.3 kcal mole^{-1}, so that $K_{assoc} = 6.5 \times 10^{12}$ M^{-1} at 4°. We thus find for carbonic anhydrase $k_r = 1.5 \times 10^{-9}$ sec^{-1}, 670 times smaller than the rate for carboxypeptidase. Since the ratio of association constants is about 10^{-3}, the values of k_f for the two systems should be roughly equal. It thus appears that the tighter binding of Zn(II) in carbonic anhydrase is primarily a reflection of a markedly slower rate of dissociation of the metal ion.

Davis[30] concluded that since EDTA does not remove Zn(II) from carbonic anhydrase at neutral pH, the association constant must be much larger than that of Zn^{2+} with EDTA. From the rate data reported here and the values of K_{assoc} given by Lindskog and Nyman[5] one can conclude that the half-time for the dissociation of Zn(II) from carbonic anhydrase is 5–6 *years* at pH 8, 0.1 *M* ionic strength, 25°. Obviously one cannot expect any detectable reaction with EDTA at pH 7–8 in experiments lasting only several days or weeks. Similarly, no appreciable exchange[31] with $^{65}Zn^{2+}$ was observed at pH 7.4, 0°, during 32-days' exposure.

Although the experiments reported in this paper have not lead to sharply defined ideas concerning the binding of Zn(II) in carbonic anhydrase, they indicate clearly that the binding is unique in several respects as compared with the interactions of Zn^{2+} with small ligands of known structure. As mentioned earlier, it is probable that the Zn(II) in the enzyme is constrained to tetrahedral coordination geometry whereas the coordination is usually octahedral with the small ligands. Furthermore one would expect the location of the Zn(II) in a cleft in the protein molecule to have a pronounced effect on the rate of its combination with the protein.

(28) D. E. Goldsack, W. S. Eberlein, and R. A. Alberty, *J. Biol. Chem.*, **241**, 2653 (1966).

(29) B. L. Vallee and J. E. Coleman in "Comprehensive Biochemistry," Vol. 12, M. Florkin and E. H. Stotz, Ed., Elsevier Publishing Co., New York, N. Y., 1964, p 204.

(30) R. P. Davis, *J. Am. Chem. Soc.*, **81**, 5674 (1959).

(31) R. Tupper, R. W. E. Watts, and A. Wormall, *Biochem. J.*, **50**, 429 (1952).