Inactivation of Bovine Carbonic Anhydrase by Dipicolinate: Kinetic Studies and Mechanistic Implications†

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ABSTRACT: A pathway for the loss of zinc by carbonic anhydrase to the chelating agent dipicolinate has been deduced through an analysis of pH–rate, inhibition, and equilibrium profiles. This information was supplemented by titration of the reaction products and further studies on the dipicolinate inhibition of *p*-nitrophenyl acetate and CO_2 activities. The rate of inactivation by dipicolinate increases 500-fold on changing the pH from 8 to 6 and then reaches a plateau with $pK_a = 5.7$. Similarly, the pH profile of the inhibition constant, obtained so as to include only the initial binding equilibrium, displayed essentially the same pH dependence, with a pK_a of 6.0 in H_2O (corresponding to a pK_a of 5.7 in 9:1 H_2O/acetone). It appears that zinc loss occurs through a protonated enzyme–dipicolinate complex of $pK_a \simeq 6.0$ and that the subsequent metal exchange is rate limiting. In addition, the inactivation rate, the initial and final equilibria, and the esterase and hydrase activities all had pH profiles exhibiting an inflection point around pH 7.0. These data require two enzymatic ionizations exhibiting pK_a values of 6.0 and 7.0, with dipicolinate binding to the most acidic form. The effect of the cosolvent acetone and the value of pK_{a1} are in agreement with an active site imidazole. The kinetic constants obtained for the initial inhibition were $K_{i1} = 20$ mM, $pK_{a1}^{enz} = 6.0$, and $pK_{a2}^{enz} = 7.0$, while for the subsequent metal exchange step $k_f^{exch} = 0.15\ s^{-1}$, $K_{exch} = 2.0 \times 10^{-4}$ M, and $k_r^{exch} = 7.5 \times 10^2\ M^{-1}\ s^{-1}$. The inactivation equilibrium constant $K'_{eq} = [Zn(dipic)_2^{2-}][\sum E_{apo}]/([\sum E_{holo}][dipic^{2-}]^2)$ increases 600-fold on changing the pH from 8 to 6 while, correspondingly, the plot of log K'_{eq} vs. pH changes slope from −2 to −1, the inflection occurring at pH 7.0. Titrations of the metallo complexes formed from the reaction [Zn(dipic)(OH_2) and $Zn(dipic)_2^{2-}$] demonstrated no ionizations in this pH region. The pH profile of K'_{eq} is thus consistent with a protonation of the holoenzyme at pH 7.0 and a protonated apoenzyme species $E_{apo}(H^+)$ that differs from the holoenzyme by +1 proton at pH <7.0 and +2 protons at pH >7.0. The apoenzyme does not display the expected titration of the three unligated histidine residues created by the loss of zinc but adds a single proton instead.

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the interconversion of CO_2 and bicarbonate (Pocker & Sarkanen, 1978). Early investigators have noted that the catalytically essential metal ion is not noticeably exchanged with radioactive zinc nor is EDTA[1] effective in its removal (Tupper et al., 1951). Consequently, the unprecedented speed of zinc removal by 2,6-pyridinedicarboxylate dianion (dipicolinate) is of much interest, both from the standpoint of convenience in the preparation of apo and alternate metal-substituted carbonic anhydrases and for the mechanistic information inherent in the dipicolinate–enzyme reaction. This paper expands the latter field, viewing dipicolinate as a powerful active site probe, which, by its very rapidity, can furnish direct information about the metal chelating properties of carbonic anhydrase, information that is uncomplicated by steric factors or by multiple competing processes, as in the case of other chelating agents (Romans et al., 1978; Kidani & Hirose, 1977).

Previously, the kinetics of the inactivation of carbonic anhydrase by dipicolinate at pH 6.6 were characterized and compared with those exhibited by 1,10-phenanthroline (Pocker & Fong, 1980). This paper derives a mechanism for the zinc removal reaction based, in the main, on the pH dependence of the inhibition constant K_i(obsd), the second-order inactivation rate constant k'', and the observed equilibrium constant for zinc removal K'_{eq}. The close fit of the kinetic scheme to the data demonstrates that the inactivation process can be divided into two parts, the initial binding equilibrium, which gives rise to the pH dependence of the reaction, and the zinc exchange portion, which is rate determining and largely pH independent.

Two new insights about the protonation behavior of carbonic anhydrase arise from this study. First, the pH profile for dipicolinate binding strongly implies that there is an ionization of $pK_a = 6.0$ in the active site of carbonic anhydrase that is not visible in the catalysis of common substrates under normal conditions but that controls the binding of the dipicolinate dianion. Second, analysis of the pH dependence of the equilibrium constant, K'_{eq} shows that the three imidazole residues produced on zinc loss do not titrate in pH region 6–8, but instead, the apoenzyme adds a single proton as a consequence of metal ion removal. This shows that the three unligated imidazoles formed in the apoenzyme must still interact very strongly, possibly through a bridged hydrogen-bonded network. The observations clarify the nature of the protein–metal ion interaction and have implications for both catalysis and anionic inhibition.

Experimental Procedures

Apparatus. All ultraviolet and visible absorbances were monitored on a Cary 210 spectrophotometer, thermostated at 25.0 ± 0.05 °C with an external constant temperature bath. Measurements of pH were made with an Orion 801 or a custom-made pH meter that was designed and constructed by the departmental electronics shop. The latter instrument was normally attached to a computer-controlled titrator and employs exceptionally sensitive electronics.

The stopped-flow apparatus, thermostated at 25.0 ± 0.05 °C, is also computer controlled. Modifications were made to a Durrum instrument to decrease the mechanical and electrical

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PNPA, *p*-nitrophenyl acetate; $dipic^{2-}$, dipicolinate dianion; NMR, nuclear magnetic resonance.

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response time and to reduce the magnitude of the mechanical shock wave (Pocker & Fong, 1980). Rather than utilize the oscilloscope display of the absorbance as the primary source of data, we obtained greatly increased accuracy for linear rates by the conversion of the signal into digital form and by performing least-squares calculations on the resulting data points.

Titrations were performed on a modified titration assembly consisting of a Radiometer ABU1 autoburet controlled by a PDP 8/E minicomputer. This apparatus operates through a large stepping motor that can be programmed to step a discrete number of steps at a specified frequency. Each step corresponds to the addition of 1/16000 mL of titrant. Values of pH, for the generation of titration curves or for feedback in the pH-stat mode of operation, are obtained through A/D conversion of a ±5-V output from our custom-made pH meter. The titration cell itself is thermostated and features variable-speed mechanical stirring and nitrogen purging. The software written to control the apparatus employs variable delay times, multiple-averaged pH readings, and control of the stirrer motor so that the observed pH electrode response is not dependent on solution inhomogeneities or stirring effects. Both the titration curve and its derivative may be displayed, analyzed, and plotted.

Materials. Imidazole (Eastman), *p*-nitrophenol, and *p*-nitrophenyl acetate (Aldrich) were purified by multiple recrystallizations. Potassium hydrogen phthalate (Aldrich Gold Label) was dried at 110 °C before use. Other materials were reagent grade and were used without further purification. The method of standardization of bovine carbonic anhydrase (Worthington) is described previously (Pocker & Fong, 1980). For the titration of the enzyme, however, this carbonic anhydrase was further purified by dialysis for several weeks in deionized, degassed water and dialysis tubing (M_r cutoff 12000–14000), the final dialyses being done over a bed of ion-exchange resin. Dithizone (Fisher) was purified according to published procedures (Kagi & Vallee, 1958). Spectral-grade acetone (Aldrich) was used for the most demanding titrations, but reagent grade was found to be satisfactory for the kinetics. Sodium hydroxide solutions were made by dilution of a saturated solution and protected from atmospheric carbon dioxide.

Buffers. *N*-Methylimidazole and γ-picoline buffers were made by the addition of sulfuric acid to the free base. Phosphate buffers were mixtures of K_2HPO_4 and NaH_2PO_4, while sodium malonate and malonic acid were components of the malonate buffers. All buffers were equalized in ionic strength with sodium sulfate. Metal-free buffers for dipicolinate kinetics were extracted 5 times with 0.02% dithizone/CCl_4. Although the alkaline buffers (pH 7.4–8.0) are more efficiently extracted by dithizone (Irving, 1977), the solubility of the material in the aqueous phase is greater and required extensive back-washing with CCl_4.

Kinetic Procedures. Titrations were performed at 25.0 °C and 50–70 mL/min nitrogen flushing, with sodium hydroxide titrant that was standardized with KHP and constant-boiling HCl solutions. The amount of residual acidity in the water (assumed to be due to CO_2) was determined by direct titration and was subtracted from all subsequent experiments. The titration program took data points at 0.1 pH intervals, allowing an electrode settling time of 8–10 s before reading the pH. With solutions of exceptionally low ionic strength, however, slower electrode response required an increase in settling time to 30–40 s.

The procedure for studying esterase activity was also used for monitoring a portion of the inactivation rates. Metal-free buffer (with or without $dipic^{2-}$) and an acetone solution of PNPA were mixed 9:1 (v/v), and the resulting absorbance changes were monitored at 348 (the isosbestic point) or 400 nm. Enzymatic reactions were initiated by the injection of small aliquots of enzyme into cells, the observed rate being corrected by the subtraction of the nonenzymatic one. At pH values lower than 5.0, a reproducible time-dependent increase in turbidity caused by enzyme denaturation (Riddiford, 1964) was observed in solutions containing buffer, acetone, and carbonic anhydrase.

A stopped-flow apparatus was used to monitor the kinetics of PNPA hydrolysis on a short enough time scale to detect inhibition caused by the initial binding of dipicolinate to the enzyme. In one syringe, PNPA dissolved in acetone or acetonitrile was added to an aqueous dipicolinate solution so that the organic solvent comprised 20% of the volume. The other syringe contained the enzyme dissolved in buffer. Upon a 1:1 mixing in the instrument, a 9:1 H_2O/organic solvent solution was obtained, and the absorbance change at 400 nm was recorded.

Stopped-flow kinetics of the enzymatic hydrolysis of CO_2 in 10% acetone utilized enzyme dissolved in 9:1 buffer/acetone in one syringe and CO_2 dissolved in 9:1 H_2O/acetone in the other. *p*-Nitrophenol was used to monitor the pH shift caused by the hydration reaction and was present in the buffer syringe. The substrate solution was made from dilutions of a saturated solution of CO_2 in 9:1 H_2O/acetone that was calibrated by the $BaCO_3$ precipitation method (Skoog & West, 1969). Buffer factors were obtained for each buffer by the addition of small known aliquots of standardized H_2SO_4 or NaOH and by measurement of the resultant absorbance change. The buffer system used for stopped-flow kinetics, upon 1:1 dilution in the apparatus, was 0.040 M in total phosphate; ionic strength $\mu = 0.14$. Similar procedures without the organic cosolvent were used to monitor the inactivation reaction.

Calculations. Rates of dipicolinate inactivation were calculated by quantifying the effect of dipicolinate on the enzymatic hydrolysis of PNPA. The concentration of the substrate was much less than K_m, so that its presence did not affect the rate of inactivation. On the other hand, the K_i for dipicolinate was included in the rate expression (eq 1 and 2). Equations

$$E_{holo} + dipic^{2-} \overset{K_i}{\rightleftharpoons} E_{holo}(dipic^{2-}) \quad (1)$$

$$E_{holo}(dipic^{2-}) \xrightarrow{k_2} E_{apo} + Zn(dipic) \quad (2)$$

1 and 2 were integrated with respect to time and the results expressed as the ratio of absorbances for PNPA hydrolysis containing no dipicolinate and the identical hydrolysis with dipicolinate present (eq 3). The second-order rate constant

$$Abs^{ref}/Abs^{dipic} = (1 + [dipic^{2-}]/K_i)\left[\frac{k''_{obsd}[dipic^{2-}]t}{1 - \exp(-k''_{obsd}[dipic^{2-}]t)}\right] \quad (3)$$

k''_{obsd} is related to the kinetic scheme by eq 4. In eq 3, the

$$k''_{obsd} = k_2/(K_i + [dipic^{2-}]) \quad (4)$$

first term $(1 + [dipic^{2-}]/K_i)$ is responsible for the initial inhibition observed at small values of t (see Figure 1B).[2] In

[2] When $k''_{obsd}[dipic^{2-}]t \ll 1$, the exponential term in eq 3, which is of the form e^{-x}, can be approximated by $1 - x$ so that

$$e^{-k''_{obsd}[dipic^{2-}]t} \simeq 1 - k''_{obsd}[dipic^{2-}]t$$

As a result, eq 3 reduces to $Abs^{ref}/Abs^{dipic} = 1 + [dipic^{2-}]/K_i$, an expression that describes the inhibition by dipicolinate observed at small values of t (Figure 1B).

eq 4, the term K_i + [dipic^{2-}] is responsible for the saturation effect observed with dipicolinate (see Figure 1C). On subsequent kinetic analysis, the saturation effect was corrected for by employment of a conventional second-order rate constant, $k'' = k_2/K_i$.

Analysis of the dipicolinate inactivation as monitored by PNPA hydrolysis was performed with the aid of an interactive curve-fitting computer program. The absorbance data were compared to the theoretical line defined by eq 3, and the best value of k''_{obsd}(av) was selected. Inclusion of the time lag between the actual mixing of reactants and the start of the recording of data points leads to significantly more accurate results.

The equilibrium constant of the inactivation reaction was calculated from the residual PNPA activity of enzyme–dipicolinate solutions. These solutions utilized the same buffers as the other kinetic experiments but contained only 9% acetone. The other 1% was injected as PNPA substrate solution to initiate the enzyme hydrolysis. Measurements were made after inactivation periods exceeding 10 half-lives.

To avoid possible confusion in regard to the species actually present, we defined the apparent equilibrium constant for dipicolinate inactivation K'_{eq} by adopting a more general nomenclature (eq 5). The present definition is the more prag-

$$K'_{eq} = [\sum E_{apo}]_{eq}[Zn(dipic)_2^{2-}]_{eq}/([\sum E_{holo}]_{eq}[dipic^{2-}]^2) \quad (5)$$

matic at higher dipicolinate concentrations ($\geq 2 \times 10^{-4}$ M) and incorporates the association constant of dipic^{2-} with Zn(dipic), $K'_{eq} = (3.4 \times 10^5)K_R$ [see Pocker & Fong (1980)]. If [dipic^{2-}] $\gg$ [E_0] and contamination by Zn^{2+} ions is negligible, then $[\sum E_{apo}]_{eq} = [Zn(dipic)_2^{2-}]_{eq}$. If one uses the rate of reaction with a substrate as a measure of $[\sum E_{holo}]_{eq}$ and notes that [E_0] = $[\sum E_{holo}]$ + $[\sum E_{apo}]$, K'_{eq} can be expressed with only [E_0], [dipic$^{2-}_0$], and f_{act}, the fraction of enzymatic substrate activity remaining after dipicolate inactivation (eq 6). The conditions

$$K'_{eq} = (1 - f_{act})^2[E_0]^2/([E_0]f_{act}[dipic^{2-}_0]^2) \quad (6)$$

for the use of eq 6 rather than the more complete expression previously reported were satisfied in the experiments reported here.

Results

Dipicolinate Inactivation. (A) Initial Inhibition and Saturation. The inhibition of CO_2 hydration by dipicolinate at pH 6.8 ([dipic^{2-}] = 125 mM) and 6.6 ([dipic^{2-}] = 25 mM) is shown in Figure 1A. The ionic strengths (equalized in the reference kinetics with Na_2SO_4) were 0.42 and 0.17, respectively, the difference causing the change in pH. The inhibition observed in both cases was competitive (K_i = 0.20 and 0.12 M) and in agreement with earlier work (at pH 6.6, K_i = 0.13 with HCO_3^-, μ = 0.35, 25 °C; Pocker & Fong, 1980).

Further studies supplementing a preliminary report on PNPA inhibition (Pocker & Fong, 1980) have shown that the K_i for dipicolinate is nearly identical if the substrates HCO_3^-, CO_2, and PNPA are used. The inhibition of PNPA hydrolysis by 120 mM disodium dipicolinate, pH 6.65, μ = 0.42, in 9:1 H_2O/acetone is shown in Figure 1B and yields K_i = 0.14 M. This value of K_i was used to evaluate the expression (1 + [dipic^{2-}]/K_i) in eq 3.

With the initial term in eq 3 thus accounted for, the dependence of k''_{obsd} on [dipic^{2-}] could be demonstrated. Figure 1C is a plot of the variation of V_{obsd} vs. [dipic^{2-}] at pH 6.65, μ = 0.42, in 9:1 H_2O/acetone ($V_{obsd} = k''_{obsd}[E_0][dipic^{2-}]$). The kinetic constants k_2 (0.035 s^{-1}) and K_i (0.12 M) were initially determined from a double-reciprocal plot ($1/k''_{obsd}$ vs. $1/[dipic^{2-}]$) and optimized through a fitting of the theoretical equation (dashed line in Figure 1C) to the velocity data. The value of the corrected second-order rate constant $k'' = k_2/K_i$ was 0.30 M^{-1} s^{-1}.[3]

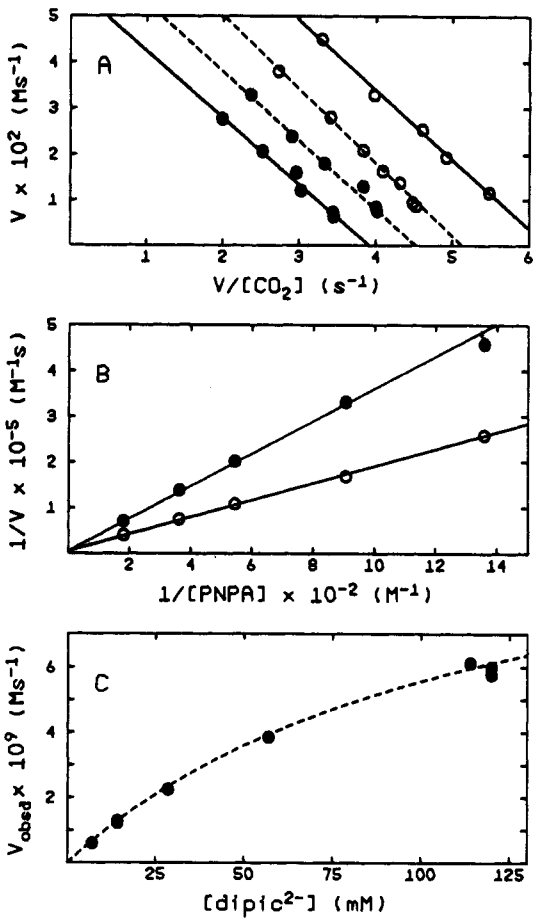

FIGURE 1: (A) Plot of inhibition by dipicolinate of the carbonic anhydrase catalyzed hydration of CO_2. The solid line (—) has [dipic^{2-}] = 125 mM, pH 6.8, μ = 0.42. The dashed line (- - -) has [dipic^{2-}] = 25 mM, pH 6.6, μ = 0.17. Kinetics with dipicolinate (●) are contrasted to the kinetics with Na_2SO_4 (○). (B) Plot of inhibition of PNPA catalysis by 120 mM dipic^{2-}, at pH 6.65, μ = 0.42, 9:1 H_2O/acetone. K_i equals 0.14 M, with dipicolinate data points (●) being compared to Na_2SO_4 data points (○). (C) Plot of observed velocity of inactivation $V_{obsd} = k''_{obsd}[E][dipic^{2-}]$, with [dipic^{2-}] = 3.6–120 mM, [E] = 3.5×10^{-7} M, at pH 6.65, 25 °C, μ = 0.42, 9:1 H_2O/acetone. The dashed line (- - -) is the theoretical curve for k''_{obsd} (see eq 4) with K_i = 120 mM and k_2 = 0.035 s^{-1}.

The variation of the inhibition constant with pH is presented in Table I. K_i was determined from the inhibition of HCO_3^- dehydration, μ = 0.53, 25 °C, in 20 mM phosphate or malonate buffers. Dipicolinate was varied from 142 mM at pH 7.4 down to 8 mM at pH 5.1; in this pH range, the mode of inhibition appeared to be competitive.

(B) pH Profiles. The logarthmic pH–rate profile for the inactivation of carbonic anhydrase by dipicolinate can be divided into three regions with slopes of 0, −1, and −2 (Figure 2). Analysis of the data (in 9:1 H_2O/acetone) revealed that the entire pH profile could be modeled by two successive ionizations, one at pH 5.7 and the other at pH 7.2. The ordinate in Figure 2 utilizes the true second-order rate constant $k'' = k_2/K_i$ (eq 1 and 2) and is corrected for the effects of saturation (Figure 1C). The maximum value for k'', on the basis of the theoretical curve, is 7.5 M^{-1} s^{-1}.

[3] The experiment employed an older batch of carbonic anhydrase and yielded a slightly lower value of k''.

Table I: Dipicolinate Inhibition Constants[a]

pH	K_i(obsd) (M)	log K_i(obsd)
5.1	0.023	−1.64
5.4	0.031	−1.51
6.05	0.048	−1.32
6.3	0.08	−1.1
6.6	0.12[b] (0.13[c])	−0.92
6.8	0.23 (0.20[d])	−0.66
7.0	0.5[e]	−0.30
7.05	0.57	−0.25
7.35	1.2	0.08

[a] μ = 0.53 unless otherwise specified. The data could be fitted to eq 12 by using pK_{a1} = 6.0, pK_{a2} = 7.0, and K_{i1} = 0.020 M. [b] μ = 0.42; 9:1 H_2O/acetone. [c] μ = 0.35; Pocker & Fong, 1980. [d] μ = 0.42; H_2O. [e] μ = 0.17; 9:1 H_2O/acetone.

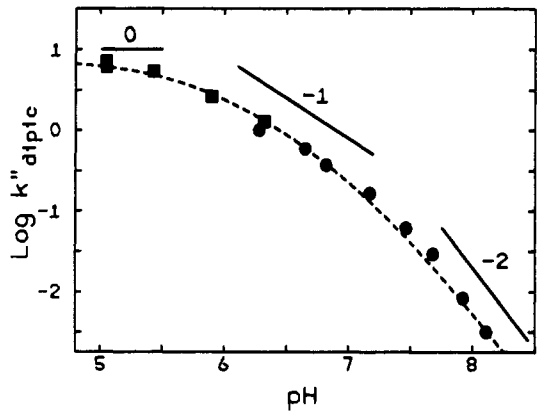

FIGURE 2: Plot of log k'', logarithm of the second-order rate constant for dipicolinate inactivation of carbonic anhydrase at 25 °C, in 9:1 H_2O/acetone. For the data from pH 5 to 7, μ = 0.14–0.15; in the pH range 7.2–8.1, μ = 0.22–0.24 (except μ = 0.45 at pH 7.8). Buffers were 36 mM malonate (■) and phosphate (●). The dashed line (---) is the theoretical curve derived from eq 13, with pK_{a2}^{enz} = 7.2, pK_{a1}^{enz} = 5.7, and k_f^{exch}/K_{i1} = 7.5 M^{-1} s^{-1}. The limiting slopes of the theoretical line are emphasized by the offset solid lines.

The dependence of the equilibrium constant for inactivation, K'_{eq}, on pH is illustrated in Figure 3. Similar to the pH profile of log k'', two distinct regions are observed from pH 6 to 8, with slopes of −1 and −2. The pH profile could be satisfactorily described by two successive ionizations having pK_a values of 5.7 and 7.0. The maximum value of K'_{eq} obtained by a best fit of the theoretical function was 3.4 × 10^3 M^{-1}.

Enzymatic Activity. In order to assess the effect of the solvent mixture (9:1 H_2O/acetone) on the inactivation kinetics, the logarithmic pH profiles for CO_2 hydration and PNPA hydrolysis were determined under identical experimental conditions (μ = 0.14, 25 °C, 9:1 H_2O/acetone) and the results are shown in Figure 4. The data of CO_2 hydration in 9:1 H_2O/acetone are plotted along with similar data obtained in water (Pocker et al., 1981). Both sets of points could be fitted by a function incorporating a pK_a of 7.0 and a maximal value of k_{cat}/K_m equal to 7.9 × 10^7 M^{-1} s^{-1}. The pH dependence resided entirely in the k_{cat} term, and the average value of K_m was 12 ± 1 mM. The kinetics for PNPA hydrolysis displayed a similar pH dependence, the data being fitted by an ionization of pK_a = 7.2 and a maximal value of k_{enz} equal to 6.7 × 10^2 M^{-1} s^{-1}.[4]

[4] We wish to emphasize that the single-ionization model based on a $pK_a \simeq 7$ describes the pH profile for the enzymatic hydrolysis of PNPA only for the usual conditions of buffered systems and moderate ionic strengths. At extremely low salt concentrations, we find the pH profile to be appreciably altered.

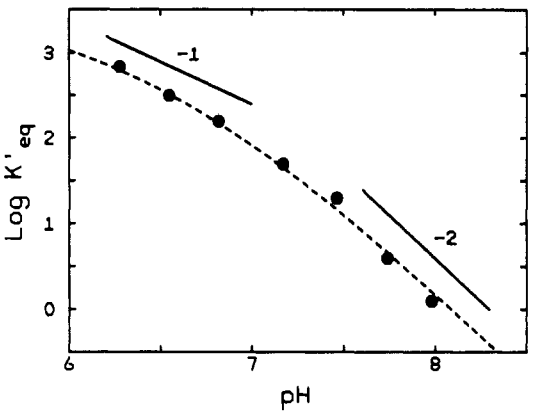

FIGURE 3: Plot of log K'_{eq}, logarithm of the equilibrium constant observed for inactivation of carbonic anhydrase by dipicolinate, from pH 6 to 8, 25 °C, in 9:1 H_2O/acetone, μ = 0.14–0.16. The dashed line (---) is the theoretical curve for two ionizations, with pK_a's of 5.7 and 7.0, and a maximum K'_{eq} value of 3.4 × 10^3 M^{-1}.

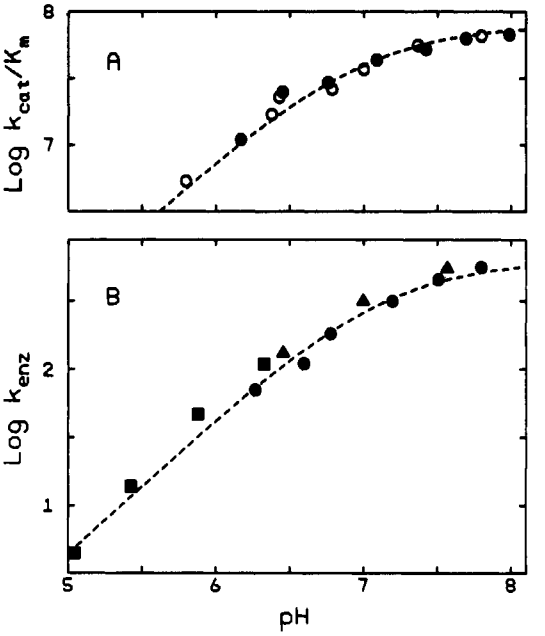

FIGURE 4: (A) Plot of log k_{cat}/K_m vs. pH for the carbonic anhydrase catalyzed hydration of CO_2 in 9:1 H_2O/acetone (●) and water (○). The mixed solvent data were collected in 36 mM phosphate buffer, μ = 0.14, 25 °C. Points in water were obtained from Pocker et al. (1981) and employed 50 mM 3-picoline, phosphate, and 1-methylimidazole buffers. The dashed line (---) is the theoretical curve for a catalyst of pK_a = 7.0 and a maximum rate constant of 7.9 × 10^7 M^{-1} s^{-1}. (B) Plot of log k_{enz} vs. pH for enzymatic hydrolysis of PNPA in 9:1 H_2O/acetone, 25 °C. Buffers were 36 mM malonate (■) and phosphate (●), μ = 0.14, and in addition 25 mM phosphate (▲), μ = 0.18–0.21. The dashed line (---) is the theoretical curve for a catalyst of pK_a = 7.2 and k_{enz}(max) = 6.7 × 10^2 M^{-1} s^{-1}.

Titration. Carbonic Anhydrase and Zinc–Dipicolinate Complexes. The titration curves of carbonic anhydrase, Zn(dipic)(OH_2), and Zn(dipic)$_2^{2-}$ in H_2O and 9:1 H_2O/acetone are shown in Figure 5. In water, carbonic anhydrase (1.8 × 10^{-5} M) loses an average of 8.2 protons when titrated from pH 5.5 to 9.3; 4.2 protons are titrated in the pH range 5.5–6.7, 2.0 protons are titrated from pH 6.7 to 7.8, and the remaining 2 protons are lost above pH 7.8. In the absence of external electrolytes, the initial pH was 5.45, a value that was reproducible after the titration by extensive dialysis. The effect of 10% acetone as a cosolvent on the titration shifts the entire titration curve alkaline by ~0.2 pH unit, but the precise value of this shift is dependent on the number of protons titrated.

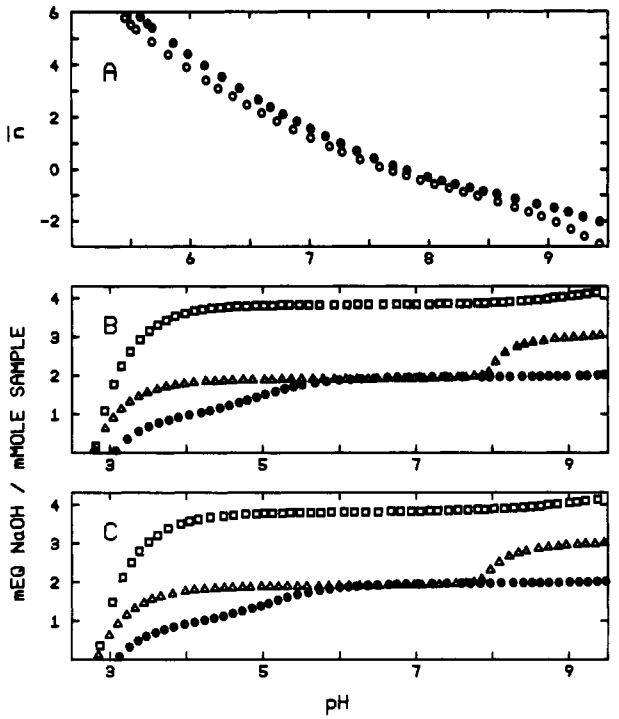

FIGURE 5: (A) Plots of titration of 1.8×10^{-5} M isoionic carbonic anhydrase with NaOH in H_2O (○) and 9:1 H_2O/acetone (●), 25 °C. (B and C) Plots of titration of 1.0 mM dipicolinic acid (●), 1.0 mM Zn(dipic)(OH_2) (△), and 0.50 mM Zn(dipic)$_2^{2-}$ (□) with NaOH, 25 °C. Titrations in water are shown in (B); titrations in 9:1 H_2O/acetone appear in (C).

The $\bar{n}$ method of Bjerrum, as reported by Petitfaux & Fournaise (1972), was used to calculate the number of titrated groups.

The titration curve of Zn(dipic)(OH_2)[5] clearly shows the loss of a proton with a pK_a value of 8.5; the species thus formed, Zn(dipic)$(OH)^-$, is stable to pH 10, the end point of the titration. The change of solvent from water to 9:1 H_2O/acetone does not affect the pK_a, in contrast to other weak acids. The titration of Zn(dipic)$_2^{2-}$, formed by addition of zinc sulfate to dipicolinic acid, displays only the neutralization of the four protons released, implying that the metallo complex is stable from pH 3 to 10, the range of the titration. Since the presence of cosolvent has no effect on either this titration or the titration of a dilute mineral acid (i.e., 1.2×10^{-2} M HCl), the hydrogen ion activity is not affected by the presence of 10% acetone (Bates, 1981). The pK_a for the second ionization of dipicolinic acid is 4.85 in H_2O and 4.93 in 9:1 H_2O/acetone, μ = 0.14, 25 °C, the increased value in the presence of organic cosolvent being in accord with the decrease in dielectric constant (Wynne-Jones, 1933).

Discussion

The inhibition of dipicolinate, as shown in Table I, is strikingly different from classical anionic inhibition. The specific nature of the inhibition implies the existence of a protonated complex E(H$^+$)(dipic) to account for the decrease in K_i(obsd) at acidic pH. While protonation of a preexisting inhibitor complex is a tempting possibility, analysis of this hypothesis requires that K_i(obsd) should then be proportional to $1/[H^+]$, which is contrary to the observed saturation behavior (at pH <5.5, K_i(obsd) ~ 0.02 M). The kinetic scheme fitting the data has an enzymatic protonation of p$K_a \simeq 6.0$, which must precede the binding of dipicolinate. This protonated form of the enzyme (EH$^+$) becomes the predominant enzyme species at pH <5.5 and accounts for the reduced pH dependence of K_i(obsd) in the acidic region. Since the pH profiles for substrate catalysis (Figure 4) display no obvious anomalies at pH ~6, the coexisting protonated enzyme species is nearly identical in catalytic behavior.

Table II: Rate and Equilibrium Constants for Dipicolinate Inactivation[a]

pK_{a1}^{enz} = 6.0[b] (5.7[c])	k_f^{exch} = 0.15 s^{-1} [d]
pK_{a2}^{enz} = 7.0[b] (7.2[c])	K_{exch} = 2.0×10^{-4} M[e]
K_{i1} = 0.20 M	k_r^{exch} = 7.5×10^2 M^{-1} s^{-1} [f]

[a] Based on the equations of Scheme I. [b] μ = 0.53; H_2O. [c] μ = 0.14–0.24; 9:1 H_2O/acetone. [d] Calculated from eq 13 by using the data at acidic pH in Figure 2 and Table I. [e] Calculated from eq 14, with $K_{di} = 3.4 \times 10^5$ (Andregg, 1960). [f] Calculated from $K_{exch} = k_f^{exch}/k_r^{exch}$.

The most likely group exhibiting an ionization of pK_a = 6.0 is a histidyl residue. This pK_a is in accord with earlier NMR and titration evidence (Campbell et al., 1977; Cohen et al., 1972; Nilsson & Lindskog, 1967), and furthermore, favorable electrostatic interaction between the imidazolium cation and the dipicolinate dianion provides a rationale for the binding of dipicolinate predominantly to the most acidic enzyme species. In addition, although the effect of the cosolvent in the 9:1 H_2O/acetone mixture is not large, the kinetics are in qualitative agreement with the effect of the medium on the titration of imidazolium chloride [for ImH$^+$, p$K_a(H_2O)$ – pK_a(9:1 H_2O/acetone) = 0.14; μ = 0.002]. The decreased pK_a in organic cosolvent is seen by a comparison of the pK_a value in Figure 2 (pK_a = 5.7, 9:1 H_2O/acetone) with that reported in Table I (pK_a = 6.0, H_2O) and also by an analysis of previously published inactivation rate constants (Pocker & Fong, 1980). At pH 6.6, the value for k'' in H_2O is 1.1 M^{-1} s^{-1} while in 9:1 H_2O/acetone it is 0.6 M^{-1} s^{-1}, implying that ΔpK_a = p$K_a(H_2O)$ – pK_a(9:1 H_2O/acetone) = 0.26.

The foregoing analysis of the variation of K_i(obsd) with pH suggests that the pH dependence for the removal of zinc from the holoenzyme by dipicolinate is entirely a function of the initial binding steps and that the zinc exchange reaction per se is pH independent. The pH profiles for K_i(obsd), the initial binding step, and k'', the rate constant for zinc removal, are nearly identical. In addition, the pH profiles for both dipicolinate kinetic and substrate catalysis had an inflection at pH 7. On the basis of these observations, a possible model for the loss of enzymatic zinc is shown in Scheme I (eq 7–11).[6]

Scheme I

$$EH^+ \overset{K_{a1}^{enz}}{\rightleftharpoons} E + H^+ \quad (7)$$

$$E \overset{K_{a2}^{enz}}{\rightleftharpoons} EOH + H^+ \quad (8)$$

$$EH^+ + dipic^{2-} \overset{K_{i1}}{\rightleftharpoons} EH^+(dipic) \quad (9)$$

$$EH^+(dipic) \underset{k_r^{exch}}{\overset{k_f^{exch}}{\rightleftharpoons}} E_{apo}(H^+) + Zn(dipic) \quad (10)$$

$$Zn(dipic) + dipic^{2-} \overset{K_{di}}{\rightleftharpoons} Zn(dipic)_2^{2-} \quad (11)$$

[5] For brevity, we have chosen to represent the 1:1 zinc–dipicolinate complex as Zn(dipic)(OH_2), displaying only the ionizable aquo ligand. There is some uncertainty, however, as to the exact number of bound water molecules (Petitfaux & Fournaise, 1972).

[6] Our choice of nomenclature for the ionization that governs the change in carbonic anhydrase activity (p$K_a \simeq 7$; p$K_b \simeq 7$) conforms to the popular zinc aquo/hydroxo hypothesis wherein the ionization is thought to leave a hydroxo ligand attached to the zinc ion in the holoenzyme, EOH (Pocker & Deits, 1981, 1982).

The pH dependence of K_i(obsd), k'', and K'_{eq} can be represented in terms of this scheme by eq 12–14. The theoretical

$$K_i(\text{obsd}) = K_{i1}\left[\frac{K_{a1}^{enz}([H^+] + K_{a2}^{enz}) + [H^+]^2}{[H^+]^2}\right] \quad (12)$$

$$k'' = k_f^{exch}/K_i(\text{obsd}) \quad (13)$$

$$K'_{eq} = K_{exch}K_{di}/K_i(\text{obsd}) \quad (14)$$

lines depicted in Figures 2 and 3 were calculated from these equations and fit the data closely. The data for K_i(obsd) in Table I also agrees well with the predicted pH dependence (eq 12). Table II contains the kinetic parameters for the inhibition and zinc exchange processes, calculated from the fitted theoretical equations.

Qualitative agreement exists between the values for the dipicolinate kinetics reported in this work and earlier reports (Harrington & Wilkins, 1980; Kidani & Hirose, 1980; Romans et al., 1978). In addition, it appears that the inactivation rate constants for chelating agents that actively remove the metal [Zn(II) or Co(II)] from carbonic anhydrase increase with increasing acidity. Likewise, the available binding constants follow this trend. The control of the dipicolinate inactivation kinetics exerted by critical ionizations within the enzyme (reported in this work) may provide a preliminary conceptual framework for the understanding of these other experimental systems.

The pH profile of the equilibrium constant for zinc removal, K'_{eq} (Figure 3), can be analyzed by determining the pH-dependent species participating in the reaction (eq 5). The titration results for $Zn(dipic)_2^{2-}$, $Zn(dipic)(OH_2)$, and dipicolinate show that in the pH range 6–8, these compounds are in a constant protonation state so that the variation of K'_{eq} with pH is caused by differences in protonation between the apo- and holoenzyme species. An inspection of Figure 3 shows that the apo- and holoenzymes differ by one proton at pH <7 and by two protons at pH >7. The one proton ionization centered at pH 7 seems wholly attributable to a group of pK_a = 7.0 present in the holoenzyme (see Figures 2 and 4). Except for this ionization, the two proteins (apo- and holoenzyme) titrate in parallel.

Earlier measurements of the protons released on addition of zinc acetate to apocarbonic anhydrase (Lindskog, 1963) qualitatively support our determination of the proton requirements accompanying apoenzyme formation. Similar to our results, at pH 6, 1.3 protons were released while at pH 9 2.3 protons were given off, the inflection point of the titration occurring roughly around pH 7.8. Although we find the second proton is added at pH 7.0, a pH somewhat more acidic than Lindskog's value, the larger uncertainty in his titrations, the internal consistency of our chelation data, and especially the close match of the pK_a controlling enzymatic activity (Figure 4) with the pK_a affecting the dipicolinate enzyme equilibrium (Figure 2) compel us to favor the catalytic pK_a of 7.0 as controlling the ionization in question.

The observation that from pH 6 to 8 the apoenzyme and holoenzyme titrate in tandem, except for an ionization of pK_a = 7 in the holoenzyme, contrasts sharply with the expected titration of the three unligated histidine residues produced on metal ion removal. In light of this unexpected behavior, it appears that the three imidazoles interact strongly, preferring to share a proton through a hydrogen-bonded network, much as they formerly shared a metal ion. This circumstance may help explain the general failure to detect conformational changes in the enzyme upon loss of zinc. Interestingly, this behavior is mimicked, in part, by active site analogues containing bis(imidazole) and tris(imidazole) groupings in which the residues are covalently bound in close proximity (Drey & Fruton, 1965; Tang et al., 1978). The correct positioning of the ligating histidine residues, as reflected by their unusual pK_a's, may be an important factor contributing to both the unique rapidity of the enzymatic catalysis (Pocker & Deits, 1982) and the conformational stability of the apoenzyme.

Registry No. *p*-Nitrophenyl acetate, 830-03-5; carbon dioxide, 124-38-9; zinc, 7440-66-6; dipicolinic acid, 499-83-2; carbonic anhydrase, 9001-03-0.

References

Andregg, G. (1960) *Helv. Chim. Acta 43*, 414.

Bates, R. (1981) *CRC Crit. Rev. Anal. Chem. 10*, 247.

Campbell, I., Lindskog, S., & White, A. (1977) *Biochim. Biophys. Acta 484*, 443.

Cohen, J., Yim, C., Kandel, M., Gornall, A., Kandel, S., & Freedman, M. (1972) *Biochemistry 11*, 327.

Drey, C. N. C., & Fruton, J. (1965) *Biochemistry 4*, 1.

Harrington, P. C., & Wilkins, R. G. (1980) *J. Inorg. Biochem. 12*, 107.

Irving, H. M. N. H. (1977) *Dithizone*, p 42, The Chemical Society, London.

Kagi, J., & Vallee, B. (1958) *Anal. Chem. 30*, 1951.

Kidani, Y., & Hirose, J. (1977) *J. Biochem. (Tokyo) 81*, 1383.

Kidani, Y., & Hirose, J. (1980) *Biochim. Biophys. Acta 622*, 71.

Lindskog, S. (1963) *J. Biol. Chem. 238*, 945.

Nilsson, A., & Lindskog, S. (1967) *Eur. J. Biochem. 2*, 309.

Petitfaux, C., & Fournaise, R. (1972) *Bull. Soc. Chim. Fr.*, 914.

Pocker, Y., & Sarkanen, S. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47*, 149–274.

Pocker, Y., & Fong, C. T. O. (1980) *Biochemistry 19*, 2045.

Pocker, Y., & Deits, T. L. (1981) *J. Am. Chem. Soc. 103*, 3949.

Pocker, Y., & Deits, T. L. (1982) *J. Am. Chem. Soc. 104*, 2424.

Pocker, Y., Deits, T. L., & Tanaka, N. (1981) in *Advances in Solution Chemistry* (Bertini, I., Lunazzi, L., & Dei, A., Eds.) pp 253–274, Plenum, New York.

Riddiford, L. M. (1964) *J. Biol. Chem. 239*, 1079.

Romans, A. Y., Graichen, M. E., Lochmuller, C. H., & Henkens, R. W. (1978) *Bioinorg. Chem. 9*, 217.

Skoog, D. A., & West, D. M. (1969) *Fundamentals of Analytical Chemistry*, p 310, Holt, Rinehart & Winston, New York.

Tang, C. C., Davalian, D., Huang, P., & Breslow, R. (1978) *J. Am. Chem. Soc. 100*, 3918.

Tupper, R., Watts, R. W. E., & Wormall, G. (1951) *Biochem. J. 50*, 429.

Wynne-Jones, W. F. K. (1933) *Proc. R. Soc. London, Ser. A 140*, 440.