Matthews, R. G., Arscott, L. D., & Williams, C. H., Jr. (1974) *Biochim. Biophys. Acta 370*, 6–38.
McBurney, M. W., & Whitmore, G. F. (1974) *Cell 2*, 183–188.
Poe, M., Breeze, A. S., Wu, J. K., Short, C. R., Jr., & Hogsteen, K. (1979) *J. Biol. Chem. 254*, 1799–1805.
Ratner, S., Blanchard, M., & Green, D. E. (1946) *J. Biol. Chem. 164*, 691–701.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Selhub, J., Savin, M. A., Sakami, W., & Flavin, M. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 312–314.
Taylor, R. T., & Hanna, M. L. (1977) *Arch. Biochem. Biophys. 181*, 331–344.
Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62D*, 185–198.

Kinetics of Inactivation of Erythrocyte Carbonic Anhydrase by Sodium 2,6-Pyridinedicarboxylate†

Y. Pocker* and Conrad T. O. Fong

ABSTRACT: The inactivation of bovine carbonic anhydrase by sodium 2,6-pyridinedicarboxylate (sodium dipicolinate) has been studied at pH 6.6, 25 °C. The catalytically essential zinc ion of the enzyme is removed with unprecedented speed by this chelating agent, producing inert apoenzyme and a zinc dipicolinate complex. This zinc complex rapidly reacts further with dipicolinate, forming the more stable $Zn(dipic)_2^{2-}$ species. The partitioning of zinc ion between enzyme and dipicolinate chelates was measured by separation of the two species using ultrafiltration and determination of their respective zinc concentrations by atomic absorption. The concentration of catalytically active enzyme in the presence of dipicolinate was measured by using either bicarbonate ion or *p*-nitrophenyl acetate as substrate. The rates of disappearance of catalytically active enzyme and appearance of zinc ions in the form of zinc dipicolinate chelates were measured in parallel runs and found to be identical. The exponential decay of enzymatic activity with dipicolinate in excess was analyzed by an integrated rate equation, and the resultant time dependence of the inactivation process was linear for nearly four half-lives. The apparent rate constant, k_{app}, was found to be directly proportional to the dipicolinate concentration. The second-order rate constant for inactivation, $k'' = k_{app}/[\text{dipicolinate}]$, with bicarbonate ion as the substrate was 1.1 M^{-1} s^{-1} at pH 6.6. Inactivation experiments employing *p*-nitrophenyl acetate as the substrate for determining enzymatic activity were performed in 10% v/v acetone. Under these conditions, the results were $k'' = 0.6$ M^{-1} s^{-1} at pH 6.5 and $k'' = 0.3$ M^{-1} s^{-1} at pH 6.9. The equilibrium constant at pH 6.6 for the reaction of enzyme and dipicolinate to produce apoenzyme and $Zn(dipic)_2^{2-}$ calculated from the residual enzymatic activity is $K_{eq} = 1 \times 10^2$ M^{-1}. The formal binding constant of sodium dipicolinate to the enzyme was measured as K_i with bicarbonate and CO_2 as substrates under initial conditions so that no inactivation could occur through loss of zinc. Competitive and noncompetitive behavior were observed, respectively, with $K_i = 0.1$ M for both substrates. The formal activation parameters for the extraction of zinc by dipicolinate at pH 6.6 and 25 °C are $\Delta H^{\ddagger} = 24$ kcal, $\Delta S^{\ddagger} = 22$ eu, and $\Delta G^{\ddagger} = 18$ kcal. By way of comparison, 1,10-phenanthroline, commonly used to produce the apoenzyme, is 5×10^3 slower than dipicolinate at pH 6.6 and 25 °C.

Salts of 2,6-pyridinedicarboxylic acid (dipicolinic acid) are present in large amounts in the spores of microorganisms (Leanz & Gilvarg, 1973; Woodruff et al., 1974). Dipicolinic acid has been studied as an inducer of sporulation (Fukuda et al., 1969), an enzymatic inhibitor (Mann & Byerrum, 1974; Tochikubo, 1974), and a herbicide (Naik et al., 1972). Recently it has been discovered that sodium dipicolinate is able to remove the catalytically essential zinc ion from the metalloenzyme carbonic anhydrase (Kidani et al., 1976).

The present paper reports on the kinetics and mechanism of the reaction between bovine erythrocyte carbonic anhydrase and sodium dipicolinate. Carbonic anhydrase catalyzes the interconversion of bicarbonate and carbon dioxide. Appropriate conditions for the monitoring of bicarbonate dehydration and CO_2 hydration have been delineated in some detail (Pocker & Bjorkquist, 1977; Pocker & Miksch, 1978). Accordingly, the inactivation of carbonic anhydrase by dipicolinate was studied by monitoring these physiological reactions at pH 6.6 and 25 °C. Additional data are presented, utilizing the hydrolysis of the synthetic substrate *p*-nitrophenyl acetate to measure the loss of enzymatic activity (Pocker & Stone, 1967, 1968). 1,10-Phenanthroline, a chelating agent commonly used with carbonic anhydrase for the removal of zinc at acid pH (Lindskog & Malmstrom, 1962), was also studied. We find that 2,6-pyridinedicarboxylate is much more efficient since it reacts 5×10^3 times faster at pH 6.6. This unprecedented speed of zinc removal allowed us to develop a facile procedure for the replacement of the zinc ion in carbonic anhydrase by other metal ions.

The inactivation of carbonic anhydrase by a chelating agent can be visualized as occurring through an initial binding equilibrium followed by a ligand exchange process, whereby the metal ion is transferred from the enzyme to the chelating agent through an intermediate which contains the zinc simultaneously bound to both. A comparison of $\Delta G^{\ddagger}$ for the dipicolinate-assisted removal of enzymatic zinc and the spontaneous dissociation [$(E)Zn \rightarrow E_{apo} + Zn^{2+}(aquo)$] reveals that $\Delta G^{\ddagger}$ is 6 kcal lower for dipicolinate. This substantial

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lowering of the free energy of activation is not the result of a favorable binding equilibrium since initial binding to the enzyme is rather weak but represents a stabilization of the transition state for the exchange process. 1,10-Phenanthroline has the same binding constant to the enzyme as dipicolinate and a near equal association constant with zinc (Sillen & Martell, 1971). The large disparity in their respective inactivation rates ($\Delta\Delta G^{\ddagger} = 5$ kcal)) demonstrates the sensitivity of ligand exchange to the structure of the chelating agent and underscores the uniqueness of dipicolinate. The unusual speed of zinc removal from carbonic anhydrate by dipicolinate may further clarify the metal coordination properties of this enzyme and additionally prove useful in the study of other metalloenzymes.

Materials and Methods

Materials. *p*-Nitrophenol (Eastman) was purified by recrystallization. *N*-Methylimidazole (Aldrich) was distilled under reduced pressure before use and stored in a nitrogen atmosphere at 5 °C. Bovine carbonic anhydrase was obtained from Worthington Biochemicals and a single batch (CA 36N642) was used for all kinetic determinations. The active concentration was determined by the slope of a graph of bicarbonate dehydrase activity vs. [acetazolamide], where [E] $= 5 \times 10^{-7}$ M > K_i (acetazolamide). This concentration was identical with that obtained by the absorbance at 280 nm, with $\epsilon = 54000$. All other chemicals were reagent grade and were used without further purification.

Apparatus. Measurements of pH were made with either an Orion Model 801 or a Beckman Research pH meter. Ultraviolet and visible absorbances were measured on a Beckman DU-2 or Varian 635 spectrophotometer, both of which were thermostated at 25.0 °C. Atomic absorption was determined with a Perkin-Elmer 403 instrument equipped with an HGA-2000 graphite furnace.

The stopped-flow apparatus is a Durrum instrument. Modifications include a novel pneumatic drive system, a sophisticated photomultiplier amplifier, and an A/D interface to a PDP 8/L minicomputer. The drive system was reconstructed for low inertia, and, in addition, a solid-state timer was incorporated into the actuation mechanism so that uniform pressure could be applied for the duration of the reaction. The circuitry constructed for the amplification and normalization of the signal from the photomultiplier tube utilizes two switchable filter constants in order to faithfully track the initial mixing transient and yet provide optimal noise reduction. The output of the amplifier is attached to an A/D converter which, after a mixing-time delay, is sampled 125 times by the computer during the selected observation period. These readings are converted to percent transmittance by comparison with a voltage scale established by 0% *T* (slit closed) and 100% *T* (distilled water) standards. After conversion to absorbance, the least-squares line through them is determined. The slope of this line, representing the change of absorbance during the observation period, is the primary instrumental output. Additional information such as the correlation coefficient of the line (>0.99), the initial absorbance of the reaction mixture, and a visual display of the data on a calibrated storage oscilloscope is also presented to the operator.

Buffer and Substrate Solutions. Distilled deionized water was used for all buffer solutions. *N*-Methylimidazole (NMI)[1] buffers were prepared with sulfuric acid. Phosphate buffers were mixtures of K_2HPO_4 and Na_2HPO_4.

Buffers employed for stopped-flow bicarbonate or CO_2 kinetics consisted of 80 mM NMI, 60 mN H_2SO_4, and 1.00×10^{-4} M *p*-nitrophenol. In the case of stopped-flow PNPA hydrolysis, both 80 mM NMI and 100 mM phosphate buffers were used. These concentrations are lower by a factor of two during the reaction with the substrate because of the 1:1 dilution in the apparatus. The pH of buffers for stopped-flow work was measured both under conditions of use (1:1 dilution) and in the concentrated reactant form, and the differences were found to be negligible.

Buffers used in the conventional spectrophotometric procedure for measuring PNPA hydrolysis were 50 mM in phosphate and contained 10% v/v acetone. This amount of cosolvent caused a pronounced shift in the pH values of the buffer system. All pH values include this effect.

Buffers for the atomic-absorption experiment contained 10 mM NMI and 7.5 mequiv of H_2SO_4. The lack of sodium sulfate and the lower concentration as compared with other buffers reduced the amount of zinc contamination contributed by the buffer components.

Substrate solutions for bicarbonate dehydration contained 12–80 mM $NaHCO_3$ and sufficient Na_2SO_4 so that the ionic strength was 0.2 when diluted 1:1 with buffer solution. CO_2 solutions were made by diluting a saturated CO_2 stock solution maintained at 30.0 °C. Stopped-flow substrate solutions of PNPA were made with 20% v/v acetone, equalizing the ionic strength with Na_2SO_4.

Kinetic Procedure

Zinc Removal. The rate of removal of zinc from the enzyme by dipicolinate was monitored by following the increase in solution of zinc species which were not bound to the enzyme. The separation of zinc–enzyme chelates from dipicolinate–zinc ones was accomplished by an Amicon 202 ultrafiltration apparatus fitted with a UM-2 (1000 mol wt cutoff) membrane. A large volume of *N*-methylimidazole buffer containing both enzyme and dipicolinate was placed in the chamber above the filter. At regular intervals, a small aliquot of the solution was forced through the filter, and the concentration of zinc was determined by atomic absorption at 214 nm. The membrane was shown to be impermeable to enzyme and freely permeable to zinc dipicolinate species. An extensive series of standards was run which demonstrated that neither the enzyme nor the buffer components affected the atomic absorption of zinc in the sample. Careful technique was necessary to ensure a low background level of zinc in the solutions (Struempler, 1973).

Bicarbonate and CO_2. Rates of bicarbonate dehydration or CO_2 hydration were determined on a modified Durrum stopped-flow apparatus at 25.0 °C. The change in pH caused by the reaction ($H^+ + HCO_3^- \rightleftharpoons CO_2 + H_2O$) was monitored by the change in the absorbance of *p*-nitrophenol at 400 nm. The proportionality between A_{400} and $[H^+]$ was independently determined through the addition of known amounts of acid or base. The change in absorbance with time, dA/dt, could thus be converted to $d[H^+]/dt$, the velocity of the reaction. Nonenzymatic rates were subtracted from the observed total velocity. This correction was small (initially <1%) in the case of inactivation kinetics and from 1 to 10% for the great bulk of the equilibrium activity (K_R) measurements. The instrumental parameters were optimized for each experiment to ensure that (1) only the initial linear pseudo-zero-order portion of the reaction was being observed, (2) sufficient resolution was present to accurately determine the observed absorbance change, and (3) the observed absorbance change represented an insignificant pH change in the reaction mixture (<0.05 pH).

[1] Abbreviations used: NMI, *N*-methylimidazole; PNPA, *p*-nitrophenyl acetate; dipic, 2,6-pyridinedicarboxylate (dipicolinate).

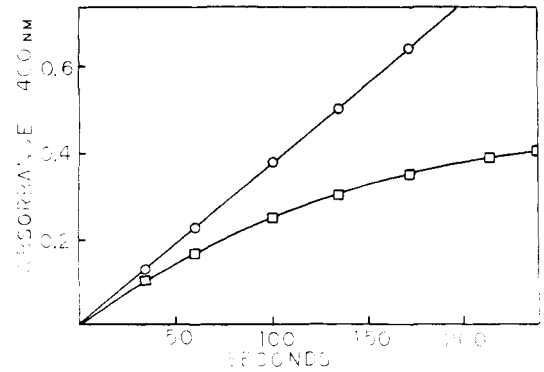

FIGURE 1: Plot of initial hydrolysis of 5.52 mM PNPA by 0.33 μM carbonic anhydrase, pH 7.0, 25 °C, 10% v/v acetone (○). With the addition of 45 mM sodium dipicolinate (□).

Inactivation. The inactivation of bovine carbonic anhydrase was followed by using bicarbonate and PNPA as substrates. In the case of bicarbonate, sodium dipicolinate and the enzyme were mixed together in the buffer solution before insertion into the stopped-flow apparatus. At known times after the initial mixing, the bicarbonate dehydrase activity was determined in the manner previously described. The time required to determine the activity was sufficiently short (30 ms) that the amount of active enzyme remained unchanged. A limitation of the technique, however, was that due to the delay before activity measurements could be made the inactivation by dipicolinate concentrations greater than a few millimolar was too fast to follow.

An alternative method utilizing PNPA extended the inactivation rate measurements to higher dipicolinate concentrations. In this procedure, the zero-order portion of the enzymatic hydrolysis of PNPA was monitored on a conventional spectrophotometer by the appearance of the *p*-nitrophenolate anion at 400 nm (ϵ 19 000). A comparison of the hydrolysis rates in the presence and absence of dipicolinate but with the same initial concentration of enzyme is shown in Figure 1. At any arbitrary time t, the ratio of the slope of tangents drawn to the absorbance plots will give the ratio of the velocities of the two reactions. Since the velocity is proportional to the catalytically active enzyme concentration, the ratio of the velocities was used to calculate the amount of active enzyme remaining in the dipicolinate reaction mixture at time t. A second, more convenient method of analysis was also employed, utilizing the ratio of the absorbances at regular time intervals. The rate equations for the production of *p*-nitrophenolate in the presence and absence of dipicolinate were integrated. The ratio of A_{400} at time t is a function only of the relative concentration of active enzyme and, with the same initial concentration of carbonic anhydrase, is

$$A_{400}^{\text{ref}}/A_{400}^{\text{dipic}} = k''[\text{dipic}^{2-}]t/(1 - \exp(-k''[\text{dipic}^{2-}]t)) \quad (1)$$

The average second-order rate constant of inactivation (k'') over a period of 2–4 reaction half-lives was calculated with the aid of a curve-fitting computer program. The rate constants were identical when the same data were analyzed by using both the tangent and the absorbance ratio methods.

Equilibrium Constants. The initial binding constant of dipicolinate was determined on the stopped-flow apparatus, using both bicarbonate and CO_2 as substrates. In these experiments, dipicolinate was in contact with the enzyme for 10 ms before the measurement of the reaction velocity. The measurement time was kept short enough so that inactivation of the enzyme due to zinc removal was negligible. The rate of the reaction in the presence of dipicolinate was compared to a standard, and the results were expressed as K_i.

The equilibrium constant for the zinc removal reaction was defined as

$$K_R = \frac{[E_{apo}]_{eq}[Zn(dipic)]_{eq}}{[(E)Zn]_{eq}[dipic^{2-}]_0} \quad (2)$$

since in the present work $[dipic^{2-}] > [E_{apo}]_{eq}$, $[dipic^{2-}]_{eq}$ can be approximated by $[dipic^{2-}]_0$. The concentration of active enzyme at equilibrium, $[(E)Zn]_{eq}$, was determined by the bicarbonate dehydrase activity after a time period exceeding ten half-lives of inactivation. In evaluating K_R, the equilibrium constants among Zn^{2+}, Zn(dipic), and $Zn(dipic)_2^{2-}$ determined by G. Andregg were used. Further, the amount of zinc in the buffer components measured by atomic absorption spectroscopy was included. Noting that $[E_{apo}]_{eq} = [(E)Zn]_0 - [(E)Zn]_{eq}$

$$K_R = \frac{[(E)Zn]_0 - [(E)Zn]_{eq}}{[(E)Zn]_{eq}[dipic^{2-}]_0} \times \left[\frac{[(E)Zn]_0 - [(E)Zn]_{eq} + (Zn^{2+})_{buffer}}{1 + 3.4 \times 10^5[dipic^{2-}]_0 + (2.2 \times 10^6[dipic^{2-}]_0)^{-1}}\right] \quad (3)$$

The equilibrium constant between (E)Zn and the more stable $Zn(dipic)_2^{2-}$ complex is $(3.4 \times 10^5)K_R$.

Calculations. The integrated rate equation for the inactivation of carbonic anhydrase by dipicolinate was derived by using the following two relationships:

$$(E)Zn + dipic^{2-} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E_{apo} + Zn(dipic) \quad (4)$$

$$Zn(dipic) + dipic^{2-} \overset{K_{di}}{\rightleftharpoons} Zn(dipic)_2^{2-} \quad (5)$$

The reaction of Zn(dipic) with another dipicolinate (eq 5) was assumed to be fast as compared with the back reaction k_r. As a consequence, the zinc mono- and bis(dipicolinate) complexes are in equilibrium throughout the time course of the reaction. In addition, due to the concentration of chelating agent, the concentration of free zinc ion can be neglected. Thus, one can write

$$[Zn(dipic)]_t/[Zn(dipic)]_{eq} = [E_{apo}]_t/[E_{apo}]_{eq} \quad (6)$$

Combining eq 6 and eq 2, noting from the form of eq 4 that $k_f/k_r = K_R$, and solving for k_r lead to

$$k_r = \frac{k_f[E_{apo}]_t[(E)Zn]_{eq}[dipic^{2-}]_0}{[E_{apo}]_{eq}^2[Zn(dipic)]_t} \quad (7)$$

The rate of the inactivation reaction is

$$\frac{-d[(E)Zn]}{dt} = k_f[dipic^{2-}]_0[(E)Zn]_t - k_r[E_{apo}]_t[Zn(dipic)]_t \quad (8)$$

Substituting the expression for k_r (eq 7) into eq 8 and employing eq 9 yield an expression which may be readily integrated. The result after integration is

$$(E_{apo})_t = [Zn(dipic)]_t + [Zn(dipic)_2^{2-}]_t \quad (9)$$

$$-k_f(dipic)_0t = \frac{([(E)Zn]_0 - [(E)Zn]_{eq})^2}{[(E)Zn]_{eq}^2 - [(E)Zn]_0^2} \times \ln \frac{[(E)Zn]_0^2 - [(E)Zn]_{eq}[(E)Zn]_t}{[(E)Zn]_t - [(E)Zn]_{eq}} + C \quad (10)$$

Reaction Products. The reaction between bovine carbonic anhydrase and sodium dipicolinate was found to be reversible, to produce metal-free enzyme and zinc–dipicolinate chelates

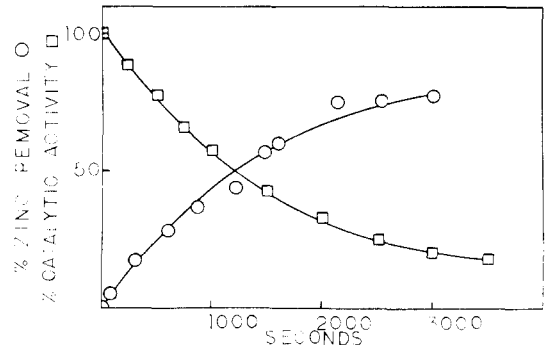

FIGURE 2: Plot of loss of bicarbonate dehydration activity (□) and the removal of zinc (○) after the addition of 0.50 mM sodium dipicolinate at pH 6.6, 25 °C. [(E)Zn] = 1.0 μM for the loss of enzymatic activity and [(E)Zn] = 0.20 μM for the removal of zinc.

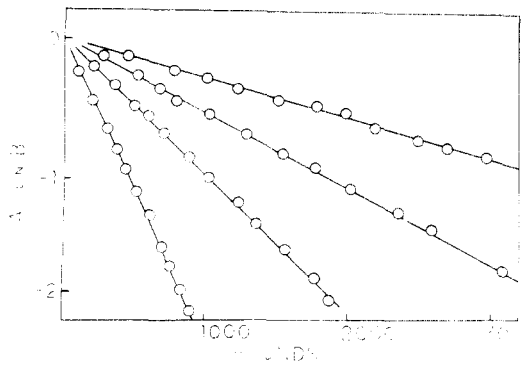

FIGURE 3: Plots of loss of bicarbonate dehydration activity of carbonic anhydrase vs. time due to sodium dipicolinate at 25 °C, I = 0.18, using the integrated equation

$$k' = \frac{1}{t}\left(\frac{([(E)Zn]_0 - [(E)Zn]_{eq})^2}{[(E)Zn]_{eq}^2 - [(E)Zn]_0^2}\right) \times \ln\left(\frac{[(E)Zn]_0^2 - [(E)Zn]_{eq}[(E)Zn]_t}{[(E)Zn]_t - [(E)Zn]_{eq}}\right) \quad (11)$$

The last term is abbreviated in the drawing as $A \ln B$. The vertical axis of the drawing spans three half-lives. Reading from the upper line down: sodium dipicolinate = 0.25, 0.50, 1.0, and 2.15 mM; carbonic anhydrase = 1.0, 1.0, 0.20–1.6, and 1.0 μM.

as the reaction products, and to allow the facile interchange of cobalt for zinc in the active site of the enzyme. First, the inactivation of the enzyme by dipicolinate was reversible upon addition of sufficient zinc to saturate the added dipicolinate. An equilibrium mixture containing 1.0×10^{-6} M enzyme and 5.0×10^{-4} M dipicolinate and possessing 5.6% activity was restored to 93% activity within 0.5 h by the addition of 5.0×10^{-4} M $ZnSO_4$. Full activity was restored by the addition of excess $ZnSO_4$. Second, the rate of appearance in solution of zinc dipicolinate chelates was found to be equal to the rate of loss of enzymatic activity. Figure 2 illustrates this point. The stoichiometry of the reaction is such that the percent of zinc bound to the enzyme (calculated from the percent bicarbonate dehydrase activity) and the percent of zinc appearing in the solution as dipicolinate chelates (calculated from the atomic absorption of filterable zinc) add to give 100% of the zinc initially present. Third, the reaction with dipicolinate can be used to prepare carbonic anhydrase with cobalt as the metal in the active site. Cobalt sulfate was added to samples of zinc–enzyme which had been subjected to multiple dialyses with dipicolinate. After dialyses with water to remove the excess cobalt, a catalytically active preparation was obtained whose visible spectra matched published specifications (Lindskog & Malmstrom, 1962; Hunt et al., 1977).

Inactivation Experiments. The bicarbonate dehydrase activity of bovine carbonic anhydrase declined exponentially with time when mixed with sodium dipicolinate (Figure 2). Calculation of the pseudo-first-order ([dipic^{2-}] > [(E)Zn]) rate constant of inactivation was initially performed with graphs of ln (% activity$_t$ − % activity$_\infty$) vs. time, which is valid if the back reaction can be neglected (eq 4). The results, however, suggested that this approximation was breaking down at the lowest dipicolinate concentrations. The rate expression for the proposed mechanism (eq 4 and 5) could be integrated with the inclusion of the reverse reaction if the velocity of the reaction of Zn(dipic) with dipic^{2-} was significantly greater than with E_{apo}. This supposition was easily justifiable since [dipic^{2-}] > [E_{apo}] and, additionally, the estimated rate constant[2] for the reaction of dipic^{2-} with Zn(dipic) was greater than that found for the reaction with E_{apo}.

The newly integrated equation gave rise to identical values of the second-order rate constant (k'') at high dipicolinate concentrations and abolished the deviation observed at the lower concentrations. Graphs of inactivation data utilizing this equation had good linearity (correlation coefficient ≥ 0.99%) throughout the measurement period, a minimum of two half-lives, and, in some experiments, almost four half-lives (Figure 3). The pseudo-first-order rate constants (k_{app}) obtained from the inactivation data were found to be invariant with respect to enzyme and directly proportional to dipicolinate concentration. The second-order rate constant, $k'' = k_{app}/$[dipic^{2-}], remained constant over a wide range of dipicolinate concentrations. The rate constant at pH 6.6 obtained by the bicarbonate method (1.1 M^{-1} s^{-1}) is in reasonable agreement with that obtained at pH 6.55, 10% v/v acetone, employing *p*-nitrophenyl acetate (0.6 M^{-1} s^{-1}). At pH 6.9, 10% acetone, k'' was 0.3 M^{-1} s^{-1} with PNPA. The range of dipicolinate concentrations was 0.25–2.0 mM with HCO_3^- at pH 6.6, 1.2–45 mM with PNPA at pH 6.5, and 1.2–220 mM with PNPA at pH 6.9.

Equilibrium Constants. The initial binding equilibrium between dipicolinate and carbonic anhydrase was determined by the extent of inhibition of CO_2, HCO_3^-, and PNPA. Competitive inhibition was observed with bicarbonate (K_i = 0.13 M) and noncompetitive inhibition was observed with CO_2 (K_i = 0.15 M). On the other hand, PNPA was not observably inhibited by 45 mM dipicolinate at pHs ranging from 6.5 to 7.5.

The average value of K_R, the equilibrium constant for the enzyme–dipicolinate reaction (eq 2), was $3 \pm 1 \times 10^{-4}$. This resulted from 32 determinations with [(E)Zn] = 0.20–2.0 μM and [dipic^{2-}] = 0.05–2.0 mM, which produced amounts of inactivation ranging from 40 to greater than 99.5%. The constancy of the equilibrium constant over a wide range of concentrations supports the proposed scheme for the reaction.

Temperature Dependence. The temperature dependence of the inactivation reaction was measured by monitoring PNPA hydrolysis. At pH 6.5 from 20 to 30 °C the reaction was found to double in rate for each increase of 5 °C. The formal activation parameters are $\Delta H^\ddagger$ = 24 kcal, $\Delta S^\ddagger$ = 22 eu, and $\Delta G^\ddagger$ = 18 kcal.

1,10-Phenanthroline. Experiments similar to those described for dipicolinate gave a second-order constant at 2.4×10^{-4} M^{-1} s^{-1} for the inactivation of carbonic anhydrase by 1,10-

[2] The Ni(II) monodipicolinate complex has a rate constant of 2.4×10^4 M^{-1} s^{-1} for the formation of the (dipic^{2-})$_2$ complex (Cassat & Wilkins, 1968). The analogous rate constant for the zinc complex was estimated from the behavior of other ligands to be at least two orders of magnitude faster (Hague, 1977).

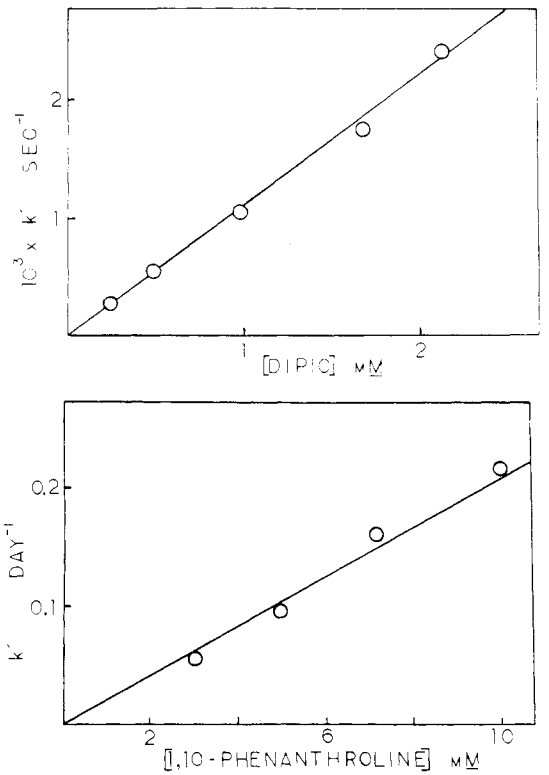

FIGURE 4: (Top) Plot of pseudo-first-order rate constant (k') of inactivation by dipicolinate vs. [dipicolinate] at pH 6.6, 25 °C. (Bottom) Plot of pseudo-first-order rate constant (k') of inactivation of 1,10-phenanthroline vs. [1,10-phenanthroline] at pH 6.6, 25 °C.

phenanthroline at 25 °C, pH 6.6. As depicted in Figure 4, this rate constant was independent of the concentration of the chelating agent from 3.0 to 10 mM. K_i of 1,10-phenanthroline measured with bicarbonate as the substrate was approximately 0.1 M.

Discussion

The formal initial binding equilibrium of dipicolinate to carbonic anhydrase was measured by inhibition studies under conditions before the loss of zinc could occur. The competitive and noncompetitive behavior observed, respectively, with bicarbonate and CO_2 as substrates and the constancy in the value of K_i (0.1 M) were similar to the inhibition properties of the halides (Pocker & Tanaka, 1978) and paralleled the behavior of acetate ion, which was concurrently studied. In view of this, the most likely binding site M^{-1} the dipicolinate dianion is, in accord with other anions, at the zinc ion of the enzyme. Catalysis of the hydration of the pyridine aldehydes by carbonic anhydrase (K_m = 0.1 M) would imply that effective association between the enzymatic zinc and appropriate pyridine derivatives occurs (Pocker & Meany, 1967).

The rate of removal of zinc ion from the enzyme by dipicolinate was found to obey classical second-order kinetics. Both bicarbonate and PNPA were used to monitor the dipicolinate reaction, and comparable rate constants were observed for the inactivation (1.1 and 0.6 M^{-1} s^{-1}). In the case of bicarbonate, the characterization of the zinc removal reaction as second order does not conflict with the observation of an initial binding equilibrium since the experimentally accessible dipicolinate concentrations were too small to cause saturation effects. Likewise, with PNPA, the observed initial inhibition constant and the lack of saturation effects are self-consistent, albeit slightly different in absolute magnitude than with bicarbonate. The difference in reaction media (10% acetone with PNPA) may be responsible for the observed deviations.

Table I: Zinc Removal from Carbonic Anhydrase by Dipicolinate and 1,10-Phenanthroline[d]

chelator	$K^{-1}_{binding}$ (M)	k_f (M^{-1} s^{-1})	rel rate[c]
	pH 6.6, 25 °C[a]		
dipicolinate	0.13	1.1	4.6×10^3
1,10-phenanthroline	0.08	2.4×10^{-4}	
	pH 5.0, 0 °C[b]		
dipicolinate	0.0043	3.2×10^{-1}	2.5×10^1
1,10-phenanthroline	0.0026	1.3×10^{-2}	

[a] This work; $K^{-1}_{binding}$ obtained by inhibition studies and k_f by the loss of bicarbonate dehydration activity. [b] Kidani & Hirose, 1977; $K^{-1}_{binding}$ and k_f were obtained from kinetic analysis of the loss of PNPA hydrolysis activity. [c] $k_{f,dipic}/k_{f,1,10\text{-phenanthroline}}$. [d] Binding parameters for the separate chelators are available in studies by Romans et al., 1978 (1,10-phenanthroline, pH 5.5, 4 °C; $K^{-1}_{binding}$ ~0.03 M; our analysis of the data therein) and Kidani et al., 1976 (dipicolinate, pH 7.0, 0 °C; $K^{-1}_{binding}$ = 0.01 M). In addition, work on carboxypeptidase at pH 7.0, 25 °C (Billo, 1979), has shown that, similar to the results reported in this paper, both 1,10-phenanthroline and dipicolinate exhibit no observable saturation in their rate of zinc removal.

The equilibrium constant for the reaction of carbonic anhydrase with dipicolinate to form the apoenzyme and a Zn(dipic) complex was measured over a wide range of enzyme and dipicolinate concentrations and found to be constant. From this measurement ($K_R = 3 \times 10^{-4}$) and the association constant of zinc with dipicolinate (Andregg, 1960) a value of 1×10^{10} M^{-1} was calculated for the association constant of zinc with the apoenzyme. This agrees with the value of 2×10^{10} M^{-1} at pH 6.6 obtained from measurements of zinc dissociation and association (Romans et al., 1978) and demonstrates that the presence of dipicolinate does not alter the affinity constant of the apoenzyme for zinc. The concentration of dipicolinate was sufficiently high that the predominant zinc–dipicolinate reaction product was the $Zn(dipic)_2^{2-}$ complex, which has an equilibrium constant for formation from enzyme and dipicolinate of 1×10^2 M^{-1}.

The comparison of the rate constants of zinc removal for 1,10-phenanthroline and dipicolinate studied under identical conditions is presented in Table I. The difference in rate between the two chelating agents is considerably larger under the conditions of the present study than at acidic pH and low temperatures. Kidani & Hirose (1977) have reported that dipicolinate is 30 times faster than 1,10-phenanthroline, whereas in the present study the difference is 5×10^3. The high discrimination between the two chelating agents by the enzyme in the less strenuous conditions of this study underscores the uniqueness of dipicolinate. In addition, both dipicolinate and 1,10-phenanthroline show strong saturation effects in the rate of zinc removal at pH 5.0, 0 °C, whereas none were observed at pH 6.6, 25 °C. In view of these differences, it appears that the metal-removal reaction may be a sensitive probe of the environment of the metal in a metalloenzyme.

Both dipicolinate and 1,10-phenanthroline in excess combine with carbonic anhydrase at pH 6.6, 25 °C, in a pseudo-first-order reaction, resulting in the ligand exchange of the zinc ion of the enzyme. The initial binding equilibria between chelator and enzyme, measured by inhibition methods, are nearly equal and rather weak (0.1 M) for both reagents. The large difference in the observed rates of zinc removal, therefore, seems attributable to the relative rates of reaction of the initial enzyme–chelator complex to give apoenzyme and a zinc

chelate. This is noteworthy because dipicolinate and 1,10-phenanthroline have nearly identical first and second affinity constants for zinc ion (Sillen & Martell, 1971) and neither affects the equilibrium constant for the spontaneous dissociation of zinc from the holoenzyme (this work; Lindskog & Malmstrom, 1962).

It is difficult to compare the pseudo-first-order rates of these ligand-assisted dissociations with the so-called "spontaneous dissociation" of zinc from the enzyme because the latter rate, normally assumed to be first order, may in fact be pseudo-first-order due to the participation of water (solvent) in the reaction. A pragmatic comparison of the differences among these processes can be made if the former are defined relative to a 1 M standard state and the latter is assumed to be first order. A $\Delta G^{\ddagger}$ of about 24 kcal can be calculated for the rate of the spontaneous dissociation by using the association rate constant (Henkens & Sturtevant, 1968) and the equilibrium constant. Upon comparison with the $\Delta G^{\ddagger}$ of 18 kcal found for dipicolinate and the $\Delta G^{\ddagger}$ of 23 kcal calculated for 1,10-phenanthroline, it can be seen that dipicolinate is much more efficient.

References

Andregg, G. (1960) *Helv. Chim. Acta 52*, 414.

Billo, E. J. (1979) *J. Inorg. Biochem. 10*, 331.

Cassatt, J. C., & Wilkins, R. G. (1968) *J. Am. Chem. Soc. 90*, 6045.

Fudaka, A., Gilvarg, C., & Lewis, J. (1969) *J. Biol. Chem. 244*, 5636.

Hague, D. N. (1977) in *Molecular Biology, Biochemistry, and Biophysics* (Pecht, I., & Rigler, R., Eds.) Vol. 24, Springer-Verlag, New York.

Henkens, R. W., & Sturtevant, J. M. (1968) *J. Am. Chem. Soc. 90*, 2669.

Hunt, J. B., Rhee, M. J., & Storm, C. B. (1977) *Anal. Biochem. 79*, 614.

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

Kidani, Y., Hirose, J., & Koike, H. (1976) *J. Biochem. (Tokyo) 79*, 43.

Leanz, G., & Gilvarg, C. (1973) *J. Bacteriol. 144*, 455.

Lindskog, S., & Malmstrom, B. G. (1962) *J. Biol. Chem. 237*, 1129.

Mann, D. F., & Byerrum, R. U. (1974) *J. Biol. Chem. 249*, 6817.

Naik, M. N., Jackson, R. B., Stoakes, J., & Swaby, R. J. (1972) *Soil Biol. Biochem. 4*, 313.

Pocker, Y., & Meany, J. E. (1967) *J. Am. Chem. Soc. 89*, 631.

Pocker, Y., & Stone, J. T. (1967) *Biochemistry 6*, 668.

Pocker, Y., & Stone, J. T. (1968) *Biochemistry 7*, 4139.

Pocker, Y., & Bjorquist, D. W. (1977) *Biochemistry 16*, 5698.

Pocker, Y., & Miksch, R. R. (1978) *Biochemistry 17*, 1119.

Pocker, Y., & Tanaka, N. (1978) *Science 199*, 907.

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

Sillen, L. G., & Martell, A. E. (1971) *Stability Constants of Metal Ion Complexes*, Special Publication No. 25, The Chemistry Society, London.

Struempler, A. W. (1973) *Anal. Chem. 45*, 2251.

Tochikubo, K. (1974) *J. Bacteriol. 117*, 1017.

Woodruff, W. H., Spiro, T. G., & Gilvarg, C. (1974) *Biochem. Biophys. Res. Commun. 58*, 197.

Effects of Phospholipid Fatty Acid Composition and Membrane Fluidity on the Activity of Bovine Brain Phospholipid Exchange Protein†

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ABSTRACT: The interaction of bovine brain phospholipid exchange protein with membranes has been investigated as a function of membrane phospholipid fatty acid composition. Single bilayer vesicles were prepared by sonication, centrifugation, and molecular sieve chromatography and were used as acceptor membranes in the exchange protein catalyzed transfer of phosphatidylinositol from rat liver microsome donor membranes. For the series egg phosphatidylcholine and dioleoyl-, and dielaidoyl-, and dimyristoylphosphatidylcholine, initial rates of phosphatidylinositol transfer were highest with the two cis-unsaturated species and lowest with the saturated species, the trans-unsaturated species being intermediate. A progressive decrease in transfer rate was noted with vesicles containing a mixture of egg phosphatidylcholine and dimyristoylphosphatidylcholine as the molar proportion of the latter phospholipid increased. Apparent Michaelis constants for the interaction between exchange protein and different vesicle preparations decreased in the order saturated > trans unsaturated > cis unsaturated. Maximum velocities were independent of fatty acid composition. The fluorescence polarization of diphenylhexatriene in vesicle preparations also decreased in the same order, under conditions well above the thermotropic gel to liquid-crystalline phase transition of all phospholipids studied. These results suggest that the fatty acid composition, the degree of unsaturation, and in particular the hydrocarbon fluidity of the membrane are important determinants in the activity of bovine brain phospholipid exchange protein.

In recent years the physicochemical characterization of biological and artificial membranes has been advanced through the use of phospholipid exchange proteins. Among the applications which have been described are the insertion of spectroscopic probes into membranes, the determination of membrane topography, and the measurement of transbilayer mobility (Rothman & Dawidowicz, 1975; Barsukov et al., 1967; Bloj & Zilversmit, 1976; Shaw et al., 1977; Brophy et al., 1978; Sandra & Pagano, 1978; van den Besselaar et al., 1978; DiCorleto & Zilversmit, 1979). Each of these depends upon selective associations between exchange proteins and transferable phospholipid molecules, such as the specificity of bovine liver exchange protein for PtdCho[1] (Kamp et al., 1977).

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