Plant Carbonic Anhydrase. Properties and Bicarbonate Dehydration Kinetics†

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ABSTRACT: Spinach carbonic anhydrase subjected to gel chromatography revealed two active fractions. One corresponds to the previously identified hexameric form of the enzyme while the second transient fraction has a molecular weight corresponding to a dimeric form. The dehydrase activity of the enzyme with respect to HCO_3^- was investigated using a stopped-flow technique. Values of the Michaelis-Menten kinetic parameters K_m and k_{cat} ($\equiv k_2$) were determined as a function of pH between 6.2 and 7.6 in phosphate and *N*-methylimidazole buffers. Values of K_m remained essentially constant around 34 mM in *N*-methylimidazole buffers, but increased significantly in phosphate buffers as the pH decreased. The behavior of the turnover number k_{cat} with respect to pH was found to be consistent with a titratable activity linked group having an apparent pK_a of 7.7 as was shown earlier for CO_2 hydration catalyzed by this enzyme (Pocker, Y., & Ng, J. S. Y. (1973) *Biochemistry 12,* 5127), but with dehydrase activity depending on the acidic form. In *N*-methylimidazole buffers as the pH decreased the value of k_{cat} increased toward a high plateau value of 1.4×10^5 s^{-1} in the pH region below 7.2. In phosphate buffers the value of k_{cat} increased continuously as the pH decreased, reaching a value of 3.4×10^5 s^{-1} at the lowest pH value studied, 6.23. These values were calculated per mole of monomer of molecular weight 26 500. The corresponding values of k_{cat} calculated per mole of hexameric enzyme of molecular weight 160 000 are 8.7×10^5 and 20.1×10^5 s^{-1}, respectively. For both buffer systems the value for log (k_{cat}/K_m) was constant in the plateau region below pH 7.2 and declined for pH values above 7.2 approaching a line whose slope was approximately one. It appears that the acidic species in phosphate buffers, $H_2PO_4^-$, is acting to further increase both the turnover number k_{cat} and the formal enzyme-HCO_3^- dissociation constant. These results are discussed in relation to the possible physiological roles of plant carbonic anhydrase.

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) activity in plants was first observed by Neish (1939). However, unlike the more extensively studied carbonic anhydrases isolated from mammalian erythrocytes (several detailed reviews are available; see, for example, Coleman, 1971; Lindskog et al., 1971; Pocker & Sarkanen, 1978), those from higher plants have only recently been isolated and characterized. These include spinach (Rossi et al., 1968; Pocker & Ng, 1973, 1974; Jacobson et al., 1975), parsley (Tobin, 1968, 1970), pea leaves (Atkins et al., 1972b; Kisiel & Graf, 1972), and lettuce leaves (Walk & Metzner, 1975) as well as *Tradescantia albiflora* Kunth (Atkins et al., 1972b).

The carbonic anhydrases isolated from higher plants appear to consist of two types. One type is found in monocotyledon species and has a suggested molecular weight of 42 000 (Atkins et al., 1972b). The other type, found in dicotyledon species, possesses a hexameric structure and contains 6 g-atoms of zinc per molecule. The molecular weight of this enzyme has been reported to be near 180 000. In some cases, the existence of isozymes has been reported (Graham et al., 1971; Atkins et al., 1972a; Walk & Metzner, 1975).

It is only in relatively recent years that serious inquiry has been directed toward elucidating the exact physiological role of the enzyme in plants (Graham et al., 1971; Everson, 1971; Werden & Heldt, 1972; Randall & Bouma, 1973; Jacobson et al., 1975). In dicotyledon species, carbonic anhydrase appears to be localized exclusively within the chloroplast (Graham et al., 1971; Werden & Heldt, 1972; Jacobson et al., 1975; Walk & Metzner, 1975). Differential centrifugation studies have shown that carbonic anhydrase activity is not associated with either the chloroplast envelope membrane or the lamellar membranes and hence must reside within the chloroplast stroma (Poincelot, 1972; Jacobson et al., 1975). Proposed explanations of the physiological role of carbonic anhydrase must take cognizance of these facts.

All of the components in the reaction catalyzed by carbonic anhydrase (eq 1)

$$CO_2 + 2H_2O \rightleftharpoons H_3O^+ + HCO_3^- \qquad (1)$$

are obviously of fundamental importance when one considers that the chloroplast is the site of the carbon dioxide assimilating reactions of plants. In particular, within the chloroplast, the bicarbonate ion has been cited as having several important roles: (1) stabilization and possible activation of the primal carbon dioxide fixating enzyme in C_3 plants, ribulose-1,5-diphosphate carboxylase (Laing et al., 1975; see, however, Lorimer et al., 1976); (2) stimulation of the Hill reaction, well-documented, but recently more clearly delineated as acting at or near the oxygen evolving site of photosystem II (Stemler & Govindjee, 1973a,b, 1974; Stemler et al., 1974); and (3) stimulation of photophosphorylation (Batra & Jagendorf, 1965) perhaps due to bicarbonate mediated effects on the reversible ATPase known as coupling factor 1 (Nelson et al., 1972). In light of the continuing ambiguity surrounding the physiological role of carbonic anhydrase and the recognition that the bicarbonate ion may exert a controlling influence at several sites within the chloroplast, we deemed it important to examine in detail the interaction between the spinach enzyme and bicarbonate. We present here a kinetic study of the

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enzyme-catalyzed dehydration of bicarbonate as a function of both pH and buffer composition.

Experimental Section

Materials. *p*-Nitrophenol (Eastman) was purified by sublimation under vacuum. *N*-Methylimidazole (Aldrich) was purified by distillation under reduced pressure. Bio-Gel A-0.5m was purchased from Bio-Rad Laboratories. All other chemicals were either analytical or reagent grade and were used without further purification.

Apparatus. All pH measurements were made using an Orion Model 801 digital pH meter. For gel chromatography, two 2.5 × 40 cm columns (Pharmacia) in series were used. Elution by gravity was regulated by employing a constant pressure Mariotte eluent flask. Additional apparatus and procedures have been described previously (Pocker & Ng, 1973).

Methods: Preparation of Spinach Carbonic Anhydrase. Carbonic anhydrase was isolated and purified as previously described (Pocker & Ng, 1973) with the following modifications. After fractionation using ammonium sulfate, the entire enzyme sample was passed through a short bed of DEAE-cellulose to remove those contaminants which bind irreversibly to the ion-exchange medium. Afterward this portion of DEAE-cellulose was discarded and the enzyme sample was chromatographed as described previously on a larger DEAE-cellulose column.

The presence of a yellow contaminant lead to the appendage of an additional step to the procedure. The pooled and concentrated enzyme recovered after ion-exchange chromatography was introduced onto an agarose gel column, 2.5 × 80 cm (Bio-Rad Bio-Gel A-0.5m). The flow rate was approximately 20 mL per h. In some cases the gel column evidenced the surprising ability to resolve the carbonic anhydrase activity into two separate fractions, which were collected separately and reduced in volume using an Amicon ultrafiltration cell. The appearance of the second fraction appeared to be related to the length of time the enzyme was allowed to remain in dilute solution after column chromatography using DEAE-cellulose. The elution volumes of the two fractions were compared with those of trypsin, pepsin, ovalbumin, bovine serum albumin, and yeast alcohol dehydrogenase.

Buffer Components and Solutions. Deionized distilled water was used exclusively for the preparation of buffer solutions. The total buffer concentration was maintained constant at 0.04 M throughout the pH range studied. Phosphate buffers were prepared by accurate mixing of stock solutions of Na_2HPO_4 and NaH_2PO_4 to obtain the desired buffer ratio. *N*-Methylimidazole buffers were prepared by acidifying appropriate stock solutions of *N*-methylimidazole with sulfuric acid. All buffers contained 0.02 M NaCl and 0.002 M EDTA to stabilize the enzyme. Furthermore, all buffers were maintained at a constant ionic strength of 0.26 by addition of appropriate amounts of Na_2SO_4.

Bicarbonate substrate solutions were prepared by dissolving carefully weighed samples of $NaHCO_3$ in deionized distilled water that had been degassed by boiling for a minimum of 30 min. All substrate solutions were maintained at a constant ionic strength of 0.14 by addition of appropriate amounts of Na_2SO_4, so that after 1:1 solution under stopped-flow conditions the combined ionic strength of the buffer (0.26/2) and the substrate (0.14/2) would be constant at 0.2. The resultant substrate solutions were stored in air-tight syringes and were discarded if not used within 2 days of preparation.

Kinetic Procedure and Calculations. The kinetics of the uncatalyzed and enzyme catalyzed dehydration of HCO_3^- were studied using a Durrum-Gibson stopped-flow spectrophotometer. The reaction was initiated by the rapid mixing of a solution of bicarbonate with the chosen buffer (with or without enzyme) in a 1:1 ratio in the stopped-flow spectrophotometer. The uptake of protons by the dehydration reaction results in a change in pH which may be monitored by observing the change in absorbance of an appropriate indicator. The change in absorbance with time is related to the actual rate of dehydration by a buffer factor. An excellent account of the theory and experimental limitations of this stopped-flow pH indicator technique can be found in the work of Khalifah (1971).

For this work, *p*-nitrophenol was the indicator used for both *N*-methylimidazole and phosphate buffers, since the experimentally determined pK_a' of 7.03 for *p*-nitrophenol (at an ionic strength of 0.2) was close to both that of phosphate at 6.75 and *N*-methylimidazole at 7.19. As explained by Khalifah for the indicator stopped-flow technique the indicator and buffer should have pK_a values as close together as possible. Absorbance changes were monitored at 400 nm.

In some cases a small correction factor was applied for the essentially instantaneous uptake of protons by species present in the bicarbonate substrate solutions. A small amount of bicarbonate is converted to carbonic acid according to eq 2.

$$HCO_3^- + H_3O^+ \rightarrow H_2CO_3 + H_2O \qquad (2)$$

In addition, a small amount of carbonate ion initially present is converted into bicarbonate according to eq 3.

$$CO_3^{2-} + H_3O^+ \rightarrow HCO_3^- + H_2O \qquad (3)$$

The uptake of protons results in a small change in the initial pH of the reaction mixture which varies with substrate concentration. This results in a change in the initial absorbance of the reaction mixture. To offset this change, very small aliquots of acid were added to the buffer in proportion to the strength of the bicarbonate solution being used to produce an equivalent change in absorbance in the opposite direction. The efficiency of this correction was monitored by checking the consistency of the initial absorbance with varying substrate concentrations.

The output from the photomultiplier tube of the stopped-flow spectrophotometer was fed into a PDP-8L computer. The time of observation of the reaction was kept small compared with the half-life of the reaction. Under these conditions the substrate concentration remained essentially invariant and the change in absorbance with time appeared linear (pseudo-zero-order). Values of dA/dt for each run were calculated by a least-squares method computer program written by Mr. Don Moore in our laboratories.

The program allows the operator to select desired data output and to set variable experimental parameters. Data output selected for this work were the value of dA/dt, the initial absorbance and the correlation coefficient for each run. In addition to the observation time, a variable mixing time was set so that the computer would wait a predetermined time after the reaction was initiated before observing data. Effects due to the turbulent mixing of the two solutions could thus be obviated. The mixing time was generally on the order of 20 ms.

Values of dA/dt were converted to values of V_{dehyd} by multiplication by a buffer factor ($\equiv d[H^+]/dA$). Buffer factors, calculated from the formula given by Gibbons & Edsall (1963), were found to agree with experimentally determined values generally to within 5%. Each value of V_{dehyd} was usually the average of six kinetic runs. For most runs the correlation coefficient was better than 0.99.

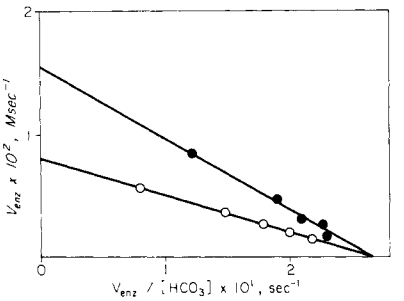

FIGURE 1: Representative Woolf-Augustinsson-Hofstee plots of spinach carbonic anhydrase catalyzed dehydration of HCO_3^- at 25.0 °C. (●) With 0.02 M phosphate, 0.010 M NaCl, 0.001 M EDTA, $[E]_0 \approx 8.71 \times 10^{-8}$ M, pH 6.82; (○) 0.02 M N-methylimidazole, 0.010 M NaCl, 0.001 M EDTA, $[E]_0 = 9.79 \times 10^{-8}$ M, pH 6.88.

Since the bicarbonate concentration remained essentially invariant under the conditions of the experiment, it was included in calculations of the ionic strength. Background rates without enzyme were performed, V_{buffer}, and these nonenzymatic rates were subtracted from those in the presence of enzyme, $V_{enz} = V_{dehyd} - V_{buffer}$. Although solutions of $NaHCO_3$ contain small quantities of CO_2 (approximately 1% of the bicarbonate concentration), the correction for the back reaction was negligible. Values of K_m and k_{cat} ($\equiv V_{max}/[E]$) were obtained from plots of V_{enz} vs. $V_{enz}/[HCO_3^-]$. A typical plot is shown in Figure 1.

Results

The separation of the carbonic anhydrase activity into two fractions by the gel chromatography was unexpected. The predominant fraction had an elution volume corresponding to a protein with an apparent molecular weight of 200 000. This apparent molecular weight should be considered at best as a measure of the upper limit for the true molecular weight. Gel chromatography provides comparative measurements of the apparent Stokes' radii of molecules. For spherical proteins the apparent molecular weight will correspond well with the true molecular weight, but for nonspherical proteins the apparent Stokes' radius will correspond to a spherical protein larger in size than the protein being observed and the apparent molecular weight will be too large (Andrews, 1970).

We have noted that, although the molecular weight for dicotyledon plant carbonic anhydrase is generally reported as near 180 000, in fact the molecular weight determinations for the hexameric form of the enzyme have varied widely and the molecular weight determinations for single subunits have averaged somewhat less than 30 000 (Tobin, 1970; Atkins et al., 1972a; Kisiel & Graf, 1972; Pocker & Ng, 1973). In the present paper we have used a value of 160 000 for the molecular weight of the hexamer and a value of 26 500 for the molecular weight of a single subunit.[1]

The second fraction eluted from our gel column was transient, in that it was not always detectable, and its specific activity varied from zero to one-quarter of the specific activity

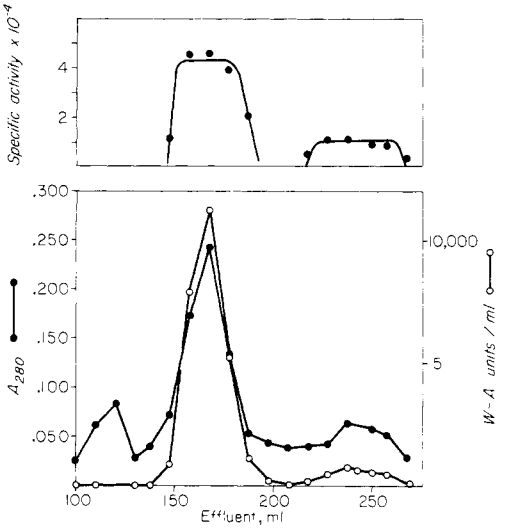

FIGURE 2: Results of reloading previously collected predominant fraction on Bio-Gel A-0.5m gel column. Buffer is 0.02 M phosphate-0.1 M NaCl-0.001 M EDTA, pH 6.82. Specific activity was approximated by the ratio of enzymatic activity, measured in Wilbur-Anderson (W-A) units as applied by Rickli et al. (1963), to the absorbance at 280 nm.

of the predominant fraction. The elution volume of the second fraction corresponds to a protein with an apparent molecular weight of 55 000, or approximately twice the molecular weight of a single subunit of the hexameric form of the enzyme. The elution of the yellow contaminant overlapped the elution of the minor fraction.

When reintroduced separately onto the gel column, both fractions were again resolved into two separate fractions. In each case most of the activity was eluted at the same point as that collected previously, and in each case some activity was observed to elute at a point corresponding to the other fraction. When the minor fraction was reintroduced onto the gel column, approximately one-quarter of the protein had recombined to form the more active high molecular weight enzyme. Similar results were observed when the predominant fraction was reintroduced onto the gel column as is shown in Figure 2. Subsequent efforts to handle the enzyme more carefully by reducing its exposure to conditions of high dilution and temperature fluctuation were rewarded by the virtual disappearance of the minor fraction. For kinetic studies the more carefully handled predominant fraction was used exclusively.

The kinetics of bicarbonate dehydration, both uncatalyzed and enzyme catalyzed, was studied over the pH region 6.2 to 7.6. Since a fresh solution of bicarbonate normally has a pH of around 8.25, mixing it with buffers whose pH values exceed 7.6 does not lead to changes in substrate concentration great enough to allow accurate kinetic measurements. For the nonenzymatic acid catalyzed dehydration reaction, a value for $k_{HCO_3^-}^{H_3O^+}$ of 5.56×10^4 M^{-1} s^{-1} was obtained, in excellent agreement with the value of 5.5×10^4 M^{-1} s^{-1} reported by Gibbons & Edsall (1963).

The catalysis of the dehydration of HCO_3^- by carbonic anhydrase appears to follow the Michaelis-Menten mechanism, as indicated by the linearity of plots of V_{enz} vs. $V_{enz}/[HCO_3^-]$. This method of plotting, superior to Lineweaver-Burk plots (Doud & Riggs, 1965), produces a line whose slope is equal in magnitude to the Michaelis-Menten constant K_m, and whose intercept is equal to V_{max} (see Figure 1). It is, of course, recognized that the rate constants determined experimentally may be considerably more complex than those indicated by this simplified scheme.

[1] Sedimentation equilibrium experiments kindly performed by Mr. Donald Senear of the Department of Biochemistry on our enzyme preparation revealed a homogeneous protein with a molecular weight of 157 000. After the present paper was submitted for publication we were able to calculate a molecular weight of 26 500 per subunit based on the amino acid composition of spinach carbonic anhydrase reported in a manuscript submitted for publication by M. Kandel, A. G. Cornall, D. C. Cybulsky, & S. I. Kandel, who kindly allowed us a preview. Our experimental molecular weight of 157 000 is in excellent agreement with the rounded value of 160 000 calculated for the hexamer.

TABLE I: The Influence of Buffer Composition and pH on the Michaelis-Menten Parameters of HCO_3^- Dehydration Catalyzed by Spinach Carbonic Anhydrase.

Buffer[a]	pH	$V_m \times 10^2$ [b] ($M\ s^{-1}$)	$K_m \times 10^3$ [b] (M)	$k_{cat}^{hexamer} \times 10^5$ [c] (s^{-1})	$k_{cat}^{monomer} \times 10^5$ [c] (s^{-1})	$\log(k_{cat}^{monomer}/K_m)$
Phosphate	6.23	2.31	98.0	20.1	3.4	6.535
	6.44	2.30	95.2	20.1	3.4	6.546
	6.64	1.68	68.4	14.7	2.5	6.554
	6.82	1.63	62.6	14.2	2.4	6.578
	7.03	1.10	44.0	9.6	1.6	6.560
	7.22	0.81	36.9	7.0	1.2	6.501
	7.40	0.42	26.6	3.7	0.6	6.361
Phosphate[d]	6.23	3.23	58.9	14.1	2.3	6.601
	6.44	2.45	53.8	10.7	1.8	6.520
	6.64	2.53	46.3	11.0	1.8	6.599
	6.82	2.22	46.2	9.7	1.6	6.543
	7.03	2.02	46.7	8.8	1.5	6.497
	7.22	1.41	35.5	6.2	1.0	6.460
	7.40	0.88	29.2	3.8	0.6	6.342
	7.62	0.49	30.5	2.5	0.4	6.135
N-Methylimidazole	6.35	11.1	38.3	8.7	1.4	6.574
	6.51	11.2	34.4	8.7	1.4	6.651
	6.88	8.0	29.9	6.2	1.0	6.540
	7.07	7.1	33.8	5.5	0.9	6.436
	7.28	6.1	35.0	4.7	0.8	6.351
	7.37	2.5	34.6	1.9	0.3	5.971

[a] Total buffer concentration under stopped-flow conditions was kept at 2.0×10^{-2} M. Na_2SO_4 was added to maintain an ionic strength of 0.2. In addition, 0.01 M NaCl and 0.001 M EDTA were added to stabilize the enzyme. [b] Each value is obtained from a plot of five different concentrations of HCO_3^- and each V_E value used in the plot is generally the average of six runs. [c] In evaluating the expression $k_{cat} = V_{max}/[E]$, values for [E] were expressed both as per mole of hexamer of molecular weight 160 000 and per mole of monomer of molecular weight 26 500. The latter values facilitate a more direct comparison with the erythrocyte enzyme and were used exclusively in the text unless otherwise indicated. [d] This phosphate buffer system incorporates the correction factor for proton uptake by bicarbonate substrate solutions as described in the text.

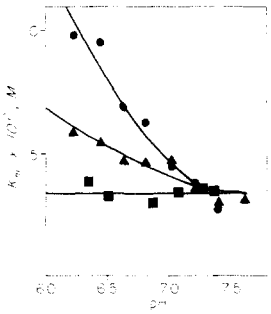

FIGURE 3: Values of K_m for the spinach carbonic anhydrase catalyzed dehydration of HCO_3^- as a function of pH at 25.0 °C and ionic strength 2×10^{-1}. Total buffer concentration, 2.0×10^{-2} M: (●) phosphate; $[E]_0 = 8.71 \times 10^{-8}$ M; (▲) phosphate incorporating correction factor (see text), $[E]_0 = 1.74 \times 10^{-8}$ M; (■) *N*-methylimidazole, $[E]_0 = 9.79 \times 10^{-8}$ M. In addition, buffers contained 0.01 M NaCl and 0.001 M EDTA to stabilize enzyme.

The variations in the Michaelis-Menten parameters K_m and k_{cat} ($\equiv V_{max}/[E]$) are tabulated in Table I. Values of k_{cat} were calculated both per mole of hexamer and per mole of monomer. Throughout this paper we have used the latter values to facilitate comparison with the more extensively studied erythrocyte carbonic anhydrase. There are two sets of data for phosphate buffers. The set reflecting the generally lower values for K_m and k_{cat} incorporates the correction factor for the essentially instantaneous proton uptake by some components of the bicarbonate substrate solution. Practically speaking, this correction seemed to have little effect on the observed kinetics. The trend toward lower values for the Michaelis-Menten parameters is not due to this correction, which should act in the opposite direction, but is attributed to the lower activity of the enzyme sample since a period of 8 months separates the two sets of data for phosphate buffers.

It can be seen in Figure 3 that the value of K_m in *N*-methylimidazole buffers is essentially constant over the pH region studied. It is also apparent that as one moves toward lower pH values there is a pronounced increase in the value of K_m in phosphate buffers.

The variation in k_{cat} with pH is shown in Figure 4. It can be seen that the changes in K_m with pH are reflected in concurrent changes in the value of k_{cat}. This is especially evident if one examines the change in $\log(k_{cat}/K_m)$ with pH, as shown in Figure 5. Despite significant differences in the values observed for K_m and k_{cat} over the pH range 6.2 to 7.0, the value of $\log(k_{cat}/K_m)$ appears to remain constant. In the pH region above 7.2 the value of $\log(k_{cat}/K_m)$ appears to decline and approaches a line with a slope of approximately 1 at the highest pH values examined. Closer examination of phosphate as a buffer constituent showed that when the pH was held constant at 7.03 both K_m and k_{cat} appeared to increase linearly with increasing phosphate concentration, as shown in Figure 6.

Discussion

Previous work in these laboratories demonstrated that when spinach carbonic anhydrase was incubated with sodium dodecyl sulfate for a period of 2 min, successive polyacrylamide gel electrophoresis revealed multiple states of aggregation of subunits ranging from monomers to hexamers in increments of one subunit (Pocker & Ng, 1973). In addition, Atkins et al. (1972a) reported that when they performed gel electrophoresis on crude extracts from a number of higher plants some dicotyledon species showed an active band corresponding to a low molecular weight protein. The band was not observed consistently and the proportion of this protein varied between extracts

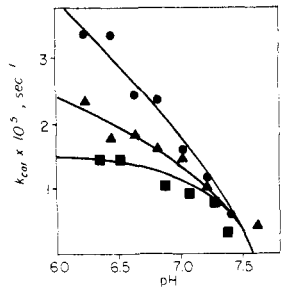

FIGURE 4: Values of k_{cat} as a function of pH. Conditions are the same as in Figure 3.

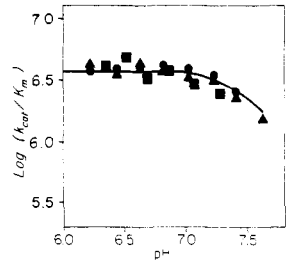

FIGURE 5: Values of $\log(k_{cat}/K_m)$ as a function of pH. Conditions are the same as in Figure 3.

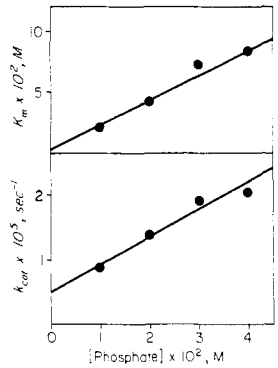

FIGURE 6: Effects of phosphate concentration on K_m and k_{cat} at pH 7.03. Ionic strength maintained at 0.2 using Na_2SO_4. In addition, 0.01 M NaCl and 0.001 M EDTA added to stabilize enzyme.

of a single species. Subsequent characterization by sodium dodecyl sulfate gel electrophoresis of more highly purified enzyme from the dicotyledon *Pisum sativum* revealed two proteins having molecular weights of 28 000 and 56 500, corresponding respectively to monomers and dimers (Atkins et al., 1972b).

We believe that our data are best explained by assuming that at least two states of aggregation of subunits, corresponding to hexamers and dimers, are catalytically active. In addition, the two states are interconvertible to some extent, as shown by their reintroduction onto the gel column where each active fraction was again resolved into two active forms.

It is probable that the active dimeric form observed represents a breakdown product of the native enzyme. However, the accumulation of data presented above could be explained by the existence of variable states of aggregation of subunits within the plant, possibly mediated by both temperature and ionic strength effects. The apparent lower specific activity of the dimeric form suggests that conditions favoring its formation within the plant could constitute a form of regulatory control of carbonic anhydrase activity. Conditions which might favor the formation of the dimeric form are being actively investigated in our laboratories.

At the most elementary level, the enzyme catalyzed dehydration of bicarbonate can be viewed in terms of the Michaelis–Menten scheme as shown in eq 4.

$$EH^+ + HCO_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH^+\cdot HCO_3^- \xrightarrow{k_2} E + CO_2 + H_2O \quad (4)$$

In *N*-methylimidazole buffers we find K_m, defined as $(k_{-1} + k_2)/k_1$, to be a constant with a value of 34 mM over the pH range 6.2 to 7.4, while the overall enzymatic activity is governed by k_{cat} ($\equiv V_{max}/[E] = k_2$) which is approximately constant at lower pH values and decreases in magnitude above pH 7. These results are in agreement with earlier work showing that for CO_2 hydration catalyzed by spinach carbonic anhydrase the pH profile for the turnover number k_{cat} is consistent with a titratable activity linked group in the enzyme having an apparent pK_a of 7.7 (Pocker & Ng, 1973). Similarly, a recent investigation of the dehydration of bicarbonate catalyzed by bovine erythrocyte carbonic anhydrase showed that K_m for bicarbonate remained constant over the pH range 5.5 to 7.8 while k_{cat} for that enzyme showed a sigmoidal profile with an inflection at pH 6.9 (Pocker & Bjorkquist, 1977). Although the dehydration reaction cannot be investigated using stopped-flow techniques over a pH region encompassing the apparent pK_a of 7.7 for spinach carbonic anhydrase (see Experimental Section), we hope to extend this region to higher values using a pH-stat technique.

In phosphate buffers in the pH region 6.2 to 7.2, the turnover number k_{cat} increases with decreasing pH, but this change is accompanied by a concurrent increase in the corresponding value of K_m. It appears that the buffer species $H_2PO_4^-$ is simultaneously affecting both k_{cat} and K_m. The combined ability of the enzyme to bind and convert substrate to product can be expressed by the kinetic constant k_{enz}, defined in eq 5 (Pocker & Storm, 1968).

$$k_{enz} = V_{enz}/[E][S] = k_{cat}/(K_m + [S]) \quad (5)$$

In the pH region below 7.2 values of the ratio k_{cat}/K_m are approximately constant and have the same value in both *N*-methylimidazole and phosphate buffers (for a plot of $\log(k_{cat}/K_m)$ against pH, see Figure 5). Thus, at low substrate concentrations the values of k_{enz} and, hence, V_{enz} in each buffer are nearly equal. However, with increasing substrate concentration one observes higher V_{enz} values in phosphate relative to *N*-methylimidazole buffers, as is evident from an inspection of Figure 1. It appears that for the dehydration reaction phosphate buffers serve to increase the range of substrate concentrations that the enzyme is sensitive to.

These results may be combined with earlier work on the enzyme catalyzed hydration of CO_2.[2] Pocker & Ng (1973)

[2] A quantitative comparison can be made using the Haldane relationship (eq 6) which relates the Michaelis–Menten parameters to the equilibrium constant, K_{eq}, for the reaction given in eq 1.

$$k_{cat}^{CO_2}K_m^{HCO_3^-}/k_{cat}^{HCO_3^-}K_m^{CO_2} = K_{eq}/[H^+] \quad (6)$$

Although the Haldane relationship is strictly true only for systems at equilibrium, while our values were obtained from initial rates, it is still useful to see if it applies. Making reasonable corrections for the relative efficiency of two different enzyme preparations in catalyzing the dehydration of bicarbonate under identical conditions and for the presence of additional inhibitory Cl^- in the buffer systems used to study CO_2 hydration (Pocker & Ng, 1973), one can calculate at pH 6.82 an apparent equilibrium constant of 5.6×10^{-7}, which is in fair agreement with a value of 4.8×10^{-7} in the absence of enzyme. A more complete investigation of the compliance of spinach carbonic anhydrase with the Haldane relationship is currently being pursued in our laboratories.

found that monobasic phosphate, $H_2PO_4^-$, reduced the apparent affinity of the enzyme for CO_2 while at higher pH values dibasic phosphate, HPO_4^{2-}, and other basic buffer species enhanced the turnover number k_{cat} for CO_2.

The effect of inorganic phosphate on spinach carbonic anhydrase is highly significant. From the combined results it appears that the buffer species are involved in the proton transfer that differentiates product and substrate in this system. Similar effects have been observed with erythrocyte carbonic anhydrase (Christiansen & Magid, 1970; Silverman & Tu, 1972, 1975, 1976; Tu & Silverman, 1975; Pocker & Bjorkquist, 1977; Pocker & Tanaka, 1978). Although it is not certain that the plant enzyme operates by the same mechanism as the erythrocyte enzyme, simple mechanisms are clearly insufficient to explain the results. For the erythrocyte enzyme a reaction scheme accounting for the observed buffer effects has recently been advanced (Pocker & Tanaka, 1978). It remains to be seen whether or not this scheme is applicable in the case of the plant enzyme. Whatever the mechanism, it should be born in mind that phosphate is a prominent species in the chloroplast stroma. As such the effect of phosphate on spinach carbonic anhydrase would be expected to be of physiological significance.

Several physiological roles have been proposed for plant carbonic anhydrase, most notably that it could facilitate CO_2 diffusion into the chloroplast (Enns, 1967; Nelson et al., 1969; Brown et al., 1970; Everson, 1971; Graham et al., 1971) or that it could catalyze the conversion of bicarbonate to the active form of carbon utilized by ribulose diphosphate carboxylase (Everson, 1970; Zelitch, 1971; Poincellot, 1972). Jacobson et al. (1975) have presented a mathematical argument against the former and have argued that the intrachloroplastic bicarbonate pool is too small to support the latter role. They found that ethoxzolamide, an inhibitor of plant carbonic anhydrase, had no significant effect on electron flow for coupled or uncoupled chloroplasts and had only minimal effect on the reduction of CO_2. Previous work using acetazolamide could be in doubt since in addition to carbonic anhydrase inhibition acetazolamide appears to inhibit photosynthetic electron transport by structurally mimicking other known inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Swader & Jacobson, 1972).

It has been suggested that the role of carbonic anhydrase might be to mediate large transient changes in pH such as might be induced by intermittent changes in light intensity caused by leaves shading each other. By moderating local pH changes, carbonic anhydrase would play a protective role in preventing the denaturation of proteins (Jacobson et al., 1975). Additionally, we think that in view of the potential bicarbonate mediated control sites mentioned in the introductory section carbonic anhydrase could shorten the response time at these sites by hastening the attainment of new equilibrium concentration of CO_2 and HCO_3^- when the pH changes rapidly.

It certainly has not escaped our attention that the role of carbonic anhydrase may be to catalyze reactions other than the interconversion of CO_2 and HCO_3^-. The catalytic versatility of erythrocyte carbonic anhydrase is well-established with respect to hydration of aldehydes (Pocker & Meany, 1965, 1967; Pocker & Dickerson, 1969) and hydrolysis of esters (Tashian et al., 1964; Pocker & Stone, 1965, 1967; Verpoorte et al., 1967; Pocker & Watamori, 1971; Pocker & Guilbert, 1972, 1974). For example, it has been demonstrated that the aldolase and the isomerase were specific for the keto rather than the hydrated form of glyceraldehyde 3-phosphate (Reynolds et al., 1971). We are actively investigating the behavior of plant carbonic anhydrase toward a variety of "unnatural" substrates.

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Inactivation of Electric Eel Acetylcholinesterase by Acylation with *N*-Hydroxysuccinimide Esters of Amino Acid Derivatives†

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ABSTRACT: A series of *N*-hydroxysuccinimide esters of substituted amino acids has been examined as a potential class of acylating agents for covalent modification of acetylcholinesterase from the electric eel. Such esters inactivate acetylcholinesterase, and those which contain bulky aromatic groups are particularly effective. Thus, the *N*-hydroxysuccinimide ester of *N*-acetyl-*p*-(2,4-dinitroanilino)-L-phenylalanine at 25 μM causes, under appropriate conditions, 50% inactivation of the enzyme within 1 min and 96% inactivation after 20 min. Certain quaternary ammonium salts accelerate the inactivation process, some retard it, and others have little effect. Pyridine-2-aldoxime methiodide fails to reactivate the enzyme modified sequentially with diethyl fluorophosphate and the active ester, indicating that inactivation by the ester results from modification at a site distinct from the active site serine. The rate of inactivation is greatly enhanced upon raising the pH from 7.0 to 8.9, suggesting the modification of an amino acid side chain with a high pK_a. Deacylation of the inactivated enzyme by 0.5 M hydroxylamine regenerates activity at a rate indicative of the modification of a tyrosine residue. Absorption spectra of the modified enzyme before and after deacylation with hydroxylamine show that almost complete inactivation is achieved by covalent reaction of one molecule of reagent per catalytic subunit of acetylcholinesterase. The experimental results are interpreted to indicate inactivation of acetylcholinesterase by acylation of a single tyrosine residue near the active site of the enzyme.

Anticholinesterase agents such as organophosphates, carbamates, and sulfonyl halides are generally believed to exert their effects by forming a stable covalent bond with the serine in the active site of the enzyme (Aldridge & Reiner, 1972). We wish to describe the action on acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AcChE)[1] of a different class of acylating agents, *N*-hydroxysuccinimide esters of amino acid

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[1] Abbreviations used are: AcChE, acetylcholinesterase; ONSu, succinimido-oxy; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PAM, pyridine-2-aldoxime methiodide; PTMA, phenyltrimethylammonium; MAC, *N*-methylacridinium; PMI, pyridine methiodide; DEFP, diethyl fluorophosphate; Dnp, 2,4-dinitrophenyl; DnpNH, 2,4-dinitroanilino; DMF, *N,N*-dimethylformamide; Moc, methyloxycarbonyl; DapA, 4-dimethylaminophenylazo; Dns, 5-dimethylaminonaphthalene-1-sulfonyl (dansyl). All other abbreviations are according to IUPAC–IUB rules; see, for example: (1972) *J. Biol. Chem. 247,* 977.

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