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Plant Carbonic Anhydrase. Properties and Carbon Dioxide Hydration Kinetics†

Y. Pocker* and Joan S. Y. Ng‡

ABSTRACT: Carbonic anhydrase from spinach is shown to be an hexameric enzyme of mol wt 180,000 and to contain six Zn atoms per molecule. In the presence of sodium dodecyl sulfate it can be dissociated into a mixture of pentamers, tetramers, trimers, dimers, and monomers as identified by electrophoresis on polyacrylamide gel. The monomer subunits can be reassociated into active enzyme if the latter is dissociated in guanidine hydrochloride rather than in sodium dodecyl sulfate. In contrast to all previous reports, a sulfhydryl reducing agent is not needed for stabilizing the enzyme. 5,5′-Dithiobis(2-nitrobenzoate) does not react with the intact enzyme; however, in the presence of guanidine hydrochloride the dissociated enzyme is shown to contain 12 sulfhydryl groups. The enzyme strongly catalyzes the hydration of CO_2. The enzyme–substrate interaction appears to follow a Michaelis–Menten mechanism with a Hill constant of 1.02. Hydrase activity rises continuously but nonuniformly with increasing pH between 6.0 and 9.0. The pH profile of k_{cat} ($\equiv k_2$) exhibits a simple sigmoidal curve with an inflection at pH 7.7. Phosphate, and to a lesser extent other buffers, have significant effects on both kinetic parameters but especially on K_m. When extrapolated to zero buffer concentration, values of K_m are pH independent. It appears that the acidic form, $H_2PO_4^-$, lowers the CO_2 affinity of the plant enzyme by increasing the value of K_m while the basic form, HPO_4^{2-}, behaves as a mild activator in that it increases the value of k_{cat}. The possible role of the enzyme in promoting photosynthesis is discussed.

Plant carbonic anhydrase was first obtained from leaf cytoplasm by Neish (1939). This carbonate hydrolyase (EC 4.2.1.1) is present in chloroplasts of species which carry out photosynthesis *via* the Calvin cycle (Everson and Slack, 1968), often in close association with ribulose diphosphate carboxylase, an enzyme which uses CO_2 as the C_1 substrate for the carboxylase reaction (Poincelot, 1972a). Recently, the plant enzyme was isolated from spinach (Rossi *et al.*, 1968), parsley (Tobin, 1968, 1970), and pea leaves (Kisiel and Graf, 1972) as well as from *Tradescantia albiflora* Kunth (Atkins *et al.*, 1972) and is reported to exhibit fundamentally different properties from the one isolated from erythrocytes. Both the pea and parsley enzymes were found to be hexameric, mol wt

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TABLE I: Summary of Preparation.

Fraction	[Protein][a] (mg/ml)	Enzyme[b] (Units/ml)	Specific[c] Activity
Crude extract	10.5	97	73
30–55% $(NH_4)_2SO_4$ fractionation	12.7	1,400	1,100
Pooled effluent from DE-52 column	0.77	1,850	24,100

[a] Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. [b] Carbonic anhydrase activity was assayed by the method of Wilbur and Anderson (1948). A unit of activity is defined as $(T_b - T_e)/T_e$ where T_b is the time for the uncatalyzed and T_e is the time for the enzyme-catalyzed reaction. [c] The specific activity was calculated according to the formula $10[(T_b/T_e) - 1]$/mg of protein (Rickli *et al.*, 1964).

180,000, and to contain 6 g-atoms of zinc per molecule, whereas the spinach enzyme was reported to have a mol wt 150,000 and to contain no zinc. The *Tradescantia* enzyme (monocotyledon species) was recently estimated to have a mol wt of 42,000 ± 2,000, with a subunit size of 27,500 ± 2,200; the number of Zn atoms per molecule is uncertain. Acetazolamide inhibition is significantly smaller toward the plant enzyme (Tobin, 1970; Everson, 1970; Y. Pocker and J. Ng, unpublished results, 1971) than toward the erythrocyte enzyme.

While strong catalysis of CO_2 hydration is observed with the plant enzyme, no detailed kinetic study has been reported so far. However, since the enzyme plays an essential role in photosynthesis (Waygood *et al.*, 1969; Everson, 1970; Poincelot, 1972a) we deemed it important to carry out a detailed characterization of its catalytic properties. The terms of reference dictating the scope and purpose of this study were twofold: (1) to characterize more closely the spinach enzyme and (2) to provide a molecular basis for the regulatory effect of this enzyme. In this work, we report the isolation to homogeneity and characterization of the spinach enzyme. A detailed study of the enzyme-catalyzed hydration of CO_2 as a function of pH and buffer composition is also presented.

Experimental Section

Materials. DE-52 was obtained from Reeve Angel and prepared according to the laboratory manual from the company. Sephadex G-25 (medium) was purchased from Pharmacia. Guanidine hydrochloride of Ultra Pure grade was obtained from Schwarz/Mann and 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) was obtained from Aldrich. Both were used without further purification. *p*-Nitrophenol (Eastman) was purified by sublimation under vacuum. Sodium dodecyl sulfate was kindly supplied by Dr. Anna Pocker. 1,2-Dimethylimidazole (Aldrich) was further purified by vacuum distillation. Imidazole was recrystallized three times from benzene. All other chemicals were either analytical or reagent grade and were used without further purification. Chemicals for disc gel electrophoresis were obtained from Canalco.

Apparatus. All pH measurements were done at 25° with a Corning glass electrode (476022) using a Beckman research pH meter. For column chromatography, a 5 × 90 cm column was obtained from Glenco Scientific Inc. The flow rate was regulated by a polystaltic action pump (Buchler Instruments) with four pumping channels. Fractions collected from chromatography were monitored by an Automatic Fraction Collector, Model 1205D-3 (Research Specialties Co.) with a 10-ml volumetric siphon. Disc gel electrophoresis was performed on a Canalco Model 12 with Model 1400 as power source. Zn determination was carried out on an Atomic Absorption spectrophotometer, Model 153 (Instrumentation Laboratory, Inc.), with a Zn lamp, λ 213.9 nm. Carbon dioxide hydration kinetics were performed on a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp.) consisting of an optical path length of 2 cm. The instrument was equipped with two equal size syringes and a Teflon mixing chamber. The insulated bath compartment consisting of a specially constructed circulating device was thermostated at 25.0 ± 0.02° by a Sargent Thermonitor Model SW (S-82055). Buffer factors were determined on a Beckman DU spectrophotometer thermostated at 25.0 ± 0.02° by a Sargent Thermonitor.

Methods. Preparation of Spinach Carbonic Anhydrase. The following separation procedure carried out at 4° yielded *ca.* 1 g of enzyme from 20 lb of fresh spinach. Step 1: Fresh spinach was washed and the stems were discarded. The leaves were then cut and blended with buffer in a Waring Blender. Approximately 1.5 l. of buffer (0.02 M sodium phosphates–0.1 M sodium chloride–0.001 M EDTA (pH 6.8)) was required for each kilogram of leaves. Step 2: The suspension was filtered through a Buchner funnel and the pulp discarded. Step 3: The filtrate was brought up to 30% saturation with $(NH_4)_2SO_4$ and stirred for at least 1 hr before centrifuging at 8 krpm for 30 min with a Lourdes centrifuge. The pellets were discarded. Step 4: To the supernatant liquid, more $(NH_4)_2SO_4$ was added so that the final concentration would be 55% saturation. The precipitate was recovered by centrifugation and dissolved in phosphate buffer. Step 5: After dialysis to get rid of excess $(NH_4)_2SO_4$, the protein solution was loaded onto a 5 × 90 cm DE-52 column. The flow rate was 45 ml/hr. Fractions with specific activity, defined by Rickli *et al.*, greater than 20,000 were pooled. The colorless solution was then concentrated in an Amicon Ultrafiltration Cell and stored in refrigerator at 5°. The results are summarized in Table I. Polyacrylamide disc gel electrophoresis of the final enzyme solution in 7% polyacrylamide gel (pH 9.5) showed a single component of carbonic anhydrase.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. A slightly modified method of Weber and Osborn (1969) on molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis was used. In two instances, the incubation time for the protein was changed from 2 hr to 2 min at 37° in 0.01 M phosphate buffer containing 1% sodium dodecyl sulfate–1% β-mercaptoethanol (pH 7.0). For standardization the following proteins were used: bovine carbonic anhydrase, pepsin, trypsin, bovine serum albumin, and catalase. The experiments were performed in 7% acrylamide gel with 0.1% sodium dodecyl sulfate at room temperature.

Enzyme Dissociation and Reassociation. Intact spinach carbonic anhydrase (5 mg) dissolved in 6 ml of phosphate buffer was mixed with 3 ml of 9 M guanidine hydrochloride. The solution was left to stand at room temperature for 30 min until no activity could be detected. It was then mixed with 60 ml of phosphate buffer containing 0.1 M β-mercaptoethanol and 2×10^{-3} M $ZnCl_2$ (pH 6.8). The final concentration of guanidine hydrochloride was approximately 0.5 M. The pro-

tein was concentrated in an Amicon Ultrafiltration Cell before the enzyme activity was tested.

Sulfhydryl Group Determination. Thiol group titrations were performed with Nbs_2 according to Ellman (1959). The spinach enzyme (0.77 mg) was diluted with 1 ml of H_2O at room temperature. The difference in the absorbance at 412 nm between the enzyme solution and the blank was determined. Aliquots of 20 μl containing 10 mM Nbs_2 were added to each cuvette and the difference in absorbance was determined again; ϵ 1.36 $\times$ 10^4 M^{-1} cm^{-1} was used for the 2-carboxy-3-nitrothiophenol anion. This process was repeated with the enzyme treated for 0.5 hr with 6 M guanidine hydrochloride.

Substrate Preparation. Saturated CO_2 solutions were prepared by bubbling CO_2 gas from a gas cylinder (Airco) to a thermostated (25.0 $\pm$ 0.02°) deionized distilled water reservoir at 1 atm pressure. After saturation was reached, these solutions were allowed to flow by gravity directly into the stopped-flow syringe. Solutions with different CO_2 concentrations were prepared by diluting measured amounts of saturated CO_2 solution in an air-tight syringe containing different known quantities of deionized distilled water. The solution was brought to equilibrium by oscillating a glass bead in the syringe. The concentrations of CO_2 in all final solutions were determined by precipitating with standardized $Ba(OH)_2$ solution and back-titration against standardized HCl by means of a Radiometer Automatic Titrator, using glass electrode Type 202C. The end points of the titrations were determined from recorded titration curves obtained from the instrument. This method is found to be superior to the classical titration with phenolphthalein indicator. The latter has large error percentage due to the atmospheric CO_2 and the indecisive and incorrect end point. Since $Ba(OH)_2$ reacts rapidly with atmospheric CO_2, precautions are necessary. This was done by storing the $Ba(OH)_2$ solution in a two-necked flask in a desiccator containing KOH pellets. One neck of the flask was connected to a drying tube filled with ascarite and the other neck was stoppered by a serum cap. Upon use, a syringe is introduced to the solution through the serum cap. Using such method, the $Ba(OH)_2$ solution could be kept for months without any trace of $BaCO_3$ contamination. Each concentration of CO_2 used was the average of four titrations which agreed with each other within less than 2%. Values obtained for the saturated solutions at 15.0, 25.0, and 35.0° agree with the values reported in the CRC Handbook of Chemistry and Physics to within 1%.

Buffer Components and Solutions. The total buffer concentration used in this work, unless otherwise stated, was maintained constant at 0.03 M throughout the pH range studied. Phosphate buffer was prepared by accurately weighing samples of Na_2HPO_4 and NaH_2PO_4 to the desired buffer ratio. 1,2-Dimethylimidazole buffer was made by acidifying 0.03 M 1,2-dimethylimidazole with hydrochloric acid to the desired pH. Tris buffer was found to be unsuitable for CO_2 hydration kinetics due to the possible side reaction between the substrate and buffer component leading to carbamate formation. Unless otherwise stated, all buffer solutions were brought to an ionic strength of 0.15 by adding an appropriate amount of NaCl. Furthermore, 0.001 M EDTA was added to each solution to stabilize the enzyme.

Kinetic Procedure and Calculations. In the stopped-flow spectrophotometer, the hydration reaction was initiated by 1:1 rapid mixing of CO_2 solution and a buffer solution containing an indicator with or without the enzyme. The indicators used were *p*-nitrophenol (pK_a = 7.1) for phosphate buffer of pH range 6.14–7.59 and *m*-cresol purple (pK_a = 8.3) for 1,2-dimethylimidazole buffer of pH range 7.76–9.03. They are chosen such that their pK_a's are close to those of the buffer systems. The absorbance change due to the small change of proton concentration in the course of the reaction was recorded spectrophotometrically at wavelength λ 400 nm for *p*-nitrophenol and 578 nm for *m*-cresol purple. The concentration of the enzyme was chosen such that the rate in the presence of enzyme would be at least five times greater than the uncatalyzed rate.

The method of calculation for the pseudo-first-order rate constant was described by Gibbons and Edsall (1963). Carbon dioxide hydration can be written as

$$CO_2 + H_2O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H^+ + HCO_3^-$$

The rate equation is then

$$-d[CO_2]/dt = k_1[CO_2] - k_{-1}[H^+][HCO_3^-]$$

When pH is greater than 7, the reaction

$$CO_2 + OH^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HCO_3^-$$

has to be taken into account. At the very first instance of the reaction, the rate is directly proportional to the initial CO_2 concentration. Thus

$$(d[P])/dt_{t=0} = (k_1 + k_2[OH^-])[CO_2]_0$$

where $[CO_2]_0$ is the initial $[CO_2]$ and [P] is the product concentration.

In the stopped-flow kinetics, one measures the rate of change of absorbance. When the initial time scale is small, a plot of log $(A - A_e)$ against time gives a straight line indicating that the reaction is pseudo first order. The value of $(dA/dt)_{t=0}$ is related to $(dP/dt)_{t=0}$ by a buffer factor, Q_0.

$$(dP/dt)_{t=0} = -Q_0(dA/dt)_{t=0} = \lim_{t=0} [k_{app}Q_0(A_0 - A_e)]$$

It can be shown that

$$\log (A - A_e) = \log (A_0 - A_e) - k_{app}t/2.3$$

This apparent rate coefficient, k_{app}, can be obtained from the limiting slope of a plot of log $(A - A_e)$ *vs.* time.

The buffer factor, Q_0, was introduced because in the hydration reaction it is the release of the proton that causes the color change of the indicator, and the rate of the reaction is directly related to the protons released. Hence it is necessary to know the effect of the addition of protons on the absorbance of the buffer–indicator system in order to calculate the initial rate of the reaction. Consequently, Q_0 is defined as the ratio of (moles of H^+ added per liter)/(absorbance change). For small quantities of acid added, Q_0 = d(Acid)/dA, and is experimentally determined by adding aliquots of standardized HCl to the buffer system and observing the change of absorbance in the Beckman DU spectrophotometer at 25°.

Values of k_{app} were calculated by a CDC 6400 digital computer with a Fortran IV computer program of least-squares method written by Dr. N. Watamori. In most runs the correlation coefficient of the slope was better than 0.997.

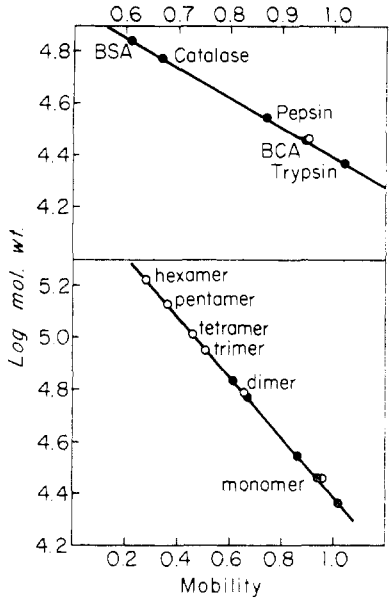

FIGURE 1: Determination of the molecular weight of spinach carbonic anhydrase by acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. Gels were 7% acrylamide. The mobility is defined as $(ab)/(cd)$ where a is the distance of protein migration, b is the length of gel before staining, c is the distance of dye migration, and d is the length of gel after destaining. Incubation temperature 37°. In both lines the marker proteins (●) are trypsin, bovine carbonic anhydrase B, pepsin, catalase, and bovine serum albumin after an incubation time of 2 hr. Upper line: spinach carbonic anhydrase (○) as the monomeric form following an incubation time of 2 hr. Lower line: spinach carbonic anhydrase (○) as a mixture of oligomers following an incubation time of only 2 min. The molecular weight of the monomer is 30,000 and of the hexamer 180,000.

Results

Molecular Weight Determinations. Figure 1 shows a calibrated curve for polyacrylamide gel electrophoresis in sodium dodecyl sulfate. After complete dissociation (upper line), the spinach enzyme exhibits a molecular weight near 30,000. However, changing the incubation time from 2 hr to 2 min at 37° (lower line) produced a mixture of hexamer, pentamer, tetramer, trimer, dimer, and monomer corresponding to molecular weights of 180,000, 150,000, 120,000, 90,000, 60,000, and 30,000, respectively.[1] Attempts to reassociate the enzyme by dialyzing the sodium dodecyl sulfate dissociated enzyme solution in phosphate buffer were unsuccessful.

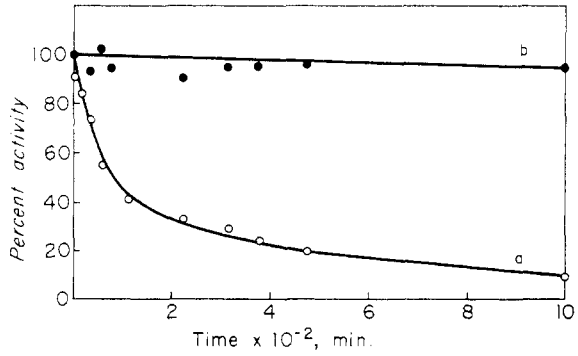

FIGURE 2: Comparison of the activities of spinach carbonic anhydrase in two different buffer mediums at room temperature. Curve a represents the instability of the enzyme in a buffer containing 0.02 M phosphate–0.01 M β-mercaptoethanol. Curve b represents the stability of the enzyme in 0.02 M phosphate–0.1 M NaCl–0.001 M EDTA. Activities were determined by the Wilbur–Anderson method.

[1] Atkins *et al.* (1972) observed a band corresponding to the dimer of Pisum enzyme during their sodium dodecyl sulfate gel electrophoresis.

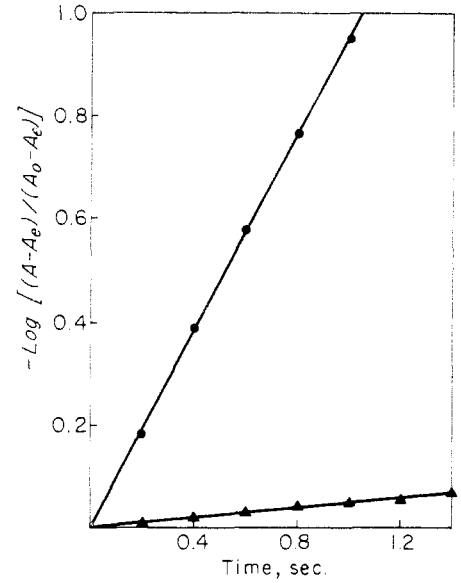

FIGURE 3: Typical first-order rate plots of CO_2 hydration with and without spinach carbonic anhydrase at 25.0°: $[CO_2]_0 = 1.39 \times 10^{-2}$ M, $[E]_0 = 3.12 \times 10^{-8}$ M in phosphate buffer (pH 6.82) (0.02 M phosphate–9×10^{-3} M NaCl–9×10^{-5} M EDTA–5×10^{-5} M *p*-nitrophenol) followed at 400 nm.

Stability and the Sulfhydryl Groups of the Enzyme. Ever since Bradfield (1947) claimed that the plant enzyme contains –SH groups, all workers in this field have been using sulfhydryl reducing agents to protect the enzyme. However, it has been found in these laboratories that the enzyme is stable in the absence of such agents. Two batches of enzyme were prepared in different buffer media, one containing 0.02 M phosphate–0.01 M β-mercaptoethanol and the other containing 0.02 M phosphate–0.1 M sodium chloride–0.001 M EDTA. We found that the activity of the enzyme in the first buffer system decreased rapidly whereas that in the second buffer medium decreased only very slightly after 50 hr at room temperature (Figure 2), and less than 30% of activity was lost after 1 year of storage at 4°.

Tests with Nbs_2 show that Nbs_2 is inactive toward the intact enzyme since no color was developed after 2 hr of standing at room temperature and no significant loss in activity was found after 24 hr at 4°. However, if the enzyme is treated with 6 M guanidine hydrochloride for 0.5 hr before adding Nbs_2, a color is developed. From the absorbance measured at 412 nm, we obtained 12 –SH groups (to the nearest integer) per molecule of enzyme. This result indicates that although –SH groups are present in the dissociated enzyme, it is not involved in the catalytic activity.

Dissociation and Reassociation of the Enzyme. Approximately 65% of original activity was recovered after the reconstitution. It is found that the presence of both β-mercaptoethanol and $ZnCl_2$ is necessary for the reassociation. This indicates that –SH groups in the enzyme are exposed upon dissociation and must be protected for successful reassociation. Thus this result is in agreement with the Nbs_2 experiment.

Zn Determination. All precautions were taken to get rid of Zn ions in the buffer and all glass wares used. Standard curves of zinc acetate solutions containing 0.05–2 ppm of Zn were determined before and after the measurement of the enzyme solution. Readings were integrated for every 10 sec. The re-

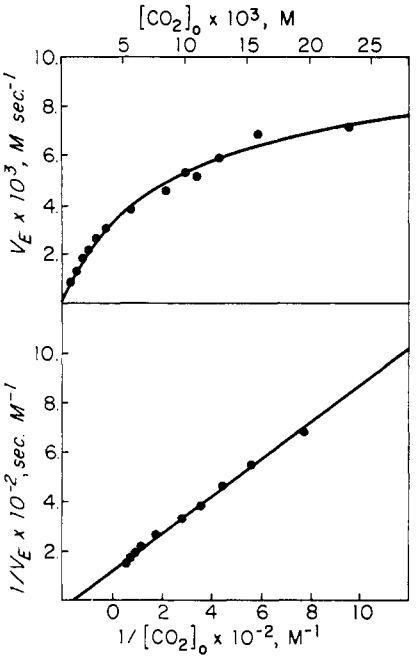

FIGURE 4: Representative plots for a typical spinach carbonic anhydrase catalyzed hydration of CO_2 at 25.0°: $[E]_0 = 3.92 \times 10^{-8}$ M; 0.02 M phosphate–9 $\times 10^{-3}$ M NaCl–9 $\times 10^{-5}$ M EDTA–5 $\times 10^{-5}$ M *p*-nitrophenol (pH 6.82); upper curve, plot of V_E *vs.* $[CO_2]_0$; lower curve, Lineweaver–Burk plot.

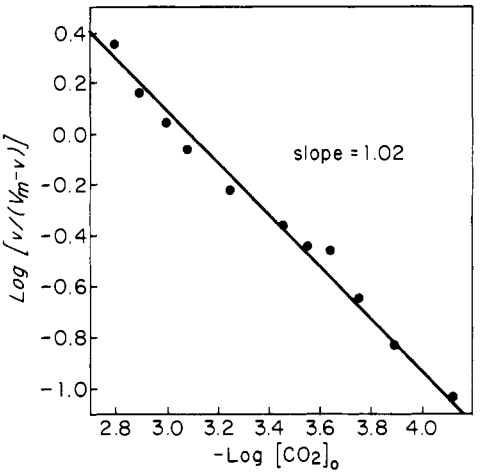

FIGURE 5: Hill plot of CO_2 hydration catalyzed by spinach carbonic anhydrase. Conditions are the same as in Figure 4.

sult shows that Zn is present in the enzyme and it gives a 1:6 ratio of moles of enzyme to moles of Zn. This is in agreement with the parsley (Tobin, 1970) and pea enzyme (Kisiel and Graf, 1972).

Kinetic Results. A typical first-order hydration of CO_2 with and without spinach carbonic anhydrase in phosphate buffer at 25° is shown in Figure 3. A plot of rate against substrate concentration together with its corresponding Lineweaver–Burke plot is depicted in Figure 4. The enzyme–substrate interaction follows the Michaelis–Menten mechanism as indicated by the linearity of the Lineweaver–Burke plot. In order to determine the number of molecules of substrate binding to the enzyme, a Hill plot (Hill, 1913) is used (Figure 5). Hill proposed an interaction mechanism for a multiple-site enzyme and assumed that the rate-determining step was the breakdown of the EA_n complex to give products. The resulting kinetic equation is

$$\frac{v}{V_{max}} = \frac{1}{1 + (K/A)^n}$$

where n = the number of ligand binding sites per molecule of protein and K = the binding constant. Thus a plot of log $v/(V_{max} - v)$ *vs.* log A would yield a straight line with a slope n which is directly related to the number of sites. The slope of our line is 1.02 (Figure 5) indicating that one CO_2 molecule is bound to each molecule of the enzyme or to each active site of

$$E + nA \rightleftharpoons EA_n \longrightarrow E + nP$$

TABLE II: Effects of pH on the Rate of Hydration.

Buffer[a]	pH	Indicator $\times 10^5$ [b] (M)	$V_m \times 10^2$ [c] (M sec^{-1})	$K_m \times 10^2$ [c] (M)	$k_2 \times 10^{-5}$ (sec^{-1})	log (k_2/K_m)
Phosphate	6.25	10 *p*-NP	0.25	1.79	0.58	6.50
	6.46	5 *p*-NP	0.25	0.93	0.59	6.80
	6.67	5 *p*-NP	0.35	0.76	0.83	7.04
	6.87	5 *p*-NP	0.33	0.37	0.77	7.32
	7.00	5 *p*-NP	0.37	0.41	1.09	7.42
	7.25	5 *p*-NP	0.58	0.16	1.35	7.92
	7.43	2.5 *p*-NP	0.73	0.18	1.69	7.98
1,2-Dimethylimidazole	7.76	5 *m*-CP	1.27	0.24	2.86	8.08
	7.96	5 *m*-CP	1.84	0.21	3.86	8.26
	8.17	5 *m*-CP	1.66	0.10	3.84	8.57
	8.39	2.5 *m*-CP	1.88	0.10	4.37	8.68
	8.70	1 *m*-CP	1.84	0.17	4.26	8.40
	8.88	1 *m*-CP	2.06	0.15	4.77	8.51
	9.03	1 *m*-CP	2.30	0.12	5.32	8.72

[a] Total buffer concentration was kept at 1.5×10^{-2} M. NaCl was added to the buffer system to maintain an ionic strength of 0.075. In addition to this 5×10^{-4} M of EDTA was added to the solution to stabilize the enzyme. [b] The indicators used were: *p*-nitrophenol (*p*-NP) and *m*-cresol purple (*m*-CP). [c] Each value is obtained from a Lineweaver–Burke plot of five different concentrations of CO_2 and each V_E value used in the plot is the average of four runs; $[E]_0 = 4.32 \times 10^{-8}$ M.

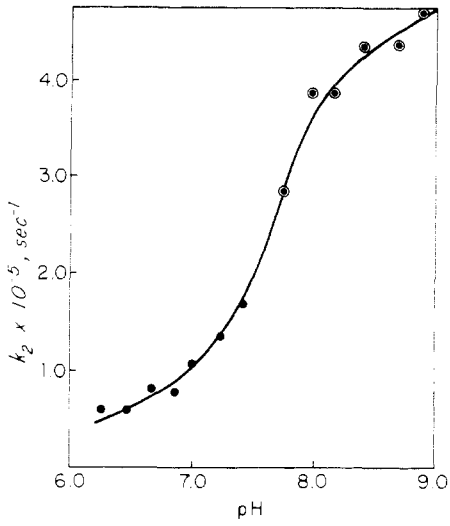

FIGURE 6: The turnover number k_2 in sec^{-1} for the spinach carbonic anhydrase catalyzed hydration of CO_2 as a function of pH at 25.0° and ionic strength 7.5×10^{-2}. Total buffer concentration, 1.5×10^{-2} M: (●) phosphate; (◉) dimethylimidazole.

the enzyme molecule provided that the active sites are noninteracting with each other.

The study of CO_2 hydration activity of spinach carbonic anhydrase as a function of pH is highly instructive. Values of K_m and k_2 were evaluated over the entire pH range. Different buffer systems were used and the ionic strength of all solutions was kept at 0.075 with sodium chloride. The results are given in Table II.

The turnover number of the enzyme–substrate complex, k_2, in the simplified mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

is found to increase with pH (Figure 6); a tenfold increase is observed in going from pH 6.0 to 9.0. The plot of $\Delta k_2/\Delta$pH *vs.*

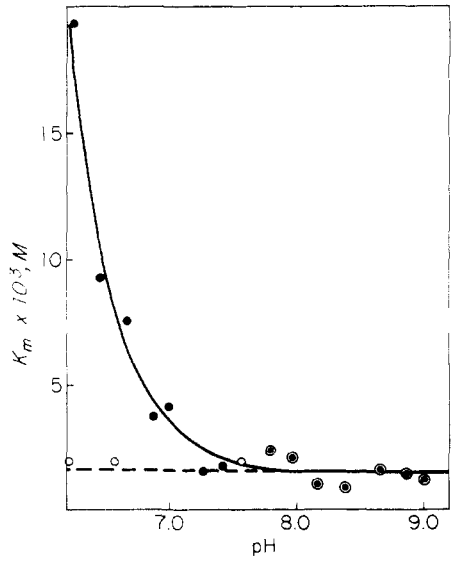

FIGURE 7: Values of K_m as a function of pH in (●) phosphate and (◉) dimethylimidazole buffers at 25.0°. Conditions are the same as those given in the legend to Figure 6; (○) values of K_m extrapolated to zero buffer concentration. Solid line is a curve through the experimental points. Broken line passes through the extrapolated values of K_m.

TABLE III: Buffer Effects on the Kinetic Parameters of CO_2 Hydration Catalyzed by Spinach Carbonic Anhydrase.[a]

Buffer	[Buffer]$_{Total}$ $\times 10^2$ (M)	pH	$K_m \times 10^2$ (M)	$k_2 \times 10^{-5}$ (sec^{-1})
Phosphate	2.5	6.26	2.44	1.05
	2.0	6.26	1.71	0.87
	1.5	6.26	1.79	0.58
	1.25	6.26	1.06	0.45
	2.0	6.62	0.87	1.12
	1.5	6.62	0.76	0.83
	1.25	6.62	0.55	0.61
Imidazole	2.5	6.74	1.21	0.71
	1.5	6.74	0.79	0.62
	2.5	7.11	0.78	1.00
	2.0	7.11	0.48	0.94
	1.5	7.11	0.39	0.85

[a] Ionic strength of the buffers was kept at 7.5×10^{-2} by adding NaCl. In addition, 5×10^{-4} M EDTA was added to stabilize the enzyme; $[E]_0 = 4.32 \times 10^{-5}$ M.

pH defines the inflection of the sigmoidal k_2 profile as occurring at pH 7.7. This result is compatible with the activity of a basic group responsible for hydration, and the pK_a of which is 7.7 as compared to 6.9 in the case of the bovine enzyme.

The dependence of K_m on pH (Figure 7) shows two distinct regions. At pH <7.4, K_m decreases as pH increases and at pH >7.4, K_m is independent of pH. This may be due to the different buffer systems used to study this wide range of pH. Inasmuch as K_m increases with phosphate concentration at a constant pH the CO_2 affinity of the enzyme is clearly affected by the binding of buffer components to the enzyme rather than by the state of protonation alone. This result is tabulated in Table III. To distinguish between the phosphate effect and the chloride effect, 5×10^{-2} M NaCl was added to each buffer system to stabilize the enzyme and the ionic strength is maintained at 0.15 with Na_2SO_4 instead of NaCl. The same buffer effect was observed (Figure 8). In other words, phosphate causes the enzyme to bind CO_2 less efficiently. This effect decreases as the pH increases. Extrapolating to zero buffer concentration, K_m at low pH appears to be the same as the K_m at high pH, namely, K_m is a constant (1.5 mM) in the

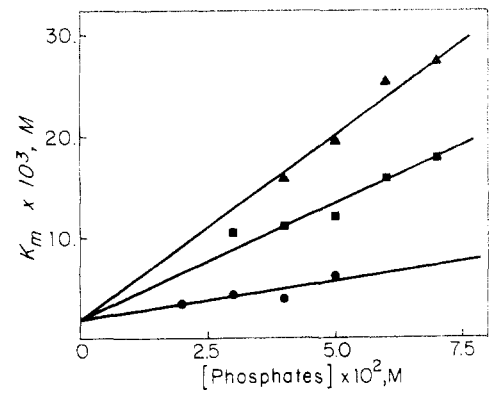

FIGURE 8: The effect of buffer concentration on K_m at different values of pH. Ionic strength of all solutions maintained at 1.5×10^{-1} with Na_2SO_4; 5×10^{-2} M NaCl and 5×10^{-4} M EDTA were added to each solution to stabilize the enzyme. Values of K_m (in M^{-1}) at: (▲) pH 6.14; (■) pH 6.57; and (●) pH 7.55.

pH range 6.1–9.0. This constancy in K_m with varying pH is similar to the one observed with the bovine (Kernohan, 1965) and human enzymes (Khalifah, 1971).

On the other hand, the turnover number k_2 increases with phosphate concentration (Figure 9). This effect increases as pH increases. Since the buffer systems contain both the monobasic and dibasic phosphates, we suggest that the monoanion plays the role at the lower pH, affecting the binding constant whereas the dianion is responsible for the effect on k_2 at the high pH. In short, at lower pH, $H_2PO_4^-$ acts as an inhibitor while at high pH, HPO_4^{2-} acts as an activator.

Similar but less pronounced effects on K_m and k_2 are also found with imidazole as buffer. The results are given in Table III. When extrapolated to zero buffer concentration, the K_m values at pH 6.74 and those at pH 7.11 converge to a constant value of around 1.5 mM, which is in good agreement with the value obtained from the phosphate buffers.

Both acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) and azide were found to inhibit spinach carbonic anhydrase in the stopped-flow spectrophotometric experiments as well as in the Wilbur–Anderson (1948) assays. At pH 6.82 azide is found to be a stronger inhibitor, $K_i = 3.3 \times 10^{-5}$ M, than acetazolamide, $K_i = 2 \times 10^{-4}$ M. Thus the spinach enzyme differs markedly from the mammalian enzymes where acetazolamide inhibition, $K_i = 2 \times 10^{-8}$ M, is much more potent than the azide inhibition, $K_i \sim 10^{-5}$ M.

Discussion

Spinach, a dicotyledon species, is shown to possess a carbonic anhydrase similar to that found in parsley and pea. The enzyme has a molecular weight around 180,000 and contains six tightly bound atoms of zinc. It is an hexameric enzyme as confirmed by the dodecyl sulfate gel electrophoresis in which different aggregation stages of the subunits can be identified. Atkins *et al.* (1972) claimed the existence of isozymes in pisum. They observed two active enzyme bands in the gel electrophoresis. However, our results show only one band in the gel electrophoresis and agree with the similar observations made by Tobin (1968, 1970) and Rossi *et al.* (1968). Furthermore, in contradistinction to all previous reports, the sulfhydryl groups in the enzyme do not react readily with an oxidizing agent. When Nbs_2, a specific reagent for –SH groups, is added to the enzyme solution in the absence of a sulfhydryl protecting agent, no loss of enzyme activity is observed even after 24 hr at 5°. Also, no reduction of Nbs_2 takes place. However, after being dissociated in 6 M guanidine hydrochloride, the enzyme rapidly reduces Nbs_2. This indicates that the –SH groups in the intact enzyme are not exposed to the environment, as previously assumed. *p*-Chloromercuribenzoate is known to inhibit the enzyme. Rossi *et al.* (1968) in their *p*-chloromercuribenzoate titration, found approximately eight –SH groups per molecule of enzyme whereas we find 12 in our Nbs_2–guanidine hydrochloride experiment. The results of Rossi *et al.* are understandable since *p*-chloromercuribenzoate is also known to be a dissociating agent (Leitzmann *et al.*, 1970). The fact that Nbs_2 by itself does not oxidize the –SH groups in the intact enzyme shows that the function of these 12 –SH groups is to maintain its structural integrity. Moreover, Tobin (1970) found seven cysteine groups per monomer in his amino acid analysis. Thus, besides the 12 –SH groups involved in association, there are quite a large number of cysteine groups well protected and well hidden in the enzyme. Perhaps one of the most striking results to come out from the present work is

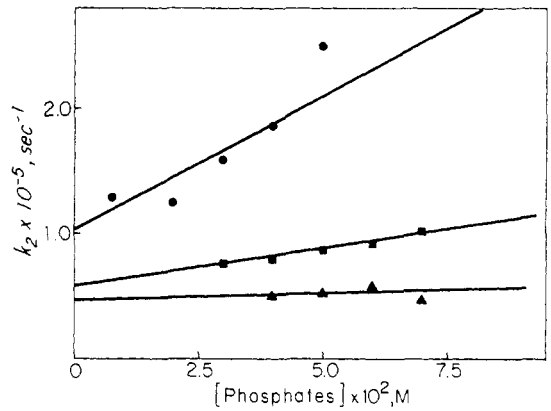

FIGURE 9: Effects of phosphate concentration on k_2 at different values of pH. Conditions are the same as those given in the legend to Figure 8.

that the reassociation of the six subunits can be achieved provided the conditions of the dissociation are carefully controlled. These include use of guanidine hydrochloride rather than dodecyl sulfate as dissociating agent, coupled with the minimum exposition of the subunit to the action of the dissociating agent (no longer than 30 min). In fact, long contact with guanidine hydrochloride leads first to an irreversible dissociation and finally to a partial precipitation of the protein.

From the result of the Hill plot, it is concluded that the enzyme binds with CO_2 in a 1:1 ratio. However, since Hill's assumption was based on infinite cooperativity (Koshland, 1970), *i.e.*, the binding of the first molecule of ligand is immediately followed by the binding of all subsequent molecules of ligand, two plausible conclusions can be drawn. Either (a) there is only one CO_2 molecule binding to one molecule of enzyme or (b) the active sites of the enzyme are noninteracting. If conclusion (b) is true, then it means that either the binding of the substrate does not induce any conformational change and no direct site–site interactions or the induced conformational change is not transmitted to the neighboring subunits.

Using a simplified model, the enzyme-catalyzed hydration of CO_2 can be depicted as

$$H_2O + CO_2 + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\displaystyle OH_2}{E{-}CO_2} \xrightarrow{k_2} E + H^+ + HCO_3^-$$

At zero buffer concentration, K_m, defined as $(k_{-1} + k_2)/k_1$, is a constant with a value of 1.5 mM in the pH range of 6.0–9.0. This indicates that the CO_2 binding site in the enzyme does not ionize in this pH range. Similar results were obtained with erythrocyte carbonic anhydrase where K_m remains essentially constant while $V_m/[E]_0$ dictates the overall change in enzymatic activity (Kernohan, 1965; Pocker and Meany, 1965a,b; Pocker and Stone, 1967; Pocker and Dickerson, 1968; Pocker and Storm, 1968; Pocker and Watamori, 1971; Khalifah, 1971). The K_m values with respect to CO_2 hydration are 12 mM for the bovine enzyme, 4 mM for the human B, and 9 mM for the human C. Thus the affinity of the spinach enzyme for CO_2 substrate is larger than that of the erythrocyte species. However, this affinity is greatly affected by $H_2PO_4^-$ in the pH range of 6.0–7.4. The $H_2PO_4^-$ inhibits the binding of CO_2 to the enzyme. The inhibition is most pronounced when the activity linked group in the enzyme is in its acidic form. This inhibition decreases as pH increases. Since such an effect is exerted on K_m alone with little effect on k_2, two possible conclusions can be reached. Either (a) the $H_2PO_4^-$ binds to the same site as CO_2 or (b) the anion binds to a site which

strongly affects the binding site for CO_2 but has little effect on the turnover mechanism. We believe that the striking effect of $H_2PO_4^-$ is highly significant and our working hypothesis is that phosphates may have a regulatory effect on the plant enzyme similar to that found with 2,3-diphosphoglycerate and hemoglobin (Benesch and Benesch, 1969). Clearly, the organic phosphates undergoing carboxylation during photosynthesis may themselves provide a subtle control of the CO_2 affinity of the plant enzyme, a problem which is being actively pursued in these laboratories.

The pH profile of k_2 is consistent with the mechanism in which the activity linked group in the enzyme is most active in its basic form and weakly active in its acidic form. The pK_a of this group is 7.7. Comparing the k_2 values at high activity plateau (pH ~9), we have $k_2 = 4.7 \times 10^5$ sec^{-1} for the spinach enzyme whereas the bovine enzyme gives $k_2 = 10^6$ sec^{-1} and the human C gives $k_2 = 1.4 \times 10^6$ sec^{-1}. Whether the similarity in the magnitude of the turnover number is of any mechanistic significance is still unclear since the amino acid composition of the plant enzyme and the erythrocyte species is quite different. Furthermore, the amino acid residues in the active site of the spinach enzyme are still unknown. Nonetheless, in view of the similarities between the spinach and the bovine enzymes with respect to the pH profiles of their kinetic parameters, K_m and k_2, it is very likely that the CO_2 hydration mechanism is similar in the two enzymes. In the hydration mechanism of the erythrocyte enzyme, Zn and a coordinated water molecule have been unambiguously demonstrated as essential components. Some investigators (Lindskog *et al.*, 1971) suggest that the pK_a of 6.9 obtained from the pH profiles of k_2 and log (k_2/K_m) is due to the dissociation of the zinc-bound water molecule to form a zinc-bound hydroxide ion. Others (Pocker and Meany, 1965a,b, 1967; Pocker and Stone, 1965; Pocker and Dickerson, 1968; Pocker and Storm, 1968) contend that around physiological pH a general base having a pK_{BH^+} of about 7.0 in the enzyme promotes the transfer of a proton from the zinc-bound water. Such a general base could be the imidazole of the histidine residue at or near the active site of the enzyme. This mechanism, if applicable to the plant enzyme, would explain the activating effect of an external general base, the dibasic phosphate ion, which affects the value of k_2. Indeed, the activation increases with increasing pH.

The physiological role of carbonic anhydrase in the plant still remains to be elucidated. Evidence that the enzyme is essential for maximum rate of photosynthesis in spinach chloroplasts is provided by Everson (1970). It was found by Poincelot (1972b) to be located in the stromal fraction of the chloroplast[2] in plants, *e.g.*, spinach and peas, which carry out the photosynthesis with the Calvin cycle. Its distribution pattern in these plants parallels that of ribulose diphosphate carboxylase. On the other hand, the enzyme is found in the cytoplasm of certain panicoid grasses in which the C_4-dicarboxylic acid pathway operates (Everson and Slack, 1968). The quantity of carbonic anhydrase in such plants is only one-fifth to one-tenth that of species in which the normal Calvin cycle is operative.[3] Hatch and Slack (1966) have shown that phosphoenolpyruvate carboxylase is implicated in the primary photosynthesis carboxylation reaction in these panicoid grasses. Thus the function of the enzyme in both species may be to convert HCO_3^- or CO_2 into the active species of "CO_2" which is then utilized by either ribulose diphosphate carboxylase or phosphoenolpyruvate carboxylase.

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[2] Recently, Enns (1967) has demonstrated by using a model that carbonic anhydrase will enhance the rate of transport of CO_2 across the membrane by as much as 100-fold. Since the enzyme is found in stroma, another of its functional roles could be to facilitate the transport of CO_2 across the envelope membrane, as pointed out by Poincelot (1972b).

[3] Carbonic anhydrase may also play a role in photorespiration (Decker, 1955). Thus Waywood *et al.* (1969) suggested that this enzyme may act as a "trap" for CO_2, preventing its escape into the atmosphere *via* the action of glycollate oxidase in photorespiration.