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(22) We wish to point out that we previously and unintentionally published the K_D for the *p*-nitrophenol–cyclohexaamylose complex as 5.3×10^{-2} M. This error escaped our attention. The number should read 5.23×10^{-3} M and therefore sodium *p*-nitrophenolate binds 13.0 and not 130 times more tightly in the cyclohexaamylose cavity than *p*-nitrophenol does.

Bifunctional Substrates of Erythrocyte Carbonic Anhydrase. Enzyme-Catalyzed Hydration and Hydrolysis of Pyruvate Esters

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Abstract: The present investigation shows that carbonic anhydrase from bovine erythrocytes catalyzes both the *hydration* and the *hydrolysis* of pyruvate esters. Both enzymatic processes exhibit sigmoidal pH–rate profiles with a point of inflection around neutrality. Both obey Michaelis–Menten kinetics. For a given ester, under similar experimental conditions, turnover numbers for the enzymatic hydration are much larger than those for the corresponding hydrolysis. However, it was observed that the values of K_m for hydrolysis were significantly smaller than those for hydration. Both reactions of the bifunctional substrates appear to be strongly inhibited by acetazolamide. The experimental inhibition constants, however, differ widely: K_i(hydrolysis) $< 3 \times 10^{-7}$ M vs. K_i(hydration) $= 2 \times 10^{-5}$ M. It is suggested that the dual function of bovine carbonic anhydrase on one and the same substrate may involve somewhat different modes of binding and hence different courses of enzyme action despite a number of kinetic similarities between enzymatic hydration and hydrolysis.

Erythrocyte carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) possesses wide catalytic versatility both in terms of its binding capacity and turnover efficiency. The enzyme acts both as a hydrase[2a–d] and an esterase.[3] However, never before has a single substrate served to demonstrate both types of activity.

Alkyl pyruvate esters possess a carbonyl which undergoes hydration (reaction 1) and an adjacent alkyl carboxylate group which undergoes hydrolysis (reaction 2).[4]

$$CH_3COCO_2R + H_2O \rightleftharpoons CH_3C(OH)_2CO_2R \quad (1)$$

$$\begin{bmatrix} CH_3COCO_2R \\ H_2O \uparrow\downarrow -H_2O \\ CH_3C(OH)_2CO_2R \end{bmatrix} + H_2O \longrightarrow \begin{bmatrix} CH_3COCO_2^- \\ H_2O \uparrow\downarrow -H_2O \\ CH_3C(OH)_2CO_2^- \end{bmatrix} + H^+ + ROH \quad (2)$$

In our earlier work, we have compared the catalytic properties of bovine carbonic anhydrase (BCA) as a hydrase[2b] with its catalytic properties as an esterase.[3] The present work shows that BCA catalyzes not only the hydration (reaction 1), but also the hydrolysis (reaction 2) of methyl and ethyl pyruvate. These bifunctional substrates offer a unique opportunity to investigate the enzymatically catalyzed consecutive reactions. Thus, a common substrate allows the direct comparison of kinetic parameters associated with the known hydrase and esterase activities of the enzyme in the presence and absence of certain inhibitors. A detailed knowledge of the kinetic behavior of pyruvate systems is made even more important by the use of some related compounds as enzyme modification agents, e.g., bromopyruvate, and by the very special properties of the N^{τ}-carboxyketoethylated histidine residue in the enzyme.[5,6]

For reactions 1 and 2, the experimental techniques necessary to obtain accurate rate data are far simpler than those involved in similar studies pertaining to the reversible hydration of carbon dioxide. Furthermore, the solubilities of the low molecular weight alkyl pyruvates in water are such that conveniently measured amounts of substrate can be added to buffered enzyme solutions to obtain accurate Michaelis parameters.

Experimental Section

Lyophilized BCA was obtained from Mann Research Laboratories. The methods of purification[6] and standardization[7] of the enzyme solutions were described in earlier publications. The substrates (Aldrich Chemical Co.) were distilled through a Vigreux column: bp (methyl pyruvate) 43 °C (19 Torr); bp (ethyl pyruvate) 54 °C (19 Torr). Buffer solutions were prepared in deionized water from reagent grade buffer components. The ionic strength of all solutions was adjusted by adding the appropriate quantities of sodium sulfate. Acetazolamide was obtained from American Cyanamid (Lederle Laboratory Division). The instruments employed for spectrophotometric and pH measurements and for temperature control were described in an earlier publication.[4]

The reactions were initiated by adding the appropriate volumes of the pyruvate esters to 3 mL of the reaction solution by means of a calibrated Hamilton syringe. The initial equilibration between the pyruvate esters and their respective hydrates (eq 1) results in a relatively rapid diminution of absorbancy ($\tau_{1/2} < 2.0$ s at 25.0 °C) at 340 nm. The pyruvate ion formed in the much slower subsequent hydrolysis step has a considerably lower fraction of hydration, $\chi^{25\,°C}_{\text{pyruv. ion}} = 0.06$,[8] than that of the pyruvate esters, $\chi^{25\,°C}_{MP} = 0.74$,[4] $\chi^{25\,°C}_{EP} = 0.70$.[4] Thus, the hydrolyses of the pyruvate esters were monitored spectrophotometrically at 340 nm by the subsequent increase in absorbancy which occurs with the formation of the less hydrated pyruvate ion.[4,8] The observed velocities (in M min^{-1}), $v_{obsd} = v_{buffer} + v_{enz}$, were calculated by dividing the initial increasing slopes associated with the hydrolysis by the difference in extinction coefficients between the pyruvate ion and the ester under consideration: $\Delta\epsilon^{MP}_{340nm} = 16.1$ M^{-1} cm^{-1}, $\Delta\epsilon^{EP}_{340nm} = 15.5$ M^{-1} cm^{-1}. The initial slopes of increasing

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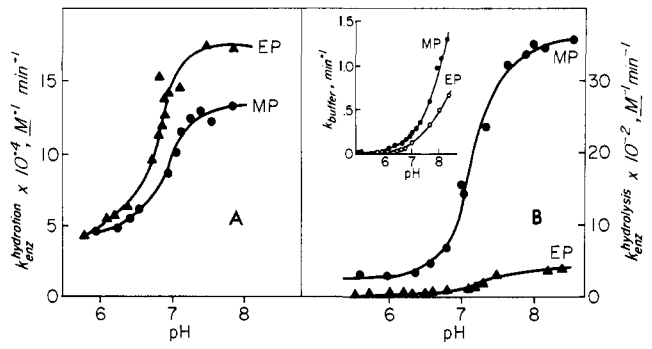

Figure 1. (A) The BCA-catalyzed hydration of pyruvate esters (see ref 2c). (B) The BCA-catalyzed hydrolysis of pyruvate esters. The hydrolysis reactions were carried out in 0.5 M phosphate, μ = 3.5 (Na_2SO_4), at 25.0 °C; [methyl pyruvate] = 0.076 M; [ethyl pyruvate] = 0.18 M.

absorbancy were taken over short periods of time, such that no detectable change in pH occurred. Duplicate runs were generally carried out under each set of experimental conditions. The UV spectra of the hydrolyzed ester solutions were shown to be identical with that of a solution of the equivalent concentration of pyruvate ion, $CH_3COCO_2^- + H_2O \rightleftharpoons CH_3C(OH)_2CO_2^-$. Reaction velocities were also monitored over longer periods of time by using a Radiometer titrator, Type TTlc. Kinetic data obtained spectrophotometrically and titrimetrically were strictly in accord.

The observed rate constants, k_{obsd}, include the catalytic components associated with the enzyme and the buffer system employed:

$$k_{obsd} = \frac{v_{obsd}}{[\text{pyruvate ester}]} = k_{buffer} + k_{enz}[enz] \quad (3)$$

The pH-rate profile for the buffer-catalyzed reaction component is shown in Figure 1B (insert). The catalytic coefficient for the enzyme was calculated by subtracting, from k_{obsd}, the catalytic contribution by the buffer:

$$k_{enz} = (k_{obsd} - k_{buffer})/[enz] \quad (4)$$

Results and Discussion

The sigmoidal pH-rate profiles for both the enzymatic hydrations (Figure 1A) and the hydrolyses (Figure 1B) show inflections around neutrality suggesting that a group (or groups) of pK_a around 7.0 plays an important role in the catalytic activity of the enzyme.[9] The enzymatic hydrations and hydrolyses[1c] both strictly obey Michaelis-Menten kinetics. For both reactions, K_m appears to remain constant with pH while k_2 reflects the variation of k_{enz} with pH.[10,11]

The turnover numbers calculated from Figure 1B, k_2^{MP} = 330 min^{-1} and k_2^{EP} = 20 min^{-1}, at neutral pH are comparable to those for the *p*-nitrophenyl ester series previously studied in these and in other laboratories.[3] Although k_2 for the enzymatic hydration of methyl pyruvate[2c] is larger than that for its enzymatic hydrolysis, it must be noted that the spontaneous hydration[4] is ca. 1000 times more rapid than the spontaneous hydrolysis. This taken into account, a more meaningful comparison of hydrase and esterase activities at the respective plateau regions for methyl pyruvate would be: $(k_2/k_0)^{MP}_{hydrase}$ = 25 000[12a] vs. $(k_2/k_0)^{MP}_{esterase}$ = 150 000, where k_0 represents the spontaneous rates of hydration and hydrolysis, respectively.[12b] Thus, in terms of rate enhancement, BCA is a more effective esterase than hydrase for methyl pyruvate.

As in the case of the enzymatic hydrations, the turnover number favors methyl pyruvate over ethyl pyruvate at a given pH, while the Michaelis constants are larger for the former substrate (see Table I). Since for both hydration and hydrolysis K_m is insensitive to changes in k_2, the reciprocals of the Michaelis constants should at least approximate the binding constants between the respective substrates and BCA.[11] Thus, for both reactions, it would appear that ethyl pyruvate, the more hydrophobic substrate, binds more strongly than does methyl pyruvate. Parallel observations have been made earlier regarding the binding of BCA to a series of esters[3] and to a series of aliphatic aldehydes.[2b-d]

Table I. Comparison of Michaelis Parameters for the BCA-Catalyzed Hydration and Hydrolysis of Pyruvate Esters

	Substrate	pH	K_m, M[b]	k_2, min^{-1}
Hydration[a]	MP	6.52	0.39	24 000
	MP	6.96	0.39	34 000
	MP	7.40	0.39	50 000
	MP[c]	6.97	0.39	17 300
	EP	6.96	0.19	25 400
Hydrolysis[d]	MP	6.02	0.15	58
	MP	6.66	0.16	160
	MP	7.03	0.15	330
	MP[e]	6.66	0.15	116
	EP	7.03	0.04	20

[a] See ref 2c. [b] For hydrolysis, K_m values based on total ester concentration. [c] In the presence of 4.2×10^{-5} M acetazolamide and 5.2×10^{-5} M BCA; $K_i = 2 \times 10^{-5}$ M. [d] All hydrolysis series run with 2.75×10^{-5} M BCA. [e] In the presence of 8.01×10^{-6} M acetazolamide.

The hydrase and esterase activities of BCA on the alkyl pyruvates are both strongly inhibited by acetazolamide, a specific inhibitor of BCA which is known to interact at or near the zinc atom in the enzyme molecule.[13]

There are many similarities in the manifestation of hydrase and esterase activities on a given pyruvate ester. It is also worthwhile, however, to note some of the significant differences. The ratio of k_2^{MP}/k_2^{EP} is much larger for the hydrolysis than for the hydration. This is perhaps to be expected, since in comparison to hydrase activity, the turnover for esterase activity requires enzyme interaction much closer to the alkoxy group (OMe vs. OEt).

The ratio of K_m^{MP}/K_m^{EP} is considerable larger for hydrolysis than for hydration. Furthermore, the K_m values for the hydrolysis of both esters are considerably smaller than the corresponding values for the hydration. Indeed, the values K_m for hydrolysis are in reality even smaller than the apparent values given in Table I if account is taken of the fact that these experimental (apparent) K_m values are based on total ester concentration, $[CH_3COCO_2R] + [CH_3C(OH)_2CO_2R]$.[14] Thus, the activation of an alkyl pyruvate toward the two re-

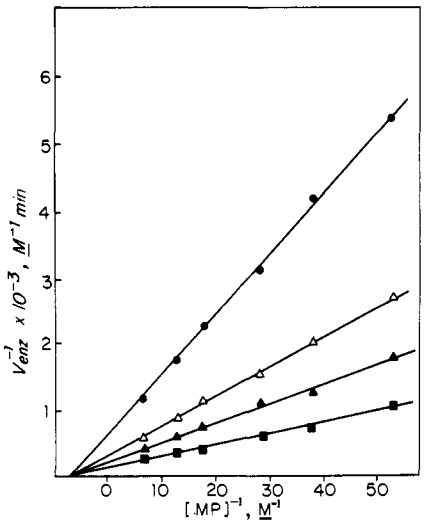

Figure 2. Lineweaver-Burke plots of the BCA (2.75×10^{-5} M) catalyzed hydrolysis of methyl pyruvate: (■) pH 7.03; (▲) pH 6.66; (△) pH 6.66 in the presence of 8.01×10^{-6} M acetazolamide; (●) pH 6.02. The values of K_m determined from these plots are based on total ester concentration, $[CH_3COCO_2Me] + [CH_3C(OH)_2CO_2Me]$.

actions may involve separate binding of the substrate in slightly different conformations. There is the additional possibility that *different* forms of the pyruvate, e.g., unhydrated vs. hydrated, serve as the preferential substrates for the enzymatic hydration and hydrolysis reactions, respectively.

From Figure 2, it can be shown that the acetazolamide inhibition of the hydrolysis of methyl pyruvate (pH 6.66) is essentially stoichiometric at the concentrations of enzyme and inhibitor employed.[15a] Virtually total inhibition was observed using about one and one-half times as much acetazolamide as BCA. For this system, enzyme–inhibitor binding is so tight that it is not possible to obtain a numerical value for K_i. However, by applying Henderson's method of kinetic analysis,[15b] it was estimated that $K_i <$ ca. 3×10^{-7} M. The inhibition of the esterase activity, therefore, is much more potent than that of hydrase activity ($K_i = 2 \times 10^{-5}$ M).[2c] Thus, for pyruvate esters, it would appear that BCA functions more "normally" in its esterase activity than in its hydrase activity. We also observed for the pyruvate esters that the residual esterase activity around pH 6 is considerably smaller than that for hydrase activity. The rather noticeable residual activity for the enzymatic hydration of methyl pyruvate at low pH[2c] and the relatively large dissociation constant observed for acetazolamide inhibition may be accounted for by the existence of a secondary catalytic site.[2c] Recent evidence from these laboratories shows that, for certain substrates of BCA, the apoenzyme may also exhibit some activity even at pH 6, an activity which is relatively much less sensitive to acetazolamide inhibition.[16] If, indeed, a secondary catalytic site exists, it would appear from the present work that it is less important for the enzymatic hydrolysis of alkyl pyruvates than for their respective hydrations.

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(1) (a) Visiting Scholar, summer 1975; Fellow of the National Science Foundation Science Faculty Professional Development Program, 1977–1978; (b) Fellow of the Washington State Heart Association, 1974–1975; (c) Undergraduate Research Participant.

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(9) (a) It is recognized that pH–rate profiles rarely fit theoretical titration curves exactly. The titration of an enzyme indeed would be expected to cause distortion in the chemical environment of a given functional group. In the present case, since the curve appears to predominantly reflect changes in k_2 as a function of pH, the inflection more properly results from the titration of a group or groups associated with the turnover of the active enzyme–substrate complex. It has further been suggested that the active site on BCA contains two important ionizing groups of similar pK_a which are both titrated around neutrality.[9b] (b) Y. Pocker and S. Sarkanen, *Adv. Enzymol. Relat. Areas Mol. Biol.*, in press.

(10) Overall enzymatic catalysis refers to $k_{enz} = k_2/(K_M + [S])$. It would perhaps be more instructive to compare the k_{enz} values for the hydrolysis of methyl pyruvate and ethyl pyruvate using lower substrate concentrations. In this connection, several values for k_{enz} on the pH–rate profile were determined for [ethyl pyruvate] = 0.06 M. Consistently, these k_{enz} values were observed to exceed those at 0.18 M by a factor of ca. 2.2.

(11) The constants, k_1, k_{-1}, and k_2, are the specific rate constants in the simplified mechanism:

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

It is realized that the kinetic parameters determined experimentally are considerably more complex than indicated by the above formulation.[7]

(12) (a) Ratio taken at 5.0 °C. (b) $k_0 = 3.6 \times 10^{-3}$ min^{-1} for the hydrolysis of methyl pyruvate at 25.0 °C.

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(14) If it could be shown that the unhydrated esters were the preferential substrates for enzymatic hydrolyses, the experimental K_m values would have to be corrected to correspond to the fraction of unhydrated ester. In the case of methyl pyruvate, $K_m(CH_3COCO_2R) = (1 - \chi)K_m^{app} = 0.039$ M, and in the case of ethyl pyruvate, $K_m(CH_3COCO_2R) = 0.012$ M. On the other hand, it can be shown that if the *hydrated* esters serve as the preferential substrates of enzymatic hydrolysis $K_m(CH_3C(OH)_2CO_2R) = 0.11$ M (methyl pyruvate) and $K_m(CH_3C(OH)_2CO_2R) = 0.028$ M (ethyl pyruvate).

(15) (a) It must be noted that when inhibition is essentially stoichiometric, it is not possible to distinguish between competitive and noncompetitive inhibition from such analysis. (b) P. J. F. Henderson, *Biochem. J.*, **127**, 321 (1972).

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