Use of pH Studies To Elucidate the Catalytic Mechanism of Rabbit Muscle Creatine Kinase†

Paul F. Cook,* George L. Kenyon,* and W. Wallace Cleland*

ABSTRACT: The pH variation of the kinetic parameters for rabbit muscle creatine kinase has been determined in order to deduce the chemical mechanism and mode of binding of the substrates. A cationic acid (probably histidine) on the enzyme with a pK near 7 must be unprotonated for phosphorylation of creatine and protonated for the reverse reaction, and thus acts as an acid–base catalyst. The binding of creatine in the E–MgADP–creatine and E–MgADP–creatine–nitrate complexes (E = enzyme, ADP = adenosine diphosphate) is not sensitive to the protonation state of this histidine, but creatine alone does not form a binary complex with enzyme when this group is protonated. A neutral acid (probably carboxyl) with a pK near 6 must be ionized for binding of either creatine or phosphocreatine under all conditions. This group probably forms an ionic bond with the tertiary nitrogen of the guanidinium group, thus localizing the positive charge away from the nitrogen that is phosphorylated. The binding of nitrate ion to enzyme–MgADP is pH independent (although 70-fold tighter with creatine also present), and thus any groups hydrogen bonding to the oxygens of the γ-phosphate group of adenosine triphosphate must have pKs above 10. The shape of the $V/K_{\text{P-creatine}}$ profile (a distinct hollow was present at both 12 and 25 °C, but not at 35 °C) and an isotope trapping experiment at pH 7 show that at the pH optimum phosphocreatine is sticky and reacts to give creatine 4–6 times faster than it dissociates from the central complex. However, phosphocreatine dissociates from the binary enzyme–phosphocreatine complex 70–80 times faster than the maximum velocity.

Creatine kinase catalyzes the reversible phosphorylation of creatine by MgATP. At pH 8, the kinetic mechanism appears rapid equilibrium random in both directions on the basis of both initial velocity studies (Morrison & James, 1965) and the patterns of isotope exchange at equilibrium (Morrison & Cleland, 1966), while at pH 7 initial velocity studies indicate a rapid equilibrium ordered mechanism for phosphorylation of creatine, with MgATP adding to the enzyme prior to creatine (Schimerlik & Cleland, 1973).

Although the kinetic mechanism for this enzyme is well documented, little is known about the enzymatic residues necessary for auxillary catalytic and binding roles. James & Cohn (1974) have suggested that the planar anions formate and nitrate are bound to the ϵ-amino group of an active site lysine and that the transferred phosphoryl group is similarly bound. An arginine in the active site has been implicated by the work of Borders & Riordan (1975). The presence of a histidine in the active site has been indicated by the diethyl pyrocarbonate inactivation of creatine kinase with a stoichiometry of two histidines per dimer (Pradel & Kassab, 1968; Clarke & Price, 1979). Reaction of an active-site thiol with iodoacetate leads to inactivation (Watts & Rabin, 1962), but conversion to the methyl disulfide with methyl methanethiosulfonate leads to partially active enzyme (Smith & Kenyon, 1974; Maggio et al., 1977), so the thiol does not appear to participate directly in catalysis. Herein, we report pH studies that indicate that a cationic acid group (probably histidine) acts as an acid–base catalyst in the reaction and that a neutral acid that is probably a carboxyl group appears to bind the positively charged guanidinium groups of both creatine and phosphocreatine. Quantitative analysis of the pH profiles and the use of the isotope trapping method of Rose et al. (1974) also indicate that at pH 7 phosphocreatine reacts to give creatine faster than it dissociates, so that the mechanism is not a rapid equilibrium one in this direction at the pH optimum.

Materials and Methods

Chemicals. [^{14}C]Phosphocreatine was prepared from 0.44 mM [^{14}C]creatine (23 Ci/mol from Amersham Searle) by using 100 mM Tris-HCl, pH 8, 3 mM ATP, 10 mM MgOAc, and 1.9 μM creatine kinase sites in 5 mL. ATP was regenerated in situ with 3 mM phosphoenolpyruvate, 10 mM KCl, and 520 U/mL pyruvate kinase (Sigma), and this regeneration also served to make the creatine kinase reaction irreversible. After 1 h, the reaction mixture was vortexed with several drops of CCl_4, centrifuged for 15 min at 6500g to remove enzymes, applied to a 1.2 × 30 cm column of Dowex 1-X8 equilibrated with 0.1 N sodium formate and 100 mM Tris-HCl, pH 8, and eluted with a 0.1–1 N gradient of the same buffer. The resulting 55 μM phosphocreatine solution (10 mL) was adjusted to 20 mM with unlabeled phosphocreatine to give a final specific activity of 0.25 Ci/mol. Creatine kinase was purified from rabbit muscle by the method of Kuby et al. (1954) and had a specific activity of 60 IU.

Initial Velocity Studies. Initial velocity studies were carried out with a Beckman DU monochromator and a Gilford OD converter by monitoring change in the absorbance at 340 nm. In the direction of creatine phosphorylation, creatine kinase was coupled to pyruvate kinase and lactate dehydrogenase. Reaction mixtures contained 75 mM buffer (all were neutralized with KOH, so that sufficient K^+ was present to activate pyruvate kinase), 5 mM MgOAc (except 10 mM below pH 6), 3 mM ATP (except 6 mM below pH 6), 0.4 mM DPNH, 0.4 mM phosphoenolpyruvate, 143 U/mL lactate dehydrogenase, 82 U/mL pyruvate kinase, and variable creatine. The level of creatine kinase was varied from 0.48

† From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70112 (P.F.C.), the Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California, San Francisco, California 94143 (G.L.K.), and the Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706 (W.W.C.). *Received August 5, 1980.* This work was supported by a National Institutes of Health postdoctoral fellowship (1F 32GM 05940-02) to P.F.C. and National Institutes of Health grants to W.W.C. (GM 18938) and G.L.K. (AM 17323). G.L.K. is a U.S. Public Health Service Research Career Development Awardee, AM 00014, 1975–1980.

0006-2960/81/0420-1204$01.25/0

μM at pH 5 to 4.8 nM at pH 9.7. At pH values of 6.5 and below, DPNH, lactate dehydrogenase, and pyruvate kinase were added to the reaction mixture just prior to the addition of creatine kinase to prevent degradation of DPNH and loss of coupling enzyme activity. The levels of coupling enzymes required to give correct initial velocities without excessive lags were determined at pH 5.05, and these enzyme levels were used for the entire pH range.

In the direction of ADP phosphorylation, creatine kinase was coupled to hexokinase and glucose-6-P dehydrogenase. Reaction mixtures contained 75 mM buffer, 5 mM MgOAc (except 10 mM below pH 6), 3 mM ADP (except 6 mM below pH 6), 3 mM glucose, 1 mM TPN, 3.3 U/mL hexokinase, 6.6 U/mL glucose-6-P dehydrogenase, and variable phosphocreatine. The levels of creatine kinase used varied from 4.8 nM at pH 5.4 and 8.8 to 0.48 nM at pH 7.3. At pH values of 8 and above, TPN was added to the reaction mixture just prior to the addition of creatine kinase to prevent degradation, while at pH 6.5 and below, phosphocreatine, hexokinase, and glucose-6-P dehydrogenase were added just prior to the addition of creatine kinase to prevent degradation of phosphocreatine and loss of coupling enzyme activity. The required levels of coupling enzymes were the same at pH 8.78 and 5.0, and these levels were used over the entire pH range.

In experiments with creatine and nitrate as simultaneous competitive inhibitors vs. phosphocreatine, the assay conditions were as described above with phosphocreatine varied around its Michaelis constant in the absence of inhibitors and in the presence of several levels of nitrate minus creatine, several levels of creatine minus nitrate, and several levels of creatine at several nitrate levels. For example, at pH 6.7 the concentrations used were the following: creatine (at zero nitrate), 50 and 100 mM; nitrate (at zero creatine), 75 and 150 mM; creatine (at 10 and 20 mM nitrate), 10 and 20 mM.

pH Studies. Determination of V and V/K for both creatine and phosphocreatine and the K_i's of both nitrate and creatine as competitive inhibitors of phosphocreatine as a function of pH were carried out at saturating concentrations of nucleotide by varying the levels of substrates and inhibitors as discussed above. Buffers (75 mM) were used over the following pH ranges: acetate, 5.0–5.4; Mes,[1] 5.4–6.4; Pipes, 6.37–7.3; Hepes, 7.4–8.2; Taps, 8.4–9.3; Ches, 9.3–9.7. All buffers were titrated to pH with KOH. In all cases, sufficient overlaps were obtained between buffers either to rule out or to make minor corrections for buffer effects. When the kinetic parameters were determined as a function of pH at 12, 25, and 35 °C, the temperature was maintained with a circulating water bath with capacity to heat and cool and thermospacers for the cell compartment. Assay mixtures were incubated for at least 10 min in the water bath and then for 5 min in the cell compartment prior to initiation of the reaction. The required levels of coupling enzymes were redetermined at 12 and 35 °C, and these enzymes were routinely added to the reaction mixture just prior to addition of creatine kinase at 35 °C.

In solvent perturbation studies in which the pH dependence of V and $V/K_{\text{P-creatine}}$ was determined with or without 25% dimethylformamide, the profiles were determined either with the above cationic acid buffers or with the following neutral acid buffers. A mixture 25 mM each in acetate and cacodylate was used from pH 5.5 to 7, 25 mM cacodylate and 12.5 mM each of acetate and borate from pH 7 to 8.2, and 25 mM each of cacodylate and borate from pH 8.1 to 9. A small amount of inhibition was observed above pH 7, probably caused by adduct formation between borate and TPN, but sufficient overlaps were available between uninhibited and inhibited regions for corrections to be made. The required levels of coupling enzymes were redetermined in neutral acid buffers and in both neutral and cationic acid buffers in the presence of 25% dimethylformamide. In the presence of 25% dimethylformamide at pH 7.4, V in the direction of ADP phosphorylation was 60% of the V in the absence of the solvent.

Isotope-Trapping Studies. A solution containing 1.4 mM creatine kinase (based on ϵ_{280} of 8.96 for a 1% solution) and 10 mM [^{14}C]phosphocreatine (2.25 Ci/mol) in 0.05 mL was added to 5 mL of a rapidly stirring solution of 50 mL of Tris-HCl, pH 7, MgOAc (6 mM at 5 mM ADP, 20 mM at 10 mM ADP, and 50 mM at 40 mM ADP), 20 mM unlabeled phosphocreatine, and 5, 10, or 40 mM ADP. The reaction was stopped after 5 s by addition of EDTA (30, 40, and 60 mM final concentrations for 5, 10, and 40 mM ADP), and acid-washed, heat-activated charcoal was added (0.25, 0.5, and 0.7 g for 5, 10, and 40 mM ADP). This mixture was then filtered through a bed of Celite in a sintered glass funnel, and the filtrate was applied to a 1.2 × 30 cm column of Dowex 1-X8 equilibrated with 0.1 N sodium formate and 100 mM Tris-HCl, pH 8, and developed with a 0.1–1 N linear gradient of the same buffer. The fractions were counted to determine the amount of [^{14}C]creatine formed. At each ADP concentration, controls were run in which creatine kinase was added to a reaction mixture containing all components listed above, including the [^{14}C]phosphocreatine, and the reaction was stopped with EDTA and analyzed in the usual way. The cpm for the control and experimental systems were 2658 and 6090 at 5 mM ADP, 2295 and 7743 at 10 mM ADP, and 2816 and 11096 at 40 mM ADP.

Data Processing. Reciprocal initial velocities (or amounts of [^{14}C]phosphocreatine trapped as creatine) were plotted against reciprocal substrate concentrations, and all plots were linear. The data were fitted to appropriate equations with the Fortran programs of Cleland (1979). The individual saturation curves used to obtain pH profiles were fitted to eq 1. Data for pH profiles which showed a decrease in log V, log V/K, or pK_i with a slope of 1 as the pH was decreased or a slope of −1 as the pH was increased were fitted to eq 2 or 3 (H is [H$^+$]). When log V and log V/K decreased at both low and high pH, the data were fitted to eq 4. Data for the $V/K_{\text{P-creatine}}$ profile which exhibited three pH dependent terms as the pH was increased were fitted to eq 5, while data for the V/K_{creatine} profile which decreased with a final slope of 2 as the pH was decreased were fitted to eq 6. Data for the $V/K_{\text{P-creatine}}$ profile

$$v = VA/(K + A) \quad (1)$$

$$\log y = \log [C/(1 + H/K_1)] \quad (2)$$

$$\log y = \log [C/(1 + K_1/H)] \quad (3)$$

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (4)$$

$$\log y = \log [C(1 + K_1/H)/(1 + (K_2/H)(1 + K_3/H))] \quad (5)$$

$$\log y = \log [C/(1 + H/K_2 + H^2/(K_1K_2))] \quad (6)$$

$$pK = \Delta H_{ion}/(2.303RT) + B \quad (7)$$

$$v = VA[K(1 + I/K_{is}) + A] \quad (8)$$

$$v = VA/[K(1 + I/K_{il} + J/K_{iJ} + IJ/(\alpha K_{il}K_{iJ})) + A] \quad (9)$$

[1] Abbreviations used: Mes, 2-(N-morpholino)ethanesulfonic acid; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Taps, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Ches, 2-(N-cyclohexylamino)ethanesulfonic acid; epoxycreatine, N-(2,3-epoxypropyl)-N-amidinoglycine.

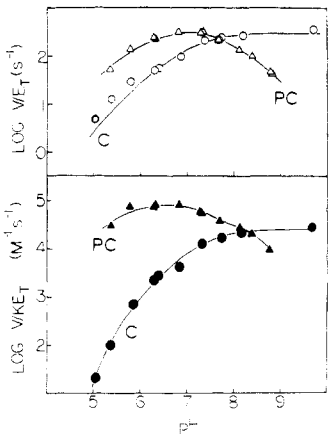


FIGURE 1: pH dependence of $V_{creatine}$ (○), $V_{P\text{-}creatine}$ (△), $V/K_{creatine}$ (●), and $V/K_{P\text{-}creatine}$ (▲) for creatine kinase. In the direction of creatine phosphorylation, MgATP was maintained at 3 mM above pH 6 and 6 mM at pH 6 and below, while in the direction of MgADP phosphorylation, MgADP was maintained saturating at 3 mM above pH 6 and 6 mM at pH 6 and below. Solid lines are theoretical curves for fits to eq 2, 4, and 6, and a combination of eq 4 and 5 for $V/K_{P\text{-}creatine}$ as discussed under Materials and Methods.

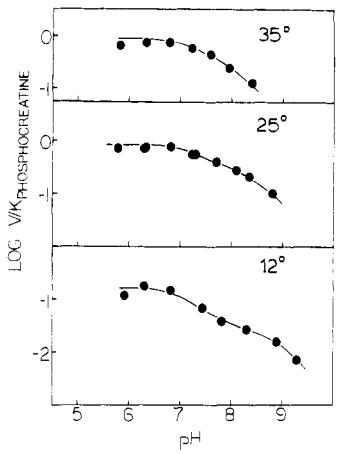


FIGURE 2: Dependence of the $V/K_{P\text{-}creatine}$ pH profile on temperature. Conditions are listed in the legend to Figure 1. Solid lines are theoretical fits to eq 3 for 35 °C and eq 5 for 12 and 25 °C. V/K values are relative (not V/KE_t).

shown in Figure 1 were fitted to eq 4 from pH 5.3 to 7.3 and to eq 5 from pH 5.8 to 8.8, and the curves were normalized to one another. In eq 2–6, K_1, K_2, and K_3 represent the dissociation constants for groups on the enzyme, y is V, V/K, or $1/K_i$, and C is the pH independent value of y. Apparent pK values as a function of temperature were fitted to eq 7. Data for linear competitive inhibition by one or by two inhibitors at the same time were fitted to either eq 8 or eq 9, where K_{is} is the slope inhibition constant for a single inhibitor, K_{iI} is the constant for I in the absence of J, K_{iJ} is the constant for J in the absence of I, αK_{iI} and αK_{iJ} are the slope inhibition constants for I and J in the presence of J or I, and α is the synergism factor whose reciprocal equals the increase in affinity of I and J for EJ and EI relative to their affinity for E.

Results

pH, Temperature, and Solvent Dependence of Kinetic Parameters. As shown in Figure 1, $V_{creatine}$ decreases below a pK of 7.01 ± 0.05 while $V/K_{creatine}$ decreases at low pH with pKs of 7.40 ± 0.04 and 5.57 ± 0.04. $V_{P\text{-}creatine}$ decreases below a pK of 6.16 ± 0.02 and above a pK of 7.87 ± 0.02, while $V/K_{P\text{-}creatine}$ decreases below a pK of 5.56 ± 0.08 and exhibits three pH dependent terms as the pH is increased, so that when the data were fitted to eq 5 pK_1 = 7.6 ± 0.5, pK_2 = 7.2 ± 0.3, and pK_3 = 8.3 ± 0.2.

The temperature dependence of the $V/K_{P\text{-}creatine}$ profile above pH 6 is shown in Figure 2. While the hollow in the profile corresponding to eq 5 is visible at 25 °C and is even more apparent at 12 °C, it is not seen at 35 °C, and the data were fitted to eq 3. For comparison, the other two profiles were also fitted to eq 3 (this gives the intersection point of the asymptotes of the curve), and pK values of 7.93 ± 0.09, 7.78 ± 0.05, and 7.61 ± 0.07 were obtained at 12, 25, and 35 °C. A fit of these pK values to eq 7 gave a ΔH_{ion} of 5.5 ± 0.7 kcal/mol. The pK values obtained similarly from the temperature dependence of the $V_{P\text{-}creatine}$ profile were 8.24 ± 0.05, 7.87 ± 0.02, and 7.48 ± 0.02 at 12, 25, and 35 °C. These values correspond to a ΔH_{ion} of 13.2 ± 1.4 kcal/mol.

When the pH dependence of $V/K_{P\text{-}creatine}$ was determined in neutral and cationic acid buffers in the presence and absence of 25% dimethylformamide, the data in Table I were obtained.

The pH dependence of p$K_{i\,creatine}$, p$\alpha K_{i\,creatine}$, p$K_{i\,nitrate}$, p$\alpha K_{i\,nitrate}$, and $1/\alpha$ from fits to eq 9 (where α is the synergism factor, K_i is a dissociation constant from the E–MgADP–creatine or E–MgADP–nitrate complex, and αK_i is a dissociation constant from the E–MgADP–creatine–nitrate complex) is shown in Figure 3. The values of α (0.014), $K_{i\,nitrate}$ (125 mM), and $\alpha K_{i\,nitrate}$ (2 mM) are pH independent, while p$K_{i\,creatine}$ and p$\alpha K_{i\,creatine}$ decrease below a pK of 6.02 ± 0.03. The pH independent values of $K_{i\,creatine}$ and $\alpha K_{i\,creatine}$ above the pK are 65 mM and 0.9 mM, respectively. The pK observed in the pK_i profile was 5.99 ± 0.01 at 12 °C and 6.10 ± 0.09 at 35 °C, and a fit of these pKs to eq 7 gave a ΔH_{ion} of −1.8 ± 0.8 kcal/mol.

Table I: Apparent pK Shifts for the $V/K_{P\text{-}creatine}$ Profile Induced by 25% Dimethylformamide in Neutral and Cationic Acid Buffers[a]

neutral acid buffers[b]			cationic acid buffers[b]		
−DMF	+DMF	Δ	−DMF	+DMF	Δ
c	c		5.56 ± 0.08	6.04 ± 0.04	0.48 ± 0.09
7.70 ± 0.03	6.51 ± 0.03	−1.19 ± 0.04	7.79 ± 0.05	6.97 ± 0.02	−0.82 ± 0.05

[a] pK values calculated from fits to eq 4 in cationic acid buffer but eq 3 in neutral acid ones. [b] See Materials and Methods for the buffers used. [c] Profiles not carried out at low enough pH to determine the acid side pK in neutral acid buffers.

Isotope Trapping. A binary complex of labeled phosphocreatine and creatine kinase was diluted into a solution containing a large excess of unlabeled phosphocreatine and variable MgADP and allowed to react for 5 s at pH 7 in the isotope-trapping method of Rose et al. (1974). From a reciprocal plot of [^{14}C]creatine formed vs. MgADP, the maximum amount of phosphocreatine trapped as creatine was 0.86 ± 0.04 mM, and the K'_{ADP} (apparent Michaelis constant for trapping) was 9.4 ± 1.1 mM.

Discussion

Interpretation of the pH Profiles. The pH profiles in Figure 1 suggest that there is a single group with a pK near 7 which acts as an acid–base catalyst and must be unprotonated in the direction of creatine phosphorylation and protonated for MgADP phosphorylation. The apparent pK of this group is observed in both V and V/K profiles. In addition, another group with a pK near 6 must be unprotonated for activity in either direction.

Above pH 7, the $V/K_{P\text{-}creatine}$ profile shows the shape which has been termed a "hollow" by Cleland (1977). In the

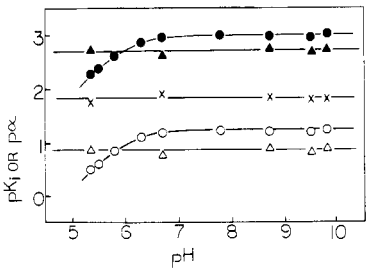
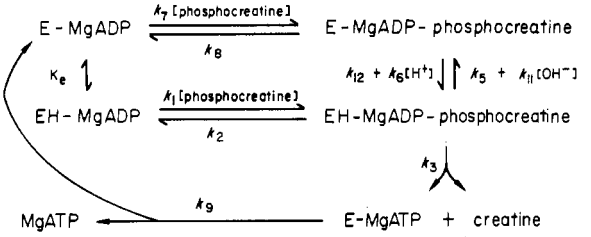


FIGURE 3: Dependence of $1/K_{i\,creatine}$ (○), $1/\alpha K_{i\,creatine}$ (●), $1/K_{i\,nitrate}$ (△), $1/\alpha K_{i\,nitrate}$ (▲), and $1/\alpha$ (×), where creatine and nitrate are nonmutually exclusive competitive inhibitors of phosphocreatine and α is the synergism factor. MgADP was maintained saturating, as in the legend to Figure 1. Solid lines for $1/K_{i\,creatine}$ and $1/\alpha K_{i\,creatine}$ are theoretical curves for fits to eq 2, while for $1/K_{i\,nitrate}$, $1/\alpha K_{i\,nitrate}$, and $1/\alpha$, the straight line represents the average value for the parameter calculated from the data for pH values from 5.3 to 9.8.

Scheme I



mechanism shown in Scheme I in the presence of saturating MgADP, the expression for $V/K_{phosphocreatine}$ is[2]

$$V/KE_t = \{[k_1k_3/(k_2 + k_3)][1 + (k_7aK_e/(k_1k_5k_8H)) \times (1 + k_2k_7k_{12}K_e/(k_1aH))]\}/\{[1 + K_e/H][1 + (k_2k_7bK_e/(k_1(k_2 + k_3)k_5k_8H))(1 + k_2k_7k_{12}K_e/(k_1bH))]\} \quad (10)$$

where $H = [H^+]$ and

$$a = k_2k_{12} + k_2k_8 + k_5k_8 \quad (11)$$

$$b = (k_2 + k_3)k_{12} + (k_2 + k_3 + k_5)k_8 \quad (12)$$

A hollow will be observed in the V/K profile when both the substrate and the proton in EH–MgADP–phosphocreatine are sticky (that is, k_3 must be greater than or equal to k_2, and k_5 and k_{12} must be less than or equal to k_2 and k_8). If $k_3 < k_2$, the large terms in both numerator and denominator factor out, and a simple profile with pK_e is seen. This apparently occurs when dimethylformamide is added.

Equation 10 is more complex than eq 5, but, if we assume[2] that because the pK is around 7 and $k_6 = k_{11}$ that $k_5 = k_{12}$ and we further assume that k_5, $k_{12} < k_2$, k_8 (that is, the proton on the enzyme is sticky), eq 10 reduces to

$$V/KE_t = \{[k_1k_3/(k_2 + k_3)][1 + (k_2k_7K_e/(k_1k_5H)) \times (1 + k_5k_7K_e/(k_1k_8H))]\}/\{[1 + K_e/H][1 + (k_2k_7K_e/(k_1k_5H))(1 + k_2k_5k_7K_e/(k_1(k_2 + k_3)k_8H))]\} \quad (13)$$

If k_1 and k_7 are similar while $k_5 < k_2$, k_8, eq 13 will closely resemble eq 5, with the values of K_1, K_2, and K_3 in eq 5 being $k_5k_7K_e/(k_1k_8)$, K_e, and $k_2k_5k_7K_e/[k_1(k_2 + k_3)k_8]$. The V/K profile will begin to drop at pK_e, level out log (k_8/k_5) pH units above pK_e, and then begin to decrease log $(1 + k_3/k_2)$ pH units still higher. From the fits to eq 5, we can thus estimate that log (k_8/k_5) is 0.4 at 25 °C and 0.97 at 12 °C, so that the k_8/k_5 ratios are 2.5 and 9.3, respectively. In view of this trend, it is not surprising that at 35 °C k_8 is probably less than k_5, and, thus, no hollow is seen. The rate constant that is highly temperature sensitive appears to be k_5 (or k_{12}, since we have assumed them to be equal).

The value of log $(1 + k_3/k_2)$ appears to be 0.7 at 25 °C and 1.09 at 12 °C from the difference between pK_1 and pK_3 from fits to eq 5. Thus, k_3/k_2 is 4.0 at 25 °C and 11.3 at 12 °C, and phosphocreatine, as well as the proton, is stickier at low than at high temperatures. At 35 °C, the lack of stickiness of the proton eliminates the hollow, but it is clear that the substrate is still sticky since the apparent pK is seen at 7.61, which is clearly above the pK of the acid–base catalyst. If pK_e is taken as 7.2, $k_3/k_2 = 1.6$ at 35 °C, while if pK_e is 7.0, $k_3/k_2 = 3.1$. While these calculations are only approximate, from the trends it appears that k_2 is more temperature dependent than k_3. Thus, the catalytic reaction has the lowest ΔH value, the conformational change that permits substrate release has the next highest ΔH value, and the conformational change which permits proton transfer between water and the bound proton on the acid–base catalyst has the highest ΔH value.[3]

Solvent perturbation of apparent pKs allows one to determine whether the groups involved are neutral or cationic acids. Cationic acids such as the nitrogen bases exhibit little change in pK when organic solvent is added, since a net positive charge is present before and after dissociation of the acid. Neutral acids such as carboxyl groups will show an increase in pK, however, since a new positive and negative charge are present after dissociation and the solvent impedes dissociation even when, as with dimethylformamide or dimethyl sulfoxide, the dielectric constant of the water–solvent mixtures is not very different from that of pure water. These experiments are best carried out by measuring pH values before addition of organic solvent and comparing apparent pK values with and without the organic solvent in both neutral and cationic acid buffers (Cleland, 1977). When the buffer is a cationic acid, organic solvent does not change its pK or the pH in the solution. A neutral acid group on the enzyme thus appears to have its pK increased by the organic solvent, but a cationic acid group shows no change. Conversely, when the buffer is a neutral acid, its pK is elevated by the solvent, and so, as a result, is the pH of the solution. Organic solvent thus introduces a frame shift in the pH scale (since pH values are measured before solvent addition), and the pK of a cationic group will be seen at a lower apparent pK. A neutral acid group appears to show no change since its pK is elevated along with that of the buffer.

[2] It is important to include in the interconversion of E–MgADP–phosphocreatine and EH–MgADP–phosphocreatine rate constants for both protonation by H^+ (k_6) and proton release to H_2O (k_5) and also those for proton transfer from water (k_{12}) and deprotonation by OH^- (k_{11}). The relationship between these rate constants is $k_5/k_6 = k_{11}K_w/k_{12}$ where $K_w = 10^{-14}$ M^2. For carboxyl, imidazole, and similar groups in aqueous solution where both rate constants are limited solely by diffusion, k_6 and k_{11} are essentially equal (Eigen, 1964), although this is not the case for certain groups like thiols and might not hold true when access to a group on the protein which is covered by a substrate is restricted. If we assume k_6 and k_{11} are equal, k_5 and k_{12} will be of similar size if pK_e is 7, but $k_5 > k_{12}$ when it is less than 7, and $k_5 < k_{12}$ when it is greater than 7. Since pK_e is close to 7 in the present case, the full equation must be employed.

[3] Note that when the substrate is sticky one cannot determine the temperature dependence of pK_e from the intersection points of the two asymptotes of the profile ($pK_2 + pK_3 - pK_1$ from a fit to eq 5) since the factor log $(1 + k_3/k_2)$ may also be temperature sensitive, as here. In theory, one could analyze the temperature dependence of pK_2 from fits to eq 5, but, in view of the limited data, the number of assumptions and approximations involved, and the uncertainties in the fits to eq 5, it would be preferable to analyze the temperature dependence of the pK in the V/K profile for a slow, and thus nonsticky, substrate. Slow substrates are known for creatine kinase (Rowley et al., 1971; McLaughlin et al., 1972), so such experiments could readily be carried out.

The data in Table I show a marked decrease in the apparent pK of the acid–base catalytic group in both neutral and cationic acid buffers when solvent was added. The most likely reason for most of the decrease in the pK in cationic acid buffers is a loosening of the enzyme's tertiary structure by the dimethylformamide so that phosphocreatine is no longer sticky. A pK of 7 in the solvent perturbation experiment is in reasonable agreement with the value of 7.2 obtained for pK_2 in the $V/K_{\text{phosphocreatine}}$ profile. The additional decrease in the pK from 7 to 6.5 in neutral acid buffers plus organic solvent then argues that the acid–base catalyst is a cationic acid. Since its pK is near 7, it is probably a histidine, but a lysine with a low pK such as that seen for acetoacetate decarboxylase (Schmidt & Westheimer, 1971) cannot be ruled out.

It should be noted that the binding of creatine as a binary E–creatine complex is sensitive to the state of protonation of this group. At pH 8, such a binary complex forms since the mechanism is rapid equilibrium random (Morrison & James, 1965; Morrison & Cleland, 1966), but, at pH 7 or below, it does not form since the mechanism becomes equilibrium ordered [Schimerlik & Cleland (1973), and confirmed at pH 6 in the present work]. On the other hand, the profiles in Figure 3 show that when a nucleotide is present creatine binding is insensitive to the protonation state of the acid–base catalyst. Presumably, the repulsive interactions of the protonated histidine and guanidinium groups are neutralized by the negative charges of the nucleotide.

The pK around 5.6–6 of the group which must be ionized for creatine binding as either a substrate (V/K_{creatine} in Figure 1) or inhibitor (p$K_{\text{i creatine}}$ or p$\alpha K_{\text{i creatine}}$ in Figure 3) increases in cationic acid buffers in the presence of organic solvent (Table I), as expected if the group were a neutral acid (the profiles obtained in neutral acid buffers were not extended to low enough pH to determine this pK accurately). Its low ΔH_{ion} (-1.8 ± 0.8 kcal/mol) suggests it is a carboxyl group, and it is likely the carboxyl group labeled by the epoxycreatine affinity label (Marletta & Kenyon, 1979).

Figure 3 shows that the K_{i} for the planar nitrate ion, which mimics the transferrable phosphoryl group by binding to the same site as this putative phosphoryl group on creatine kinase (Milner-White & Watts, 1971; Reed & Cohn, 1972; Reed et al., 1978), exhibits no pH dependence. Thus the pK of any lysine involved in the binding of nitrate as proposed by James & Cohn (1974) must be higher than 10 in both the E–MgADP and E–MgADP–creatine complexes.

Determination of the Stickiness of Phosphocreatine by the Isotope-Trapping Technique. To corroborate the conclusion from the pH studies that phosphocreatine was sticky from the ternary complex, we used the isotope-trapping technique of Rose et al. (1974) at pH 7. Since the dissociation constant of phosphocreatine from the E–phosphocreatine complex is high (3.9 mM; Schimerlik & Cleland, 1973), the mechanism shown in reaction 14 and eq 15 and 16 (Cleland, 1977) were used to treat the data. k_{13} and k_2 represent the rate constants

$$\begin{array}{ccccc} & & \text{E} + \text{MgADP} + \text{creatine}^* & & \\ & & \uparrow k_3 & & \\ \text{E-phosphocreatine}^* & \overset{\text{MgADP}}{\rightleftharpoons} & \text{E-phosphocreatine}^*\text{-MgADP} & & \cdot\ (14) \\ \downarrow k_{i3} & & \downarrow k_2 & & \\ \text{E} + \text{phosphocreatine}^* & & \text{E-MgADP} + \text{phosphocreatine}^* & & \end{array}$$

$$k_2/k_3 = (E_t/[\text{creatine}^*_{\text{max}}])/(1 + K_{\text{i P-creatine}}/[\text{P-creatine}]) - 1 \quad (15)$$

$$(K'_{\text{ADP}}/K_{\text{ADP}})(E_t/[\text{creatine}^*_{\text{max}}])/(1 + K_{\text{i P-creatine}}/[\text{P-creatine}]) \geq k_{13}/k_3 \geq K'_{\text{ADP}}/K_{\text{ADP}} \quad (16)$$

for dissociation of phosphocreatine from the binary and ternary complexes (once labeled molecules dissociate, they are diluted by the large pool of unlabeled substrate and contribute only to the same extent as the blank) and k_3 is the net rate constant for catalysis and release of products. For our experiments, $E_t = 1.4$ mM, [creatine$^*_{\text{max}}$] = 0.86 mM, $K_{\text{i P-creatine}} = 3.9$ mM, [phosphocreatine] free in the original incubation mixture along with E–phosphocreatine* was 8.6 mM, K'_{ADP} (the apparent Michaelis constant for isotope trapping) was 9.4 mM, and K_{ADP} (the Michaelis constant for the chemical reaction at pH 7) was 0.14 mM (Schimerlik & Cleland, 1973). Substituting these values into eq 15 and 16 gives $k_2/k_3 = 0.16$ and $78 \geq k_{13}/k_3 \geq 67$, so that $k_3/k_2 = 6$ and $0.015 \geq k_3/k_{13} \geq 0.013$.

The value of 6 for k_3/k_2 agrees with the value of 4 estimated from the pH profiles and confirms that phosphocreatine is sticky from the ternary E–MgADP–phosphocreatine complex at pH 7. However, phosphocreatine dissociates 70–80 times faster from the binary E–phosphocreatine complex than the net rate constant for catalysis and product release. As the pH is raised, however, the stickiness of phosphocreatine is eliminated since catalysis is slowed, but reactant release is not. Thus, at pH 8, the isotope-exchange studies of Morrison & Cleland (1966) showed that the kinetic mechanism was a rapid equilibrium one in which neither substrate was sticky. We would also expect the stickiness to disappear below pH 6, but this has not been tested.

Chemical Mechanism and Geometry of the Active Site. The actual chemical reaction catalyzed by creatine kinase can be written in a generalized form

$$^{-+}\text{R}{=}\text{NH}_2 + \text{MgATP}^{2-} \leftrightarrow {}^{-+}\text{R}{=}\text{NH}{-}\text{PO}_3^{2-} + \text{MgADP}^- + \text{H}^+ \quad (17)$$

where R is the rest of the creatine molecule. Clearly, an acid–base catalyst is needed to accept the proton from the guanidinium group during the phosphorylation of creatine and donate a proton during the reverse reaction, and this group has been identified in the present work as a cationic acid, probably histidine. By contrast, for beef liver fructokinase (Raushel & Cleland, 1977) and yeast hexokinase (Viola & Cleland, 1978), the acid–base catalyst has been identified as a carboxyl group [confirmed for hexokinase as an aspartate by the X-ray studies of Anderson et al. (1978)]. The reasons for the difference may be 2-fold. First, fructokinase and hexokinase have the sole physiological role of catalyzing phosphorylation. The low pK of near 6 of the carboxyl is thus no disadvantage, and a negatively charged group is clearly a better base to use for proton removal than is a neutral histidine. Creatine kinase, on the other hand, must catalyze both the phosphorylation of creatine in the resting state in muscle and the rapid rephosphorylation of MgADP to MgATP during periods of active muscle work. In order to play both roles, the acid–base catalyst must have a pK closely matching the pH of the cell, and this is easier to accomplish with histidine than with a carboxyl group. Second, the phosphorylation of a positively charged guanidinium group presents special problems since it is necessary for one nitrogen to act as a nucleophile in attacking the γ phosphorus of ATP, which carries a partial positive charge as the result of coordination of one oxygen of the γ-phosphate by Mg^{2+} and hydrogen bonding of the other two oxygens by positively charged lysines or arginines. If the NH_2 group being phosphorylated formed a hydrogen bond to an ionized carboxyl group, the negative charge of the carboxyl would attract a good portion of the positive charge of the guanidinium group to this nitrogen and thus make it a much poorer nucleophile. A neutral histidine does not produce the same effect.

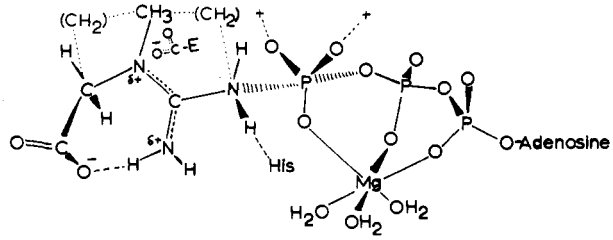


FIGURE 4: Possible structure of the transition state for phosphoryl transfer in the creatine kinase reaction. His is the hypothetical acid–base catalyst (shown here above the plane of the guanidinium group), and E–COO^- is the hypothetical carboxyl group (shown here below the plane) which must be ionized for binding of creatine or phosphocreatine and which presumably reacts with epoxycreatine, which has an ethylene oxide ring attached to the *N*-methyl group. The CH_2 groups in parentheses indicate where methylene bridges can be inserted to yield cyclic substrates with high substrate activity.

The role of the carboxyl group that must be ionized for the binding of creatine and phosphocreatine now becomes clearer. Since there are no attractive ionic interactions between the histidine, which is the acid–base catalyst and the guanidinium group, other interactions are necessary to hold the guanidinium group in place. Further, if the nitrogen which is to attack the phosphorus is to be a good nucleophile, essentially all of the positive charge of the guanidinium group must be localized on the other two nitrogens. We propose that the carboxyl group in the active site forms an ionic interaction with one of these guanidnium nitrogens, and, since epoxycreatine[1] is a substrate as well as an active site directed inhibitor for this carboxyl group (Marletta & Kenyon, 1979),[4] we suggest that it is the tertiary nitrogen with which it interacts.

This interaction, as well as the probable geometry of the reactants in the active site, is shown in Figure 4. We have shown the carboxyl on one side of the plane of the guanidinium group and the histidine on the other, but the stereochemistry is arbitrary in both cases and remains to be determined. The stereochemistry of the creatine molecule is, however, that deduced from the high substrate activity of molecules bridged with methylene groups, as shown by the dotted lines (McLaughlin et al., 1972; Dietrich et al., 1980). We have shown a hydrogen bond from the carboxyl of creatine to the free NH_2 of the guanidinium group, but this also is speculative; it would assist in localizing the positive charge away from the nitrogen being phosphorylated.

The geometry of the tripolyphosphate chain is that deduced from the Δ screw sense specificity with β,γ-bidentate CrATP by Dunaway-Mariano & Cleland (1980) and the Δ screw sense specificity with α-S-ADP by Burgers & Eckstein (1980). In the structure shown, which is the transition state for phosphoryl transfer, it is not known whether the α-phosphate is coordinated closely to the metal as shown (about 2.0 Å) or loosely to the metal, as in the crystal of Zn(bpy)ATP (2.4–2.7 Å; Orioli et al., 1980) (bpy = 2,2′-bipyridyl), or does not become coordinated until after phosphoryl transfer, as suggested by the substrate activity with Δ β,γ-bidentate CrATP. Three waters are shown coordinated to Mg, as indicated by the recent EPR studies of Reed & Leyh (1980). The arrangement in space of the adenosine is not shown; insufficient distance measurements are available to define this structure. The position of the Mg^{2+} is on the same side of the overall structure as the carboxyl group of creatine but as far as possible from the *N*-methyl group since distance measurements by NMR relaxation methods indicate that the metal is about 9 Å from the CH_2 or CH_3 groups of creatine in the E–MgADP–creatine complex (McLaughlin et al., 1976) and only 8 Å from the carboxyl carbon (Reed & McLaughlin, 1973). The distances in Figure 4 are about 7 Å, suggesting that the conformation changes which lead to the catalytic configuration bring the substrates closer together by 3 Å. A similar situation occurs with hexokinase, where the distance from the expected γ-phosphate position to C-6 of glucose is also at least 2 Å too far (Steitz et al., 1977). Kinases will probably all display conformation changes of this kind since it is necessary to displace water from the active site prior to creating the correct catalytic geometry, or the γ-phosphate of ATP will react with water instead of the intended substrate.

[4] We presume that this is the carboxyl derivatized by this inhibitor. If this carboxyl hydrogen bonded to the other primary nitrogen of the guanidinium group, it would be too far from the epoxy group of epoxycreatine to react with it, unless the latter were adsorbed in some different fashion during the inactivation process. However, it seems unlikely that epoxycreatine is bound differently for inactivation than for phosphorylation.

Acknowledgments

We thank Dr. G. H. Reed for useful discussions and sharing of unpublished data.

References

Anderson, C. M., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207.
Borders, C. L., Jr., & Riordan, J. F. (1975) *Biochemistry 14*, 4699.
Burgers, P. M. J., & Eckstein, F. (1980) *J. Biol. Chem. 255*, 8229.
Clarke, D. E., & Price, N. C. (1979) *Biochem. J. 181*, 467.
Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Dietrich, R. F., Miller, R. B., Kenyon, G. L., Leyh, T. S., & Reed, G. H. (1980) *Biochemistry 19*, 3180.
Dunaway-Mariano, D., & Cleland, W. W. (1980) *Biochemistry 19*, 1506.
Eigen, M. (1964) *Angew Chem., Int. Ed. Engl. 3*, 1.
James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 2599.
Kuby, S. A., Noda, L., & Lardy, H. A. (1954) *J. Biol. Chem. 209*, 191.
Maggio, E. T., Kenyon, G. L., Markham, G. D., & Reed, G. H. (1977) *J. Biol. Chem. 252*, 1202.
Marletta, M. A., & Kenyon, G. L. (1979) *J. Biol. Chem. 254*, 1879.
McLaughlin, A. C., Cohn, M., & Kenyon, G. L. (1972) *J. Biol. Chem. 247*, 4382.
McLaughlin, A. C., Leigh, J. S., Jr., & Cohn, M. (1976) *J. Biol. Chem. 251*, 2777.
Milner-White, E. J., & Watts, D. C. (1971) *Biochem. J. 122*, 727.
Morrison, J. F., & James, E. (1965) *Biochem. J. 97*, 37.
Morrison, J. F., & Cleland, W. W. (1966) *J. Biol. Chem. 241*, 673.
Orioli, P., Cine, R., Donati, D., & Mangani, S. (1980) *Nature (London) 283*, 691.
Pradel, L. A., & Kassab, R. (1968) *Biochim. Biophys. Acta 167*, 317.
Raushel, F. M., & Cleland, W. W. (1977) *Biochemistry 16*, 2176.
Reed, G. H., & Cohn, M. (1972) *J. Biol. Chem. 247*, 3073.
Reed, G. H., & McLaughlin, A. C. (1973) *Ann. N.Y. Acad. Sci. 222*, 118.
Reed, G. H., & Leyh, T. S. (1980) *Biochemistry 19*, 5472.

Reed, G. H., Barlow, C. H., & Burns, R. A., Jr. (1978) *J. Biol. Chem. 253*, 4153.
Rose, I. A., O'Connell, E. L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249*, 5163.
Rowley, G. L., Greenleaf, A. L., & Kenyon, G. L. (1971) *J. Am. Chem. Soc. 93*, 5542.
Schimerlik, M. I., & Cleland, W. W. (1973) *J. Biol. Chem. 248*, 8418.
Schmidt, D. E., Jr., & Westheimer, F. H. (1971) *Biochemistry 10*, 1249.
Smith, D. J., & Kenyon, G. L. (1974) *J. Biol. Chem. 249*, 3317.
Steitz, T. A., Anderson, W. F., Fletterick, R. J., & Anderson, C. M. (1977) *J. Biol. Chem. 252*, 4494.
Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111.
Watts, D. C., & Rabin, B. R. (1962) *Biochem. J. 85*, 507.

Interactions between Apoaspartate Aminotransferase and Pyridoxal 5′-Phosphate. A Stopped-Flow Study†

D. Vergé* and M. Arrio-Dupont

ABSTRACT: The fast kinetics and mechanism of the reconstitution reaction of holoaspartate aminotransferase from apoenzyme and pyridoxal 5′-phosphate were investigated by the stopped-flow method. When the absorbance change was monitored at 362 nm, the process was shown to involve three steps. The dependence of the three relaxation times on pyridoxal 5′-phosphate concentration and the analysis of the amplitudes enabled us to propose a mechanism in which the initial reversible binding step was followed by two irreversible isomerization steps. The rate constants and the extinction coefficients at 362 nm of the intermediate species were determined. Studies of the reconstitution under the stoichiometric conditions at various wavelengths confirmed the occurrence of at least three steps, and especially of the last decoupled step, but strongly suggest that the actual mechanism is more complex.

During the formation of the active structure of an enzyme, it is interesting to study the last steps of the process to understand the formation of a catalytic structure. Stable structures are encountered for which no activity can be observed and for which some conformational changes are necessary to obtain the catalytic form. The best examples of these phenomena are the activation of zymogens to give active enzymes by proteolytic cleavage and the interaction of coenzymes with apoenzymes to give active holoenzymes. Pyridoxal phosphate enzymes are especially suitable for this kind of study because spectroscopic characteristics of free and bound coenzyme are very different and allow the easy observation of the binding of the coenzyme.

We chose to study aspartate aminotransferase,[1] for which the binding of pyridoxal phosphate is quasi-irreversible and for which important differences have been observed [cf. the review by Braunstein (1973)] between free and bound coenzyme. In this enzyme, conformational changes between apo- and holoenzyme are evidenced from experiments based upon stability toward urea denaturation (Ivanov et al., 1973), fluorescence of aromatic residues (Arrio-Dupont, 1978), and hydrogen–deuterium exchange (Abaturov et al., 1968). It has been shown that the kinetics of reconstitution of the apoenzyme are very dependent on the mode of preparation of the apoenzyme (Arrio-Dupont, 1972). An apoenzyme prepared in the presence of phosphate according to Scardi (1963) is slowly reactivated. After phosphate removal by precipitation with $(NH_4)_2SO_4$ (Furbish et al., 1969), the rate of reconstitution is increased, and it has been shown by Fonda & Auerbach (1976) that the kinetics follow a two-step process. The rate of reconstitution of an apoenzyme prepared according to Wada & Snell (1962), i.e., without phosphate, is faster than that of the apoenzyme prepared according to Furbish et al. (1969) (Arrio-Dupont, 1972). In this paper, the binding process of pyridoxal phosphate to apoenzyme prepared without inorganic phosphate is studied and some spectroscopic characteristics of the intermediary species are described.

Experimental Procedures

Materials

Enzymes. The α form of holoaspartate aminotransferase was prepared from pig heart cytosol according to Martinez-Carrion et al. (1967). Apoenzyme free of inorganic phosphate was obtained as previously described by a method adapted from Wada & Snell (1962) (Arrio-Dupont, 1972). Protein concentrations were calculated from spectrophotometric measurements using $\epsilon_{280} = 1.32\ 10^5\ M^{-1}\ cm^{-1}$ for the apoenzyme dimer and $\epsilon_{280} = 1.40\ 10^5\ M^{-1}\ cm^{-1}$ for the holoenzyme dimer. Enzyme activities were determined as previously described (Cournil et al., 1975) according to the method of Karmen (1955). The remaining activity of the apoenzyme was found to be less than 4% of the holoenzyme activity in all experiments, and the enzyme recovered full activity after addition of PLP. After preparation, the apoenzyme was used within 24 h.

Chemicals. Pyridoxal 5′-phosphate (PLP) ($\epsilon_{388} = 6.2\ 10^3\ M^{-1}\ cm^{-1}$ in 0.1 N NaOH) of the highest purity available, L-cysteinesulfinic acid, and NADH were purchased from

† From the Laboratoire d'Enzymologie Physico-chimique et moléculaire, Groupe de Recherche No. 13 du C.N.R.S., Batiment 433, Université de Paris-Sud, Centre d'Orsay 91405, France. *Received July 18, 1980.* This work was supported by grants from the Centre National de la Recherche Scientifique. This and previously published work (Vergé et al., 1979) has been carried out as a partial fulfillment of a doctoral thesis to be submitted by D.V.

[1] Abbreviations used: aspartate aminotransferase, L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1); PLP or pyridoxal-P, pyridoxal 5′-phosphate; TEA, triethanolamine.

0006-2960/81/0420-1210$01.25/0