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A Reappraisal of the Reaction Pathway of Pyruvate Carboxylase

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The reaction pathway catalysed by pyruvate carboxylase was re-examined by using two independent experimental approaches not previously applied to this enzyme. To avoid the variable stoicheiometry associated with oxaloacetate formation, the reaction rate was measured by following release of P_i. Initial velocities, when plotted as a function of varying concentrations of either $MgATP^{2-}$ or HCO_3^-, at fixed concentrations of pyruvate, gave in double-reciprocal-form families of straight intersecting lines. Further, when the reaction velocity was determined as a function of varying $MgATP^{2-}$ concentrations by using pyruvate, 3-fluoropyruvate and 2-oxobutyrate as alternative carboxyl-acceptor substrates, the slopes of the double-reciprocal plots were significantly different. Both results support a sequential reaction pathway.

The data obtained from initial-velocity, product-inhibition and isotope-exchange studies on pyruvate carboxylase (EC 6.4.1.1) isolated from rat liver (McClure *et al.*, 1971*a*,*b*), chicken liver (Scrutton *et al.*, 1965; Barden *et al.*, 1972) and sheep kidney (Ashman & Keech, 1975) have been interpreted as showing that these enzymes catalyse a reaction with a non-classical Ping Pong Bi Bi Uni Uni sequence. That is, the reaction consists of two partial reactions:

$$MgATP^{2-} + HCO_3^- + \text{Enz·biotin} \rightleftharpoons \text{Enz·biotin·}CO_2 + MgADP^- + P_i \quad (1)$$

$$\text{Enz·biotin·}CO_2 + \text{Prv} \rightleftharpoons \text{Enz·biotin} + OxAcO^- \quad (2)$$

Eqns. (1) and (2) predict a family of parallel lines when the dependence of initial velocity is plotted in double-reciprocal form as a function of varying $MgATP^{2-}$ (or HCO_3^-) concentrations at various fixed pyruvate concentrations. For the enzyme isolated from rat (McClure *et al.*, 1971*a*,*b*) or sheep mitochondria (Ashman & Keech, 1975), the data obtained are inconsistent with this prediction, since the slopes of these lines vary from almost parallel to converging with changes in the pyruvate concentration. Further, in view of the non-classical pyruvate saturation curve obtained for the vertebrate enzymes (Taylor *et al.*, 1969), it appears that the pyruvate concentration range used in the study of the reaction pathway of chicken pyruvate carboxylase (Barden *et al.*, 1972) was too narrow to allow unequivocal interpretation of the results.

By using the enzyme isolated from pig liver, Warren & Tipton (1974*a*,*b*) postulated a sequential reaction pathway in which pyruvate binding occurred before all the products were released. To resolve these conflicting views, we have sought new ways to elucidate the reaction pathway catalysed by this enzyme. The results of this investigation are entirely consistent with a sequential mechanism.

Abbreviations used: Enz·biotin, enzyme–biotin; Enz·biotin·CO_2, enzyme–biotin–CO_2 complex; Prv, pyruvate; $OxAcO^-$, oxaloacetate.

Methods and Materials

Pyruvate carboxylase was purified from freeze-dried sheep liver mitochondria by the method previously described (Easterbrook-Smith *et al.*, 1976*b*) to a specific activity of 12 units/mg of protein. Each assay contained 0.06 enzyme unit. When the velocity of the enzymic reaction was determined by measuring the rate of release of $[^{32}P]P_i$ from [γ-^{32}P]ATP, the assay conditions were the same as previously described (Easterbrook-Smith *et al.*, 1976*b*). The reaction was quenched by the addition of 50μl of 6M-HCl. Carrier P_i (20μmol) was added, and the P_i in a 0.25ml sample was extracted into the organic phase of a separation system consisting of water-saturated 2-methylpropan-1-ol (4ml) and ammonium molybdate (1ml) (40mM in 1.25M-H_2SO_4). Duplicate samples of the organic phase were taken and their radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer with the counting medium described by Patterson & Greene (1965) containing 30% Triton X-100. Non-specific $MgATP^{2-}$ hydrolysis was corrected for by using a control in which the pyruvate carboxylase had been specifically inactivated by previous treatment with a 100-fold excess of avidin. $H^{14}CO_3^-$ fixation was assayed as described previously (Taylor *et al.*, 1969).

[γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964), and 3-fluoropyruvate and 2-oxobutyrate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The purity of these compounds was checked by t.l.c. of their dinitrophenylhydrazones on cellulose thin-layer plates (Eastman–Kodak, Rochester, NY, U.S.A.) in butan-1-ol/water/ethanol (5:4:1, by vol.) as the developing solvent. No contaminants were detected by this technique.

Results and Discussion

Under conditions where HCO_3^- is present at saturating concentrations, the Ping Pong reaction pathway may be represented by eqns. (3) and (4):

$$MgATP^{2-} + Enz\cdot biotin \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Enz\cdot biotin(HCO_3^-, MgATP^{2-}) \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} Enz\cdot biotin\cdot CO_2 + MgADP^- + P_i \quad (3)$$

$$Enz\cdot biotin\cdot CO_2 + Prv \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} Enz\cdot biotin(CO_2\cdot Prv) \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} Enz\cdot biotin + OxAcO^- \quad (4)$$

Assuming initial-velocity conditions, eqn. (5),

$$\frac{[E_t]}{v} = K_\alpha \cdot \frac{1}{[MgATP^{2-}]} + K_\beta \cdot \frac{1}{[Prv]} + \left(\frac{1}{k_{+2}} + \frac{1}{k_{+4}}\right) \quad (5)$$

describes the dependence of the reaction rate (v) on the concentrations of the substrates, where $[E_t]$, [Prv], K_α and K_β represent total enzyme concentration, pyruvate concentration, the slope term $[(k_{-1}+k_{+2})/k_{+1}k_{+2}$ when $MgATP^{2-}$ is the varied substrate], and $(k_{-3}+k_{+4})/k_{+3}k_{+4}$, respectively. If an alternative oxo acid substrate, A, is used, the rate constants k_{+3}, k_{-3} and k_{+4} will assume new values i.e., k_{+3}', k_{-3}' and k_{+4}', and eqn. (5) will become

$$\frac{[E_t]}{v'} = K_\alpha \cdot \frac{1}{[MgATP^{2-}]} + K_\beta' \cdot \frac{1}{[A]} + \left(\frac{1}{k_{+2}} + \frac{1}{k_{+4}'}\right) \quad (6)$$

where v' and K_β' represent the rate of synthesis of the alternative carboxylated product and $(k_{-3}'+k_{+4}')/k_{+3}'k_{+4}'$ respectively.

Similarly, the sequential reaction pathway may be represented by eqns. (7) and (8):

$$Enz\cdot biotin + MgATP^{2-} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Enz\cdot biotin(HCO_3^-, MgATP) \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} Enz\cdot biotin(HCO_3^-, MgATP\cdot Prv) \quad (7)$$

$$Enz\cdot biotin(HCO_3^-, MgATP^{2-}, Prv) \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} Enz\cdot biotin(OxAcO^-, MgADP, P_i) \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} Enz\cdot biotin + OxAcO^- + MgADP + P_i \quad (8)$$

Assuming initial-velocity conditions eqn. (9) describes the dependence of the rate (v) of carboxylated product synthesis on the concentrations of substrates where K_α'' and K_β'' represent

$$\left(1 + \frac{k_{-1}}{k_{+2}} \cdot \frac{1}{[Prv]} + \frac{k_{-1}k_{-2}}{k_{+2}k_{+3}} \cdot \frac{1}{[Prv]}\right) \quad \text{and} \quad \left(1 + \frac{k_{-2}}{k_{+3}}\right)$$

respectively:

$$\frac{[E_t]}{v} = K_\alpha'' \cdot \frac{1}{k_{+1}[MgATP^{2-}]} + K_\beta'' \cdot \frac{1}{[Prv]} + \left(\frac{1}{k_{+3}} + \frac{1}{k_{+4}}\right) \quad (9)$$

In this case, if an alternative oxo acid substrate A is used, rate constants k_{+2}, k_{-2}, k_{+3} and k_{-3} will assume different values, and therefore the numerical values of both K_α'' and K_β'' will change.

Both 2-oxobutyrate and 3-fluoropyruvate have been shown to be alternative oxo acid acceptor substrates for pyruvate carboxylase (Keech & Utter, 1963; Cheung & Walsh, 1976). Therefore eqns. (5) and (6) predict that, if the proposed Ping Pong reaction sequence is correct, plots of $1/v$ versus $1/[MgATP^{2-}]$ for fixed concentrations of pyruvate or one of the above alternative substrates should have identical slopes of numerical value K_α. Conversely, the double-reciprocal plots for a sequential reaction pathway should have different slopes, since the slope term in eqn. (9), i.e., K_α'', is a function of k_{-1}, k_{+2}, k_{-2} and k_{+3} which in turn depend on the oxo acid substrate used. The results, presented in Table 1, for experiments of this type show that the

Table 1. *Alternative substrate kinetic constants*

The dependence of oxaloacetate synthesis on $MgATP^{2-}$ concentration for each oxo acid substrate was determined. The $MgATP^{2-}$ concentration range was 0.02–1.8mM, and the oxo acid and HCO_3^- concentrations were 10mM. The slopes and intercepts of the lines were obtained from a least-mean-squares analysis. The values shown represent the means obtained from duplicate experiments. The slope ratio represents the slope of the line relative to that with pyruvate as the oxo acid substrate. By analysis of covariance (Snedecor & Cochran, 1967) the slopes are significantly different. *Variance ratio (F_1, 31) = 6.4; $P<0.05$; **variance ratio (F_1, 32) = 383.9; $P<0.001$.

Oxo acid substrate	Slope	Slope ratio	$V_{max.}$ (%)
Pyruvate	0.035±0.0004	1.00	100
2-Oxobutyrate	0.076±0.004*	2.17±0.14	4
3-Fluoropyruvate	0.350±0.014**	10.28±0.52	5

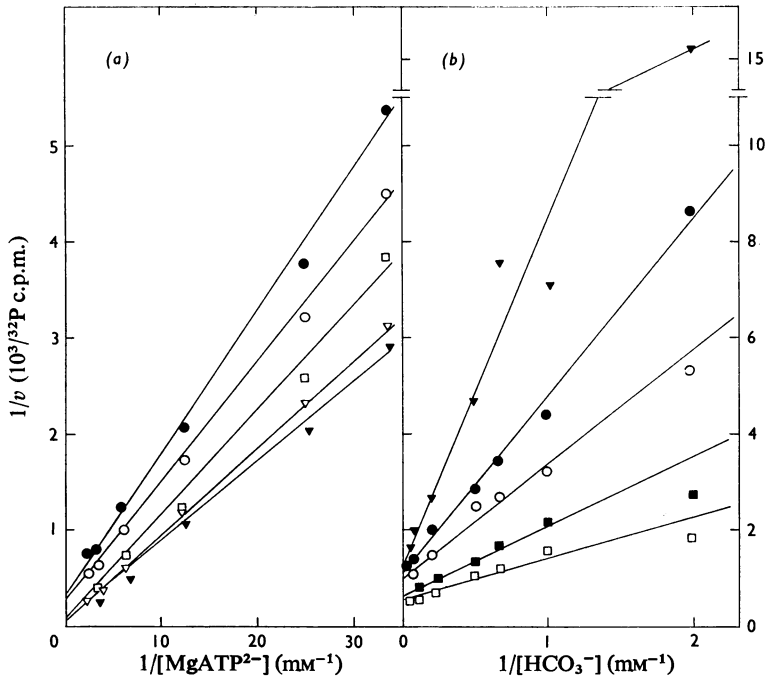

Fig. 1. *Reciprocal plots of P_i release as a function of varying either (a) $MgATP^{2-}$ concentration or (b) HCO_3^- concentrations at several fixed concentrations of pyruvate*

(*a*) The pyruvate concentrations used were 0.5mM (●), 0.8mM (○), 5.0mM (□), 10mM (▽) and 20mM (▼), and the specific radioactivity of the [γ-^{32}P]ATP was 3500c.p.m./nmol. (*b*) The pyruvate concentrations used were 0.5mM (▼), 0.8mM (●), 1.0mM (○), 5.0mM (■) and 20mM (□). The concentration of the [γ-^{32}P]ATP was 1.0mM (1100c.p.m./nmol).

slopes of the lines for the alternative substrates are significantly different. Such data clearly eliminate the proposed Ping Pong reaction sequence as a viable mechanism of sheep liver pyruvate carboxylase. An approach similar to that outlined above was used by Webb *et al.* (1976) in their investigation of the reaction pathway of acetate kinase isolated from *Escherichia coli*.

Kinetic data obtained from pyruvate carboxylase at various concentrations of pyruvate by using the rate of oxaloacetate synthesis as a measure of reaction velocity are characterized by downward-concave double-reciprocal plots (Taylor *et al.*, 1969; McClure *et al.*, 1971*a*; Barden *et al.*, 1972; Ashman & Keech, 1975). The non-linear profiles arise because oxaloacetate synthesis reflects only the rate of pyruvate

carboxylation, which is not stoicheiometric with the rate of ATP hydrolysis at non-saturating concentrations of pyruvate (Easterbrook-Smith *et al.*, 1976*b*). A possible explanation for this phenomenon is that after pyruvate binds to the enzyme, it is not necessarily committed to catalysis but can be released without being carboxylated (Easterbrook-Smith *et al.*, 1976*b*; Cheung & Walsh, 1976). Therefore to avoid the complication of non-linear reciprocal plots, we have re-examined the dependence of initial velocity as a function of varying the $MgATP^{2-}$ (or HCO_3^-) concentrations at various fixed pyruvate concentrations by monitoring the rate of P_i release. As Fig. 1 shows, these experiments resulted in families of linear intersecting lines consistent with a sequential reaction pathway similar to that proposed by Warren & Tipton (1974*a*,*b*).

It is appreciated that any proposition supporting a sequential pathway for this enzyme must also provide some explanation for the fact that the enzyme catalyses isotopic-exchange reactions in the absence of components of the other partial reaction. This contingency can be met if it is assumed that $MgADP^-$ and P_i are released at a significant rate only after the binding of pyruvate. This requirement provides a plausible explanation for the very low rate of the ATP $\rightleftharpoons$ $[^{32}P]P_i$ isotopic-exchange reaction (McClure *et al.*, 1971*b*; Scrutton *et al.*, 1965; Ashman & Keech, 1975). With regard to the very rapid [2-^{14}C]-pyruvate $\rightleftharpoons$ oxaloacetate exchange reaction (McClure *et al.*, 1971*a*; Ashman & Keech, 1975), this is not inconsistent with a sequential pathway if the active site is considered to consist of two spatially distinct subsites, as is suggested from product-inhibition studies (McClure *et al.*, 1971*a*; Barden *et al.*, 1972; Ashman & Keech, 1975), and as may be inferred from nuclear-magnetic- and electron-spin-resonance studies (Mildvan *et al.*, 1966; Reed & Scrutton, 1974).

Further, affinity labelling of the $MgATP^{2-}$-binding site (Easterbrook-Smith *et al.*, 1976*a*) resulted in inhibition of the ATP $\rightleftharpoons$ $[^{32}P]P_i$ exchange reaction without affecting the [2-^{14}C]pyruvate $\rightleftharpoons$ oxaloacetate exchange reaction. The point here is that, although in the overall reaction release of $MgADP^-$ and P_i occurs after pyruvate binding, the separation of the site implies that there is no mechanistic (in the sense of chemical interactions between the substrates and the active-site amino acids residues) requirement for this order of release to be maintained.

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