Pig Liver Pyruvate Carboxylase

THE REACTION PATHWAY FOR THE CARBOXYLATION OF PYRUVATE

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1. The reaction pathway for the carboxylation of pyruvate, catalysed by pig liver pyruvate carboxylase, was studied in the presence of saturating concentrations of K^+ and acetyl-CoA. 2. Free Mg^{2+} binds to the enzyme in an equilibrium fashion and remains bound during all further catalytic cycles. $MgATP^{2-}$ binds next, followed by HCO_3^- and then pyruvate. Oxaloacetate is released before the random release, at equilibrium, of P_i and $MgADP^-$. 3. This reaction pathway is compared with the double displacement (Ping Pong) mechanisms that have previously been described for pyruvate carboxylases from other sources. The reaction pathway proposed for the pig liver enzyme is superior in that it shows no kinetic inconsistencies and satisfactorily explains the low rate of the $ATP \rightleftharpoons [^{32}P]P_i$ equilibrium exchange reaction. 4. Values are presented for the stability constants of the magnesium complexes of ATP, ADP, acetyl-CoA, P_i , pyruvate and oxaloacetate.

Pig liver pyruvate carboxylase has been purified to homogeneity as described in the preceding paper (Warren & Tipton, 1974a) and, in common with pyruvate carboxylases from other sources, catalyses the following reaction:

where M^+ is a univalent cation (typically K^+).

The carboxylation of pyruvate by the pig liver enzyme was subjected to a thorough kinetic analysis in an attempt to delineate the reaction pathway. The analysis was carried out in the presence of saturating concentrations of K^+ (approx. 70 mM; K_m for $K^+ = 1.1$ mM) and acetyl-CoA (100 μ M; K_s for acetyl-CoA = 22 μ M).

Materials

Pyruvate carboxylase, coupling enzymes and other assay components were obtained in the manner described in the preceding paper (Warren & Tipton, 1974a). α -Oxopentanoic acid, α -oxobutyric acid and *p*-hydroxyphenylpyruvic acid were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Methods

Assays for pyruvate carboxylase

Pig liver pyruvate carboxylase was assayed at 30°C by using a coupled assay for oxaloacetate (the

oxaloacetate-coupled assay). The assay system contained 100 mM-triethanolamine hydrochloride-KOH buffer, pH 8.0, 5 mM-potassium pyruvate, 1 mM-ATP, 5 mM-MgSO₄, 15 mM-KHCO₃, 0.1 mM-acetyl-CoA, 0.1 mM-NADH and 10 units of malate dehydrogenase in a total volume of 2.0 ml. The reaction was started by the addition of 10–50 μ g of pyruvate carboxylase. The coupling time required before the rate of NADH oxidation reached 99% of the steady-state rate of pyruvate carboxylation was less than 3 s (McClure, 1969). The *I* of the assay medium was maintained at 150 $\pm$ 15 mM by the addition of 1 M-KCl; variation within these limits had no detectable effect on the reaction rate.

When oxaloacetate was used as a product inhibitor, pyruvate carboxylase was assayed in a system (the oxaloacetate assay) similar to that described above, with the omission of NADH and malate dehydrogenase. The synthesis of oxaloacetate was monitored by the increase in absorption at 290 nm and corrections were applied for the spontaneous decarboxylation of oxaloacetate. The value of ϵ for oxaloacetate under these conditions was 570 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹.

The results from initial-velocity studies were plotted by the method of Lineweaver & Burk (1934). Kinetic constants were evaluated by the method of Florini & Vestling (1957) and inhibition constants were normally determined by the method of Dixon (1953).

Stability constants for magnesium complexes

Pig liver pyruvate carboxylase requires both $MgATP^{2-}$ and free Mg^{2+} , so that kinetic analyses

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which seek to vary these two parameters require a knowledge of the magnesium-chelating capacity of the components of the assay system. The stability constant for MgATP^{2-} (and other magnesium complexes) is sensitive to pH, I , temperature and the concentration of univalent cations such as K^+ (Melchior & Melchior, 1958; Burton, 1959; O'Sullivan & Perrin, 1964). These parameters were therefore maintained in the assays for pyruvate carboxylase described above, and in the systems used to determine the stability constants given in Table 1. Three methods were used to determine the stability constants. The first method was that described by Burton (1959) and was used if the stability constant was greater than 1000M^{-1} . The second method (Watanabe *et al.*, 1963) was adapted for use with a Gilford 240 spectrophotometer, and since this method was very insensitive, it was only superior to the method of Burton (1959) when the stability constant was less than 1000M^{-1} . However, as shown in Table 1, both methods were in good agreement for the stability constants of MgATP^{2-} and MgADP^- . Thirdly, the stability constant for the magnesium complex of oxaloacetate was determined by utilizing the absorption of this complex at 290nm.

The magnesium complexes of the following compounds were not considered because of their low magnesium-chelating capacity and/or because of their low concentration in the assay medium: adenosine (Phillips, 1966), HCO_3^- (Raaflaub, 1960), NADH (Duggleby & Dennis, 1970) and malate dehydrogenase.

The information given in Table 1 was used to construct a FORTRAN program which calculated the total quantities of Mg^{2+} and ATP that had to be added to the assay system to yield a fixed concentration of free Mg^{2+} and MgATP^{2-} . Naturally, a fixed concentration of these two parameters necessitated a variation in the concentration of ATP^{4-} .

Table 1. *Stability constants for the magnesium complexes of assay components*

Methods 1: Burton (1959); 2: Watanabe *et al.* (1963); 3: measurement of the magnesium complex of oxaloacetate at 290nm. Further details of these methods are given in the text.

Assay component	Stability constant (M^{-1})	Method
ATP	17000	1
	23000	2
ADP	1300	1
	1180	2
Acetyl-CoA	900	2
P_i	52	2
Pyruvate	15	2
Oxaloacetate	4	3

However, because of the high stability of the MgATP^{2-} complex, the concentration of ATP^{4-} never rose above 20% of that of MgATP^{2-} during any of the kinetic studies described in the present paper.

Kinetic studies using HCO_3^-

All solutions were freshly prepared in de-ionized de-gassed water and were de-gassed for 5–10min. Where possible, the solution was maintained at $\text{pH} \leq 6.0$; otherwise the solution was adjusted to the appropriate pH by using freshly prepared 1M-KOH. The concentration of endogenous HCO_3^- was determined by measuring the activity of pyruvate carboxylase at several concentrations of added KHCO_3 and constructing a linear reciprocal plot by using the highest added concentrations of KHCO_3 . The K_m and V values determined by this method were in good agreement with those obtained by using a FORTRAN program which incremented the added KHCO_3 by small amounts until the most linear reciprocal plot was obtained. The values of K_m and V were used to calculate the endogenous concentration of HCO_3^- which was typically 0.2mM at the start of the experiment and 0.3mM at the end. This small change in the endogenous concentration of HCO_3^- did not affect the results to any significant extent.

All other methods used in this paper have been described in the preceding paper (Warren & Tipton, 1974a).

Results

All of the results presented in this paper are consistent with the reaction pathway depicted in Scheme 1. The most important feature of this pathway is the lack of any feature that would render the mechanism double displacement (Ping Pong) in nature. The lack of a classical or non-classical (Northrop, 1969) double-displacement mechanism is in distinct contrast with the reaction pathways that have been postulated for pyruvate carboxylase (Barden *et al.*, 1972; McClure *et al.*, 1971b; Feir & Suzuki, 1969).

Substrate studies

The results of these studies are summarized in Table 2.

Free Mg^{2+} . The rate equation for the ordered, steady-state addition of two substrates (A, B) would be

$$v = \frac{V}{1 + \frac{K_m^A}{[A]} + \frac{K_m^B}{[B]} + \frac{K_s^A \cdot K_m^B}{[A] \cdot [B]}} \quad (1)$$

Scheme 1. Reaction pathway for the carboxylation of pyruvate by pig liver pyruvate carboxylase

The section enclosed within the dashed lines is in rapid equilibrium. E_{CO₂} represents the carboxybiotin form of pyruvate carboxylase.

Table 2. Apparent kinetic constants for pig liver pyruvate carboxylase

Changing fixed substrate (A)	Variable substrate (B)	Observed pattern	Kinetic constants (mM)			Conditions*
			K_m^A	$\frac{K_s^B \cdot K_m^{A\dagger}}{K_m^B}$	$K_s^{\dagger\dagger}$	
Mg ²⁺ _{free}	MgATP ²⁻	Intersecting†	0		0.48	—
	HCO ₃ ⁻	Intersecting	0.18		0.30	0.8 mM-MgATP ²⁻
	Pyruvate	Intersecting	<0.25		<0.02	0.5 mM-MgATP ²⁻
MgATP ²⁻	Mg ²⁺ _{free}	Intersecting	0.87	∞		—
	HCO ₃ ⁻	Intersecting	0.18		0.34	5 mM-Mg ²⁺ _{free}
	Pyruvate	Intersecting	0.13		0.026	5 mM-Mg ²⁺ _{free}
HCO ₃ ⁻	Mg ²⁺ _{free}	Intersecting	0.93	2.1		0.8 mM-MgATP ²⁻
	MgATP ²⁻	Intersecting	1.25	2.4		5 mM-Mg ²⁺ _{free}
	Pyruvate	Intersecting	0.83		1.27	2 mM-MgATP ²⁻
Pyruvate	Mg ²⁺ _{free}	Intersecting	0.14	0.011		0.5 mM-MgATP ²⁻
	MgATP ²⁻	Intersecting	0.30	0.059		5 mM-Mg ²⁺ _{free}
	HCO ₃ ⁻	Intersecting	0.17	0.26		2 mM-MgATP ²⁻

* Other conditions are those specified in the text for the assay medium.
† Intersecting on 1/v axis.
‡ Differentiated by assuming the order of addition of substrates and effectors given in Scheme 1.

If, however, A was bound to the free enzyme (E) at thermodynamic equilibrium, an ‘infinite’ concentration of B would decrease the concentration of free enzyme to zero since all of the EA would have

been converted into EAB; moreover, A would only be required in amounts stoichiometric with the enzyme. The value of K_m^A should be determined, theoretically, at an ‘infinite’ concentration of B;

Fig. 1. Reciprocal plot of the carboxylation of pyruvate as a function of free Mg^{2+} concentration at several fixed concentrations of MgATP^{2-}

Pyruvate carboxylase (25 μg ; 2.6 units/mg) was added to the oxaloacetate-coupled assay (see the text). The concentrations of MgATP^{2-} used were: 1.0 mM ($\circ$), 0.5 mM (Δ), 0.33 mM ($\square$), 0.25 mM ($\bullet$), 0.20 mM ($\blacktriangle$), 0.17 mM ($\blacksquare$), 0.13 mM (∇) and 0.10 mM ($\blacktriangledown$). Inset: the variation of the slopes ($\bullet$) and intercepts ($\blacktriangle$) as a function of MgATP^{2-} concentration.

Fig. 2. Reciprocal plot of the carboxylation of pyruvate as a function of MgATP^{2-} concentration at several fixed concentrations of free Mg^{2+}

The enzyme concentration and assay method were the same as in Fig. 1. The concentrations of free Mg^{2+} used were: 1.33 mM ($\circ$), 0.67 mM (Δ), 0.44 mM ($\square$), 0.33 mM ($\bullet$), 0.27 mM ($\blacktriangle$), 0.22 mM ($\blacksquare$) and 0.19 mM (∇). Inset: the variation of the slopes as a function of Mg^{2+} concentration.

since the concentration of E available to combine with A would be zero under these conditions, the value of K_m^A must also be zero. Hence eqn. (1) becomes

$$v = \frac{V}{1 + \frac{K_m^B}{[B]} \left(1 + \frac{K_s^A}{[A]} \right)} \quad (2)$$

In practical terms, an infinite concentration of B will remove any experimentally observed dependence on A. Further, the asymmetry of eqn. (2) allows the order of substrate addition to be determined, merely by examination of the primary plots.

Figs. 1 and 2 suggest that free Mg^{2+} binds to pig liver pyruvate carboxylase at equilibrium, followed by the binding of MgATP^{2-} . When the concentration of free Mg^{2+} was varied at several fixed concentrations of MgATP^{2-} (Fig. 1) the slope replot (slope versus $1/[\text{MgATP}^{2-}]$) passed through the origin; this means that an 'infinite' concentration of MgATP^{2-} would have given a reciprocal plot that was parallel to the $1/[\text{Mg}^{2+}]$ axis. The independence of the reaction rate on free Mg^{2+} under these conditions was also shown by the converse primary plot (Fig. 2) in which the lines intersected on the $1/v$ axis.

HCO_3^- and pyruvate bind to pig liver pyruvate carboxylase after the binding of MgATP^{2-} . If either compound could have bound to EMg (Scheme 1) the rate equation would have been as follows:

$$v = \frac{V}{1 + \frac{K_m^{\text{MgATP}}}{[\text{MgATP}^{2-}]} + \frac{K_m^X}{[X]} + \frac{K_s^{\text{MgATP}} \cdot K_m^X}{[\text{MgATP}^{2-} \cdot X]} \left(1 + \frac{K_s^{\text{Mg}}}{[\text{Mg}^{2+}]_{\text{free}}} \right)} \quad (3)$$

Fig. 3. Reciprocal plot of the carboxylation of pyruvate as a function of $MgATP^{2-}$ concentration at several fixed concentrations of pyruvate

Pyruvate carboxylase (13 μ g; 2.8 units/mg) was added to the oxaloacetate-coupled assay. The pyruvate concentrations used were: 2.2 mm (○), 0.36 mm (Δ), 0.23 mm (□), 0.115 mm (●), 0.057 mm (▲), 0.038 mm (■), and 0.029 mm (▽). Inset: the variation of the slopes (●) and intercepts (▲) as a function of pyruvate concentration.

Fig. 4. Reciprocal plot of the carboxylation of pyruvate as a function of $MgATP^{2-}$ concentration at several fixed levels of pyruvate

Pyruvate carboxylase (45 μ g; 2.6 units/mg) was added to the oxaloacetate-coupled assay. The HCO_3^- concentration was 200 mm and the I was maintained at approx. 300 mm by the addition of 1.0 M-KCl. The pyruvate concentrations used were: 0.228 mm (○), 0.057 mm (Δ), 0.032 mm (□), 0.023 mm (●), 0.018 mm (▲) and 0.014 mm (■). Inset: the variation of the intercepts as a function of pyruvate concentration. The value of $K_m^{pyruvate}$ determined from this plot was 0.22 mm.

Fig. 5. Reciprocal plot of the carboxylation of pyruvate as a function of HCO_3^- concentration at several fixed concentrations of $MgATP^{2-}$

Pyruvate carboxylase (16 μ g; 2.7 units/mg) was added to the oxaloacetate-coupled assay. The mixture contained 100 mm-potassium pyruvate. The $MgATP^{2-}$ concentrations used were: 0.50 mm (○), 0.125 mm (Δ), 0.063 mm (□), 0.042 mm (●), 0.031 mm (▲) and 0.025 mm (■). Inset: the variation of the slopes (○) and intercepts (Δ) as a function of $MgATP^{2-}$ concentration. The values of the kinetic constants determined from these plots were: $K_m^{MgATP} = 0.11$ mm; $K_s^{MgATP} = 0.20$ mm; $K_m^{HCO_3^-} = 0.60$ mm; and $\frac{K_s^{MgATP} \cdot K_m^{HCO_3^-}}{K_m^{MgATP}} = 1.09$ mm.

where X is either HCO_3^- or pyruvate. Eqn. (3) would predict rapid-equilibrium ordered kinetics with respect to free Mg^{2+} and either $MgATP^{2-}$ and HCO_3^- or $MgATP^{2-}$ and pyruvate. This was clearly not the case (Table 2).

Free Mg^{2+} binds to pig liver pyruvate carboxylase

and remains bound during all further catalytic cycles (Scheme 1); the evidence for this postulate is derived from product and dead-end inhibition studies described below.

$MgATP^{2-}$. This substrate was bound to EMg (Scheme 1) in a steady-state fashion, because reciprocal plots involving $MgATP^{2-}$ and HCO_3^- did not yield patterns characteristic of rapid-equilibrium

Table 3. *Product inhibition constants for pig liver pyruvate carboxylase* K_i values were determined by the method of Dixon (1953).

Product inhibitor	Varied substrate	Inhibition pattern	Apparent K_i (mM)	Conditions†
MgADP ⁻	MgATP ²⁻	Competitive	0.30	5 mM-Mg ²⁺
	HCO ₃ ⁻	Mixed	0.55	0.1 mM-MgATP ²⁻
	Pyruvate	Mixed	6.5	0.2 mM-MgATP ²⁻
P _i	MgATP ²⁻	Competitive	22.0	
	HCO ₃ ⁻	Mixed	—*	0.025 mM-MgATP ²⁻
	Pyruvate	Mixed	—*	0.05 mM-MgATP ²⁻
Oxaloacetate	MgATP ²⁻	Non-competitive	0.070	0.25 mM-pyruvate
	HCO ₃ ⁻	Mixed	0.034	0.25 mM-pyruvate
	Pyruvate	Competitive	0.050	

* K_i values could not be obtained because of non-linear Dixon plots; see the text.

† Other conditions are those specified in the text for the assay medium.

ordered addition (Table 2). This same argument applies to the binding of HCO₃⁻ and pyruvate.

HCO₃⁻. HCO₃⁻ binds to pyruvate carboxylase before pyruvate. This was demonstrated by using the supersaturation technique first used by Frieden (1959). For an ordered reaction involving three substrates (A, B, C), reciprocal plots involving A and C will yield intersecting patterns. If, however, the concentration of B is raised to approx. $100 \times K_m^B$ the intersecting pattern will become parallel. Since supersaturation with either A or C will not have this effect, the method can be used to determine the middle substrate in an ordered three-substrate reaction. Reciprocal plots involving MgATP²⁻ and pyruvate were intersecting at normal, saturating (15 mM) concentrations of HCO₃⁻ (Fig. 3). The pattern became parallel when the level of HCO₃⁻ was raised to 200 mM (Fig. 4). This effect was not caused by the high I (approx. 300 mM), which necessarily prevailed in these experiments, because 200 mM-HCO₃⁻ could be replaced by 185 mM-KCl-15 mM-HCO₃⁻ and the results were then similar to those presented in Fig. 3. On the other hand, supersaturation with pyruvate (Fig. 5) did not render the normal intersecting lines (Table 2) parallel. Unfortunately, pyruvate binds to the free form of chicken liver pyruvate carboxylase, although it shows a marked preference for the carboxybiotin form of the enzyme (see below). If this situation also prevails with the pig liver enzyme, supersaturation with pyruvate could help this substrate to 'jump the queue' in what would otherwise be an ordered mechanism. Confirmation of the ordered binding of HCO₃⁻ and pyruvate was demonstrated by product inhibition studies discussed below.

Pyruvate. Non-linear reciprocal plots involving pyruvate have been observed for pyruvate carboxylase purified from chicken liver (Scrutton *et al.*, 1965), rat liver (McClure *et al.*, 1971a,b; Seufert *et al.*, 1971), sheep kidney (Taylor *et al.*, 1969) and

Bacillus stearothermophilus (Cazzulo *et al.*, 1970), and the pig liver enzyme was no exception. However, no non-linearity was observed up to 5 mM-pyruvate when the concentration of acetyl-CoA was greater than 20 μ M, so that this complication could be ignored in these studies.

Although pyruvate can bind to the free form of chicken liver pyruvate carboxylase (Mildvan *et al.*, 1966), it shows a marked preference for the carboxybiotin form of the enzyme (Scrutton *et al.*, 1965). If this applies to the pig liver enzyme then pyruvate will bind after the formation of carboxybiotin (see Scheme 1). Classical double-displacement kinetics are not observed with this enzyme because the two products, MgADP⁻ and P_i, are not released before the binding of pyruvate.

Product inhibition studies

These results are summarized in Table 3.

MgADP⁻ and P_i. MgADP⁻ (Fig. 6) and P_i (Fig. 7) were competitive inhibitors with respect to MgATP²⁻ and mixed inhibitors with respect to HCO₃⁻ and pyruvate (Table 3). This strongly suggests that MgADP⁻ and P_i are the last products to leave the enzyme and that free Mg²⁺ binds to the free enzyme and then remains bound during all subsequent catalytic cycles (see Scheme 1). Unfortunately, the results obtained with P_i are not as clear cut as those obtained with MgADP⁻. The I of the assay medium was necessarily maintained at the high value of 350 mM because of the polyanionic nature of this compound together with the high K_i values obtained (22 mM; Table 3). This change in the value of I adversely affected the stability constant for the magnesium complex of P_i. Rather than recalculate the latter value it became clear that this apparently unfavourable situation could be turned to advantage. If the concentration of free Mg²⁺ was calculated by ignoring the concentration of P_i then the effect of

Fig. 6. Product inhibition of pyruvate carboxylation by MgADP^- as a function of the concentration of MgATP^{2-}

Pyruvate carboxylase ($30\mu\text{g}$; 2.7units/mg) was added to the oxaloacetate-coupled assay. The concentrations of MgADP^- used were: 0 ($\circ$), 0.20mm (Δ), 0.40mm ($\square$), 0.60mm ($\bullet$), 0.80mm ($\blacktriangle$) and 1.00mm ($\blacksquare$). Inset: a Dixon (1953) plot of these data. The concentrations of MgATP^{2-} were: 0.10mm ($\blacktriangle$), 0.125mm ($\bullet$), 0.167mm ($\square$), 0.025mm (Δ) and 0.50mm ($\circ$).

Fig. 7. Product inhibition of pyruvate carboxylation by P_i as a function of the concentration of MgATP^{2-}

Pyruvate carboxylase ($50\mu\text{g}$; 2.7units/mg) was added to the oxaloacetate-coupled assay. The concentration of free Mg^{2+} was not corrected for changes in the concentration of P_i (see the text). The I was maintained at approx. 350mm by the addition of 1.0M-KCl . The following concentrations of P_i were used: 0 ($\circ$), 20mm (Δ), 40mm ($\square$), 60mm ($\bullet$), 80mm ($\blacktriangle$) and 100mm ($\blacksquare$). Inset: the variation of the slopes as a function of P_i concentration.

Vol. 139

Fig. 8. Product inhibition of pyruvate carboxylation by P_i as a function of the concentration of HCO_3^-

Pyruvate carboxylase ($110\mu\text{g}$; 2.6units/mg) was added to the oxaloacetate-coupled assay. The concentration of free Mg^{2+} was not corrected for changes in the concentration of P_i (see the text) and I was maintained at approx. 350mm by the addition of 1.0M-KCl . The following concentrations of P_i were used: 2mm ($\bullet$), 22mm ($\blacktriangle$), 42mm ($\blacksquare$), 62mm ($\circ$), 82mm (Δ) and 102mm ($\square$). Inset: the variation of the slopes (Δ) and intercepts ($\bullet$) as a function of P_i concentration.

Fig. 9. Product inhibition of pyruvate carboxylation by oxaloacetate as a function of the concentration of pyruvate

Pyruvate carboxylase ($50\mu\text{g}$; 2.7units/mg) was added to the oxaloacetate assay. The concentrations of oxaloacetate used were: 0 ($\circ$), $54\mu\text{M}$ (Δ), $109\mu\text{M}$ ($\square$), $163\mu\text{M}$ ($\bullet$) and $217\mu\text{M}$ ($\blacktriangle$). Inset: a Dixon (1953) plot of these data. The concentrations of pyruvate were: 0.10mm ($\blacksquare$), 0.12mm ($\blacktriangle$), 0.6mm ($\bullet$), 0.25mm ($\square$), 0.49mm (Δ) and 0.99mm ($\circ$).

P_i would be twofold; first, it would bind to EMg (Scheme 1) in a manner competitive with MgATP^{2-} . Secondly, by sequestering free Mg^{2+} , P_i would appear to be a competitive inhibitor with respect to free Mg^{2+} . Under normal conditions this dual effect would make P_i a mixed or non-competitive inhibitor with respect to MgATP^{2-} . However, if free Mg^{2+} binds to the enzyme in an equilibrium fashion then an 'infinite' concentration of MgATP^{2-} will eliminate

Table 4. *Dead-end inhibition constants for pig liver pyruvate carboxylase* K_i values were determined by the method of Dixon (1953).

Dead-end inhibitor	Varied substrate	Inhibition pattern	Apparent K_i (mM)	Conditions†
Adenosine	MgATP ²⁻	Competitive	0.090	
	HCO ₃ ⁻	Mixed	0.12	0.1 mM-MgATP ²⁻
	Pyruvate	Mixed	0.69	0.2 mM-MgATP ²⁻
α -Oxobutyrate	MgATP ²⁻	Uncompetitive	6.0*	0.1 mM-Pyruvate
	HCO ₃ ⁻	Mixed	5.7	0.025 mM-Pyruvate
	Pyruvate	Mixed	3.5	
α -Oxopentanoate	Pyruvate	Mixed	2.7	
<i>p</i> -Hydroxyphenylpyruvate	Pyruvate	Mixed	2.3	

* K_i value determined from the replot of the intercepts.

† Other conditions are those specified in the text for the assay medium.

Fig. 10. *Dead-end inhibition of pyruvate carboxylation by α -oxobutyrate as a function of the concentration of pyruvate*

Pyruvate carboxylase (35 μ g; 2.4 units/mg) was added to the oxaloacetate-coupled assay. The concentrations of α -oxobutyrate used were: 0.6 mM ($\circ$), 2.1 mM (Δ), 4.3 mM ($\square$), 6.4 mM ($\bullet$) and 8.6 mM ($\blacktriangle$). Inset: a Dixon (1953) plot of these data. The concentrations of pyruvate were: 0.10 mM ($\blacksquare$), 0.12 mM ($\blacktriangle$), 0.16 mM ($\bullet$), 0.24 mM ($\square$), 0.48 mM (Δ) and 0.95 mM ($\circ$).

both the real and the apparent inhibition by P_i at the binding sites for MgATP²⁻ and free Mg²⁺ respectively. The observed competitive patterns in Fig. 7 support the equilibrium addition of free Mg²⁺ to pig liver pyruvate carboxylase. Further, the non-linear nature of the slope replot is a witness to the dual effect of P_i in this system. This dual effect will be felt further down the reaction pathway, so that the slope and intercept replots of primary plots involving P_i and either HCO₃⁻ (Fig. 8) or pyruvate will be concave upwards.

In the absence of added P_i , the reciprocal plot is always the 'odd line out' (Figs. 7 and 8). This is most easily explained if the P_i sequesters free Mg²⁺ which was, in fact, inhibitory to the enzyme.

Oxaloacetate. This product is a linear competitive inhibitor with respect to pyruvate (Fig. 9) and a mixed inhibitor with respect to MgATP²⁻ and

HCO₃⁻ (Table 3). The interaction of pyruvate and oxaloacetate with the enzyme suggests a Theorell-Chance type of mechanism (see Scheme 1) and provides further evidence for the binding of pyruvate after the binding of HCO₃⁻.

Dead-end inhibition

These results are summarized in Table 4.

Adenosine. Adenosine is a linear dead-end competitive inhibitor with respect to MgATP²⁻ and a mixed inhibitor with respect to HCO₃⁻ and pyruvate (Table 4). This strongly suggests that HCO₃⁻ and pyruvate bind to the enzyme after the addition of MgATP²⁻.

α -Oxobutyrate. Contrary to results obtained with pyruvate carboxylase from rat liver (McClure *et al.*, 1971b), α -oxobutyrate is a mixed inhibitor with respect to pyruvate (Fig. 10). This is true over a wide range of concentrations if due allowance is made for the low rate of utilization of α -oxobutyrate by pyruvate carboxylase and lactate dehydrogenase, which is present at low activity in the purified pig liver enzyme; α -oxopentanoate and *p*-hydroxyphenylpyruvate are also mixed inhibitors with respect to pyruvate (Table 4), and the similarity of the K_i values for all three methyl-substituted pyruvate analogues suggests that the methyl group of pyruvate is not 'buried' in the active site during the carboxylation process. The locus of action of α -oxobutyrate has been investigated by determining the patterns of inhibition with respect to MgATP²⁻ and HCO₃⁻ (Table 4). α -Oxobutyrate would appear to bind to the enzyme after the binding of MgATP²⁻ and either before or randomly with HCO₃⁻. The inhibitor therefore binds to the free biotin form of the enzyme. This agrees with results obtained by using the chicken liver enzyme in which α -oxobutyrate, unlike pyruvate, does not show any preference for the carboxybiotin form of pyruvate carboxylase (Mildvan *et al.*, 1966).

Discussion

Pyruvate carboxylase from chicken liver (Keech & Utter, 1963), rat liver (McClure *et al.*, 1971a; Seufert *et al.*, 1971), sheep kidney (Keech & Barritt, 1967; Bais & Keech, 1972), yeast (Cazzulo & Stoppani, 1967, 1969), *Aspergillus niger* (Feir & Suzuki, 1969) and *Bacillus stearothermophilus* (Cazzulo *et al.*, 1970) has been shown to require MgATP^{2-} and Mg^{2+} in excess of that required to complex ATP^{4-} .

Some workers (see, e.g., Cazzulo & Stoppani, 1969) have suggested that free Mg^{2+} does not activate pyruvate carboxylase directly, but acts by removing inhibitory concentrations of ATP^{4-} . If it is assumed for the present that free Mg^{2+} itself has no direct effect on the activity of pyruvate carboxylase and ATP^{4-} is a competitive inhibitor with respect to MgATP^{2-} , the appropriate rate equation (at saturating concentrations of the other assay components) would be

$$v = \frac{V}{1 + \frac{K_m^{\text{MgATP}}}{[\text{MgATP}^{2-}]} \left(1 + \frac{[\text{ATP}^{4-}]}{K_i^{\text{ATP}}} \right)} \quad (4)$$

Since

$$K = \frac{[\text{MgATP}^{2-}]}{[\text{Mg}_{\text{free}}^{2+}] \cdot [\text{ATP}^{4-}]}$$

where K is the stability constant for MgATP^{2-} , substitution into eqn. (4) gives

$$v = \frac{V}{1 + \frac{K_m^{\text{MgATP}}}{[\text{MgATP}^{2-}]} + \frac{K_m^{\text{MgATP}}}{K_i^{\text{ATP}} \cdot K \cdot [\text{Mg}_{\text{free}}^{2+}]}} \quad (5)$$

Eqn. (5) would predict that reciprocal plots involving MgATP^{2-} and free Mg^{2+} would be families of parallel lines, and this is clearly not the case for pyruvate carboxylase from rat liver (McClure *et al.*, 1971a) and pig liver (Figs. 1 and 2). Hence free Mg^{2+} is not activating pyruvate carboxylase solely by complexing free ATP^{4-} , but activates the enzyme in its own right.

The precise order of binding of free Mg^{2+} and MgATP^{2-} to pyruvate carboxylase is still a matter of some dispute. Blair (1969), in his re-interpretation of the work of Keech & Barritt (1967) on sheep kidney pyruvate carboxylase, has suggested random-order binding and this has been supported by further work on this enzyme by Bais & Keech (1972). However, at pH 8.0 free Mg^{2+} binds to rat liver (McClure *et al.*, 1971a) and pig liver (Figs. 1 and 2) pyruvate carboxylase in a rapid-equilibrium ordered fashion followed by the binding of MgATP^{2-} . Moreover, a more careful scrutiny of the work of Bais & Keech (1972) would suggest this possibility

for the enzyme from sheep kidney. The slope replotted of the primary plot of $1/v$ against $1/[\text{Mg}^{2+}]$ at several fixed concentrations of MgATP^{2-} could pass through the origin and the converse primary plot [which was not shown by Bais & Keech (1972)] does intersect on the $1/v$ axis. The equilibrium binding of free Mg^{2+} is followed by the steady-state addition of MgATP^{2-} and HCO_3^- to the pig liver enzyme. This result is in agreement with the results obtained with the enzyme from rat liver (McClure *et al.*, 1971a), although the authors' conclusions (McClure *et al.*, 1971b) appear to contradict their own experimental results.

The reaction pathway of the pig liver enzyme is not double displacement, and this contrasts with the mechanisms that have been proposed for the enzyme from *Aspergillus niger* (Feir & Suzuki, 1969), rat liver (McClure *et al.*, 1971a,b) and chicken liver (Barden *et al.*, 1972). The latter three mechanisms are in error to differing degrees. Feir & Suzuki (1969) studied the interaction between the three substrates ATP, HCO_3^- and pyruvate, and out of six possible primary plots only two yielded families of parallel lines. For a double-displacement mechanism which involves three substrates in the reaction mechanism, it is difficult (if not impossible) to conceive of a mechanism that will yield fewer than four primary plots comprising families of parallel lines in the absence of any reaction products. Further, Feir & Suzuki (1969) applied the method of King & Altman (1956) to the steady-state reactions which occur at each of the three independent binding sites for ATP, HCO_3^- and pyruvate and then synthesized these results into one rate equation. Although this rate equation predicts the peculiar primary plots discussed above, it is quite incorrect to predict steady-state reaction fluxes in isolation and then suggest that the reaction flux is the effective sum of these individual components. The mechanisms for the rat and chicken liver enzymes are based on the 'non-classical Ping Pong mechanism' proposed for transcarboxylase (Northrop, 1969); this mechanism allows the formation of ternary complexes without affecting the parallel-line plots which normally characterize such mechanisms. To achieve this situation the binding sites for the two partial reaction components must bind their substrates at equilibrium and must be totally independent of each other. This latter point invalidates the mechanism proposed for the rat liver enzyme because bound pyruvate is believed to affect the binding of HCO_3^- (McClure *et al.*, 1971b). Barden *et al.* (1972) assume, on the basis of no published evidence, that HCO_3^- and MgATP^{2-} randomly bind to the enzyme at equilibrium (presumably after the equilibrium binding of free Mg^{2+}). If chicken liver pyruvate carboxylase binds MgATP^{2-} and HCO_3^- in the steady-state fashion described above it could not obey a non-classical Ping Pong mechanism.

In contrast with the mechanisms that have been proposed for other pyruvate carboxylases, the mechanism for the pig liver enzyme shows no kinetic inconsistencies and also provides an explanation for the low rate of the $\text{ATP} \rightleftharpoons [^{32}\text{P}]\text{P}_i$ equilibrium exchange reaction (Scrutton *et al.*, 1965; Cazzulo & Stoppani, 1967; McClure *et al.*, 1971c). In the mechanism proposed for the pig liver enzyme, MgADP^- and P_i are only released after the release of oxaloacetate. This suggests that the presence of enzyme-bound carboxybiotin (but not biotin) somehow 'traps' the MgADP^- and P_i at their respective binding sites. Evidence in support of this view comes from the stimulation of this exchange reaction by α -oxobutyrate (McClure *et al.*, 1971c). The interaction of pyruvate and oxaloacetate with pig liver pyruvate carboxylase has been depicted as obeying a Theorell-Chance mechanism. This is in accord with n.m.r. studies on the chicken liver enzyme, which have shown that these two α -keto acids move into and out of the co-ordination sphere of the bound manganese at a rate which is several orders of magnitude greater than the rate of the overall reaction (Mildvan & Scrutton, 1967; Scrutton & Mildvan, 1970).

A quantitative description of the reaction mechanism of pyruvate carboxylase demands a knowledge of the kinetic parameters for the decarboxylation of oxaloacetate. This will be discussed in the following paper (Warren & Tipton, 1974b).

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