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SHEEP KIDNEY MITOCHONDRIAL PHOSPHOENOLPYRUVATE CARBOXYLASE REACTION MECHANISM

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SUMMARY

Evidence pertaining to the mechanism of action of phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) has been obtained from initial velocity, product inhibition, exchange reactions and proton relaxation rate studies. The data obtained could be explained by a sequential reaction mechanism. Ternary complexes containing enzyme, Mn^{2+} and substrate were detected for phosphoenolpyruvate (PEP), IDP, ITP and oxaloacetate.

The central complex consisting of enzyme, Mn^{2+}, IDP, PEP and CO_2 is formed by a preferred pathway where IDP binds first and is followed by the random addition of PEP and CO_2. However, PEP can bind to the enzyme in the absence of IDP and CO_2. CO_2 is normally bound last (or released first, depending upon the reaction direction).

Evidence is presented to suggest that the reaction involves a two-step conversion of the central complex with the phosphoryl transfer from PEP to IDP preceding the carboxylation of the enzyme-bound 3-carbon intermediate.

It is proposed that two atoms of Mn^{2+} are involved in the reaction, one of which binds directly to the enzyme while the other is involved in charge neutralization of IDP.

INTRODUCTION

Lane and his coworkers[1,2] studied the mechanism of action of pig liver mitochondrial phosphoenolpyruvate (PEP) carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) using electron paramagnetic resonance, proton relaxation rate measurements, gel filtration, equilibrium dialysis binding and kinetic studies. They proposed that a central complex consisting of enzyme, Mn^{2+}, PEP, IDP and CO_2 was formed by a mixed ordered-random addition, *i.e.* when Mn^{2+}

Abbreviation: PEP, phosphoenolpyruvate.

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was bound, either PEP, IDP or CO_2 might bind next, but if IDP or CO_2 bound prior to PEP, the PEP site was unavailable and a dead-end complex was formed[3]. Although the binding of PEP proceeded in a random fashion with respect to Mn^{2+}, the affinity of the enzyme for PEP was greatly enhanced by the presence of Mn^{2+}. The ternary complex, enzyme–Mn^{2+}–PEP, could bind either IDP or CO_2 and this complex then in turn bound the third substrate to form the central complex. The central complex then underwent a conversion by a concerted mechanism to a new complex consisting of enzyme, Mn^{2+}, ITP and oxaloacetate.

On the other hand, Felicioli *et al.*[4], who studied the mechanism of the decarboxylation reaction catalysed by chicken liver mitochondrial PEP carboxylase, concluded that the reaction was a Bi Ter mechanism[5].

The present investigation was undertaken to elucidate the mechanism of action of sheep kidney mitochondrial PEP carboxylase and possibly to resolve the difference of opinion between these two groups.

MATERIALS AND METHODS

Unless otherwise indicated, the sources of reagents and enzyme were as previously described[6–8]. PEP carboxylase had a specific activity of 3–5 for proton relaxation rate studies while for the other studies, the specific activity was 2.5. $MnCl_2$ (Analar, B.D.H. Ltd) was treated with dithizone and standardized[9]. $CaCl_2$ (Analar, B.D.H. Ltd) was dried at 200 °C to constant weight before stock solutions were made. Imidazole was purchased from Sigma and *N*-ethylmorpholine, redistilled under reduced pressure, was purchased from Eastman Kodak.

Initial velocity studies on the carboxylation reaction

The carboxylation of PEP was followed by the incorporation of $H_2{}^{14}CO_3$ into oxaloacetate which was converted to aspartate in the presence of L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) as previously described[6] except that the concentrations of $MnCl_2$, PEP, IDP and $NaH^{14}CO_3$ were as detailed in the legends. Although the ideal situation for initial velocity studies is to use an uncoupled assay system, this was unsatisfactory with this enzyme because non-linear double reciprocal plots made interpretation impossible[8]. However, McClure[10] has shown that a coupled assay system will give an accurate measure of enzymic activities.

In the case of the product inhibition studies, conditions were as detailed above except that $MnCl_2$ was present at a constant concentration of 4 mM.

Determination of the proton relaxation rate

Measurements of the proton relaxation rate of water protons were made with a 90–180° pulsed nuclear magnetic resonance spectrometer operating at 30 MHz per s. Experiments were carried out in volumes of 0.1–0.2 ml at room temperature (20–22 °C).

The definition and determination of the enhancement, ε, of the proton relaxation rate for a solution containing Mn^{2+} in the presence of a complex species has been described[11–14]. In so far as it was possible, experiments to determine ε_t, the enhancement of ternary enzyme–metal–substrate complexes, and K_D and K_3, the constants for the dissociation of metal and substrate, respectively, from these com-

plexes, were designed so as to parallel the experiments of Miller *et al.*[1]. Thus, the graphical procedures of Miller *et al.*[1] were used for the analysis of the data. After this work had been completed, computer programs for the determination of the proton relaxation rate parameters became available[15,16] and they were applied to one of the titrations.

Processing of kinetic data

Reaction velocity data were processed using a CDC 6400 digital computer using the Fortran programs of Cleland[17]. The data were analysed in subsets consisting of velocities obtained when the concentrations of two substrates were varied at some constant saturating level of the third substrate .The data in each subset could thus be treated as a bireactant system and compared with Eqn 1 if parallel lines were obtained or with Eqn 2 if intersecting lines were obtained.

$$v = \frac{VAB}{K_bA + K_aB + AB} \tag{1}$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \tag{2}$$

The nomenclature of the kinetic constants follows the definitions and notation of Cleland[5]. The initial velocity patterns were analysed by inspection[18].

Concentrations of free and complexed forms of PEP and IDP as well as free Mn^{2+} were calculated using the following dissociation constants for the Mn^{2+}–substrate complexes: $K_{Mn\_PEP} = 1.79$ mM[14,19]; $K_{Mn\_IDP} = 0.1$ mM (rounded off from that obtained by O'Sullivan and Cohn[13] and corrected for pH 6.5). However, the initial velocity patterns were the same regardless of whether the reciprocal velocity was plotted as a function of the reciprocal of free substrate, Mn–substrate or total substrate.

RESULTS

Initial velocity studies

The initial velocity patterns shown in Fig. 1 were obtained when PEP and IDP were the variable and fixed variable substrates. The family of linear parallel lines indicated an irreversible connection between the enzyme forms binding these two substrates. Plots of intercepts from the primary plots with respect to reciprocal concentrations of the fixed variable substrate are shown as dashed lines in Fig. 1 and represent standard Lineweaver–Burk plots[20] at infinite concentration of the substrates.

The initial velocity patterns obtained when the concentration of PEP and HCO_3^- were varied are shown in Fig. 2. The family of linear intersecting lines indicated a reversible connection between the enzyme forms binding these two substrates. When the concentrations of IDP and HCO_3^- were varied, the pattern of parallel lines (Fig. 3) indicated an irreversible connection between the enzyme forms binding these two substrates. When the concentrations of IDP and HCO_3^- were varied, the pattern of parallel lines (Fig. 3) indicated an irreversible connection between the enzyme forms binding these two substrates.

Product inhibition studies were restricted to using ITP as the product inhibitor

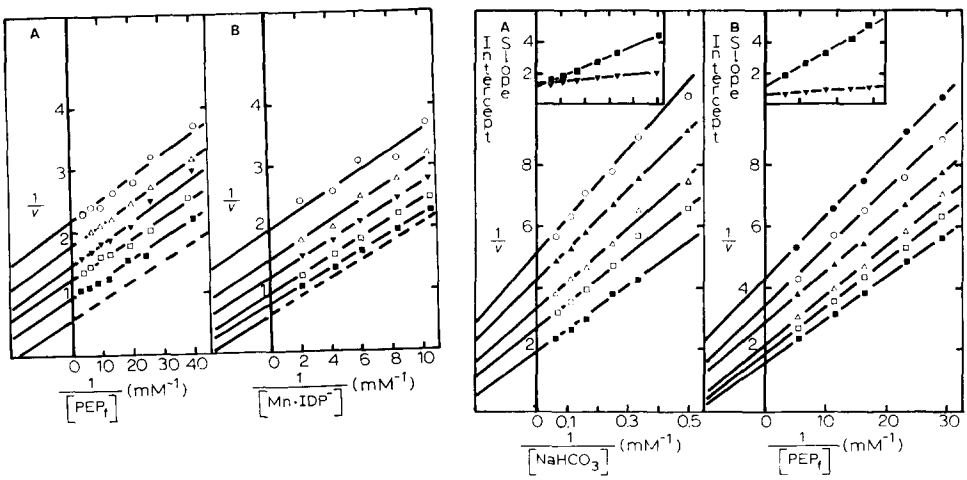


Fig. 1. Double reciprocal plots of initial velocity against free PEP (PEP_f) (A) and Mn·IDP (B). Reaction mixtures contained (in addition to standard components listed in Materials and Methods): $NaH^{14}CO_3$, 10 mM; $MnCl_2$, 2.0 mM. (A) PEP was varied at the constant Mn·IDP levels: ■—■, 0.465 mM; □—□, 0.236 mM; ▼—▼, 0.165 mM; △—△, 0.118 mM; ○—○, 0.095 mM. (B) Replots of the data in A. Mn·IDP varied at constant PEP levels: ■—■, 0.26 mM; □—□, 0.1 mM; ▼—▼, 0.049 mM; △—△, 0.037 mM; ○—○, 0.024 mM. – – –, secondary plots of intercepts from the related primary plots.

Fig. 2. Double reciprocal plots of initial velocity against $NaHCO_3$ (A) and free PEP (PEP_f) (B). Reaction mixtures contained (in addition to standard components listed in Materials and Methods) $MnCl_2$, 2.0 mM; Mn·IDP, 0.485 mM. (A) $NaHCO_3$ was varied at the constant PEP_f levels: ■—■, 0.18 mM; □—□, 0.087 mM; △—△, 0.06 mM; ▲—▲, 0.043 mM; ○—○, 0.034 mM. (B) Replot of data in A. PEP_f was varied at the constant $NaHCO_3$ levels: ■—■, 16.0 mM; □—□, 9.0 mM; △—△, 6.0 mM; ▲—▲, 4.0 mM; ○—○, 3.0 mM; ●—●, 2.0 mM. Insets secondary plots of intercepts ■—■)(and slopes (▼—▼) from the related primary plots *versus* the reciprocal concentrations of substrate.

of the carboxylation reaction. Oxaloacetate was not used as a product inhibitor because the rate of the $^{14}CO_2$:oxaloacetate exchange reaction was about three times faster than the maximum rate of the carboxylation reaction[6]. ITP was shown to be a linear non-competitive inhibitor with respect to both PEP (Fig. 4A) and HCO_3^- (Fig. 4B). In both cases, replots of slopes and intercepts as functions of ITP concentrations were linear (insets, Figs 4A and 4B) indicating that only one molecule of ITP was bound per mole of enzyme in any reversible sequence associated with these substrates. With IDP as the variable substrate, ITP showed non-linear double reciprocal plots (Fig. 5). The kinetic constants obtained from the initial velocity studies are summarized in Table I.

Role of Mn^{2+}

The interactions between enzyme, Mn^{2+} and substrates were investigated using proton relaxation rate and initial velocity studies.

Formation of a binary Mn–enzyme complex

The variation of enhancement with constant enzyme and varying concentrations of Mn^{2+} at pH 7.5 is shown in Fig. 6. The data are presented as a straight line plot of reciprocal enhancement against total Mn^{2+} concentration in accordance with Miller *et al.*[1]. By extrapolation of this plot, ε_b, the enhancement of the Mn–enzyme

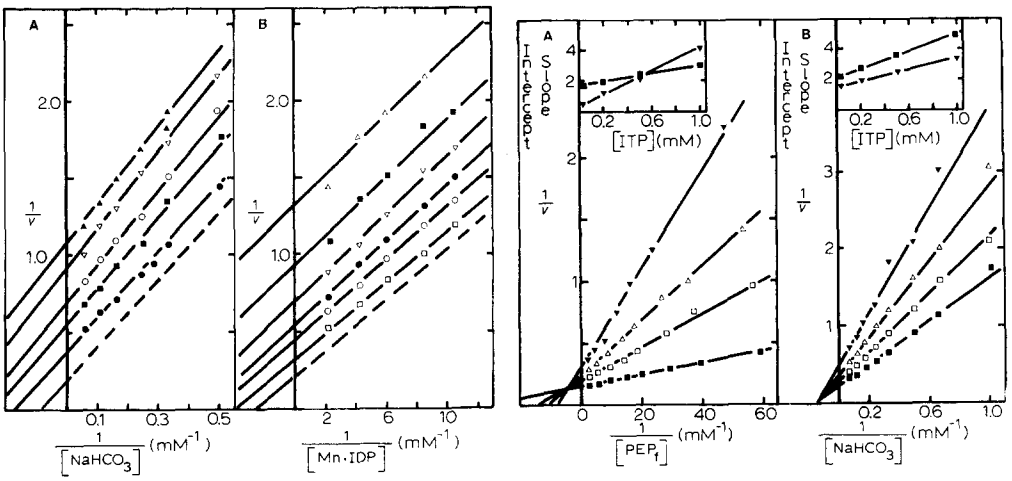


Fig. 3. Double reciprocal plots of initial velocity against $NaHCO_3$ (A) and Mn·IDP (B). Reaction mixtures contained (in addition to standard components listed in Materials and Methods): $MnCl_2$, 2.0 mM; PEP, 0.5 mM. (A) $NaHCO_3$ was varied at the constant Mn·IDP levels; ●—●, 0.465 mM; ■—■, 0.235 mM; ○—○, 0.165 mM; ▽—▽, 0.118 mM; ▲—▲, 0.094 mM. (B) Replot of the data in A. Mn·IDP varied at the constant $NaHCO_3$ levels; □—□, 16.0 mM; ○—○, 9.0 mM; ●—●, 6.0 mM; ▽—▽, 4.0 mM; ■—■, 3.0 mM; △—△, 2.0 mM; – – –, secondary plots of intercepts from the related primary plots.

Fig. 4. Inhibition patterns caused by the presence of product. Reaction mixtures contained (in addition to standard components listed in Materials and Methods): $MnCl_2$, 4.0 mM; Mn·IDP, 0.485 mM, $NaH^{14}CO_3$, 12 mM (A); PEP, 0.5 mM (B). (A) Free PEP (PEP_f) was varied at the constant Mn·ITP levels: ■—■, 0.0 mM; □—□, 0.2 mM; △—△, 0.5 mM; ▼—▼, 1.0 mM. (B) $NaHCO_3$ was varied at the constant Mn·ITP levels; ■—■, 0.0 mM; □—□, 0.2 mM; △—△, 0.5 mM; ▼—▼, 1.0 mM. Insets, secondary plots of intercepts (■—■) and slopes (▲—▲) from the primary plots *versus* the concentration of Mn·ITP.

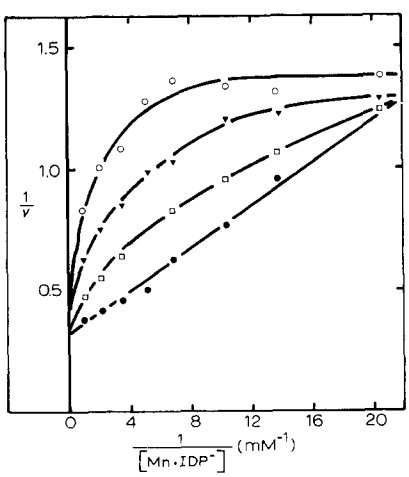


Fig. 5. Inhibition patterns caused by the presence of product. Reaction mixtures contained (in addition to standard components listed in Materials and Methods): $MnCl_2$, 4.0 mM; $NaH^{14}CO_3$, 12.0 mM; PEP, 0.5 mM. Mn·IDP was varied at the constant Mn·ITP levels: ●—●, 0.0 mM; □—□, 0.2 mM; ▼—▼, 0.5 mM; ○—○, 1.0 mM.

TABLE I

KINETIC CONSTANTS FOR PEP CARBOXYLASE

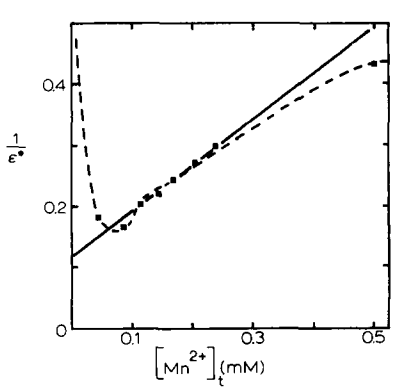
(*a*) Average of the values determined from the secondary plots of the initial velocity data; (*b*) Values obtained from the initial velocity data using the Fortran programs of Cleland[17]. The value for oxaloacetate obtained from data presented in ref. 7.

Substrate	*K_m values (M)*	
	(a)	*(b)*
PEP_{free}	$8.19 \cdot 10^{-5} \pm 0.58 \cdot 10^{-5}$	$7.76 \cdot 10^{-5} \pm 0.73 \cdot 10^{-5}$
$NaHCO_3$	$5.64 \cdot 10^{-3} \pm 0.53 \cdot 10^{-3}$	$8.78 \cdot 10^{-3} \pm 0.86 \cdot 10^{-3}$
$Mn \cdot IDP^-$	$3.56 \cdot 10^{-4} \pm 0.35 \cdot 10^{-4}$	$3.63 \cdot 10^{-4} \pm 0.31 \cdot 10^{-4}$
$Mn^{2+}{}_{free}$	$8.82 \cdot 10^{-5} \pm 0.24 \cdot 10^{-5}$	$7.56 \cdot 10^{-5} \pm 0.75 \cdot 10^{-5}$
Oxaloacetate	$4.45 \cdot 10^{-4} \pm 0.02 \cdot 10^{-4}$	

complex, was determined as 8.3. It was noted, however, that the points fell on the - type of curve (dotted in Fig. 6) predicted by Danchin[21] on theoretical grounds. Computer analysis[15] of the data was carried out with n, the number of moles of Mn^{2+} bound per mole of enzyme, varied from 1 to 4. This analysis gave a best fit to the data for n equal to 1, with ε_b of 6.6 and K_D, $1.4 \cdot 10^{-5}$ M with a percentage standard deviation of 5.9%.

A Scatchard[22,23] plot of these data (Fig. 7) gave a straight line cutting the axis at 1.0 (the number of Mn^{2+} binding sites) and for a K_D of $1.7 \cdot 10^{-5}$ M. This plot also indicated that a reasonable range of the titration curve had been covered. (This very critical criterion in the analysis of titration curves has been emphasised by Deranleau[24,25] and others[15,16].) A titration of constant Mn^{2+} with varying enzyme concentration was also carried out and graphical analysis gave resultts similar to the above.

Similar titrations were carried out at pH 6.5, the pH at which the kinetic studies were carried out. Increased enhancements on the addition of enzyme to Mn^{2+}



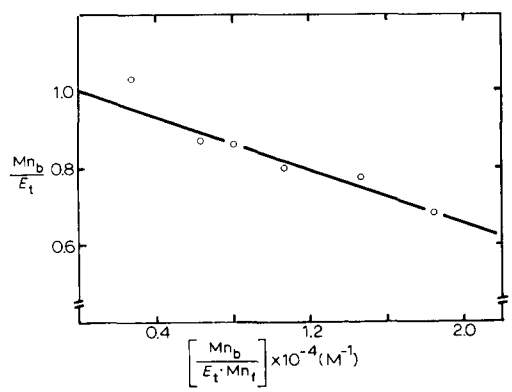


Fig. 6. Titration of a constant amount of PEP carboxylase with Mn^{2+} at pH 7.5. The reciprocal of the enhancement of the proton relaxation rate (ε^*) was plotted against Mn^{2+} concentration. Each solution contained 0.08 M *N*-ethylmorpholine (Cl^-), pH 7.5, 0.115 mM enzyme and Mn^{2+} as indicated. Temperature was 20 °C — — —, theoretical curve obtained according to Danchin[21].

Fig. 7. Scatchard[22,23] plot of the results of Fig. 6. The intercept on the ordinate gives n, the number of Mn^{2+} binding sites per mole, and the intercept on the abscissa gives $1/K_D$, where K_D is the dissociation constant for enzyme Mn^{2+} complex.

were observed, again indicative of complex formation. However, the experimental values were much lower than at pH 7.5 and the experiments were further hampered by a tendency for the protein to precipitate at this pH at the concentrations used. Graphical methods[1] were used to obtain estimates of 3.1 and $6 \cdot 10^{-5}$ M for ε_b and K_D, respectively. Because of the experimental difficulties, it is not possible to assert that these represent significant variations from the results at pH 7.5.

Ternary enzyme–Mn^{2+}–PEP complex formation

The addition of PEP to the binary enzyme–Mn^{2+} complex at pH 7.5 caused a decrease in the observed enhancement, indicating the formation of a ternary complex containing enzyme, Mn^{2+} and PEP. Neither IDP nor HCO_3^- was required for the formation of this complex. Titration of the binary complex with PEP was carried out at three enzyme concentrations and using the procedure of Miller *et al.*[1] a value for ε_t of 6.4 was obtained from the data. A change in enhancement was also observed at pH 6.5; this was smaller and in the opposite direction to the results at pH 7.5. The formation of a similar complex consisting of enzyme, Mn^{2+} and oxaloacetate was also demonstrated by proton relaxation rate.

Ternary enzyme–Mn^{2+}–IDP complex formation

The formation of a complex consisting of enzyme, Mn^{2+} and IDP was indicated by a small decrease in the observed enhancement on the addition of 0.1 mM IDP to the binary complex at pH 7.5. However, at pH 6.5, there was a larger increase in the proton relaxation rate and, using the procedure of Miller *et al.*[1] the results of titrations of enzyme with IDP at constant Mn^{2+} concentration yielded a value for ε_t of 4.0. Even smaller effects were seen with ITP and the data could not be analysed.

Table II shows some of the proton relaxation rate results, together with the corresponding data of Miller *et al.*[1].

TABLE II

SUMMARY OF RESULTS FROM PROTON RELAXATION RATE EXPERIMENTS

Conditions and interpretation discussed in the text. Values in parentheses from Miller *et al.*[1]. (Note that their results for IDP were obtained at pH 7.5.)

Substrate	*pH*	ε_b	ε_t	K_D *(M)*	K_3 *(M)*
Mn^{2+}	7.5	6.6–8.3		$1.4 \cdot 10^{-5}$	
		(14.2)		$(4 \cdot 10^{-5})$	
	6.5	3.0		$6 \cdot 10^{-5}$	
Mn^{2+} + PEP	7.5		6.4		$1 \cdot 10^{-5}$
			(6.7)		$(5 \cdot 10^{-6})$
Mn^{2+} + IDP	6.5		4.0		$2 \cdot 10^{-5}$
			(10.4)		$(3.4 \cdot 10^{-5})$

Inhibition by Ca^{2+}

Cohn[26] has classified metal ion activated enzymes into two groups based on their response to Ca^{2+}. The basis of this classification is that Ca^{2+} activates only those enzymes where the activating cation is not bound directly to the enzyme, *e.g.* enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40). Since the previous data showed that Mn^{2+} was bound by sheep kidney mitochondrial PEP carboxylase, it would be expected that Ca^{2+} would inhibit the enzymic activity. In fact Ca^{2+} proved to be a weak

TABLE III

EFFECT OF Ca^{2+} ON PEP CARBOXYLASE ACTIVITY

Assay solutions (0.5 ml) contained (in μmoles); imidazole (Cl^-), pH 6.5 (adjusted at 30 °C), 50; metal ion as indicated; IDP, 0.5; PEP, 0.4; $NaH^{14}CO_3$ ($3 \cdot 10^5$ cpm per μmole) 5; GSH, 0.8; sodium glutamate, 5; pyridoxal phosphate, 0.02; aspartate transaminase, approx. 4.5 units. Incubation was for 5 min at 30 °C. The reaction was stopped with 0.25 ml of a 10% (w/v) trichloroacetic acid solution.

Ca^{2+} (mM)	*Mn^{2+} (mM)*	*Activity (μmoles)*	*% of maximal activity*
0.0	3.0	0.052	100
0.8	3.0	0.049	94
3.0	3.0	0.047	90
3.0	0.0	0.00	0

inhibitor in the presence of Mn^{2+} (Table III) and furthermore it failed to activate the enzyme.

A possible explanation for these observations could be the involvement of two metal ion species, *viz.* Ca^{2+} functioning *via* a Ca–IDP$^-$ complex and Mn^{2+} activating via the enzyme–Mn^{2+} complex. This hypothesis was tested kinetically by evaluating the nature of the inhibition by Ca^{2+} with respect of Mn^{2+}. With pyruvate kinase, where the evidence indicates that only one metal ion is required for catalysis. Ca^{2+} is strictly a competitive inhibitor with respect to Mn^{2+} and it competes with Mn^{2+} in the formation of the enzyme–metal complex as shown by a diminution of the proton relaxation rate enhancement of the binary complex[12]. However, with sheep kidney mitochondrial PEP carboxylase, Ca^{2+} was a mixed type inhibitor with respect to Mn^{2+} (Fig. 8). This is consistent with the involvement of two metal ions in the enzymic reaction.

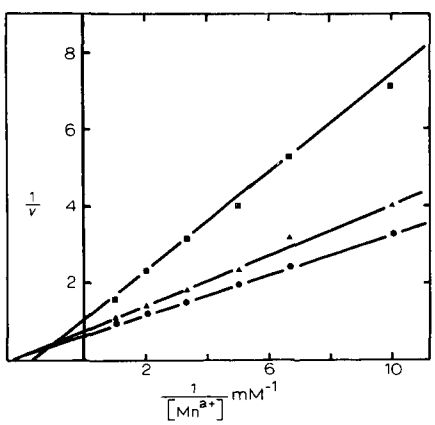


Fig. 8. Inhibition patterns caused by the presence of Ca^{2+}. Reaction mixtures contained (in addition to standard components listed in Materials and Methods): PEP, 0.5 mM; IDP, 0.5 mM; $NaH^{14}CO_3$, 12 mM; Mn^{2+} and Ca^{2+} as indicated. Mn^{2+} was varied at the constant Ca^{2+} levels ●—●, 0.0 mM; ▲—▲, 1.0 mM; ■—■, 6.0 mM.

DISCUSSION

Analysis of the initial velocity data presented here indicates an irreversible connection between the enzyme forms binding the substrate pairs, IDP and PEP, and IDP and HCO_3^-, but a reversible sequence between those binding PEP and HCO_3^-. In addition to these restrictions, any proposed mechanistic scheme must account for the following observations: (a) both IDP and ITP bind tightly to the enzyme in the absence of other substrates[6]; (b) Mn^{2+} binds strongly to the enzyme in the absence of other substrates (R. J. Barns and D. B. Keech, in preparation); (c) ITP, IDP, PEP and oxaloacetate bind independently to the enzyme in the presence of Mn^{2+}; (d) the enzyme catalyses a $^{14}CO_2$:oxaloacetate exchange reaction in the presence of Mn^{2+}. This reaction is stimulated by IDP and ITP while Mg^{2+} can only partially replace Mn^{2+} (ref. 7); (e) ITP is a linear non-competitive inhibitor of the carboxylation reaction with respect to both PEP and CO_2; (f) ternary complexes consisting of enzyme, Mn^{2+} and substrate were detected for PEP, IDP, ITP and oxaloacetate.

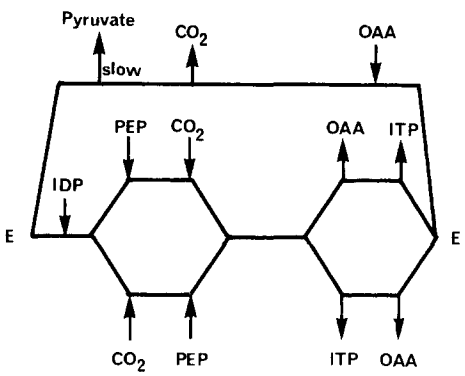


Fig. 9. The reaction sequence of sheep kidney mitochondrial PEP carboxylase showing the preferred pathway to the central complex. IDP binds first followed by the random addition of PEP and CO_2. The outer sequence shows the sequence whereby the nucleotide-independent $^{14}CO_2$: oxaloacetate reaction occurs. OAA, oxaloacetate.

Considering initially only the organic components of this system, since Mn^{2+} was always at saturating levels and need not be considered, the carboxylation system becomes one of three substrates and two products. To incorporate all the known kinetic and binding properties of the enzyme, a scheme is proposed whereby IDP binds first followed by the random addition of PEP and CO_2 (Fig. 9). It would therefore follow that, under the conditions of the kinetic analysis reported here, when IDP was a variable substrate the second substrate to bind in this scheme would be that substrate which is held at a fixed saturating level. Finally, the second variable substrate would be bound. Therefore, this scheme would predict non-sequential initial velocity patterns when IDP is a variable substrate because the presence of the non-varied saturating component would create an irreversible connection between the addition of IDP and the varied non-saturating substrate (*cf.* Figs 1 and 3). On the other hand, because of the random addition of CO_2 and PEP, the enzyme forms binding these substrates would always be reversibly connected and sequential kinetic

patterns result (Fig. 2). The scheme shown in Fig. 9 is in fact a preferred pathway for substrate addition as the proton relaxation rate studies showed that PEP can bind in the absence of IDP.

Furthermore, one would predict that with such a sequential scheme, ITP would be a linear non-competitive inhibitor with respect to PEP and CO_2. This was in fact observed (Fig. 4). It is interesting to note that in the case of ITP inhibition with respect to IDP, non-linear initial velocity plots were observed (Fig. 5). These plots are similar to and have the same inflection point as the non-linear initial velocity plots obtained with an uncoupled carboxylation assay system[8]. Thus, it can be argued that the interactions observed with the uncoupled assay and induced by oxaloacetate have now been introduced into the product inhibition system by ITP, although much higher levels are needed, assuming $Mn \cdot ITP^{2-}$ to be the active species. Thus, the scheme depicted in Fig. 9 is in accord with all the observed binding properties and the initial velocity and product inhibition studies. This scheme also shows the sequence whereby the nucleotide-independent $^{14}CO_2$:oxaloacetate exchange reaction occurs[7]. In the absence of nucleotide, oxaloacetate is decarboxylated to yield the enzyme-bound 3-carbon intermediate which dissociates slowly to yield pyruvate. This sequence is stimulated by IDP but, in the presence of ITP, phosphorylation of the intermediate occurs at a faster rate than the dissociation of the intermediate, thus producing PEP.

A nucleotide-independent $^{14}CO_2$:oxaloacetate exchange reaction and an enzyme bound intermediate would normally imply a Ping Pong[5] mechanism. However, Moffet and Bridger[27] have reported that *Escherichia coli* succinyl-CoA synthetase exhibits kinetic patterns characteristic of a sequential mechanism (intersecting initial velocity patterns) despite the fact that catalytic participation of a phospho-enzyme intermediate has been established. Similarly, PEP carboxylase shows 'sequential' kinetic patterns.

Role of Mn^{2+}

Evidence was obtained from the proton relaxation rate studies that the sheep kidney mitochondrial enzyme binds one mole of Mn^{2+} per mole. The data indicated a higher enhancement for the binary complex at pH 7.5 than at pH 6.5, though the results at the latter pH were hampered by the tendency of the protein to precipitate. As anticipated from the work of Miller *et al.*[1], this enzyme would appear to fit into the Type II category[28] where the metal ion at the active site has the potential capacity to act as a 'metal-bridge' between enzyme and substrate; substantial evidence for such a structure has been put forward for two Type II enzymes, pyruvate kinase[29,30] and pyruvate carboxylase[30]. We should note, however, that one of the criteria of a Type II enzyme[28] that $\varepsilon_t > \varepsilon_b$, was only just met since the enhancement for the ternary complexes with both PEP and IDP was of the same order as for the binary complex (see Table II). Indeed, at pH 6.5 the addition of IDP caused an increase in the enhancement. However, any significance attached to the numerical values of the enhancements should be done with caution, since in only one case, Mn–enzyme at pH 7.5, was it established that the data could be rigorously analysed. For the other complexes the enhancements were too small or it was not possible to cover a sufficiently wide range of conditions. (It might be noted that these cautionary remarks also apply to nearly all of the published work on enhancement of proton relaxation

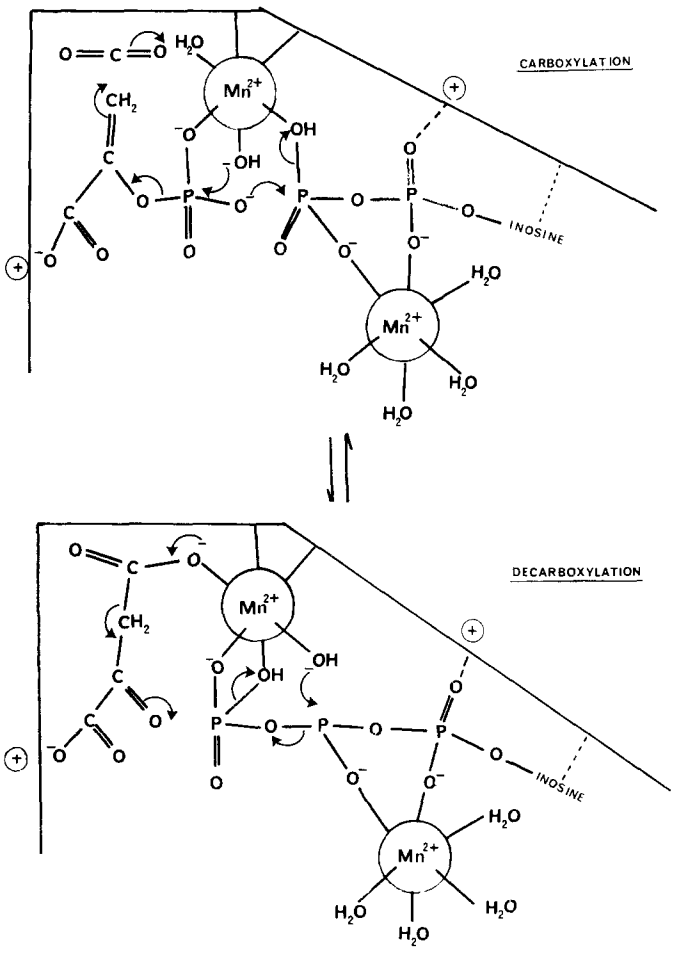


Fig. 10. Proposed scheme for the mechanism of PEP carboxylase including both Mn^{2+} and organic components.

rate, including the work of Miller *et al.*[1]). Independent evidence for the binding of Mn^{2+} to PEP carboxylase was obtained by virtue of the protection it afforded against inactivation of the enzyme by 1-fluoro-2,4-dinitrobenzene (R. J. Barns and D. B. Keech, in preparation) and diazobenzene sulphonate (unpublished).

Evidence in favour of the involvement in the reaction mechanism of a metal ion in addition to that which is bound to the enzyme includes (i) free nucleotide is a potent inhibitor of enzymic activity, (ii) Mn^{2+} protects against inactivation by 1-fluoro-2,4-dinitrobenzene while Mg^{2+} is ineffective at a pH where Mg^{2+} is catalytically active (R. J. Barns and D. B. Keech, in preparation), (iii) the mixed-type inhibition by Ca^{2+}, (iv) the synergistic effect of Mn^{2+} and Mg^{2+} shown for the decarboxylation reaction by both mitochondrial and cytosol PEP carboxylase from guinea pig liver[31] and by the rat cytosol[32] enzyme, (v) the involvement of two metal ions in the related enzyme, PEP carboxytransphosphorylase[33], and (vi) complexes of the type, enzyme–Mn^{2+}–substrate–Mn^{2+} were detected for GDP and GTP with the pig liver enzyme[1].

Therefore, from the data available, it is possible to suggest a dual role for Mn^{2+}; firstly, to provide a bridge for the binding of PEP and also to form a complex with the nucleotide to give the metal nucleotide complex, the true substrate for the enzyme, *i.e.* a role of charge neutralization is suggested as appears to be the case for creatine kinase[13,34] and adenylate kinase[35].

Reaction mechanism

A tentative mechanism, proposed as a working hypothesis for future experimentation is detailed in Fig. 10. This scheme takes into account the two-step reaction sequence and the requirement for two metal ions in the catalysis although the role of the second metal ion is probably charge neutralisation[13,34,35]. The essential feature of this mechanism is the involvement of Mn^{2+}-bound OH^- which are continuously utilized and reformed without any net uptake consistent with the lack of incorporation of ^{18}O from $H_2{}^{18}O$ (ref. 2). Also the carboxylation–decarboxylation process is not necessarily stoichiometric with the phosphoryl transfer process such that the $^{14}CO_2$: oxaloacetate exchange reaction is independent of phosphoryl transfer[7] with enzyme-bound pyruvate (or more strictly the enolate ion) as an intermediate. Carboxylation of the enzyme-bound enolpyruvate intermediate would yield the keto form of oxaloacetate, the primary carboxylation product of PEP carboxylase[36].

This mechanism is similar to that proposed by Wood *et al.*[33] for PEP carboxytransphosphorylase except that these workers proposed an enzyme-bound pyrophosphoenolpyruvate intermediate whereas the equivalent intermediate for PEP carboxylase, ITP-enolpyruvate, could not exist in accord with the $^{14}CO_2$:oxaloacetate exchange reaction being independent of phosphoryl transfer. In fact, a PP_i-independent exchange reaction which is stimulated by PP_i does appear to exist for PEP carboxytransphosphorylase so that very similar mechanisms probably exist for these two related enzymes.

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