Pyruvate carboxylase catalysis of phosphate transfer between carbamoyl phosphate and ADP

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In a reaction that is analogous to the phosphorylation of ADP from carboxyphosphate, pyruvate carboxylase catalyses the formation of ATP from carbamoyl phosphate and ADP at a rate that is about 0.3 % of the pyruvate-carboxylation reaction and about 3 % of the full reverse reaction. Acetyl-CoA stimulates the phosphorylation of ADP from carbamoyl phosphate but is not an essential requirement of the reaction. Mg^{2+} also stimulates the reaction, and in the range of Mg^{2+} concentrations considered the effect on V is much larger in the absence of acetyl-CoA than in its presence. Acetyl-CoA and Mg^{2+} may be acting in a co-operative way to stimulate the phosphorylation of ADP in a similar way to their effects on the pyruvate-carboxylation reaction. The phosphorylation of ADP by carbamoyl phosphate is also stimulated by the presence of biotin in the part of the active site where this reaction occurs, but again it is not absolutely required for the reaction to proceed. The pH profiles of the phosphorylation of ADP by carbamoyl phosphate indicate that there are at least two ionizable residues involved in the reaction, one of which probably has a role in the release of carbamate from the active site.

INTRODUCTION

Pyruvate carboxylase (EC 6.4.1.1) catalyses the following reactions:

Reactions (1) and (2) are thought to occur at spatially distinct subsites within the active site of the enzyme, with biotin acting as a mobile carboxy-group carrier between the subsites (see Attwood & Keech, 1984). Reaction (1) is thought to occur via a carboxyphosphate intermediate (Wallace *et al.*, 1985), such that HCO_3^- acts as a nucleophile to remove the γ -phosphate group from MgATP to form carboxyphosphate, from which the carboxy group is transferred to biotin, either directly or via the formation of CO_2 (see reaction 3):

Wallace *et al.* (1985) were able to isolate an enzyme complex that was capable of both phosphorylating MgADP and carboxylating pyruvate. The complex was both formed and could phosphorylate MgADP in the absence of acetyl-CoA, whereas the rate of carboxylation of pyruvate in the presence of acetyl-CoA was 15 times faster than in its absence. On the basis of this evidence, Wallace *et al.* (1985) suggested that the major role of acetyl-CoA in reaction (1) was to facilitate the transfer of the carboxy group from carboxyphosphate to biotin. The question still remains, however, does acetyl-CoA have a role in the formation of carboxyphosphate? Similarly, does the other effector of reaction (1), Mg^{2+} , play a part in carboxyphosphate formation and does biotin have a role in this reaction, if only an indirect one?

From their pH profiles of the reverse reaction of pyruvate carboxylase, Attwood & Cleland (1986) found that there were several ionizable residues involved in reaction (1), but were not

able to assign the ionizable residues to particular steps in the reaction. Thus it is not clear if any of these ionizable residues participate in the transfer of phosphate between carboxyphosphate and MgADP.

Carbamoyl phosphate ($\text{}^2\text{-O}_3\text{POCONH}_2$) is a structural analogue of carboxyphosphate and has been found to be capable of

transferring its phosphate group to MgADP in reactions catalysed by acetyl-CoA carboxylase from *Escherichia coli* (Polakis *et al.*, 1972) and sheep kidney pyruvate carboxylase (Ashman & Keech, 1975). The use of carbamoyl phosphate as a substrate analogue of carboxyphosphate makes possible the study of the part of reaction (1) that does not involve carboxylation of biotin, separate from the rest of the catalytic cycle of pyruvate carboxylase. In this way we have addressed the questions posed above about the roles of acetyl-CoA, Mg^{2+} and biotin in the formation of carboxyphosphate, and whether any of the ionizable residues observed in the pH profiles obtained by Attwood & Cleland (1986) participate in this part of reaction (1).

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MATERIALS AND METHODS

Materials

Chicken liver pyruvate carboxylase was purified as described by Goss *et al.* (1979) and then subjected to further purification on an avidin-agarose affinity column to an average specific activity of 48 units/mg of protein (1 unit of enzymic activity is defined as the amount of enzyme required to catalyse the formation of 1 μ mol of oxaloacetate/min under saturating substrate conditions at 30 °C).

Avidin, avidin-agarose, carbamoyl phosphate and oxamate were obtained from Sigma Chemical Co., and all other materials were high-purity preparations from commercial suppliers.

Pyruvate-carboxylation assay

The conditions for this assay were described by Attwood & Cleland (1986) except that the buffer used was a mixture containing 50 mM-Bistris, 25 mM-Tricine and 25 mM-glycine, pH 8.0. This buffer system was used in all of the experiments described in this paper.

ADP phosphorylation from carbamoyl phosphate

The reaction was performed at 25 °C in an assay volume of 1 ml that contained the following components unless otherwise stated: 2 mM-ADP, 1–20 mM-carbamoyl phosphate, 8 mM-MgCl₂, 0.5 mM-glucose, 0.5 mM-NADP⁺, 0.25 mM-acetyl-CoA, 1 unit of hexokinase and 5 units of glucose-6-phosphate dehydrogenase. After establishment of a baseline rate of change in absorbance at 340 nm the reaction was started by the addition of pyruvate carboxylase.

Biotin-dependence of the ADP-phosphorylation reaction

The biotin content of a sample of pyruvate carboxylase was determined by using the [¹⁴C]biotin-avidin assay described by Rylatt *et al.* (1977). One-third of the pyruvate carboxylase was incubated for 1 h at 25 °C in a solution containing avidin such that the concentration of biotin-binding sites was 10 times the concentration of biotin in the enzyme, and another one-third of the pyruvate carboxylase sample was incubated under the same conditions except that the avidin had been incubated for 1 h with a 10-fold excess of free biotin before addition to the enzyme. The last one-third of the enzyme was incubated under the same conditions as the other two samples, but in the absence of avidin. After the incubation period, the samples of pyruvate carboxylase were assayed for pyruvate-carboxylating activity. The samples were then used in the ADP-phosphorylation reaction both in the presence and in the absence of acetyl-CoA with 5 mM-MgCl₂ and 10 mM-carbamoyl phosphate.

Data analysis

All kinetic data were fitted by the least-squares method with a program written by Duggleby (1981). Individual saturation curves were fitted to eqns. (4a) and (4b), and the secondary plots of slope and intercept versus oxamate concentration were fitted to eqns. (5) and (6) respectively (see the Appendix for derivations). Eqns. (7) and (8) were fitted to the data obtained in the pH profile experiments. [In fitting eqn. (6) to the slope data, the values of V_1 and K_1' obtained from the fit of eqn. (5) to the intercept data were inserted.]

$$v = V/(1 + K/[S]) \quad (4a)$$

$$v = (V/K)/(1/K + 1/[S]) \quad (4b)$$

$$\text{Intercept} = (1 + [I]/K_1')/(V_1 + V_2 \cdot [I]/K_1') \quad (5)$$

$$\text{Slope} = K(1 + [I]/K_1')/(V_1 + V_2 \cdot [I]/K_1') \quad (6)$$

$$\log V = \log \{C_v/(1 + [H^+]/K_1 + K_2/[H^+])\} \quad (7)$$

$$\log (V/K) = \log \{C_{v/K}/(1 + [H^+]/K_3)\} \quad (8)$$

where v is the velocity of the reaction, K is the Michaelis constant, $[S]$ is carbamoyl phosphate concentration, V is the maximal velocity, $[I]$ is the concentration of oxamate, K_1' is the dissociation constant of the enzyme-oxamate complex, K_1' is the dissociation constant of the enzyme-carbamoyl phosphate-oxamate complex, V_1 is the maximal velocity in the absence of oxamate and V_2 is the maximal velocity in the presence of saturating oxamate. In eqns. (7) and (8) C_v and $C_{v/K}$ are constants and K_1 , K_2 and K_3 are the dissociation constants of protonated ionizable groups.

RESULTS AND DISCUSSION

Comparison of ADP phosphorylation by carbamoyl phosphate with pyruvate carboxylation and oxaloacetate decarboxylation

V for ADP phosphorylation by carbamoyl phosphate is only about 0.3% of that for the pyruvate-carboxylation reaction, which is in agreement with the value found by Ashman & Keech (1975) for the sheep kidney enzyme, and only about 3% of that for the oxaloacetate-decarboxylation reaction [V for the oxaloacetate reaction was calculated by using the finding by Attwood & Cleland (1986) that this reaction proceeded at 9.8% of pyruvate carboxylation]. This probably indicates that carbamoyl phosphate is a poor substrate compared with carboxyphosphate, which is a putative intermediate in the full reverse reaction of pyruvate carboxylase when oxaloacetate decarboxylation is linked to ATP formation.

Effect of acetyl-CoA and Mg²⁺ on ADP phosphorylation by carbamoyl phosphate

Fig. 1 shows Lineweaver-Burk plots of the reaction where carbamoyl phosphate was the substrate whose concentration was varied both in the presence and in the absence of acetyl-CoA, and in the presence of either 5 mM- or 20 mM-MgCl₂. In the presence of acetyl-CoA V is 14 times larger than in its absence, V/K is 7 times as great in the presence of acetyl-CoA as in its absence, and $K_{\text{acetyl-CoA}} = 1.8 K_{\text{acetyl-CoA}}$. This effect of acetyl-CoA was not due to the prevention of dilution inactivation, since in the assays without acetyl-CoA no time-dependent loss of activity was observed and the concentration of pyruvate carb-

Fig. 1. Lineweaver-Burk plots of the dependence of the velocity of the ADP-phosphorylation reaction catalysed by pyruvate carboxylase on the concentration of carbamoyl phosphate and the effects of acetyl-CoA and Mg²⁺ on the reaction

The reactions were performed as described in the Materials and methods section under the following conditions: Δ , 0.25 mM-acetyl-CoA, 5 mM-MgCl₂; $\blacktriangle$, 0.25 mM-acetyl-CoA, 20 mM-MgCl₂; $\circ$, 0 mM-acetyl-CoA, 5 mM-MgCl₂; $\bullet$, 0 mM-acetyl-CoA, 20 mM-MgCl₂. The continuous lines are derived from least-squares-regression fits to the data of eqn. (4a).

oxylase in these assays was 8.5 $\mu\text{mol}/\text{min}$ per ml (pyruvate-carboxylating activity), which is greater than the 3–4 $\mu\text{mol}/\text{min}$ per ml below which a significant time-dependent loss of activity is observed (see Attwood & Keech, 1984). These data indicate that, although acetyl-CoA is not essential, it greatly stimulates the reaction. These results are similar to the effects of acetyl-CoA on the ATP-ADP-exchange reaction in the rat liver enzyme observed by McClure *et al.* (1971), where acetyl-CoA was not absolutely required for the reaction but stimulated the rate of the reaction about 8-fold. However, the reaction mixture used by McClure *et al.* (1971) to measure the ATP-ADP exchange contained both P_i and HCO_3^- and thus in part would encompass the biotin-carboxylation reaction (see Attwood & Keech, 1984). Thus the overall observed effect of acetyl-CoA on the ATP-ADP exchange observed by McClure *et al.* (1971) comprised actions on both phosphate transfer between ATP and HCO_3^- and carboxy-group transfer between carboxyphosphate and biotin. Our results confirm the findings by Wallace *et al.* (1985) that the transfer of phosphate from ATP to HCO_3^- does not absolutely require acetyl-CoA but indicate that acetyl-CoA plays a role in this reaction in addition to its role in the transfer of the carboxy group between biotin and carboxyphosphate. The lower K for carbamoyl phosphate in the absence of acetyl-CoA may indicate that under these conditions the binding of this intermediate analogue is facilitated. This effect could be associated with the fact that acetyl-CoA induces a conformation change in the enzyme (Mayer *et al.*, 1980; Johannssen *et al.*, 1983), and in this conformation access to the active site by the analogue of an intermediate that would normally not leave the active site is more restricted than in the conformation assumed by the enzyme in the absence of acetyl-CoA.

In Fig. 1 the effect of increasing the concentration of MgCl_2 from 5 mM to 20 mM both in the presence and in the absence of acetyl-CoA is shown, and Table 1 shows the kinetic parameters derived from least-squares-regression analyses of the data as described in the Materials and methods section. In both the presence and the absence of acetyl-CoA, increasing $[\text{Mg}^{2+}]$ caused an increase in V ; however, in the presence of acetyl-CoA, at 20 mM- Mg^{2+} V was only 1.2 times greater than at 5 mM- Mg^{2+} . In the absence of acetyl-CoA, on the other hand, the effect of increasing $[\text{Mg}^{2+}]$ on V was much greater, such that at 20 mM- Mg^{2+} V was 3.4 times greater than at 5 mM- Mg^{2+} . In the presence of acetyl-CoA increasing $[\text{Mg}^{2+}]$ caused a slight decrease in K from 5.6 mM to 4.0 mM, whereas in the absence of acetyl-CoA increasing $[\text{Mg}^{2+}]$ actually caused an increase in K from 3.5 mM to

6.5 mM. In both the presence and the absence of acetyl-CoA increasing $[\text{Mg}^{2+}]$ caused V/K to increase by 64–69%. In general, therefore, increasing $[\text{Mg}^{2+}]$ enhances the ADP-phosphorylation reaction both in the presence and in the absence of acetyl-CoA. However, in the absence of acetyl-CoA Mg^{2+} has a much more marked effect on V than in its presence. This correlates quite well with the observations made by McClure *et al.* (1971) on the effects of Mg^{2+} on the ATP-ADP-exchange reaction in rat liver pyruvate carboxylase, where at saturating acetyl-CoA increasing $[\text{Mg}^{2+}]$ above 5 mM resulted in only a small stimulation of the reaction. Again, however, the effect of Mg^{2+} on the ATP-ADP-exchange reaction observed by McClure *et al.* (1971) cannot be definitively ascribed to an effect on the phosphate transfer between ATP and HCO_3^- , for the reasons discussed above. Barden & Scrutton (1974) found that $[\text{acetyl-CoA}]$ above about 15 μM had no effect on the K_a for Mg^{2+} in the pyruvate-carboxylation reaction catalysed by the chicken liver enzyme; however, below this concentration decreasing $[\text{acetyl-CoA}]$ resulted in an increase in K_a , such that at 2 μM -acetyl-CoA the K_a for Mg^{2+} was 2.5 mM compared with 0.5 mM at 50 μM -acetyl-CoA. The difference in the degree of stimulation of ADP phosphorylation at saturating carbamoyl phosphate by Mg^{2+} in the presence or in the absence of saturating acetyl-CoA may be explained by the fact that acetyl-CoA lowers the K_a for Mg^{2+} . Thus at saturating acetyl-CoA the ADP-phosphorylation reaction is close to being maximally stimulated at 5 mM- Mg^{2+} , whereas in the absence of acetyl-CoA the reaction is less than half-maximally stimulated at 5 mM- Mg^{2+} .

This effect of Mg^{2+} on V , however, is distinct from the effect on V/K , which was approximately the same in the presence or in the absence of acetyl-CoA, and the effect on K , where increasing $[\text{Mg}^{2+}]$ resulted in either an increase or a decrease of this parameter depending on whether or not acetyl-CoA was present. The increase in K for carbamoyl phosphate produced by increasing Mg^{2+} in the absence of acetyl-CoA may reflect a decrease in the affinity of the enzyme for carbamoyl phosphate caused by an Mg^{2+} -induced conformation change, perhaps somewhat similar to that induced by acetyl-CoA, for which there is some supportive evidence (Attwood *et al.*, 1986). In the presence of saturating acetyl-CoA, however, increasing $[\text{Mg}^{2+}]$ actually appears to decrease K for carbamoyl phosphate slightly, indicating that, after the induction of the conformational changes in the enzyme by acetyl-CoA and Mg^{2+} that decrease the affinity for carbamoyl phosphate, further addition of Mg^{2+} may then increase this affinity.

Apart from its interaction with acetyl-CoA stimulation of catalytic activity, Mg^{2+} is known to have two major effects in the pyruvate-carboxylation reaction. The binding of MgATP and Mg^{2+} have co-operative effects on each other's Michaelis constant (Barden & Scrutton, 1974) and the dissociation constant of the enzyme- Mg^{2+} complex is 6 mM, which is about the same order of magnitude as that seen in the current experiments. Mg^{2+} has also been shown to enhance the actual binding of nucleoside triphosphate with a K_a of about 110 μM (Attwood *et al.*, 1984a), and hence this effect would not be observed in the current experiments, where the minimum free $[\text{Mg}^{2+}]$ was greater than 3 mM. The other major effect on the pyruvate carboxylation reaction is inhibitory in that Mg^{2+} increases the affinity of carboxybiotin for the site of reaction (1) (Attwood *et al.*, 1984b), and in fact this is stimulatory in the reverse reaction (Attwood & Cleland, 1986). It is possible that this action of Mg^{2+} may apply not just to carboxybiotin but also to biotin, in which case part of the stimulatory action of Mg^{2+} on ADP phosphorylation by carbamoyl phosphate may be due to a requirement that biotin be in the part of the active site where reaction (1) occurs. This point is considered below.

Table 1. Kinetic parameters of the phosphorylation of ADP by carbamoyl phosphate catalysed by pyruvate carboxylase with carbamoyl phosphate as the substrate whose concentration was varied

Values in parentheses are standard errors of the estimates of the kinetic parameters derived from the least-squares fits of eqn. (4a) (V and K) and eqn. (4) (V/K and K) to the data.

Reaction conditions	$10^2 \times V$ ($\mu\text{mol}/\text{min}$)	$10^3 \times V/K$ ($\mu\text{mol}/\text{min}$ per mM)	K (mM)
0.25 mM-Acetyl-CoA, 5 mM- MgCl_2	2.48(± 0.04)	4.44(± 0.12)	5.6(± 0.2)
0.25 mM-Acetyl-CoA, 20 mM- MgCl_2	3.00(± 0.23)	7.52(± 1.08)	4.0(± 0.8)
0 mM-Acetyl-CoA, 5 mM- MgCl_2	0.17(± 0.07)	0.56(± 0.04)	3.1(± 0.3)
0 mM-Acetyl-CoA, 20 mM- MgCl_2	0.57(± 0.02)	0.88(± 0.04)	6.5(± 0.5)

Table 2. Effects of preincubation of pyruvate carboxylase with a 10-fold excess of avidin on the phosphorylation of ADP by carbamoyl phosphate

Reaction conditions (preincubation conditions)	$10^2 \times$ Reaction velocity ($\mu\text{mol/min}$)	Velocity (% of that in the absence of avidin)
0.25 mM-Acetyl-CoA (-avidin)	3.66	100
0.25 mM-Acetyl-CoA (+avidin + free biotin)	3.56	97
0.25 mM-Acetyl-CoA (+avidin)	0.085	2
0 mM-Acetyl-CoA (-avidin)	0.151	100
0 mM-Acetyl-CoA (+avidin-free biotin)	0.150	99
0 mM-Acetyl-CoA (+avidin)	0.085	56

Biotin-dependence of the ADP-phosphorylation reaction

Table 2 illustrates the effect of preincubation of pyruvate carboxylase with an excess of avidin on ADP phosphorylation by carbamoyl phosphate. In both the presence and the absence of acetyl-CoA, the velocity of the reaction was decreased to the same level by incubation with avidin. In the samples where avidin had been preincubated with free biotin the pyruvate carboxylase activity was essentially unaffected, and this indicated that in the other samples with avidin present the inhibition is caused by the binding of the avidin to the biotin moiety of pyruvate carboxylase. One explanation of these results is that, although not essential for the transfer of phosphate between carbamoyl phosphate and ADP, the presence of biotin does stimulate the reaction. Alternatively, the decrease in velocity in the presence of avidin may indicate that when this fairly large protein (68 000 M_r) binds to pyruvate carboxylase it may restrict access of substrates to the active site or may induce a distortion of the active site so as to inhibit its catalytic properties.

In order to try and define more clearly whether biotin has a role in ADP phosphorylation, it was decided to examine the effect of oxamate on the reaction. Fig. 2 shows the effect of increasing concentrations of oxamate on ADP phosphorylation by carbamoyl phosphate. As can be seen, oxamate was inhibitory in a non-competitive fashion; however, the re-plots of the intercepts and slopes of the Lineweaver-Burk plots were non-linear (see Figs. 2b and 2c), with the effect of oxamate concentration on these two parameters lessening at high oxamate concentrations. This indicates hyperbolic non-competitive inhibition of this type shown in Scheme 1, in which saturation of the enzyme with oxamate does not result in the complete loss of enzymic activity. The derivation of the rate equation and the equations relating slopes and intercepts of Lineweaver-Burk plots to oxamate concentration are given in the Appendix. As can be seen from Figs. 2(b) and 2(c), the continuous lines that result from fits of eqns. (5) and (6) to the intercept and slope data respectively indicate that Scheme 1 is an adequate model. These fits gave estimates of the values of K_i and K_i' the standard errors of which overlapped, and hence K_i and K_i' cannot be regarded as having significantly different values.

Oxamate is an inhibitor analogue of pyruvate and has been shown to act as a signal for the movement of biotin from the site of reaction (1) to that of reaction (2) (Goodall *et al.*, 1981; Attwood & Cleland, 1986); therefore the inhibitory effect of oxamate on the ADP-phosphorylation reaction is likely to be

Fig. 2. (a) Lineweaver-Burk plots showing the effects of increasing oxamate concentrations on the kinetics of the ADP-phosphorylation reaction catalysed by pyruvate carboxylase with carbamoyl phosphate as the substrate whose concentration was varied, and plots of (b) intercepts and (c) slopes versus oxamate concentration

The reactions were performed as described in the Materials and methods section with the following concentrations of oxamate: $\circ$, 0 mM; $\blacktriangle$, 1.82 mM; $\square$, 4.55 mM; $\bullet$, 9.09 mM; $\triangle$, 18.18 mM. The continuous lines were derived from least-squares-regression fits of eqns. (4a) and (4b) to each set of data at a particular concentration of oxamate. The values of intercepts ($\bullet$) and slopes ($\blacktriangle$) shown in (b) and (c) were also derived from these fits. The continuous lines in (b) and (c) are least-squares-regression fits of eqns. (5) and (6) to the intercept and slope data respectively, as described in the Materials and methods section.

Scheme 1. Proposed model of the inhibition by oxamate (Ox) of the phosphorylation of ADP by carbamoyl phosphate (S) catalysed by pyruvate carboxylase (E)

The derivation of the rate equation and the equations relating slopes and intercepts of Lineweaver-Burk plots to oxamate concentration are given in the Appendix.

caused by the movement of biotin away from the site of reaction (1). Biotin is not thought to be directly involved in the transfer of phosphate between ADP and carbamoyl phosphate or carboxyphosphate (Climent & Rubio, 1986), and thus the positioning of biotin at the site of reaction (1) must contribute to the

Fig. 3. pH profiles of the V (○) and V/K (△) of the phosphorylation of ADP from carbamoyl phosphate, catalysed by pyruvate carboxylase, with carbamoyl phosphate as the substrate whose concentration was varied

The continuous lines are least-squares-regression fits of eqns. (7) and (8) to the V and V/K data respectively. The pK values with standard errors derived from these fits are: V profile, $pK_1 = 7.6 (\pm 0.2)$, $pK_2 = 9.5 (\pm 0.4)$; V/K profile, $pK_3 = 7.2 (\pm 0.1)$, where the values in parenthesis are the upper bounds of the error on the pK value using the standard error (S.E.) of the estimate of K_n obtained from the least-squares-regression analyses, i.e. $\text{error} = \log[1/(K_n - \text{S.E.})] - pK_n$.

conformational arrangement of that part of the active site for the most efficient catalysis. The finding that saturation of the enzyme with oxamate does not completely inhibit the ADP-phosphorylating activity suggests that the presence of biotin at the site reaction (1) stimulates ADP phosphorylation but is not essential for the reaction to proceed. This suggestion is based on the assumption that saturation of the enzyme with oxamate results in the biotin being bound to the site of reaction (2) in all enzyme molecules. Biotin has been shown not to be essential in the transfer of phosphate between ATP and HCO_3^- , as demonstrated in biotin carboxylase from *E. coli* by Climent & Rubio (1986).

pH-dependence of the ADP-phosphorylation reaction

Fig. 3 shows the effects of pH on V and V/K where carbamoyl phosphate was the substrate whose concentration was varied. Both profiles have a slope of 1 at pH values below 7, which may be due to the protonation of an ionizable residue that is involved in a step in the reaction that affects both V and V/K . At pH values above 8.5, however, only the V profile indicates the presence of an ionizable residue. The protonation state of this residue must affect a step in the reaction outside those between the binding of carbamoyl phosphate and the dissociation of the first product, since these steps are also covered by V/K (Cleland, 1977). In the full reverse reaction of pyruvate carboxylase, Attwood & Cleland (1986) found that the V profile exhibited two pK values on the acid side and one on the alkaline side, whereas V/K exhibited only one pK value on either side. The pK values observed by Attwood & Cleland (1986) in the V and V/K profiles above pH 7.5 were 8.3 and 7.9 respectively, whereas in the V profile for the ADP-phosphorylation reaction pK_2 was 9.5. This suggests that the step in the reaction affected by the protonation state of the ionizable residue represented by pK_2 is not involved in the normal reverse reaction of pyruvate carboxylase. It is not involved in the binding of carbamoyl phosphate to the enzyme since at V this substrate is saturating. However, the release of carbamate from the active site is a step that does not occur in the full reverse reaction, and if this step were subsequent to the release of MgADP V/K would not be affected. Having thus tentatively assigned a role for the ionizable residue represented by pK_2 , this leaves no pK values apparent in the ADP-phos-

phorylation profile that correspond to the pK values observed by Attwood & Cleland (1986) in the V and V/K profiles of the full reverse reaction above pH 7.5. This lends support to the suggestion by Attwood & Cleland (1986) that these pK values represent an ionizable residue that participates in the enolization of biotin and that this step does not occur as part of ADP phosphorylation by carbamoyl phosphate.

pK_1 and pK_3 may either represent two ionizable residues that have roles in steps in the reaction that are not mutually covered by V and V/K , or represent a single ionizable residue that is required to be in the deprotonated state to participate in a step between the binding of carbamoyl phosphate and the dissociation of the first product. This uncertainty of assignment of pK_1 and pK_2 to ionizable residues involved in particular steps in the phosphorylation of ADP by carbamoyl phosphate, and the differences between these actual pK values and the corresponding pK values, obtained by Attwood & Cleland (1986) for the full reverse reaction, make comparisons between these parts of the two sets of results extremely difficult. However, Attwood & Cleland (1986) found there to be a second pK on the acid side of the V profile of the full reverse reaction, and there is no corresponding pK in either the V profile or the V/K profile of ADP phosphorylation by carbamoyl phosphate. This lends support to the assignment of this pK by Attwood & Cleland (1986) to an ionizable residue involved in the movement of biotin between the sites of reactions (1) and (2), since this step does not occur as part of the ADP phosphorylation by carbamoyl phosphate.

Conclusions

In this paper we have examined the effects of the activators, Mg^{2+} and acetyl-CoA, and the presence of biotin on the phosphorylation of ADP from carbamoyl phosphate catalysed by pyruvate carboxylase. Since this reaction is analogous to the transfer of phosphate between carboxyphosphate and ADP that is thought to occur in the normal reactions catalysed by pyruvate carboxylase, this has allowed the examination of the roles played by the effectors in this part of the overall reaction. We have shown that, although acetyl-CoA and the presence of biotin are not essential for the first half of reaction (3) to occur, both stimulate the reaction, as does Mg^{2+} . Thus at least part of the stimulatory actions of Mg^{2+} and acetyl-CoA on the pyruvate-carboxylation reaction may stem from their effects on carboxyphosphate formation.

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APPENDIX

Scheme 1 in the main paper is the proposed model of the inhibition of the phosphorylation of ADP by carbamoyl phosphate. In this model the enzyme–oxamate complex (EOx) is still capable of catalysing the reaction but at a lower rate than the free enzyme (E). In order to derive a rate equation for Scheme 1, both oxamate (Ox) and carbamoyl phosphate (S) are assumed to be in rapid equilibrium with the enzyme:

$$[E] = \frac{K[ES]}{[S]}; \quad [EOxS] = \frac{[ES][Ox]}{K_1'}; \quad [EOx] = \frac{K[ES][Ox]}{K_1[S]}$$

$$[E]_{\text{total}} = [E] + [ES] + [EOx] + [EOxS]$$

$$= [ES]\{1 + [Ox]/K_1' + K/[S](1 + [Ox]/K_1)\}$$

The velocity of the reaction is given by the following equation:

$$v = d[P]/dt = k_1[ES] + k_2[EOxS] \quad (A1)$$

Substituting for [EOxS] and [ES] in eqn. (A1):

$$v = \frac{[E]_{\text{total}}(k_1 + k_2[Ox]/K_1')}{\{1 + [Ox]/K_1' + K/[S](1 + [Ox]/K_1)\}}$$

$$= \frac{V_1 + V_2[Ox]/K_1'}{\{1 + [Ox]/K_1' + K/[S](1 + [Ox]/K_1)\}}$$

Thus in the Lineweaver–Burk plot the slope and intercept are given by the following equations:

$$\text{Slope} = \frac{K(1 + [Ox]/K_1)}{(V_1 + V_2[Ox]/K_1')}$$

$$\text{Intercept} = \frac{(1 + [Ox]/K_1')}{(V_1 + V_2[Ox]/K_1')}$$

where V_1 is the maximum velocity in the absence of oxamate and V_2 is the maximum velocity in the presence of saturating oxamate.

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