Bicarbonate-dependent ATP cleavage catalysed by pyruvate carboxylase in the absence of pyruvate

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Preparations of pyruvate carboxylase catalyse the cleavage of MgATP in the absence of pyruvate and acetyl-CoA. The rate of this cleavage is higher in the presence of HCO_3^- than in its absence. Incubation of the enzyme preparations with an excess of the pyruvate carboxylase inhibitor, avidin, completely abolishes the pyruvate carboxylating activity of the enzyme preparations but only abolishes the HCO_3^- -dependent MgATP cleaving activity, with no effect on the HCO_3^- -independent ATPase activity. The HCO_3^- -dependent MgATP cleavage is also sensitive to inhibition by a pyruvate carboxylase inhibitor, oxamate, and the dependence of the reaction on the free Mg^{2+} concentration is similar to that of the pyruvate-carboxylation reaction, whereas the HCO_3^- -independent MgATP cleavage is not dependent on the concentration of free Mg^{2+} in the range tested. This indicates that MgATP cleavage by pyruvate carboxylase is entirely dependent on the presence of HCO_3^- and that there may be a low level of ATPase contamination in the enzyme preparations. In addition, inhibition of the HCO_3^- -dependent MgATP cleavage by both avidin and oxamate indicate that although biotin does not directly participate in the reaction, its presence is required in that part of the active site of the enzyme. The rate of HCO_3^- -dependent MgATP cleavage is about 0.07% of that of the full pyruvate carboxylation reaction under similar conditions with saturating substrates. The reaction mechanism is sequential with respect to MgATP and HCO_3^- addition and Mg^{2+} adds at equilibrium before MgATP. Acetyl-CoA stimulates the HCO_3^- -dependent MgATP cleavage at low MgATP concentrations, with the stimulation being greater at low Mg^{2+} concentrations. At high levels of MgATP in the presence of acetyl-CoA, substrate inhibition is evident and is more pronounced at increasing concentrations of Mg^{2+} . This inhibition appears to be, at least in part, caused by inhibition of decarboxylation of the enzyme-carboxybiotin complex by the binding to this complex of Mg^{2+} and MgATP, which probably act to reduce the rate of movement of carboxybiotin from the site of the MgATP cleavage reaction to that of the pyruvate carboxylation reaction where it is unstable and decarboxylates.

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). In their studies on propionyl-CoA carboxylase

(Wallace *et al.*, 1985; Climent & Rubio, 1986); thus HCO_3^- is thought to act as a nucleophile removing the γ -phosphate group from MgATP to form carboxyphosphate. From this activated form of HCO_3^- , the carboxy group is transferred to biotin, either directly or via the formation of CO_2 :

using $\text{HC}^{18}\text{O}_3^-$, Kazirot *et al.* (1962) found that one of the oxygen atoms from the $\text{HC}^{18}\text{O}_3^-$ was incorporated into P_i released from MgATP, indicating a direct interaction between the γ -phosphate from MgATP and HCO_3^- . Catalysis of the phosphorylation of MgADP with carbamoyl phosphate by biotin carboxylase from *Escherichia coli* (Polakis *et al.*, 1972) and by pyruvate carboxylase (Ashman & Keech, 1975) suggested that carboxyphosphate is an intermediate in reaction (1), since carbamoyl phosphate is an analogue of carboxyphosphate. Further evidence that reaction (1) occurs via a carboxyphosphate intermediate has accumulated

Wallace *et al.* (1985) were able to isolate an enzyme complex that was capable of both phosphorylating MgADP and carboxylating pyruvate. This enzyme complex 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. 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. This, however, does not exclude the possibility that acetyl-CoA also plays a role in the formation of the putative carboxyphosphate intermediate. In addition, Mg^{2+} may exert its

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activating effect on the pyruvate carboxylation reaction by stimulating the formation of carboxyphosphate. If the reaction does proceed via a carboxyphosphate intermediate, then biotin is not directly involved in the cleavage of MgATP, but its presence in the enzyme subsite where reaction (1) occurs may still have an effect.

If reaction (1) does proceed via a carboxyphosphate intermediate, then the cleavage of MgATP should be absolutely dependent on the presence of HCO_3^- . Scrutton & Utter (1965) found that the ATP:ADP isotope exchange reaction catalysed by chicken liver pyruvate carboxylase was not dependent on added HCO_3^- , however, they did not account for the relatively high levels of endogenous HCO_3^- present in the reaction solutions (approx. 2 mM at pH 8.4). McClure *et al.* (1971a), however, found that the rate of the ATP:ADP isotope exchange reaction in rat liver pyruvate carboxylase was dependent on HCO_3^- concentration in the range 0.3–1.0 mM. To date, the total dependency of MgATP cleavage on the presence of HCO_3^- has not been demonstrated in biotin-dependent carboxylases.

Attwood & Graneri (1991) were able to study the reverse of the first part of reaction (3) indirectly using carbamoyl phosphate, an analogue of carboxyphosphate, as a substrate for the phosphorylation of MgADP. These authors showed that although the presence of both acetyl-CoA and biotin at the enzyme subsite, where reaction (3) occurs, stimulate the phosphorylation of MgADP by carbamoyl phosphate, neither are absolutely required. However, to actually study the first part of reaction (3) as it occurs in the overall pyruvate carboxylation reaction, it must be determined whether pyruvate carboxylase is capable of catalysing the cleavage of MgATP at a measurable rate in the absence of pyruvate and acetyl-CoA. If this is possible, then the effects of acetyl-CoA, Mg^{2+} and biotin on the first part of reaction (3) and the dependence of MgATP cleavage on the presence of HCO_3^- can be studied in isolation from the rest of reaction (3) and separate from reaction (2).

MATERIALS AND METHODS

Materials

Chicken liver pyruvate carboxylase was prepared to an average specific activity of 30 units/mg of protein as described by Goss *et al.* (1979), except that a DEAE-Sephacrose column was used in place of the DEAE-Sephadex column (1 unit of enzymic activity is defined as the amount of enzyme required to catalyse the formation of 1 μmol of oxaloacetate/min under saturating substrate conditions at 30 °C). The purity of the pyruvate carboxylase was estimated to be between 77% and 88%. These estimates were obtained by densitometric analysis of Coomassie Blue-stained SDS/polyacrylamide gels of pyruvate carboxylase preparations.

An aqueous solution of $\text{NaH}^{14}\text{CO}_3$ was obtained at a specific radioactivity of 55 mCi/mmol from Amersham Australia Pty. Ltd., while $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained as an aqueous solution of the triethylammonium salt at a specific radioactivity of approx. 3000 Ci/mmol. Avidin 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 as 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 7.8. This buffer system was used in all of the experiments described in this paper.

Spectrophotometric MgATP-cleavage assays

In the experiments where the MgATP concentration was varied at different, fixed concentrations of HCO_3^- , buffer solution was prepared fresh daily, containing its components at concentrations 1.053 times their final assay concentrations and MgCl_2 at 1.053 times its final assay concentration. The pH of this solution was then adjusted to between 3.5 and 4.5 with HCl and sparged with N_2 for 3–4 h, after this time the pH was re-adjusted back to 7.8 using saturated NaOH. The reaction was monitored by measuring the formation of MgADP by the action of pyruvate kinase which resulted in the formation of pyruvate which was then reduced to lactate by lactate dehydrogenase with the concomitant oxidation of NADH, this was measured by monitoring the decrease in absorbance at 340 nm. Hence, to the stock buffer solution was then added solid NADH, phosphoenolpyruvate, lactate dehydrogenase and pyruvate kinase in a volume of solution that was 0.37% of the total volume of the stock buffer. During the pH adjustment and subsequent storage, this stock buffer solution was kept under a stream of N_2 . Equimolar solutions of ATP and MgCl_2 were prepared in water that had been freshly boiled and allowed to cool under a stream of N_2 . The pH of the ATP solution was adjusted to 7.8 with saturated NaOH. The concentration of the ATP solutions were determined by end-point assay with hexokinase and glucose-6-phosphate dehydrogenase. When an assay was to be performed, 950 μl of the stock buffer solution was taken in a pipette and placed in a cuvette in the spectrophotometer, the sample compartment of which was continually flushed with N_2 . Equal volumes of ATP and MgCl_2 were added and the volume of the assay mix was made up to 990 μl with boiled water. Since there is interdependence between the concentration of free Mg^{2+} and the concentration of MgATP in the assay mix, the free Mg^{2+} concentration was taken to be the total concentration of Mg^{2+} minus that of ATP. Calculations of the actual free Mg^{2+} concentration using a dissociation constant for MgATP of 0.0143 mM (Morrison, 1979) showed that the assumed value of free Mg^{2+} was never less than 99.1% of the actual value. After a few minutes, 10 μl of chicken liver pyruvate carboxylase (approx. 8 units of pyruvate carboxylating activity) was added. Usually there was a brief burst of MgADP formation, probably due to the small amount of HCO_3^- present in the solutions of lactate dehydrogenase, pyruvate kinase and pyruvate carboxylase. A linear rate was then established, and this was the part of the reaction used to determine the velocity of the HCO_3^- -independent reaction. The final levels of the components of the reaction mixture were: 1.25–10 mM- Mg^{2+} , 0.05–1.00 mM-Mg-ATP, 0.1 mM-NADH, 0.5 mM-phosphoenolpyruvate, 3 units of pyruvate kinase and 5 units of lactate dehydrogenase, and the reactions were performed at 25 °C.

Solid NaHCO_3 was then added to the stock buffer solution to give final assay concentrations of 1.25–40 mM and the experiments in the absence of HCO_3^- were then repeated. To obtain rates of HCO_3^- -dependent MgATP cleavage, the rates of reaction in the absence of HCO_3^- at each MgATP concentration were subtracted from the corresponding rates in the presence of HCO_3^- .

The experiments where the concentration of MgATP was varied at different fixed free Mg^{2+} concentrations were performed in a similar way to that described above, except that the concentration of NaHCO_3 added to the stock buffer solution was fixed at 150 mM and the pH was then re-adjusted. Checks were made that 150 mM- Na^+ did not affect the HCO_3^- -independent reaction by including 150 mM-NaCl in the reaction mix. The effects of acetyl-CoA on HCO_3^- -dependent MgATP cleavage were studied in experiments similar to those described above

except for the inclusion of 0.25 mM-acetyl-CoA, and the range of MgATP concentrations was extended to 0.025–2 mM (in these experiments the assumed concentration of free Mg^{2+} was never less than 98.6% of the actual value).

Effect of avidin on MgATP cleavage

The biotin content of a sample of pyruvate carboxylase was estimated from the protein concentration and molecular mass of the enzyme subunit. Half of the enzyme was then incubated for 2 h at 25 °C in a solution of buffer containing avidin, such that the concentration of biotin-binding sites was 10 times the concentration of biotin in the enzyme. The other half of the enzyme was incubated under identical conditions, except that avidin was omitted. After the incubation period, the samples were assayed for biotin carboxylating activity. The MgATP-cleaving activity of both samples was then determined in the presence and absence of 20 mM- HCO_3^- , with 2 mM-MgATP and 5 mM- Mg^{2+} .

Effect of oxamate on MgATP cleavage

Since oxamate interferes with spectrophotometric assay of MgADP formation, in these experiments the cleavage of MgATP was measured by determining the release of [^{32}P]P_i from [γ - ^{32}P]ATP. The experiments were performed in a similar way to the spectrophotometric assays except that the reactions were performed in tubes in a lidded water bath at 25 °C which was constantly flushed with N₂. The reaction mixture contained the buffer mixture, 5 mM- Mg^{2+} , 0.05 mM or 2 mM-MgATP and 20 μCi of [γ - ^{32}P]ATP, in the presence or absence of 150 mM- NaHCO_3 and in the presence or absence of 20 mM-sodium oxamate. The reactions were started by the addition of 10 μl of pyruvate carboxylase (approx. 8 units of pyruvate carboxylating activity) and at 1 min intervals over a period of 6 min aliquots (50 μl) of the reaction mixture were spotted on to 2 cm squares of Whatman 31 ET Chr paper that had been pretreated with 50 μl of a solution comprising 5.7% (w/v) trichloroacetic acid, 2.9% (w/v) $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ and 29 mM-triethylamine/HCl. These paper squares containing the precipitated [^{32}P]P_i were washed to remove [γ - ^{32}P]ATP and were then prepared for scintillation counting as described by Reimann & Umfleet (1978). In addition, 10 μl aliquots of the reaction mixture were placed in 5 ml of toluene scintillation fluid containing Triton X-100 to determine the total radioactivity present, and hence the specific radioactivity of the [γ - ^{32}P]ATP in the reaction mixture. The radioactivity present on each paper square of a particular time course was plotted against time, and the rate of increase in radioactivity was determined by linear least-squares regression (in all cases the rate of increase in radioactivity was linear over the 6 min assay period). From the known specific radioactivity of the [γ - ^{32}P]ATP in the reaction mixture, the rate of increase in radioactivity on the paper squares was converted into a rate of cleavage of ATP expressed in $\mu\text{mol}/\text{min}$.

Effects of Mg^{2+} and ATP on the stability of the enzyme-carboxybiotin complex

The enzyme-[^{14}C]carboxybiotin complex was prepared from $\text{NaH}^{14}\text{CO}_3$ and isolated essentially as described by Attwood *et al.* (1984b). It was then divided into three equal portions and various additions were made to each, so that all portions contained 100 mM- NaHCO_3 . Two portions also contained 10 mM- MgCl_2 and one of these portions also contained 2 mM-ATP. The [^{14}C]carboxy group is slowly released from the enzyme-[^{14}C]carboxybiotin complex to form $\text{H}^{14}\text{CO}_3^-$ and the stability of the complex at 10 °C in each of the three portions was measured essentially as described by Attwood & Wallace (1986).

Briefly, the ^{14}C -labelled carboxy group of the enzyme-[^{14}C]carboxybiotin complex in 50 μl aliquots taken at various times from the main solutions of the complex, was transferred to pyruvate to form [^{14}C]oxaloacetate which was stabilized by the addition of semicarbazide/HCl. This solution was then spotted on to Whatman 3MM paper squares, which were dried and placed in scintillation fluid for counting. After 165 min, the radioactivity in aliquots from the portion of the enzyme-[^{14}C]carboxybiotin complex containing only 100 mM- NaHCO_3 did not decrease further, indicating the presence of a small amount of non-labile ^{14}C and the corresponding c.p.m. (799) were subtracted from all the c.p.m. values of all samples. The radioactivity remaining in the enzyme-[^{14}C]carboxybiotin complex at each time point was expressed as a percentage of that present at zero time.

Data analysis

All kinetic data were fitted using a least-squares program written by Duggleby (1981). Kinetic constants and nomenclature are essentially as suggested by Cleland (1979). Both eqn. (4), which describes a pattern of lines in the Lineweaver-Burk plot that intersect off the ordinate (sequential mechanism), and eqn. (5), which describes a pattern of lines that intersect on the ordinate (equilibrium-ordered mechanism), were fitted to the results from the experiments where the MgATP concentration was varied, either at different, fixed HCO_3^- or free Mg^{2+} concentrations. The best fit was deemed to be the equation that gave the lowest sum of the squares of the residuals. Eqn. (6), which describes substrate inhibition, was fitted to each set of data at a particular free Mg^{2+} concentration. Eqn. (7) was fitted to the data describing the decay of the enzyme-[^{14}C]carboxybiotin complex.

$$v = \frac{VAB}{K_a B + K_b A + AB + K_{ia} K_b} \quad (4)$$

$$v = \frac{VAB}{K_b A + AB + K_{ia} K_b} \quad (5)$$

$$v = \frac{VA}{K_a + A + A^2/K_i} \quad (6)$$

$$\% \text{E}^{14}\text{CO}_2^- = 100e^{-k_{\text{obs}} t} \quad (7)$$

In eqn. (7), % $\text{E}^{14}\text{CO}_2^-$ is the % of the enzyme-[^{14}C]carboxybiotin complex remaining at time t and k_{obs} is the measured pseudo-first-order rate constant.

RESULTS AND DISCUSSION

Cleavage of MgATP by pyruvate carboxylase preparations and its dependence on HCO_3^- , Mg^{2+} and biotin

The preparations of pyruvate carboxylase studied were found to catalyse both HCO_3^- -independent and HCO_3^- -dependent MgATP cleavage. The rate of HCO_3^- -independent cleavage of MgATP varied from one-fifth to twice that of the HCO_3^- -dependent rate, depending on the concentrations of MgATP, HCO_3^- and free Mg^{2+} . The rate of HCO_3^- -independent MgATP cleavage at a particular MgATP level was not dependent on free Mg^{2+} concentration in the range 1–20 mM, whereas the HCO_3^- -dependent rates showed very marked dependencies on free Mg^{2+} concentration (see section below on effects of free Mg^{2+} concentration on HCO_3^- -dependent rates). The velocity of HCO_3^- -dependent MgATP cleavage at saturating concentrations of HCO_3^- and MgATP was found to be 0.07% of the rate of

pyruvate carboxylation under similar conditions with saturating substrates and activators.

In the absence of avidin, the rate of MgATP cleavage ($-\text{HCO}_3^-$) was 2.86 nmol/min and in the presence of 20 mM- HCO_3^- the rate was 7.54 nmol/min. In the presence of avidin, however, the rate of MgATP cleavage ($-\text{HCO}_3^-$) was 2.83 nmol/min, while with 20 mM- HCO_3^- the rate was 2.86 nmol/min. This shows that incubation with avidin completely abolished the HCO_3^- -dependent rate of MgATP cleavage, but did not affect the HCO_3^- -independent rate. Since the action of avidin is to bind to biotin, this suggests that the HCO_3^- -dependent cleavage of MgATP was caused by the biotin-containing pyruvate carboxylase, whereas the HCO_3^- -independent cleavage of MgATP was caused by the presence of non-biotin-dependent, ATPase. As avidin is a fairly large protein (M_r -68000) it is possible that on binding to pyruvate carboxylase, it restricts the access of substrates to the active site, or induces a distortion in the active site that reduces its catalytic properties. On the other hand, biotin may be required at the part of the active site of pyruvate carboxylase where reaction (1) occurs in order for catalysis of MgATP cleavage to take place. This possibility was tested by examining the effects of oxamate on the reaction. The result of the experiment was that oxamate inhibited HCO_3^- -dependent MgATP cleavage such that in the presence of 20 mM-oxamate the rate was only 2% of that in its absence. Oxamate is an inhibitor analogue of pyruvate and its binding to the enzyme 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). There is little likelihood that oxamate could act as a carboxyphosphate analogue inhibitor because of the structural dissimilarity between the two compounds. In addition, Attwood & Graneri (1991) found that oxamate was a non-competitive inhibitor with respect to the carboxyphosphate analogue carbamoyl phosphate, and thus oxamate does not bind at the same site on the enzyme as carboxyphosphate. Therefore the inhibitory effect of oxamate on HCO_3^- -dependent MgATP cleavage is very likely to be caused by the removal of biotin from the site of reaction (1). Since biotin is not thought to participate directly in the cleavage of MgATP (Climent & Rubio, 1986), its requirement at the site of reaction (1) may be necessary to complete the conformational arrangement of that part of the active site in order for HCO_3^- -dependent MgATP cleavage to occur. Similar effects of avidin and oxamate were found on the phosphorylation of ADP by carbamoyl phosphate catalysed by pyruvate carboxylase (Attwood & Graneri, 1991); however, the presence of avidin only reduced the rate of this reaction to 56% of that in its absence. In addition, oxamate exhibited hyperbolic non-competitive inhibition, with maximum inhibition of $V_{\max}$ being 69%. Attwood & Graneri (1991) concluded that although the presence of biotin at the site of reaction (1) stimulated the rate of phosphorylation of ADP by carbamoyl phosphate, this was not essential for the reaction to proceed. From the results of Attwood & Graneri (1991), it is calculated that 20 mM-oxamate would produce 92% of maximal inhibition of ADP phosphorylation, and this compares reasonably well with the 98% inhibition of HCO_3^- -dependent MgATP cleavage observed with 20 mM-oxamate. This indicates that saturation with oxamate would produce 100% inhibition of HCO_3^- -dependent MgATP cleavage and hence suggests that the presence of biotin at the site of reaction (1) is essential for MgATP cleavage. Although the results of Attwood & Graneri (1991) suggest that biotin is not essential for the reverse reaction, i.e. the carboxylation of MgADP by carboxyphosphate, one must be cautious in drawing such conclusions when using substrate analogues, since in these experiments the reaction may not proceed in exactly the same way as with the actual substrate.

Fig. 1. Kinetics of HCO_3^- -dependent cleavage of MgATP with MgATP as the varied substrate at different, fixed concentrations of HCO_3^- in the presence of 10 mM free Mg^{2+} at 25 °C, pH 7.8

Conditions were as described in the Materials and methods section. The concentrations of HCO_3^- used were: ○, 2.5 mM; ▲, 5 mM; □, 10 mM; ●, 20 mM; and △, 40 mM. The lines were derived from a least-squares fit of eqn. (4) to the non-reciprocal form of the data.

Table 1. Apparent kinetic constants for MgATP and HCO_3^- at 2 mM and 10 mM free Mg^{2+} for HCO_3^- -dependent MgATP cleavage

Values in parentheses are standard errors of the estimates of the kinetic parameters obtained by a least-squares fit of eqn. (4) to the data in Fig. 1 (10 mM free Mg^{2+}) and to similar data at 2 mM free Mg^{2+} .

Free Mg^{2+} concn. (mM)	Kinetic constants (mM)			
	K_{MgATP}	K_{IMgATP}	$K_{\text{HCO}_3^-}$	$K_{\text{IHCO}_3^-}$
2	0.25 (±0.05)	0.36 (±0.23)	3.4 (±1.3)	5.0 (±2.6)
10	0.027 (±0.012)	0.42 (±0.18)	2.6 (±0.9)	42 (±24)

Kinetics of HCO_3^- -dependent MgATP cleavage and the effects of HCO_3^- and free Mg^{2+} concentrations

Fig. 1 shows the kinetics of HCO_3^- -dependent MgATP cleavage at a free Mg^{2+} concentration of 10 mM with MgATP as the varied substrate at different, fixed concentrations of HCO_3^- . The lines correspond to a fit of eqn. (4) to the data, which indicates that the reaction is sequential, with either ordered or rapid random equilibrium addition of MgATP and HCO_3^- . This pattern is similar to that seen in the overall pyruvate carboxylation reaction for MgATP and HCO_3^- , although there is some disagreement as to the mechanism of addition of MgATP and HCO_3^- . McClure *et al.* (1971b) and Barden *et al.* (1972) concluded that the addition of the two substrates was random in the rat and chicken liver enzymes, whereas Warren & Tipton (1974) concluded that for the pig liver enzyme, the addition was ordered, with MgATP adding first. Table 1 shows the apparent kinetic parameters calculated from the data in Fig. 1 and also from similar data at 2 mM free Mg^{2+} (results not shown). In going from 2 mM to 10 mM free Mg^{2+} , K_{MgATP} is greatly reduced, while $K_{\text{HCO}_3^-}$ is not changed to any great extent. The values of $K_{\text{HCO}_3^-}$ and K_{MgATP} are similar to the literature values obtained for these kinetic parameters in the overall, pyruvate carboxylation reaction (McClure *et al.*, 1971b; Barden *et al.*, 1972; Warren & Tipton, 1974).

Fig. 2 shows the kinetics of HCO_3^- -dependent MgATP cleavage where the concentration of MgATP was varied at different, fixed concentrations of free Mg^{2+} in the presence of saturating HCO_3^- . When eqns. (4) and (5) were fitted to the data, the best fit was given by eqn. (5). Analysis of individual sets of data at each free Mg^{2+} concentration gave a value for the intercept ($1/v$)

Fig. 2. Kinetics of HCO_3^- -dependent cleavage of MgATP with MgATP as the varied substrate at different, fixed concentrations of free Mg^{2+} in the presence of 150 mM- HCO_3^- at 25 °C, pH 7.8

Conditions were as described in the Materials and methods section. The concentrations of free Mg^{2+} were: ○, 1.5 mM; ▲, 2.0 mM; □, 3.0 mM; ●, 5.0 mM and △, 10.0 mM. The lines were derived from a least-squares fit of eqn. (5) to the non-reciprocal form of the data.

of the reciprocal plot of each data set, these intercepts did not increase with $1/[\text{free Mg}^{2+}]$ in the way that would be expected if eqn. (4) was correct. Thus, the addition of MgATP and Mg^{2+} appear to be equilibrium-ordered, with Mg^{2+} adding first, at equilibrium. This is in agreement with the way that Mg^{2+} and MgATP add to the enzyme in the overall pyruvate carboxylation reaction (McClure *et al.*, 1971b).

What then, is the role of Mg^{2+} in the cleavage of MgATP? Attwood *et al.* (1984a) showed that Mg^{2+} enhanced the binding of nucleoside triphosphate with a K_a of about 110 μM; however, in the present work the minimum free Mg^{2+} concentration used was 1 mM and thus this effect would not be observed. The other known effect of Mg^{2+} is that it enhances the binding of carboxybiotin to the site of reaction (1) (Attwood *et al.*, 1984b), and it may be that Mg^{2+} also has the same effect on biotin. Since from the experiments with avidin and oxamate, biotin appears to be required at the site of reaction (1) in order for HCO_3^- -dependent MgATP cleavage to occur, the enhancement of the binding of biotin to this site may account for the stimulatory effect of Mg^{2+} on the reaction. The effect of Mg^{2+} is similar to that seen on the phosphorylation of MgADP by carbamoyl phosphate (Attwood & Graneri, 1991) and the above explanation of its effect was suggested to apply to this reaction.

In general, the kinetics of HCO_3^- -dependent MgATP cleavage are in agreement with the kinetic patterns of MgATP, HCO_3^- and Mg^{2+} observed in the overall pyruvate carboxylation reaction. This indicates that MgATP cleavage in the absence of pyruvate occurs in a very similar way to that in the overall pyruvate carboxylation reaction. Mg^{2+} adds at equilibrium before MgATP and the fact that changing the free Mg^{2+} concentration from 2 mM to 10 mM changes the apparent values of K_{MgATP} and $K_{\text{HCO}_3^-}$, but not those of $K_{\text{HCO}_3^-}$ or K_{MgATP} suggests that the addition of HCO_3^- and MgATP is ordered, with HCO_3^- adding last.

Effects of acetyl-CoA on the HCO_3^- -dependent cleavage of MgATP

Fig. 3 shows the effect of 0.25 mM-acetyl-CoA on the kinetics of the HCO_3^- -dependent cleavage of MgATP at saturating HCO_3^- concentrations and three different concentrations of Mg^{2+} . Clearly, at high concentrations of MgATP, substrate inhibition was evident in all three curves and apparently became more pronounced as free Mg^{2+} concentrations increased. No such inhibition was seen in the HCO_3^- -independent MgATP cleavage. This is illustrated in Table 2, which shows the estimates of kinetic parameters obtained by fitting eqn. (6) to the data.

Fig. 3. Kinetics of HCO_3^- -dependent cleavage of MgATP with MgATP as the varied substrate at different, fixed concentrations of free Mg^{2+} in the presence of 100 mM- HCO_3^- and 0.25 mM-acetyl-CoA at 25 °C, pH 7.8

Conditions were as described in the Materials and methods section. The concentrations of free Mg^{2+} were: ○, 1 mM; □, 5 mM and △, 10 mM. The lines were derived from a least-squares fits of eqn. (6) to the non-reciprocal form of each set of data at a particular concentration of free Mg^{2+} .

Table 2. Apparent kinetic parameters for HCO_3^- -dependent MgATP cleavage in the presence of acetyl-CoA at different free Mg^{2+} concentrations

Values in parentheses are standard errors of the estimates of the kinetic parameters obtained by a least-squares fit of eqn. (6) to the data in Fig. 3.

Free Mg^{2+} concn. (mM)	$10^3 \times V_{\text{max}}$ (μmol/min)	K_{MgATP} (mM)	K_i (mM)
1	3.80 (±0.31)	0.013 (±0.005)	1.66 (±0.47)
5	2.95 (±0.21)	0.008 (±0.005)	1.05 (±0.23)
10	2.50 (±0.36)	0.012 (±0.007)	0.50 (±0.18)

Table 3. Comparison of velocities of HCO_3^- -dependent MgATP cleavage in the presence and absence of acetyl-CoA at low and saturating MgATP concentrations

Presence of 0.25 mM-acetyl-CoA	Free Mg^{2+} concn. (mM)	$10^3 \times V_{\text{max}}$ at 0.05 mM-MgATP (μmol/min)	$10^3 \times V_{\text{max}}$ (μmol/min)
—	1	0.14	5.71
+	1	2.80	3.80
—	5	1.33	5.71
+	5	2.20	2.30

With increasing free Mg^{2+} concentrations, the apparent K_i decreased, as did the apparent V_{max} . Table 3 shows comparisons between the velocities of MgATP cleavage under identical conditions in the presence or absence of acetyl-CoA at 1 or 5 mM free Mg^{2+} . This shows that while the apparent V_{max} (at infinite MgATP concentration) was higher in the absence of acetyl-CoA than that calculated in its presence at both 1 and 5 mM free Mg^{2+} , the presence of acetyl-CoA greatly enhanced the velocity of the reaction at low concentrations of MgATP. In addition, the enhancement of the velocity was greater at 1 mM free Mg^{2+} than at 5 mM free Mg^{2+} , this is similar to the effect of acetyl-CoA on the pyruvate carboxylase-catalysed phosphorylation of MgADP by carbamoyl phosphate (Attwood & Craneri, 1991). These authors suggested that, as in the overall pyruvate carboxylation reaction (Barden & Scrutton, 1974), acetyl-CoA lowers the apparent K_a for Mg^{2+} . This may well mean that acetyl-CoA

enhances the binding of Mg^{2+} to the enzyme and, as a result, the reaction is close to maximally stimulated at both 1 mM and 5 mM free Mg^{2+} in the presence of acetyl-CoA at 0.05 mM-MgATP (see Table 3). Whereas in the absence of acetyl-CoA, 1 mM- Mg^{2+} produces less than 10 % of the maximal stimulation and 5 mM- Mg^{2+} produces more than 40 % of the maximal stimulation. As discussed in the section above on the effects of free Mg^{2+} levels on the reaction, a possible mode of action for Mg^{2+} is to enhance the binding of biotin to the site of reaction (1) in the same way that it is known to enhance the binding of carboxybiotin to this site. Thus if acetyl-CoA enhances the binding of Mg^{2+} to the enzyme, this in turn enhances the binding of biotin to the site of reaction (1) and stimulates the reaction.

It should be noted, however, that as the free Mg^{2+} concentration increased there was inhibition of HCO_3^- -dependent MgATP cleavage at all concentrations of MgATP (see Fig. 3). According to the evidence of Wallace *et al.* (1985) the carboxy group from the putative carboxyphosphate intermediate is transferred, in the presence of acetyl-CoA, to biotin to form carboxybiotin. Attwood *et al.* (1984b) have shown that the effect of increasing the free Mg^{2+} concentration is to stabilize the enzyme-carboxybiotin complex by holding the carboxybiotin at the site of reaction (1). If the decarboxylation of carboxybiotin is rate limiting for HCO_3^- -dependent MgATP cleavage in the presence of acetyl-CoA, then the inhibitory effect on this reaction of increasing the free Mg^{2+} concentration is probably due to the stabilization of the enzyme-carboxybiotin complex. The question then arises as to the cause of the substrate inhibition seen at high concentrations of MgATP that is compounded by increasing the free Mg^{2+} concentration, as seen by the decrease in K_i that this produces (see Table 2). It is possible that under these reaction conditions MgATP can bind to the enzyme-carboxybiotin complex and further stabilize it. To test this hypothesis, the effect of MgATP on the stability of the enzyme-carboxybiotin complex was examined. The results of these experiments (see Fig. 4), show that 10 mM- Mg^{2+} reduced the pseudo-first-order rate constant for the decay of the enzyme- ^{14}C carboxybiotin complex to 43 % of that in the absence of added Mg^{2+} , while the presence of 2 mM-ATP and 10 mM- Mg^{2+} reduced the rate constant for decay

to 30 % of that in the absence of both ATP and Mg^{2+} . This effect of ATP in the presence of Mg^{2+} was not caused by the re-formation of enzyme- ^{14}C carboxybiotin complex, since any $\text{H}^{14}\text{CO}_3^-$ derived from the decay of the complex was completely swamped by the large concentration of $\text{H}^{14}\text{CO}_3^-$ (100 mM) present in the reaction mixture, and hence any re-formed complex was almost entirely enzyme- ^{14}C carboxybiotin which did not form radioactive oxalacetate on reaction with pyruvate. Thus it is likely that the substrate inhibition seen in the HCO_3^- -dependent cleavage of MgATP at high MgATP concentrations is, at least in part, due to stabilization of the enzyme-carboxybiotin complex by binding of MgATP, making the decarboxylation of this complex rate limiting in the catalytic cycle.

Summary and general discussion

Preparations of pyruvate carboxylase catalysed both HCO_3^- -dependent and HCO_3^- -independent cleavage of MgATP. The HCO_3^- -dependent cleavage was inhibited by avidin and oxamate, which are inhibitors of pyruvate carboxylation, whereas the HCO_3^- -independent reaction was not affected. The kinetics of HCO_3^- -dependent MgATP cleavage showed similar dependence on the concentrations of MgATP, HCO_3^- and free Mg^{2+} as the kinetics of the overall pyruvate carboxylation reaction. On the other hand, the HCO_3^- -independent cleavage of MgATP showed no dependence on free Mg^{2+} concentration in the range used, and showed no substrate inhibition at high concentrations of MgATP as was the case with the HCO_3^- -dependent reaction in the presence of acetyl-CoA. All of the evidence clearly indicates that pyruvate carboxylase is responsible for the HCO_3^- -dependent cleavage of MgATP, while the HCO_3^- -independent reaction is probably caused by a low level of contamination of the preparation with an ATPase. Apart from the differences in V_{max} values which are discussed below, the similarity between the kinetic parameters determined for the cleavage of MgATP in the absence of pyruvate compared with those determined in the overall pyruvate carboxylation reaction indicates that the same reaction occurs in each case. Thus the conclusions drawn from the present study of MgATP cleavage in the absence of pyruvate are also applicable to MgATP cleavage in the complete pyruvate carboxylation reaction.

The high sensitivity of the HCO_3^- -dependent cleavage of MgATP to inhibition by both avidin and oxamate suggests that there is an absolute requirement for the presence of biotin at the site of reaction (1) in order for MgATP cleavage to occur. This is somewhat unexpected in view of the commonly accepted mechanism of biotin carboxylation that initially involves the formation of a carboxyphosphate intermediate by direct reaction between MgATP and HCO_3^- [see reaction (3)]. This reaction does not directly involve biotin, and in biotin carboxylase Climent & Rubio (1986) showed that the presence of biotin was not essential for the HCO_3^- -dependent cleavage of MgATP. In his review on the mechanism of biotin-dependent enzymes, Knowles (1989) discussed the three proposed mechanisms for biotin carboxylation that account for the direct interaction of the HCO_3^- with γ -phosphate of MgATP. One of those was essentially that depicted in reaction (3) in which biotin plays no direct role in the cleavage of MgATP. In the other two mechanisms and *o*-phosphobiotin intermediate was proposed. The second of these mechanisms proposed that HCO_3^- attacks the phosphate of *o*-phosphobiotin to form the intermediate carboxyphosphate which then carboxylates biotin. Knowles (1989) ruled out this mechanism on the basis that it predicts that the reaction should proceed with retention of stereochemical configuration at the phosphorus atom, whereas in biotin carboxylase the reaction was found to proceed with inversion at phosphorus (Kluger *et*

Fig. 4. Decay of the enzyme- ^{14}C carboxybiotin complex at 10 °C, pH 7.8, in 0.1 M-N-ethylmorpholine/HCl buffer, with no further additions (○); with 10 mM- MgCl_2 (□) or with 10 mM- MgCl_2 + 2 mM-ATP (△)

Note that each symbol represents the average of three measurements from three separate experiments. The reactions were performed as described in the Materials and methods section and the amount of enzyme- ^{14}C carboxybiotin complex remaining at time t was expressed as a percentage of that present at $t = 0$ min. The lines were derived from least-squares fits of the eqn. (7) to each set of data, and the pseudo-first-order rate constants calculated from these fits were: $0.0425 (\pm 0.0011) \text{ min}^{-1}$ (no additions); $0.0186 (\pm 0.0008) \text{ min}^{-1}$ (10 mM- MgCl_2) and $0.0126 (\pm 0.0003) \text{ min}^{-1}$ (10 mM- MgCl_2 + 2 mM-ATP) (values in parentheses are standard errors of the estimates of the rate constants derived from the least-squares fitting program).

al., 1979). The last mechanism was based on that suggested by Kluger & Adawadkar (1976) in which the *o*-phosphobiotin undergoes a concerted reaction with HCO_3^- in which an oxygen from HCO_3^- is transferred to P_i and the biotin is carboxylated. This mechanism directly involves biotin in MgATP cleavage, but does not account for the kinetically competent enzyme complex isolated by Wallace *et al.* (1985) which contains an activated carboxy group that is not carboxybiotin. This leaves reaction (3) as being the best of the proposed mechanisms, and suggests that biotin is needed to bind at the site of reaction (1) so that that part of the active site of the enzyme is in the correct conformation for MgATP cleavage to occur, even though biotin itself does not chemically participate in the cleavage.

Assuming that reaction (3) is correct, in the absence of acetyl-CoA HCO_3^- -dependent MgATP cleavage results in the formation of the enzyme-carboxyphosphate complex which must then decompose, presumably with the formation of HCO_3^- and P_i , to complete the catalytic cycle [see reaction (8)]:

In the presence of acetyl-CoA, however, HCO_3^- -dependent cleavage of MgATP results in the formation of the enzyme-carboxybiotin complex and it is this species of the enzyme that must decarboxylate to complete the catalytic cycle [see reaction (9)]:

The results given in the present paper suggest that, in the presence of acetyl-CoA, the rate-limiting step in the catalytic cycle is the decarboxylation of this enzyme-carboxybiotin complex. What, then is the rate-limiting step in the absence of acetyl-CoA? In the absence of pyruvate, the steady-state cleavage of MgATP proceeds at a much lower V_{max} than that for the overall pyruvate carboxylation reaction. This indicates that the rate-limiting step in reaction (8) lies somewhere in the second part of the reaction, perhaps the dissociation of MgADP from the

enzyme. The rate-limiting step may, however, be the decomposition of the carboxyphosphate intermediate, which although estimated to be extremely labile in solution (Sauers *et al.*, 1975) may well be greatly stabilized in the active site of the enzyme, as indicated by the isolation of a putative enzyme-carboxybiotin complex by Wallace *et al.* (1985).

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REFERENCES

- Ashman, L. K. & Keech, D. B. (1975) *J. Biol. Chem.* **250**, 14–21
 Attwood, P. V. & Cleland, W. W. (1986) *Biochemistry* **25**, 8191–8196
 Attwood, P. V. & Graneri, B. D. L. A. (1991) *Biochem. J.* **273**, 443–448
 Attwood, P. V. & Keech, D. B. (1984) *Curr. Top. Cell. Regul.* **23**, 1–55
 Attwood, P. V. & Wallace, J. C. (1986) *Biochem. J.* **235**, 359–364
 Attwood, P. V., Coates, J. H. & Wallace, J. C. (1984a) *FEBS Lett.* **203**, 191–196
 Attwood, P. V., Wallace, J. C. & Keech, D. B. (1984b) *Biochem. J.* **219**, 243–251
 Barden, R. E. & Scrutton, M. C. (1974) *J. Biol. Chem.* **249**, 4829–4838
 Barden, R. E., Fung, C.-H., Utter, M. F. & Scrutton, M. C. (1972) *J. Biol. Chem.* **247**, 1323–1333
 Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–138
 Climent, I. & Rubio, V. (1986) *Arch. Biochem. Biophys.* **251**, 465–470
 Duggleby, R. G. (1981) *Anal. Biochem.* **110**, 9–18
 Goodall, G. J., Baldwin, G. S., Wallace, J. C. & Keech, D. B. (1981) *Biochem. J.* **199**, 603–609
 Goss, N. H., Dyer, P. Y., Keech, D. B. & Wallace, J. C. (1979) *J. Biol. Chem.* **254**, 1734–1739
 Kaziro, Y., Hass, L. F., Boyer, P. D. & Ochoa, S. (1962) *J. Biol. Chem.* **237**, 1460–1468
 Kluger, R. & Adawadkar, P. D. (1976) *J. Am. Chem. Soc.* **98**, 3741–3742
 Kluger, R., Davis, P. P. & Adawadkar, P. D. (1979) *J. Am. Chem. Soc.* **101**, 5995–6000
 Knowles, J. R. (1989) *Annu. Rev. Biochem.* **58**, 195–221
 McClure, W. R., Lardy, H. A. & Cleland, W. W. (1971a) *J. Biol. Chem.* **246**, 3584–3590
 McClure, W. R., Lardy, H. A., Wagner, M. & Cleland, W. W. (1971b) *J. Biol. Chem.* **246**, 3579–3583
 Morrison, J. F. (1979) *Methods Enzymol.* **63**, 257–294

- Polakis, S. E., Guchait, R. B. & Lane, M. D. (1972) *J. Biol. Chem.* **247**, 1335–1337
 Reimann, E. M. & Umfleet, R. A. (1978) *Biochim. Biophys. Acta* **523**, 516–521
 Sauers, C. K., Jencks, W. P. & Groh, S. (1975) *J. Am. Chem. Soc.* **97**, 5546–5553
 Scrutton, M. C. & Utter, M. F. (1965) *J. Biol. Chem.* **240**, 3714–3723
 Wallace, J. C., Phillips, N. B., Snoswell, M. A., Goodall, G. J., Attwood, P. V. & Keech, D. B. (1985) *Ann. N.Y. Acad. Sci.* **447**, 169–188
 Warren, G. B. & Tipton, K. F. (1974) *Biochem. J.* **139**, 311–320