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Factors that influence the translocation of the *N*-carboxybiotin moiety between the two sub-sites of pyruvate carboxylase

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The active site of pyruvate carboxylase, like those of all biotin-dependent carboxylases, is believed to consist of two spatially distinct sub-sites with biotin acting as a mobile carboxy-group carrier oscillating between the two sub-sites. Some of the factors that influence the location and rate of movement of the *N*-carboxybiotin were studied. The rate of carboxylation of the alternative substrate, 2-oxobutyrate, was measured at 0°C in an assay system where the isolated enzyme–[^{14}C]carboxybiotin was the carboxy-group donor. The results are consistent with the hypothesis that the location of the carboxybiotin in the active site is determined by the presence of Mg^{2+}, acetyl-CoA and the oxo acid substrate. The presence of Mg^{2+} favours the holding of the complex at the first sub-site, whereas α-oxo acids induce the complex to move to the second sub-site. At low concentrations pyruvate induces this movement but does not efficiently act as a carboxy-group acceptor; hydroxypyruvate, glyoxylate and oxamate, though not carboxylated, still induce the movement. The allosteric activator acetyl-CoA exerts only a slight stimulation on the rate of translocation to the second sub-site, and this stimulation arises from an increase in the dissociation constant for Mg^{2+}.

The current view of the active site of the biotin-dependent enzyme pyruvate carboxylase (EC 6.4.1.1) is that it consists of two spatially distinct sub-sites. At the first sub-site, the fixation of CO_2 to form an *N*-carboxybiotin complex is coupled to the hydrolysis of ATP:

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

Once formed, the *N*-carboxybiotin intermediate acts as a mobile carboxy-group carrier and transports the activated carboxy group to the second sub-site, where pyruvate binds and the second partial reaction occurs:

$$\text{Enzyme–biotin–}CO_2^- + \text{pyruvate} \rightleftharpoons \text{Enzyme–biotin} + \text{oxaloacetate} \quad (2)$$

Initial-velocity and product-inhibition studies have been used to elucidate the order of substrate binding and product release (McClure *et al.*, 1971*a*; Barden *et al.*, 1972; Warren & Tipton, 1974; Easterbrook-Smith *et al.*, 1978), but these investigations provide very little information about the nature of the interactions and intermediate steps that give rise to product formation. Isotope-exchange procedures have been used to provide some insight into the sequence of events occurring at each of the two sub-sites (McClure *et al.*, 1971*b*; Scrutton & Utter, 1965; Ashman & Keech, 1975). However, the only information available on the translocation of the carboxybiotin from the first sub-site to the second sub-site comes from the study by Easterbrook-Smith *et al.* (1976). They showed that pyruvate acts as a signal to summon carboxybiotin to the second sub-site.

In the present investigation we examined other factors that influence the location of carboxybiotin by using the enzyme isolated in its *N*-carboxybiotin form.

Methods and materials

Preparation of pyruvate carboxylase

Pyruvate carboxylase was prepared from freeze-dried sheep liver mitochondria by the procedure described by Goss *et al.* (1979), except that a DEAE-Sepharose column (69 cm × 2.5 cm) replaced the DEAE-Sephadex column. The enzyme was purified to specific activities in the range 17–35 units/mg of protein, where 1 unit of enzyme catalysed the formation of 1 μmol of oxaloacetate/min at 30°C.

Isolation of the Enzyme–[^{14}C]carboxybiotin complex

Pyruvate carboxylase was incubated in 0.5 ml of a

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solution containing 100mM-*N*-ethylmorpholine/HCl buffer, pH7.8, 5mM-$MgCl_2$, 0.25mM-acetyl-CoA, 2.5mM-ATP and 2.5mM-$NaH^{14}CO_3$ (57mCi/mmol). After 10min at 0°C, the reaction was terminated by the addition of 0.5ml of 200mM-EDTA, pH7.0. After standing for 4h at 0°C, the Enzyme–[^{14}C]carboxybiotin complex was transferred to a 100mM-*N*-ethylmorpholine/HCl buffer solution, pH7.8, by centrifuging through Sephadex G-25 as described by Helmerhorst & Stokes (1980).

Measurement of the translocation rate

Samples (200μl, containing 100000c.p.m./ml) of the isolated Enzyme–[^{14}C]carboxybiotin were incubated for 10min at 0°C in the presence of the compound to be tested. The measurement of the translocation rate was initiated by the addition of 20mM-2-oxobutyrate. At various time intervals, 20μl samples were transferred to 40μl of 2M-semicarbazide hydrochloride. A 50μl portion of this solution was applied to a 2cm × 2cm square of Whatman 3MM paper, which was heated at 105°C for 5min to remove any $^{14}CO_2$ not transferred to 2-oxobutyrate, and the radioactivity of the paper was counted in 3ml of toluene scintillation fluid (Bousquet & Christian, 1960).

At the beginning and end of each experiment, the total concentration of the Enzyme–[^{14}C]carboxybiotin complex was measured by transfer of the [^{14}C]carboxy group to pyruvate. A 20μl sample was transferred to 20μl of 20mM-pyruvate in 0.1M-*N*-ethylmorpholine/HCl buffer, pH7.8, for 5min. The product, oxaloacetate, was stabilized with 20μl of 2M-semicarbazide hydrochloride and its radioactivity determined as described above.

Data analysis

Lines were fitted by the weighted least-squares method with the aid of the GLIM system (Baker & Nelder, 1978) to determine the simplest model structure and to obtain maximum likelihood estimates of parameters.

Materials

$MgCl_2$ solution was prepared from spectroscopically pure Mg as described previously (Bais & Keech, 1972) and standardized by atomic absorption spectrophotometry. $NaH^{14}CO_3$, *N*-ethylmorpholine (redistilled), sodium pyruvate and 2-oxobutyric acid were as previously described (Easterbrook-Smith *et al.*, 1978).

Results and discussion

Time course for the carboxylation of 2-oxobutyrate from Enzyme–[^{14}C]carboxybiotin

Since the transfer of the activated carboxy group from the Enzyme–carboxybiotin form to pyruvate is very rapid, an analogue of pyruvate, 2-oxobutyrate, and a low temperature (0°C) were used to slow down the transfer process. By using this assay system it was possible to study the factors controlling the release of the *N*-carboxybiotin complex from the first sub-site.

Fig. 1 presents the results obtained when the isolated Enzyme–[^{14}C]carboxybiotin complex was treated with 10mM-EDTA and the time course for the transfer of the activated carboxy group to 2-oxobutyrate was determined. Although the reaction appears to obey pseudo-first-order kinetics, the line of best fit does not extrapolate to 100% when $t = 0$. This result was interpreted to indicate that there are two forms or states of the Enzyme–[^{14}C]carboxybiotin complex at equilibrium with each other; one state is proposed to be enzyme with carboxybiotin bound at the first sub-site (State I), and the other state (State II) is unbound, presumably in the vicinity of the first sub-site (certainly not in the second sub-site, where the carboxybiotin is

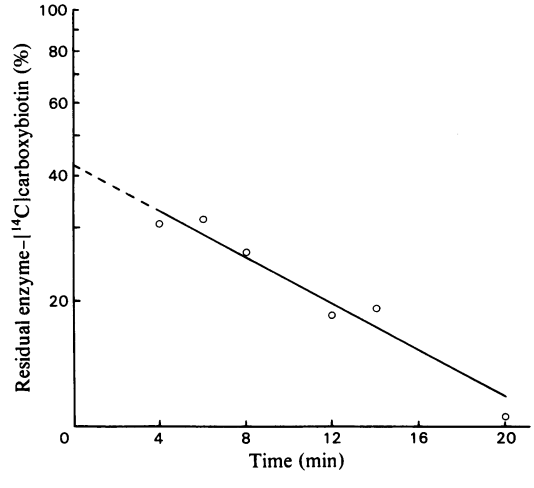


Fig. 1. *Time course of the translocation of the* [^{14}C]*-carboxybiotin complex and the transfer of the activated carboxy group to 2-oxobutyrate by pyruvate carboxylase*

The assay solution contained: 100mM-*N*-ethylmorpholine/HCl buffer, pH7.8, Enzyme–[^{14}C]-carboxybiotin complex (10^5c.p.m./ml) and 10mM-EDTA at 0°C. The reaction was initiated by the addition of 20mM-2-oxobutyrate. At specific time intervals, 20μl samples were transferred to 40μl of 2M-semicarbazide hydrochloride, and the amount of acid-stable radioactivity was determined. The total amount of radioactivity available at any time was determined by transferring any unreacted [^{14}C]-carboxybiotin to pyruvate, as described in the Methods and materials section.

known to be unstable; Easterbrook-Smith *et al.*, 1976):

$$\underset{\text{(bound)}}{\text{State I}} \rightleftharpoons \underset{\text{(unbound)}}{\text{State II}} \qquad (3)$$

After the initial burst of transferring activity (Fig. 1), presumably due to the transfer of the activated carboxy group from State II to the acceptor molecule (eqn. 4), a second, lower, rate of transfer is observed:

$$\text{State I} \rightleftharpoons \text{State II} \xrightarrow[\text{Acceptor}]{\text{Product}} \text{Enzyme–biotin} \qquad (4)$$

Since the transfer from State II to acceptor is very rapid, the second slower phase has been interpreted to represent the rate of dissociation of carboxybiotin bound at the first sub-site (State I) to form State II.

Effect of Mg^{2+} on the Enzyme–[^{14}C]carboxybiotin complex

Any treatment that perturbs the equilibrium between the two states will affect both the size of the 'burst' and the observed rate of the transfer process. Results presented in Fig. 2 show that Mg^{2+} has a pronounced effect on both processes. The decrease in the 'burst' size with increasing concentrations of Mg^{2+} indicates that the equilibrium between the two states has been shifted in favour of State I, whereas the decrease in the observed transfer rate indicates that in the presence of Mg^{2+} State I carboxybiotin is bound more tightly to the first sub-site (eqn. 5):

$$\underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}}\ \underset{\text{Mg}^{2+}}{\text{Mg}^{2+}\text{–State I}} \qquad \text{State I} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{State II} \xrightarrow[\text{Acceptor}]{\text{Product}} \text{Enzyme–biotin} \qquad (5)$$

The effects of increasing Mg^{2+} concentration observed in Fig. 2 cannot be attributed to changes in Cl^- concentration or ionic strength because the time course for the transfer to 2-oxobutyrate in the presence of 18mM-NaCl is similar to that obtained in the presence of 10mM-EDTA. Furthermore, the half-life of the Enzyme–[^{14}C]carboxybiotin complex prepared under the same conditions as the experiments described above and stored in the absence of a CO_2^--acceptor molecule (i.e., in the absence of either pyruvate or 2-oxobutyrate) was determined to be 170min, whereas in the presence of 10mM-$MgCl_2$ the half-life was 700min. The 4-fold increase in the half-life time of the complex indicates that the carboxybiotin moiety is more stable in the first sub-site and is probably complexed to the Mg^{2+}.

In order to develop this model in quantitative terms, the following assumptions were made: (*a*) in the absence of a carboxy-group acceptor molecule (eqn. 5), the [^{14}C]carboxybiotin moiety adopts an equilibrium distribution between State I and State II, i.e.:

$$k_{+2}[\text{State I}] = k_{-2}[\text{State II}] \qquad (6)$$

and

$$k_{-1}[\text{Mg}^{2+}][\text{State I}] = k_{+1}[\text{Mg}^{2+}\text{–State I}] \qquad (7)$$

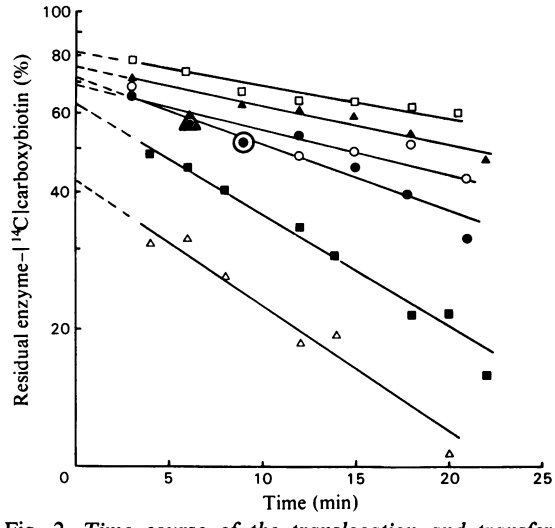


Fig. 2. *Time course of the translocation and transfer processes by pyruvate carboxylase in the presence of Mg^{2+}*

The experimental conditions were the same as those described in Fig. 1 legend, except that Mg^{2+} was added as follows: 2mM (■); 4mM (●); 6mM (○); 8mM (▲); 10mM (□). The control contained no Mg^{2+}, but 10mM-EDTA (△).

(*b*) the conversion of State I into State II is slow, i.e. k_{+1} and $k_{-1} > k_{+2}$, and $k_{+3} \gg k_{+2}$; (*c*), 2-oxobutyrate is present at saturating concentrations, i.e. doubling the concentration of 2-oxobutyrate does not change either the slope or the intercept of the line shown in Fig. 1. Thus the fraction of total [^{14}C]carboxybiotin converted into stable products in the rapid-burst phase of the reaction, i.e. the fractional burst, equals:

$$\frac{[\text{State II}]}{[\text{Mg}^{2+}\text{–State I}] + [\text{State I}] + [\text{State II}]} = \frac{[\text{State II}]}{\frac{k_{-1}k_{-2}}{k_{+1}k_{+2}}[\text{Mg}^{2+}][\text{State II}] + \frac{k_{-2}}{k_{+2}}[\text{State II}] + [\text{State II}]}$$

and

$$\frac{1}{\text{Fractional burst}} = \frac{k_{-1}k_{-2}}{k_{+1}k_{+2}}[\text{Mg}^{2+}] + \frac{k_{-2}}{k_{+2}} + 1 \qquad (8)$$

After the rapid phase of the reaction, virtually all remaining [^{14}C]carboxybiotin is bound at the first sub-site, i.e. [State II]$\rightarrow$0. Furthermore, from the assumptions (*b*) and (*c*), k_{+2} is the rate-limiting step during the conversion of [^{14}C]carboxybiotin into stable products during the slow phase, so that eqn. (5) simplifies to:

$$[Mg^{2+}\text{–State I}] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [\text{State I}] \xrightarrow[\text{Acceptor}]{k_{+2}} \underset{Mg^{2+}}{\text{Products}} \quad \text{Enzyme–biotin} \qquad (9)$$

with k_{+2} rate-limiting.

From assumption (*b*), at time *t*:

$$k_{+1}[Mg^{2+}\text{–State I}] = k_{-1}[Mg^{2+}][\text{State I}]$$

and total [^{14}C]carboxybiotin at time *t*:

$$= [Mg^{2+}\text{–State I}] + [\text{State I}]$$

$$= \frac{k_{-1}}{k_{+1}}[Mg^{2+}][\text{State I}] + [\text{State I}]$$

$$= [\text{State I}]\left(\frac{k_{-1}}{k_{+1}}[Mg^{2+}] + 1\right)$$

Thus:

$$\frac{d([^{14}C]\text{carboxybiotin})}{dt} = k_{+2}[\text{State I}] = -\frac{k_{+2}[^{14}C]\text{carboxybiotin}}{1 + \frac{k_{-1}}{k_{+1}}[Mg^{2+}]} \qquad (10)$$

Integration gives:

$$\ln\left(\frac{[^{14}C]\text{carboxybiotin}_t}{[^{14}C]\text{carboxybiotin}_0}\right) = -\frac{k_{+2}}{1 + \frac{k_{-1}}{k_{+1}}[Mg^{2+}]} \qquad (11)$$

Thus the apparent first-order rate constant is:

$$\frac{k_{+2}}{1 + \frac{k_{-1}}{k_{+1}}[Mg^{2+}]}$$

so that:

$$\frac{1}{\text{Rate}} = \frac{k_{-1}}{k_{+2}k_{+1}}[Mg^{2+}] + \frac{1}{k_{+2}} \qquad (12)$$

Thus a plot of 1/rate against [Mg^{2+}] should be linear with slope of $k_{-1}/k_{+2}k_{+1}$ and intercept of $1/k_{+2}$, whereas a plot of 1/fractional burst against [Mg^{2+}] should also be linear but with slope of $k_{-1}k_{-2}/k_{+1}k_{+2}$ and intercepts of $1 + k_{-2}/k_{+2}$. [Graphs of this form are shown in Figs. 4 and 5 and fit the prediction of linearity ($P > 10\%$, χ^2 test; Bliss, 1970). From a fractional burst plot it can be calculated that in the absence of Mg^{2+} the equilibrium between the two states, i.e. k_{+2}/k_{-2}, is 0.85 ± 0.08.]

Effect of acetyl-CoA on the Enzyme–[^{14}C]carboxybiotin complex

In order to ascertain whether the allosteric activator of pyruvate carboxylase, acetyl-CoA, influenced events involved in the translocation process, an experiment similar to that shown in Fig. 2 was performed in the presence of 250μM-acetyl-CoA (Fig. 3). The effects are seen most readily by

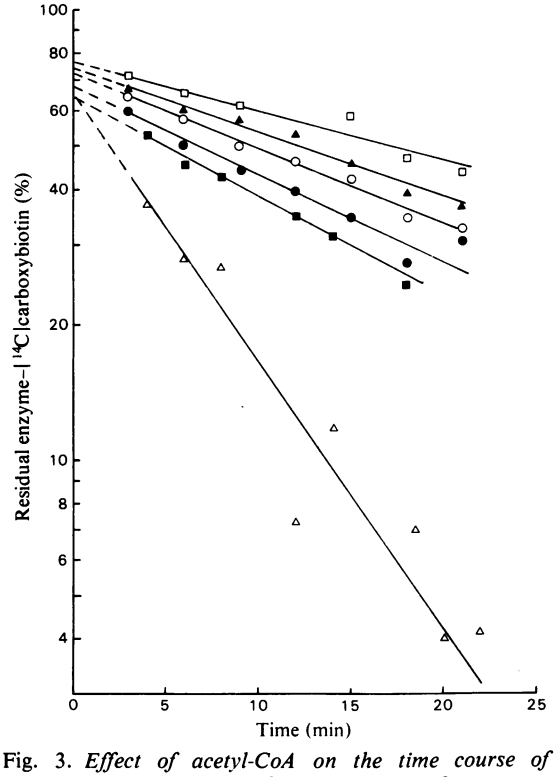


Fig. 3. *Effect of acetyl-CoA on the time course of translocation and transfer processes of pyruvate carboxylase at various concentrations of Mg^{2+}*

The experimental conditions were the same as those described in Fig. 2 legend, except that 0.25mM-acetyl-CoA was present in all assay solutions.

examination of the secondary plots (Figs. 4 and 5) based on eqns. (8) and (12). In both cases the presence of acetyl-CoA affects the slope of the plot but not the intercept on the ordinate, thus indicating that the dissociation constant for Mg^{2+} (k_{+1}/k_{-1}) is increased. The effect of this is to increase the concentration of the State II form, which provides more *N*-carboxybiotin for transfer to the acceptor substrate.

On the other hand, acetyl-CoA has no significant effect on k_{+2}/k_{-2}, i.e. on the rate of translocation between the first sub-site and the second sub-site.

Factors that affect the location of carboxybiotin by binding at the second sub-site

During the course of the overall reaction catalysed by pyruvate carboxylase, it is necessary for the carboxybiotin to be released from the first sub-site and moved to the second sub-site. Here it transfers its activated carboxy group to the acceptor molecule. In the present study a number of compounds possessing structural features resembling pyruvate were examined to determine what features of the pyruvate molecule affect the release of carboxybiotin from the first sub-site and the translocation to the second sub-site. The compounds used fall into three groups: (*a*) those that are substrates for pyruvate carboxylase (i.e. pyruvate, fluoropyruvate and 2-oxobutyrate), (*b*) those that are inhibitors (hydroxypyruvate, oxamate and glyoxylate) and (*c*) those that have no effect on the enzyme (propionate, lactate and acetaldehyde).

Although the transition from State I to State II is slow enough to be measured with 2-oxobutyrate as the acceptor substrate, the transition is too fast to be measured under similar conditions if either pyruvate or fluoropyruvate is used as acceptor (G. J. Goodall, unpublished work). This means that both pyruvate and fluoropyruvate initiate the release of the carboxybiotin from the first sub-site, but the addition of a methyl group at the C-3 position (as in 2-oxobutyrate) drastically decreases the efficiency of the releasing process. On the other hand, compounds, such as propionate, lactate and acetaldehyde, that lack either the carboxy or the oxo group of pyruvate are neither substrates nor inhibitors of pyruvate carboxylase. Furthermore, they do not perturb the equilibrium between State I and State II. Thus both the carboxy and the oxo group of pyruvate appear to be essential both for binding and for initiating the transfer process.

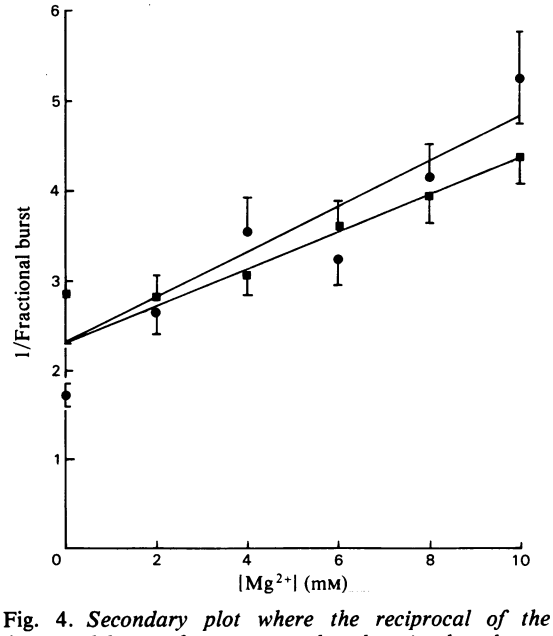


Fig. 4. *Secondary plot where the reciprocal of the fractional burst of pyruvate carboxylase is plotted as a function of Mg^{2+} concentration*

The reciprocal of the ordinate intercepts were obtained from Fig. 2 (●) and Fig. 3 (■) and were plotted in accordance with eqn. (8). The error bars indicate ±1 S.D.

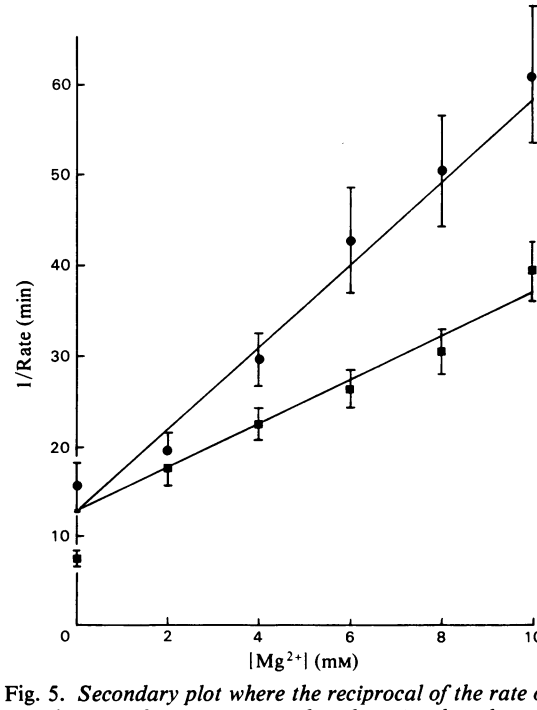


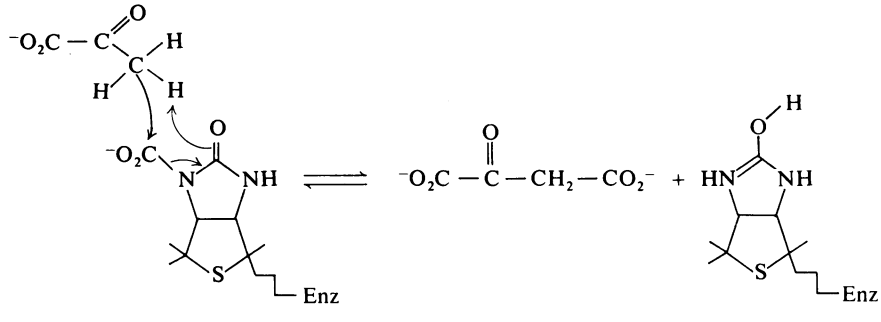
Fig. 5. *Secondary plot where the reciprocal of the rate of translocation by pyruvate carboxylase is plotted as a function of Mg^{2+} concentration*

The reciprocals of the rates were obtained from Fig. 2 (●) and Fig. 3 (■) and were plotted in accordance with eqn. (12). The error bars indicate ±1 S.D.

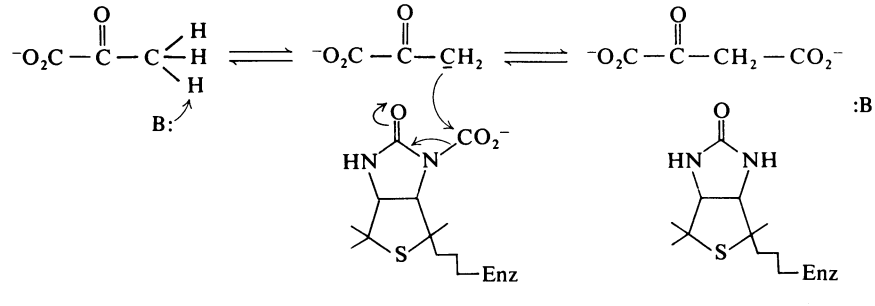
It has been shown prevously (Easterbrook-Smith *et al.*, 1976) that pyruvate carboxylase exhibits a hydrolytic leak (i.e. an abortive hydrolysis of the Enzyme–carboxybiotin complex) at low concentrations of pyruvate. The explanation for this phenomenon put forward by these authors is that the binding of pyruvate shifts the carboxybiotin complex to the pyruvate-binding site in readiness for the carboxylation of pyruvate. Previously, the n.m.r. studies by Mildvan *et al.* (1966) had shown that the binding of pyruvate is a rapid equilibrium process and that pyruvate moves into and out of the pyruvate-binding site at a rate that is two orders of magnitude faster than the rate of the overall reaction. Therefore, if the carboxybiotin arrives at the pyruvate-binding site and finds the site unoccupied, a likely event at non-saturating concentrations of pyruvate, some of the complex may spontaneously hydrolyse to biotin and CO_2. The study on the isotopic effects on pyruvate carboxylase catalysis by Cheung & Walsh (1976) supports this explanation. They concluded that 'after the pyruvate molecule binds, it is only about 50% committed to catalysis, i.e. it will come back off the enzyme without reacting one out of two times it binds.'

This explanation is further supported in the present investigation by measurements of the stability of isolated Enzyme–[^{14}C]carboxybiotin complex in the presence of certain inhibitors of the enzyme. These inhibitors are competitive with respect to pyruvate. To perform these experiments, the Enzyme–[^{14}C]carboxybiotin complex was isolated at 0°C, and 6mM-$MgCl_2$ was added to ensure that at least 75% of the complex was in the first sub-site (see Fig. 2). The stability of the complex was monitored by transfer of the [^{14}C]carboxy group to pyruvate, as described in the Methods and materials section. Fig. 6 shows that without any addition there was negligible loss of the [^{14}C]carboxy group over 6min. However, in the presence of hydroxypyruvate, oxamate or glyoxylate there was a rapid loss of radioactivity from the *N*-[^{14}C]carboxybiotin complex. Obviously, these compounds act as very efficient translocation signals, attracting the carboxybiotin complex into the second sub-site, but, since they are not carboxylated (Fig. 6), the complex spontaneously decarboxylates. It should be noted that the rate of translocation and decomposition of the complex in the presence of these compounds is much higher than the rate of translocation in the presence of 2-oxobutyrate.

(*a*) Concerted mechanism



(*b*) Carbanion mechanism



Scheme 1. *Previously proposed mechanisms for biotin-dependent carboxylation reactions*

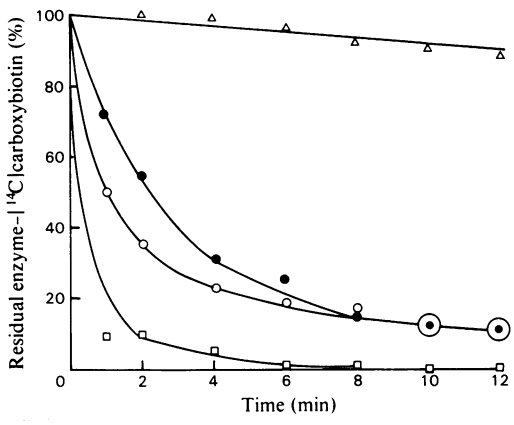


Fig. 6. *Effect of α-oxo acid derivatives on the stability of the [^{14}C]carboxybiotin complex of pyruvate carboxylase*

The assay solutions contained: 100mM-*N*-ethylmorpholine/HCl buffer, pH 7.8, 6mM-$MgCl_2$ and Enzyme–[^{14}C]carboxybiotin (3×10^4 c.p.m./ml). The reaction was started by the addition of 10mM-hydroxypyruvate (□), 10mM-glyoxylate (●) or 10mM-oxamate (○). Water was added to the control (△). The residual Enzyme–[^{14}C]carboxybiotin complex was measured at the times indicated by transfer to pyruvate as described in the Methods and materials section.

The behaviour of these compounds, i.e. hydroxypyruvate, oxamate and glyoxylate, is consistent with the previous conclusion that the carboxy and oxo groups of pyruvate are necessary for binding and initiating the translocation of the carboxybiotin. However, the fact that glyoxylate acts as a very efficient signal suggests that the methyl group is not required for these processes.

It has been proposed by various workers (Rétey & Lynen, 1965; Mildvan *et al.*, 1966; Prescott & Rabinowitz, 1968; Moss & Lane, 1971; Rose *et al.*, 1976) that the reactions catalysed by biotin-dependent carboxylases occur in a concerted manner (Scheme 1*a*). More recently, Stubbe *et al.* (1980) have proposed a mechanism involving the formation of a carbanion as an intermediate (Scheme 1*b*). Our observation that glyoxylate labilizes the carboxybiotin, resulting in the rapid decomposition of carboxybiotin, does not fit either of these mechanisms. In the concerted mechanism, a proton is transferred from the substrate to the carbonyl oxygen of biotin. Obviously, glyoxylate cannot supply a proton. Furthermore, glyoxylate cannot form a carbanion, as required in Scheme 1(*b*). Unfortunately, our results do not permit us to advance any mechanism of action, although it is obvious that in the first sub-site the ureido nitrogen is a good nucleophile and the carboxybiotin is relatively stable. In the second sub-site, since the carboxy derivative is so unstable, it can be assumed that the environment there renders the ureido group a very poor nucleophile.

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References

Ashman, L. K. & Keech, D. B. (1975) *J. Biol. Chem.* **250**, 14–21

Bais, R. & Keech, D. B. (1972) *J. Biol. Chem.* **247**, 3255–3261

Baker, R. J. & Nelder, J. A. (1978) *The GLIM System Manual Release 3*, Numerical Algorithms Group, Oxford

Barden, R. E., Fung, C., Utter, M. F. & Scrutton, M. C. (1972) *J. Biol. Chem.* **247**, 1323–1333

Bliss, C. J. (1970) *Statistics in Biology*, vol. 2, p. 68, McGraw-Hill, New York

Bousquet, W. F. & Christian, J. E. (1960) *Anal. Chem.* **32**, 722–723

Cheung, Y. & Walsh, C. (1976) *Biochemistry* **15**, 3749–3754

Easterbrook-Smith, S. B., Hudson, P. J., Goss, N. H., Keech, D. B. & Wallace, J. C. (1976) *Arch. Biochem. Biophys.* **176**, 709–720

Easterbrook-Smith, S. B., Wallace, J. C. & Keech, D. B. (1978) *Biochem. J.* **169**, 225–228

Goss, N. H., Dyer, P. Y., Keech, D. B. & Wallace, J. C. (1979) *J. Biol. Chem.* **254**, 1734–1739

Helmerhorst, E. & Stokes, G. B. (1980) *Anal. Biochem.* **104**, 130–135

McClure, W. R., Lardy, H. A., Wagner, M. & Cleland, W. W. (1971*a*) *J. Biol. Chem.* **246**, 3579–3583

McClure, W. R., Lardy, H. A. & Cleland, W. W. (1971*b*) *J. Biol. Chem.* **246**, 3584–3590

Mildvan, A. S., Scrutton, M. C. & Utter, M. F. (1966) *J. Biol. Chem.* **241**, 3488–3498

Moss, J. & Lane, M. D. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol.* **35**, 321–442

Prescott, D. J. & Rabinowitz, J. L. (1968) *J. Biol. Chem.* **243**, 1551–1557

Rétey, J. & Lynen, F. (1965) *Biochem. Z.* **342**, 256–271

Rose, I. A., O'Connell, E. L. & Solomon, F. (1976) *J. Biol. Chem.* **251**, 902–904

Scrutton, M. C. & Utter, M. F. (1965) *J. Biol. Chem.* **240**, 3714–3723

Stubbe, J., Fish, S. & Abeles, R. H. (1980) *J. Biol. Chem.* **255**, 236–242

Warren, G. B. & Tipton, K. F. (1974) *Biochem. J.* **139**, 311–320