The carboxybiotin complex of chicken liver pyruvate carboxylase

A kinetic analysis of the effects of acetyl-CoA, Mg^{2+} ions and temperature on its stability and on its reaction with 2-oxobutyrate

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The enzyme– $[^{14}C]$ carboxybiotin complex of chicken liver pyruvate carboxylase has been isolated and shown to be relatively stable, with a half-life at 0 °C of 342 min. The kinetic properties of the decay of this complex, in both the presence and the absence of the substrate analogue, 2-oxobutyrate, have been examined. The data for the reaction with 2-oxobutyrate at 0 °C fitted a biphasic exponential decay curve, enabling the calculation of rate constants for both the fast and slow phases of the reaction at this temperature. The effect of temperature on the observed pseudo-first-order rate constant for the slow phase of the reaction with 2-oxobutyrate, and that for the decay of the enzyme– $[^{14}C]$ carboxybiotin complex alone, have been examined. Arrhenius plots of these data revealed that the processes being studied in each type of experiment were single reactions represented by one rate constant in each case. For the decay of the enzyme– $[^{14}C]$ carboxybiotin complex in the absence of 2-oxobutyrate, the rate-determining process may be the movement of carboxybiotin from the site of the first partial reaction to the site of the second. The calculated thermodynamic activation parameters indicate that this reaction is accompanied by a large change in protein conformation. With 2-oxobutyrate present, the observed process in the slow phase of the reaction was probably the dissociation of the carboxybiotin from the first subsite. Here, the activation parameters suggest that a much smaller change in protein conformation accompanies this reaction. Both sets of experiments were also performed in the presence of acetyl-CoA, but this activator had little effect on the measured thermodynamic activation parameters. However, in both cases the observed pseudo-first-order rate constants in the presence of acetyl-CoA were about 75% of those in its absence. The effects of Mg^{2+} on the reaction kinetics of the enzyme– $[^{14}C]$ carboxybiotin complex with 2-oxobutyrate were similar to those observed with the sheep enzyme by Goodall, Baldwin, Wallace & Keech [(1981) *Biochem. J.* **199**, 603–609].

INTRODUCTION

Pyruvate carboxylase (EC 6.4.1.1) is proposed to catalyse the formation of oxaloacetate from pyruvate, HCO_3^- and ATP in two partial reactions at spatially distinct sub-sites as follows:

(for more details see the review by Attwood & Keech, 1984).

Goodall *et al.* (1981) were able to isolate the enzyme– $[^{14}C]$ carboxybiotin complex of sheep liver pyru-

vate carboxylase, which proved to be sufficiently stable at 0 °C that the kinetics of the transfer of the $[^{14}C]$ carboxy group to the substrate analogue, 2-oxobutyrate, could be examined. These investigators

found that the reaction with 2-oxobutyrate was biphasic with a very rapid ‘burst’ phase preceding a slower, measurable, pseudo-first-order reaction. They also dis-

Scheme 1. Transfer of the carboxy group from the enzyme–carboxybiotin complex to 2-oxobutyrate

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covered that the amplitudes of both the 'burst' phase and the observed pseudo-first-order rate constant decreased in the presence of increasing Mg^{2+} concentrations. From these observations Goodall *et al.* (1981) proposed the reaction scheme shown in Scheme 1. In State I the carboxybiotin is bound to the site of the first partial reaction, and in State II the carboxybiotin is not found at the first subsite but is in its vicinity. In addition, the following relationships between the rate constants were found to apply in the slow phase of the reaction:

$$\frac{1}{k_{obs.}} = \frac{k_{-1}[Mg^{2+}]}{k_{+1}k_{+2}} + \frac{1}{k_{+2}} \quad (3)$$

Thus in the absence of Mg^{2+} the observed rate constant is in fact k_{+2} , which Goodall *et al.* (1981) proposed to represent the dissociation of the carboxybiotin from the site of the first partial reaction. The first phase occurs because $k_{+3} \gg k_{+2}$, so that before addition of 2-oxobutyrates States I and II are at equilibrium. However, as soon as the substrate is added, all the State II, reacts rapidly, leaving, in the absence of Mg^{2+} , all the enzyme- $[^{14}C]$ carboxybiotin in the form of State I. The rate-limiting step in the transfer of the $[^{14}C]$ carboxy group is then governed by k_{+2} . The effect of Mg^{2+} is to reduce the amount of enzyme- $[^{14}C]$ carboxybiotin complex in the form of State II and thus reduce the amplitude of the first phase. The effect on the observed rate constant is demonstrated in eqn. (3).

In a later investigation by Attwood *et al.* (1984) the kinetics of decay of enzyme- $[^{14}C]$ carboxybiotin at 0 °C and the kinetics of the transfer of the $[^{14}C]$ carboxy group to pyruvate were examined. In both cases the reactions were monophasic and obeyed pseudo-first-order kinetics. On the basis of these results the reaction schemes shown in Schemes 2 and 3 were proposed.

In both cases the results were consistent with k_{+2} and k_{-2} being $> k_{+3}$, such that States I and II are in equilibrium throughout the reaction and k_{+3} is the rate-limiting step. These authors proposed that the step represented by k_{+3} was the movement of carboxybiotin from the site of the first partial reaction to the site of the second partial reaction.

All of the above studies with the sheep liver enzyme were performed at 0 °C. However, chicken liver pyruvate carboxylase is labile below approx. 15 °C (Scrutton *et al.*, 1965; Irias *et al.*, 1969), and concomitant with the loss of overall activity is the loss of the pyruvate:oxaloacetate exchange reactivity (Scrutton *et al.*, 1965). Irias *et al.* (1969) found that saturating concentrations of acetyl-CoA prevented cold inactivation. Thus the enzyme- $[^{14}C]$ -

Scheme 2. Decay of enzyme- $[^{14}C]$ carboxybiotin

Scheme 3. Transfer of the carboxy group from the enzyme-carboxybiotin complex to pyruvate

carboxybiotin complex of the chicken enzyme may not be very stable at the low temperatures required for kinetic observations. Also, the ability of the enzyme- $[^{14}C]$ carboxybiotin complex to transfer its $[^{14}C]$ carboxy group to 2-oxobutyrates may be impaired.

In this investigation we set out to: (i) isolate the enzyme- $[^{14}C]$ carboxybiotin complex of chicken liver pyruvate carboxylase; (ii) examine the kinetics of decay of enzyme- $[^{14}C]$ carboxybiotin complex in the presence and absence of acetyl-CoA; (iii) examine the kinetics of the reaction between the enzyme- $[^{14}C]$ carboxybiotin complex and 2-oxobutyrates in the presence and absence of acetyl-CoA and the effects of Mg^{2+} on the reaction; and (iv) observe the effects of temperature on the observed rate constants for both types of reaction and thereby to calculate the thermodynamic activation parameters for the movement of carboxybiotin between subsites and the dissociation of carboxybiotin from the first subsite.

MATERIALS AND METHODS

Materials

Chicken liver pyruvate carboxylase (EC 6.4.1.1) was purified as described by Goss *et al.* (1979) to a specific activity of 20–25 units/mg of protein (1 unit of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1 μ mol of oxaloacetate/min). The catalytic activity of the enzyme was assayed using the spectrophotometric assay described by Duggleby *et al.* (1982).

$MgCl_2$ was prepared from spectroscopically pure Mg and redistilled HCl, and standardized by atomic absorption spectroscopy. *N*-Ethylmorpholine (redistilled) and sodium pyruvate were as described previously by Easterbrook-Smith *et al.* (1978). $NaH^{14}CO_3$ was obtained as an aqueous solution of 2 mCi/ml at a specific radioactivity of 55 mCi/mmol from Amersham (Australia) Pty. Ltd. Acetyl-CoA was prepared from CoA and purified as described previously (Ashman *et al.*, 1972).

Isolation of the enzyme- $[^{14}C]$ carboxybiotin complex

This was performed as described by Attwood *et al.* (1984), using about 24 units of enzyme in the reaction volume of 0.5 ml. The reaction was terminated in the present experiments by the addition of 0.5 ml of ice-cold 14 mM-EDTA, pH 7.0. In those experiments performed in the presence of acetyl-CoA, the Sephadex G-25 used to isolate the enzyme- $[^{14}C]$ carboxybiotin complex from the other components of the reaction mixture was equilibrated with 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, containing 0.25 mM-acetyl-CoA. Associated with each unit of enzyme were 30 000–50 000 c.p.m. of radioactivity.

Stability of the enzyme- $[^{14}C]$ carboxybiotin complex

Generally the solutions containing the enzyme- $[^{14}C]$ carboxybiotin complex were diluted 1:1 with 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, which had been equilibrated at the temperature at which the experiment was to be performed. Where specified, this buffer also contained 0.25 mM-acetyl-CoA. The final solutions were allowed to equilibrate at the required temperature for at least 10 min before the first 20 μ l portion was removed and added to 20 μ l of buffer containing 20 mM-sodium pyruvate. This mixture was left

for 3–4 min to ensure complete transfer of the available [^{14}C]carboxy group to pyruvate from the enzyme–[^{14}C]carboxybiotin complex. The resultant [^{14}C]oxaloacetate was stabilized by the addition of 20 μl of 1.5 M-semicarbazide hydrochloride. The radioactivity present in these oxaloacetate solutions was measured using the method described by Attwood *et al.* (1984) by spotting 50 μl of the above solutions on to squares of Whatman 3MM paper.

Transfer of [^{14}C]carboxy group from the enzyme–[^{14}C]carboxybiotin complex to 2-oxobutyrates

The experiments to determine the effect of temperature and acetyl-CoA on the reaction were performed in a similar way to the stability experiments. Here, however, the enzyme–[^{14}C]carboxybiotin complex solutions were diluted 1:1 with buffer containing 20 mM-2-oxobutyrates. At various time intervals after this dilution 40 μl portions were removed and added to 20 μl of 1.5 M-semicarbazide hydrochloride and the radioactivity, present in this case as 3-methyloxaloacetate, was measured as described above. The total radioactivity available was measured by transferring any unreacted [^{14}C]carboxy group to pyruvate as described by Goodall *et al.* (1981).

In the experiments involving Mg^{2+} , to 300 μl of the enzyme–[^{14}C]carboxybiotin in complex solutions were added 100 μl of 60 mM-2-oxobutyrates, the required volume of 0.1 M- MgCl_2 and the volume of the reaction mixture was made up to 600 μl with 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8. The experiment was then performed in the same way as described above, transferring 40 μl portions to 20 μl of 1.5 M-semicarbazide hydrochloride at various time intervals after the addition of the 2-oxobutyrates. All the experiments involving Mg^{2+} were performed at 0 °C and the results for all the experiments where 2-oxobutyrates was a substrate were calculated and expressed as described by Goodall *et al.* (1981).

Data analysis

All lines and curves were fitted to the data on the CBM 4016 computer, by using the non-linear regression program written by Duggleby (1981).

RESULTS AND DISCUSSION

Stability of the enzyme–[^{14}C]carboxybiotin complex

Fig. 1 shows the decay time courses for the enzyme–[^{14}C]carboxybiotin complex at various temperatures. As can be seen, the process is first order and similar to that seen for the sheep liver enzyme–[^{14}C]carboxybiotin complex (Attwood *et al.*, 1984). However, if the pseudo-first-order rate constants for the decay of the enzyme–[^{14}C]carboxybiotin complexes of the chicken and sheep enzymes at 0 °C are compared [i.e. $k_{\text{obs.}}$ (0 °C, chicken) = 0.0021 min^{-1} ; $k_{\text{obs.}}$ (0 °C, sheep) = 0.0202 min^{-1} (Attwood *et al.*, 1984)] it can be seen that the carboxybiotin complex in the chicken enzyme is much more stable than in the sheep enzyme.

Fig. 2 shows the Arrhenius plots of the observed pseudo-first-order rate constants for the decay of the enzyme–[^{14}C]carboxybiotin complex in both the presence and the absence of saturating concentrations of acetyl-CoA. The thermodynamic activation parameters at 0 °C,

Fig. 1. Time courses for the decay of the enzyme–[^{14}C]carboxybiotin complex at 0 °C (●), 4 °C (□), 8 °C (■), 12 °C (△) and 16 °C (○) in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8

The lines represent least squares fits to the data of single first-order exponential decay curves.

calculated from Fig. 2, are shown in Table 1. As is clear from Fig. 2 and Table 1, acetyl-CoA has little effect on the activation energy of the reaction. However, the rate constants in the presence of acetyl-CoA are only 71–72% of those in its absence. This effect can be seen in the somewhat lower activation entropy of the reactions in the presence of acetyl-CoA. This effect of acetyl-CoA might be taken as an indication that acetyl-CoA stabilizes the enzyme–[^{14}C]carboxybiotin complex by preventing cold inactivation. However, the effect of acetyl-CoA on the decay of the carboxybiotin complex is still observed at 16 °C, at which temperature cold inactivation does not appear to occur (Irias *et al.*, 1969). Thus it seems that acetyl-CoA exerts a direct effect on the kinetic process of the decay of the enzyme–[^{14}C]carboxybiotin complex.

The linearity of the Arrhenius plots indicates that a single rate-limiting step is observed over this temperature range (i.e. 0–16 °C). The large activation energy calculated for this reaction suggests that a substantial change in protein conformation is involved in this process. This is supported by the large positive activation entropy (Table 1), indicating some disruption in the protein and its interaction with the solvent. As mentioned in the Introduction, in their studies on the carboxybiotin complex of the sheep liver enzyme Attwood *et al.* (1984) proposed that the rate-limiting step in the decay of the carboxybiotin complex was the movement of carboxybiotin from the vicinity of the site of the first partial reaction to the site of the second partial reaction. If this is the case, movement of the biotin prosthetic group

Fig. 2. Arrhenius plots of the observed pseudo-first-order rate constants for the decay of the enzyme- $[^{14}\text{C}]$ carboxybiotin complex in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, in the presence (○) and the absence (▲) of 0.25 mM-acetyl-CoA

Error bars indicate ± 1 S.E.M. as calculated in the non-linear regression program of Duggleby (1981).

between subsites would therefore seem to involve appreciable changes in the protein structure.

Effects of temperature on the transfer of the $[^{14}\text{C}]$ carboxy group from the enzyme- $[^{14}\text{C}]$ carboxybiotin complex to 2-oxobutyrates

Fig. 3 demonstrates the effect of temperature on the kinetics of the reaction of the enzyme- $[^{14}\text{C}]$ carboxybiotin complex with 2-oxobutyrates in the absence of acetyl-CoA. At 0 °C, part of the fast phase of the reaction was monitored as well as the slow phase and the rate constant for this part of the reaction was estimated to be 2.9 min^{-1} from a fit of the data to a biphasic exponential decay curve. Thus the rate constant for the fast phase of the reaction is 23 times larger than that for the slow phase. At 4 °C the biphasic curve did not give a significantly better fit to the data than the single exponential decay.

Fig. 3. Time courses for the decay of the enzyme- $[^{14}\text{C}]$ carboxybiotin complex at 0 °C (▲), 4 °C (●), 8 °C (□), 12 °C (△) and 16 °C (○) in 0.1 M-*N*-ethylmorpholine/HCl buffer, pH 7.8, containing 10 mM-2-oxobutyrates

The lines represent least squares fits to the data of single first-order exponential decay curves at 4–16 °C and to a biphasic curve of the form $Ae^{-k_1t} + (100 - A)e^{-k_2t}$ at 0 °C.

From 8 to 16 °C no fits to the biphasic curve were obtained, since the estimates for the rate constant for the fast phase tended to infinity. In the presence of acetyl-CoA, the rate constant for the fast phase of the reaction at 0 °C was found to be 3.3 min^{-1} , but there were no other valid fits to a biphasic curve for the data obtained at the other temperatures. It is clear that the observed pseudo-first-order rate constant of the slow phase increases with increasing temperature. However, the effect on the fractional burst, as indicated by the

Table 1. Thermodynamic activation parameters for the decay of the enzyme- $[^{14}\text{C}]$ carboxybiotin complex and its reaction with 10 mM-2-oxobutyrates at 0 °C in the presence and absence of 0.25 mM-acetyl-CoA calculated from the Arrhenius plots shown in Figs. 2 and 4

Type of experiment	Experimental conditions	E_a (kJ/mol)	$\Delta H^\ddagger$ (kJ/mol)	$\Delta G^\ddagger$ (kJ/mol)	$\Delta S^\ddagger$ (J/mol per K)
Decay of enzyme- $[^{14}\text{C}]$ -carboxybiotin complex	+ Acetyl-CoA	130.1	127.8	88.5	143.7
	– Acetyl-CoA	131.6	129.3	88.0	151.1
Reaction with 2-oxobutyrates	+ Acetyl-CoA	79.9	77.6	80.8	–11.7
	– Acetyl-CoA	94.4	92.2	80.5	+42.8

Fig. 4. Arrhenius plots of the observed pseudo-first-order rate constants for the slow phase of the reaction between the enzyme-[^{14}C]carboxybiotin complex and 10 mM-2-oxobutyrates, in 0.1 M-N-ethylmorpholine/HCl buffer, pH 7.8, in the presence (O) or absence (Δ) of 0.25 mM-acetyl-CoA

Error bars indicate ± 1 S.E.M. as calculated in the non-linear regression program of Duggleby (1981).

intercepts of the lines at zero time, is not so clear, but on the whole there seems to be little change.

Fig. 4 shows Arrhenius plots of the observed first-order rate constants of the slow phase of the reaction using data obtained from experiments similar to those shown in Fig. 3, except that for one temperature range the reaction was performed in the presence of a saturating concentration of acetyl-CoA. The thermodynamic activation parameters at 0 °C calculated from these plots are given in Table 1.

In the presence of acetyl-CoA $E_a = 79.9 \pm 14.9$ kJ/mol and in its absence $E_a = 94.4 \pm 9.9$ kJ/mol. As can be seen from the calculated standard errors on these estimates, the effect of acetyl-CoA on this parameter is not significant. However, acetyl-CoA, in general, reduces the observed first-order rate constants to about 76–78% of those in its absence. Acetyl-CoA does not appear to affect the amplitude of the burst phase of the reaction. The effect on the observed rate constant is manifested in a lower activation entropy in the presence of acetyl-CoA, when in fact it becomes negative compared with a positive value in its absence. The effect of acetyl-CoA in reducing the

rate constants is seen across the entire temperature range (0–16 °C). Thus, as before, the effect of acetyl-CoA on the kinetics of the transcarboxylation reaction is not related to its ability to prevent cold inactivation.

The linearity of the Arrhenius plots indicates that a single rate-limiting step is observed over this temperature range. Goodall *et al.* (1981) proposed that the rate-limiting step of the slow phase of the reaction with 2-oxobutyrates is the dissociation of carboxybiotin from the site of the first partial reaction. Thus it would appear that acetyl-CoA directly influences this process. However, Goodall *et al.* (1981) found that, with the sheep liver enzyme, the only effect acetyl-CoA had was to increase the dissociation constant of the Mg^{2+} -enzyme-carboxybiotin complex by modifying the k_{+1}/k_{-1} ratio (see Scheme 1).

If the processes observed in the two types of experiments correspond to those proposed by Goodall *et al.* (1981) and Attwood *et al.* (1984) then the data presented here indicate that the activation energy associated with the dissociation of the carboxybiotin from the first subsite is appreciably lower than that associated with the movement of the carboxybiotin between the subsites. In addition, the entropy of activation of the dissociation step is small compared with that of the movement of carboxybiotin from the first to the second subsite. Both of these factors indicate that there is much less perturbation of the structure of the protein when the carboxybiotin dissociates from the first subsite than when it moves to the second subsite.

Effects of Mg^{2+} on the reaction of the enzyme-[^{14}C]carboxybiotin complex and 2-oxobutyrates

The effects of Mg^{2+} on this reaction (results not shown) are qualitatively similar to those observed on the reaction of the enzyme-[^{14}C]carboxybiotin complex of the sheep liver enzyme by Goodall *et al.* (1981). Increasing concentrations of Mg^{2+} reduce the size of the 'burst' phase and decrease the observed rate constant of the slow phase in accordance with the equations derived by Goodall *et al.* (1981) for Scheme 1. However, the calculated values of k_{+2} (i.e. 0.152 min^{-1}) and k_{-2} (i.e. 0.626 min^{-1}) are both higher than those obtained for the sheep enzyme, with k_{+2} being 2.6 times greater and k_{-2} 9 times greater. Overall, this results in the carboxybiotin being more tightly bound to the first subsite in the chicken enzyme. On the other hand, the dissociation constant for the Mg^{2+} -State I complex of the chicken enzyme (i.e. 3.2 mM) is similar to that of the sheep enzyme.

General discussion

Clearly, exposure of the enzyme-carboxybiotin complex to low temperatures does not affect its stability or its ability to transfer its carboxyl group to 2-oxobutyrates. Thus, after exposure to these temperatures, the carboxybiotin is able to make contact with the sites of both partial reactions within the active site of the enzyme. The conditions under which these experiments were performed were similar to those used by Scrutton *et al.* (1965) and Irias *et al.* (1969), where loss of both overall enzymic activity and the pyruvate:oxaloacetate exchange activity were observed. In addition, Scrutton *et al.* (1965) also found that the ATP:P_i exchange activity was lost concomitantly with the overall enzymic activity. Since both of the exchange reactions involve the participation of the biotin group (see eqns. 1 and 2) one possible

explanation of the loss of the enzymic activities on cold inactivation is that the biotin can no longer reach the sites of the partial reactions. The implication of this explanation is that on exposure to low temperatures the active site of the enzyme opens out, thus moving apart the sites of the partial reactions. However, the results of the present investigation seem to contradict this explanation of cold inactivation. Nevertheless, it should be remembered that in the present experiments it is the carboxylated form of the enzyme which is dealt with and that in its reaction with 2-oxobutyrates it only has to undergo one turnover. It is possible that, because the carboxybiotin is able to stay at the first subsite, this may restrain the movement of the subsites apart and thus prevent the structural changes which might otherwise occur when the enzyme alone is exposed to low temperatures. After the loss of the carboxyl group from the biotin, these changes may then occur but are not detected in these experiments.

The effect of acetyl-CoA both on the dissociation of the carboxybiotin from the first subsite and on its movements to the second subsite is to reduce the activation entropy. This may be related to the effect acetyl-CoA has on the structure of the enzyme. Mayer *et al.* (1980) found the enzyme molecules prepared in the presence of acetyl-CoA did not exhibit the clefts that run along the long axis of the subunits in molecules prepared in its absence. In addition, Attwood *et al.* (1985) found that acetyl-CoA causes enzyme molecules to adopt a tight, tetrahedron-like, conformation. It would seem therefore that acetyl-CoA binding produces a tighter, more ordered, molecule, which may be less flexible than the enzyme alone. Thus acetyl-CoA may constrain perturbations in the protein/solvent system that might occur in its absence, during the dissociation and movement of the carboxybiotin from the first subsite. In view of the dramatic effect of acetyl-CoA on the catalytic activity of chicken liver pyruvate carboxylase it would appear unlikely from the results presented here that the second partial reaction (eqn. 2) is the rate-limiting step in the overall reaction.

If the assignment of reaction steps in Schemes 1 and 2 accurately reflects actual changes in the position of carboxybiotin, then, as one might intuitively expect, the dissociation of the carboxybiotin from the first subsite appears to be accompanied by a much smaller protein-conformation change than the movement of the carboxybiotin to the second subsite. This large change associated with the movement of carboxybiotin presumably may affect the binding of the products of the first partial reaction, thus causing their release. From the work of Attwood *et al.* (1984), it is clear that pyruvate has a large effect on this process in that the rate constant for

this step is larger in the presence of pyruvate than in its absence. It would be interesting therefore in future work to examine the activation parameters of the reaction between the enzyme-carboxybiotin complex and pyruvate.

Finally, as we have seen from the kinetic studies presented here and by Goodall *et al.* (1981), the chicken enzyme appears overall to have very similar characteristics to the sheep enzyme in the reactions of the enzyme-carboxybiotin complex. Thus the reaction schemes proposed by Goodall *et al.* (1981) and Attwood *et al.* (1984) should be applicable to pyruvate carboxylases derived from a variety of sources.

The present paper has demonstrated that the enzyme-carboxybiotin complex of the chicken liver enzyme can be isolated and is stable at 0 °C. In addition, the kinetic properties of its reaction with 2-oxobutyrates have been examined. By observing the effects of temperature on this reaction and on the stability of the enzyme-carboxybiotin complex it has been possible to calculate the activation parameters of what appear to be the dissociation of the carboxybiotin from the first subsite and its movement to the second subsite.

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