The Atypical Velocity Response by Pyruvate Carboxylase to Increasing Concentrations of Acetyl-Coenzyme A

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An investigation was made of the interaction of pyruvate carboxylase with its allosteric effector, acetyl-CoA, and the velocity profile of the deacylation of acetyl-CoA as a function of acetyl-CoA concentration indicated that this ligand does not bind to this enzyme in a positive homotropic co-operative manner. An examination was therefore made of the factors that contribute to the sigmoidicity of the rate curves obtained for pyruvate carboxylation with various concentrations of acetyl-CoA. Hill coefficients for acetyl-CoA obtained with both sheep and chicken liver pyruvate carboxylases were found to be dependent on the fixed pyruvate concentration used in the assay solution. Thus, by varying the acetyl-CoA concentration, the degree of saturation of the enzyme by pyruvate was also changed. A further consequence of non-saturating concentrations of pyruvate was that the non-productive hydrolysis of the enzyme–carboxybiotin complex increased, resulting in an under-estimate of the reaction velocity measured by oxaloacetate formation. Another factor contributing to the sigmoidicity is that, at non-saturating concentrations of acetyl-CoA, the enzyme undergoes inactivation upon dilution to low protein concentrations, again resulting in an under-estimate of the reaction velocity. Under conditions where none of the above factors was operating and the only effect of varying acetyl-CoA concentrations was to alter the proportion of the enzyme catalysing the carboxylation reaction at acetyl-CoA-dependent and -independent rates, the sigmoidicity of the acetyl-CoA velocity profile was completely eliminated.

Since the discovery of pyruvate carboxylase (Utter & Keech, 1960), the mechanism of activation of the enzyme by acetyl-CoA has been the subject of some speculation. With the first reports of a sigmoidal velocity response to increasing concentrations of acetyl-CoA (Barritt *et al.*, 1966; Scrutton & Utter, 1967) it was assumed that the activator bound to the enzyme in a homotropic co-operative manner. Direct evidence for co-operative binding was reported by Frey & Utter (1977), but the high enzyme concentration used by those authors involved a rate of deacylation of acetyl-CoA that renders an unequivocal interpretation of their data difficult. Other evidence suggests that homotropic co-operativity of binding is not necessarily the explanation of the non-classical kinetic response, namely active monomers of rat liver pyruvate carboxylase exhibit a sigmoidal velocity profile (Nakashima *et al.*, 1975), suggesting that inter-subunit interaction is not involved, and it is reported (Utter *et al.*, 1975) that only one molecule of acetyl-CoA binds per monomer.

Furthermore, when theoretical curves for pyruvate carboxylase activity were compared with the data of

Barritt *et al.* (1976) for the dependence of the enzyme's activity in intact isolated mitochondria on the intra-mitochondrial concentration of acetyl-CoA, the best fit was obtained when a Hill coefficient of unity (indicating lack of co-operativity) was used in the expression for acetyl-CoA saturation (J. R. Griffiths, personal communication).

To resolve these conflicting views, an examination was made of the factors which contribute to the sigmoidicity of the velocity profiles obtained for pyruvate carboxylase with various concentrations of acetyl-CoA.

Methods and Materials

Pyruvate carboxylase was purified from freeze-dried mitochondria as previously described (Easterbrook-Smith *et al.*, 1976), to specific activities in the range 17–32 units/mg (1 unit catalyses the formation of 1 μ mol of oxaloacetate/min at 30°C). The assay systems and procedures used for measuring the overall acetyl-CoA-dependent activity of sheep and chicken liver pyruvate carboxylases, either as the

amount of P_i released or as the amount of oxaloacetate synthesized, were as previously described (Easterbrook-Smith *et al.*, 1976), with details specific to the experiments herein being given in the Figure legends.

The assay conditions used for measuring the rate of acetyl-CoA deacylation are presented in the legend to Fig. 1. After incubation of the reaction mixture for the appropriate time, the reaction was stopped by the addition of 0.05 ml of 6M-HCl. Carrier sodium acetate (15 μ mol) was added, followed by 30 mg of activated charcoal in 0.3 ml of 1M-HCl. The resulting suspension was allowed to stand for 5 min. The charcoal was removed by centrifuging and the radioactivity determined in samples of the supernatant.

Other methods and materials used were as described previously (Ashman *et al.*, 1972; Ashman *et al.*, 1973; Easterbrook-Smith *et al.*, 1976). Where appropriate, data were analysed by using a FORTRAN linear regression programme. The sigmoidal profiles obtained from velocity measurements at various acetyl-CoA concentrations were fitted to the Hill equation by

Fig. 1. Dependence of the rate of acetyl-CoA deacylation on acetyl-CoA concentration for sheep and chicken liver pyruvate carboxylases

Sheep liver pyruvate carboxylase (○—○): the assay solution contained in a final volume of 0.4 ml (μ mol), Tris chloride, pH 8.4, 40; ATP, 1.0; $MgCl_2$, 2.6; sodium pyruvate, 4; $[1-^{14}C]$ acetyl-CoA (5.6×10^2 c.p.m./nmol) as indicated; 2.5 units of enzyme (specific activity 19 units/mg); the reaction time was 20 min at 30°C. Chicken liver pyruvate carboxylase (△---△): the assay solution contained, in a final volume of 0.4 ml (μ mol): Hepes (K^+ form), pH 7.2, 4; KCl, 80; EDTA, 0.4; $[1-^{14}C]$ acetyl-CoA (1.34×10^3 c.p.m./nmol) as indicated; 10 units of enzyme (specific activity 25 units/mg); the reaction time was 8 min at 30°C.

using the computer program developed by Vaughn *et al.* (1976).

Results

Dependence of acetyl-CoA deacylation on acetyl-CoA concentration

Preparations of pyruvate carboxylase of high specific activity, isolated from either sheep or chicken liver mitochondria, catalyse the slow deacylation of acetyl-CoA to CoA and acetate (Scrutton & Utter, 1967). The deacylase activity co-purifies with pyruvate carboxylase during both ion-exchange chromatography and gel filtration; it is also stimulated by the presence of the substrates of the carboxylation reaction. These facts imply that both the deacylase and carboxylase activities are associated with pyruvate carboxylase and that the deacylase activity is not due to a separate contaminating enzyme. Thus the deacylase activity provides a means of studying the interaction between acetyl-CoA and the enzyme.

Fig. 1 presents, in double-reciprocal form, data obtained from experiments designed to measure the rate of deacylation as a function of acetyl-CoA concentration in the presence of $MgATP^{2-}$, Mg^{2+} and pyruvate. The slope of the line (0.147 ± 0.004) and the vertical intercept (0.36 ± 0.03) yielded a K_m value of $40.9 \pm 4.7 \mu M$ for acetyl-CoA in this reaction and under these conditions. The relatively low standard errors of the parameters of the straight line suggest that acetyl-CoA does not bind to the enzyme in a positive homotropic co-operative manner.

The results presented in Fig. 1 further indicate that, under the conditions used by Frey & Utter (1977), there is not a sigmoidal response of the rate of deacylation by chicken pyruvate carboxylase to increasing acetyl-CoA concentrations. Moreover, the K_m value of $54 \mu M$ obtained from a linear regression analysis of the data may be used in conjunction with the maximum rate of deacylation (4 nmol/min per mg of protein) reported by Frey & Utter (1977) to estimate the extent of deacylation occurring during a binding study experiment. At an initial concentration of $10 \mu M$ $[1-^{14}C]$ acetyl-CoA, a protein concentration of 2.5 mg/ml and a 2.5 min time interval, it is possible to calculate, with the K_m and V values above, that approximately 35% of the radioactivity was present as $[1-^{14}C]$ acetate by the time the unlabelled acetyl-CoA was added. Given that the rate of diffusion of a molecule in solution is inversely proportional to its average radius, the $[1-^{14}C]$ acetate would be expected to diffuse across the binding cell membrane many times faster than the rate of the $[1-^{14}C]$ acetyl-CoA diffusion. This in turn would lead to an underestimate of the binding of acetyl-CoA by pyruvate carboxylase, particularly at low concentrations of the ligand, and hence it is difficult to assess the

significance of the h value of 1.9 reported by Frey & Utter (1977).

Elimination of the sigmoidal velocity profile for the carboxylation reaction

Since the results above suggest that acetyl-CoA did not bind to pyruvate carboxylase in a positive homotropic co-operative manner, the sigmoidal velocity profile obtained in the carboxylation reaction when the variable ligand was acetyl-CoA must be due to some special features of the assay system. The phenomenon of irreversible inactivation of the enzyme on dilution below approximately 4 units/ml (Ashman *et al.*, 1972) was considered to be one factor contributing to the atypical kinetic response. Acetyl-CoA protects the enzyme against this inactivation process, but below 40 μM -acetyl-CoA a rapid loss of enzymic activity occurs and is not recovered by the addition of more acetyl-CoA. The effect of this phenomenon on the velocity curve is that a falsely reduced rate of enzymic activity is observed at low concentrations of acetyl-CoA. To eliminate this factor as far as possible, an experiment was carried out with an enzyme concentration raised from the normal 0.1 unit/ml to 5.2 units/ml. To ensure that the reaction rate was linear with time, the incubation

period was reduced to 30s. In addition, the concentrations of all reaction components were raised to levels that were saturating regardless of the acetyl-CoA concentration (Ashman *et al.*, 1972). The results obtained (Fig. 2) in double-reciprocal form show a concave curving line, the opposite of the parabola expected with positive homotropic co-operativity. An explanation for this unexpected but reproducible result may be that, under these conditions, the only effect of varying the acetyl-CoA concentration would be on the proportion of enzyme catalysing the reaction at the acetyl-CoA-dependent rate compared with the acetyl-CoA-independent rate.

The fractions of total enzyme (E_T) with and without acetyl-CoA (E_A and E_0 respectively) bound are given by the expressions in eqns. (1) and (2), where A is the concentration of acetyl-CoA.

$$E_A = E_T/[1 + (K_a/A)] \quad (1)$$

$$E_0 = E_T/[1 + (A/K_a)] \quad (2)$$

If the velocity constants of the acetyl-CoA-dependent and -independent reactions are k_a and k_b respectively, then the observed velocity (v) will be given by the expression:

$$\begin{aligned} v &= k_a E_A + k_b E_0 \\ &= \frac{k_a E_T}{1 + \frac{K_a}{A}} + \frac{k_b E_T}{1 + \frac{A}{K_a}} \end{aligned} \quad (3)$$

Now $k_a E_T$ and $k_b E_T$ are the maximum velocities of the acetyl-CoA-dependent and -independent reactions respectively, therefore:

$$v = \frac{V^{(D)}}{1 + \frac{K_a}{A}} + \frac{V^{(I)}}{1 + \frac{A}{K_a}} \quad (4)$$

where $V^{(D)}$ and $V^{(I)}$ are the maximum velocities for the dependent and independent activities.

The theoretical curves generated from eqn. (4) by calculating the dependence of reaction velocity on acetyl-CoA concentration are presented in Fig. 3. The similarity of the double-reciprocal plot to the experimental data presented in Fig. 2 implies that the scheme presented here is a plausible model of acetyl-CoA interaction with pyruvate carboxylase.

Effect of pyruvate concentration on the velocity response curve for various acetyl-CoA concentrations

To reconcile the data shown in Fig. 2 with the previous reports of a sigmoidal velocity profile in the carboxylation reaction (Barritt *et al.*, 1966; Scrutton & Utter, 1967), it is possible to provide, in part, a

Fig. 2. Reciprocal of reaction velocity versus reciprocal acetyl-CoA concentration at saturating concentrations of all other reaction components with sheep pyruvate carboxylase

The assay contained, in a final volume of 0.5 ml (μmol): Tris chloride, pH 8.4, 40; ATP, 4; MgCl_2 , 17; pyruvate, 36; NH_4Cl , 83; $\text{NaH}^{14}\text{CO}_3$ (9.86×10^5 c.p.m./ μmol), 38; 2.6 units of enzyme. The reaction time was 30s.

plausible explanation by extending the studies of Ashman *et al.* (1972). Their investigations suggest that, when acetyl-CoA is the variable ligand, the fixed concentration of pyruvate, although saturating at high concentrations of acetyl-CoA, will become non-saturating as the concentration of acetyl-CoA is decreased. Hence one would expect the degree of sigmoidicity to be reduced as the concentration of pyruvate is raised. The family of curves shown in Fig. 4, where acetyl-CoA is the variable ligand and pyruvate is the fixed variable, confirms the above prediction. It can be seen that, at high concentrations of pyruvate, the deviation from linearity is least, but as the fixed pyruvate concentration is decreased the deviation from linearity increases. The data obtained from both sheep and chicken liver pyruvate carboxylases showing the effect of different concentrations of pyruvate on the h value for acetyl-CoA are summarized in Table 1.

Effect of hydrolysis of the enzyme-carboxybiotin complex on h for acetyl-CoA

Since the abortive hydrolysis of the enzyme-carboxybiotin complex is maximal at non-saturating concentrations of pyruvate (Easterbrook-Smith *et al.*, 1976), it might be expected that this could also be a factor contributing to the sigmoidicity of the velocity profile. The results shown in Fig. 5, where the reaction velocity was monitored by measuring both the P_i released and oxaloacetate synthesized,

Fig. 3. Theoretical curves generated from eqn. (4) in the text by calculating the dependence of reaction velocity on acetyl-CoA concentration

Curve A: the simulation of the acetyl-CoA-dependent term alone. Curve B: the simulation of the acetyl-CoA-independent term alone. Curve C: the simulation of the sum of both terms. The values of K_a , $V^{(D)}$ (i.e. the maximum velocity of the acetyl-CoA-dependent reaction) and $V^{(I)}$ (the maximum velocity of the acetyl-CoA-independent reaction) were $12 \mu\text{M}$, 10.0 and 2.5 respectively. Inset: the double reciprocal plot of curve (c).

Fig. 4. Effect on sheep pyruvate carboxylase of various acetyl-CoA concentrations at different fixed concentrations of pyruvate

The concentrations of pyruvate were (mM): 1.0 (●); 3.0 (○); 5.0 (▼); 10 (△); 20 (■).

Table 1. Effect of various pyruvate concentrations on the Hill coefficient (*h*) for acetyl-CoA

Concn. of pyruvate (mM)	<i>h</i>	
	Sheep enzyme	Chicken enzyme
1.00	3.40 ± 0.05	4.20 ± 0.19
3.00	2.77 ± 0.23	3.48 ± 0.17
5.00	2.15 ± 0.15	—
10.00	1.82 ± 0.04	2.24 ± 0.21
20.00	1.95 ± 0.15	2.22 ± 0.14
30.00	—	1.84 ± 0.20

Fig. 5. Acetyl-CoA kinetics of sheep pyruvate carboxylase. Reaction velocity was measured as the amount (μmol) of both P_i released (Δ) and oxaloacetate synthesized ($\bullet$).

demonstrate first of all that the velocity of the reaction differs for the two products. Secondly, the *h* value obtained by using the rate of oxaloacetate synthesis (h_{OAA}) as a measure of the reaction velocity was 2.08 ± 0.04 , whereas the value based on P_i released (h_{P_i}) was 1.68 ± 0.05 . (The values obtained with chicken liver pyruvate carboxylase were: h_{OAA} , 2.76; h_{P_i} , 2.43.) It can be seen from Fig. 5 that the ratio of P_i released to oxaloacetate synthesized varies as a function of acetyl-CoA concentration. Since the h_{OAA} value is higher than the h_{P_i} value it is concluded that the hydrolysis of the enzyme-substrate complex at low concentrations of acetyl-CoA is a factor contributing to the sigmoidicity of the velocity profile when the velocity is measured in terms of oxaloacetate production.

Effect of MgATP^{2-} and K^+ concentration on *h* for acetyl-CoA

The effect of varying non-saturating concentrations

Table 2. Effect of various MgATP^{2-} concentrations on the Hill coefficient (*h*) and apparent K_s for acetyl-CoA

The rate of oxaloacetate synthesis was determined as described by Easterbrook-Smith *et al.* (1976), 0.07 unit of sheep liver pyruvate carboxylase being used per assay. The acetyl-CoA concentrations used were $4\text{--}75 \mu\text{M}$; concentrations of Tris chloride, pH 8.4, pyruvate, bicarbonate and free Mg^{2+} were maintained at 90, 20, 20, and 2.5 mM respectively.

MgATP^{2-} (mM)	Apparent $K_s (\mu\text{M})$	<i>h</i>
2.5	11.1 ± 0.5	1.81 ± 0.14
5.0	14.4 ± 0.5	1.66 ± 0.19
7.5	15.9 ± 0.6	1.78 ± 0.11
10.0	18.3 ± 0.9	1.90 ± 0.13
12.5	24.0 ± 0.5	1.75 ± 0.04

of reaction components on the Hill coefficient was extended to include the effects of various fixed concentrations of MgATP^{2-} and K^+ . The significant point here is that Ashman *et al.* (1972) found that the apparent K_m for MgATP^{2-} was not influenced by the presence or absence of acetyl-CoA. Therefore the MgATP^{2-} concentration would remain saturating regardless of the acetyl-CoA concentration, and so varying the fixed MgATP^{2-} concentration would not be expected to influence the sigmoidicity of the acetyl-CoA velocity profile. The results of the experiments carried out to test this prediction are summarized in Table 2, and show that the MgATP^{2-} concentration used in the assay has no significant effect on *h* for acetyl-CoA activation of the enzyme.

The effect of the univalent cation concentration on the shape of the acetyl-CoA velocity curve was also investigated and in the range 0–15 mM had no significant effect on *h*. This finding was consistent with the observation of Barden & Scrutton (1974) that the apparent K_s for K^+ was independent of the acetyl-CoA concentration.

Discussion

Since the development of the concept of co-operativity of binding (Monod *et al.*, 1965; Koshland *et al.*, 1966) the observation of non-classical saturation kinetics for a given enzyme has usually been explained by asserting that the ligand binding is a co-operative process. However, on occasions it has been overlooked that the relationship between the equations describing non-classical binding and non-classical kinetics is not necessarily simple. This point has been demonstrated by the theoretical studies of a number of investigators (e.g., Dalziel, 1968; Goldbetter, 1974). Furthermore, alternative explanations are possible, including the multiple-reaction-pathway model (Sweeney & Fisher, 1968; Wells *et al.*, 1976), the

polymerizing enzyme schemes (Nichol *et al.*, 1967) and the mnemonical enzyme concept (Rabin, 1967; Ricard *et al.*, 1974). In addition, Fisher & Keleti (1975) have shown that if an enzyme is partially inactivated during the course of the reaction velocity determination, and if the substrate alters the rate of inactivation, then a sigmoid substrate saturation curve will be obtained. Examples of this phenomenon are available from studies with threonine deaminase (Harding, 1969) and the K^+ -stimulated AMP aminohydrolase (Hemphill *et al.*, 1971).

The results presented here indicate that part of the non-classical kinetic behaviour observed with acetyl-CoA can be explained by the ability of this ligand to protect pyruvate carboxylase against inactivation on dilution. A major cause of non-classical saturation behaviour for acetyl-CoA was the effect this ligand has on pyruvate binding and in turn on the non-productive hydrolysis of $[E_{CO_2}^{biotin}]$ (Easterbrook-Smith *et al.*, 1976). In view of (i) the lack of unequivocal experimental evidence to support the concept of positive homotropic co-operative binding of acetyl-CoA, (ii) the data presented here, these observations provide a plausible alternative explanation for the non-classical velocity profile obtained with this ligand. This conclusion is strongly supported by the fact that, when all of these anomalous phenomena have been eliminated, the kinetic response to increasing concentrations of acetyl-CoA is no longer sigmoidal but in fact shows a hyperbolic double-reciprocal response curve.

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