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SUBSTRATE-INHIBITION BY ACETYL-CoA IN THE CONDENSATION REACTION BETWEEN OXALOACETATE AND ACETYL-CoA CATALYZED BY CITRATE SYNTHASE FROM PIG HEART

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Summary

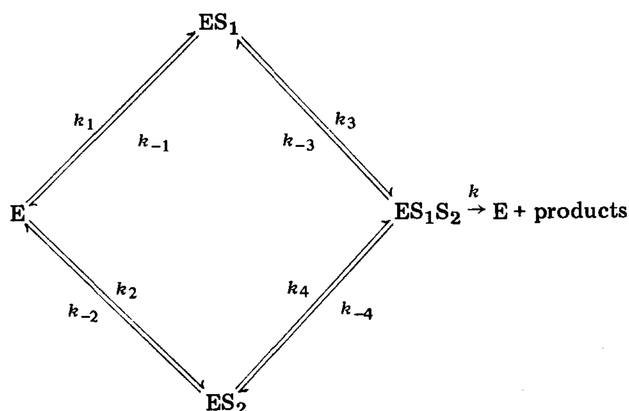
Deviations from Michaelis-Menten kinetics in the pig-heart citrate synthase (citrate-oxaloacetate-lyase(*pro*-3*S*-CH_2 · COO^- → acetyl-CoA), EC 4.1.3.7) system have been characterized and analyzed in view of the kinetic theory described in the preceding paper. The enzymic condensation reaction between acetyl-CoA and oxaloacetate is subject to substrate-inhibition by acetyl-CoA. This can be attributed to the formation of a productive enzyme-acetyl-CoA complex with a dissociation constant of 110 μM. The binding of acetyl-CoA to the enzyme decreases the on-velocity constant for oxaloacetate-binding from 4000 $min^{-1} \cdot \mu M^{-1}$ to 1700 $min^{-1} \cdot \mu M^{-1}$. The affinity of citrate synthase for oxaloacetate increases at least 20-fold on the binding of acetyl-CoA. The latter cooperativity effect can be attributed to a more than 45-fold decrease of the off-velocity constant for oxaloacetate-binding.

Introduction

Citrate synthase (citrate-oxaloacetate-lyase(*pro*-3*S*-CH_2 · COO^- → acetyl-CoA), EC 4.1.3.7) which catalyzes the initiating condensation reaction between acetyl-CoA and oxaloacetate in the citric acid cycle, operates by a ternary-complex mechanism [1—6]. Conversion of the ternary enzyme-substrate complex into a ternary enzyme-product complex has been suggested to be rate-limiting during catalysis [7,8]. Binary enzyme-substrate complexes are known to be formed with both oxaloacetate [6,9] and acetyl-CoA [10,11], indicating that the catalytic reaction proceeds basically by a random-order mechanism (Scheme 1). Under conditions where Michaelis-Menten kinetics prevail with respect to both substrates, however, the enzyme operates by an effectively ordered mechanism with oxaloacetate adding first to the enzyme [11,12]. Examination of the Michaelis-Menten behaviour of the system has, therefore, provided information mainly about the pathway for ternary-complex forma-

tion involving the binary enzyme-oxaloacetate complex [11].

According to the kinetic theory presented in the preceding paper, information about the alternative pathway (Scheme 1) to the productive ternary-complex might be obtained by examination of deviations from Michaelis-Menten kinetics possibly exhibited by the system.



Scheme 1. The random-order ternary-complex mechanism in view of which the present kinetic data are interpreted. E, S_1 and S_2 denote pig-heart citrate synthase, acetyl-CoA and oxaloacetate, respectively.

Such information would by valuable from a mechanistic point of view, as it might contribute to the understanding of cooperativity effects observed in the process of substrate-binding to the enzyme [10—12]. For this reason, and in order to test the applicability and validity of the relationships derived in the preceding paper, the second-degree rate-behaviour of citrate synthase from pig heart has now been characterized and analyzed.

Materials and Methods

Citrate synthase from pig heart was obtained from Boehringer Mannheim GmbH (Mannheim, G.F.R.) as a crystalline suspension in 2.2 M ammonium sulphate, and was used without further purification. The specific activity of the enzyme preparation used agreed within 5% with that of pure enzyme [13]. Acetyl-CoA was prepared by the method of Simon and Shemin [14], reaction products being separated by gel chromatography on Sephadex G-15. Other reagents used were of analytical grade.

Steady-state reaction velocities of the enzyme-catalyzed reaction between acetyl-CoA (5—2500 μM) and oxaloacetate (5—2500 μM) were determined at 27°C with a Zeiss PM QII spectrophotometer, using the colorimetric assay described by Srere et al. [1]. Reaction solutions contained about 0.2 nM enzyme, 250 μM 5,5′-dithiobis-(2-nitrobenzoic acid), and varied amounts of substrates in 0.1 M Tris/acetate buffer, pH 8.2.

Statistical procedures were routinely applied for evaluation of the kinetic data. Regression curves and parameter estimates were computed by iterative non-linear regression techniques described previously [15].

Results

The steady-state velocity of the condensation reaction between acetyl-CoA and oxaloacetate, catalyzed by citrate synthase from pig heart, was determined by standard spectrophotometric techniques over a wide range of substrate concentrations (5—2500 μM). Fig. 1 shows typical results in the form of Lineweaver-Burk plots with respect to acetyl-CoA at different fixed concentrations of oxaloacetate. As has been reported previously [6], approximately linear plots are obtained for acetyl-CoA concentrations below 60 μM. At higher concentrations of acetyl-CoA deviations from linearity typical of substrate-inhibition are observed, inhibition being most pronounced at low concentrations of oxaloacetate. When reactions were carried out in the presence of 500 μM oxaloacetate, no significant deviations from linearity could be detected even at the highest (2.5 mM) acetyl-CoA concentration tested.

Lineweaver-Burk plots with respect to oxaloacetate were found to be linear over the entire range of substrate concentrations used (Fig. 2).

The mechanistic information inherent in the asymptotically linear part of the Lineweaver-Burk plots in Fig. 1 has been considered previously [6,11,12], and the present kinetic analysis was directed towards the observed deviations from linearity. For each fixed concentration of oxaloacetate, linear asymptotes in Fig. 1 were defined by weighted least-squares fitting of a straight line to the data obtained for acetyl-CoA (S_1) concentrations between 5 and 60 μM. Differences (D_1) between the experimentally observed reciprocal reaction velocity and the value predicted by the linear asymptote were then calculated for acetyl-CoA concentrations above 200 μM. Fig. 3 shows that experimental points in plots of $1/D_1$ vs. $1/[S_1]$ fall well along straight lines at fixed concentrations of oxaloacetate. Fig. 4 indicates that slopes and intercepts of the regression lines in Fig. 3 are proportional to the concentration of oxaloacetate. Intercepts and curvature of the graphs in Fig. 4 were found to be of statistically insignificant magnitude.

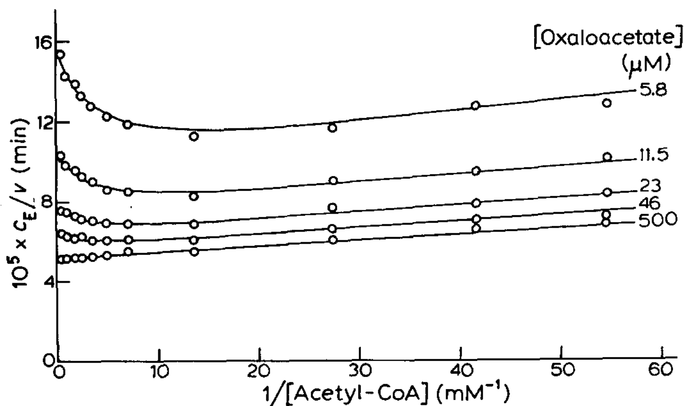


Fig. 1. Lineweaver-Burk plots with respect to acetyl-CoA for the citrate synthase-catalyzed reaction between acetyl-CoA and oxaloacetate. Reactions were performed at 27°C in 0.1 M Tris/acetate buffer, pH 8.2, containing about 0.2 nM enzyme and varied concentrations of substrates. Each experimental point represents the mean of 3—5 independent determinations of the molar enzymic reaction volocity (v/c_E).

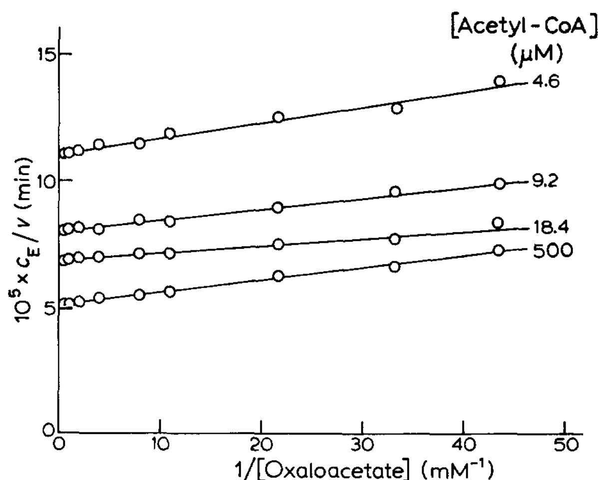
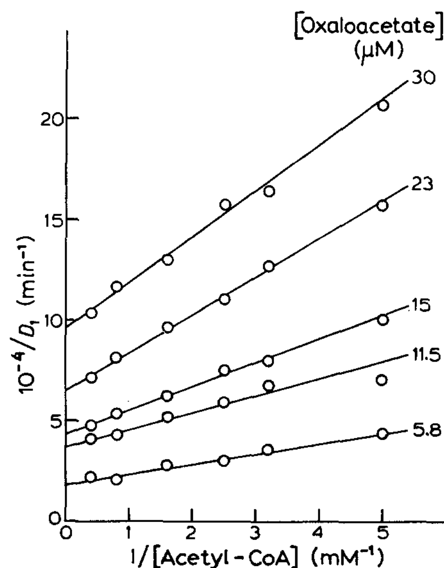


Fig. 2. Lineweaver-Burk plots with respect to oxaloacetate for the citrate synthase-catalyzed reaction between acetyl-CoA and oxaloacetate. Conditions as in Fig. 1.

These results establish that deviations from linearity in Lineweaver-Burk plots with respect to acetyl-CoA (S_1) at fixed concentrations of oxaloacetate (S_2) conform to the relationship

$$\frac{1}{D_1} = \frac{[S_2]}{\epsilon_1}\left(1 + \frac{K_{app}}{[S_1]}\right) . \tag{1}$$

Further, they show that K_{app} exhibits no significant variation with $[S_2]$ for



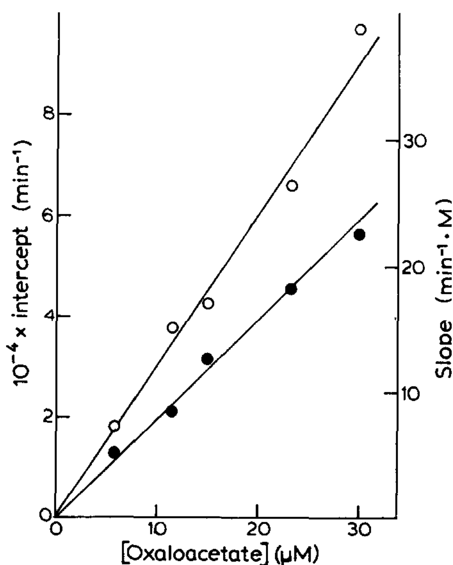


Fig. 3. Double-reciprocal plots of deviations (D_1) from linearity in Lineweaver-Burk plots with respect to acetyl-CoA at fixed low concentrations of oxaloacetate. Conditions as in Fig. 1.

Fig. 4. Replots of intercepts (o) and slopes (•) of the straight lines in Fig. 3 vs. the concentration of oxaloacetate.

oxaloacetate concentrations between 5 and 25 μM. Preliminary estimates of the inhibition parameters ϵ_1 and K_{app} were calculated from the slopes of the linear replots in Fig. 4. Final estimates were computed statistically by fitting Eqn. 1 to the total number of experimental observations, which gave $\epsilon_1 = 0.33$ ($\pm$0.03) min · nM and $K_{app} = 260$ ($\pm$40) μM.

Fig. 4 shows that deviations from Michaelis-Menten kinetics with respect to acetyl-CoA decrease towards zero with increasing concentrations of oxaloacetate. This observation provides direct evidence that deviations are caused by complex-formation between acetyl-CoA and free enzyme, which is the enzyme species to which oxaloacetate binds when Michaelis-Menten kinetics prevail. The data in Fig. 3, further, establish that the enzyme retains a definite activity at infinite concentration of acetyl-CoA. This means that the complex formed between free enzyme and acetyl-CoA is a productive one, i.e. it may combine with S_2 to form the productive complex ES_1S_2 in Scheme 1. It may be concluded that substrate-inhibition by acetyl-CoA is attributable to the utilization of alternative pathways for formation of the productive ternary-complex.

Consequently, the relationships derived in the preceding paper can be applied for evaluation of ϵ_1 and K_{app} in terms of rate constants in Scheme 1. For the ordered case considered here, these inhibition parameters were shown to be given by

$$\epsilon_1 = \frac{1}{k_3} - \frac{1}{k_2} \quad (2)$$

$$K_{app} = \frac{k_{-1}k_2}{k_1k_3} + \frac{k_2[S_2]}{k_1} \quad (3)$$

Assuming that $k_2 = 4000$ min^{-1} · μM^{-1} [11], the present estimate of ϵ_1 yields $k_3 = 1700$ ($\pm$150) min^{-1} · μM^{-1}. The concentration-independent estimate of K_{app} then corresponds to $k_{-1}/k_1 = 110$ ($\pm$15) μM. Further, it follows from the concentration-independence of K_{app} that $k_{-1} >> k_3[S_2]$ over the range of oxaloacetate concentrations considered in the determinations of K_{app} ($[S_2] <$ 30 μM). This means that $k_{-1} >> 50000$ min^{-1} and $k_1 >> 500$ min^{-1} · μM^{-1}.

Discussion

The second-degree rate-behaviour of citrate synthase from pig heart is, in all respects tested, consistent with that described in the preceding paper for an effectively ordered case of the random-order ternary-complex mechanism in Scheme 1. No realistic alternative mechanism of substrate-inhibition can be envisaged. The kinetic estimate of k_{-1}/k_1 obtained from the substrate-inhibition data (110 μM) agrees excellently with previous equilibrium determinations of the dissociation constant for the binary enzyme-acetyl-CoA complex (about 100 μM [10]; 130 μM [11]). This result provides a final confirmation of the reliability of the present interpretation of the kinetic data, and establishes the validity of the kinetic theory on which the interpretation is based.

The present investigation confirms the previous conclusion that citrate synthase from pig heart operates by an effectively ordered mechanism when

Michaelis-Menten kinetics prevail, oxaloacetate adding first to the enzyme under such conditions [11,12]. High concentrations of acetyl-CoA cause a shift of reaction flow from the pathway involving the enzyme-oxaloacetate complex to the pathway involving the enzyme-acetyl-CoA complex. This results in substrate-inhibition, since the rate of binding of oxaloacetate is higher in the former route (k_2 = 4000 min^{-1} · μM^{-1}) than in the latter one (k_3 = 1700 min^{-1} · μM^{-1}). According to Eqn. 2, substrate-activation would be obtained by the same basic mechanism if the presence of acetyl-CoA at the active center of the enzyme increases the on-velocity constant for oxaloacetate-binding ($k_3 > k_2$) instead of decreasing it. This provides a reasonable explanation for the mechanistic origin of the substrate-activation pattern observed at high concentrations of acetyl-CoA with citrate synthase from rat kidney and rat brain [16]. The present results thus suggest that citrate synthases from various animal sources (which yield closely similar parameter values for the Michaelis-Menten rate-behaviour [16]) operate by the same basic ternary-complex mechanism, with oxaloacetate binding first to the enzyme under Michaelis-Menten conditions.

Examination of the Michaelis-Menten behaviour of pig-heart citrate synthase has provided estimates of rate constants (k, k_2, k_{-2}, and the term $(k + k_{-4})/k \cdot k_4$) in the pathway involving the binary enzyme-oxaloacetate complex [11]. The present investigation provides estimates of rate and equilibrium constants in the alternative pathway to the ternary-complex, which makes it meaningful to discuss cooperativity effects in the process of substrate-binding to the enzyme. Introducing the magnitude of k_{-4} in relation to k as a variable α by putting $k_{-4} = \alpha k$, magnitudes of rate constants in Scheme 1 can be expressed as summarized in Table I. The cooperativity factor is then given by ($K_i = k_{-i}/k_i$)

$$\frac{K_1}{K_4} = \frac{K_2}{K_3} \approx 20\left(1 + \frac{1}{\alpha}\right) \qquad (4)$$

Eqn. 4 shows that acetyl-CoA and oxaloacetate are bound at least 20 times more firmly in the ternary enzyme-substrate complex than in the respective

TABLE I

KINETIC ESTIMATES OF RATE CONSTANTS IN SCHEME 1 FOR THE CONDENSATION REACTION CATALYZED BY CITRATE SYNTHASE

Data refer to the reaction at 27°C in 0.1 M Tris/acetate buffer, pH 8.2. Estimates have been calculated from Dalziel rate coefficients (ϕ_i), reported and interpreted previously [6,11], and from the substrate-inhibition parameters ϵ_1 and K_{app} determined in the present work. The parameter α is defined by $k_{-4} = \alpha k$.

Rate constant	Estimate	Determined from
k_1	>>500 min^{-1} · μM^{-1}	K_{app}
k_2	4 000 min^{-1} · μM^{-1}	ϕ_2
k_3	1 700 min^{-1} · μM^{-1}	ϵ_1
k_4	3 450(1 + α) min^{-1} · μM^{-1}	ϕ_1
k	20 400 min^{-1}	ϕ_0
k_{-1}	110 · k_1 min^{-1}	K_{app}
k_{-2}	20 000 min^{-1}	ϕ_{12}
k_{-3}	460α/(1 + α) min^{-1}	$K_1K_3 = K_2K_4$
k_{-4}	20 400α min^{-1}	Undetermined

binary complexes. The cooperativity factor becomes 20 when $\alpha >> 1$, but may be considerably larger if α is less than unity.

There are, in fact, some arguments favouring the possibility that α is less than unity ($k_{-4} < k$). Citrate synthase has the metabolic function of catalyzing the condensation reaction between acetyl-CoA and oxaloacetate unidirectionally. The potential catalytic capacity of the enzyme for the forward reaction is best utilized when $k >> k_{-3}$, k_{-4}, i.e. when the rate of breakdown of the ternary-complex into free enzyme and products greatly exceeds the rates of dissociation of substrates from the complex. Such conditions, according to the data in Table I, certainly prevail for oxaloacetate ($k_{-3} \leqslant 460$ min$^{-1} << k$), and would also be catalytically favourable as concerns the rate of dissociation of acetyl-CoA.

It may, further, be noted that on-velocity constants determined for the binding of substrates to enzymes of a size comparable to that of citrate synthase frequently have been found to be of a magnitude between 1000 and 6000 min^{-1} · μM^{-1}, consistent with that expected for a diffusion-controlled reaction [17—19]. The previous estimate k_2 = 4000 min^{-1} · μM^{-1} [11] may thus be typical for a diffusion-controlled specific binding of small ligands to the active center of citrate synthase. The possibility that $\alpha >> 1$ then seems rather unlikely, as it would imply that $k_4 >> 4000$ min^{-1} · μM^{-1} (see Table I). A value of α less than unity, on the other hand, would give a k_4 value closely similar to that of k_2. The idea that binding rates for both substrates are mainly controlled by diffusion factors, i.e. that k_1, k_2, k_3, and k_4 are all of similar orders of magnitude, is attractive and consistent with the present results. The fact that k_3 for closely related citrate synthases may be either larger or smaller than k_2, yielding a kinetic pattern of either substrate-activation or substrate-inhibition, can then be understood in terms of minor changes of the effective surface for enzyme-oxaloacetate encounter, caused by the presence of acetyl-CoA at the active center of the enzyme.

Curves in Fig. 1, which fit well to the experimental data, have been calculated from the full rate-equation corresponding to Scheme 1 [20] using rate constants according to Table I with the assumption that $k_1 = k_4$ and $\alpha = 1$. Closely similar curves are obtained over a fairly wide range of α values larger or smaller than unity. Similarly, the magnitude of k_1 can be varied considerably without any significant effect on the fit of the curves. No firm conclusion as to the magnitude of k_1 and k_{-4} can, therefore, be drawn from the experimental data available at present.

The precise magnitude of the cooperativity factor (Eqn. 4) for substrate binding to the enzyme thus remains uncertain. It can be concluded from Eqn. 4, however, that the affinity of citrate synthase for either substrate increases at least 20-fold on binding of the second substrate. In the case of oxaloacetate, the present determination of k_3 makes it possible to draw the additional conclusion that this cooperativity effect cannot be attributed to an increased magnitude of the on-velocity constant (which actually decreases from 4000 to 1700 min^{-1} · μM^{-1}), but derives from an at least 45-fold decrease in magnitude of the off-velocity constant (from 20000 to less than 460 min^{-1}). A similar situation is likely to be at hand as concerns the cooperative effect of oxaloacetate on the binding of acetyl-CoA.

Kinetic and equilibrium studies of the binding of substrate analogues to citrate synthase might be expected to provide more detailed information about the mechanistic background for the cooperativity effects observed. Such work is in progress.

References

1 Srere, P.A., Brazil, H. and Gonen, L. (1963) Acta Chem. Scand. 17, S129—S134
2 Shepherd, D. and Garland, P.B. (1969) Biochem. J. 114, 597—610
3 Hochachka, P.W. and Lewis, J.K. (1970) J. Biol. Chem. 245, 6567—6573
4 Moriyama, T. and Srere, P.A. (1971) J. Biol. Chem. 246, 3217—3222
5 Smith, C.M. and Williamson, J.R. (1971) FEBS Lett. 18, 35—38
6 Johansson, C.J., Mahlén, A. and Pettersson, G. (1973) Biochim. Biophys. Acta 309, 466—472
7 Bové, J., Martin, R.O., Ingraham, L.L. and Stumpf, P.K. (1959) J. Biol. Chem. 234, 999—1003
8 Kosicki, G.W. and Srere, P.A. (1961) J. Biol. Chem. 236, 2566—2570
9 Srere, P.A. (1966) J. Biol. Chem. 241, 2157—2165
10 Weidman, S.W., Drysdale, G.R. and Mildvan, A.S. (1973) Biochemistry 12, 1874—1883
11 Johansson, C.J. and Pettersson, G. (1974) Eur. J. Biochem. 42, 383—388
12 Johansson, C.J. and Pettersson, G. (1974) Eur. J. Biochem. 46, 5—11
13 Singh, M., Brooks, G.C. and Srere, P.A. (1970) J. Biol. Chem. 245, 4636—4640
14 Simon, E.J. and Shemin, D. (1953) J. Am. Chem. Soc. 75, 2520—2524
15 Pettersson, G. and Pettersson, I. (1970) Acta Chem. Scand. 24, 1275—1286
16 Matsuoka, Y. and Srere, P.A. (1973) J. Biol. Chem. 248, 8022—8030
17 Hammes, G.G. (1968) Adv. Protein Chem. 23, 1—57
18 Wong, J.T.F. (1975) Kinetics of Enzyme Mechanisms, pp. 187—226, Academic Press, London
19 Gutfreund, H. (1972) Enzymes: Physical Principles, pp. 159—228, Wiley Interscience, London
20 Pettersson, G. (1972) Biochim. Biophys. Acta 276, 1—11