Biochimica et Biophysica Acta, 309 (1973) 466–472

BBA 66920

KINETIC STUDIES ON CITRATE SYNTHASE FROM PIG HEART

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(Received January 15th, 1973)

SUMMARY

The kinetic properties of citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating) EC 4.1.3.7) from pig heart have been investigated. Inconsistencies in previous results concerning the enzyme-catalyzed reaction between acetyl-CoA and oxaloacetate were found to be due to an overestimation of the stability constant for the binary enzyme–oxaloacetate complex. Studies of the protective effect of oxaloacetate on the urea-induced denaturation of citrate synthase gave evidence for the presence of two oxaloacetate-binding sites per enzyme molecule. The kinetic data obtained are most readily interpreted in terms of a rapid-equilibrium type of ternary-complex mechanism with non-cooperative substrate binding. A compulsory-order type of mechanism in which oxaloacetate adds first to the enzyme cannot, however, be definitely excluded.

INTRODUCTION

Citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) occupies a key position in the metabolism of most organisms, and several investigations have been directed towards the kinetic and regulatory properties of this enzyme. There is strong evidence that the citrate synthase-catalyzed condensation reaction between acetyl-CoA and oxaloacetate proceeds by a ternary-complex mechanism, and kinetic results have usually been interpreted as being indicative of a rapid-equilibrium type of random-order mechanism in which the binding of each substrate is unaffected by the presence of the other substrate[1–5]. The main evidence claimed to support this idea is the observation that the K_m value for each substrate is independent of the concentration of the co-substrate.

The steady-state rate behaviour of enzymes operating by a random-order ternary-complex mechanism is, however, fairly complex, and the mechanistic significance of concentration-independent K_m values has not been analyzed in a generalized way. Furthermore, Srere[6] has reported that a binary complex is formed between citrate synthase and oxaloacetate and that complex formation results in an increased stability towards urea denaturation. The dissociation constant obtained

from urea denaturation experiments was found to be one order of magnitude lower than the K_m value for oxaloacetate. This observation is not consistent with a rapid-equilibrium type of mechanism and, in fact, not consistent with any special case of a ternary-complex mechanism (see Discussion).

It was, therefore, considered of great interest to reinvestigate the rate behaviour of citrate synthase with particular reference to the above inconsistency, and to examine the mechanistic significance of the kinetic data in view of generalized relationships derived by the steady-state kinetic asymptote theory[7]. Initial-velocity parameters have now been determined for the enzymatic reaction between acetyl-CoA and oxaloacetate, and binding constants for the latter substrate were estimated by the urea denaturation technique, using an experimental approach which permits an evaluation of the number of substrate-binding sites in citrate synthase to be made.

EXPERIMENTAL

Materials

Citrate synthase from pig heart was obtained from Boehringer Mannheim as a crystalline suspension in 2.2 M $(NH_4)_2SO_4$, and was used without further purification. The specific activity of the protein preparations used was between 87 and 95% of that of pure enzyme[8]. Other reagents used were of analytical grade.

Methods

The initial-velocity behaviour of citrate synthase was determined at 26.5 °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 various amounts of substrates in 0.1 M Tris–acetate buffer (pH 8.2). Empirical rate equations were established by standard graphical and statistical methods described elsewhere[9].

Urea denaturation experiments were performed at 26.5 °C in 0.1 M Tris–acetate buffer (pH 8.2) according to the procedure described by Srere[6]. The concentration of enzyme, calculated on the basis of specific activity measurements, was varied from 1 to 10 μM, whereas the urea concentration was kept at 6.5 M. Concentrations of oxaloacetate were varied between 0 and 65 μM. Apparent first-order rate constants for the urea-induced exposure of sulphydryl groups in the protein were determined graphically.

RESULTS

Kinetic experiments

The kinetics of the pig heart citrate synthase-catalyzed reaction between acetyl-CoA and oxaloacetate were studied over a fairly wide range of substrate concentrations (5–500 μM acetyl-CoA and 10–1000 μM oxaloacetate) using about 0.2 nM enzyme. Lineweaver–Burk graphs with respect to oxaloacetate were invariably found to be linear, whereas the corresponding graphs with respect to acetyl-CoA exhibited a curvature characteristic of substrate inhibition at acetyl-CoA concentrations above 60–100 μM. Substrate inhibition was most pronounced at low levels of oxaloacetate.

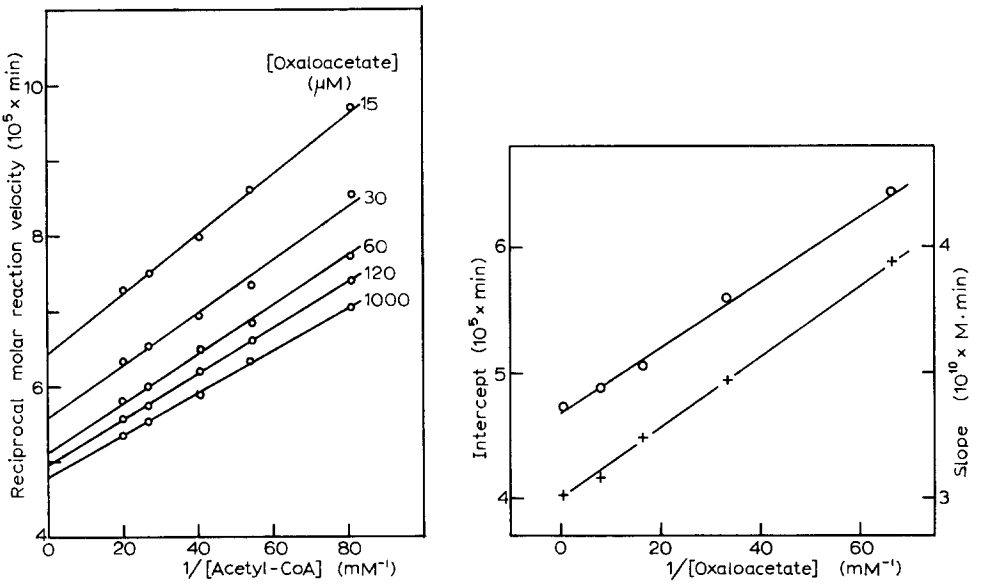


Fig. 1. Lineweaver–Burk plots with respect to acetyl-CoA for the citrate synthase-catalyzed reaction between acetyl-CoA (5–60 μM) and oxaloacetate (15–1000 μM). Reactions were performed at 26.5 °C in 0.1 M Tris–acetate buffer (pH 8.2), using about 0.2 nM enzyme.

Fig. 2. Replots of slopes (+) and intercepts (○) of the straight lines obtained in Fig. 1 *vs* the reciprocal oxaloacetate concentration.

For these reasons, quantitative evaluation of the kinetic data was restricted to observations made at acetyl-CoA concentrations below 60 μM.

Fig. 1 shows Lineweaver–Burk graphs with respect to acetyl-CoA (below 60 μM) at various fixed concentrations of oxaloacetate. Replots of slopes and intercepts of the straight lines obtained in Fig. 1 *vs* the reciprocal oxaloacetate concentration were linear (Fig. 2), indicating that the reaction conforms to an asymptotic rate equation of the Dalziel type[10]

$$1/v = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2] + \Phi_{12}/[S_1][S_2] \qquad (1)$$

where v denotes the molar enzymatic reaction velocity, and S_1 and S_2 stand for acetyl-CoA and oxaloacetate, respectively. Preliminary estimates of the Dalziel coefficients Φ_i were obtained graphically using the plotting methods indicated by Figs 1 and 2. Consistent estimates were obtained from Lineweaver–Burk graphs with respect to oxaloacetate at fixed concentrations of acetyl-CoA. Final estimates of Dalziel coefficients were computed statistically by weighted fitting of Eqn 1 to the experimental material, which gave $\Phi_0 = 4.9 \cdot 10^{-5}$ min, $\Phi_1 = 2.9 \cdot 10^{-10}$ min·M, $\Phi_2 = 2.5 \cdot 10^{-10}$ min·M and $\Phi_{12} = 1.5 \cdot 10^{-15}$ min·M^2.

For a comparison with previous kinetic results referring to citrate synthase[1–5], which have been evaluated in terms of a Michaelis–Menten equation, it may be observed that K_m for S_1 at fixed concentrations of S_2 is given by

$$K_m(S_1) = (\Phi_1 + \Phi_{12}/[S_2])/(\Phi_0 + \Phi_2/[S_2]) \qquad (2)$$

If $K_m(S_1)$ is independent of $[S_2]$ over a range of substrate concentrations where none

of the four terms in Eqn 1 can be completely neglected, the numerator and denominator on the right hand side of Eqn 2 must contain a common $[S_2]$-dependent factor, which implies that

$$\Phi_0 \Phi_{12} = \Phi_1 \Phi_2 \tag{3}$$

and that $K_m(S_1)$ equals the quotient Φ_{12}/Φ_2. It can, similarly, be shown that K_m for S_2 under Condition 3 becomes independent of $[S_1]$ and equals the quotient Φ_{12}/Φ_1. In the present investigation the ratio $\Phi_0\Phi_{12}/\Phi_1\Phi_2$, calculated on the basis of statistical estimates of Dalziel coefficients for a series of experiments, was found to vary between 0.8 and 1.2, which appears to confirm that K_m for each substrate is independent of the concentration of the co-substrate. The K_m values corresponding to the Dalziel coefficients given above are 6 μM for acetyl-CoA and 5 μM for oxaloacetate, and agree well with those reported previously for the pig heart enzyme[1,2].

Urea-denaturation experiments

Srere[6] has shown that the protective effect of oxalocaetate on the urea-induced denaturation of citrate synthase can be used to estimate the stability constant K_2 for the binary enzyme–oxaloacetate complex from the relationship

$$\log (k_0/k - 1) = \log K_2 + n \log [S_2] \tag{4}$$

where k_0 and k denote the apparent first-order rate constant for exposure of sulphydryl groups on denaturation of the enzyme in the absence and presence, respectively, of oxaloacetate. The integer n stands for the number of oxaloacetate molecules bound per active site of the protein, and was found to equal unity. The stability constant calculated by Srere from double-logarithmic plots corresponding to Eqn 4 was $K_2 = 1.6\,\mu\text{M}^{-1}$.

A modified and more sensitive method for determination of K_2 from urea-denaturation data was employed in the present work. A preliminary analysis of the experimental results, using the double-logarithmic plotting method of Srere, confirmed that n in Eqn 4 equals unity, which means that Eqn 4 reduces to

$$k_0/k - 1 = K_2[S_2] \tag{5}$$

Assuming that each enzyme molecule contains m non-cooperative oxaloacetate-binding sites, the following relationships involving the apparent equilibrium concentration $[ES_2]$ of the enzyme–oxaloacetate complex are valid

$$[ES_2] = K_2[E][S_2] \tag{6}$$

$$c_E = [E] + [ES_2] \tag{7}$$

$$c_{S_2} = [S_2] + m\cdot[ES_2] \tag{8}$$

where c_E and c_{S_2} stand for the total concentration of enzyme and oxaloacetate, respectively. Eqns 6–8 may be solved for $[S_2]$ with elimination of $[E]$ and $[ES_2]$, and insertion of the solution into Eqn 4 yields

$$k_0/k - 1 = 0.5\,[\sqrt{1(+ mK_2c_E - K_2c_{S_2})^2 + 4K_2c_{S_2}} - (1 + mK_2c_E - K_2c_{S_2})] \tag{9}$$

For large values of c_{S_2} Eqn 9 approaches the linear relationship

$$k_0/k - 1 = K_2c_{S_2} - mK_2c_E \tag{10}$$

i.e. a graph of k_0/k—1 *vs* c_{S_2} approaches a linear asymptote with the slope K_2 and an intercept equal to mc_E.

Fig. 3 shows a plot of k_0/k—1 *vs* c_{S_2} for a typical experiment in which a moderately high enzyme concentration was used ($c_E = 2.49\ \mu M$). The curve drawn in Fig. 3 corresponds to $m = 2$ and $K_2 = 0.153\ \mu M^{-1}$, which were the best-fit parameter values in Eqn 9 calculated by iterative non-linear regression analysis of the experimental data for integer values of m. Similar experiments were performed at various enzyme concentrations between 1 and 10 μM, and consistently gave best fits for $m = 2$, strongly suggesting that there are two non-cooperative oxaloacetate-binding sites per enzyme molecule. The mean value of K_2 obtained from these experiments was 0.17 ($\pm$ 0.03) μM^{-1}. The reason for the discrepancy between the present estimate of K_2 and that obtained by Srere[6] is not clearly understood, but may at least partly be due to the use of a more precise model (Eqn 9) in the present investigation.

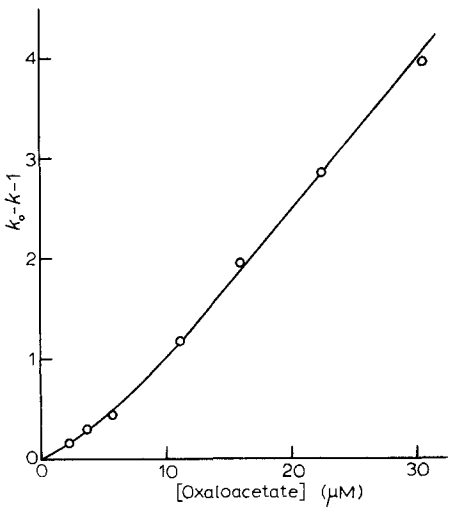


Fig. 3. The protective effect of oxaloacetate on the urea-induced denaturation of citrate synthase. The apparent first-order rate constant for exposure of sulphydryl groups in the absence (k_0) and presence (k) of various amounts of oxaloacetate was determined at 26.5 °C using 6.5 M urea in 0.1 M Tris–acetate buffer (pH 8.2) containing 2.49 μM enzyme and 0.25 mM 5,5′-dithiobis-(2-nitrobenzoic acid). The curve drawn was calculated by iterative regression analysis, and corresponds to Eqn 9 with $m = 2$ and $K_2 = 0.153\ \mu M^{-1}$.

DISCUSSION

The rate-behaviour of enzymes operating by the generalized ternary-complex mechanism shown in Scheme 1 has recently been analyzed by application of the steady-state kinetic asymptote theory[11,12], and it was found that Dalziel coefficients for such reactions are interrelated through

$$\Phi_1 = \Phi_{12}K_2(1 + P_1) \quad (11)$$

$$\Phi_2 = \Phi_{12}K_1(1 + P_2) \quad (12)$$

where $K_i = k_i/k_{-i}$ and P_i are mechanism-characterizing quantities which may attain any positive value[7]. A rapid-equilibrium type of mechanism corresponds to $P_1 =$

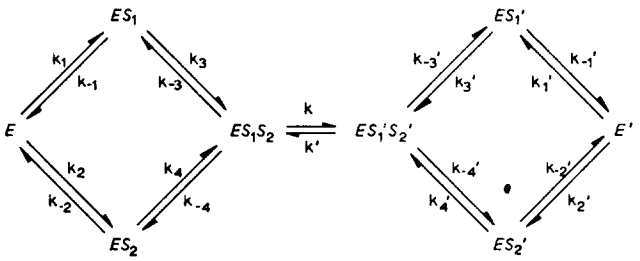
$P_2 = 0$, while a compulsory-order mechanism in which S_1 (S_2) adds first to the enzyme is characterized by $P_1 \gg 1$ and $P_2 = 0$ ($P_1 = 0$ and $P_2 \gg 1$). Eqns 11 and 12 and Condition 3 imply that

$$K_m(S_1) = \Phi_{12}/\Phi_2 = 1/K_1(1 + P_2) \leq 1/K_1 \quad (13)$$

$$K_m(S_2) = \Phi_{12}/\Phi_1 = 1/K_2(1 + P_1) \leq 1/K_2 \quad (14)$$

showing that concentration-independent K_m values in a ternary-complex mechanism always equal or are less that the dissociation constant for the corresponding binary enzyme–substrate complex.

As was pointed out in the introduction, previous results concerning the citrate synthase-catalyzed reaction between acetyl-CoA and oxaloacetate are inconsistent with this implication of the asymptote theory in yielding a K_m for oxaloacetate which exceeds the dissociation constant for the enzyme–oxaloacetate complex by a factor of about 10. There are three possible explanations for this inconsistency: The magnitude of K_m for oxaloacetate may have been overestimated, the magnitude of the corresponding dissociation constant may have been underestimated, and the reaction may proceed by an entirely different mechanism than the one shown in Scheme 1.



Scheme 1. The generalized ternary-complex mechanism in view of which the present kinetic results are discussed. E, S_1 and S_2 stand for citrate synthase, acetyl-CoA and oxaloacetate, respectively.

The steady-state kinetic parameter values obtained in the present investigation are fully consistent with those reported previously[1,2]. The dissociation constant ($1/K_2$) for the binary enzyme–oxaloacetate complex, on the other hand, was found to be considerably larger than the value of 0.6 μM reported by Srere[6] and, within the actual experimental precision, agrees well with $K_m = 5$ μM for oxaloacetate. The present results thus remove the previous inconsistency, and there is no reason to doubt that citrate synthase operates by a ternary-complex mechanism.

Previous suggestions that citrate synthase operates by a rapid-equilibrium type of ternary-complex mechanism have been based on the observation that K_m for either substrate is independent on the concentration of the co-substrate[3,4]. Concentration-independent K_m values are obtained as soon as Condition 3 is fulfilled, and in the case of a ternary-complex mechanism merely indicate that

$$(A + B)(A + R) = (1 + P_1)(1 + P_2)K_1/K_4 \quad (15)$$

in the terminology defined elsewhere[7]. Even though Eqn 15 is consistent with a rapid-equilibrium mechanism ($P_1 = P_2 = 0$) in which interconversion of ternary complexes is slow in comparison to other reaction steps ($A \gg B, R$) and in which

substrate-binding to the enzyme is non-cooperative ($K_1 = K_4$), there are various other conditions under which Eqn 15 is valid. The mere fact that concentration-independent K_m values are obtained does not, therefore, exclude that the mechanism is of a compulsory-order type. The present observation that K_m for oxaloacetate agrees with the dissociation constant for the binary enzyme–oxaloacetate complex, on the other hand, provides direct evidence that P_1 in Eqn 11 is of negligible magnitude, for which reason a compulsory-order mechanism in which acetyl-CoA adds first to the enzyme can be ruled out. Unfortunately, there is no similar direct information about the magnitude of P_2; the formation of a binary complex between citrate synthase and acetyl-CoA has not, hitherto, been experimentally demonstrated, and the magnitude of the corresponding dissociation constant is unknown.

It may be concluded that the present results are most readily interpreted in view of a ternary-complex mechanism of the rapid-equilibrium type with non-cooperative substrate-binding. It should be emphasized, however, that neither the present, nor previous, kinetic results definitely exclude that citrate synthase operates by a compulsory-order mechanism in which oxaloacetate adds first to the enzyme. Such a mechanism is not implausible. Combination of oxaloacetate to the enzyme appears to induce conformational changes[6,13] which may facilitate the binding of acetyl-CoA. There is also strong evidence that binding of oxaloacetate is necessary before citrate synthase exerts any labilizing effect on acetyl-CoA[14]. Whether or not kinetic data for the effect of inhibitors on citrate synthase catalyzed reactions are in consistence with a compulsory-order mechanism remains to be shown.

ACKNOWLEDGEMENT

This work was supported by grants from the Swedish Natural Science Research Council. Thanks are due to Prof. P. A. Srere, Dallas, for valuable advice and discussions during the course of this investigation.

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