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IMMOBILIZED CITRATE SYNTHASE

AMAL MUKHERJEE[1] and PAUL A. SRERE

Pre-Clinical Science Unit of Veterans Administration Hospital
and
Department of Biochemistry
University of Texas Health Science Center
Dallas, Texas

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Kinetic properties of pig heart citrate synthase immobilized on Sepharose were determined. Compared to the free enzyme the K_m values for both acetyl-CoA and oxalacetate were increased. The kinetic pattern of the Lineweaver–Burk plots of both substrates for the immobilized enzyme was that of lines intersecting on the x axis. This is the same as that obtained for the free enzyme and indicates that there is no change in the kinetic mechanism of the reaction. The pH response and the Arrhenius plot of both the immobilized and free enzyme were the same. The enzymes show a break in their Arrhenius plots. The immobilized enzyme exhibits greater heat stability than does the free enzyme.

INTRODUCTION

In a recent paper, Srere et al. (1) showed that an immobilized three-enzyme system of malate dehydrogenase, citrate synthase, and lactate dehydrogenase which catalyzed the overall reaction

$$\text{Ac-CoA} + \text{malate} + \text{pyruvate} \rightarrow \text{citrate} + \text{CoA} + \text{lactate}$$

operated at about two times the rate of a comparable soluble enzyme system. The model was prepared and studied in an attempt to assess differences in the kinetics that might arise when enzymes which may be normally immobilized *in situ* were studied *in vitro*.

Since the rate-limiting step in this immobilized system was citrate synthase, we have studied the kinetics of this immobilized enzyme alone in order to better assess the results of the more complex enzyme system. This paper presents the results of our studies which compare the steady state kinetics, pH behavior, and thermal stability of pig heart citrate synthase *in vitro* and immobilized on Sepharose beads.

[1] Present address: Department of Internal Medicine, University of Texas Health Science Center, 5323 Harry Hines Blvd., Dallas, Texas 75235.

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MATERIALS AND METHODS

CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Oxalacetic acid was obtained from Calbiochem, and CoA was purchased from P-L Biochemicals. Citrate synthase (from pig heart) in ammonium sulfate was purchased from Boehringer Knoll (Germany), and ATP was a product of Sigma Chemical Company (St. Louis, Missouri). Acetyl-CoA was prepared by a modification of the procedure of Simon and Shemin (2).

Preparation of Immobilized Citrate Synthase

One gram of activated Sepharose 4B was swollen in 100 ml of 1 mM HCl for about an hour. It was then washed with 150–200 ml 1 mM HCl. The gel was washed with 100–150 ml of water and then with 200 ml of cold 0.1 M $NaHCO_3$. Citrate synthase (25 μl of the 10-mg/ml ammonium sulfate suspension) was dissolved in 10 ml of cold 0.1 M $NaHCO_3$. About 1 g of the washed activated Sepharose was added to this solution and the suspension was incubated overnight at 4°C in a rotating test tube. The enzyme–gel preparation was then washed for 1 h with each of the following cold solutions: 0.1 M $NaHCO_3$, 0.5 M NaCl, 0.1 M $NaHCO_3$, and finally with water. The immobilized enzyme was stored at 4°C. For some studies the preparation was lyophilized and stored at 4°C. For enzyme assay, an aliquot of diluted immobilized enzyme (while stirring) was used. Linearity in enzyme activity was obtained with increase in the volume of the aliquot.

The assay for immobilized citrate synthase was the same as that described by Srere et al. (1). The soluble enzyme was assayed as described previously (3). The reactions were carried out at 25°C and initiated by the addition of oxalacetate. The measurements were made in a Hitachi No. 124 double beam spectrophotometer with the attachment of a Hitachi 165 recorder. One unit of enzyme is the amount of enzyme that catalyzes the liberation of 1 μmol of CoA per minute.

Initial velocity measurements were plotted in the Lineweaver–Burk manner, and the line was obtained by the least squares method.

RESULTS

The apparent Michaelis constants for both substrates were determined for both the free enzyme and immobilized systems from the Lineweaver–Burk plots (Figs. 1 and 2). Variation of one substrate at a series of fixed levels of cosubstrate gave apparent Michaelis constants for acetyl-CoA (AcCoA)

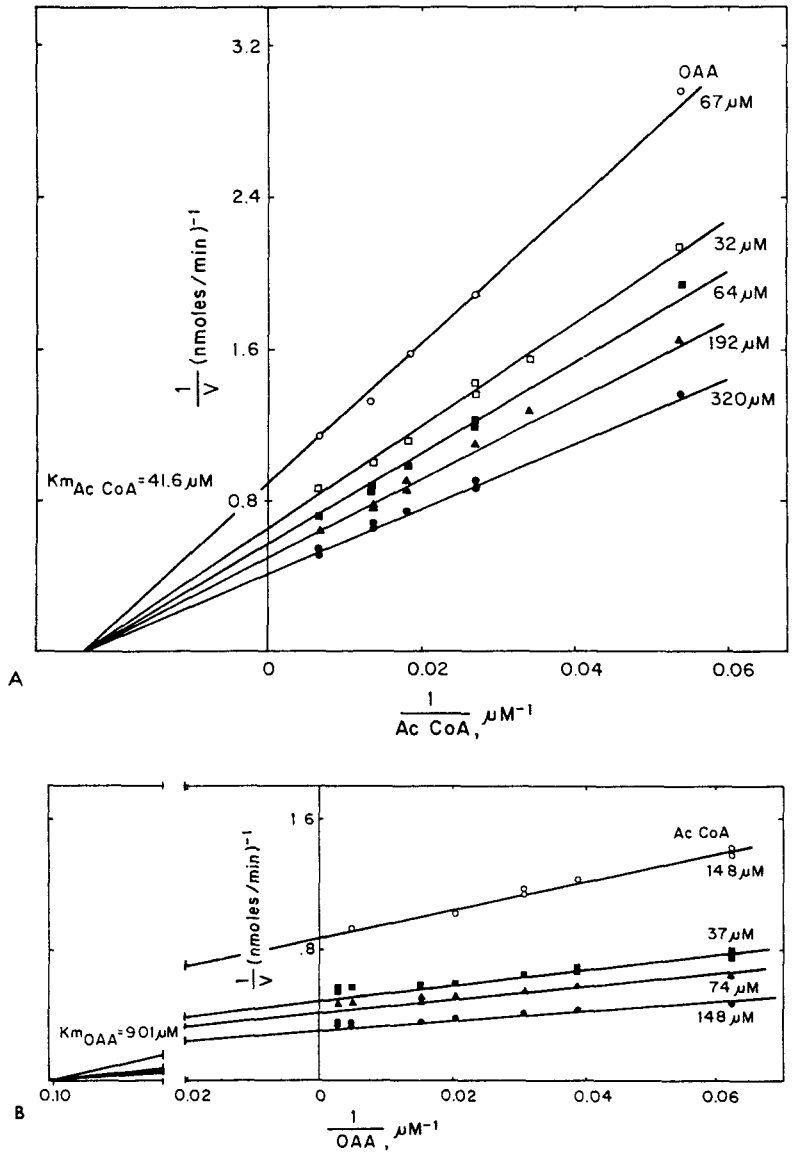

FIG. 1. (a) Double reciprocal plots of the initial velocities for the immobilized pig heart citrate synthase with AcCoA as the varied substrate. The reaction was carried out at 25°C in a total volume of 11.9 ml with 100 mM Tris-Cl buffer, pH 8.1, 0.2 mM DTNB, 18.5–148 μM AcCoA, and 6.7–320 μM OAA. The reaction was initiated by addition of OAA. (b) Double reciprocal plots of the initial velocities for the immobilized pig heart citrate synthase with OAA as the varied substrate. The reaction was carried out at 25°C in a total volume of 11.9 ml with 100 mM Tris-Cl buffer, pH 8.1, 0.2 mM DTNB, 16–320 μM OAA, and 14.8–148 μM AcCoA. The reaction was initiated by addition of OAA.

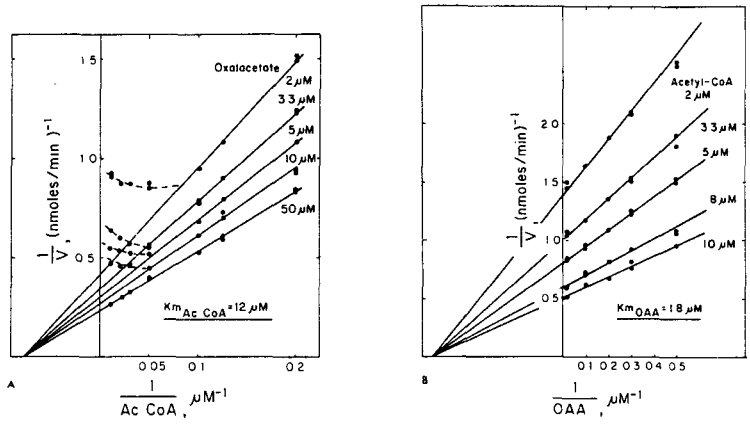

FIG. 2. (a) Double reciprocal plots of the initial velocities for the free pig heart citrate synthase with AcCoA as the varied substrate. The reaction was carried out at 25°C in a total volume of 1.0 ml with 100 mM Tris-Cl buffer, pH 8.1, 0.1 mM DTNB, 2–50 μM OAA, 5–100 μM AcCoA, and 0.15 μg of the enzyme. The reaction was initiated by addition of OAA. (b) Double reciprocal plots of the initial velocities for the free pig heart citrate synthase with OAA as the varied substrate. The reaction was carried out at 25°C in a total volume of 1.0 ml with 100 mM Tris-Cl buffer, pH 8.1, 0.1 mM DTNB, 2–50 μm OAA, 2–10 μM AcCoA; and 0.15 μg of the enzyme. The reaction was initiated by addition of OAA.

and oxalacetate (OAA) that were independent of the concentration of the cosubstrate. For immobilized pig heart citrate synthase, the apparent K_m for AcCoA was 41.6 μM as determined with OAA concentrations of 67–320 μM (Fig. 1a), and the apparent K_m for OAA was 9.0 μM as determined with AcCoA concentrations of 14.8–148 μM (Fig. 1b). Using the (unbound) pig heart citrate synthase, the apparent K_m for AcCoA was 12 μM as determined with OAA concentrations of 2–50 μM (Fig. 2a), and the apparent K_m for OAA was 1.8 μM as determined with AcCoA concentrations of 2–10 μM (Fig. 2b).

Both free and immobilized pig heart citrate synthase are inhibited by ATP. Plots of the reciprocal initial reaction velocity of immobilized citrate synthase against ATP concentration at AcCoA concentrations of 14.8–74 μM and a concentration of 32 μM OAA showed that the inhibition was the competitive type with respect to AcCoA with a K_i of 2.35 mM (Fig. 3). A K_i of 1.1 mM was calculated for ATP for free pig heart citrate synthase from the data of Kosicki and Lee's experiment (4).

The plot of activity against pH of an immobilized enzyme did not reveal any change in either the position of the pH optimum or in the shape of the curve when compared to the pH–activity curve of the free enzyme. The optimum activity range is broad, and for both forms of the enzyme is located around pH 8.7 (Fig. 4).

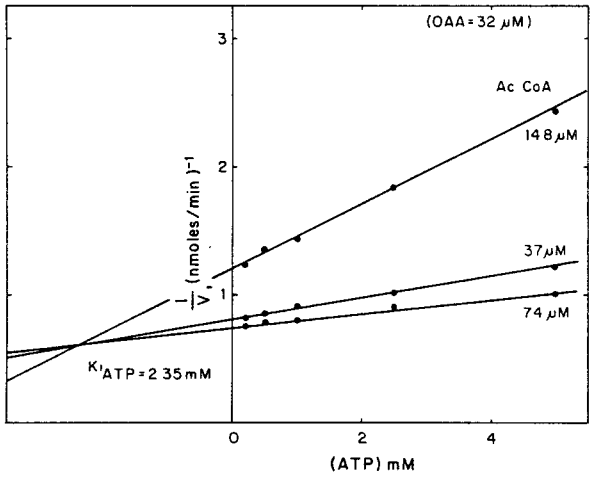

FIG. 3. Plot of reciprocal initial reaction velocity of immobilized pig heart citrate synthase against the ATP concentration. The reaction was carried out at 25°C in a total volume of 11.9 ml with 100 mM Tris-Cl buffer, pH 8.1, 0.2 mM DTNB, 32 μM OAA, 14.8–74 μM AcCoA, and varied concentration of ATP as shown in the figure. The reaction was initiated by the addition of AcCoA.

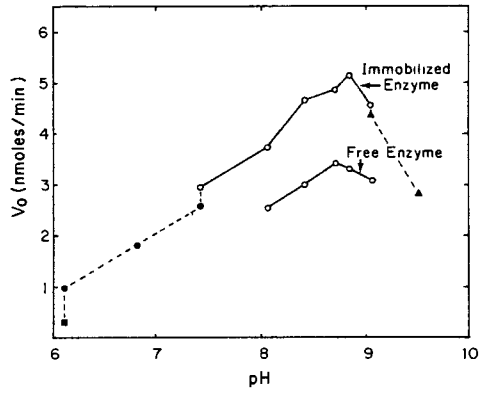

FIG. 4. The plot of the initial velocity against pH for the immobilized and free citrate synthases. The reaction was carried out at 25°C in a total volume of 11.9 ml with 100 mM buffer, 0.2 mM DTNB, 148 μM AcCoA, and 320 μM OAA. The reaction was started by addition of AcCoA. ○, Tris-Cl buffer; ●, KPO_4 buffer; ▲, sodium bicarbonate buffer; ■, imidazole acetate buffer.

Only small differences were detected when the activities of immobilized and free enzyme systems were studied as a function of temperature (Fig. 5). A plot of the logarithm of enzyme activity as a function of the reciprocal of the absolute temperature (Arrhenius plot) showed an apparent break in the plot at a temperature of about 20°C for both the immobilized enzyme system (Fig. 5a) and free enzyme system (Fig. 5b). For the immobilized enzyme, the activation energy (E) calculated from the Arrhenius function $(d \ln k)/dT = -E/RT^2$ is higher (12,000 cal/mol) at low temperatures than at high temperatures (5700 cal/mol), whereas for the free enzyme the corresponding activation energies are 1300 and 5100 cal/mol, respectively.

Thermal stability studies showed some differences between the free and immobilized enzymes. When the logarithm of the percentage of the enzyme activity remaining was plotted as a function of time, the decrease in free enzyme activity was biphasic at 38°C, indicating two different rate constants, 0.12 and 0.023 min^{-1}. The immobilized enzyme decreased in activity with a single rate constant, 0.04 min^{-1}. This shows that immobilized enzyme is more stable against heat denaturation than is the free enzyme. OAA partially protected both the free and immobilized enzyme from thermal inactivation.

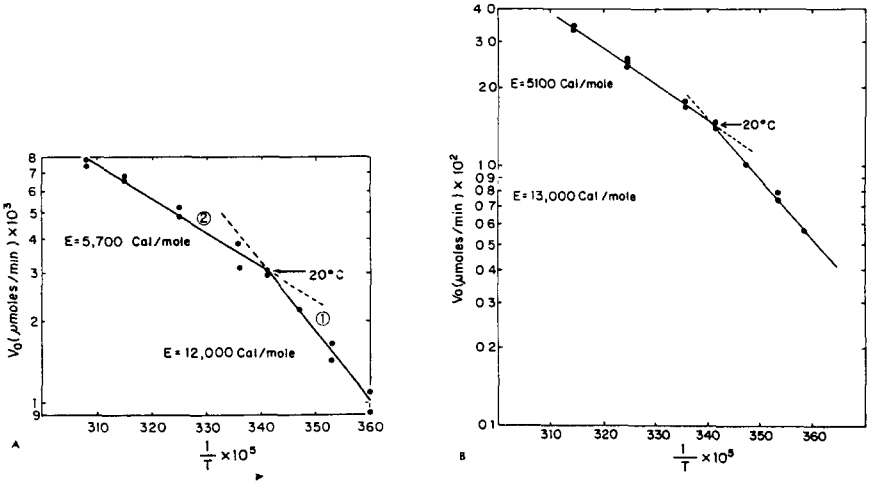

FIG. 5. (a) The plot of the logarithm of initial velocity of immobilized citrate synthase as a function of the reciprocal of the absolute temperature. The reaction was carried out with 100 mM Tris-Cl buffer, pH 8.1, 0.2 mM DTNB, 320 μM OAA, and 148 μM AcCoA in a total volume of 11.9 ml. The reaction was started by addition of OAA. (b) The plot of the logarithm of initial velocity of free citrate synthase as a function of the reciprocal of the absolute temperature. The reaction was carried out with 100 mM Tris-Cl buffer, pH 8.1, 0.2 mM DTNB, 320 μM OAA, and 148 μM AcCoA in a total volume of 11.9 ml. The reaction was started by addition of OAA.

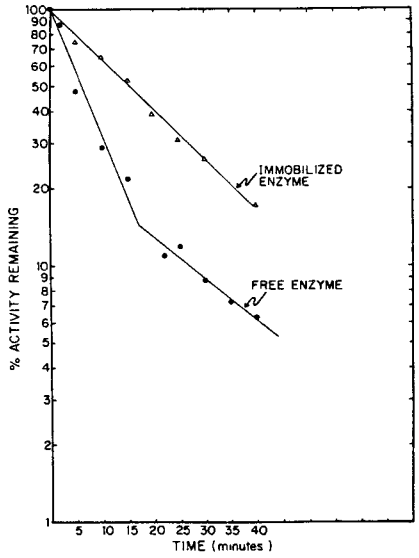

FIG. 6. The plot of the logarithm of the percentage of activity of citrate synthase remaining as a function of time. Enzyme in 100 mM Tris-Cl buffer, pH 8.1, was incubated alone or with 500 μM OAA at 38°C, and at varied intervals an aliquot was assayed for activity. The reaction was carried out with 100 mM Tris-Cl buffer, pH 8.1, 0.2 mM DTNB, 312 μM OAA, and 148 μM AcCoA in a total volume of 11.9 ml. The reaction was started by addition of OAA.

DISCUSSION

The apparent K_m values for AcCoA and OAA are almost four times higher for the immobilized enzyme than for the free enzyme. An increase in the value of K_m for substrates has been reported for a number of other immobilized enzymes (5–8). The change in the apparent K_m has been explained in a number of different ways. One possibility is that the ionic charge on the support can influence the K_m. In the present case one would expect an increase in positive charge to give an increase in the substrates concentration (they are both negatively charged) in the enzyme microenvironment. This would result in a lowering of the apparent K_m values, not an increase as observed here. Another effect that has been observed with immobilized enzymes is that of diffusion–limited reactions due to an unstirred layer of water near the insoluble support. This effect would cause an increase in the apparent K_m values. Finally, it is possible that during the covalent attachment of the enzyme to the Sepharose, modification of the active site occurs which results in a change in the K_m. This latter possibility has not been examined.

For both the free and the immobilized enzyme systems for each of the substrates, and intersection on the x axis in the Lineweaver–Burk plot occurs, indicating $K_a = K_{i_a}$. These results eliminate a Ping-Pong mechanism and suggest that the mechanism is sequential involving ternary complexes.

Although from our kinetic data K_m equals K_{diss} for both the substrates in pig heart, rat kidney (9), and rat heart and liver citrate synthase (10), a direct measure of K_{diss} of AcCoA with pig heart enzyme by Weidman et al. (11) and by Johansson and Pettersson (12) indicates that K_m is different from K_{diss}. Johansson and Pettersson suggest that these results rule out a random rapid equilibrium mechanism (12). An ordered mechanism for citrate synthase reaction has been considered a number of times, since it is known that OAA causes an apparent conformation change in the pig heart enzyme (13, 14) and that S-malate (an analog for OAA) induces an AcCoA enolase activity in the enzyme (15). Results of Johansson and Pettersson (16) and Weidman and co-workers (17), indicating that OAA affects the binding of AcCoA or spin label analog of AcCoA to pig heart citrate synthase, strongly suggest an ordered mechanism for citrate synthase. Our recent studies on the mechanism of rat heart citrate synthase by biospecific adsorption–elution technique (18) in which the enzyme could only be eluted from Sepharose-ATP column by OAA and CoA is also consistent with the idea that the reaction could be of ordered type. However, this situation is analogous to the situation for yeast hexokinase, which Cleland describes as a random mechanism involving several rate-limiting ternary complex conformation changes during its course (19).

Many workers have shown that several Krebs cycle intermediates affect the activity of citrate synthase and have discussed the interactions in terms of control of Krebs cycle activity. Thus, Hathaway and Atkinson (20) showed in yeast that ATP inhibits citrate synthase. This inhibition by ATP was shown to be a competitive type with respect to AcCoA. From the data of Kosicki and Lee (4) the K_i for ATP on pig heart citrate synthase was calculated to 1.1 mM, which is nearly one-half that found for the immobilized enzyme. The increase in K_i could be caused by the same factors which result in a change in the apparent K_m values.

Previous studies with other immobilized enzymes showed that the position of optimum pH activity may be displaced to higher or lower pH values or not at all when compared to the free enzyme (5,21,28). Our present data do not reveal any change in the pH response of immobilized citrate synthase when compared to its free state.

The data presented here show that only small differences exist between the immobilized and free forms of citrate synthase activity with respect to the effect of temperature on their activity.

Enhanced thermal stability of immobilized enzymes has been shown by many others for different enzymes (29–32). It has been shown previously that enzyme–substrate binary complex formation with OAA protects citrate synthase from heat denaturation (13,14). Immobilized enzyme was also partially protected from heat denaturation when OAA was present in the

medium. One significant difference in the thermal stability study was that when the logarithm of the percentage of the activity was plotted against time, the free enzyme had a break in the slope, indicating two different rate constants, whereas immobilized enzyme did not show any such break. At present, we are unable to assess the actual mechanism for the biphasic activity of free citrate synthase, but one explanation may be that heat inactivation occurs in two stages, first the dissociation to form monomers, then an unfolding to complete denaturation.

It is possible that citrate synthase occurs in cells in association with the inner membrane of mitochondria, and thus its behavior may be similar to that of the immobilized enzyme rather than of the free enzyme. Thus, recent kinetic experiments of citrate synthase *in situ* (33) using toluene-treated rat liver mitochondria showed an increased K_m for AcCoA. The differences observed here therefore may have some physiological implications.

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