Purification of and Mechanism Studies on Citrate Synthase

USE OF BIOSPECIFIC ADSORPTION-ELUTION TECHNIQUES*

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Affinity chromatography already has proven to be the most powerful single method for the isolation and purification of proteins. Other methods of protein separation usually depend upon bulk properties of proteins (charge, size, solubility), and because of the overlap in the properties of the thousands of proteins in most cellular extracts, most methods are unable to effect a separation of the mixture into more than a few fractions (usually never more than 10). Since each protein usually has an extraordinary specificity to bind a single specific ligand, it has been possible by the method of affinity chromatography to effect spectacular purifications and recoveries of individual proteins.

One recent modification of the affinity chromatography method, introduced by Mosbach and his co-workers (1), is the specific elution of an enzyme bound to an immobilized general ligand. A general ligand is a material such as an adenine nucleotide which can bind to a large number of proteins. Specificity in purification is partially accomplished by the binding step and often completely accomplished by use of a specific elution technique. In the experiments of Mosbach et al. (1) a Sepharose-AMP column was used, and lactate dehydrogenase (EC 1.1.1.27) was specifically eluted with a combination of pyruvate and NAD. The latter compounds form a tight "dead end" complex with the enzyme and are thus highly specific in eluting only lactate dehydrogenase from the column.

In the present work we have applied this elution principle to the purification of citrate synthase (EC 4.1.3.7) from rat heart. A Sepharose-"ATP" (2) column was chosen, since eukaryotic citrate synthases are inhibited by ATP. The inhibition by ATP is competitive with acetyl-CoA, which suggests that ATP binds to the same site on the enzyme as does acetyl-CoA (3). Citrate synthase is one of many proteins which bind to such a column, but it can be specifically eluted with its dead end complex-forming compounds, CoA and oxalacetate. Neither of the substrates of the citrate synthase reaction alone nor the other putative dead end complex formers, citrate and acetyl-CoA, could elute the enzyme from the column.

We also have used the results of these studies and the elution of citrate synthase with substrate analogs in an attempt to reconcile the different mechanisms proposed for the enzyme from steady state kinetic data.

MATERIALS AND METHODS

CNRs-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals, Upsala, Sweden. Adipic acid dihydrazide was a product of Eastman Kodak Co. Oxalacetic acid and Coenzyme A were purchased from Calbiochem and P L Biochemicals, respectively. Citrate synthase (from pig heart) was purchased from Boehringer Mannheim, Germany. ATP, (R)- and (S)-malate, α-ketoglutarate, and 6,6′-dithiobis(2-nitrobenzoic acid) were products of Sigma Chemical Co.

Sepharose 4B-adipic acid dihydrazide conjugate was prepared, and the periodate-oxidized ATP was coupled to it according to the method of Lamed et al. (2). This material is referred to as Sepharose-"ATP." The amount of periodate-oxidized ATP bound to the column was determined by ashing the material in Mg(NO₃)₂ and then determining the phosphorus as inorganic phosphate (4). This value was 3.7 μmol of periodate-oxidized ATP/ml of packed column volume. Crystalline Escherichia coli citrate synthase was purified by a modification of our published procedure (5). The preparation of acetyl-CoA was based on the procedure developed by Simon and Shenin (6). Citrate synthase activity was determined by measuring the rate of CoA-SH formation in the presence of 5,5'-dithiobis(2-nitrobenzoate) as described by Srere et al. (7). One unit of the enzyme was the amount of enzyme that catalyzes the liberation of 1 μmol of CoA/min.

Acrylamide disc gel electrophoresis of the enzyme was carried out according to the procedure of Davies (8). Electrophoresis was performed in 5.0% acrylamide gel in 0.05 M Tris-glycine buffer, pH 8.3, containing 10 mM citrate. For sodium dodecyl sulfate gel electrophoresis of the enzyme, the procedure of Weber and Osborn (9) was used, with 10% acrylamide gels and a running buffer of 0.05 M sodium phosphate/0.1% sodium dodecyl sulfate, pH 7.0. The gels were stained in 0.2% Coomassie blue.

Protein was assayed in column eluates using the method of Warburg and Christian (10). The protein of pure preparations was determined by the method of Lowry et al. (11) using crystalline bovine serum albumin as a standard.

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RESULTS

Purification of Citrate Synthase from Rat Heart: Homogenization—Frozen rat hearts (26 g), washed free of blood in cold 0.9% NaCl, were homogenized in 5 volumes of 20% alcohol and 0.4 M KCl. Homogenization was performed in a Waring Blender for 10 ½-min periods at full speed, cooling the mixture between homogenization periods in a salt-ice bath. The homogenate was centrifuged at 20,000 × g for 40 min at 4°, and the precipitate was discarded. The supernatant fluid was dialyzed overnight against 5 liters of cold 5 mM KPO₄ buffer, pH 7.4, with one change of buffer.

Ammonium Sulfate Fractionation—The dialyzed supernatant fluid was centrifuged, and the precipitate was discarded. The supernatant fluid was brought to 50% saturation of ammonium sulfate with the solid salt (29.1 g/100 ml). The precipitate was removed by centrifugation, and the supernatant solution was brought to 75% saturation of ammonium sulfate (15.9 g/100 ml), stirred for 1 hour, and centrifuged again as described above. The precipitate was dissolved in a small quantity of 5 mM KPO₄ buffer, pH 7.4, and dialyzed against 2 liters of the same buffer at 4° for 24 hours with three changes of buffer.

Sepharose-“ATP” Column Chromatography—The supernatant solution (after centrifugation to remove a small amount of precipitate) was applied to a Sepharose-“ATP” column previously equilibrated with 5 mM KPO₄ buffer, pH 7.4. The column was washed with the same buffer until no more protein was eluted. Solutions containing 200 μM potassium citrate, 200 μM acetyl-CoA, 100 μM potassium citrate + 100 μM acetyl-CoA, 200 μM oxalacetate, and 200 μM CoA, each in 5 mM KPO₄ buffer, pH 7.4, were successively applied to the column, but no enzyme was eluted. The enzyme was eluted with a mixture of 100 μM CoA and 100 μM oxalacetate in 5 mM KPO₄ buffer, pH 7.4. When no further enzyme activity appeared in the eluate, the column was washed with 200 mM KCl, which resulted in the elution of protein that contained no citrate synthase activity. The elution profile of the enzyme from the column is shown in Fig. 1. Fractions having the highest activities were pooled, and the protein was precipitated with (NH₄)₂SO₄. The precipitate was collected by centrifugation and dissolved in 20 mM KPO₄ buffer, pH 7.4. The results of the purification procedure are summarized in Table I.

Fig. 2, a and b, shows the disc gel electrophoresis patterns of the proteins in the original ammonium sulfate fraction applied to the column and in the final preparation. The eluate obtained with the CoA and oxalacetate wash from the Sepharose-“ATP” column shows a single protein band. Sodium dodecyl sulfate gel electrophoresis (Fig. 2c) of the enzyme also shows a single protein band. Comparison with standard proteins indicated a subunit $M_r$ of 40,000 to 50,000, in agreement with earlier results (3).

The column could be regenerated for use in another purification procedure by washing it with 200 mM KCl followed by equilibration with 5 mM KPO₄ buffer, pH 7.4.

Elution Behavior of Citrate Synthase—In additional experiments the order and concentration of eluants used was varied. The enzyme can be eluted immediately with a mixture of CoA and oxalacetate without prior washes with the individual substrates, that is to say the behavior of the enzyme on the Sepharose-“ATP” is not affected by previous noneluting conditions. If lower concentrations of either CoA or oxalacetate are used, then a slow elution of citrate synthase occurs yielding rather broad peaks of activity. Instead of complete elution in 2 or 3 tubes, 20% of the activity is found in a total of 10 or more tubes. CoA levels as high as 1 mM failed to displace the enzyme from the column. A mixture of acetyl-CoA (50 μM) and oxalacetate (50 μM) cannot elute the enzyme from the column.

Elution with Oxalacetate Analogues—If the oxalacetate is replaced with a-ketoglutarate in the elution mixture with CoA, no enzyme is eluted. However, a slow elution is obtained with 200 μM (S)-malate and 100 μM CoA, and a rapid elution with 200 μM (R)-malate and 100 μM CoA (Fig. 3). Similarly (data not shown) when the elutions were carried out separately with

| Step | Total activity | Total protein | Specific activity |
|------|---------------|---------------|------------------|
| Homogenate in 0.4 M KCl + 20% alcohol | 2250 | 1320 | 1.7 |
| 50 to 75% (NH₄)₂SO₄ | 2050 | 300 | 6.8 |
| Sepharose- “ATP” | 1680 | 14.5 | 116 |
column (0.4 x 5 cm) of Sepharose-"ATP." All eluting materials were in column.

(R)-malate and CoA, and (S)-malate and CoA, then the former either (R)- or (S)-malate does not elute the enzyme from the combination results in a much more rapid elution of the enzyme than does the latter. Acetyl-CoA in combination with Sepharose-"ATP." This is expected, since E. coli citrate synthase binds to synthase is only poorly inhibited by ATP (12). On the other hand, citrate synthases from human heart and yeast were prepared in a manner similar to that described for Sepharose-"ATP" column, only citrate synthase forms a complex with both CoA and oxalacetate, so that it is the only protein eluted from the column by this mixture. This confirms the usefulness of the technique of elution by "dead end" complex formation (14, 15), which has been used so successfully on lactate dehydrogenase.

The binding of a protein to an immobilized ligand is undoubtedly a more complex process than simple ligand-protein interaction (16). After the initial binding step there is probably a secondary interaction between the hydrophobic spacer arm and the protein, and perhaps even a tertiary interaction between the matrix and the bound protein. It is difficult to determine the extent of the latter two interactions. If, in a control experiment, unmodified Sepharose or Sepharose with the spacer arm is used, the interactions with the protein cannot be the same as those that occur when the immobilized ligand is present. When the protein is bound through the ligand, then regions of the protein are brought in close proximity with the spacer arm and matrix so that the secondary and tertiary interactions may occur. In the absence of the initial interaction between active site on the protein and ligand, the probability of secondary and tertiary bindings is small. If secondary and tertiary interactions occur to an important extent, then the following discussion would have to be modified.

We can consider two different explanations for the observed elution patterns. First, it is possible that the enzyme is bound to the ligand column not only at its adenine nucleotide (acetyl-CoA or CoA) site but also at its oxalacetate site. It would be necessary therefore to have both the substrates present to elute the enzyme from the column. On the other hand, the enzyme may be bound to ATP through just one binding site (the CoA site), and the binding of oxalacetate to its own site on the enzyme may cause a conformation change which enhances the binding of CoA to the enzyme without a concomitant increase in ATP binding.

The results of experiments on the elution of rat heart and pig heart citrate synthase from Sepharose-"ATP" are summarized in Table II. It is difficult to interpret all of these results with a single hypothetical mechanism. The difference between the pig heart and rat heart enzyme, i.e. the ability of the pig heart enzyme to be eluted with single substrates, may be due to a difference in the dissociation constants for the substrates and the two enzymes. Yet the kinetic behavior and kinetic constants for the two enzymes as measured in the steady state are...
elute the enzyme may be due to the rapid enzymic conversion of the acyl-CoA substrates behave identically with the enzyme from rat tissue. The dissociation constants of 10^6 and 30 PM with the rat heart enzyme are consistent with a mechanism in which the binding of oxalacetate increases the ability of the enzyme to bind acetyl-CoA.

An ordered mechanism for the citrate synthase reaction has been considered a number of times, since it is known that oxalacetate causes an apparent conformation change in the pig heart enzyme (19, 20) and that (S)-malate, an analog for oxalacetate, but not (R)-malate, induces an acetyl-CoA-enolase activity in the enzyme (21). We have shown also that oxalacetate and (S)-malate (but not (R)-malate) can protect citrate synthase against urea denaturation (20), again in contrast to the results of Weidman et al. (18) (see above) and our elution data.

The steady state kinetics of citrate synthase has been studied thoroughly only for the rat tissue and pig heart enzymes. Our kinetic analysis of the rat enzyme as well as partial kinetic analyses on other citrate synthases indicated that the mechanism may be a random one in both the forward and reverse direction, with two “dead end” complexes (E-AcCoA-Cit and E-CoA-OAA) formed in a kinetically significant manner (13). The kinetic data for both the pig heart and rat citrate synthases showed that oxalacetate did not affect the K_m of acetyl-CoA, and that acetyl-CoA did not affect the K_m of oxalacetate. Such data indicate that K_m for the substrates should equal their K_diss. Whereas the K_m for oxalacetate (5 μM) is equal to K_diss for oxalacetate (5 μM), the K_m of acetyl-CoA (5 μM) has been shown to be quite different from its K_diss. A direct measure of K_diss of acetyl-CoA with pig heart enzyme by Weidman et al. (22) yielded a value of 140 μM (K_m = 5 μM). Similar results were reported by Johansson and Pettersson (23) who reported a K_diss of acetyl-CoA and pig heart enzyme measured directly by a gel equilibrium diffusion technique of 130 μM. Johansson and Pettersson suggest that these results rule out a random rapid equilibrium mechanism (23).

The data presented here are consistent with the idea that oxalacetate does affect the binding of the acetyl-CoA. 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 (24).

The observation that *E. coli* citrate synthase does not bind to the Sepharose-“ATP” column, compared to pig heart, rat heart, rat liver, human heart, and yeast citrate synthases, all of which bind to the column, reflect the known difference between the poor ability of ATP to inhibit the *E. coli* enzyme compared to its ability to inhibit the eukaryotic enzymes. Further, the fact that the eukaryotic citrate synthases can be eluted from Sepharose-AMP columns with a single substrate is probably related to the observation that AMP is a poor inhibitor of citrate synthase. Since identical results were obtained with Sepharose-“ATP” where the linkage is through the ribose portion of the ATP molecule, and with Sepharose-ATP (according to Mosbach et al. (1)) where the linkage is through the N-6 group, it seems unlikely that strong interaction occurs through those parts of the ATP molecule and the enzyme.

Although our present results do not yield an unequivocal mechanism, it appears that affinity chromatography can serve both as a useful tool for the purification of the enzymes and as an interesting method for the study of the reaction mechanisms (25). In the present case the results for the rat citrate synthase are consistent with a mechanism in which the binding of oxalacetate increases the ability of the enzyme to bind acetyl-CoA.
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