Structure of ATP Citrate Lyase from Rat Liver

PHYSICOCHEMICAL STUDIES AND PROTEOLYTIC MODIFICATION*

(Received for publication, March 25, 1976)

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ATP citrate lyase was purified by two different procedures from the livers of rats first starved and then fed with a fat-deficient and high carbohydrate-glycerol diet. These enzyme preparations were judged homogeneous by sedimentation equilibrium and polyacrylamide gel electrophoresis. The molecular weight of the native enzyme was around $4.4 \times 10^5$ as determined by sedimentation equilibrium. On sodium dodecyl sulfate gel electrophoresis the enzyme usually showed a single protein band with an estimated molecular weight of $1.2 \times 10^5$. A similar value for the molecular weight of the subunit was obtained by gel filtration on 6% agarose in the presence of 6 M guanidinium chloride. The molecular weight of this polypeptide chain was estimated by sedimentation equilibrium to be around $1.1 \times 10^5$. These results indicated that ATP citrate lyase has a subunit structure of four polypeptides of similar size.

The extinction coefficient of the dry protein and its amino acid composition are also reported.

Some batches of fully active enzyme, judged to be homogeneous by sedimentation equilibrium and polyacrylamide gel electrophoresis, showed two additional major polypeptides ($M_r \approx 7.1 \times 10^4$ and $5.5 \times 10^4$) on sodium dodecyl sulfate gel electrophoresis. Studies on the polypeptides produced by proteolytic modification of the native enzyme by trypsin indicated that the additional protein bands observed on sodium dodecyl sulfate gel electrophoresis with some of the batches of enzyme could have been formed by limited proteolysis ("nicking") of the original $1.1 \times 10^5$ subunit. Trypsin treatment of the native enzyme did not affect the enzyme activity, whereas chymotrypsin and pronase treatment inactivated the enzyme. The trypsin-treated enzyme, which contained only the two smaller polypeptides, did not differ significantly from the untreated enzyme with respect to sedimentation behavior, phosphorylation by ATP, $K_m$ for citrate, and immunoreactivity, but it was more heat-labile than the untreated enzyme. The phosphate group on the phosphorylated "nicked" enzyme was located on the larger polypeptide fragment.

ATP citrate lyase (EC 4.1.3.8) is a cytosolic enzyme which catalyzes the following reaction:

\[
\text{Citrate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{oxaloacetate} + \text{ADP} + P_i + \text{acetyl-CoA}
\]

The enzyme is widely distributed in animal tissues (1) and has been reported in the mango fruit (2), a yeast (3), and a mold (4). It has been obtained from rat liver in a crystalline form (5). Based on its sedimentation and diffusion coefficients, a molecular weight of 500,000 was estimated for the rat liver enzyme (5). Our preliminary results from sedimentation equilibrium experiments gave a value of 400,000 (6). Extensive studies have been carried out on its kinetics, mechanism of action, and its possible role in the generation of extramitochondrial acetyl-CoA needed for various metabolic activities of the cell (6).

* This work was supported by grants from the Veterans Administration to (P. A. S. and E. G. R.) and the United States Public Health Service (AM-11913 to P. A. S. and HL-14938 to E. G. R.).

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It has been shown that the enzyme is phosphorylated when incubated with ATP in the presence of Mg$^{2+}$, and 2 mol of PO$_4$ were estimated to be bound per mol of phosphoenzyme on the basis of a molecular weight of 500,000 (7, 8). However, in spite of these extensive studies on various aspects of the enzyme, little is known about its structure.

In this paper we report the results of our physicochemical studies of the ATP citrate lyase from rat liver. In the course of these studies we observed that some of our enzyme preparations which were judged to be homogeneous by sedimentation equilibrium and polyacrylamide gel electrophoresis gave rise to three major protein bands on SDS gel electrophoresis. However, variations in the relative amounts of these protein bands (designated as polypeptides I, II, and III in decreasing order of size) led us to investigate the possibility that limited proteolysis ("nicking") of the native enzyme gave rise to the two

The abbreviations used are: SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, N'-p-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride.
smaller polypeptides (II and III). The action of three proteases on the native enzyme was studied. Trypsin treatment yielded a modified enzyme with only the two smaller protein hands on SDS gel electrophoresis. The enzymatic properties of the trypsin-treated and the native enzymes were essentially the same.

**EXPERIMENTAL PROCEDURE**

**Materials**

Analytical grade reagents and materials were obtained from the following commercial sources: DEAE-cellulose (DE52) from REeve Angel; Bio-Gel A-1.5m and A-5m (both 200 to 400 mesh) from Bio-Rad Laboratories; Sephadex G-25 and blue dextran from Pharmacia Fine Chemicals; SDS, NADH, ATP, NADP, L-1-tosylamide-2-phenyleryl chloromethyl ketone, Nα-p-tosyl-L-lysine chloromethyl ketone, phenylmethylsulfonyl fluoride, soya trypsin inhibitor, phosphorylase a, and chymotrypsin from Sigma Chemicals; diithiothreitol and pronase from Calbiochem; Aquasol, Protosol, and [γ-32P]ATP (17.9 mCi/mmol) from New England Nuclear; malate dehydrogenase, citrate synthase, hexokinase, and glucose-6-phosphate dehydrogenase from Boehringer Mannheim; trypsin (TPCK-treated) from Worthington, and CoA from P-L Biochemicals. Purified β-galactosidase from Escherichia coli was a gift from Dr. I. Zabin of the University of California at Los Angeles.

**Enzyme Assay**

ATP citrate lyase was assayed by the malate dehydrogenase-coupled procedure as described earlier (1, 9). The assay mixture contained 100 μmol of Tris/HCl, pH 8.7; 20 μmol of potassium citrate; 10 μmol of ATP; and the enzyme was precipitated by the addition of solid ammonium sulfate (50% saturation). The precipitate was collected by centrifugation and dissolved in a minimum volume of the same buffer. The fractions containing the enzyme with high specific activity were pooled. The pooled fractions were applied to a DEAE-cellulose column (1.5 × 10 cm) as described earlier. This procedure yielded on enzyme having a specific activity of about 7 with an overall recovery of about 11%.

**Polyacrylamide Gel Electrophoresis of Native ATP Citrate Lyase**

Disc gel electrophoresis of the native enzyme was carried out in 4.5% polyacrylamide gels with Tris/glycine buffer, pH 8.3, according to Daves (11), and the protein was stained with Coomassie blue.

**SDS Gel Electrophoresis**

The procedure of Weber and Osborn (12) was used with a running buffer of 0.05 M sodium phosphate, pH 7.0, containing 0.1% SDS. Gels with 7% as well as 4.5% polyacrylamide were used. The details of preparing the samples have been described earlier (13), and protein was stained with Coomassie blue.

**Ultracentrifugation Studies**

All ultracentrifugation studies were performed in a Beckman Spinco model E ultracentrifuge equipped with electronic speed control. Sedimentation velocity runs were carried out using schlieren optics, and high speed sedimentation equilibrium studies were according to Yphantis (14) with the use of interference optics. The photographic plates were read on a semi-automated plate reader as described earlier (15).

**Dry Weight and Extinction Coefficient of ATP Citrate Lyase**

The enzyme was desalted on a Sephadex G-25 column (1.5 × 20 cm) and the protein concentration was calculated from the absorbance at 279 nm (ɛ1% = 11.4, see below). The desalted protein was hydrolyzed, and the amino acid composition of the hydrolyzed sample was determined on a Beckman 120-C amino acid analyzer as described earlier (15). The amount of half-cystine was determined by titration with 5,5-dithiobis(2-nitrobenzoate) after NaBH₄ reduction according to the method of Habeeb (16). Tryptophan was determined by the spectrophotometric method described by Benzec and Schmid (17).

**Amino Acid Analysis**

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**Method 2**—Since, in some of the steps of Method 1, there were conditions favoring the release and activation of lysosomal proteases, another procedure which eliminates these conditions was developed to purify ATP citrate lyase from fresh livers. Fresh livers were minced and suspended in 0.25 M sucrose containing 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol and homogenized in a Dounce homogenizer. The homogenate was centrifuged in a refrigerated centrifuge at 30,000 × g for 30 min. The supernatant fluid was collected and then fractionated by the addition of solid ammonium sulfate. The precipitate between 25 and 45% saturation of ammonium sulfate, which contained more than 90% of the native enzyme activity, was collected by centrifugation. It was dissolved in a minimum volume of 5 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol. It was immediately desalted on a Sephadex G-25 column (5 × 50 cm) equilibrated with the same buffer. The desalted enzyme solution was put on a DEAE-cellulose column (2.5 × 40 cm) equilibrated with the same buffer, washed with the buffer and finally eluted with a KC1 gradient (0 to 0.4 M). The fractions with high specific activity were pooled and the enzyme was precipitated by the addition of solid ammonium sulfate (50% saturation). The precipitate was collected by centrifugation and dissolved in a minimum volume of the same buffer. The enzyme solution was applied to a Bio-Gel A-1.5m column (2.5 × 96 cm) equilibrated with the same buffer. The fractions containing enzyme with high specific activity were pooled. The pooled fractions were applied to a DEAE-cellulose column (1.5 × 10 cm) as described earlier. This procedure yielded on enzyme having a specific activity of about 7 with an overall recovery of about 11%.

**Treatment of ATP Citrate Lyase with Proteases**

The native enzyme (about 1 mg) was treated with trypsin, chymotrypsin, and pronase in the presence of 0.1 M Tris/HCl, pH 7.4, containing 1 mM dithiothreitol at room temperature. Suitable aliquots were assayed for ATP citrate lyase activity at various time periods of incubation. In some experiments the ratio of the protease to the ATP citrate lyase was varied from 0.1 to 2% (w/w).

For SDS gel analysis the protease action was stopped by the addition of 4% SDS, 10% glycerol, 0.1% β-mercaptoethanol, 0.05% SDS, and 0.05% bromophenol blue, mixed with an equal volume of SDS gel sample buffer, and heated to 95°C for 3 min. Suitable aliquots of the incubation mixture before the addition of SDS were used to determine the dry weight of protein by the procedure of Goodrich and Reithel (18).
Reisolation of Trypsin-treated ATP Citrate Lyase

ATP citrate lyase (10 mg) was treated with 0.1 mg of trypsin for 20 min at room temperature. Soy trypsin inhibitor (1 mg) was added to the solution and the mixture was passed through a Bio-Gel A-1.5m column (1.5 x 90 cm) equilibrated with 50 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol. The enzyme was eluted with the same buffer, and the fractions having enzyme activity were pooled and concentrated by ammonium sulfate precipitation (75% saturation). This preparation was used as the trypsin-treated enzyme in subsequent studies.

Kinetic Studies

The kinetic behavior of the native enzyme and the trypsin-treated enzyme was studied by varying the concentrations of citrate in two different assay conditions. One assay was that of Sere (1, 9) as described earlier and the other assay contained a higher concentration of Cl (0.25 M KCl) together with the other assay components (19).

Phosphorylation of Native and Trypsin-treated ATP Citrate Lyase

The enzyme was incubated with γ-32P]ATP and MgCl₂ in 0.1 M Tris/HCl, pH 8.1, for 20 min at room temperature (9). In some experiments, the phosphoenzyme was precipitated by the addition of cold trichloroacetic acid and collected by filtration on Millipore filters. For the preparation of [32P]phosphoenzyme used in SDS gel analysis, the enzyme was incubated with γ-32P]ATP and MgCl₂, and the reaction mixture was passed through a Sephadex G-25 column to separate the small molecular weight compounds from the [32P]phosphoenzyme.

Determination of Radioactivity

The radioactivity of the protein solutions and the precipitates on Millipore filters was measured with a Nuclear Chicago liquid scintillation spectrometer, using Aquasol as the scintillation fluid. Gel slices were digested with Protosol (20) before the determination of radioactivity.

Heat Stability of Native and Trypsin-treated ATP Citrate Lyase

Native and trypsin-treated ATP citrate lyase preparations were diluted in 50 mM Tris/HCl, pH 8.1, for 20 min at room temperature (9). In some experiments, the phosphoenzyme was precipitated by the addition of cold trichloroacetic acid and collected by filtration on Millipore filters. For the preparation of [32P]phosphoenzyme used in SDS gel analysis, the enzyme was incubated with γ-32P]ATP and MgCl₂, and the reaction mixture was passed through a Sephadex G-25 column to separate the small molecular weight compounds from the [32P]phosphoenzyme.

Several preparations of the enzyme obtained by the two methods described were judged homogeneous by the results of high speed sedimentation equilibrium studies (Fig. 1). The linearity of the plot of In J versus Δρ (Fig. 1A) suggested that the enzyme was homogeneous. Fig. 1B is a plot of the apparent molecular weight averages versus the protein concentration in fringes. On the basis of a partial specific volume of 0.73 ml/g (calculated from the amino acid composition), the molecular weight of the native enzyme was estimated as 4.4 x 10⁶.

Further evidence for the homogeneity of the preparation was provided by the results of polyacrylamide gel electrophoresis (Fig. 2). A single protein band was observed when the electrophoresis was carried out in the absence of any dissociating agents (Gel a), indicating the homogeneity of the native enzyme. Different enzyme preparations, each of which gave a single band under the conditions of electrophoresis as in Fig. 2a, gave variable results when subjected to SDS gel electrophoresis (Gels b and c). Gel b showed a single major protein band, whereas Gel c had three major protein bands, the slowest moving protein band having a similar mobility to the major protein band of Gel b. The relative proportions of the two faster moving polypeptides (when they were present) with respect to the slow moving one varied widely in different batches of enzyme, each of which was judged homogeneous by other criteria. We were unable to find any correlation between the amounts of the faster moving protein bands and (a) the

Fig. 1. High speed sedimentation equilibrium of ATP citrate lyase. The enzyme purified by Method 1 (0.61 mg/ml) in 0.1 M Tris/HCl, pH 7.4, containing 1 mM EDTA and 10 mM dithiothreitol was subjected to high speed sedimentation equilibrium at 4°C and a speed of 9000 rpm for 23 h. A, In J versus Δρ. B, apparent molecular weight averages versus protein concentration as fringes. O, Mₐ, weight average molecular weight and O, Mₜ, number average molecular weight.

Fig. 2. Polyacrylamide gel electrophoresis of ATP citrate lyase. a, disc gel electrophoresis of the native enzyme on a 4.5% polyacrylamide gel according to Davies (11). b and c, SDS gels of two different batches of enzyme on 7% polyacrylamide according to Weber and Osborn (12). All the gels were stained with Coomassie blue.
method of preparation of the enzyme, (b) the use of frozen or fresh tissue, (c) the presence or absence of the protease inhibitors, TLCK and PMSF during the preparation of the enzyme and subsequent SDS treatment.

The specific activity of pure ATP citrate lyase was 7 to 8 units/mg of protein as calculated from the protein concentration determined by the method of Warburg and Christian (21). On the basis of dry protein (E1%279 = 11.4 at 279 nm, see below), a specific activity of 5.5 to 6.3 was observed for different preparations.

Ultraviolet Absorption Spectrum and Dry Weight of ATP Citrate Lyase

The absorption spectrum of the enzyme showed a maximum at 279 nm and a minimum at 250 nm. The ratio of the absorbances at 280 and 260 nm was 1.61, there was no maximum in the visible range, and the ratio of the absorbances at 320 and 279 nm was 0.08. Based upon the dry weight analysis of the enzyme, its absorptivity was 11.4 for E1%279 at 279 nm.

Amino Acid Composition of ATP Citrate Lyase

The amino acid composition of the enzyme is presented in Table I. The recovery of amino acids was about 94% of the dry weight of protein. In the report on the amino acid analysis, Inoue et al. (5) obtained an 87% recovery (calculated by us). Our results showed significantly higher values for half-cystine, histidine, lysine, proline, serine, and tryptophan, and a lower value for glutamic acid than those reported by Inoue et al. (5).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mean ± SD</th>
<th>μmol/mg</th>
<th>μg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.643 ± 0.011</td>
<td>45.7</td>
<td>47.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.332 ± 0.009</td>
<td>51.8</td>
<td>48.8</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.692 ± 0.015</td>
<td>76.2</td>
<td>78.2</td>
</tr>
<tr>
<td>1/2-Cystine</td>
<td>0.798</td>
<td>88.7</td>
<td>67.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.657 ± 0.023</td>
<td>84.8</td>
<td>92.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.710 ± 0.021</td>
<td>40.5</td>
<td>42.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.189 ± 0.004</td>
<td>25.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.470 ± 0.012</td>
<td>53.2</td>
<td>52.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.630 ± 0.012</td>
<td>71.4</td>
<td>71.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.572 ± 0.002</td>
<td>73.3</td>
<td>56.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.224 ± 0.005</td>
<td>22.4</td>
<td>29.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.320 ± 0.003</td>
<td>47.1</td>
<td>47.3</td>
</tr>
<tr>
<td>Proline</td>
<td>0.508 ± 0.046</td>
<td>49.4</td>
<td>39.9</td>
</tr>
<tr>
<td>Serine</td>
<td>0.473</td>
<td>41.2</td>
<td>34.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.424</td>
<td>42.9</td>
<td>42.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.095</td>
<td>17.7</td>
<td>7.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.277 ± 0.010</td>
<td>45.2</td>
<td>46.9</td>
</tr>
<tr>
<td>Valine</td>
<td>0.549 ± 0.020</td>
<td>54.3</td>
<td>54.1</td>
</tr>
<tr>
<td>Total</td>
<td>938.7</td>
<td>88.0</td>
<td>88.0</td>
</tr>
</tbody>
</table>

* A, present results; B, results of Inoue et al. (5).
* Determined by titration with 0.5 M dithiobis(2-nitrobenzoate) after NaBH₄ reduction (16).
* Extrapolated to zero time hydrolysis.
* Determined by the spectrophotometric method of Benzce and Schmid (17).
* Calculated by us from the data of Inoue et al. (5).

FIG. 3. Molecular weight of polypeptide chains of ATP citrate lyase by SDS gel electrophoresis. The enzyme and the marker proteins were denatured by heating for 5 min in a boiling water bath in the presence of 0.1% SDS and 2 M β-mercaptoethanol as described earlier (13), and electrophoresis was carried out in a 4.5% acrylamide gel in the presence of 0.1% SDS. *standard molecular weight markers: A, bovine serum albumin (dimer) (136,000); B, β-galactosidase (130,000); C, phosphorylase a (94,000); D, bovine serum albumin (monomer) (68,000); E, pig heart citrate synthase (50,000). (I, II and III), polypeptide chains I, II, and III of ATP citrate lyase.

FIG. 4. Gel filtration of ATP citrate lyase on 6% agarose in 6 M guanidinium chloride. About 5 mg of ATP citrate lyase were dialyzed against 6 M guanidinium chloride containing 10 mM sodium phosphate buffer, pH 7.0, and 10 mM dithiothreitol and subjected to gel filtration on a Bio-Gel A-5 m column (1.5 x 90 cm) as described in the text. Fractions of 1 ml were collected and protein was determined by the fluorescence intensity. The same column was standardized on a separate run by using the following markers: blue dextran for the void volume (V₀); dimethyl sulfoxide arginine for the internal volume (Vᵥ); bovine serum albumin (dimer) (136,000); bovine serum albumin (monomer) (68,000); ovalbumin (45,000); and ovalbumin (45,000).
of 6 M guanidinium chloride (as in Fig. 4), are presented in Fig. 5. The linearity of the plot of $\ln J$ versus $\Delta r^2$ (Fig. 5 A) indicates that the preparation was homogeneous. Fig. 5 B is the plot of the apparent molecular weight averages ($M_a$ and $M_b$) versus the protein concentration. On the basis of a partial specific volume of 0.73 ml/g (assuming no change in this solvent), a molecular weight of $1.1 \times 10^5$ was calculated for this polypeptide chain.

A summary of the molecular weight values obtained for the native enzyme and the three polypeptide chains is presented in Table II.

**Effect of Proteases on Activity and Structure of ATP Citrate Lyase**

The variations observed in the results of SDS gel electrophoresis of different batches of enzyme (Fig. 2) could have been due to the presence of variable amounts of an intact subunit (polypeptide chain I) and its cleavage products (chains II and III). This hypothesis was tested by treating homogeneous preparations of the enzyme with trypsin, chymotrypsin and pronase. Chymotrypsin and pronase treatment inactivated the enzyme, whereas trypsin treatment had no effect on the activity of the enzyme (Fig. 6). SDS gel electrophoresis of the protease-treated samples (Fig. 7) showed that polypeptide chain I was absent in all of them. Only two major protein bands (having the same mobilities as polypeptide chains II and III) were present in the trypsin-treated sample, whereas chymotrypsin and pronase treatment resulted in a number of additional protein bands.

**Effect of Trypsin Concentration on Activity and Structure of ATP Citrate Lyase**

The effect of trypsin concentration on the activity of ATP citrate lyase in the range of 0.1 to 2% (w/w) was studied.
Structure of ATP Citrate Lyase

The concentration of chloride ion in the reaction mixture is known to influence the kinetic behavior of ATP citrate lyase (19). The $K_m$ for citrate was obtained from Lineweaver-Burk plots under two assay conditions, viz., "low" chloride (usual assay condition) and "high" chloride (additional 0.25 M KCl). At the low chloride concentration the $K_m$ values for citrate of the untreated and the trypsin-treated enzymes were $1.7 \times 10^{-4}$ and $2.1 \times 10^{-4}$ M, respectively. At the high chloride concentration the corresponding values were $1.7 \times 10^{-4}$ and $1.8 \times 10^{-4}$.

Kinetic Studies with Native and Trypsin-treated Enzymes

Fig. 9. Sedimentation velocity of trypsin-treated and untreated ATP citrate lyase. ATP citrate lyase was treated with trypsin and reisolated from a Bio-Gel A-1.5m column as described in the text. The native enzyme as well as the trypsin-treated enzyme was equilibrated with 50 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and 1 mM dithiothreitol by gel filtration on Sephadex G-25 columns (1.5 x 20 cm) equilibrated and eluted with the above buffer and centrifuged at 60,000 rpm at 8.2°C. The photograph was taken 24 min after attaining the speed. Upper pattern, the native enzyme at 2.3 mg/ml; lower pattern, the trypsin-treated enzyme at 2.8 mg/ml.

Immunological Studies

Rabbit antisera against ATP citrate lyase were placed in the center wells of Ouchterlony plates, and the trypsin-treated enzyme and the untreated enzyme were placed in the outer wells. After 24 h at room temperature fused precipitation bands were observed for both the enzymes (Fig. 10).

Effect of Trypsin Treatment on Heat Stability of Enzyme

The heat stabilities of the trypsin-treated and the untreated enzymes were compared. At protein concentrations of 0.18 mg/ml and a temperature of 45°C, the trypsin-treated enzyme rapidly lost its activity with a $t_{1/2}$ of about 5 min, whereas the untreated enzyme was more stable with a $t_{1/2}$ of about 30 min (Fig. 11A). At higher protein concentrations of 0.5 mg/ml and a lower temperature of 40°C, there was no appreciable loss of activity with the untreated enzyme up to a period of 60 min, whereas the trypsin-treated enzyme lost 50% of its activity in about 16 min (Fig. 11B).

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Fig. 11. Heat stability of native and trypsin-treated ATP citrate lyase. One milliliter each of the native as well as the reisolated trypsin-treated enzyme in 0.1 M Tris/HCl pH 7.4., containing 1 mM EDTA and 1 mM dithiothreitol was kept in A, 45° water bath at a protein concentration of 0.18 mg/ml; B, 40° water bath at a protein concentration of 0.5 mg/ml. Suitable aliquots were taken at the indicated time periods for the assay of the remaining enzyme activity. ○, native enzyme; ●, trypsin-treated enzyme.

Table III

Effect of trypsin treatment on phosphorylation of ATP citrate lyase

ATP citrate lyase was treated with trypsin and reisolated (as in Fig. 9). The native and the trypsin-treated enzyme were incubated in separate tubes with 2.5 nmoi of [γ-32P]ATP for 20 min at room temperature. In addition, the reaction mixture consisted of 50 μmol of Tris/HCl, pH 8.1, 5 nmol of MgCl₂ and 0.5 μmol of dithiothreitol in a total volume of 0.2 ml. The reaction was stopped by the addition of 5 ml of 5% ice cold trichloroacetic acid. The mixture was filtered through a Millipore filter and further washed with more cold trichloroacetic acid. The precipitate retained on the filter was counted as described earlier. The specific activity of [γ-32P]ATP was 8.43 × 10⁵ cpm/nmol, for which the ATP was determined by the hexokinase/glucose-6-PO₄, dehydrogenase-coupled system (22). The molar ratios were calculated on the basis of a molecular weight of 4.4 × 10⁵ and a specific activity of 6.3 units/mg of dry protein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP citrate lyase</th>
<th>32P incorporated</th>
<th>PO₄ incorporated</th>
<th>mol PO₄/mol enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>1.6</td>
<td>13.1</td>
<td>1.6</td>
<td>2.7</td>
</tr>
<tr>
<td>2. 1% Trypsin (w/w) for 20 min</td>
<td>1.2</td>
<td>8.9</td>
<td>1.1</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Phosphorylation of Native and Trypsin-treated Enzymes

The trypsin-treated enzyme was phosphorylated to almost the same extent as the untreated enzyme (Table III). A molar ratio of 2.7 mol of PO₄ per mol of enzyme was obtained for the native enzyme and the corresponding value for the trypsin-treated enzyme was 2.5.

In another experiment, [32P]phosphoenzymes were isolated on Sephadex G-25 columns, and the 32P-labeled enzyme was subjected to SDS gel electrophoresis. In the case of the native enzyme most of the radioactivity was, of course, in polypeptide chain I. A small amount of 32P was found in chain II and none with chain III (Fig. 12A). All the radioactivity was associated with polypeptide chain II in the case of the trypsin-treated enzyme (Fig. 12B).

Discussion

The molecular weight of ATP citrate lyase was estimated to be (4.4 ± 0.06) × 10⁵ from six sedimentation equilibrium experiments. This value is lower than the value of 500,000 reported by Inoue et al. (5), a value based on a sedimentation coefficient of 13.5 S and a diffusion coefficient of 2.62 × 10⁻⁷ cm²/s and a V of 0.75 ml/g (assumed). Our estimation of the molecular weight was based on a V of 0.73 ml/g calculated from the amino acid composition of the enzyme. Another factor contributing to the difference in the molecular weights could be the different techniques employed in these studies. Molecular weights obtained by the sedimentation velocity-diffusion technique are subject to greater uncertainty than those by the high speed sedimentation equilibrium (23).

Inoue et al. (5) reported a specific activity of 5.94 for their crystalline preparation, based on a protein value determined by the method of Lowry et al. (24) and an enzyme assay carried out at 37° and a pH of 8.4. Our specific activity values of 5.5 to 6.3 were based on an assay at 25° and a pH of 8.7 and protein values by dry weight analysis. Under the conditions of their enzyme assay and protein determination, the specific activity of our preparations was determined to be 8.7 to 10. In addition, we obtained a lower ratio of the absorbances at 320 and 279 nm (0.08) than that obtained by these workers (0.24) (calculated...
by us from their data (5)), which indicates the absence of a contaminating chromophore in our preparation.

The observation of multiple protein bands on SDS gel electrophoresis of some of the preparations which were judged homogenous by two different criteria did not indicate that these bands were nonidentical subunits of ATP citrate lyase for the following reasons. (a) The faster moving polypeptide chains (II and III) were completely absent in the SDS gels of many of our preparations obtained by employing either of the two methods described here. (b) The amounts of these polypeptides (when present) varied widely in these preparations. (c) The complete conversion of the largest polypeptide (chain I) into the two smaller chains (II and III) by mild treatment with trypsin indicated that the latter two chains were fragments of the large polypeptide generated by the protease. (d) The sum of the estimated molecular weights of polypeptide chains II and III is approximately equal to the value for chain I. The faster moving polypeptide chains (II and III) observed on SDS gel electrophoresis could not be artifacts of this procedure since their presence was also independently demonstrated by the technique of dissociating the enzyme in 6 M guanidinium chloride and subsequent gel filtration (Fig. 4). Moreover, SDS gel electrophoresis of different samples from the same batch of enzyme, dissociated by SDS in the presence and absence of protease inhibitors, TLCK and PMSF, gave the same results.

We have not attempted further characterization of the three polypeptide chains. But, on the basis of SDS gel electrophoresis, the two polypeptides obtained after trypsin treatment of the enzyme have the same molecular weight as the two smaller polypeptides (chains II and III) observed in the SDS gels of some of the preparations. Therefore, the polypeptide chains of the trypsin-treated enzymes are also referred to as chains II and III, although there might be some differences between these two chains and chains II and III observed with some of the enzyme preparations which had not been treated with trypsin, and presumably formed by some other endogeneous liver protease.

We have studied the size of the polypeptide chain I by three different techniques. The SDS gel electrophoresis and the gel filtration technique gave the same value of 1.2 \( \times 10^5 \) daltons, whereas the high speed sedimentation equilibrium method gave a value of 1.1 \( \times 10^5 \) daltons. In spite of the obvious drawback of using an assumed \( V \) for the polypeptide in guanidinium chloride in the calculation of the molecular weight by the sedimentation equilibrium method, the value of 1.1 \( \times 10^5 \) is preferred, since reliable marker proteins in the range above 100,000 daltons are not available. It can be concluded that ATP citrate lyase consists of four polypeptides of similar size. Whether these polypeptide chains are identical or not remains to be determined.

Based on the results of SDS gel electrophoresis and the gel filtration on agarose in the presence of 6 M guanidinium chloride, average values of 7.1 \( \times 10^4 \) and 5.5 \( \times 10^4 \) were calculated as the molecular weights of the two polypeptides.

The work of Wakil and his co-workers (25) on mammalian fatty acid synthetase and that of Numa and his group (26) on the mammalian acetyl-CoA carboxylase have demonstrated the presence in liver of proteases which modify these enzymes to give rise to multiple protein bands on SDS gel analysis. It is conceivable that the same proteases might be responsible for the nicking observed with ATP citrate lyase.

It seems likely that the nicking occurred during the isolation procedure since we have obtained a number of batches of enzyme without appreciable nicking. Studies with batches of enzyme prepared in the presence of TLCK and PMSF did not give conclusive evidence as to whether the nicking occurred during the isolation or in the course of an in vivo process of protein turnover.

The results of the proteolysis of native ATP citrate lyase by trypsin give some insight into the tertiary structure of the subunits. One can postulate the existence of two separate compact “domains” bridged by an exposed protease-sensitive chain. Such a structure would be similar to that of a variety of enzymes as described by Rossman and Liljas (27). In the case of ATP citrate lyase the only observed differences in the trypsin-treated enzyme were in the SDS gel patterns and the decreased heat stability, leaving unaffected the \( K_m \) for citrate, phosphorylation by ATP, immunoreactivity, and sedimentation behavior. Thus, even after cleavage of the linking region, there remains sufficient interaction between the two domains to maintain the structural features essential for enzymatic activity as well as for quaternary structure.

The reaction catalyzed by this enzyme is quite complex, and studies in several laboratories have led to the proposal of a six-step mechanism (6). In the light of this proposal, the present identification of two fragments of the subunit of the enzyme by tryptic digestion, of which only one is phosphorylated, may be of great significance. It is conceivable that these fragments representing different regions of the native enzyme might be involved in the catalysis of distinct partial reactions. Studies on the reconstitution of the isolated fragments may provide further insight into the mechanism of this reaction.

Acknowledgments—The skilled technical assistance of Carolyn Stewart, Mary Canon, and D. Rockholt is gratefully acknowledged. We thank Dr. F. J. Reithel for the dry weight determination and Dr. K. Wildenthal and Susan Jasinski of the Department of Physiology, The University of Texas Health Science Center at Dallas, for the amino acid analysis.

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