

The Reductive Tricarboxylic Acid Cycle of Carbon Dioxide Assimilation: Initial Studies and Purification of ATP-Citrate Lyase from the Green Sulfur Bacterium *Chlorobium tepidum*

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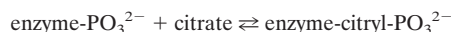
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Carbon dioxide is fixed largely by the reductive tricarboxylic acid (RTCA) cycle in green sulfur bacteria. One of the key enzymes, ATP-citrate lyase, was purified to apparent homogeneity from the moderately thermophilic green sulfur bacterium *Chlorobium tepidum*. The molecular weight of the native enzyme was about 550,000, and the preponderance of evidence indicated that the protein is composed of identical subunits (M_r of $\cong 135,000$) which degraded to two major proteins with M_r s of $\cong 65,000$ and $\cong 42,000$. Western immunoblots and in vitro phosphorylation experiments indicated that these two species could have been the result of proteolysis by an endogenous protease, similar to what has been observed with mammalian, yeast, and mold ATP-citrate lyase. In addition to apparent structural similarities, the catalytic properties of *C. tepidum* ATP-citrate lyase showed marked similarities to the eukaryotic enzyme, with significant differences from other prokaryotic ATP-citrate lyases, including the enzyme from the closely related organism *Chlorobium limicola*. Phosphorylation of *C. tepidum* ATP-citrate lyase occurred, presumably on a histidine residue at the active site, similar to the case for the mammalian enzyme. In contrast to the situation observed for other prokaryotic ATP-citrate lyase enzymes, the *C. tepidum* enzyme was not able to replace ATP and GTP for activity or use Cu^{2+} to replace Mg^{2+} for enzyme activity. Given the apparent structural and catalytic similarities of the enzyme from *C. tepidum* and its eukaryotic counterpart, the *C. tepidum* system should serve as an excellent model for studies of the enzymology and regulation of this protein.

Many anaerobic bacteria, and virtually all archaea, assimilate carbon dioxide by pathways that are distinct from the Calvin-Benson-Basham reductive pentose phosphate scheme (10). Green sulfur photosynthetic bacteria, e.g., *Chlorobium* species, employ interesting ferredoxin-linked CO_2 fixation reactions, and this finding, along with a preponderance of other enzymological and metabolic evidence, resulted in the formulation by Evans et al. (7) of a reductive tricarboxylic acid (RTCA) pathway of CO_2 fixation (Fig. 1). One complete turn of this cycle yields one molecule of oxaloacetate from four molecules of CO_2 , or in a variation from the original scheme, two CO_2 molecules can form a molecule of acetate. Confirmation of this scheme, however, was not provided until a citrate lyase activity was detected in crude extracts of *Chlorobium limicola* (14). In contrast to prokaryotic citrate lyases obtained from bacteria growing anaerobically on citrate, the *C. limicola* enzyme was found to be both ATP and coenzyme A (CoA) dependent, and until its discovery in *C. limicola*, ATP-dependent citrate lyase enzymes had been identified only in eukaryotes, being found in all animal tissues (30), some oleaginous yeasts and molds (11, 18), plants (9), and green algae (5). The function of this enzyme in eukaryotes is to provide cytosolic acetyl-CoA for biosynthesis of fats, cholesterol, and gangliosides (29, 30). In bacteria, however, ATP-citrate lyase has been found only in organisms which employ the RTCA cycle to assimilate CO_2 into cell material. ATP-citrate lyases have now

been identified in several groups of prokaryotes (proteobacteria, green sulfur bacteria, and thermophilic Knallgas bacteria) (14, 25, 27), in sulfur-dependent archaea (3), and in the eukaryotic green alga *Chlamydomonas reinhardtii* (5). It is apparent (Fig. 1) that ATP-citrate lyase is the pivotal enzyme of the RTCA cycle, catalyzing the conversion of the two molecules of CO_2 fixed by the ferredoxin-dependent α -ketoglutarate and pyruvate synthase enzymes to acetyl-CoA, with the concomitant regeneration of the acceptor molecule of the cycle, oxaloacetate. ATP-citrate lyase catalyzes one of the most complex reactions attributed to a single protein, namely, the magnesium-specific ATP (MgATP)-dependent conversion of CoA and citrate to oxaloacetate and acetyl-CoA. Indeed the overall reaction is in actuality the sum of several partial reactions:



The mechanism of the mammalian enzyme has been studied in some detail and is thought to proceed by phosphorylation of a histidine residue at the active site, followed by formation of a freely diffusible citryl-phosphate intermediate (15). Current evidence supports a direct attack on this phosphoanhydride intermediate to form a noncovalent enzyme-citryl-CoA complex, which is subsequently cleaved to form acetyl-CoA and oxaloacetate (38). Another characteristic shared by all eukaryotic ATP-citrate lyases is their apparent instability and sensitivity to cleavage by an endogenous protease activity during purification. This proteolysis has been shown to produce sim-

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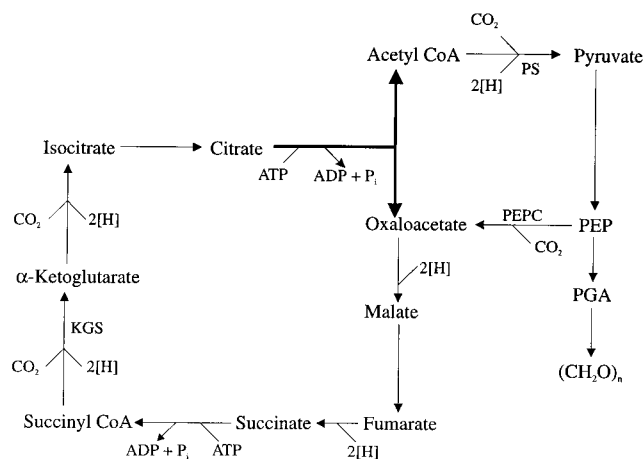


FIG. 1. The RTCA cycle of CO_2 fixation. The reaction catalyzed by ATP-citrate lyase is highlighted; other key reactions are catalyzed by pyruvate synthase (PS), α -ketoglutarate synthase (KGS), and PEP carboxylase (PEPC). PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate.

ilar-size fragments in both the mammalian and yeast enzymes (12, 26, 28), indicating similarities in their quaternary structures, and has inhibited attempts to purify the enzyme in some strains of yeasts and molds.

To date, little is known of the structure and biochemical properties of prokaryotic ATP-citrate lyases. The enzyme was recently purified and partially characterized from an aerobic extremely thermophilic, hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* (13); in addition, partial purification of the enzyme from *C. limicola* was reported some time ago (2). The related organism *Chlorobium tepidum*, a moderate thermophile (33), has proven to be a very versatile organism for biochemical and physiological studies, as it grows rapidly and is quite amenable to genetic manipulation (34). In this paper, we report the first purification and characterization of ATP-citrate lyase from an anoxygenic bacterium. Our findings indicate that the enzyme from *C. tepidum* may be more similar to its eukaryotic counterpart than to the prokaryotic enzyme from *H. thermophilus* or to the partially purified enzyme from the closely related green sulfur bacterium *C. limicola*.

MATERIALS AND METHODS

Organisms and growth conditions. *C. tepidum* TLS was grown phototrophically in Pf-7 medium as previously described (33). For enzyme purification, high cell yields and maximum enzyme production were obtained by the addition of acetate (0.1%, vol/vol) and pyruvate (0.05%, vol/vol) to the basic Pf-7 medium. At early exponential growth phase, *C. tepidum* cultures were harvested by centrifugation under either anaerobic or aerobic conditions; the cells were washed two times in 50 mM Tris-HCl (pH 7.5)–1 mM EDTA (pH 8.0), and the cell pellet was stored at -70°C prior to purification.

Enzyme assays. ATP-citrate lyase activity was routinely determined spectrophotometrically at room temperature, using the malate dehydrogenase coupled assay (28). The assay mixture contained, in a volume of 1 ml, 4 mM MgCl_2 , 5 mM dithiothreitol (DTT), 2 mM ATP, 0.2 mM CoA, 4 mM sodium citrate, 0.25 mM NADH, 0.5 U of malate dehydrogenase, and ATP-citrate lyase, all in 100 mM Tris-HCl (pH 8.5). Enzyme assays for all kinetic studies were performed at 48°C or at temperatures specified in the figure legends.

ATP-citrate lyase was also assayed in polyacrylamide gels after electrophoresis of the purified enzyme in nondenaturing (6% acrylamide) gels (22); the gels were then incubated in the reaction mixture described above (20 ml, total volume) with the following additions: 0.4 mg of nitroblue tetrazolium per ml, 0.24 mg of phenazine methosulfate per ml, and 180 U of malate dehydrogenase. Gels were incubated in the dark at room temperature for 12 to 18 h. A gel incubated in an assay cocktail lacking citrate was used as a negative control. The polyacrylamide gel assay reaction couples the cleavage of citrate by ATP-citrate lyase to the reduction of oxaloacetate by NADH via malate dehydrogenase. Oxidation of NADH was visualized as a clear band against a dark blue background.

For kinetic analyses, Michaelis constants were determined from Lineweaver-

Burk plots, derived from the results of experiments performed at the optimum pH and temperature of the *C. tepidum* ATP-citrate lyase.

Enzyme purification. Frozen cells (ca. 80 g [wet weight]) were thawed in 450 ml of extract buffer (100 mM phosphate [pH 7.2], 3 mM MgCl_2 , 10 mM β -mercaptoethanol [BME]). Protease inhibitors were included in different purification experiments, either alone or as a cocktail at the following concentrations: 1 mM phenylmethylsulfonyl fluoride, 0.5 mg of leupeptin per ml, 1 mM benzamide, and 1 mg of antipain per ml. The cells were broken by two passages through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 15,400 lb/in². Cell debris was removed by low-speed centrifugation (20 min at 17,000 $\times g$), followed by ultracentrifugation (1 h at 100,000 $\times g$). The cleared supernatant was precipitated with ammonium sulfate to 40% saturation, and the precipitate was discarded following centrifugation (10 min at 17,000 $\times g$). To the remaining supernatant, solid ammonium sulfate was added to 55% saturation; precipitated proteins were collected by centrifugation, resuspended in 30 ml of extract buffer, and then dialyzed overnight against 4 liters of the same buffer. The dialyzed solution was loaded onto 25-ml sucrose gradients (5 to 20%) in 3-ml amounts and centrifuged in a swinging-bucket rotor (Beckman SW28) for 20 h at 120,000 $\times g$. Fractions (1 ml) from each gradient were collected and assayed for enzyme activity. Active fractions were pooled and dialyzed overnight against 4 liters of buffer (100 mM phosphate [pH 7.2], 3 mM MgCl_2 , 10 mM BME, 0.5 M sucrose). This solution (about 80 ml, total volume) was placed on a DE-52 DEAE-cellulose column (2.5 by 20 cm; Whatman, Kent, England) which had been previously equilibrated with the same buffer. The column was washed with 500 ml of this buffer, and the enzyme was eluted with a 600-ml linear gradient of KCl (from 0 to 0.3 M). Active fractions were pooled and concentrated to 5 ml in an Amicon (Lexington, Mass.) ultrafiltration cell (model 52) and then dialyzed extensively against buffer A (10 mM phosphate [pH 7.2], 3 mM MgCl_2 , 10 mM BME). Approximately one half of this dialyzed solution (about 5 to 10 mg) was loaded onto a hydroxyapatite column (1 by 5 ml; Bio-Rad Econo-Pac HTP) attached to a fast protein liquid chromatography (FPLC) system (Pharmacia) which had been equilibrated in buffer A (10 mM phosphate [pH 7.2], 3 mM MgCl_2 , 10 mM BME). The enzyme was eluted with a 180-ml linear gradient (10 to 400 mM phosphate). Active fractions were examined for purity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and were stored in 20% glycerol at -70°C .

Preparation of protein samples for SDS-PAGE. Several methods were used in attempts to prevent proteolysis of protein samples prior to SDS-PAGE analysis (35).

(i) **Method 1 (incubation in SDS at 100°C).** Buffer containing 9 parts of 10 mM sodium phosphate (pH 7.0)–1% SDS–1% BME was heated to 100°C in a boiling water bath, after which 1 part of the protein solution (0.05 to 1.0 mg/ml) was added. Following incubation for 2 to 5 min, samples were cooled to room temperature and analyzed on SDS-gels.

(ii) **Method 2 (denaturation in guanidine hydrochloride).** A tube containing 1 ml of 0.1 M Tris-HCl (pH 8.5) (in 7 to 8 M guanidine hydrochloride) was heated to 100°C , after which the protein sample (0.2 to 1 mg) and 15 μl of BME were added and the tube was incubated for an additional 3 to 5 min. The tube was then transferred to 37°C for 2 h, and then the sample alkylated by the iodoacetic acid procedure (35). Before SDS-gel analysis, the sample was dialyzed first against 9 M urea (in 0.1 M Tris-HCl [pH 8.0]) then against 10 mM sodium phosphate (pH 7.0) containing 0.1% SDS.

(iii) **Method 3 (performic acid oxidation).** A solution containing 1 part of 30% H_2O_2 and 9 parts of 88% formic acid was prepared and incubated for 2 h at room temperature. The protein sample (200 to 500 μg) was added to 1 ml of this solution and incubated at 0°C for 1 to 2 h. The mixture was diluted 10-fold with water, frozen, and lyophilized. The lyophilized powder was dissolved in 0.2 M sodium phosphate (pH 8.0) containing 1% SDS and incubated at 37°C for 4 h. Before SDS-PAGE analysis, the solution was dialyzed against 10 mM sodium phosphate (pH 7.0) containing 0.1% SDS (35).

PAGE and immunoblotting. PAGE was performed for both nondenaturing (6%) and SDS-denaturing (10 to 15%) gels as described by Laemmli (16). Molecular weight markers for SDS-PAGE were from Bio-Rad. Proteins were visualized with Coomassie brilliant blue R stain (0.1% in 25% ethanol–5% acetic acid). The phosphorylation labeling experiment using [γ - ^{32}P]ATP (111 TBq/mmol; Dupont NEN Research, Boston, Mass.) employed a modification of the Laemmli method for alkaline SDS-PAGE (32). The SDS digestion buffer for this protocol contained 0.2 M Tris-HCl (pH 8.5), 25% glycerol, 5% SDS, 50 mM DTT, 4 mM phenylmethylsulfonyl fluoride, and 0.01% bromophenol blue. The buffer was added to protein samples in a 1:1 ratio. Gels were electrophoresed for 2.5 h at 4°C and treated for 20 min in 25% isopropanol–0.5% Na_2CO_3 followed by 20 min in 10% isopropanol–0.5% Na_2CO_3 . The gels were dried between cellophane sheets (Hoefer Pharmacia Biotech, Inc., San Francisco, Calif.) on an SE 1200 Easy Breeze air gel dryer (Hoefer Scientific Instruments), and radioactivity bands were then visualized with a Storm 840 imaging system (Molecular Dynamics, Sunnyvale, Calif.). Protein was measured by the Lowry method (18) with bovine serum albumin as a standard.

For Western immunoblot analyses, SDS-PAGE proteins were transferred to HA membranes (Millipore Corp., Bedford, Mass.) in 10 mM CAPS (3-cyclohexyl-amino-1-propanesulfonic acid) buffer, which contained methanol (10%, vol/vol) at pH 11, using a Trans-Blot semidry transfer cell (Bio-Rad). The membrane was washed three times in phosphate-buffered saline (PBS) (24)

containing 0.05% Tween 20. Polyclonal antisera raised against purified *C. tepidum* ATP-citrate lyase (the primary antibody) was diluted 100-fold in washing buffer, agitated gently for 2 to 18 h at room temperature, and then washed three times in PBS-Tween 20. Secondary antibody (goat anti-rabbit immunoglobulin G antibody conjugated with alkaline phosphatase) was diluted 1:3,000 and incubated 2 to 18 h. The blot was washed three times in the same buffer and one time in 0.1 M Tris-acetate (pH 9.5). The membrane was developed in the dark by incubation in a 0.1 M Tris-acetate (pH 9.5) buffer solution containing 10 mM MgCl₂, nitroblue tetrazolium (0.2 mg/ml), and 5-bromo-4-chloro-3-indolyl phosphate (0.2 mg/ml) (dissolved in 0.1 ml of dimethyl formamide). Bands appeared in about 5 min, and the reaction was then stopped by rinsing the membrane in deionized water.

In vitro phosphorylation of ATP-citrate lyase. *C. tepidum* ATP-citrate lyase (3 μg) was incubated with [γ -³²P]ATP (1 μCi) in a reaction mixture (50 μl, total volume) containing 50 mM Tris-Cl (pH 8.5), 3 mM MgCl₂, and 1 mM DTT. The reaction mixtures were incubated at 37°C for 10 min prior to alkaline SDS-PAGE. Following the labeling reaction, some samples were incubated for an additional 10 min at 37°C under the conditions described in Results.

N-terminal amino acid sequencing. Proteins were transferred electrophoretically from SDS-polyacrylamide (10%) gels to polyvinylidene difluoride membranes (Millipore) in CAPS buffer and then stained with a dilute Coomassie brilliant blue stain solution (50% methanol, 0.1% Coomassie blue) for 5 min. Bands of interest were cut from the membrane and sequenced with an Applied Biosystems model 120A protein/peptide sequencer.

Determination of molecular weight. The molecular weight of the native enzyme was determined by gel filtration chromatography on a Superose 6 HR 10/30 column attached to an FPLC system (Pharmacia). The column was equilibrated with 80 mM phosphate buffer (pH 7.2) containing 3 mM MgCl₂ and 10 mM BME. The following molecular weight markers were used: blue dextran (M_r of 2×10^6), thyroglobulin (M_r of 669,000), apoferritin (M_r of 443,000), and alcohol dehydrogenase (M_r of 150,000). Elution was performed with the same phosphate buffer, and 0.5-ml fractions were collected and assayed for ATP-citrate lyase activity. Subunit molecular weights and proteolytic products were determined by SDS-PAGE using Bio-Rad molecular weight markers.

RESULTS

Purification, homogeneity, and stability of ATP-citrate lyase from *C. tepidum*. ATP-citrate lyase was purified to apparent homogeneity by using the protocol described in Materials and Methods. Enzyme activity eluted as a broad peak from sucrose gradient and DEAE-cellulose columns but as a single sharp peak following hydroxyapatite chromatography (Fig. 2), with an overall purification of 98-fold and a yield of 8% (Table 1). For convenience during the purification steps, assays were performed aerobically and at ambient temperature; the specific activities in Table 1 were calculated under these conditions. It was found that the specific activity of purified preparations increased three- to fourfold when assayed at the optimum growth temperature of *C. tepidum* (48°C). No significant differences in absolute activity or in the properties of the enzyme was observed by purifying the enzyme under anaerobic conditions. The SDS-PAGE profile of proteins at each step of the purification is shown in Fig. 3. SDS-PAGE analysis of the purified enzyme from most preparations showed two major species (M_r s of ~65,000 and 42,000; Fig. 3A, lane 6); in addition, a minor component with an M_r of about 135,000 was often observed. Nondenaturing gel electrophoresis of this preparation yielded a single species (Fig. 3B), indicative of a potential homogeneous preparation. Furthermore, polyacrylamide gel activity assays on nondenaturing gels showed a citrate-dependent oxidation of NADH (Fig. 3C), confirming that this protein had associated ATP-citrate lyase activity. The elution of this component from a nondenaturing gel, followed by SDS-PAGE, produced the same 65- and 42-kDa species as observed prior to nondenaturing PAGE (results not shown).

During the course of establishing the above-described purification protocol, we found that the overall yield of ATP-citrate lyase disturbingly varied in different preparations, sometimes resulting in almost a complete loss of activity following DEAE-cellulose and hydroxyapatite column chromatography. Eventually, it was found that the inclusion of sucrose in the buffers during DEAE-cellulose column chromatography helped to sig-

nificantly stabilize the enzyme. It was also apparent that variations in enzymatic activity observed during purification correlated with the relative amount of the 135- and 42-kDa species observed on SDS-polyacrylamide gels. In addition, the relative proportions of two polypeptides (i.e., 65 and 42 kDa) also varied widely in different enzyme preparations. These observations suggested the possibility of proteolytic processing of the enzyme during purification by some endogenous protease(s), as has often been observed with eukaryotic ATP-citrate lyases, which are notoriously unstable (12, 26, 28). Several variables relating to sample preparation were evaluated in attempts to either stabilize the enzyme or determine the relationship between these three polypeptides and ATP-citrate lyase activity. First, a variety of protease inhibitors (see Materials and Methods) were included in the buffers during purification, with no discernible difference in the levels of activity. Second, a variety of sample preparation and digestion methods (Materials and Methods) were attempted to prevent potential proteolysis during the time the protein was denatured prior to SDS-PAGE, including injecting samples into boiling SDS, denaturation in guanidine hydrochloride and alkylation, and performic acid oxidation (35). Again, no discernible difference in the pattern of electrophoretic products was observed. Third, the enzyme was purified under anaerobic conditions, with no effect. Finally, glycerol, citrate, and ATP were included in the purification buffers, with no obvious differences in purification results. In fact, ATP tended to destabilize the enzyme in both crude extracts and purified preparations, resulting in a rapid loss of enzyme activity. This situation has also been observed during the purification of ATP-citrate lyase from the oleaginous yeast *Rhodotorula gracilis* (26). The purified *C. tepidum* enzyme, stored in 20% glycerol at -70°C, was relatively stable for at least 6 months while retaining approximately 65 to 75% of its original activity.

Western immunoblot analyses. The 135-kDa protein that was often not visible on the stained SDS-gel (Fig. 3A, lane 6) was identified in Western blot analyses using antibodies prepared against the apparently homogeneous native protein (Fig. 3B). Immunoblot analysis after SDS-PAGE of proteins present at each stage of this enzyme preparation clearly showed the presence of the prominent 65- and 42-kDa bands, along with an additional higher-molecular-mass protein of about 135 kDa (Fig. 3, lanes 3 to 5). A few proteins intermediate in size also reacted with these antibodies (Fig. 3D, lanes 3 to 5), indicating the presence of each of these polypeptides in the apparent single species observed on the nondenaturing gel (Fig. 3B). In some preparations (results not shown), there was a low-molecular-weight (M_r of $\approx 16,000$ to 18,000) protein which reacted with these antibodies.

Molecular weight of ATP-citrate lyase. The purified ATP-citrate lyase yielded an estimated molecular weight of about 550,000, based on both its elution from a Superose 6 gel filtration column (Fig. 4) and its migration relative to standards on a nondenaturing gel (results not shown). Three peaks were observed in most gel filtration experiments (Fig. 4), with ATP-citrate lyase activity corresponding to the 550,000- M_r peak (Fig. 4, fraction 14). SDS-PAGE analysis of this fraction again produced the 65- and 42-kDa proteins, whereas no observable proteins were present when fractions 18 and 24 were subjected to SDS-PAGE (data not shown). Interestingly, some gel filtration experiments showed a weak ATP-citrate lyase activity present in fraction 18 (M_r of $\approx 260,000$). This activity represented only about 20% of the maximum activity detected in the 550,000- M_r peak and suggested that fraction 18 contained a possible cleavage product of the high-molecular-weight protein which still retained some enzymatic activity. Molecular weight

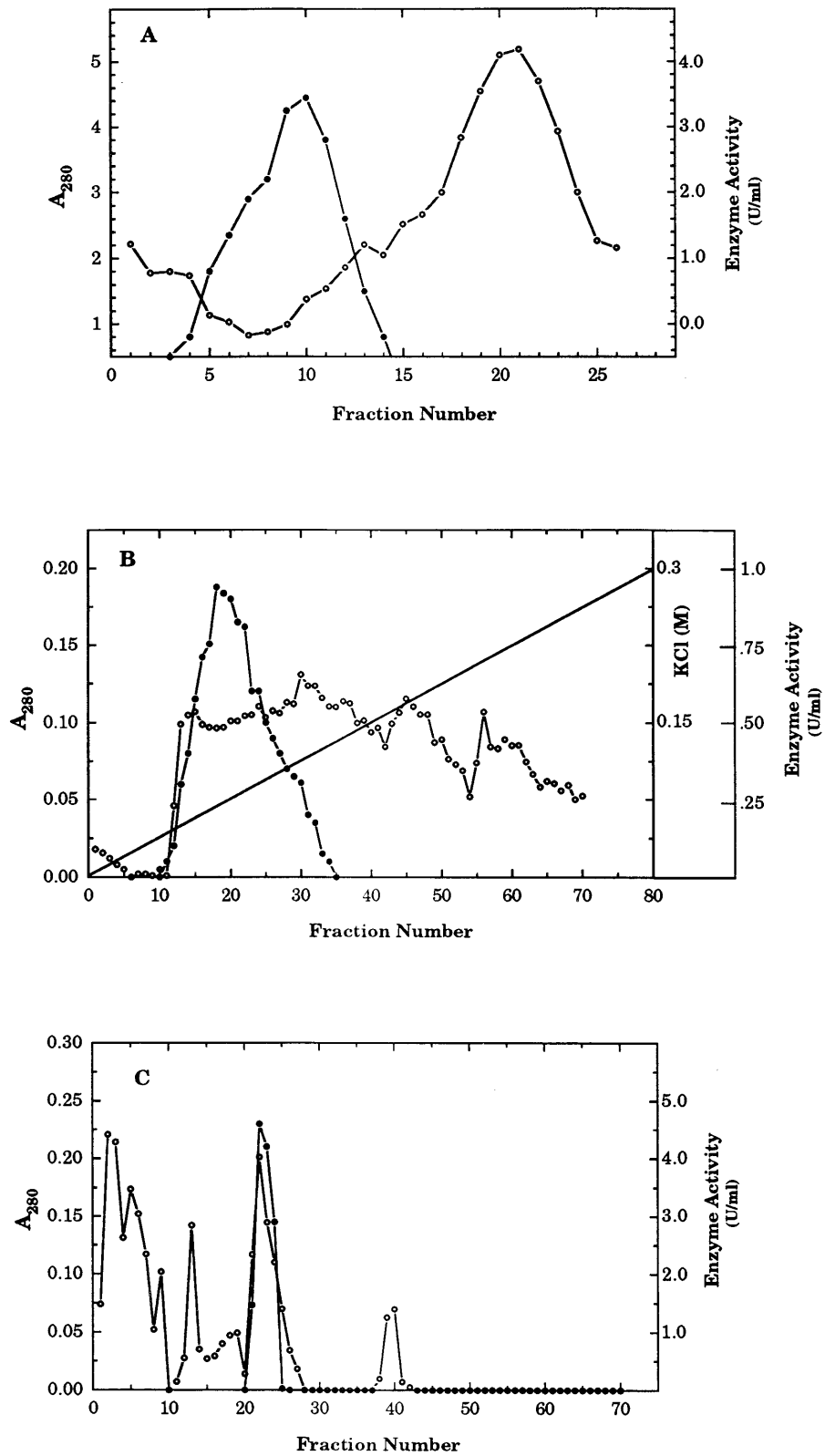


FIG. 2. Fractionation of ATP-citrate lyase on sucrose gradients (A), DEAE-cellulose (B), and hydroxyapatite (C). ○, A_{280} ; ●, enzyme activity. The solid diagonal line in panel B delineates the KCl gradient.

TABLE 1. Purification of ATP-citrate lyase from *C. tepidum*

Step	Total protein (mg)	Total activity (U ^a)	Sp act (U/mg)	Purification (fold)	Yield (%)
High-speed supernatant	2,084	119	0.057		100
Ammonium sulfate fractionation (40–55%)	744	92	0.124	2.2	78
Sucrose gradient centrifugation	139	42	0.300	5.3	35
DEAE column (0–0.3 M KCl)	56.4	35	0.624	11	30
Hydroxyapatite column (10–400 mM phosphate)	1.7	9.5	5.6 (14.4 ^b)	98	8

^a One unit is the amount of enzyme required for the ATP-dependent conversion of 1 μ mol of citrate to 1 μ mol of product at ambient temperature.

^b Specific activity at 48°C.

calibration curves on SDS-polyacrylamide gels indicated that the three polypeptides had molecular weights of about 135,000, 65,200, and 42,000, with variations in the smaller polypeptide ranging from 42,000 and 47,000 (data not shown). With the known history of this protein from eukaryotes, it is possible that the high-molecular-weight protein seen on SDS-polyacrylamide gels represented the intact subunit of *C. tepidum* ATP-citrate lyase, with the two smaller polypeptides resulting from cleavage of this protein during purification. If confirmed, this would indicate that the enzyme was a homotetramer (M_r of ~550,000), consisting of a subunit size of around 135,000.

Phosphorylation of *C. tepidum* ATP-citrate lyase. Additional experiments were initiated to analyze the quaternary structure of ATP-citrate lyase and to determine if the 65- and 42-kDa polypeptides were likely generated from an endogenous protease activity. Phosphorylation of eukaryotic ATP-citrate lyases has been shown to be autocatalytic, with the γ -phosphate from ATP shown to bind to a histidine residue at the active site (31). Incubation of the *C. tepidum* enzyme with [γ -³²P]ATP resulted in a major phosphorylated protein of 65 kDa and a labeled minor species of about 135 kDa (Fig. 5A). The incorporation of ³²P into the 135- and 65-kDa polypeptides was acid labile (Fig. 5A, lanes 1 and 3) and base stable (Fig. 5A, lanes 7, 8.5, and 14), which is consistent with phosphorylation at a histidine residue. Labeling of the 135-kDa protein was clearly visible only at pH 7.0. Hydroxylamine hydrolyzes phosphohistidines, phosphoaspartic acids, and phosphoglutamic acids, whereas sodium borohydride hydrolyzes phosphoglutamic acids and phosphoaspartic acids but not phosphohistidines (36, 37). Incubation of the *C. tepidum* ATP-citrate lyase with hydroxylamine and sodium borohydride yielded results compatible with the formation of phosphohistidine (Fig. 5B). In addition, boiling of the labeled enzyme from *C. tepidum* for 2 min completely removed the ³²P label (Fig. 5B, lane 2), again compatible with the known thermolability of phosphohistidines. To determine if phosphate incorporation was specific, *C. tepidum* ATP-citrate lyase protein samples were incubated with ADP and citrate following incubation with [γ -³²P]ATP. Removal of ³²P from the enzyme with both ADP and citrate occurred (Fig. 5B, lanes 5 and 6), while oxaloacetate had no effect (Fig. 5B, lane 7). The ability to chase the phosphorylated label with the enzyme's substrates clearly indicated that the phosphate was most likely incorporated at an active-site residue, presumably histidine, and that this site was present on both the 135- and 65-kDa polypeptides of the *C. tepidum* ATP-citrate lyase. Furthermore, the ³²P label which appeared as a smear between the positions of the 135- and 65-kDa bands was also chased with ADP and citrate (Fig.

5). These results suggested that a specific degradation of the *C. tepidum* enzyme had occurred, as opposed to the retention of some nonspecific background signal on the gel. Furthermore, the radioactive signal was rapidly lost from the 65-kDa fragment upon further proteolysis to produce the 42-kDa fragment.

Catalytic properties of *C. tepidum* ATP-citrate lyase. The Michaelis constants for various components of the *C. tepidum* ATP-citrate lyase reaction at 48°C were as follows: citrate, 210 μ M; ATP, 310 μ M; CoA, 24 μ M; and Mg²⁺, 4 mM (Table 2). The values for ATP and CoA differed significantly from those reported from a partially pure preparation of the enzyme from the closely related mesophile *C. limicola* (Table 2) (2). *C. tepidum* ATP-citrate lyase exhibited typical Michaelis-Menten kinetics for these substrates, with no evidence obtained for allosteric interactions.

Nucleotide and divalent metal specificity. The activity of the enzyme was absolutely dependent on the presence of divalent cations (Table 3). Mn²⁺ could nearly replace the requirement for Mg²⁺ for activity, giving 85% of the activity observed in the presence of Mg²⁺. In contrast to the enzyme from *C. limicola*, Cu²⁺ could not replace Mg²⁺ for activity at the concentrations tested. Also differing from the *C. limicola* ATP-citrate lyase was the ability of the *C. tepidum* enzyme to utilize CTP, but not GTP, to partially replace ATP for enzyme activity (Table 3). These results are similar to those obtained for mammalian ATP-citrate lyase (31), which is able to bind GTP but is unable to utilize this nucleotide to support activity. Until this study, the situation with the prokaryotic enzyme appeared to be quite different, with ATP-citrate lyase from both *H. thermophilus*

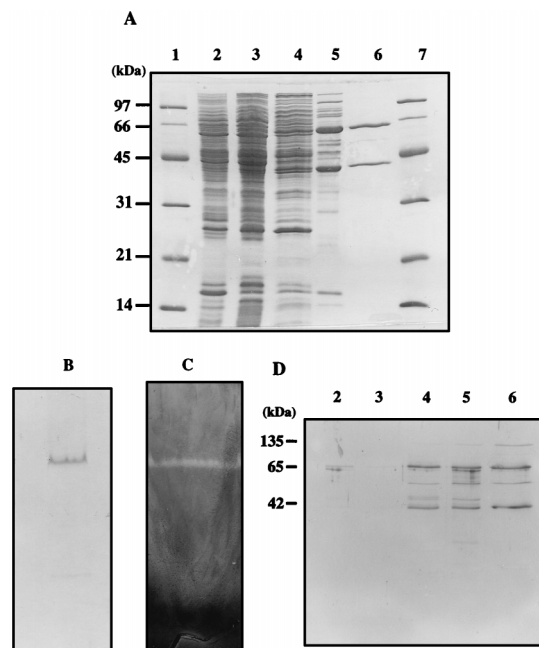


FIG. 3. SDS-PAGE analysis (A) and Western immunoblot analysis (D) of enzyme preparations at each stage during purification. Electrophoresis was carried out on 15% polyacrylamide gels. Lanes 1 and 7, molecular weight standards; lane 2, cell lysate; lane 3, ammonium sulfate pool; lane 4, sucrose gradient pool; lane 5, DE-52 column pool; lane 6, hydroxyapatite fraction. The hydroxyapatite fraction was also electrophoresed on a nondenaturing (6%) gel (B). (C) Gel assay of ATP-citrate lyase on the nondenaturing gel. The assay was performed as described in Materials and Methods, and the substrate-dependent ATP-citrate lyase activity was indicated by a clear band against the dark background. Molecular masses are indicated on the left in panels A and D.

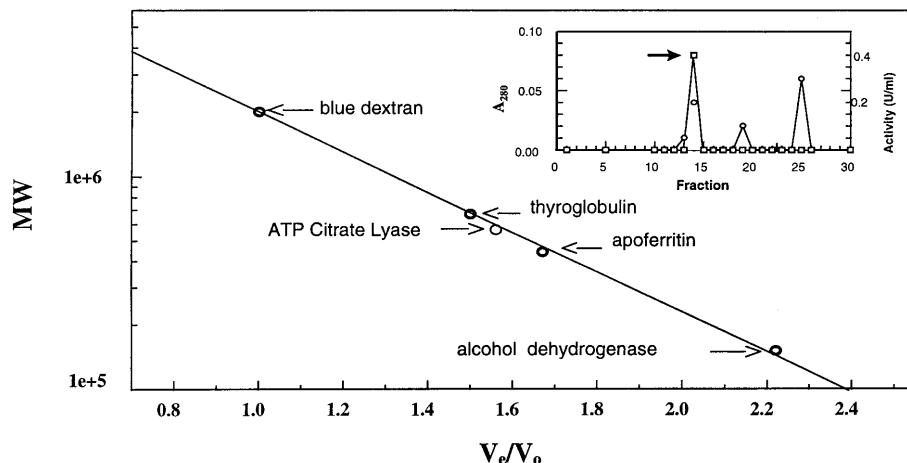


FIG. 4. Gel filtration and molecular weight determination of ATP-citrate lyase. A Superose 6 gel filtration column was calibrated by using the following as molecular weight (MW) standards: blue dextran (M_r of 2×10^6), thyroglobulin (M_r of 669,000), apoferritin (M_r of 443,000), and alcohol dehydrogenase (M_r of 150,000). ATP-citrate lyase activity (\square) eluted as a single peak with an M_r of about 550,000 (inset). Weak enzyme activity, corresponding to an M_r of 260,000, was observed in some gel filtration experiments.

and *C. limicola* capable of partially replacing ATP with GTP (2, 13). These results suggest that the *C. tepidum* enzyme may be more similar to its eukaryotic counterpart than to the prokaryotic ATP-citrate lyases isolated thus far.

Effect of pH and temperature. The pH optimum for enzyme activity was 8.5 (Table 2); however, maximum stability was observed at pH 7 to 7.2. The optimum temperature for ATP-citrate lyase activity was the same as the growth temperature optimum for the organism (48°C) but fell off sharply above 50°C (Fig. 6). Enzyme activity could be detected up to 60°C in purified preparations (Fig. 6) and up to 65°C in crude extracts or with purified enzyme containing citrate (10 mM) (data not shown). The addition of citrate to the purified enzyme prior to incubation at 65°C increased the time interval before loss of activity from 5 to 20 min. However, this stabilizing effect appeared to be related only to temperature and did not prevent proteolysis of the enzyme, as discussed earlier.

DISCUSSION

ATP-citrate lyase from the anoxic thermophilic green sulfur bacterium *C. tepidum* was purified to apparent homogeneity. Purification was difficult due to the highly labile nature of the enzyme during preparation, and significant losses of enzyme units were observed at different stages of the purification protocol. Apparently eukaryotic ATP-citrate lyase is also notorious for its lability, making it difficult to purify as well (4, 9, 21, 23, 26, 30). The stability problems with the *C. tepidum* and eukaryotic ATP-citrate lyase enzymes are interesting in comparison to ATP-citrate lyase from the aerobic, hydrogen-oxidizing thermophilic bacterium *H. thermophilus*. The enzyme from this bacterium is the only other prokaryotic ATP-citrate lyase to be purified to date and was found to be completely stable in the absence of any sulfhydryl compounds (13). However, as discussed below, the enzyme from this bacterium appears to be different in several respects from that of the *C. tepidum* and eukaryotic enzymes.

The results of gel filtration experiments yielded an estimated molecular weight for the *C. tepidum* ATP-citrate lyase of $545,000 \pm 6,500$. Preparations of *C. tepidum* ATP-citrate lyase were judged to be homogeneous by nondenaturing gel electrophoresis but consistently produced two to three polypeptides on SDS-gels. The two major components (65 and 42 kDa) were

initially thought to represent different subunits of the enzyme, with the high-molecular-weight species (size of ca. 135 kDa) and other visible trace species perhaps representing minor contaminant(s) which coeluted with the *C. tepidum* enzyme during purification. However, several lines of evidence argued against the two lower-molecular-weight polypeptides representing distinct subunits of *C. tepidum* ATP-citrate lyase and supported their identification as proteolytic fragments arising from the high-molecular-weight polypeptide. First, variations in the amount and size of the lower-molecular-weight polypeptide (average size, 42 kDa; range, 38 to 47 kDa) were observed in different purification preparations, and in two cases, purified

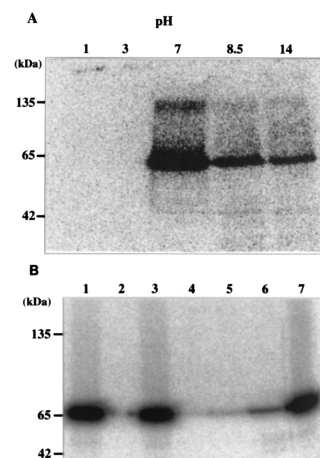


FIG. 5. SDS-PAGE analysis of the phosphorylation pattern of purified ATP-citrate lyase following labeling with [γ - 32 P]ATP. Incubation mixtures (50 μ l) contained ATP-citrate lyase (3 μ g), 50 mM Tris-Cl (pH 8.5), 3 mM MgCl₂, 1 mM DTT, and 0.2 μ Ci of [γ - 32 P]ATP. ATP and the enzyme were incubated at 37°C for 10 min. For analysis of the pH stability of ATP-citrate lyase (A), the labeled reactions were added to the appropriate buffer solutions, incubated for an additional 10 min at 37°C, and neutralized prior to SDS-PAGE. In panel B, labeled samples were incubated at pH 8.5 for an additional 10 min at -20°C (lane 1), at 100°C (lane 2), with borohydride (lane 3), with 0.8 M hydroxylamine (lane 4), with 25 μ M ADP (lane 5), with 10 mM citrate (lane 6), and with 10 mM oxaloacetate (lane 7). Samples were electrophoresed on SDS-10% polyacrylamide gels, and the results were analyzed with a Storm 840 Molecular Dynamics phosphorimager.

TABLE 2. Comparison of kinetic properties of ATP citrate lyases from various organisms

Organisms	Optimum pH	K_m				Sp act (U/mg)	Mol wt (10^3)
		ATP (μ M)	Citrate (μ M)	CoA (μ M)	Mg ²⁺ (mM)		
Rat	8.4	172	560	ND ^b	ND	6.25	440–500
<i>Penicillium spiculisporum</i>	8.0	90	180	1–3	ND	3.0	>200 ^c
<i>Lipomyces starkeyi</i>	8.4	135	70	ND	ND	0.92	510 ^c
<i>Rhodotorula gracilis</i>	8.4	175	2,630	ND	ND	5.88	520
<i>Hydrogenobacter thermophilus</i>	6.7–6.9	650	6,250	40.8	8.0	26.7	260
<i>Chlorobium limicola</i>	8.5	65	230	77	ND	1.10	240 ^c
<i>Chlorobium tepidum</i>	8.5	310 (340 ^a)	210 (300)	20 (24)	ND	14.4 (5.6)	520–550.

^a Value for experiments performed at room temperature compared to the growth temperature optimum of *C. tepidum* (48°C).

^b ND, not determined.

^c Estimation based on partial purification of the enzyme.

fractions in which this protein was completely absent still showed normal enzymatic activity. Second, immunoblots using antibodies prepared against the native protein also reacted with the higher-molecular-weight polypeptide (M_r of \approx 135,000), in addition to the two lower-molecular-weight polypeptides. Given that this preparation migrated as a single band on non-denaturing gels, it seemed likely that the lower-molecular-weight polypeptides arose from proteolytic events occurring during isolation. The appearance of these two proteins did not appear to be generated during SDS-PAGE since several methods were used to prevent proteolysis during the preparation of the sample for SDS-PAGE, including incubation in hot SDS, denaturation in guanidine hydrochloride, carboxymethylation, and performic acid oxidation (35). Third, and most importantly, in vitro phosphorylation experiments showed specific labeling of 135- and 65-kDa polypeptides, with the majority of the label found associated with the 65-kDa polypeptide. The ³²P label,

which also appeared as a smear between the 135- and 65-kDa polypeptides, could be selectively chased with citrate and ATP but not with oxaloacetate or succinate, thus providing further evidence to support a high-molecular-weight origin of the two lower-molecular-weight bands. Fourth, Western immunoblots showed strongly reacting bands at 65, 42 to 47, and in some cases 16 to 18 kDa, the sum of which approximately equals the molecular mass of the putative *C. tepidum* ATP-citrate lyase subunit (135 kDa).

We attempted to purify the 135-kDa protein in order to compare the proteolytic products generated from trypsin digestion with those produced by the putative endogenous protease activity present in *C. tepidum* enzyme preparations. This strategy had been used successfully with the mammalian enzyme, where a pure preparation of the enzyme subunit was obtained by using α_2 -macroglobulin in the purification buffers (17). Subsequent treatment of the mammalian protein with trypsin yielded fragments similar in molecular weight to those produced by the endogenous protease activity. Unfortunately, we were unable to obtain enough intact 135-kDa protein from *C. tepidum* preparations for digestion with trypsin. We also attempted to immunoprecipitate this protein, but without success. Similar instabilities and proteolysis profiles have been observed with all eukaryotic ATP-citrate lyases which have

TABLE 3. Nucleotide and metal dependency of ATP-citrate lyase activity from *C. tepidum*

Metabolite	Concn (mM)	Enzyme activity ^a (% maximum)
ATP	2.0	100
	0.5	95
CTP ^b	2.0	23
	0.5	5
GTP ^b	2.0	NA ^d
	0.5	NA
Mg ²⁺ ^c	1.0	36
	4.0	100
Mn ²⁺ ^c	1.0	80
	2.0	85
Cu ²⁺	0.1	NA
	1.0	NA
Zn ²⁺	0.1	NA
	1.0	NA

^a Assays were performed at 46 to 48°C, using 0.07 U of purified ATP-citrate lyase. ATP or Mg²⁺ was replaced by one of the compounds listed.

^b The concentration of CTP (0.5 to 2.0 mM) represents the concentration that yielded the lowest and highest activities, respectively. GTP at 0.1 to 10 mM did not support any activity.

^c Maximum activities for Mg²⁺ and Mn²⁺ were obtained at 4 and 2 mM, respectively.

^d NA, no activity.

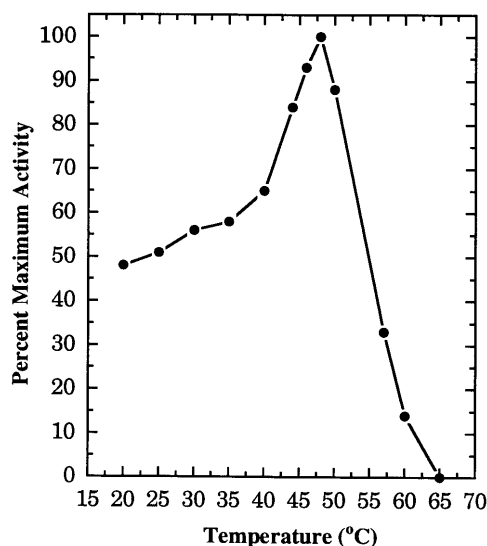


FIG. 6. Effect of temperature on ATP citrate lyase activity. *C. tepidum* ATP-citrate lyase (0.06 U) was incubated for 5 min at each temperature prior to assay of enzymatic activity.

been purified to date (4, 9, 26, 28); thus, an analogous series of events may occur during preparation of the enzyme from *C. tepidum*. In support of these findings is the fact that a trypsin-treated enzyme preparation of rat liver ATP-citrate lyase, which completely cleaved the 120-kDa subunit of this enzyme into two protein bands of 71 and 55 kDa, migrated identically to the intact enzyme in gel filtration and sedimentation velocity experiments (28). Indeed, attempts to obtain homogeneous preparations of ATP-citrate lyase from several other eukaryotic organisms have failed due to the highly labile nature of these enzymes.

On the basis of these results, we conclude that the *C. tepidum* ATP-citrate lyase is a homotetramer with a subunit M_r of $135,000 \pm 2,800$. The structure and proteolysis profile of *C. tepidum* ATP-citrate lyase indicated surprising similarities with the mammalian and yeast enzymes. *H. thermophilus* ATP-citrate lyase, the only other prokaryotic enzyme to be purified to homogeneity, was reported to be a hexamer of 43,000- M_r subunits with a native molecular weight of about 260,000 (12). This extremely thermophilic, aerobic, H_2 -oxidizing eubacterium is phylogenetically unrelated to *Chlorobium*, and consequently differences in the structure and properties of analogous enzymes would not be considered unusual. Surprisingly, sucrose gradient centrifugation of a partially purified preparation of the enzyme from *C. limicola* gave an estimated molecular weight of 240,000 (2), about half that of its close relative *C. tepidum*. Considering the unstable nature of these enzymes, and the fact that the subunit composition of the *C. limicola* enzyme could not be determined since it was only partially purified (2), the activity observed in the 240,000- M_r peak following sucrose gradient centrifugation may represent a lower state of aggregation or may in fact be a degradation product of the *C. limicola* holoenzyme. A weak but detectable ATP-citrate lyase activity was observed in some gel filtration experiments of the *C. tepidum* enzyme, indicative of a 260,000- M_r species, although the major peak of activity, along with the results from nondenaturing PAGE, indicated a molecular weight of the intact enzyme of about 550,000. Enzyme preparations from rat liver and from the oleaginous yeast *R. gracilis* have been shown to retain about 75% of their original activity following digestion with trypsin (26, 28). In these organisms, extended treatment with trypsin resulted in the complete disappearance of the high-molecular-weight subunit band (size of 120 kDa) and the appearance of two lower-molecular-weight polypeptides. This appears to be analogous to the situation with the *C. tepidum* enzyme. Given the fact that treatment of rat ATP-citrate lyase with trypsin did not affect phosphorylation with [γ - ^{32}P]ATP or its K_m for citrate, immunoreactivity, or sedimentation behavior, interactions essential for enzyme activity and quaternary structure were therefore maintained to some degree following proteolysis (28). Again, it is apparent from the results presented here that a very analogous proteolysis occurs, with activity associated with the 65,000- M_r polypeptide. This conclusion is primarily supported by the phosphorylation experiments where incubation of the *C. tepidum* ATP-citrate lyase with [γ - ^{32}P]ATP resulted in the phosphorylation of a major band with an M_r of about 65,000 and a minor band with an M_r of 135,000. Evidence discussed above indicated that this larger polypeptide represents the intact subunit of the enzyme. The active site was obviously associated with the 135,000- M_r protein as well as the 65,000- M_r cleavage product, since the ^{32}P was specifically chased from both proteins with ADP and citrate. As observed with the mammalian enzyme, the bound phosphate of the *C. tepidum* enzyme was acid labile, base stable, temperature sensitive, and hydrolyzed by hydroxylamine but not sodium borohydride. Taken together,

these properties indicate that *C. tepidum* ATP-citrate lyase is autophosphorylated, presumably on a histidine residue, at its active site. Further proteolysis of the *C. tepidum* enzyme to produce the 42-kDa fragment resulted in loss of the ^{32}P label. This pattern is strikingly similar to what was observed with the rat hepatic ATP-citrate lyase, where treatment of the purified 123-kDa ^{32}P -labeled protein with trypsin produced a 57-kDa ^{32}P -containing fragment nearly indistinguishable on SDS-gels from the endogenously generated proteolytic fragment (1). The ^{32}P label was subsequently lost from the rat enzyme following further proteolysis with trypsin, thus lending additional support for structural similarities between the mammalian and *C. tepidum* enzymes.

Analyses of other properties showed similarities of *C. tepidum* ATP citrate lyase to the eukaryotic enzyme. Except for the *H. thermophilus* enzyme, the pH optima (about pH 8.5) of purified enzymes from all other sources are very similar; moreover, all of the enzymes have native molecular weights of about 500,000, and all are presumably homotetramers of a single large subunit which is proteolytically cleaved. The structural similarity of these enzymes was also reflected by their responses to various metals and nucleotides. The mammalian enzyme is known to bind GTP, and this nucleotide could act as a donor for histidine autophosphorylation of ATP-citrate lyase, yet GTP does not support enzymatic activity (31). Similarly, the *C. tepidum* ATP-citrate lyase could not use GTP to replace ATP for activity, but it has not been determined whether GTP could support the autophosphorylation reaction. The results with GTP are particularly interesting when contrasted with the bacterial enzymes from *C. limicola* and *H. thermophilus*, both of which were able to use GTP to support enzymatic activity (2, 13). These results suggest some significant difference(s) in the nucleotide binding sites of the enzymes from *C. tepidum* and its close relative, *C. limicola*. No evidence for allosteric regulation of *C. tepidum* ATP-citrate lyase was obtained; after a long history of no apparent allosteric regulation of the mammalian (6, 28) and yeast (4, 8) enzymes, there is now some indication that the rat enzyme might show allosteric control (20). Studies suggesting a redox control of enzyme activity, based on the reversibility of the oxidized/inactive and reduced/active forms of the enzyme, have also been inconclusive (38, 39). Succinyl-CoA synthetase, an enzyme structurally and catalytically similar to ATP-citrate lyase, exhibits cooperative kinetics with various compounds (40), and a more recent study has indicated possible regulation of rat ATP-citrate lyase via both phosphorylation and allosteric control (20). However, much further work is needed to identify and elucidate possible regulatory mechanisms. Given the apparent structural similarities of the enzyme from the thermophilic green sulfur bacterium *C. tepidum* and its mammalian counterpart, further studies of this crucial enzyme of carbon metabolism from these diverse systems should be most interesting. As a key and cornerstone enzyme of the RTCA cycle of CO_2 fixation in organisms such as *C. tepidum*, studies of the regulation of ATP-citrate lyase are particularly cogent. Our results thus far point to complex phenomena related to diverse posttranslational control processes. Final confirmation of the quaternary structure and subunit composition of the *C. tepidum* ATP-citrate lyase will be greatly assisted by knowledge of its deduced primary structure. In this connection, specific oligonucleotides, constructed from N-terminal and internal amino acid sequences of the 65- and 42-kDa fragments, have identified regions of the *C. tepidum* chromosome which hybridize strongly to these probes. Experiments are in progress to clone and sequence these regions, with the eventual goal of relating

the apparent proteolytic processing of this enzyme to specific amino acid sequences and defined regulatory events.

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