The native form of ATP citrate lyase (2 mol of phosphate/tetramer) and the dephospho-ATP citrate lyase (phosphate-free) purified to homogeneity from rat liver, are phosphorylated by ATP and by the catalytic subunit of cAMP-dependent protein kinase from rabbit muscle. A total of 2 mol of phosphate/tetramer were incorporated into native enzyme, while with the dephospho form, 4 mol of phosphate were incorporated. The phosphopeptides resulting from trypsin treatment which were isolated from phosphorylated forms of both native enzyme and the dephospho enzyme were similar. The ATP citrate lyase, phosphorylated to an extent of 4 mol of phosphate/tetramer, has the same V_max as the native enzyme (2 mol of phosphate/tetramer). Native ATP citrate lyase, trypsin-treated to remove the phosphopeptide, could not be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase from rabbit muscle, suggesting a common trypsin-sensitive specific phosphorylation site. The phosphorylation rate varied with pH in potassium phosphate, imidazole/HCl, and Tris/HCl buffers. Divalent cations were essential for the activity of the protein kinase. The apparent K_m value for ATP was found to be 50 \mu M.

Several years ago, we showed that ATP citrate lyase isolated from rat liver contained 2 serine phosphates (structural) per tetramer (M_r = 440,000) (1, 2). Following that report, we and others demonstrated that when hepatocytes are incubated with glucagon, a stimulation of the incorporation of 32P as structural phosphate into ATP citrate lyase occurred (3, 4). Every action of glucagon thus far studied has indicated that the hormone acts by stimulating adenylate cyclase, with a resultant increase in cAMP in the cell, which causes an increase in the amount of the active catalytic subunit of the cAMP-dependent protein kinase. The protein kinase catalyzes the phosphorylation of a number of "target" proteins, causing a change in the properties of the protein (e.g., catalytic activity) which results in the final expression (change in metabolism) of the hormone action (5–7).

It is commonly held that the action of a catalytic subunit of the cAMP-dependent protein kinase is the same, in terms of specificity, from tissue to tissue, and, although it is known that rat liver contains such an activity, it is more convenient to use cAMP-dependent protein kinase from rabbit muscle. A recent report by Guy et al. (8) had shown that a rat mammary gland ATP citrate lyase (specific activity = 2) with a structural phosphate content of 0.2 ± 0.1 mol/monomer could be phosphorylated to a phosphate content of 0.7 ± 0.2 mol/monomer using a rabbit muscle catalytic subunit of the cAMP dependent protein kinase. The article by Guy et al. (8) did not report any change in the properties (catalytic or other) of the ATP citrate lyase when it was phosphorylated. We have shown that rat liver ATP citrate lyase (specific activity = 10) with a content of 2.0 structural phosphates/tetramer had the same kinetic properties as dephospho-ATP citrate lyase (9).

We therefore believed that it was necessary to determine whether: 1) the rat liver enzyme with 2 phosphates/tetramer could be phosphorylated to a greater extent; 2) the dephosphoenzyme could be phosphorylated to the same extent; 3) the phosphate groups put on by the kinase were on the same peptide as the phosphate groups normally present; 4) there is any activity change of ATP citrate lyase after more extensive (4 mol/tetramer) phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Analytical grade reagents and materials were obtained from the following sources: SDS, histone (Type VII-S), NADH, ATP, heat-stable cAMP-dependent protein kinase inhibitor, and soya trypsin inhibitor from Sigma; dithiothreitol from Calbiochem; malate dehydrogenase from Boehringer Mannheim; trypsin (Tosylphenylalanyl chloromethyl ketone-treated) from Worthington; CoA from PL Biochemicals; [γ-32P]ATP from New England Nuclear; Sephacryl S-200 from Pharmacia Fine Chemicals; TLC plates precoated with cellulose MN900 from BINKMANN Instruments; x-ray film XR-2 (8 × 10 inch) from Kodak.

ATP citrate lyase and dephospho-ATP citrate lyase were prepared by previously described procedures (9) and were assayed by the malate dehydrogenase-coupled procedure as described earlier (10, 11); 1 unit of enzyme is defined as the amount of enzyme necessary to catalyze the oxidation of 1 nmol of NADH/min under the assay conditions. The concentration of ATP citrate lyase was determined from its absorbance at 279 nm (E_1% = 11.4). Catalytic subunit of cAMP-dependent protein kinase from rabbit muscle was a gift from Dr. E. Krebs of the University of Washington, Seattle, WA. The protein kinase fraction used had a specific activity of 780 units/mg, where 1 unit is defined as that amount of enzyme catalyzing the transfer of 1 nmol of phosphate from [γ-32P]ATP to histone per min at 30°C under the given conditions. The isolation of trypsin-treated ATP citrate lyase was done by the following method. ATP citrate lyase (10 mg) was treated with 0.1 mg of trypsin for 20 min at room temperature. Soya trypsin inhibitor (1 mg) was added to the solution and the mixture was passed through a Bio-Gel A-1.5m column (2.5 × 40 cm) equilibrated with 50 mM Tris/HCl, pH 7.2, containing 1 mm dithiothreitol and 0.1 mM EDTA. The enzyme was eluted with the same buffer, and the fractions having enzyme activity were pooled and concentrated by ammonium sulfate precipitation (30 g/100 ml). This preparation was used as the trypsin-treated enzyme.

**Phosphorylation Assay**—The phosphorylation of ATP citrate lyase by the catalytic subunit of the cAMP-dependent protein kinase was carried out at 30°C in an assay mixture containing 50 mm imidazole/HCl buffer, 0.4 mm EDTA, 0.1 mm ethylene glycol bis(β-aminoethly ether)-NN,N',N''-tetraacetic acid, 2.0 mm magnesium ace-
Phosphorylation of ATP Citrate Lyase by Catalytic Subunit

Isolation of Phosphopeptide—Both highly purified phospho- and dephospho-ATP citrate lyase (6.5 mg/ml) were incubated separately with the catalytic subunit of the cAMP-dependent protein kinase plus \( \gamma^{32} \)P ATP for 60 min at 30°C. After the incubation period, the samples were loaded on a micro column according to Penefsky (12), except that Sephacryl S-200 was used instead of G-50 to remove most of the free \( \gamma^{32} \)P ATP and kinase. Ninety per cent of the enzyme was recovered by this method.

Histidine phosphate (the catalytic phosphate) is formed during the catalytic activity of the enzyme, and this site is phosphorylated when ATP citrate lyase is incubated with \( \gamma^{32} \)P ATP (13). In order to remove the catalytic phosphate from the histidyl residue quantitatively, without deactivation of the enzyme, the enzymes phosphorylated by the catalytic subunit were treated with 1 mM CoA and 10 mM citrate for 15 min at 30°C and then were dialyzed against 100 mM Tris/HCl, pH 7.2, containing 1 mM diithiothreitol and 0.1 M EDTA after the Sephacryl S-200 step. The dialyzed samples were treated with 0.1 mg of trypsin and incubated at 30°C for 1 h. After the incubation, 1.0 mg of trypsin inhibitor was added to stop the tryptic action and an aliquot taken for peptide mapping. Peptide mapping and detection was done on TLC plates precoated with cellulose MN300 as described elsewhere (14). Electrophoresis was performed for 2 h at 300 V using a buffer composed of pyridine:acetic acid:water (24:1:350) at pH 6.5. Chromatography was done to develop the second dimension using 1-butanol/pyridine:acetic acid:water (15: 10:3:12).

RESULTS

Phosphorylation of Both Phospho- and Dephospho-ATP Citrate Lyase by the Catalytic Subunit of Protein Kinase—The ATP citrate lyase as normally isolated from rat liver contains 2 mol of structural phosphate/mol of enzyme tetramer (2). When this enzyme is incubated with the catalytic subunit of the cAMP-dependent protein kinase, 2 additional mol of phosphate are incorporated, bringing the total phosphate content to 4 mol enzyme tetramer (Fig. 1). In the phosphorylation method described under “Experimental Procedures,” the relative rates of phosphorylation by the kinase were found to be 280 pmol/min for histone (0.2 mg/ml) as against 10 pmol/min for ATP citrate lyase (2 mg/ml). Phosphorylation of dephospho-ATP citrate lyase resulted in the final incorporation of 4 mol of phosphate/mol of enzyme tetramer (Fig. 1). In addition, the extent of phosphorylation is the same for a 50-fold range in lyase concentration. The specific heat-stable inhibitor (15) of the catalytic subunit of the cAMP-dependent protein kinase, when present in the incubation mixture, inhibited the phosphorylation of ATP citrate lyase (Fig. 1).

The Effect of Catalytic Subunit Concentration—The dependence of the rate of phosphorylation of ATP citrate lyase on the amount of catalytic subunit used was investigated. This rate of \( \gamma^{32} \)P phosphate incorporation into either the phospho or dephospho form of the lyase was found to be proportional to the catalytic subunit concentration up to 750 units/ml (data not shown). In addition, the final stoichiometry of the acid-stable phosphate bound to ATP citrate lyase was found to be constant (4 mol/tetramer) as long as the catalytic subunit was present in “catalytic” quantities. Occasionally, at relatively high kinase/lyase ratios, we found slightly more than 4 mol of phosphate bound. Whether this “extra” phosphate was a result of specific or nonspecific phosphorylation by the high concentration of catalytic subunit was not determined.

Effect of pH on Phosphorylation—The relationship of the phosphorylation reaction to pH in different buffer systems was investigated. The buffers used were potassium phosphate, pH 6.0 to 8.5; Tris/HCl, pH 6.0 to 9.0; and imidazole/HCl, pH 6.0 to 9.0. The final buffer concentration in each experiment was 50 mM. ATP citrate lyase was found to be stable in each of these buffers for the incubation period. At lower pH values, the phosphorylation rate was low in all the buffers. Above neutral pH, the phosphorylation rate of ATP citrate lyase increased in all the buffers. At even higher pH values, the phosphorylation rate decreased, except in imidazole/HCl buffer, where the rate was fairly high even at pH 9.0. The overall phosphorylation rate was better in Tris/HCl and imidazole/HCl systems compared to phosphate (data not shown).

Effect of Divalent Cations—The presence of divalent cations for the activity of protein kinases is well documented (16, 17). Magnesium ions had the maximum stimulatory effect at both low (2 mM) and high (20 mM) concentrations (Table I). The phosphorylation rate of ATP citrate lyase decreased slightly with magnesium acetate concentrations greater than 5 mM (data not shown). It is also evident that Co\(^{2+}\) and Mn\(^{2+}\) to some extent could replace magnesium. The phosphorylation did not occur when Mg\(^{2+}\) was replaced with Ca\(^{2+}\). However, Ca\(^{2+}\) added to Mg\(^{2+}\) did not change the phosphorylation rate (data not shown).

Influence of Substrate Concentration—The rate of phosphorylation of ATP citrate lyase was studied at ATP concentrations varying from 0.01 to 0.8 mM. A Lineweaver-Burk plot of the results obtained is shown in Fig. 2. The apparent \( K_m \) value for ATP when dephospho-ATP citrate lyase was used as substrate was 50 \( \mu \)M.

In another experiment, the concentration of the protein substrate was varied while the concentration of ATP was kept constant at 0.25 mM. With dephospho-ATP citrate lyase, a straight line was obtained in a double reciprocal plot, with an apparent \( K_m \) of 5.0 \( \mu \)M (Fig. 3).

Phosphorylation Site—It was important to determine whether the sites of phosphorylation of the phospho- (native)
TABLE I

Effect of cations on kinase reaction

Dephospho-ATP citrate lyase (3.0 mg/ml) was incubated in 50 mM imidazole/HCl buffer, pH 7.0, with 0.25 mM [γ-32P]ATP and 75 units/ml of the catalytic subunit of cyclic AMP-dependent protein kinase and divalent cations at the concentrations indicated. The reaction was followed for 5 min at 30 °C. The values are the average of three separate experiments. Chloride forms were used in all the experiments.

| Cation  | Concentration | Rate of phosphorylation |
|---------|---------------|-------------------------|
|         | mM            | pmol/min                |
| Mg2+    | 2             | 1.25                    |
|         | 20            | 0.85                    |
| Mn2+    | 5             | 0.80                    |
|         | 20            | 0.15                    |
| Co2+    | 5             | 0.75                    |
|         | 20            | 0.12                    |
| Ca2+    | 5             | 0                       |
|         | 20            | 0                       |
| None    |               | 0.10                    |

FIG. 2. Influence of ATP concentration on the rate of the protein kinase reaction with dephospho-ATP citrate lyase as substrate. The conditions were as described under "Experimental Procedures" with 75 units/ml of catalytic subunit of cyclic AMP-dependent protein kinase.

and dephospho-ATP citrate lyase are the same. Native ATP citrate lyase which had the phosphopeptide removed by trypsin treatment could not be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase from rabbit muscle, suggesting that this same trypsin-sensitive site was the region involved in the catalytic subunit-catalyzed phosphorylation. Also, autoradiography of the peptide map of the tryptic digests of both the native ATP citrate lyase and the dephospho-ATP citrate lyase after phosphorylation by catalytic subunit showed only one major radioactive spot. The minor spots may represent intermediates of the trypsin degrading sequence on ATP citrate lyase (2) (Fig. 4).

The major 32P-peptide spot is not reactive with ninhydrin (data not shown). Only a faint radioactive spot remained at the origin, indicating that tryptic nicking of ATP citrate lyase was essentially complete. The phosphate content of the peptides could not be chemically determined from the TLC scrapings, due to the lack of sufficient material for phosphate analysis. However, since the concentration of protein used in the phosphorylation reaction and the amount of sample taken for peptide mapping were the same for both native and dephospho-ATP citrate lyase, the increased intensity of the peptide spot from dephospho-ATP citrate lyase could be correlated to the increased incorporation of phosphate (4 mol of phosphate/tetramer) into the protein.

Fig. 3. The effect of dephospho-ATP citrate lyase (ATP-CL) concentration in the protein kinase reaction. The final ATP concentration was 0.25 mM. The conditions otherwise were same as those described under "Experimental Procedures" and the amount of catalytic subunit of cyclic AMP-dependent protein kinase used was 75 units/ml.

Fig. 4. Radioautographs of peptide maps of the tryptic digests of the [32P]phosphorylated form of native ATP citrate lyase (A) and dephospho-ATP citrate lyase (B) by the catalytic subunit of the cAMP-dependent protein kinase as described under "Experimental Procedures." The arrows indicate the points of application of the samples. The major 32P spots correspond to the phosphopeptide as normally isolated.
DISCUSSION

It has been suggested that the regulatory point in the fatty acid biosynthetic pathway is at acetyl-CoA carboxylase. This enzyme is inhibited by products of the reaction (18) as well as phosphorylated by a CAMP-dependent protein kinase (19). It was surprising, therefore, when we found that ATP citrate lyase contained 2 mol of serine phosphate/tetramer. We ruled out a simple catalytic regulatory function for these 2 phosphates, because their removal with a phosphatase, partially purified from rat liver, yielded a dephosphoenzyme which was kinetically indistinguishable from the phospho-ATP citrate lyase (9). It was soon found, however, that glucagon stimulated the incorporation of $^{32}$P into the ATP citrate lyase of rat hepatocytes (3). Further, it was shown that a tryptic phosphopeptide isolated from the glucagon-stimulated hepatoocytes was similar (though not yet proven identical) to the tryptic phosphopeptide isolated from the rat liver enzyme (20).

At the same time Alexander et al. (21) and Ramakrishna and Benjamin (22) identified a protein band ($M_\text{r} = 123,000$) from rat hepatocytes and adipocytes as ATP citrate lyase. They had previously shown that the band, whose identity was unknown to them, was phosphorylated to an increased extent when hepatocytes or adipocytes were incubated with either glucagon or insulin and that the combination of the two hormones gave an additive effect. The results were unexpected, since the actions of glucagon and insulin in many metabolic pathways were antagonistic. When the protein band was identified as a menomer of ATP citrate lyase, the result was more surprising, since it is known specifically that insulin and glucagon have opposite effects on fatty acid synthesis.

In light of the glucagon stimulation, it was to be expected that the phosphorylation would be stimulated by the CAMP-dependent protein kinase. At present, all of the hormone action of glucagon can be explained by the activation of adenylate cyclase, the subsequent rise of cAMP in cells, followed by the release of the catalytic subunit of the protein kinase as the CAMP binds the regulatory subunit of this enzyme.

It has been shown by Guy et al. (9) that a rat mammary gland preparation of ATP citrate lyase (specific activity = 2) with a phosphate content of 0.2 ± 0.1 mol of phosphate/monomer could be phosphorylated to the extent of 0.7 ± 0.2 mol of phosphate/monomer of ATP citrate lyase by the action of the catalytic subunit of the CAMP-dependent protein kinase from rabbit muscle. They showed further that the phosphorylation was inhibited by the specific protein kinase inhibitor.

In the present communication, we have confirmed and extended their observation. Using rat liver ATP citrate lyase (specific activity = 10 and 2.1 mol of phosphate/tetramer) and dephospho-ATP citrate lyase (specific activity = 10), we have shown that a total of 4 mol/tetramer can be maximally incorporated into the enzyme by a rabbit muscle preparation of the catalytic subunit of the CAMP-dependent protein kinase. In addition, the phosphorylation of ATP citrate lyase by this preparation can be inhibited by the specific protein kinase inhibitor. This is the first time it has been shown that dephospho-ATP citrate lyase can be phosphorylated in vitro and that stoichiometric quantities of phosphate can be incorporated into dephospho-ATP citrate lyase.

We have shown previously that trypsin treatment of $[^{32}P]$ ATP citrate lyase cleaves the enzyme into two domains and produces a small phosphopeptide (20). This was shown to be a single major phosphopeptide. The amino acid composition of this peptide showed the presence of at least 3 serine residues. It is yet to be determined whether glucagon stimulates the phosphorylation of ATP citrate lyase at the same site that is phosphorylated in vitro by the catalytic subunit of cyclic AMP-dependent protein kinase. However, it is significant to note that the acid-stable $^{32}$P radioactivity found in ATP citrate lyase after administration of $^{32}$P, in vivo (2) and the phosphorylation observed in vitro with the catalytic subunit of cyclic AMP-dependent protein kinase are on a common trypsin-sensitive phosphorylating site. It is also shown in the present work that the tryptic $[^{32}P]$ phosphopeptide produced from the respective enzyme after the in vitro phosphorylation of both native and dephospho-ATP citrate lyase by the catalytic subunit of the CAMP dependent protein kinase are similar (or identical) and correspond to the phosphopeptide isolated from the rat liver enzyme after $^{32}$P injection.

The data suggest that 4 phosphates are incorporated into each tetramer of ATP citrate lyase. The corresponding phosphopeptide that has been subsequently isolated contains 3 to 4 serines and 1 threonine (20). In spite of the fact that endogenous phosphates are on a serine residue, it is possible to imagine a number of different phosphopeptides indistinguishable by the separation and identification procedure used here, but containing no more than 1 phosphate/monomer.

Specific sequencing work on the phosphopeptide is in progress.

The apparent $K_m$ value for ATP is 50 μM using dephospho-ATP citrate lyase as substrate. The apparent $K_m$ values for ATP using other substrates are reported to be 10 to 20 μM for protein kinases in general (23). Again, the variation in the degree of phosphorylation may depend on the relative activities of CAMP-dependent protein kinases and the purity of the enzyme substrate tried. Using dephospho-ATP citrate lyase as the substrate, the apparent $K_m$ was found to be 5 μM, and the corresponding $V_{max}$ value was 9.0 pmol/min. This is in contrast to the phosphorylation of pyruvate kinase, where the phosphorylation rate did not reach a plateau within the concentration range used (24).

Finally, the problem of the effect of phosphorylation on the activity of ATP citrate lyase must be considered. We have shown previously that dephospho-ATP citrate lyase and tryptic-digested ATP citrate lyase (where the entire phosphopeptide has been removed) have kinetic behavior identical with the native enzyme (1, 20). When ATP citrate lyase is phosphorylated by the catalytic subunit of the CAMP-dependent protein kinase to an extent of 4 mol of phosphate/tetramer, it has the same $V_{max}$ as the normally isolated (native) enzyme (2 mol/tetramer). No explanation of the phosphorylation as a regulatory mechanism is immediately available. Recently, Furuya and Uyeda (25, 26) have shown that the phosphorylation of rat liver phosphofructokinase does not control its activity but does control the binding of an activation factor which does indeed control the activity of phosphofructokinase. It is possible that a similar mechanism is operating with ATP citrate lyase. A number of years ago, Dunnaway and Segal (27) and Osterlund Bridger (28) isolated from rat liver a stabilizing factor for ATP citrate lyase. Recently, Osterlund et al. (29) have shown that this factor also protects ATP citrate lyase from inactivation by proteases extracted from lysosomes. One could imagine that the binding of the factor is affected by phosphorylation of ATP citrate lyase, and thereby the protection against lysosomal proteases.

Also, it has been recently noted that ATP citrate lyase, though a cytosolic enzyme, apparently binds to some organelle or membrane within the cell (30). If this binding is physiologically relevant, it is possible that it is controlled by the phosphorylation state of ATP citrate lyase. It seems unlikely to us that this specific and hormone-modulated phosphorylation of ATP citrate lyase has no relevance to the activity of the enzyme in vivo.
Phosphorylation of ATP Citrate Lyase by Catalytic Subunit

Acknowledgments—Our thanks are due to Dr. James Stull of the University of Texas Health Science Center at Dallas for his help ininitiating this study. This work could not have been completed without the generous gift of protein kinase from Dr. Edwin G. Krebs of the University of Washington.

Addendum—After having completed this work, we became aware of a recent publication (31) which reports some similar studies using lactating rat mammary gland ATP citrate lyase.

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J. Biol. Chem. 1982, 257:698-702.

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