Dependence of ATP-citrate Lyase Kinase Activity on the Phosphorylation of ATP-citrate Lyase by Cyclic AMP-dependent Protein Kinase*

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ATP-citrate lyase from rat liver and adipose tissue is phosphorylated by either ATP-citrate lyase kinase or catalytic subunit of cyclic AMP-dependent protein kinase to 0.5–0.6 mol/subunit. We previously demonstrated that the site phosphorylated by ATP-citrate lyase kinase (peptide B) is different from that phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase (peptide A) (Ramakrishna, S., Pucci, D. L., and Benjamin, W. B. (1981) J. Biol. Chem. 256, 10213–10216). ATP-citrate lyase phosphorylation by both protein kinases added simultaneously was increased synergistically. When ATP-citrate lyase was first phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase, the net phosphorylation of the fragments subsequently phosphorylated by lyase kinase increased about 6-fold. However, when ATP-citrate lyase was first phosphorylated by lyase kinase, there was no effect on the subsequent phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. Alkaline phosphatase-dephosphorylated ATP-citrate lyase was phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase to 0.9–1.0 mol/subunit. However, dephospho-ATP-citrate lyase was not phosphorylated by lyase kinase. The addition of both protein kinases simultaneously phosphorylated ATP-citrate lyase up to 2 mol/subunit. Phosphorylation of dephospho-ATP-citrate lyase first by catalytic subunit of cyclic AMP-dependent protein kinase and ATP enabled the lyase to be phosphorylated by lyase kinase. Peptide mapping and phosphoamino acid analysis of dephospho-ATP-citrate lyase phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase and/or lyase kinase conclusively showed that phosphorylation of ATP-citrate lyase by ATP-citrate lyase kinase was completely dependent on peptide A phosphorylation by cyclic AMP-dependent protein kinase. Furthermore, increased phosphorylation when both protein kinases were added simultaneously was due to increased phosphorylation at peptide B.

Mammalian ATP-citrate lyase is phosphorylated in response to the action of insulin, glucagon, and β-adrenergic agonist (1–5). The glucagon- and β-adrenergic agonist-mediated phosphorylation is via the adenylate cyclase-cyclic AMP-dependent phosphorylation system (6). Since insulin action also stimulates ATP-citrate lyase phosphorylation and experimental evidence suggests a cyclic AMP-independent pathway for this phosphorylation (2), we proposed that an early event in insulin action is the stimulation of an insulin-sensitive protein kinase which phosphorylates ATP-citrate lyase and possibly other physiological substrates. Subsequently, we isolated from liver a cyclic AMP-independent protein kinase which phosphorylates ATP-citrate lyase in vitro to 0.5–0.6 mol/subunit (7). In addition, rat liver and fat ATP-citrate lyase could be phosphorylated by cyclic AMP-dependent protein kinase also to 0.5–0.6 mol/subunit (with endogenous structural phosphate content of 0.5 mol/subunit) (8–10), while rat mammary gland ATP-citrate lyase with endogenous structural phosphate content of 0.2 mol/subunit could be phosphorylated to 0.7 mol/subunit (11). However, the function of these ATP-citrate lyase phosphorylations on enzyme activity and metabolism is not known.

We have shown (8) that in vitro the ATP-citrate lyase kinase-responsive phosphorylation of lyase is different from the cyclic AMP-dependent protein kinase-responsive system since different sites in the lyase molecule are phosphorylated by the two different protein kinases. Serine is the only amino acid phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase, while lyase kinase phosphorylates both serine and threonine residues.

Recently, we have noted that incubation of ATP-citrate lyase simultaneously with both protein kinases for longer periods of time than previously reported (8) resulted in the synergistic phosphorylation of the lyase. We therefore asked whether, (a) ATP-citrate lyase phosphorylation by one protein kinase facilitates phosphorylation by the other protein kinase, and (b) ATP-citrate lyase dephosphorylation by alkaline phosphatase alters the amount of phosphorylation or the sites of phosphorylation by these protein kinases?

Here we present evidence that the ability of lyase kinase to phosphorylate ATP-citrate lyase at a unique peptide sequence (peptide B) is absolutely dependent on the lyase's prior phosphorylation at the site phosphorylated by cyclic AMP-dependent protein kinase (peptide A). With increasing lyase phosphorylation by cyclic AMP-dependent protein kinase, lyase kinase increasingly is able to phosphorylate ATP-citrate lyase at peptide B. Therefore, the phosphorylation of the enzyme by both protein kinases added together is more than the sum of the phosphorylation of native ATP-citrate lyase when it is incubated with each protein kinase separately.

EXPERIMENTAL PROCEDURES

Materials—ATP-citrate lyase (specific activity, 8–10 units/mg of...
protein) was prepared from rat adipose tissue1 and liver by modification (12) of a published method (13). Lipoamide, guanidine hydrochloride, ATP, SDS, Tris, *Escherichia coli* alkaline phosphatase, and other biochemicals were purchased from Sigma. TPCK-trypsin2 was from Worthington. [γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham Corp. ATP-citrate lyase kinase was purified from rat liver as described previously (7) with some modification. Catalytic subunit was purified as described in this method II of Bechtel et al. (14) with an additional hydroxylapatite chromatography step. TLC cellulose plates were from E. Merck. X-ray films XAR-2 were from Kodak.

**Phosphorylation Assay**—ATP-citrate lyase phosphorylation by kinase or catalytic subunit was measured by incubating these at 30 °C in a reaction mixture (50 μl) containing 50 mM MES buffer (pH 6.7), 5 mM 2-mercaptoethanol, 8 mM magnesium acetate, 0.3 mM EGTA, 0.5 mM EDTA; 0.05 mM [γ-32P]ATP (100–300 cpm/pmol), and 1 mg/ml of bovine serum albumin. The reaction was terminated by adding sample buffer (17 μl) and boiling for 3 min. Protein samples (40 μl) were analyzed by SDS-gel electrophoresis (15). Gels were treated with 1 N HCl, 7% acetic acid for 1 h to remove catalytic phosphate (histidine phosphate) (10). HCl was washed from the gel with 7% acetic acid, 10% methanol and stained with 0.1% Coomassie blue G 5% methanol, 7% acetic acid. Gels were destained with 10% methanol, 7% acetic acid and dried. ATP-citrate lyase bands were cut from the dried gels and radioactivity was determined (8).

**HPLC of ATP-citrate Lyase**—ATP-citrate lyase phosphorylated by either protein kinase or both kinases was reduced, carboxymethylated, and digested with TPCK-trypsin (8). Two-dimensional separation of the peptides and phosphoamino acid analysis have been described (8). Tryptic (trypsin/substrate, 1:50)-digested ATP-citrate lyase was chromatographed on a Waters reversed-phase 5Bondapak C18 column (0.39 × 30 cm). Sample (40 μl) was injected onto the column equilibrated with 5% acetonitrile and 0.1% trifluoroacetic acid. The column was washed for 5 min with 5% acetonitrile, and peptides were eluted with a linear gradient of 5–40% acetonitrile in 0.1% trifluoroacetic acid for 70 min followed by 40% acetonitrile in 0.1% trifluoroacetic acid for 5 min. The flow rate was 1 ml/min and peptides were monitored by absorbance at 210 nm. Radiolabeled phosphopeptides were located by collecting 1-min fractions and measuring their Cerenkov radiation. Chromatography of a completely digested sample of catalytic subunit phosphorylated lyase gave a single radiolabeled phosphopeptide with *Rt* = 26 min (peptide A, Fig. 1B). With incomplete digestion, another phosphopeptide with a retention time of 36 min (peptide A') was found. Pierce et al. (16) have shown and we in this study confirmed that further digestion of phosphopeptide A' with trypsin yielded phosphopeptide A on rechromatography. Peptides A and A' on acid hydrolysis contained only 32P-labeled phosphoserine. Chromatography of lyase kinase-phosphorylated ATP-citrate lyase, however, gave a major radiolabeled phosphopeptide with *Rt* = 72 min and three minor radiolabeled phosphopeptides with *Rt* = 49, 55, and 67 min (Fig. 1A, 1). All minor peptides and peptide B contained both phosphoserine and phosphothreonine labeled with 32P. Fractions of each phosphopeptide peak were pooled, desalted with Sep-Pak C18 (Waters), and lyophilized. Phosphopeptides were dissolved in 40% acetonitrile containing 0.2 M phosphate buffer and chromatographed on a calibrated column of (0.38 × 60 cm) Spherogel TSK-2000 SW (Beckman) at a flow rate of 0.2 ml/min.

**Preparation of Dephospho- and Phosphorylated ATP-citrate Lyase**—ATP-citrate lyase was dephosphorylated by incubating 1–1.5 mg/ml of homogenous liver ATP-citrate lyase with 0.4 mg/ml of *E. coli* alkaline phosphatase for 2–6 h at 30 °C in a reaction mixture containing 20 mM Tris-HCl (pH 8.5), 0.2 mg/ml of bovine serum albumin, and 2 mM MgSO4. The reaction mixture was passed through a Sephadex G-150 superfine column (0.7 × 30 cm), and ATP-citrate lyase free of ATP and the added protein kinase was collected in the void volume. Approximately equal amounts of these phosphorylated and dephosphorylated samples were compared when assayed either at pH 8.7 or 7.5, suggesting that during these procedures the enzyme did not undergo extensive denaturation at least in the region of the complex active site(s). Stained SDS gels did not show any increase in ATP-citrate lyase fragments in phosphorylated and dephosphorylated samples compared to native enzyme, suggesting proteolysis was negligible during alkaline phosphatase treatment and phosphorylation experiments. ATP-citrate lyase was phosphorylated with unlabeled ATP using catalytic subunit or lyase kinase. The reaction mixture (200 μl) was passed through a Sephadex G-150 superfine column (0.7 × 30 cm), and ATP-citrate lyase free of ATP and the added protein kinase was collected in the void volume.

**RESULTS**

The rate of native ATP-citrate lyase phosphorylation by lyase kinase, catalytic subunit, or combinations of both kinases is shown in Fig. 2. When lyase was phosphorylated by lyase kinase, phosphate incorporation was rapid and linear for 30 min, reaching about 0.25 mol of P/subunit incorporated at 3 h. When lyase was phosphorylated by catalytic subunit, phosphate incorporation was linear and rapid for the first 30 min, with 0.62 mol of P/subunit incorporated at 3 h (Fig. 2). The calculated phosphate incorporation into ATP-citrate lyase, if the protein kinases were added together using the previous data determined when each protein kinase was added separately, should be 0.87 mol of P/subunit. However, when ATP-citrate lyase was incubated with lyase kinase and catalytic subunit together, the phosphate incorporation was linear for about 3 h and reached 1.55 mol of P/subunit at 3 h. The simultaneous incorporation of 32P into ATP-citrate lyase at the catalytic subunit phosphorylation site is shown in Fig. 3. The calculation of tryptic fragments of [32P]ATP-citrate lyase purified ATP-citrate lyase (100 μg) phosphorylated in situ as described under "Experimental Procedures" was digested with TPCK-trypsin (trypsin/substrate, 1:50). The tryptic digest was lyophilized, dissolved in 50 μl of 0.1% trifluoroacetic acid, and centrifuged at 10,000 × g for 5 min. 40 μl of sample were processed by HPLC as described under "Experimental Procedures." A, tryptic digest of lyase phosphorylated by ATP-citrate lyase kinase; B, tryptic digest of lyase phosphorylated by catalytic subunit.

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1. S. Ramakrishna and W. B. Benjamin, manuscript in preparation.
2. The abbreviations used are: TPCK-trypsin, 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin; MES, 2-(N-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography; *Rt*, retention time; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethylether)N,N′,N′,N′-tetraacetic acid.

![Fig. 1. HPLC maps of tryptic fragments of [32P]ATP-citrate lyase.](http://www.jbc.org/content/9/3/4591.full)
tion was approximately 20% higher than expected during the incorporation. This represents approximately a 6-fold increase in phosphorylation by both protein kinases, ATP-citrate lyase was phosphorylated first with unlabelled ATP-citrate lyase kinase (PK-A) and catalytic subunit (PK-C), and combination of the two protein kinases (PK-A2PK-C). The phosphates incorporated in ATP-citrate lyase by lyase kinase and catalytic subunit were added (- - -) to calculate the expected phosphorylation by the addition of both protein kinases to the assay.

To demonstrate whether phosphorylation first by one protein kinase increases phosphorylation by the other or vice versa, ATP-citrate lyase was phosphorylated first with unlabeled ATP for various times using catalytic subunit or lyase kinase as the protein kinase (Table I). The phospho-ATP-citrate lyase was isolated and then phosphorylated for 0.5, 1.5, and 3 h with [γ-32P]ATP and the other appropriate protein kinase. Lyase kinase phosphorylated control ATP-citrate lyase to 0.13-0.21 mol of P/subunit. However, phospho-ATP-citrate lyase generated by prior phosphorylation with catalytic subunit for 1 h was further phosphorylated by lyase kinase to 0.58 mol of P/subunit at 0.5 h and to 0.96 mol of P/subunit at 3 h. When phospho-ATP-citrate lyase prepared by 4 h of incubation with catalytic subunit was incubated for an additional 3 h with lyase kinase, 1.26 mol of P/subunit was incorporated. This represents approximately a 6-fold increase in phosphorylation by lyase kinase after the substrate was first phosphorylated by catalytic subunit. Control and phospho-ATP-citrate lyase (phosphorylated by lyase kinase) incubated with catalytic subunit phosphorylated up to 0.17 mol of P/subunit at 0.5 h and 0.42 mol of P/subunit at 3 h. No significant differences were observed between control and phospho-ATP-citrate lyase first phosphorylated by lyase kinase when these samples were used as substrates for further phosphorylation by catalytic subunit. These results indicate that ATP-citrate lyase phosphorylation by catalytic subunit enhances the ability of lyase kinase to phosphorylate ATP-citrate lyase, while ATP-citrate lyase phosphorylation by lyase kinase had no effect on the ability of catalytic subunit to phosphorylate the lyase.

| Time of incubation  | Phosphate incorporated | Protein kinase | Catalytic subunit | Lyase kinase |
|---------------------|-----------------------|----------------|------------------|--------------|
| 0.5 h               | 0.13                  | PK-A           | 0.16             | 0.14         |
| 1.5 h               | 0.16                  | PK-A           | 0.16             | 0.14         |
| 3.0 h               | 0.18                  | PK-A           | 0.18             | 0.14         |

To study the effect of the dephosphorylation of ATP-citrate lyase on its subsequent phosphorylation by lyase kinase and catalytic subunit, ATP-citrate lyase was dephosphorylated and the experiments outlined in Table II were performed. Native liver ATP-citrate lyase was phosphorylated by lyase kinase to 0.5 mol of P/subunit, by catalytic subunit to 0.65 mol of P/subunit, and by the combination of the two protein kinases to 1.75 mol of P/subunit (Table II). Native fat ATP-citrate lyase was phosphorylated to 0.64, 0.59, and 1.83 mol of P/subunit by lyase kinase, catalytic subunit, and the combination of the two protein kinases, respectively. When liver ATP-citrate lyase was first phosphorylated for 1 h by lyase kinase and then by catalytic subunit for an additional 1 h, the phosphate incorporation was 1.45 mol of P/subunit. If the order of protein kinase addition was reversed, 1.77 mol of P/subunit were incorporated. Liver or fat dephospho-ATP-citrate lyase was phosphorylated by catalytic subunit to 1.0 mol of P/subunit, consistent with recent reports (10, 16, 17). However, the dephospho-ATP-citrate lyase either from liver or adipose tissue was not phosphorylated at all by lyase kinase. Incubation of dephospho-ATP-citrate lyase by simultaneous addition of lyase kinase and catalytic subunit phosphorylated the lyase to 2.18 mol of P/subunit for liver dephosphoenzyme and to 1.84 mol of P/subunit for fat dephosphoenzyme. Sequential addition of lyase kinase and then catalytic subunit phosphorylates ATP-citrate lyase's further phosphorylation by lyase kinase but the analogous reverse experiment is without effect, then ATP-citrate lyase dephosphorylation may affect its phosphorylation by each protein kinase differently. In a separate set of experiments, alkaline phosphatase treatment for 1 h, as described under "Experimental Procedures," removed all the radiolabel from 32P-labeled ATP-citrate lyase that had been prepared using catalytic subunit and/or lyase kinase followed by gel filtration. SDS-gel electrophoresis of these dephosphorylated samples followed by autoradiography demonstrated that no new radiolabeled fragments were generated during the incubation with alkaline phosphatase. These results were consistent with the stain pattern of the dephosphorylated lyase samples on SDS gels, which also did not show an increase in ATP-citrate lyase fragments, suggesting that there was no detectable proteolysis of the lyase during these incubation procedures.

![ATP-citrate Lyase Phosphorylation by Lyase Kinase](http://www.jbc.org/)

**Table I**

| First addition | Second addition | Phosphate incorporated in second incubation at 0.5 h | 1.5 h | 3.0 h |
|----------------|-----------------|-----------------------------------------------------|-------|-------|
| Buffer 1 PK-A | PK-A            | 0.13                                               | 0.14  | 0.15  |
| Buffer 2 PK-A |                 | 0.16                                               | 0.18  | 0.21  |
| Buffer 4 PK-C | 1 PK-A          | 0.14                                               | 0.16  | 0.19  |
| PK-C           | 2 PK-A          | 0.58                                               | 0.83  | 0.96  |
| PK-C           | 4 PK-A          | 0.67                                               | 0.92  | 1.08  |
| Buffer 1 PK-C | 2 PK-C          | 0.73                                               | 1.04  | 1.26  |
| Buffer 2 PK-C |                 | 0.21                                               | 0.21  | 0.41  |
| Buffer 4 PK-C |                 | 0.18                                               | 0.29  | 0.43  |

PK-A, catalytic subunit of cyclic AMP-dependent protein kinase; PK-C, ATP-citrate lyase kinase.

If catalytic subunit-mediated lyase phosphorylation poten-
incorporated 1.19–1.40 mol of P/subunit into the dephosphoenzyme. The amount of phosphate incorporated during the second incubation was phosphorylated with unlabeled ATP plus one protein kinase, consistent with phosphorylation being mostly due to the action of catalytic subunit. If the order of addition was reversed, 1.98–2.20 mol of P/subunit were incorporated, again demonstrating the potentiating effect of catalytic subunit in native and dephospho-ATP-citrate lyase samples. The dephospho-ATP-citrate lyase was isolated as described under "Experimental Procedures." The native and dephospho-ATP-citrate lyases from liver (0.4 mg/ml) and fat (0.015 mg/ml) were phosphorylated for 2 h with lyase kinase (70 µg/ml) or catalytic subunit (20 µg/ml) for 1 h with one protein kinase followed by an additional 1 h with the addition of the second protein kinase. The values are the average of three to five experimental determinations ± S.D.

**TABLE II**

| Substrate                              | PK-A* | PK-C | PK-A + PK-C | PK-C (first), PK-C (second) |
|----------------------------------------|-------|------|-------------|-----------------------------|
| Rat liver ATP-citrate lyase            | 0.49 ± 0.05 | 0.65 ± 0.06 | 1.75 ± 0.05 | 1.45 ± 0.06 |
| Liver ATP-citrate lyase (alkaline phosphatase, 3 h) | 0.00 | 0.93 ± 0.05 | 2.18 ± 0.25 | 1.38 ± 0.02 |
| Liver ATP-citrate lyase (alkaline phosphatase, 5.5 h) | 0.00 | 0.93 ± 0.02 | 2.17 ± 0.07 | 1.19 ± 0.10 |
| Fat ATP-citrate lyase                  | 0.64 ± 0.03 | 0.59 ± 0.02 | 1.83 ± 0.17 | ND |
| Fat ATP-citrate lyase (alkaline phosphatase, 3 h) | 0.00 | 0.90 ± 0.07 | 1.84 ± 0.20 | ND |
| Fat ATP-citrate lyase (alkaline phosphatase, 5.5 h) | 0.00 | 0.98 ± 0.06 | 1.62 ± 0.02 | ND |

* PK-A, ATP-citrate lyase kinase; PK-C, catalytic subunit of cyclic AMP-dependent protein kinase.

**TABLE III**

| First phosphorylation | Second phosphorylation | Phosphate incorporated in second incubation at 0.5 h | 2 h |
|-----------------------|-----------------------|---------------------------------|-----|
| Buffer                | PK-C                 | 0.46                            | 0.95|
| PK-A                  | PK-C                 | 0.51                            | 0.87|
| Buffer                | PK-A                 | 0.00                            | 0.00|
| PK-C                  | PK-A                 | 0.30                            | 0.55|

* PK-C, catalytic subunit of cyclic AMP-dependent protein kinase; PK-A, ATP-citrate lyase kinase.

**FIG. 3** HPLC map of tryptic fragments of [32P]ATP-citrate lyase phosphorylated by the addition of both catalytic subunit and lyase kinase. [32P]ATP-citrate lyase (160 µg) phosphorylated by lyase kinase plus catalytic subunit as described in the legend to Table V was trypsin-digested (1:50), and the sample was processed by HPLC as described under "Experimental Procedures." Retention times of peaks A, A', and B are 26, 36, and 72 min, respectively. Peak V is the radioactivity not retained on the column. This peak after acid hydrolysis contained little radioactive phosphoamino acids.

To examine critically whether phosphorylation by catalytic subunit potentiates phosphorylation by lyase kinase, we performed the following experiment. Dephospho-ATP-citrate lyase was isolated as described. Dephospho-ATP-citrate lyase was phosphorylated with unlabeled ATP plus one protein kinase. The phosphorylated lyase was treated under "Experimental Procedures." As predicted, the amount of phosphate incorporated during the second stage phosphorylation is shown in Table III. As predicted, dephospho-ATP-citrate lyase was phosphorylated by catalytic subunit to the same extent whatever or not it was first incubated with lyase kinase (0.97 and 0.87 mol of P/subunit, respectively). As expected, the dephosphoenzyme was not phosphorylated by lyase kinase. Dephospho-ATP-citrate lyase first phosphorylated by catalytic subunit was phosphorylated to 0.55 mol of P/subunit by lyase kinase.

To determine if ATP-citrate lyase first phosphorylated by catalytic subunit is a better substrate for lyase kinase phosphorylation by increasing phosphate incorporation at the lyase kinase-specific site (peptide B) or at a new site, ATP-citrate lyase phosphorylated in vitro with both protein kinases was digested with trypsin and the radioactive phosphopeptides were analyzed by HPLC (Fig. 3). Phosphopeptides A and A', generated by catalytic subunit phosphorylation alone, and phosphopeptide B and the minor peptides (labeled ○), generated by lyase kinase phosphorylation alone (Fig. 1), were present in the lyase phosphorylated by the combination of the two protein kinases (Fig. 3). No new phosphopeptides were detected on the chromatogram.

To determine whether the dephosphorylation of ATP-citrate lyase opened up new phosphorylation sites for either protein kinase, the trypsin-digested samples of phosphorylated native and dephospho-ATP-citrate lyase samples were analyzed by two-dimensional peptide mapping. The results suggest that the same site was phosphorylated by catalytic subunit in native and dephospho-ATP-citrate lyase. Similarly, lyase kinase phosphorylated the same site in native and dephosphoenzyme. Two-dimensional tryptic maps of native and dephosphoenzyme phosphorylated by the addition of the two protein kinases together were identical. Irrespective of the order of addition, sequential addition of the two protein...
kinases gave qualitatively similar peptide maps. However, when lyase kinase addition was preceded by catalytic subunit, lyase kinase-specific peptide was phosphorylated to a greater extent than if the order of addition of the protein kinases was reversed. Phosphoamino acid analysis of some of these samples is given in Table IV. When native or dephospho-ATP-citrate lyase was phosphorylated by catalytic subunit, only serine was phosphorylated. Lyase kinase phosphorylated both serine and threonine almost equally in native ATP-citrate lyase. Absolutely no phosphorylation was detected in the dephosphoenzyme incubated with lyase kinase. When the two protein kinases were added together, serine and threonine were phosphorylated to 73 and 27%, respectively, in the native ATP-citrate lyase and 70 and 30%, respectively, using dephospho-ATP-citrate lyase. Since lyase kinase phosphorylated serine and threonine residues of peptide B and minor fragments (Fig. 1A), labeled ⊗, it is possible that the two phosphorylated amino acids reside within a small sequence or reflect two quite distant phosphorylation sites within a much larger fragment. To study this, lyase kinase-phosphorylated ATP-citrate lyase was treated with increasing amounts of trypsin (Fig. 4). At a ratio of trypsin to substrate of 1:2, peptide B was quantitatively converted to fragments with the same retention time as the minor fragment produced by less exhaustive trypsin treatment. Note that as the radioactivity associated with peak B decreased, radioactivity associated with the radiolabeled fragments increased (Fig. 4c). Indeed, the per cent of the total radioactivity associated with the defined lyase kinase-phosphorylated peptides were similar (Fig. 4, a–c). The molecular weights of peptides A, A', B, and B' were determined by gel permeation chromatography to be 1000, 1800, 8500, and 2100, respectively. The molecular weight of phosphopeptide (R, 53 min) was 5400, suggesting it is a precursor of peptide B'. However, we do not have an estimate of the molecular weight of phosphopeptide (R, 48 min). In another series of experiments using a new batch of trypsin, when ATP-citrate lyase phosphorylated with lyase kinase was treated with trypsin (1:2), two additional phosphopeptides were found on HPLC analysis which eluted with R, less than threonine B' with M, 1600 and 1200. The molecular weight of peptide A in this report is similar to the molecular weight of the phosphopeptide generated by trypsin treatment of ATP-citrate lyase from rat liver cells (18) and 3T3-L1 cells (17). In addition, it should be noted that acid hydrolysis of peptides B and B' and phosphopeptides (Rf, 48 and 53 min) showed that they all contain phosphoserine and phosphothreonine.

To study whether a given peptide phosphorylation is potentiated by prior phosphorylation of the other peptide, we normalized the results by setting the radioactivity incorporated by each protein kinase at its unique peptide as 100% (Table V). ATP-citrate lyase incubated with both protein kinases incorporated phosphate into both peptides. Incorporation of [32P]phosphate into peptide B increased about 267% compared to that incorporated by lyase kinase alone, while there was no change in the incorporation of phosphate at the catalytic subunit site (94%).

To evaluate the possible significance of ATP-citrate lyase phosphorylation on enzyme activity, its activity was determined after phosphorylation or dephosphorylation. Under normal assay conditions (18) at both pH 8.7 and 7.5, enzyme activity was unchanged by its prior phosphorylation by either protein kinase or by the combination of both. Enzyme activity also remained unaltered by the dephosphorylation of the lyase by alkaline phosphatase treatment or rephosphorylation of the dephosphoenzyme by catalytic subunit or by both protein kinases added together.

### Table IV

**Distribution of[^32P]-labeled serine and threonine residues in phosphorylated native and dephospho-ATP-citrate lyase**

| Treatment                    | Radioactivity in | % |
|------------------------------|------------------|---|
|                              | Serine           | Threonine |
| Native ATP-citrate lyase + PK-C* | 100             | 0  |
| Native ATP-citrate lyase + PK-A     | 45              | 55 |
| Native ATP-citrate lyase + PK-C + PK-A | 73            | 27 |
| Dephospho-ATP-citrate lyase + PK-C     | 100             | 0  |
| Dephospho-ATP-citrate lyase + PK-A     | 0               | 0  |
| Dephospho-ATP-citrate lyase + PK-C + PK-A | 70          | 30 |

* PK-C, catalytic subunit of cyclic AMP-dependent protein kinase; PK-A, ATP-citrate lyase kinase.

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**Fig. 4.** HPLC map of tryptic fragments of[^32P]-ATP-citrate lyase generated by various concentrations of trypsin. ATP-citrate lyase (100 μg) was phosphorylated by lyase kinase as described in the legend to Table V. ATP-citrate lyase (500 μg) was added as carrier, and the sample was divided into three equal parts and treated with TPCK-trypsin for 24 h at 37°C in a trypsin to lyase ratio of 1:100 (a), 1:8 (b), and 1:2 (c). Sample preparation and HPLC chromatography were as described in the text with the following exceptions. Aqueous phase was 0.2 M phosphate buffer (pH 3.5) and organic phase was acetonitrile. Retention time of peaks B' and B were 43 and 73 min, respectively, slightly different from the retention times given in Figs. 1A and 2 due to the change in buffer. The lyase kinase-defined peaks in this figure are the same as those described in the other figures. Peak V is the radioactivity not retained on the column. Radioactivity injected onto the column was 13,640 (a), 11,570 (b), and 11,300 cpm (c). Per cent radioactivity recovered in the lyase kinase-defined phosphorylated peptides (R, 45–58 min plus R, 73–76 min) after the subtraction of peak V-associated counts was 84 (a), 85 (b), and 86 (c).
**Table V**

| Treatment           | Catalytic subunit-specific phosphorylation site | Lyase kinase-specific phosphorylation site |
|---------------------|-----------------------------------------------|--------------------------------------------|
| PK-A*               | 0                                             | 100                                        |
| PK-C                | 100                                           | 0                                          |
| PK-A + PK-C         | 94                                            | 367                                        |

*PK-A, ATP-citrato lyase kinase; PK-C, catalytic subunit of cyclic AMP-dependent protein kinase.

**DISCUSSION**

Following the discovery that rabbit muscle glycogen phosphorylase exists in a relatively inactive and active form, determined by its phosphorylation state, it has become apparent that there are many enzyme systems whose net enzyme activity depends on continuously controlled cyclic processes consisting of, in the most simple case, rates of phosphorylation and dephosphorylation. ATP-citrato lyase, which catalyzes the formation of acetyl-CoA in the cytosol for anabolic purposes, undergoes both rapid reversible phosphorylation-dephosphorylation in vitro and enzyme induction under control of the hormonal and metabolic state of the animal. ATP-citrato lyase, like acetyl-CoA carboxylase, should be a likely candidate to be controlled by interconvertible enzyme cascades (18). However, as yet no convincing evidence has been presented demonstrating that enzyme activity as measured in vitro is affected by its phosphorylation state, whether phosphorylated at the site phosphorylated by catalytic subunit or at the unique site phosphorylated by lyase kinase. Recalling the difficulties in deciphering the regulatory role played by the multisite phosphorylation of glycogen synthase and the paramount importance of allosteric effectors (19) and enzyme assay conditions (20), we thought it important to completely characterize the phosphorylation sites of ATP-citrato lyase as phosphorylated in vitro by lyase kinase and catalytic subunit.

During studies of the time course of ATP-citrato lyase phosphorylation, we noted that when both catalytic subunit and lyase kinase were added simultaneously to the reaction mixture, lyase phosphorylation was unexpectedly more than additive. Furthermore, when lyase was phosphorylated first with lyase kinase and the kinase removed and then phosphorylated with catalytic subunit, the phosphorylation was unaffected (Table I). However, when ATP-citrato lyase was phosphorylated first with catalytic subunit and the protein kinase removed and then phosphorylated with lyase kinase, there was a marked increase in the phosphorylation of the enzyme. We interpreted these data to indicate that catalytic subunit phosphorylation at its site (peptide A) increased the phosphorylation of lyase by lyase kinase at its site (peptide B).

Was peptide A phosphorylation (the site phosphorylated by catalytic subunit) absolutely necessary for peptide B phosphorylation (the site phosphorylated by lyase kinase) or was its role only to increase peptide B phosphorylation? To answer this question directly, we prepared alkali cardiophosphatase-treated liver and found ATP-citrato lyase and phosphorylated them by either protein kinase, both protein kinases added simultaneously, each protein kinase added sequentially, and in both orders. The dephosphopeptidase was not a substrate for phosphorylation by lyase kinase. When the protein kinases were added together or sequentially, phosphorylation increased when catalytic subunit was added first. The data suggested that catalytic subunit phosphorylation was an absolute requirement for lyase kinase phosphorylation. Since the first protein kinase added was not removed before the addition of the second, the results of these experiments did not rigorously prove the hypothesis since the remaining protein kinase might have had an unforeseen effect. In Table III are presented data showing that when both alkali phosphatase and the first added kinase were removed before the second addition, the phosphorylation by catalytic subunit (peptide A) is an absolute requirement for phosphorylation by lyase kinase at peptide B. Could this all or nothing result be due to an artifact produced by the denaturation or cleavage of ATP-citrato lyase by alkali phosphatase treatment? This is most unlikely because ATP-citrato lyase after dephosphorylation was isolated by gel filtration. In addition, no increase in proteolytic cleavage of the lyase was observed after alkali phosphatase treatment. Furthermore, all phosphorylation data were calculated from the radioactivity associated with the intact 115,000-dalton subunit. Therefore, it is not likely that a denatured or cleaved substrate had been rendered unresponsive to lyase kinase in a manner different from that of the dephosphoalkaline enzyme since there was no evidence that a substantial amount of the enzyme was cleaved or denatured by our dephosphorylation procedure. Furthermore, when phosphorylated ATP-citrato lyase was treated with trypsin and the percentage of radioactivity associated with each site determined, it was found that all of the increase in radioactivity associated with both protein kinases added simultaneously could be accounted for by an increase in radioactivity associated with peptide B (the lyase kinase-specific site) (Table IV). Finally, the dephosphoalkaline enzyme when assayed at either pH 7.5 or 8.7 was enzymatically equivalent to the native enzyme, suggesting that there was no extensive denaturation of the enzyme at least in the region of the catalytic site.

It was important to determine whether the specific amino acids phosphorylated after such treatment were consistent with our previous findings of serine and threonine phosphorylations (8). As shown in Table IV, native ATP-citrato lyase phosphorylated with lyase kinase phosphorylated serine and threonine almost equally. When catalytic subunit was added, the per cent serine phosphorylated increased appropriately. When the dephosphopeptidase was used as a substrate, the ratios of phosphoserine to phosphothreonine changed in accord with the results presented in Tables III and V.

Our data indicate that the lyase kinase-phosphorylated sites are subject to second site regulation. The possibility that the phosphorylation of two amino acids near each other or widely separated or even of several amino acid residues is controlled by a single phosphorylation at a cyclic-AMP-regulated site must be considered. This has been addressed directly by treating lyase kinase-phosphorylated ATP-citrato lyase with increasing concentrations of trypsin. Higher concentrations of trypsin produced a pattern of radiolabeled phosphopeptides with $R_v$ values similar to those of the minor fragments seen in chromatography after normal (1:50) trypsin-treatment. Furthermore, when peptide B was purified by HPLC, treated
with 1/2 trypsin, and rechromatographed, only radiolabeled fragments with similar R values to those shown in Fig. 1A (B) were found. These data indicate that peptide bonds unapproached by low trypsin treatment were hydrolyzed by higher concentrations of trypsin, producing smaller (less hydrophobic) fragments. Since the molecular weights of phosphopeptides B and B' were determined to be 8600 and 2100, respectively, and the radioactivity content of phosphothreonine and phosphoserine was conserved, we interpret the data to indicate that the lyase kinase-phosphorylated amino acids are all contained within the peptides B and B' sequences. Note that the peptide segment may contain more than the minimal number of two radiolabeled amino acids.

Are there precedents in the literature similar to the results reported in this paper? A similar, but opposite phosphorylation effect was found in studies on the glycogen synthase system. Glycogen synthase is phosphorylated by cyclic AMP-dependent protein kinase, and cyclic AMP-independent protein kinases, converting glycogen synthase I to the D-form (21). Phosphorylation of glycogen synthase by the simultaneous incubation with phosvitin kinase and cyclic AMP-dependent protein kinase resulted in more complete conversion of the I to the D-form (22). However, if synthase I is phosphorylated with cyclic AMP-dependent protein kinase, there is only a partial conversion of the I to the D-form. Unexpectedly, this phosphorylated glycogen synthase was not further phosphorylated by phosvitin kinase (22). Thus, the phosphorylation of glycogen synthase by cyclic AMP-dependent protein kinase altered the synthase molecule rendering it refractory to the action of phosvitin kinase and preventing its more complete conversion from the I to the D-form.

In this report, we have described an in vitro system whereby the phosphorylation at one site absolutely determines the phosphorylations at the other. There is as yet no evidence that such a control system is operative in vivo. Indeed, Swer-gold et al. (17) recently reported that insulin enhanced phosphorylation in the same ATP-citrate lyase tryptic fragment in 3T3-L1 cells as that increased by isoproterenol treatment. Pierce et al. (23) demonstrated that the same serine residue of rat liver ATP-citrate lyase is phosphorylated in response to both insulin and glucagon. These puzzling findings were contrary to expectations, since these hormones have opposite actions and have been found to phosphorylate different sites on the S-6 protein (24, 25).

If the findings presented in this paper are relevant to physiology, then the phosphorylated state of the enzyme prior to hormone action would determine the site(s) phosphorylated by the hormone action. In recent experiments studying in vivo ATP-citrate lyase phosphorylation by incubating rat fat pads with 32P and purifying the radiolabeled enzyme, we found that after trypsin-treatment and HPLC analysis, peptides with R values identical with peptides phosphorylated by ATP-citrate lyase kinase action in vitro (peptides B and B') and phosphopeptides with R values identical with peptides phosphorylated by cyclic AMP-dependent protein kinase (peptides A and A') were significantly radiolabeled in vivo.6 This evidence suggests that the observations of the dependence of “site B” phosphorylation on “site A” phosphorylation may have physiological significance and be involved in the metabolism of ATP-citrate lyase. One additional caveat should be mentioned. ATP-citrate lyase is extraordinarily protease sensitive (13). In vivo experimental results might be difficult to evaluate since selective loss of phosphorylated fragments is almost to be expected.

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