PHOSPHORYLATION OF ATP-CITRATE LYASE BY A CYCLIC AMP-INDEPENDENT PROTEIN KINASE FROM RAT LIVER

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1. Introduction

Insulin action in isolated adipocytes or hepatocytes incubated with $^{32}P_i$ rapidly increases the amount of ^{32}P radioactivity associated with a cytosolic phosphoprotein [1–4]. We isolated and purified this phosphoprotein from adipose and liver tissues and identified it as ATP-citrate lyase [5]. Concurrently, Alexander et al. [6] also found that in liver this phosphoprotein is ATP-citrate lyase. When ATP-citrate lyase is incubated with ATP, 2 mol phosphate/mol enzyme are incorporated into histidine residues at the catalytic site (referred to as catalytic phosphate) which are acid labile [5,7]. In addition, at least 2 mol acid-stable phosphate (referred to as structural phosphate), principally phosphoserine, have been found with each 1 mol enzyme [8]. Because structural site phosphorylation was affected by hormone action [1,2,5], we looked for a cyclic AMP-independent protein kinase in an insulin-sensitive tissue with specificity for phosphorylating the ATP-citrate lyase structural phosphate sites.

2. Experimental procedures

ATP-citrate lyase was purified (spec. act. 9.0–9.5 U/mg protein) from liver [8] and from adipose tissue (S. R. K., W. B. B., in preparation). ATP-citrate lyase activity was assayed as in [8] and 1 unit of activity was the amount required to catalyze the oxidation of 1 μmol NADH/min at 37°C.

Protein kinase activity was measured [9] by incubation at 30°C for 30 min in a reaction mixture (100 μl) containing 50 mM 2-(*N*-morpholino) ethane sulfonic acid buffer (pH 6.7); 10 mM 2-mercaptoethanol; 8 mM magnesium acetate; 0.3 mM EGTA; 0.5 mM EDTA; 0.1 mM [γ-^{32}P]ATP (100–200 cpm/pmol); protein substrate (casein, phosvitin or histone at 2 mg/ml); 5 μM cyclic AMP (when added); and protein kinase fractions as indicated in the legends. When ATP-citrate lyase phosphorylation was measured, 10 μg lyase and 10–20 μl protein kinase fractions were added to the reaction mixture (final vol. 50 μl) and the reaction was terminated by the addition of sample buffer (16 μl) [2] and boiling for 3 min. Protein samples (33 μl) were analyzed by SDS gel electrophoresis as in [2,10]. Gels were either dried or acid treated with 1 N HCl–10% acetic acid for 1 h to remove catalytic phosphate. HCl was washed from the gel with 10% acetic acid–10% methanol, dried and autoradiograms developed [10]. The bands containing radioactive lyase were cut out and radioactivity was determined. The heat-stable inhibitor of cyclic AMP-dependent protein kinase was purified through the DE-52 chromatography step [11]. Protein concentration was determined as in [12] or by UV absorption at 280 nm.

Liver from Sprague-Dawley rats (~150 g) was homogenized at 0°C with 750 ml 50 mM Tris–HCl buffer (pH 7.5) containing 15 mM 2-mercaptoethanol, 5 mM EDTA, 50 mM NaF and 0.5 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 25 000 × *g* for 20 min and then at 140 000 × *g* for 45 min. Ammonium sulfate was added to 60% saturation to the supernate and the precipitate was dissolved in 50 ml buffer A (50 mM Tris–HCl buffer (pH 7.5), 15 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride, 5% glycerol) and dialyzed against 2 l buffer A overnight with an additional change of fresh buffer. The dialyzed enzyme solution was applied to a phosphocellulose (P-11) column (4 ×

20 cm) equilibrated with buffer A as in [13]. The column was washed with buffer A (flowrate 15 ml/h) which removed ~95% of the protein containing cyclic AMP-dependent protein kinase. Proteins were eluted from the phosphocellulose column with linear gradients of 0–0.2 M KCl followed by 0.2–0.75 M KCl in buffer A.

3. Results

3.1. *Purification of ATP-citrate lyase phosphorylating kinase*

Fig.1 shows the protein elution profile and protein kinase activities with casein, histone and ATP-citrate lyase as substrates, of fractions from the phosphocellulose column. Note that cyclic AMP-dependent protein kinase was not retarded on this column (not shown in fig.1). Fractions containing histone kinase activity eluted first, followed by casein kinase activity. A sharp peak of cyclic AMP-independent ATP-citrate lyase phosphorylating activity was observed eluting at 0.20–0.25 M KCl. Fractions containing ATP-citrate lyase phosphorylating activity were pooled, concentrated by ammonium sulfate precipitation dialyzed against buffer A and applied to a hydroxylapatite column (1.5 × 20 cm). After washing the column with buffer A, protein was eluted with a linear gradient of 0–0.5 M potassium phosphate (pH 7.5) in buffer A. ATP-citrate lyase kinase eluted at a conductivity of 9–15 mMHO. The kinase was concentrated to 2 ml and fractionated on a Bio Gel P-100 column (1.5 × 100 cm) with buffer A containing 100 mM NaCl. Fractions with lyase kinase activity were pooled, concentrated and dialyzed against buffer A. The dialyzed protein was loaded on an ATP-hexane agarose column (bed vol. 5 ml), washed with buffer A and eluted with a linear gradient of 0–0.3 M NaCl in buffer A. Kinase activity eluted at 0.05–0.12 M NaCl. As summarized in table 1, the kinase was purified ~1500-fold with ~37% recovery. Note that inexplicably total ATP-citrate lyase phosphorylating activity at the phosphocellulose step increased ~130%, perhaps due to removal of some inhibitors.

3.2. *Phosphorylation of ATP-citrate lyase by ATP-citrate lyase phosphorylating kinase*

ATP-citrate lyase from fat was pure with minimal proteolytic degradation (fig.2B, lane 1) and could be used to probe for kinase activity. When ATP-citrate lyase was incubated without kinase, no acid-stable radioactivity was associated with the lyase (fig.2A, lane 1). With the addition of the protein kinase from

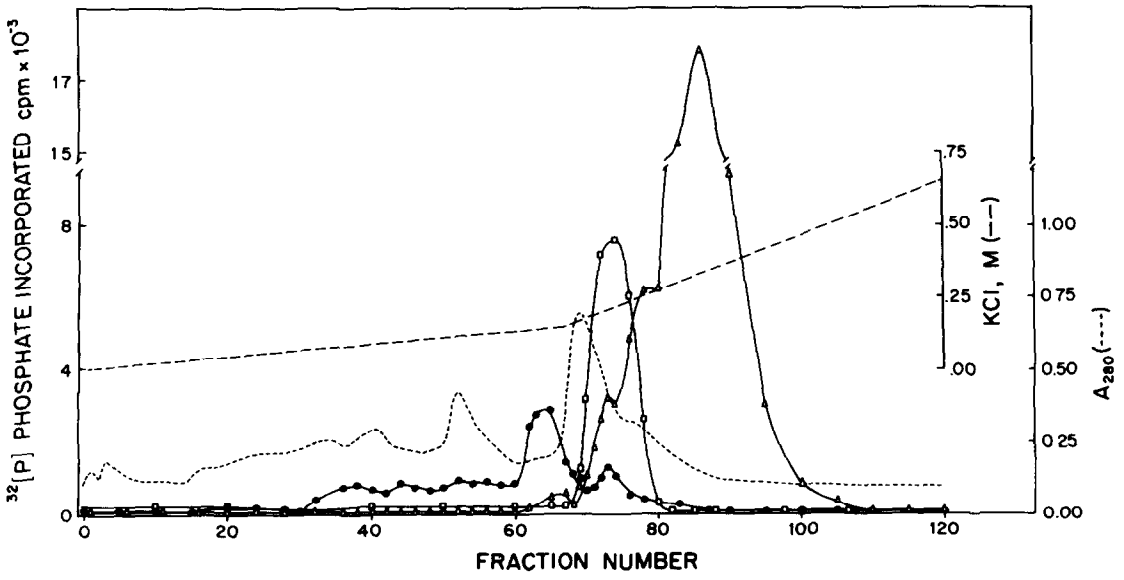

Fig.1. Purification of ATP-citrate lyase phosphorylating kinase from rat liver on phosphocellulose column. Protein (. . .) and protein kinase activity with casein (△——△), histone (●——●) or ATP-citrate lyase (□——□) as substrate is shown for the column elution after washing with buffer A. ATP-citrate lyase phosphorylating kinase was assayed by incubating 20 μl from every other tube with ATP-citrate lyase as described in the text. KCl (– – –) concentration was determined from conductivity measurements.

Table 1
Summary of purification of ATP-citrate lyase phosphorylating kinase from rat liver

Purification step	Specific activity (nmol/mg protein)[a]			Yield (%)		
	Lyase	Casein	Histone	Lyase	Casein	Histone
1. Crude extract	0.0015	0.71	1.11	100	100	100
2. Ammonium sulfate	0.003	0.63	1.56	92	95	90
3. Phosphocellulose	0.34	3.16	5.50	233[b]	4.0	4.5
4. Hydroxyl apatite	0.71	10.00	6.54	84	2.2	0.9
5. Bio-Gel P-100	1.54	7.25	3.70	81	0.7	0.23
6. ATP-hexane agarose	2.23	0.32	0.29	37	0.01	0.01

[a] Assay for kinase activity was for 15 min as described in the text
[b] The increase in the total ATP-citrate lyase phosphorylating kinase activity at this step may be due to removal of kinase inhibitors

Conditions for the assay of ATP-citrate lyase phosphorylating kinase activity may not be optimal

the phosphocellulose column there was significant acid-stable phosphorylation of ATP-citrate lyase, unaffected by cyclic AMP addition (fig.2A, lanes 2, 3). Note, that even when cyclic AMP was added to the protein kinase in the absence of lyase there was no phosphorylation of the proteins in this fraction (lanes 4,5).

Similar results were obtained with ATP-citrate lyase from liver although the presence of proteolytic fragments in these preparations complicated the phosphorylation pattern (not shown). Structural site phosphorylation could be studied quantitatively by measuring both the total radioactivity associated with ATP-citrate lyase after the kinase assay and the radioactivity associated with lyase after acid treatment of the gel. Structural site phosphorylation increased linearly for 30 min, reaching 0.4 mol phosphate incorp./subunit whereas catalytic site phosphorylation rapidly decreased (fig.3). Catalytic site phosphoryla-

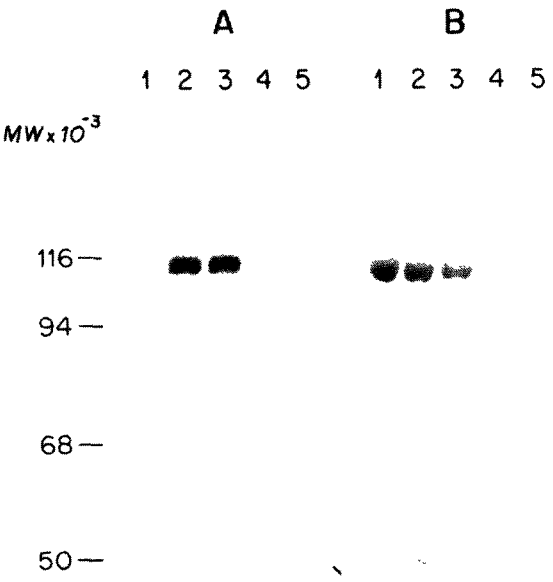

Fig.2. SDS gel electrophoretogram of ATP-citrate lyase (from fat) phosphorylated by ATP-citrate lyase phosphorylating kinase. ATP-citrate lyase (10 μg) was phosphorylated as described in the text. The kinase purified through phosphocellulose was used: (A) Autoradiograph of acid treated gel; (B) stained gel. The lanes contain: (1) ATP-citrate lyase (15); (2) ATP-citrate lyase + kinase (1456); (3) ATP-citrate lyase + kinase + cyclic AMP (1394); (4) kinase (12); (5) kinase + cyclic AMP (10). The numbers in parentheses are the radioactivity (cpm) in ATP-citrate lyase bands. Identical results were obtained using the most highly purified kinase fractions.

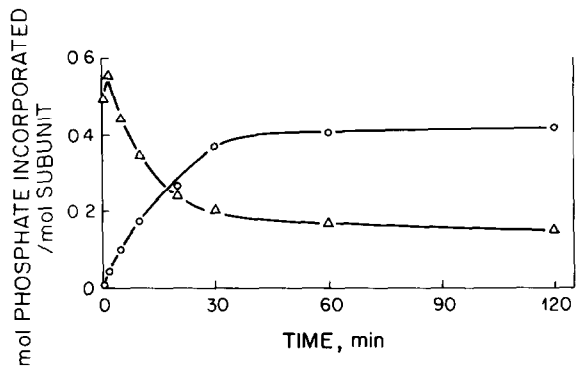

Fig.3. Phosphorylation of ATP-citrate lyase (from fat) by ATP-citrate lyase kinase. Time course of phosphorylation of ATP-citrate lyase by kinase. Purified lyase (7 μg) was phosphorylated with [γ-^{32}P]ATP with kinase as in section 2. One gel was dried directly while the other was acid-treated and dried. By subtracting radioactivity associated with ATP-citrate lyase in the acid-treated gel (structural phosphate) from the non-acid-treated gel (both catalytic and structural phosphate), structural (o——o) and catalytic site (△——△) phosphorylations were estimated. The earliest time point was 10 s at which time the catalytic site was extensively phosphorylated.

tion was rapid being complete within 1 min and stable up to 60 min even in the presence of Mg^{2+} (not shown). Protein kinase and Mg^{2+} were essential for structural site phosphorylation.

To study the effects of cyclic AMP and protein kinase inhibitor on ATP-citrate lyase structural site phosphorylation, ATP-citrate lyase was incubated either with ATP-citrate lyase phosphorylating kinase or cyclic AMP-dependent protein kinase, with or without cyclic AMP or protein kinase inhibitor as indicated (table 2). In samples incubated with cyclic AMP-dependent protein kinase, cyclic AMP markedly increased (5-fold) structural site phosphorylation. This phosphorylation was completely inhibited by protein kinase inhibitor, while in samples incubated with ATP-citrate lyase kinase and increasing amounts of protein kinase inhibitor, phosphorylation was either unaffected or in some preparations decreased only 18%.

4. Discussion

This paper presents evidence for the presence of a cyclic AMP-independent protein kinase in rat liver that phosphorylates structural phosphate sites of ATP-citrate lyase. In [14] the catalytic subunit of cyclic AMP-dependent protein kinase also phosphorylated lyase. Comparison of some of the properties of cyclic AMP-independent protein kinase described here with other kinases suggests it may be specific for ATP-citrate lyase, because ATP-citrate lyase phosphorylating ability was:

(i) Found with proteins eluting from a phosphocellulose column at 0.20–0.25 M KCl;
(ii) Unaffected by cyclic AMP;
(iii) Purified 1500-times and not associated with significant casein, histone and phosvitin kinase activities;
(iv) Minimally decreased by the heat-stable protein kinase inhibitor, which under similar conditions completely inhibited cyclic AMP-dependent protein kinase phosphorylation of ATP-citrate lyase.

Table 2
Effect of protein kinase inhibitor on structural site phosphorylation of ATP-citrate lyase

Protein kinase inhibitor (μl)	Acid-stable phosphate	
	Cyclic AMP-dependent[a] protein kinase (%)	Cyclic AMP-independent protein kinase (%)
0	100	100
5	5	93
10	1	89
20	0	82

[a] Assayed in the presence of 5 μM cyclic AMP which stimulated the rate of phosphorylation 5-fold

Phosphate incorporated into ATP-citrate lyase with cyclic AMP-dependent and -independent protein kinase was 0.11 and 0.30 mol/subunit, respectively

The relationship of the kinase studied here to previous reports of cyclic AMP-independent protein kinases with specificities for other substrates is unknown. However, we might expect this kinase to phosphorylate other phosphoproteins involved in insulin action. This kinase may be related to insulin action since this hormone increases ^{32}P-radioactivity associated with ATP-citrate lyase when adipose and liver tissues are incubated with $^{32}P_i$ in vivo [1–4]. Recent experiments (unpublished) strongly suggest that the site phosphorylated by the cyclic AMP-independent protein kinase is different from the site phosphorylated by the cyclic AMP-dependent protein kinase. The results presented in this communication support the hypothesis that insulin action increases the phosphorylation of a specific site(s) on ATP-citrate lyase by affecting a cyclic AMP-independent protein kinase.

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