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Phosphorylation by cyclic AMP-dependent protein kinase does not affect the association of ATP citrate-lyase with isolated mitochondria

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ATP citrate-lyase is known to be a substrate for various protein kinases, but the functional role, if any, of kinase-directed phosphorylation of this enzyme has not been identified. Recently, Strålfors [(1987) J. Biol. Chem. 262, 11486–11489] has suggested that effects on the association of this enzyme with mitochondria may account for the observed ability of isoproteronol or insulin to promote immobilization of ATP citrate-lyase in permeabilized cells. Here we report studies involving phosphorylation of the pure enzyme in vitro using cyclic AMP-dependent protein kinase. We show that phosphorylation has no significant effect on the fraction of the enzyme that may be bound to isolated mitochondria.

ATP citrate-lyase; Mitochondrion; cyclic AMP-dependent protein kinase

1. INTRODUCTION

ATP citrate-lyase (EC 4.1.3.8) is the enzyme that is responsible for the cleavage of citrate in the cytoplasm to supply acetyl-CoA for fatty acid biosynthesis:

$$\text{Citrate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{oxaloacetate} + \text{acetyl-CoA} + \text{ADP} + P_i$$

The enzyme can be phosphorylated in two ways. One of these is the result of formation of a phospho-enzyme intermediate in catalysis, through the transient formation of a phosphohistidine residue at the active site [1]. In addition, the enzyme may be phosphorylated at a serine residue at the 'regulatory' or structural site in vivo [2] or by the action of cyclic AMP-dependent [1,3,4] and -independent [5] protein kinases in vitro, and in response to insulin [6,7] and glucagon [4,8,9]. Curiously, phosphorylation at the structural site in response to both insulin and glucagon appears to take place at the same serine residue [9], and moreover, no change in the properties of the enzyme has been attributed to such phosphorylation [3,8,10–14]. Recently, Strålfors [15] has studied the effects of the β-adrenergic agonist isoproteronol, insulin and cyclic AMP analogs on the extent of solubilization of ATP citrate-lyase from adipocytes that have been permeabilized by treatment with digitonin. Here he has observed a correlation between phosphorylation induced by these compounds and cellular immobilization, suggesting that phosphorylation at the structural site may promote the association of the enzyme with a specific cellular organelle or membrane. In keeping with these observations, it has been reported earlier that ATP citrate-lyase from hepatocytes of starved rats is associated with mitochondria [16] and with a microsomal fraction [11]. Here, we report in vitro experiments showing that phosphorylation of the purified enzyme by cyclic AMP-dependent protein kinase has no significant effect on the degree of association of the enzyme with added mitochondria.

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2. MATERIALS AND METHODS

ATP citrate-lyase was purified to homogeneity from livers of male Sprague-Dawley rats that had been starved for 2 days and then refed a high carbohydrate diet for 3 days immediately prior to killing, essentially as in [1,17]. Enzyme activity was determined by the coupled assay outlined by Osterlund and Bridger [18].

The catalytic subunit of cyclic AMP-dependent protein kinase was purified from rabbit thigh muscle according to method II of Bechtel et al. [19]. One unit of activity was defined as that catalyzing the transfer of 1 μmol phosphate from [γ-^{32}P]ATP to histone per min at 30°C.

ATP citrate-lyase was phosphorylated at its structural site by incubation of lyase (2 mg/ml) with 5 U catalytic subunit of cyclic AMP-dependent protein kinase in 50 mM Tris-HCl (pH 8.4), 3 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM [γ-^{32}P]ATP at 30°C for varying lengths of time (see section 3), after which the reaction was stopped by the addition of EDTA to 20 mM. Any ^{32}P incorporated at the catalytic site was removed by incubation with citrate and CoA [1], and the phosphorylated lyase was isolated by gel filtration on Sephadex G-100. Preparations of ATP citrate-lyase in which the phosphoryl groups were removed from the structural sites were obtained by incubation of the phosphorylated enzyme with 0.75 U *Escherichia coli* alkaline phosphatase in 50 mM Tris-HCl (pH 8.4), 1 mM dithiothreitol for 6 h at 30°C. The dephosphorylated lyase was recovered by gel filtration as described above. In keeping with previous reports (see above), we found that the specific activity of the enzyme was unaffected by its phosphorylation.

Mitochondria were prepared from rat liver as described by Beavis et al. [20]. Binding of ATP citrate-lyase to mitochondria was tested in the following way. Enzyme (50–100 μg) was incubated with mitochondria (5 mg protein) in a solution containing 50 mM Tris-HCl (pH 8.4), 1 mM dithiothreitol and 250 mM sucrose for 90 min. The suspension was then centrifuged in an Eppendorf microcentrifuge for 5 min, and the enzyme activities in the supernatants and mitochondrial pellets were assayed.

3. RESULTS AND DISCUSSION

Fig.1 shows the rate and extent of incorporation of phosphate from ATP into the structural site of ATP citrate-lyase, as catalyzed by the catalytic subunit of cyclic AMP-dependent protein kinase. The incorporation reaches a plateau near a level of two phosphoryl groups per tetramer, a value that is consistent with an earlier suggestion [1] of half-sites reactivity for phosphorylation of this enzyme. Thus, the phosphorylated enzyme that was used

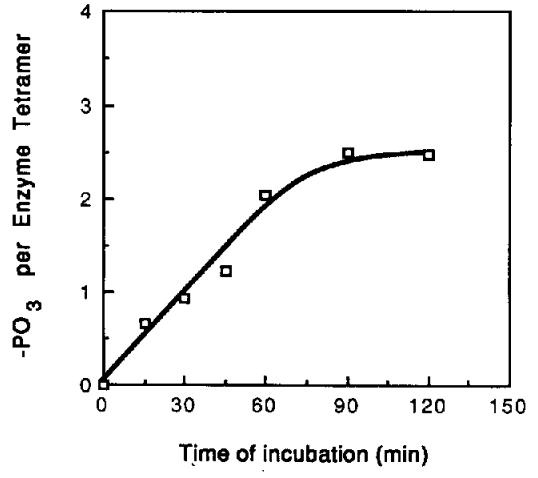

Fig.1. Time course for the structural phosphorylation of ATP citrate-lyase by the catalytic subunit of cyclic AMP-dependent protein kinase. See section 2 for experimental details.

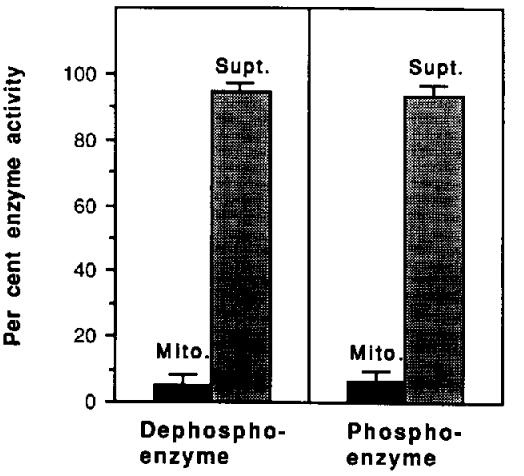

Fig.2. Lack of effect of phosphorylation on the distribution of ATP citrate-lyase between mitochondrial and soluble fractions.

routinely in this study was obtained after 90 min of incubation under these conditions, when the incorporation is near maximal.

Prompted in part by earlier reports of fractional association of ATP citrate-lyase with mitochondria [16], we undertook this direct in vitro study of the effects of phosphorylation on mitochondrial binding of the enzyme. The results, shown in fig.2, indicate no significant effect of structural phosphorylation on the small fraction of enzyme activity (~5%) that sediments with mitochondria. A similar portion of enzyme binding to mitochondria was consistently observed in these experiments; no activity was found to be associated with the isolated mitochondria before addition of pure enzyme, however, and no activity was precipitable if the mitochondria were lysed prior to use by the addition of Triton X-100 (not shown). It thus appears that a small fraction of the enzyme binds reversibly to the outer membrane of the mitochondria, but that the strength of binding is not influenced by phosphorylation. These data therefore argue against mitochondrial association as an explanation for the immobilization of enzyme that has been observed [15] in permeabilized cells that had been treated so as to increase intracellular cyclic AMP levels.

One notable characteristic of signal sequences for targetting of cytoplasmic proteins for mitochondrial translocation is their charge distribution. Survey of a selection of such signal sequences shows, in addition to the preponderance of hydrophobic and hydroxyl-containing side chains, an abundance of basic amino acids. Acidic residues seem to be generally absent from mitochondrial signal sequences (see [21] and references therein). Thus, one could argue that phosphorylation near the N-terminus of a protein, such as at the structural site of ATP citrate-lyase, could interfere with its interaction with a receptor on the cytoplasmic surface of the mitochondrial outer membrane, and that this could be a part of a regulatory mechanism governing intracellular distribution of this enzyme and perhaps others. The present results do not support such a concept.

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REFERENCES

[1] Williams, S.P., Sykes, B.D. and Bridger, W.A. (1985) Biochemistry 24, 5527–5531.
[2] Linn, T. and Srere, P.A. (1979) J. Biol. Chem. 254, 1691–1699.
[3] Guy, P.S., Cohen, P. and Hardie, D.G. (1980) FEBS Lett. 109, 205–210.
[4] Pierce, M.W., Palmer, J.L., Keutmann, H.T. and Avruch, J. (1981) J. Biol. Chem. 256, 8867–8870.
[5] Ramakrishna, S. and Benjamin, W.B. (1981) FEBS Lett. 124, 140–144.
[6] Alexander, M.C., Kowaloff, E.M., Witters, L.A., Dennihy, D.T. and Avruch, J. (1979) J. Biol. Chem. 254, 8052–8056.
[7] Pierce, M.W., Palmer, J.L., Keutmann, H.T., Hall, T.A. and Avruch, J. (1981) J. Biol. Chem. 257, 10681–10686.
[8] Janski, A.M., Srere, P.A., Cornell, N.W. and Veech, R.L. (1979) J. Biol. Chem. 254, 9365–9368.
[9] Swergold, G.D., Rosen, O.M. and Rubin, C.S. (1982) J. Biol. Chem. 257, 4207–4215.
[10] Ranganathan, N.S., Srere, P.A. and Linn, T.C. (1980) Arch. Biochem. Biophys. 204, 52–58.
[11] Alexander, M.C., Palmer, J.L., Pointer, R.H., Koumjian, L. and Avruch, J. (1981) Biochim. Biophys. Acta 674, 37–47.
[12] Avruch, J., Alexander, M.C., Palmer, J.L., Pointer, R.H., Nemenoff, R. and Pierce, M.W. (1981) Cold Spring Harbor Conf. Cell Proliferation 8, 759–770.
[13] Ranganathan, N.S., Linn, T.C. and Srere, P.A. (1982) J. Biol. Chem. 257, 698–702.
[14] Houston, B. and Nimmo, H.G. (1985) Biochim. Biophys. Acta 844, 233–239.
[15] Strålfors, P. (1987) J. Biol. Chem. 262, 11486–11489.
[16] Janski, A.M. and Cornell, N.W. (1980) Biochem. Biophys. Res. Commun. 92, 305–312.
[17] Vogel, H.J. and Bridger, W.A. (1981) J. Biol. Chem. 256, 11702–11707.
[18] Osterlund, B.R. and Bridger, W.A. (1977) Biochem. Biophys. Res. Commun. 76, 1–8.
[19] Bechtel, P.J., Beavo, J.A. and Krebs, E.G. (1977) J. Biol. Chem. 252, 2691–2697.
[20] Beavis, A.D., Branson, R.D. and Gerlid (1985) J. Biol. Chem. 260, 13424–13433.
[21] Hurt, E.C., Pesold-Hurt, B., Suda, K., Oppliger, W. and Schatz, G. (1985) EMBO J. 4, 2061–2068.