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# GTP, a Nonsubstrate of ATP Citrate Lyase, Is a Phosphodonor for the Enzyme Histidine Autophosphorylation

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ATP citrate lyase (EC 4.3.1.8.) was shown to be a major phosphorylated protein of a fraction derived from Zajdela rat hepatoma by chromatography on heparin-Ultrogel, after the incubation with [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP. Histidine was the only amino acid in the purified enzyme phosphorylated by [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP in the autocatalytic reaction which occurred apparently through an intramolecular mechanism regardless of a donor of phosphate. GTP inhibits the ATP-dependent autophosphorylation competitively despite its failure to replace ATP in the formation of acetyl-CoA catalyzed by this enzyme. 

ATP citrate lyase (EC 4.3.1.8.) catalyzes one of the most complex reactions known to be catalyzed by a single protein, the conversion of citrate and CoA into oxaloacetate and acetyl-CoA in the presence of ATP and $Mg^{3+}$ ions (1, 2). It is the source of cytosolic acetyl- CoA for biosynthetic reactions, including biosynthesis of fatty acids, cholesterol and gangliosides, the molecules which are implicated in tumorigenesis and cell growth (3). The enzyme is widespread in various mammalian tissues and regulated at the transcriptional level in close relation with nutritional changes and age (4). ATP citrate lyase (ACL)[1] is phosphorylated *in vivo* in response to several biologically active agents (5) and during cell differentiation (6). *In vitro* the phosphorylation of ACL on Ser 454 is catalyzed by cAMP-dependent protein kinase and insulin-stimulated kinase (7, 8). Ser 450 and Thr 446 are target amino acids for the phosphorylation catalyzed by glycogen synthase kinase-3 which is identical with ACL kinase (9) and may represent an important signalling mechanism (10). Besides three structural phosphorylation sites, ACL has one catalytic autophosphorylation site, histidine 760 in the sequence GHAGA (4). Phosphoprotein resulted from the ATP-dependent autophosphorylation of ACL is an intermediate in the formation of acetyl-CoA catalyzed by the enzyme (1, 2, 11).

In this paper, a novel procedure for the purification of ACL from Zajdela ascites rat hepatoma is described. Besides ATP also GTP showed to be a donor of phosphate for the autophosphorylation of purified ACL, eventhough it can not replace ATP in the catalytic activity of this enzyme.

## MATERIALS AND METHODS

### *Animals*

Wistar rats (160–190 g) were bred and Zajdela ascites hepatoma was maintained in the Institute of Molecular Genetics.

### *Chemicals and Radiochemicals*

ATP, GTP, GMP PNP, dithiothreitol (DTT), coenzyme A (CoA), phosphotyrosine, phospholysine, and phosphoarginine were from Sigma, NADH and malate dehydrogenase from Boehringer Mannheim, heparin-Ultrogel A4R was obtained from Serva, DEAE-Sepharose 6B-CL, Sephacryl S-200 and CM-Sepharose 6B-CL from Pharmacia-LKB. Precoated silica gel TLC plates were from Merck. [$\gamma$-$^{32}$P]ATP (5000 Ci/mmol) and [$\gamma$-$^{32}$P]GTP (5000 Ci/mmol) were products of Amersham and were isotopically diluted to 1 Ci/mmol. Phosphohistidine was prepared from the phosphoamidate (12).

[1] Abbreviations: DDT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; ACL, ATP citrate lyase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; GMP PNP, $\beta$-$\gamma$-methylene guanosine 5′-triphosphate.



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## *$^{32}$P-labeling*

Protein phosphorylation was assayed in reaction mixtures containing in a final volume of 50 $\mu$l 50 mM Tris-HC1 pH 7.6, 1 mM dithiothreitol, [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP and other components as specified for each experiment. Reaction mixtures were incubated for 10 min at 37°C. The reactions were initiated by the addition of labelled nucleotide. Following incubation, aliquots of the mixtures (25 $\mu$l) were withdrawn and either submitted to SDS-polyacryl-amide gel electrophoresis (SDS-PAGE) or spotted on phosphocellulose paper PW 81 (Whatman) discs immediately immersed in 75 mM phosphoric acid as described (13). The step of $H_3PO_4$ washing was slightly modified: the first wash for 5 min at 0°C was followed by two more washes in $H_3PO_4$ for 1 min at room temperature.

Reaction mixtures were analyzed by SDS-PAGE according to Fairbanks et al. (14). Gels were either stained with Coomassie blue or treated with a neutral solution of ethanol and formaldehyde in water (15), dried and autoradiographed with intensifying screens at −80°C.

## *Enzyme Assay*

ACL was assayed by the malate dehydrogenase coupled procedure (16). One unit is defined as the amount of enzyme that oxidized 1 $\mu$mol NADH per min.

## *Preparation of the Fraction Retained on Heparin-Ultrogel*

Postmitochondrial supernatant of Zajdela rat ascites hepatoma was isolated as described (17). The supernatant was applied to a heparin-Ultrogel column (2.5 × 13 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.6 containing 10% glycerol, 25 mM KCl, 5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.25 mM PMSF. The nonabsorbed protein fraction was eluted with the same buffer. The fraction retained on heparin-Ultrogel was eluted from the column with buffer A (50 mM Tris-HCl pH 7.6, 1 mM DTT, 25% (v/v) glycerol and 0.25 mM PMSF) containing 500 mM KCl, was concentrated by ultrafiltration (Amicon PM-10), dialyzed overnight against buffer A and used either directly in experiments or for further purification.

## *Purification of the Phosphoprotein of $M_r$ 122 000*

The protein was purified from the subcellular fraction retained on heparin-Ultrogel by several successive chromatographic steps. All steps were performed at 4°C.

*Chromatography on DEAE-Sepharose 6B-CL.* The dialyzed fraction (150 ml) was subjected to chromatography on a DEAE-Sepharose 6B-CL column (2.5 × 10 cm) equilibrated in buffer A. Following application of the sample, the column was washed with 800 ml equilibration buffer, and a linear gradient (700 ml) from 0 to 250 mM KCl was initiated. Fractions of 16 ml were collected at a flow rate of 40 ml/h and assayed for the phosphorylation from [$\gamma$-$^{32}$P]ATP and [$\gamma$-$^{32}$P]GTP. Labelled fractions were pooled and dialyzed overnight against buffer A.

*Rechromatography on heparin-Ultrogel A4R.* The dialyzed pool was applied to a heparin-Ultrogel column (1.5 × 20 cm) equilibrated in buffer A containing 25 mM KCl, at a flow rate of 40 ml/h and developed with a 700 ml linear gradient from 25 to 200 mM KCl in buffer A. The $^{32}$P-labelled fractions resulting from the incubation with [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP were eluted at about 100-150 mM KCl. They were pooled and dialyzed overnight against buffer A.

*Rechromatography on DEAE-Sepharose 6B-CL.* The pool of fractions was subjected to rechromatography on a DEAE-Sepharose 6B-CL column (1.5 × 15 cm) that was equilibrated in buffer A and developed at a flow rate of 25 ml/h with 500 ml of a linear gradient from 0 to 200 mM KCl in buffer A. The phosphorylated fractions resulting from the incubation of the fractions with [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP were eluted at about 80 mM KCl. They were pooled and dialyzed overnight against buffer A.

*Chromatography on CM-Sepharose 6B-CL.* The dialyzed pool (50 ml) was applied on a column (1.5 × 17 cm) of CM-Sepharose 6B-CL. Protein $M_r$ 122 000 was not absorbed on this column. The flow through fraction was concentrated by vacuum dialysis in a Sartorius SM 13200 collodion bag against buffer A, dialyzed against buffer A containing 50% glycerol, and stored at −20°C.

## *Identification of Phosphorylated Amino Acid Residues*

For the identification of phosphorylated amino acids in the phosphoprotein of $M_r$ 122 000, the reaction mixtures with $^{32}$P-labelled pp122 were hydrolyzed in 3 M KOH at 105°C for 3 hr. The hydrolysates were neutralized with 10% $HClO_4$, mixed with standard phosphoamino acids, phospholysine, phosphoarginine, and phosphohistidine and analyzed on silica gel TLC plate with two solvent cycles as described (18).

Protein concentration was determined according to Lowry et al. (19).

## RESULTS AND DISCUSSION

If a protein fraction, which was obtained from postmitochondrial supernatant of Zajdela ascites rat hepatoma by chromatography on heparin-Ultrogel was incubated with [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP, several proteins became phosphorylated. It is apparent from the SDS-PAGE of the incubation mixtures followed by autoradiography (Fig. 1) that a major phosphorylated protein band approximately of molecular mass 120–130 kDa resulted from the incubation of the fraction with a phosphate donor irrespective of whether [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP was used. This highly phosphorylated protein was purified to apparent homogeneity by a procedure described in Materials and Methods. It was developed on the basis of the finding that in the absence of $Mg^{2+}$ ions this protein was the only one protein present in the fractions eluted from the chromatography columns, which was phosphorylated after the incubation with either [$\gamma$-$^{32}$P]ATP or [$\gamma$-$^{32}$P]GTP. The method by Roskoski (16) was used to localize the phosphoprotein in the fractions eluted from the columns during the purification procedure. The method was slightly modified (Materials and Methods), since the radioactivity associated with the reaction products appeared to be rather unstable when time of the treatment with acid solutions was prolonged, suggesting that phosphoamidate linkages were formed, regardless of which phosphate donor was used. The molecular mass of the purified protein determined by SDS-PAGE was 122 kDa, and 0.2 mg protein pp122 was obtained from 1.5 g postmitochondrial supernatant of Zajdela ascites rat hepatoma.

Only histidine was identified as a phosphorylated amino acid resulting from the incubation of the purified ppl22 with [$\gamma$-$^{32}$P]GTP or [$\gamma$-$^{32}$P]ATP (Fig. 2). The phosphorylation was obviously autocatalytic, since the presence of any other proteins was not required for the reactions with both of these nucleotides. The autophosphorylation of histidine was very fast, reaching 50–70% of the saturation within 40s. Its initial rate (nM phosphorylated ppl22 per min) was determined by 20-s incubation. It was linearly proportional to the concentration of the protein over a 40-fold range (0.6–24 $\mu$g/ml) at different levels of ATP or GTP (Fig. 3). Apparently, the autophosphorylation of pp122 occurs through an intramolecular reaction, which was demonstrated for the autophosphorylation of several protein kinases (21, 22). The $K_m$ values for the pp122 autophosphorylation estimated from Lineweaver-Burk plot were 1.2–1.6 $\mu$M for ATP and 3.0–3.5 $\mu$M for GTP. Up to 0.3 mol and 0.2 mol phosphate was incorporated into 1 mol purified pp122 from [$\gamma$-$^{32}$P]ATP and [$\gamma$-$^{32}$P]GTP, respectively. Due to the acid-lability, the amount of phosphate bound to pp122 was determined by counting the radioactivity associated with the protein band that was excised from the dried gel.

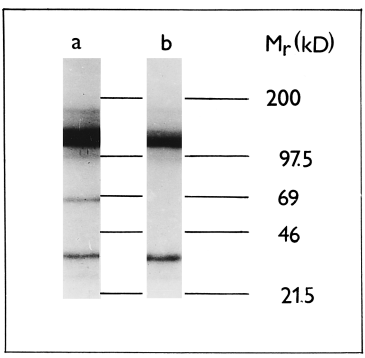


**FIG. 1.** Polyacrylamide gel electrophoretic (SDS-PAGE) analysis of the pattern of phosphorylated proteins of the fraction derived from Zajdela rat hepatoma. The positions of standard [$^{14}$C] methylated proteins, myosin ($M_r$ 200 000), phosphorylase b ($M_r$ 97 500), serum albumin ($M_r$ 69 000), ovalbumin ($M_r$ 46 000), and trypsin inhibitor ($M_r$ 21 500) are indicated on the scale. Incubation mixtures (50 $\mu$l) contained 20 $\mu$g of Zajdela rat hepatoma fraction retained on heparin-Ultrogel, 50 mM Tris buffer pH 7.6, 5 mM $MgCl_2$, 25 mM KCl, 1 mM DT, and 0.1 $\mu$Ci [$\gamma$-$^{32}$P]ATP (a) or 0.3 $\mu$Ci [$\gamma$-$^{32}$P]GTP (b).

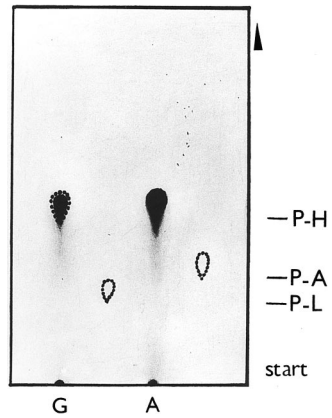


**FIG. 2.** Identification of [$^{32}$P]phosphohistidine after alkaline hydrolysis of [$^{32}$P]pp122, Protein pp122 (20 pmol) phosphorylated from 2.0 $\mu$M [$\gamma$-$^{32}$P]ATP (A) and [$\gamma$-$^{32}$P] GTP (G) was hydrolyzed for 3 hr in 3 M KOH and treated with 10% perchloric acid as it is described in Materials and Methods, and analyzed by TLC on silica gel plates in the presence of phosphoarginine, phospholysine and phosphohistidine. Phosphoamino acids were vizualized after ninhydrine staining and autoradiographed.

Since the histidine phosphorylation and molecular mass of the protein pp122 showed similarities with a subunit of ACL, we examined this enzymatic activity and found that pp122 was indeed able to catalyze acetyl-CoA formation. The specific activity of the enzyme preparations was 7–8 units/mg of protein. This finding strongly suggests that protein pp122 is a subunit of ACL, the enzyme composed of four identical subunits of approximately $M_r$ 120 000, which are autophosphorylated on histidine residues by ATP (1, 2, 22, 23).

As demonstrated in this paper, GTP appeared to be another donor of phosphate for the histidine autophosphorylation of ACL. An examination of a possible involvement of GTP also in the enzymatic activity of our preparations of ACL showed that GTP could not replace ATP in the formation of acetyl-CoA in the range of concentration 5 mM - 300 mM GTP in agreement with previous findings (2). GTP and GMP PNP, a nonhydrolysable analog of GTP, are competitive

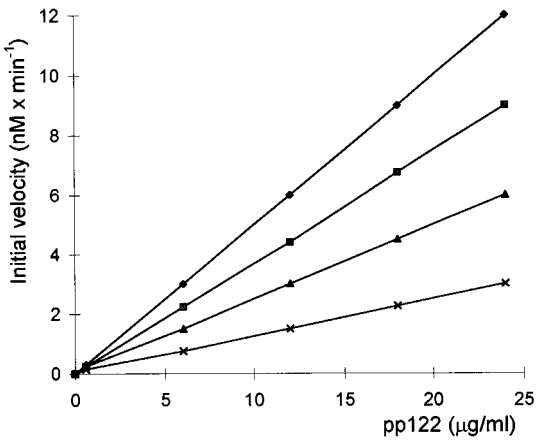


**FIG. 3.** Effect of initial concentration of pp122 on the initial velocity (nM phosphorylated pp122 × min$^{-1}$) of its autophosphorylation. Phosphorylation rates were determined by a 20-s incubation under conditions of autophosphorylation as were indicated under Materials and Methods with 0.075 $\mu$M ATP (×) and 0.3 $\mu$M ATP (◆) or 0.4 $\mu$M GTP (▲) and 0.65 $\mu$M GTP (■).

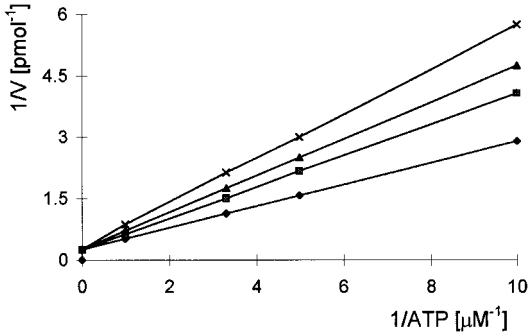


**FIG. 4.** Inhibition of the ATP-dependent autophosphorylation of pp122 in the presence of GTP and GMP PNP. Incubation mixtures contained in 25 $\mu$l 15 pmol pp122 concentrations of [$\gamma$-$^{32}$P]ATP as indicated and no inhibitor (♦), 1 $\mu$M GTP (■) or 3 $\mu$M GTP (×) or 1 $\mu$M GMP PNP (▲).

inhibitors of the ATP-dependent autophosphorylation of ACL (Fig. 4), indicating possibility that a nucleotide-binding site of ACL has a broader specificity.

Phosphorylation of histidine shows to be an important regulatory mechanism. Phosphohistidine is an intermediate in reactions involved in bacterial (24, 25) and eukaryotic cell signalling (26, 27). Mostly ATP is a donor of phosphate for histidine phosphorylation. However, GTP was found to be a donor of phosphate for histidine autophosphorylation of mitochrondrial GTP-dependent succinyl-CoA synthetase (28, 29). Acid-labile GTP-dependent autocatalytic phosphorylation of mammalian translational factors (30, 31) and $\beta$-subunit of heterotrimeric G proteins of human leukemic cells HL 60 (32) was also reported.

Physiological relevance of the GTP-dependent histidine autophosphorylation of ACL might be suggested by its apparently intramolecular mechanism. Possible physiological function of this phosphorylation remains to be established.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Linn, T. C., and Srere, P. A. (1979) *J. Biol. Chem.* **254,** 1691–1698.
2. Inoue, H., Suzuki, F., Fukunishi, K., Adachi, K., and Takeda, Y. (1966) *J. Biochem.* **60,** 543–553.
3. Sjoberg, E. R., and Varki, A. (1993) *J. Biol. Chem.* **268,** 10185–10196.
4. Elshourbagy, N. A., Near, J. C., Kmetz, P. J., Sathe, G. M., Southan, C., Strickler, J. E., Gross, M., Young, J. F., Wells, T.N. C., and Groot, P.H. E. (1990), *J. Biol. Chem.* **265,** 1450–1435.
5. Alexander, M. C., Kowaloff, E. M., Witters, L. A., Dennihy, D. T., and Avruch, J. (1979) *J. Biol. Chem.* **254,** 8052–8056.
6. Benjamin, W. B., Pentyala, S. N., Woodgett, J. R., Hod, I., and Marshak, D. (1994) *Biochem. J.* **300,** 477–482.
7. Guy, P. S., Cohen, P., and Hardie, D. G. (1980) *FEBS Lett.* **109,** 205–208.
8. Yu, K. T., Benjamin, W. B., Ramakrishna, S., Khalaf, M., and Czech, M. P. (1990) *Biochem. J.* **265,** 539–545.
9. Ramakrishna, S., D'Angelo, G., and Benjamin, W. B. (1990) *Biochemistry* **29,** 7617–7624.
10. Sutherland, C., Leighton, I. A., and Cohen, P. (1993) *Biochem. J.* **296,** 15–19.
11. Wells, T.N. C. (1991) *Eur. J. Biochem.* **199,** 163–168.
12. Wei, Y. F., and Matthews, H. R. (1991) *Methods Enzymol.* **200,** 388–414.
13. Roskoski, R. (1983) *Methods Enzymol.* **99,** 3–7.
14. Fairbanks, G., Stock, T. L., and Wallach, D.F. H. (1971) *Biochemistry* **10,** 2606–2617.
15. Fujitaki, J. M., and Smith, R. A. (1984) *Methods Enzymol.* **107,** 22–36.
16. Takeda, J., Suzuki, F., and Inoue, H. (1969) *Methods Enzymol.* **XIII,** 153–160.
17. Hradec, J., Stiborová, M., Dušek, Z., and Franěk, F. (1983) *Eur. J. Biochem.* **131,** 277–281.
18. Muñoz, G., and Marshall, S. H. (1990) *Anal. Biochem.* **190,** 233–237.
19. Lowry, O. H., Rosenbrough, N. J., and Farr, A. L. R. (1951) *J. Biol. Chem.* **193,** 265–275.

20. Hallenbeck, P. C., and Walsh, D. A. (1983) *J. Biol. Chem.* **258,** 13493–13501.
21. Meggio, F., and Pinna, L. A. (1984) *Eur. J. Bioch.* **145,** 593–599.
22. Ramakrishna, S., and Benjamin, W. B. (1979) *J. Biol. Chem.* **254,** 9232–9236.
23. Inoue, H., Suzuki, F., Tanioka, H., and Takeda, Y. (1968) *J. Biochem.* **63,** 89–100.
24. Stock, J. B., Ninfa, A. J., and Stock, A. M. (1989) *Microbiol. Rev.* **53,** 450–490.
25. Swanson, R. V., Alex, L. A., and Simon, M. J. (1994) *Trends Biol. Sci.* **19,** 485–490.
26. Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) *Nature* **369,** 242–245.
27. Crovello, C. S., Furie, B. C., and Furie, B. (1995) *Cell* **82,** 279–286.
28. Bridger, W. A. (1974) *in The Enzymes* (Boyer, P. D., Ed.) Vol. 10, pp. 581–606, Academic Press, New York.
29. Křivánek, J., and Nováková, L. (1992) *Physiol. Res.* **41,** 345–350.
30. Tuháčková, Z., and Hradec, J. (1982) *Biochem. Biophys. Res. Commun.* **105,** 1496–1502.
31. Tuháčková, Z., Ullrichová, J., and Hradec, J. (1985) *Eur. J. Biochem.* **146,** 161–166.
32. Wieland, T., Nürnberg, B., Ulibarri, J., Kaldenberg-Stasch, S., Schultz, G., and Jacobs, K. H. (1993) *J. Biol. Chem.* **268,** 18111–18118.