NATURE OF THE PHOSPHORYLATED RESIDUE IN CITRATE CLEAVAGE ENZYME*

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Summary

Citrate cleavage enzyme was phosphorylated with γ-^{32}P-ATP. A ^{32}P-compound was isolated by Dowex chromatography of an alkaline hydrolysate of the ^{32}P-phosphoenzyme. The isolated compound behaved chromatographically and electrophoretically like phosphohistidine.

Inoue et al. (1,2) and Plowman and Cleland (3) have shown that rat liver citrate cleavage enzyme (ATP citrate lyase EC 4.1.3.8.) reacts with ATP to produce a phosphoenzyme and ADP. Inoue et al. (2) also showed that the phosphoenzyme could react with citrate and CoA to produce oxaloacetate and acetyl CoA. It is possible that phosphoenzyme formation is an obligatory first step in the enzyme catalyzed reaction.

We have confirmed the results on the formation of phosphoenzyme, and have been able to isolate a ^{32}P-compound from an alkaline hydrolysate of ^{32}P-phosphoenzyme, with chromatographic and electrophoretic behavior identical to phosphohistidine. This suggests that the phosphorylation site on the enzyme may be an imidazole residue rather than a carboxyl group as postulated by Inoue et al. (2).

Preparation and Hydrolysis of ^{32}P-Phosphoenzyme

Rat liver ATP citrate lyase (4) is incubated with γ-^{32}P-ATP and magnesium chloride in 0.1 M Tris-HCl, pH 8.1. After incubation for 30 minutes at 38°

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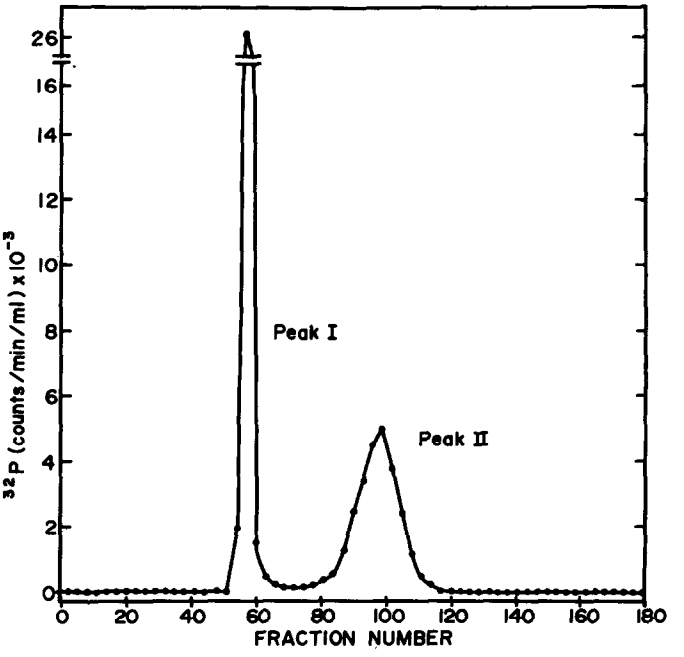
the reaction mixture is cooled and placed on a column of Bio Gel P-10 equilibrated with the same Tris buffer. Fractions are collected and assayed for enzyme activity and ^{32}P; the ratio of ^{32}P to enzyme activity being constant in each enzyme containing fraction. The average value is 2 moles of ^{32}P/mole of enzyme (molecular weight of 500,000 is assumed). Similar experiments with 8-^{14}C-ATP substituted for γ-^{32}P-ATP indicate no binding of the adenine moeity to the enzyme, in agreement with the results of Inoue et al. (1,2).



Legend for Figure 1

252 mμmoles ATP-γ-^{32}P (1.0 X 10^5 cpm/mμmole) and an equivalent amount of $MgCl_2$ were incubated with 3.5 mg of citrate cleavage enzyme in 0.1 M Tris-HCl, pH 8.2, for 30 minutes at 24° in a total volume of 0.60 ml. The ^{32}P-phosphoenzyme was isolated from excess ATP-γ-^{32}P on a Bio Gel P-10 column (1.5 X 14 cm) which had been equilibrated with 0.1 M Tris-HCl, pH 8.2. There were 11.2 X 10^5 cpm total ^{32}P bound to the enzyme. Assuming a molecular weight of 5 X 10^5 gm/mole for the citrate cleavage enzyme, there were 1.6 moles of ^{32}P bound per mole of enzyme in this particular preparation. The ^{32}P-phosphoenzyme was adjusted to 3 N NaOH then sealed in a vial. The sample was hydrolyzed 1 hour at 70° then 15 hours at 100°. After cooling the vial was opened the contents diluted approximately 50 fold and loaded on a 2.5 X 11 cm Dowex-1-(OH^-) column. The column was washed extensively with water then eluted with a linear gradient 0-1.0 M $KHCO_3$ pH 8.2. (1 liter total gradient). Fractions of 5.5 ml were collected and assayed for ^{32}P. Of the 1.0 X 10^6 cpm total ^{32}P loaded on the column 0.50 X 10^6 cpm were recovered as inorganic phosphate in Peak I and 0.34 X 10^6 cpm were recovered in Peak II.

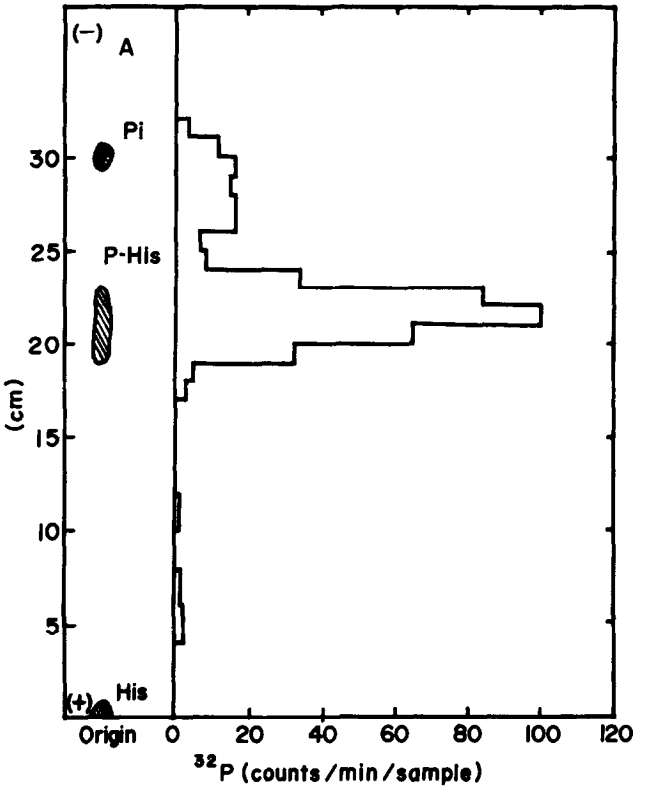
When the ^{32}P-phosphoenzyme is precipitated with cold perchloric acid and filtered rapidly on cold Millipore filters only a small percentage of the ^{32}P appears in the filtrate. If the enzyme is first treated with 1 N HCl at 100° for 1 minute or with neutral hydroxylamine (5) at 24° for 15 minutes then precipitated with perchloric acid and filtered, essentially all of the ^{32}P is found in the filtrate.

Treatment of the enzyme with 1 N KOH at 100° for 1 minute, on the other hand, solubilizes only a few percent of the enzyme ^{32}P, but a 7 minute hydrolysis in 1 N KOH at 100° solubilizes a high percentage of the ^{32}P. When an even more extensive hydrolysis in alkali (3 N NaOH at 100° for 15 hr.) is carried out and the products chromatographed on Dowex-1-(OH^-), considerable quantities of ^{32}P appear in a compound that is not inorganic phosphate (Fig. 1). The chromatographic separation procedure used was one developed by other workers (6-8) for the isolation and identification of phosphoaminoacids from alkaline hydrolysates of other phosphoproteins. The first radioactive peak eluted was identified as inorganic phosphate and the position of the second radioactive peak agreed with the elution position of phosphohistidine. Over 30% of the initial ^{32}P in the phosphoprotein was recovered, presumably as a phosphohistidine (Fig. 1).

Co-Electrophoresis of ^{32}P-peak II with 3-phosphhohistidine

The eluate corresponding to peak II was adsorbed onto another Dowex-1 (OH^-) column. The column was washed with water, then the radioactivity was eluted with 1 M ammonium carbonate. Approximately 95% of the radioactivity was recovered. The ^{32}P material obtained in this manner moved similarly to synthetic 3-phosphohistidine (9) upon co-electrophoresis in 0.05 M sodium borate buffer, pH 9.2, and in 0.1 M ammonium acetate buffer containing 10^{-3} M ethylenediamine tetraacetate at pH values of 8.25, 7.9 (Fig. 2), and 7.2.

Synthetic ε-phospholysine moved slightly farther toward the anode than the 3-phosphohistidine at pH 9.2 and had only a small percentage of the total radioactivity associated with the ninhydrin positive spot. The 1- and 3-



Legend to Figure 2

Co-Electrophoresis of Synthetic 3-phosphohistidine with ^{32}P of peak II. (Fig. 1). The 3-phosphohistidine was prepared and detected as described by Hultquist et al. (9) and Hultquist et al. (10). The synthetic 3-phosphohistidine and the ^{32}P from peak II were co-electrophoresed on Whatman 3 MM paper with 0.1 M ammonium acetate buffer, pH 7.9, containing 10^{-3} M ethylenediamine tetraacetate at 100 V/cm for 60 minutes at 10°. The inorganic phosphate was detected by spraying with ammonium molybdate and the Pauly stain was used to detect histidine and 3-phosphohistidine as described by Hultquist (10). The ^{32}P on the electropherogram was detected by cutting pieces 1 cm square then counting in 10 ml of Brays scintillation liquid. In this particular experiment, 87 percent of the ^{32}P applied to the paper was recovered.

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isomers of phosphohistidine were not clearly separated at any of the pH values, so at present we cannot unambigiously identify which isomer of phosphohistidine we have isolated.

The possibility that more than one type of residue may be phosphorylated in the ^{32}P-phosphoenzyme complex was examined by measuring the rate of hydrolysis of the ^{32}P-phosphoenzyme complex. Hydrolysis of ^{32}P from the intact ^{32}P-

Table 1

Acid Hydrolysis of ^{32}P-ATP Citrate Lyase

Conditions	k (min^{-1})
ATP-Citrate lyase-^{32}P	
46°, pH 3.4	0.0097
46°, pH 2.5	0.018
1-Phosphohistidine	
46°, pH 3.4	0.12 *
pH 2.5	0.20 *
3-Phosphohistidine	
46°, pH 3.4	0.016 *
pH 2.5	0.040 *

* Estimated from Reference 10.

The rate of hydrolysis of ^{32}P from the ^{32}P-ATP citrate lyase complex in 0.05 M phosphate buffers at the pH indicated was followed by precipitation of the protein at various times with cold 0.2 N perchloric acid. The ^{32}P remaining on the enzyme could be determined after Millipore (5μ filters) filtration and subsequent washing with perchloric acid to remove any radioactive inorganic phosphate. After the Millipore filters were dried they were dissolved in 10 mls of Bray's solution (11) and the ^{32}P remaining as an enzyme complex was determined in a Packard Tri-Carb scintillation counter. Apparent first order kinetics were obtained over at least three half times from a plot of the log of the ^{32}P-enzyme remaining vs time.

phosphoenzyme complex at several pH values is apparently first order over at least 3 half-lives and the first order rate constants are similar to those reported for 3-phosphohistidine (10) (Table 1). Although these data indicate that a single type of residue is involved we cannot as yet identify the site of phosphorylation as the 3-position of histidine rather than the 1-position, since the active site environment could effect the rate of hydrolysis of the phosphate group.

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