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Covalently bound pyruvate in phosphopantothenoylcysteine decarboxylase from horse liver

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Horse liver phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36) incorporates nonexchangeable tritium from borotritide with a decrease of the activity. Substrate prevents both tritium incorporation and the decrease in activity. Acid and base hydrolysis of the tritiated protein releases labeled lactate identified by high-voltage paper electrophoresis, paper chromatography and silicic acid chromatography. These results indicate the presence of pyruvate covalently bound through an ester linkage to phosphopantothenoylcysteine decarboxylase which is then another example of a mammalian enzyme in which pyruvate is involved in a catalytic activity.

Phosphopantothenoylcysteine; Decarboxylase; Pyruvate; (Horse liver)

1. INTRODUCTION

Phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36) catalyzes the decarboxylation of pantothenoylcysteine 4′-phosphate to pantetheine 4′-phosphate. This enzyme is a key step not only in coenzyme A biosynthesis [1,2] (the so-called Brown's route) but in the alternative cysteamine pathway of taurine biosynthesis as well [3]. Phosphopantothenoylcysteine decarboxylase is not a pyridoxal 5′-phosphate-dependent enzyme. Pyridoxal 5′-phosphate not only has not been found in the rat [4] or in the horse enzyme but it is also inhibitor [3]. The same results were recently obtained by Abeles with the *E. coli* enzyme (personal communication). However all these enzymes are inhibited by carbonyl reagents such as hydroxylamine, sodium borohydride and phenylhydrazine suggesting that a carbonyl group belonging to an unknown structure might be present.

The following qualitative experiments similar to those used by Hodgins and Abeles [5] to detect a pyruvoyl residue in the active site of an impure preparation of D-proline reductase demonstrate that pyruvate is present and functionally involved in horse liver phosphopantothenoylcysteine decarboxylase as suggested in [6,7].

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2. MATERIALS AND METHODS

Horse liver phosphopantothenoylcysteine decarboxylase was prepared as described [6,7]. The enzyme had a specific activity of about 80 nmol $CO_2 \cdot min^{-1} \cdot mg^{-1}$ assayed by using a labeled substrate [7]. Sodium borotritide (Amersham) with a specific activity of 10.5 Ci/mmol was diluted to 10 mM with 1 mM NaOH. High-voltage paper electrophoresis (Whatman 3 MM) was carried out for 20 min at 3000 V in the following buffer of pH 6.5: 24 ml glacial acetic acid, 800 ml pyridine, 7200 ml H_2O. L-Lactate (Merck) was spotted as reference and identified on the paper by spraying

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a 0.04% phenol red solution in ethanol made alkaline with NaOH. The organic acid appeared as a yellow spot on a red background. Paper strips were scanned for radioactivity with a 4π radiochromatoscanner (Packard 385). Tritium incorporation was determined by liquid scintillation counting of aliquots diluted into Aquasol (NEN) and measured on a Beckman LS 6800.

3. RESULTS

3.1. *Evidence for the presence of a carbonyl group*

The enzyme was treated with a solution of dansylhydrazine in 0.19 M acetate buffer (pH 5.6) as described by Weber and Hof [8]. After reduction with an aqueous solution of dimethylaminoborane complex followed by $NaBH_4$ the reaction mixture was filtered through a Biogel P4 column, eluted with 100 mM ammonium formate (pH 6.3) in 1 ml fractions, and scanned for absorbance at 280 nm and fluorescence at 470 nm (excitation wavelength 360 nm). Fluorescent dansylhydrazone formed was associated to the protein peak eluted.

3.2. *Tritium incorporation into the protein*

Incorporation of tritium into the protein is linearly related to the extent of inactivation as shown in fig.1: inactivation and tritium incorporation are strongly antagonized by the substrate. After this result 20 mg protein in 2 ml of 0.4 M phosphate buffer (pH 8) were treated with 100 μl of the borotritide solution and 10 μl octanol and after 30 min sodium borohydride was added to reach a final concentration of 5 mM. After a further 30 min the solution was made 50 mM in acetone and gel filtered through a 1.5 × 50 cm Biogel P4 column, eluted with water in 1.5 ml fractions. Absorbance and radioactivity were recorded: part of the latter was found in the protein peak. The radioactive protein fractions were collected and lyophilized in a Carius tube to which 1 ml of 6 N HCl was added: hydrolysis was carried out for 9 h at 110°C under pure nitrogen. The acid was removed under vacuum: the hydrolysate dissolved in 0.5 ml water was passed through a Dowex 50 WX8 column, 1 × 20 cm in H^+ form eluting with water. The acid eluant containing most of the radioactivity found in the protein was collected, lyophilized and dissolved in 200 μl water.

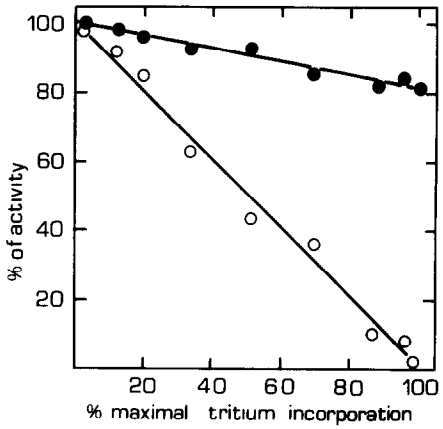

Fig.1. Relation between tritium incorporation and extent of enzyme inactivation. The enzyme (1 mg) was incubated with sodium borotritide from 1 to 5 mM for 10 min in 0.4 M potassium phosphate buffer (pH 6.5) made 0.5 M in deionized urea in a total volume of 0.5 ml. The reaction was stopped with acetone (to 0.5 M). The samples were then diluted with water and exhaustively dialyzed against 0.05 M phosphate buffer (pH 6.5): proteins, radioactivity and enzyme activity were then measured. A parallel experiment in the presence of 15 mM cold substrate (10-times its K_m value [6]) was conducted (●).

3.3. *Identification of pyruvate as the protein-bound carbonyl group*

(i) 5 μl of the dissolved lyophilizate were added to a spectrophotometric cuvette containing 3 mg NAD^+, 10 U lactic dehydrogenase (Sigma) and 0.8 ml of 0.1 M glycine buffer (pH 9.2) made 10 mM in hydrazine. After 45 min at 37°C the absorbance at 340 nm was measured against a blank containing all the reagents without the sample. NAD^+ was reduced by the sample and from its molar absorptivity an approximate content of lactate in the sample was estimated. Its rate of lactic dehydrogenase-catalysed NAD^+ reduction was found to be identical to that of a sample containing the same amount of pure L-lactate.

(ii) 150 μl were streaked on Whatman 3 MM and subjected to high-voltage paper electrophoresis for 20 min at 3000 V in buffer of pH 6.5: L-lactate was spotted as reference and identified with phenol red. The paper was then scanned for radioactivity: as reported in fig.2 a radioactive peak can be superimposed on the lactate spot. The radioactive

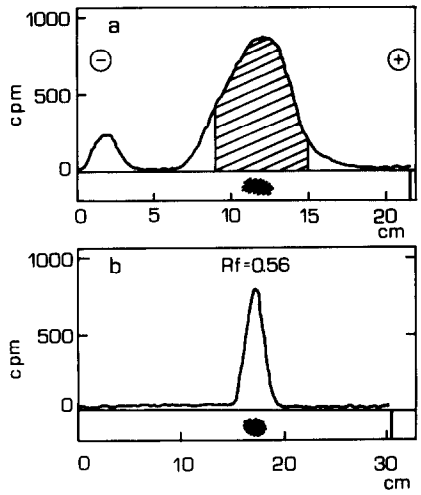

Fig.2. (a) Paper electrophoresis of tritiated hydrolysis products of the protein, treated for 9 h at 110°C and purified on Dowex 50 WX8. Part of the column eluate was electrophoresed on Whatmann 3 MM for 20 min at 3000 V in pyridine-acetic acid-water (800:24:7200, pH 6.5); (b) ascending paper chromatography on Whatmann 1 in absolute ethanol-25% NH_4OH-H_2O (8:1:1) of the eluted shaded area shown in panel a. Lower parts of each panel show the behaviour of pure L-lactate demonstrated by spraying a 0.04% phenol red solution.

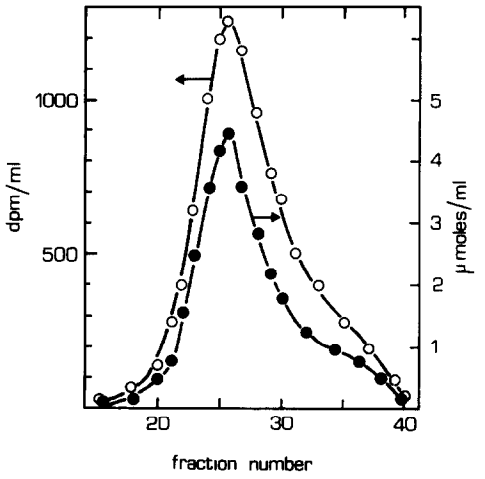

Fig.3. Silicic acid chromatography of the radioactive compound obtained from acid hydrolysis of tritiated phosphopantothenoylcysteine decarboxylase. The compound was purified by ion-exchange chromatography, paper electrophoresis and ascending paper chromatography and applied with 15 mg L-lactate to a silicic acid column (1 × 22 cm): elution was performed with chloroform-butanol (85:15) and fractions (4 ml) were collected over 3 ml water. The aqueous phase was titrated with 10 mM NaOH (●—●) and assayed for radioactivity (○—○).

zone of the sheet was eluted with water, lyophilized, dissolved in water and streaked on a strip of Whatman 1 adding in a section pure L-lactate as reference. Chromatography was carried out in absolute ethanol-25% NH_4OH-H_2O (8:1:1) and the strip was scanned for radioactivity: a single radioactive peak was obtained containing 85% of the total applied radioactivity with an R_f of 0.56, identical to that of L-lactate (fig.2b). The radioactive zone was eluted with water, lyophilized and dissolved in 0.6 ml of 0.5 M H_2SO_4 to which 15 mg L-lactate were added; the mixture was adsorbed on 1 g silicic acid (Mallinckrodt) which was transferred to the top of a silicic acid column (1 × 22 cm) with the aid of a short-stemmed funnel which, together with the container, was rinsed with 2 ml chloroform. Elution was performed with chloroform/butanol (85:15) as described by Varner [9]. Fractions of 4 ml were collected on tubes containing 3 ml water. After shaking 1 ml of the aqueous phase was titrated to pH 7 with 10 mM NaOH and 0.5 ml assayed for radioactivity. As reported in fig.3 a single radioactive peak with an elution volume corresponding to L-lactic acid was recovered: this peak contained 98% of the applied radioactivity.

3.4. *Identification of a covalent pyruvate-protein bond*

To demonstrate that pyruvate is covalently bound to the protein, the latter (25 mg) was reduced with 5 mM borotritide for 30 min at room temperature. 50 mM acetone was added and the sample dialyzed against 8 M urea in 0.01 M phosphate. No radioactivity was lost from the protein solution which was adjusted to pH 8 and treated for 60 min with 10 mM iodoacetamide. Dialyzed against water the sample was lyophilized, dissolved in 0.1 M ammonium bicarbonate and treated for 6 h with 500 μg chymotrypsin at 38°C. The sample was gel filtered on a Sephadex G-25 column 1.5 × 60 cm, eluting with 5% acetic acid and measuring absorbance at 280 nm and radioactivity. Three radioactive peaks were recovered.

Each one was divided in two parts; one was hydrolyzed with 6 N HCl at 100°C for 9 h, the other with 0.1 N NaOH at 100°C for 30 min. Both samples were passed through a Dowex 50 WX8 column and the eluants tested for lactic acid with lactic dehydrogenase. Lactic acid eluted from both the columns. The same experiments performed on bovine kidney mitochondrial aspartate aminotransferase [11] failed to give lactic acid.

4. DISCUSSION

The inhibition exerted by carbonyl reagents on phosphopantothenoylcysteine decarboxylase, the correspondence between the rate of inactivation and the rate of tritium incorporation from borotritide being strongly prevented by the substrate as well as the failure to find any carbonyl compounds other than that of pyruvate establish that pyruvate is involved in the catalytic activity of the enzyme. Random absorption of pyruvate to the protein can be ruled out by the finding that after dialysis of tritiated enzyme in 8 M urea the radioactivity was not lost and lactic acid was not recovered on treating aminotransferase with borotritide. Furthermore, separation of tritiated peptides by paper electrophoresis and the release of lactate by either acid or base hydrolysis confirm in our opinion that pyruvate is covalently bound to the protein through an ester linkage unstable to base hydrolysis, rather than through an amide linkage: in the latter case pyruvate is not released even after a long treatment in 0.1 N NaOH at 100°C [5,12]. The rate at which the tritiated product released from the protein hydrolysate reduces NAD with lactic dehydrogenase is very close to that of lactate. Nevertheless other compounds such as glycolic acid, β-hydroxylactic, and α-hydroxybutyric acid have the same reduction rate with lactic dehydrogenase [10]. To rule out this possibility, glyoxylic acid, β-hydroxypyruvic acid and α-ketobutyric acid were reduced with borotritide and submitted to either paper chromatography or silicic acid chromatography: the R_f and the elution volumes of these compounds were found to be different from that of lactate to which the compound extracted from the protein corresponds. These results are in agreement with those recently obtained by Abeles (personal communication) on the same pure enzyme extracted by *E. coli* where pyruvate is covalently bound to the protein and involved in its catalytic action.

Pyruvate has been found covalently bound to many bacterial enzymes such as histidine decarboxylase [12], proline reductase [5], phosphatidylserine [13] and *S*-adenosylmethionine decarboxylase [14]. Pyruvate has been found in the latter enzyme extracted either from yeast [15] or from rat liver [16,17]. Phosphopantothenoylcysteine decarboxylase is another example of an enzyme in which pyruvate, involved in a catalytic activity, is covalently bound to both the mammalian and the bacterial proteins.

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