Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 707-714

ON THE PHOSPHATE CONTENT OF RAT LIVER PHENYLALANINE HYDROXYLASE PURIFIED BY HYDROPHOBIC CHROMATOGRAPHY

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Received February 19,1981

SUMMARY: Rat liver phenylalanine hydroxylase purified by hydrophobic chromatography has been found to contain significantly less protein-bound phosphate than enzyme purified by more conventional procedures. Studies with purified hydroxylase of defined phosphate content suggest that phosphorylated species of phenylalanine hydroxylase possess a higher affinity for the hydrophobic matrix than does the non-phosphorylated form. This selectivity may account for the lower phosphate content in phenylalanine hydroxylase purified by hydrophobic chromatography.

INTRODUCTION: Purified rat liver phenylalanine hydroxylase is a phosphoprotein containing approximately 0.3 mol of protein-bound phosphate per mol of Mr 50,000 subunit (1, 2). The enzyme can be activated 3 to 4-fold by incubation in the presence of Mg^{2+}, ATP, cyclic AMP and cyclic AMP-dependent protein kinase. This activation is accompanied by incorporation of about 0.7 mol of phosphate per mol of Mr 50,000 subunit (1, 2).

Phenylalanine hydroxylase in extracts of rat liver can be resolved into three forms by chromatography on columns of calcium phosphate gel-cellulose (3). We have previously demonstrated that the protein kinase-catalyzed phosphorylation of phenylalanine hydroxylase *in vitro* not only activates the hydroxylase, but also leads to a marked change in its elution pattern during calcium phosphate gel-cellulose chromatography. On phosphorylation, the two major forms of the hydroxylase, forms II and III, are converted to a new form (IV) that is more tightly bound to the calcium phosphate gel than are the other forms (4, 5). Direct determination of the amounts of protein-bound phosphate in purified forms II, III and IV provided additional support for the proposal that a major structural feature determining the calcium phosphate gel-cellulose elution pattern of these forms of phenylalanine hydroxylase is their content of endogenous, protein-bound phosphate

0006-291X/81/060707-08$01.00/0

(5). In the course of additional characterization of the multiple forms of the enzyme, major differences in the phosphate content of the hydroxylase were observed depending on the method of purification of the enzymes. These differences are described in the present report.

METHODS: Phenylalanine hydroxylase activity was measured as previously described (5). The calcium phosphate gel-cellulose chromatographic medium was prepared as previously described (5) with the exception that the cellulose powder was extracted overnight with 0.5 M potassium phosphate, pH 6.8, containing 0.5 M KCl, and after washing, used immediately without drying. Preparative-scale calcium phosphate gel-cellulose chromatography was performed essentially as described previously (5). In vivo phosphorylated phenylalanine hydroxylase was prepared from rats injected with aminophylline (300 mg kg^{-1}, dissolved in phosphate-buffered saline immediately before use). Phenylalanine hydroxylase was purified by the procedure of Kaufman and Fisher (6), a modification of this procedure (5), or by the method described by Shiman et al. (7).

In order to measure protein-bound phosphate, the purified protein samples were precipitated and washed with cold 7% trichloroacetic acid. The precipitates were dissolved in 15% formic acid and aliquots were removed to estimate protein concentration. Additional aliquots were dried and then ashed with perchloric and sulfuric acids. The ashed samples were analyzed for inorganic phosphate essentially as described by Lowry et al. (8). Protein purity was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (9).

RESULTS: The purification of the two major forms of phenylalanine hydroxylase from rat liver after their separation by chromatography on calcium phosphate gel-cellulose (5) is a long, multi-step procedure modified from the original scheme described by Kaufman and Fisher (6). The recent report of a much shorter purification method which exploits the tight binding of the phenylalanine-activated hydroxylase to the hydrophobic adsorbent, phenyl sepharose (7), appeared to offer a much simpler route to the preparation of the various forms of the enzyme. Accordingly, the individual forms of phenylalanine hydroxylase were separated by calcium phosphate gel-cellulose chromatography and further purified using the procedure of Shiman et al. (7). Subsequent chemical analysis of the phosphate content of these purified forms (Table 1) showed that all of the hydroxylase species purified by this procedure had significantly lower amounts of protein-bound phosphate than previously found (5).

The purification of phenylalanine hydroxylase through the use of phenyl sepharose chromatography typically gave yields of 25-30% for the individual forms and 50-60% for enzyme purified directly from rat liver extracts. In all cases

Table 1. Phosphate Content of Purified Forms of Rat Liver Phenylalanine Hydroxylase

Source of enzyme	Phosphate content, mol Pi/mol Mr 50,000 subunit		
	Purification method A	Purification method B	Previously found
Calcium phosphate form I	0.05[1]	0.03	ND
Calcium phosphate form II	0.14[1]	0.025	0.22[a]
Calcium phosphate form III	ND	0.39	0.48[a]
Calcium phosphate form IV	ND	0.67	0.96[a]
Normal rat liver extract	0.26	0.08	0.31[b] (range 0.23-0.42)
Liver extract from aminophylline-treated rats	ND	0.55	ND
In vitro phosphorylated purified phenylalanine hydroxylase	0.97	ND	ND

[1]Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed significant contamination with other peptides.

Purification method A is that reported by Donlon and Kaufman (5).

Purification method B refers to that described by Shiman et al. (7).

[a]Reference 5.

[b]Reference 2

(ND denotes that no determination was made)

quantitative binding of the enzyme to the hydrophobic matrix was observed. The low recoveries of protein-bound phosphate and the less-than-quantitative recoveries of enzyme activity suggested that the phosphorylated species of the hydroxylase might have a greater affinity for the hydrophobic matrix and thus be selectively lost during the regular elution procedure.

In order to test this idea, phenylalanine hydroxylase purified by the procedure of Kaufman and Fisher (6) was subjected to the procedure described by Shiman et al. (7). This enzyme had an initial endogenous phosphate content of 0.26 mol of phosphate per mol of Mr 50,000 subunit. Table 2 (Experiment 1) illustrates

Table 2. Phosphate Content of Purified Phenylalanine Hydroxylase Before and After Exposure to Hydrophobic Chromatography

Sample	Activity Ratio[1] %	$6MPH_4$ Activity % recovery	Total Pi % recovery	mol Pi/mol Mr 50000 subunit (chemical analysis)	mol ^{32}P incorporated/ mol Mr 50000 subunit
Experiment 1					
initial enzyme	3.3	-	-	0.26	not determined
phenyl sepharose, 15% glycerol eluate	3.2	80.5	15.2	0.07	not determined
phenyl sepharose, 50% glycerol eluate	4.8	8.0	70.4	1.2	not determined
Experiment 2					
initial enzyme	3.0	-	-	0.28	0.72
phenyl sepharose, 15% glycerol eluate	3.4	51.0	12.5	0.10	1.06
phenyl sepharose, 50% glycerol eluate	4.3	12.0	41.1	0.47	0.82

[1]The activity ratio is the specific activity of phenylalanine hydroxylase in the presence of tetrahydrobiopterin as cofactor expressed as a percentage of the specific activity of phenylalanine hydroxylase in the presence of 6-methyltetrahydropterin ($6MPH_4$) as cofactor.

the results of this experiment. Although the bulk of the applied activity eluted under conditions described by Shiman et al. (7), only 15% of the protein-bound phosphate was recovered with this protein. When the column was subsequently eluted with a buffer containing 50% glycerol in order to further weaken hydrophobic interactions, an additional 8% of the enzyme activity was recovered. This protein contained 70% of the total protein-bound phosphate that had been applied. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that the hydroxylase in all samples was greater than 95% pure.

In order to obtain sufficient protein to further characterize these species, the experiment described above was repeated with approximately a three-fold greater amount of purified phenylalanine hydroxylase. In this case, a decrease in the recovery of the applied activity was observed (Table 2, experiment 2). However, the enzyme which eluted with buffer that contained 15% glycerol again possessed significantly less protein-bound phosphate than did the starting material. The hydroxylase which eluted with buffer that contained 50% glycerol was considerably enriched in protein-bound phosphate.

The more highly phosphorylated form possessed a greater activity than the hydroxylase species which contained less protein-bound phosphate when assayed with tetrahydrobiopterin as cofactor, in agreement with earlier observations (4, 5). Subsequent exposure of these species to in vitro phosphorylating conditions in the presence of [γ-^{32}P]-ATP resulted in different extents of incorporation of ^{32}P (Table 2). Upon complete activation by in vitro phosphorylation, the activity ratio, i.e., the specific activity of phenylalanine hydroxylase in the presence of tetrahydrobiopterin as cofactor expressed as a percentage of the specific activity of the enzyme in the presence of 6-methyltetrahydropterin as cofactor, for each species was essentially identical (approximately 9%).

The chromatographic behavior of phenylalanine hydroxylase on calcium phosphate gel-cellulose columns has been shown to depend, at least in part, on the degree of phosphorylation of the enzyme, i.e., the more highly phosphorylated enzyme is bound more tightly to the chromatographic matrix (5). When the

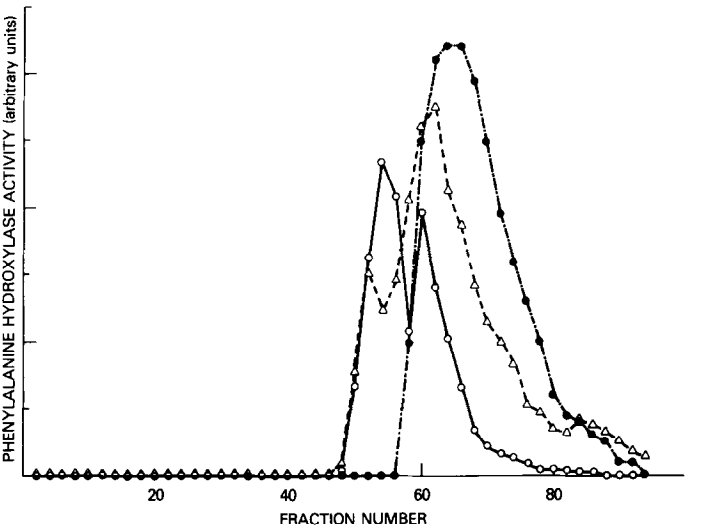

Figure 1: Calcium phosphate gel-cellulose chromatography of purified phenylalanine hydroxylase before and after exposure to hydrophobic chromatography. Approximately 600 µg of the hydroxylase species were mixed in a total volume of 1.5 ml of 30 mM Tris HCl pH 7.0 containing 0.15 M KCl and applied to 1.0 x 18 cm columns of calcium phosphate gel-cellulose. Elutions were performed with 80 ml of a linear gradient of 25-150 mM potassium phosphate, pH 6.8, containing 0.15 M KCl. The flow rates were 0.5 ml/min; 1.0 ml fractions were collected. Phenylalanine hydroxylase purified according to (6), Δ——Δ; enzyme after exposure to hydrophobic chromatography eluted with buffer that contained 15% glycerol, o——o; enzyme after exposure to hydrophobic chromatography eluted with buffer that contained 50% glycerol, ●—•—●.

For ease of comparison, activities are expressed as arbitrary units. The actual activities of the peak fractions in each case, with 6MPH$_4$ as cofactor, were: initial enzyme, 8.1 nmol tyrosine formed/min/ml; enzyme eluted from phenyl sepharose with buffer that contained 15% glycerol, 69.8 nmol tyrosine formed/min/ml; enzyme eluted from phenyl sepharose with buffer that contained 50% glycerol, 5.3 nmol tyrosine formed/min/ml.

hydroxylase which was eluted from phenyl sepharose with buffer that contained 50% glycerol was analyzed by calcium phosphate gel-cellulose chromatography, it was found to be more tightly bound to the gel than was the hydroxylase that was eluted from phenyl sepharose with buffer that contained 15% glycerol (Figure 1).

DISCUSSION: Rat liver phenylalanine hydroxylase purified by the method of Kaufman and Fisher (6) has an average endogenous phosphate content of approximately 0.3 mol per mol of Mr 50,000 subunit. This value has recently been confirmed with enzyme purified by an independent purification procedure (8). In addition, work describing the amino acid sequence at the phosphorylated site of phenylalanine hydroxylase has indicated the presence of a "substantial" amount of

endogenous phosphate in the enzyme (9). In contrast, enzyme purified by the procedure of Shiman et al. (7) contains much lower amounts of protein-bound phosphate.

The finding that essentially pure phenylalanine hydroxylase prepared by the procedure of Kaufman and Fisher (6) loses most of its protein-bound phosphate when taken through the procedure of Shiman et al. (7), and that the bulk of the missing protein-bound phosphate can be recovered from the phenyl sepharose as phenylalanine hydroxylase, indicates that phosphorylated species of the enzyme are being selectively retained by the hydrophobic matrix. This may account in part for the low phosphate values obtained with the enzyme purified by hydrophobic chromatography.

The hydroxylase species selectively retained by the hydrophobic matrix can be recovered by increasing the concentration of glycerol in the elution buffer. The material thus recovered exhibits many properties characteristic of more highly phosphorylated phenylalanine hydroxylase, such as increased specific activity in the presence of tetrahydrobiopterin as cofactor, and tighter binding to calcium phosphate gel-cellulose columns.

The finding that phosphorylated phenylalanine hydroxylase is bound more tightly to phenyl sepharose than is the less phosphorylated species indicates that activation of the enzyme by phosphorylation and activation by exposure to phenylalanine resemble each other in that they both cause changes in conformation of the enzyme that lead to its tighter binding to phenyl sepharose. Also consistent with the above conclusion is our recent finding that not only phosphorylation of phenylalanine hydroxylase, but also exposure to phenylalanine leads to its tighter binding to calcium phosphate-cellulose columns (H. Wilgus and S. Kaufman, unpublished). In addition to this similarity in physical properties, the two modes of activation produce non-independent changes in catalytic activity. Thus, native phenylalanine hydroxylase exhibits a 4-fold increase in tetrahydrobiopterin-dependent activity upon exposure to in vitro phosphorylating conditions. This fully phosphorylated enzyme can be activated an additional 3-fold by preincubation with

phenylalanine. By contrast, when the native enzyme is preincubated with phenylalanine, a 12-fold increase in activity is observed (M. Parniak and S. Kaufman, unpublished data).

The hydroxylase which eluted from phenyl sepharose with buffer containing 50% glycerol possessed greater than stoichiometric amounts of protein-bound phosphate (1.2 mol Pi per mol Mr 50000 subunit; Table 2, experiment 1). This same material obtained in the second experiment possessed less-than-stoichiometric amounts of phosphate; however, after complete activation by in vitro phosphorylation, the enzyme contained 1.3 mol Pi per Mr 50000 subunit. Other workers have defined the sequence around the serine residue which is phosphorylated in phenylalanine hydroxylase (11). The present observations raise the intriguing possibility that an additional site capable of being phosphorylated may exist in the hydroxylase molecule.

ACKNOWLEDGEMENT: We thank Ms. Mindy Kaufman for her excellent assistance in the preparation of this manuscript.

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