MODIFICATION OF THE MULTIPLE FORMS OF RAT HEPATIC PHENYLALANINE HYDROXYLASE BY in vitro PHOSPHORYLATION

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Received August 18, 1977

SUMMARY: The two major forms of rat liver phenylalanine hydroxylase have been isolated and partially purified. The tetrahydrobiopterin-dependent activity of these forms can be differentially stimulated by exposure to enzymatic phosphorylating conditions. This in vitro treatment is associated with incorporation of ^{32}P into the enzymes and generates a further, chromatographically distinct, species. These results suggest that the multiple forms of rat liver phenylalanine hydroxylase are due to different degrees of phosphorylation.

INTRODUCTION: Phenylalanine hydroxylase from extracts of rat liver (1,2) or hydrocortisone-treated Reuber H_4 hepatoma cells (3) can be resolved into three forms by chromatography on calcium phosphate (1) or hydroxylapatite columns (2,3). Only one of these forms appears to be present in untreated hepatoma cells (3) or in liver extracts from rats previously injected with p-chlorophenylalanine (2). Barranger et al. (1) have referred to these forms as isozymes and have speculated that alterations in their relative amounts may contribute to the phenotypes of phenylketonuria and hyperphenylalaninemia. Until now, however, no significant differences in the chemical, physical or kinetic characteristics of these multiple forms have been described.

Previous results from this laboratory (4) have shown that purified rat liver phenylalanine hydroxylase contains 0.3 moles of protein-bound phosphate per subunit (molecular weight = 50,000). When such preparations were incubated under phosphorylating conditions with cyclic AMP[1]-dependent protein kinase, the hydroxylase activity was stimulated 2.5- to 3.0-fold. This activation was apparent when enzyme activity was assayed with

[1]The abbreviations used are as follows: cyclic AMP, adenosine 3':5'-cyclic monophosphate; DTT, 1,4-dithiothreitol.

ISSN 0006-291X

tetrahydrobiopterin but not when assayed with a model cofactor, such as 6,7-dimethyltetrahydropterin. The activation was accompanied by the incorporation of 0.7 moles of phosphate per subunit of hydroxylase. It was suggested (4) that the multiple forms of phenylalanine hydroxylase, as separated by calcium phosphate chromatography (1), could arise from different degrees of phosphorylation. Consistent with this hypothesis, we now present evidence for (a) differential stimulation of the tetrahydrobiopterin-dependent hydroxylase activity of the two major forms of rat liver phenylalanine under *in vitro* enzymatic phosphorylating conditions and (b) the resultant modification of the chromatographic behavior of the different forms.[2]

MATERIALS AND METHODS: Theophylline, ATP, cyclic AMP and beef heart protein kinase were purchased from Sigma Chemical Co., St. Louis, Mo. 6-Methyltetrahydropterin and DTT were from Calbiochem, La Jolla, Calif. Biopterin was obtained from Regis Chemical Co., Morton Grove, Ill., and was reduced by catalytic hydrogenation (5). Catalase, DPNH, phosphocreatine and creatine kinase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. [γ-^{32}P]-ATP (26.2 Ci/mmole) was purchased from New England Nuclear, Boston, Mass. Phenylalanine hydroxylase, from livers of male Sprague Dawley rats (Taconic Farms, Germantown, N.Y.), was purified essentially as described previously (6), but with a modified calcium phosphate step (details to be described elsewhere). Form II of the enzyme was separated from Form III at the calcium phosphate step.

The components of the enzymatic phosphorylating reaction were: 25 mM potassium phosphate buffer, pH 7.4; 2 mM phosphocreatine; creatine kinase, 1 mg/ml; 4 mM theophylline; 15 mM $MgCl_2$; 0.4 mM ATP; 10 μM cyclic AMP; phenylalanine hydroxylase preparations and protein kinase in amounts stated in Table 1. After a 40 min incubation at 30° the samples were placed on ice and aliquots were assayed spectrophotometrically (7). Beef liver catalase (0.5 mg) was added to control (lacking protein kinase) and activated samples, and each mixture was freed of non-protein components by passage through columns of Sephadex G-25, medium grade, using a sample-to-bed volume ratio of 0.1 and eluting with 25 mM potassium phosphate buffer, pH 6.8, containing 0.15 M KCl. Those fractions containing phenylalanine hydroxylase were pooled and loaded onto columns (15 x 0.7 cm) of calcium phosphate uniformly mixed with wet cellulose powder (8). Elution was performed with a linear gradient of potassium phosphate, 25 to 160 mM, pH 6.8, containing 0.15 M KCl, total volume 25 ml, with a flow rate of 0.4 ml/min. Phenylalanine hydroxylase activity in these fractions was measured by fluorimetric (9) or colorimetric (10) determination of tyrosine formed in the presence of 5 mM DTT (11), 1 mM phenylalanine and 20 μM tetrahydrobiopterin. Recoveries of phenylalanine hydroxylase activity applied to the columns ranged from 70% to 80%.

[2] In contrast to others (1-3), these forms are being numbered I through IV based on their order of elution from calcium phosphate-cellulose columns. In the present study we have not investigated the properties of the minor form, which we would designate as Form I.

Table 1. Activation of phenylalanine hydroxylase Forms II and III by enzymatic phosphorylation.

Conditions	Phenylalanine hydroxylase activity (nanomoles DPNH oxidized/min/mg protein)	
	Tetrahydrobiopterin	6-Methyltetrahydropterin
Form II		
No additions	50.4	N.D.
Minus ATP	58.1	N.D.
Minus protein kinase	48.0	1776
Complete system	186.4	1721
Form III		
No additions	19.3	N.D.
Minus ATP	17.5	N.D.
Minus kinase	18.8	450.1
Complete system	41.3	385.8

Form II (0.83 mg), purified through the DEAE-cellulose step, was incubated in a volume of 0.8 ml (as described in the Methods). Beef heart protein kinase, where present, was at a concentration of 75 μg/ml.

Form III (3.5 mg), purified through the calcium phosphate step, was incubated in a volume of 1 ml. Protein kinase, where present, was at a concentration of 100 μg/ml. Hydroxylase assays were carried out on an aliquot with 1 mM phenylalanine and 20 μM tetrahydrobiopterin, and an aliquot with 5 mM phenylalanine and 250 μM 6-methyltetrahydropterin at pH 6.8 and 25°. N.D. denotes that no determination was made.

RESULTS: The data in Table 1 show that Form II of phenylalanine hydroxylase could be stimulated 4-fold by enzymatic phosphorylation, when the activity was measured in the presence of tetrahydrobiopterin. In confirmation of previous results (4), no increase in activity was seen when activity was measured with a synthetic pterin such as 6-methyltetrahydropterin. This activation was greater than the 2.5- to 3.0-fold stimulation obtained previously (4) with purified preparations which had not been fractionated to yield the various forms, or the 2.2-fold stimulation seen with Form III (Table 1). The different extent of activation by phosphorylation observed with Forms II and III is consistent with the idea that these forms of the enzyme differ in their phosphate content.

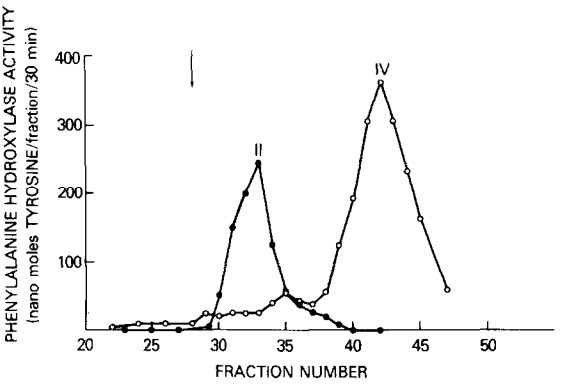

Figure 1. Calcium phosphate chromatography of phenylalanine hydroxylase Form II following incubation (see Table 1 and Methods) under control (minus protein kinase, indicated by closed circles) or phosphorylating conditions (open circles). The arrow indicates the peak of beef liver catalase included as a marker.

Following activation under phosphorylating conditions, the chromatographic behavior of Form II on calcium phosphate was modified from that of the enzyme before phosphorylation (Rf relative to catalase of 1.1) to a new and distinct species, termed Form IV, having an Rf relative to catalase of 1.5 (Fig. 1). Similarily, when Form III, whose Rf relative to catalase is 1.3, was activated under similar conditions, it also chromatographed as this new species (Fig. 2). In experiments carried out in the presence of ^{32}P-ATP this new form, produced from either Forms II or III,contained additional protein-bound phosphate (data not shown). Specifically, in this experiment a mixture of Forms II and III, containing about the same amount of tetrahydrobiopterin-dependent hydroxylase activity, was activated 3-fold in the presence of ^{32}P-ATP. Following this treatment, the hydroxylase activity and protein-bound ^{32}P co-chromatographed on the calcium phosphate column as a single form with an Rf relative to catalase of 1.5, i.e., with the same relative Rf as Form IV.

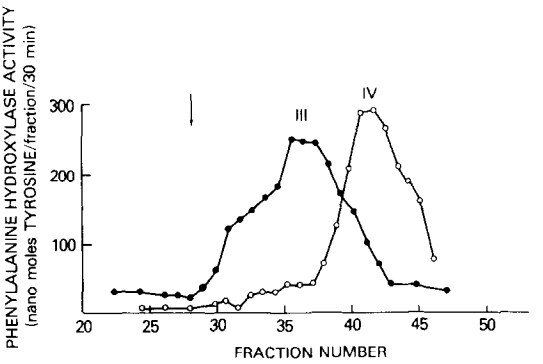

Figure 2. Calcium phosphate chromatography of phenylalanine hydroxylase Form III (the preparation used contained some residual Form II) following incubation (see Table 1 and Methods) under control or phosphorylating conditions (open circles). The arrow indicates the peak of beef liver catalase included as a marker.

DISCUSSION: Our present observations provide strong support for the idea that extent of phosphorylation is one of the structural features that is responsible for the occurrence of multiple forms of rat liver phenylalanine hydroxylase. Thus, we have shown that the two major forms, II and III, can be activated to different degrees under *in vitro* enzymatic phosphorylating conditions (Table 1). Since we have previously shown a correlation between the extent of stimulation of hydroxylase activity and increase in phosphate content (4), the results in Table 1 are consistent with the idea that the two major forms of the hydroxylase, as isolated, differ in their endogenous phosphate contents. Results of preliminary chemical analysis (12) are in accord with this conclusion: Form II contains less phosphate per mole than does Form III. That phosphate content is one of the determinants of the chromatographic separation of the different forms of the hydroxylase on columns of calcium phosphate is also supported by the results shown in Figs. 1 and 2 and the experiments with ^{32}P-ATP.

Other properties of Forms II and III that are consistent with the idea that they differ in the degree of phosphorylation are the ratios of hydroxylase activities obtained in the presence of tetrahydrobiopterin or

6-methyltetrahydropterin and the specific activities with tetrahydrobiopterin. Since phosphorylation increases hydroxylase activity in the presence of tetrahydrobiopterin but not in the presence of synthetic pterins like 6-methyltetrahydropterin (4), the ratio of phenylalanine hydroxylase activity in the presence of these two pterins varies with the extent of phosphorylation. The finding, therefore, that the ratio of activity with 6-methyltetrahydropterin to that with tetrahydrobiopterin is higher for Form II than it is for Form III (see Table 1) is in accord with the findings, mentioned above, that Form II has less phosphate than does Form III. It is also in accord with the finding that after *in vitro* phosphorylation, the ratio of these two activities for Forms II and III are the same (Table 1).[3] Likewise, the specific activities determined with tetrahydrobiopterin are consistent with these observations. Since a 4-fold purification is obtained at the DEAE-cellulose step, the data of Table 1 show that at similar states of purity Form III has higher specific activity than Form II, when assayed with tetrahydrobiopterin. The extrapolated specific activity of the activated form (Form IV) is independent of the form used to produce it.

Although our results make it likely that extent of phosphorylation is a structural determinant of the heterogeneity of rat liver phenylalanine hydroxylase, it is still possible that these forms differ in other structural features, even in their amino acid composition. If, however, extent of phosphorylation is a major determinant, and if, as seems likely, this is due to a post-translational event, then the speculation about the clinical variations in phenylketonuria being due to differences in the pattern of isozymes (1) implies that these variants are not due to genetically-determined alterations in the structure of a component of the phenylalanine

[3] The ratios of activities with 6-methyltetrahydropterin to those with tetrahydrobiopterin, observed in these experiments, are somewhat greater than those seen with samples of phenylalanine hydroxylase purified by our standard procedure (6). This discrepancy may be due to modification of the enzyme that leads to an increase in the tetrahydrobiopterin-dependent activity during the course of the standard purification.

hydroxylase system, but rather to alterations in a component of a phosphorylation or dephosphorylation system.

Finally, it should be evident that the relationship between the present results and other observations (6,13) of multiple forms of phenylalanine hydroxylase remains to be established.

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