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LIGAND EFFECTS ON THE LIMITED PROTEOLYSIS OF PHENYLALANINE HYDROXYLASE: EVIDENCE FOR MULTIPLE CONFORMATIONAL STATES

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SUMMARY: The effects of phenylalanine and tetrahydrobiopterin on the limited proteolysis of rat liver phenylalanine hydroxylase by chymotrypsin have been examined. The presence of tetrahydrobiopterin inhibits the proteolytic activation of native phenylalanine hydroxylase. In contrast, phenylalanine causes a stimulation of proteolytic activation under these conditions. Neither phenylalanine nor tetrahydrobiopterin affect the rate of hydrolysis of a synthetic substrate by chymotrypsin. Both tetrahydrobiopterin and phenylalanine inhibit the release of soluble radioactivity from [^{32}P] phosphorylated phenylalanine hydroxylase. These results confirm the existence of multiple conformational states of phenylalanine hydroxylase.

INTRODUCTION: Phenylalanine hydroxylase (PAH), which catalyses the tetrahydropterin-dependent hydroxylation of phenylalanine to yield tyrosine, is the rate-limiting step in the hepatic catabolism of phenylalanine (1). As might be anticipated, therefore, PAH is subject to metabolic regulation. Indeed, a variety of activations have been described, which can normally be observed only when the enzyme activity is measured in the presence of the natural cofactor, L-erythro-tetrahydrobiopterin (BH_4). The activity of native PAH, when assayed with BH_4, is about 3-5% of that obtained in the presence of a synthetic analog, 6-methyltetrahydropterin ($6MPH_4$). Preincubation of PAH with 1mM L-phenylalanine results in a 20-30 fold increase in the rate of BH_4-dependent activity (2,3). PAH is also activated by phospholipids such as lysolecithin (4), by sulfhydryl modification with N-ethylmaleimide (5), by partial proteolysis with chymotrypsin

Abbreviations Used:

PAH: Phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating); E.C. 1.14.16.1). BH_4: L-erythro-5,6,7,8-tetrahydrobiopterin, $6MPH_4$: DL-6-methyl-5,6,7,8-tetrahydropterin, $NaDodSO_4$: Sodium dodecyl sulfate, BSA: Bovine Serum Albumin, TCA: Trichloroacetic acid, cAMP: cyclic 3',5'-adenosine monophosphate.

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(4), and by phosphorylation (6). Furthermore, PAH, when activated by phenylalanine or lysolecithin, will bind to a hydrophobic support, and can be specifically eluted by removal of the activator from the buffer. This behavior is the basis of a simple isolation procedure, first developed by Shiman et. al.(7). These results have led to several proposals (2-9) that PAH exists in an equilibrium between a low activity conformation, which can be stabilized by BH_4, and a high activity conformation, which is stabilized reversibly by phenylalanine or lysolecithin and irreversibly by proteolysis or sulfhydryl modification.

Since the susceptibility of proteins to proteolysis can often yield valuable information about conformational adaptability (10), we decided to examine the effects of phenylalanine and BH_4 on the proteolysis of PAH by chymotrypsin. The results of these experiments provide strong support for the existence of multiple conformational states of PAH.

MATERIALS AND METHODS: Phenylalanine hydroxylase was purified from the livers of male Sprague-Dawley rats by the method of Shiman et. al. (7). Phenylalanine, α-chymotrypsin, catalase, NADH, and N-succinyl-phenylalanine-p-nitroanilide were products of Sigma Chemical Company. $6MPH_4$ was prepared by hydrogenation of 6-methylpterin (11) with PtO_2 in 1N HCl. Tetrahydrobiopterin was obtained from Dr. Schircks, and was resolved as described by Bailey and Ayling (12). Activation experiments were carried out at 25°, in a medium which contained, per ml, 50 umoles Pipes (pH 6.8), 100 umoles KCl, 200 ug purified PAH, 20 ug catalase, 1 umole dithiothreitol, and, when present, 10 or 30 ug α-chymotrypsin, 8.6 nmoles BH_4, or 200 nmoles phenylalanine. Aliquots were removed from the reaction mixtures immediately after preparation, and after various times of incubation, and they were transfered to tubes chilled in an aluminum block on dry ice. At the end of the incubation, the tubes were thawed, and a mixture containing all of the components of the assay system was added. Assays were performed for 15 minutes at 25° as described previously (13), except that BH_4-dependent assays contained 0.2 mM phenylalanine. The assay system routinely contained 0.1 mM phenylmethanesulfonylfluoride, to prevent proteolysis from occurring during the assay. In the case of incubations containing phenylalanine, the assay tubes were preincubated for 5 minutes at 25° to allow time for reversal of phenylalanine-dependent activation, in a solution containing all components except reduced pterin and phenylalanine, and the reactions were started by simultaneous addition of phenylalanine and pterin. Reactions were quenched with 12.5% trichloroacetic acid, and tyrosine formation determined by the colorimetric reaction with nitrosonaphthol (14). Activation was determined by measuring the ratio of the BH_4 to $6MPH_4$-dependent activities (13), and dividing by the ratio at zero incubation time. $NaDodSO_4$ polyacrylamide gel electrophoresis was carried out in 10% gels according to the method of Weber and Osborn (15). [^{32}P] phosphorylated PAH was prepared with cAMP-dependent kinase from bovine heart (Sigma) and separated from unreacted [γ-^{32}P] ATP (ICN) on a 0.9 x 30 cm column of Sephadex-G-25 (Fine). Incubations with chymotrypsin were carried out exactly as described above for the activation experiments, except that 1 ml of 12.5% TCA was added to each 50 ul aliquot, and 300 ug of BSA added to ensure complete precipitation. After 15 minutes at 0°, the tubes were centrifuged, and 250 ul of the supernatant removed and counted in 10 ml Hydrofluor scintillation cocktail (NEN). Control

experiments showed that greater than 95% of the bound radioactivity was collected in the protein pellet under these conditions.

RESULTS AND DISCUSSION: Incubation of native PAH with chymotrypsin results in a time-dependent increase in BH_4-dependent activity, while the $6MPH_4$-dependent activity slowly decreases. Typical results are shown in Figure 1, curve C (see figure legends for curve designations). It should be noted that the maximum activation observed in these experiments is about seven-fold, due to the use of a fixed-time assay (15 minutes); when initial rates are measured, proteolysis results in a thirty-fold activation (4). Addition of 8.6 u$\underline{M}$ BH_4 (2.1 equivalents based on subunit M_r = 50,000) to these incubations completely abolishes this activation

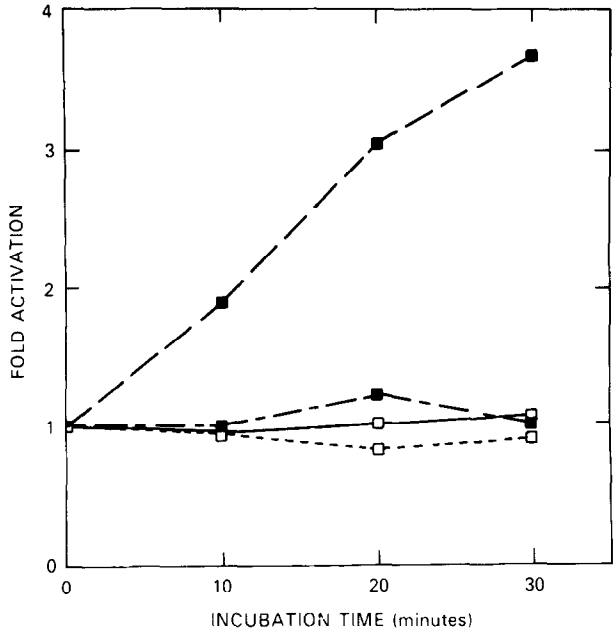

FIGURE 1

EFFECT OF BH_4 ON ACTIVATION OF PAH BY CHYMOTRYPSIN: Phenylalanine hydroxylase (200 ug/ml) was incubated as described in Methods, in the absence (open squares) or in the presence of chymotrypsin (filled squares). At the indicated times, aliquots were removed for activity measurements. The assay system contains, in a total volume of 0.50 ml, 50 umoles potassium phosphate, pH 6.8, 50 ug catalase, 250 nmoles NADH, sheep liver dihydropterin reductase in excess, 50 nmoles phenylmethanesulfonylfluoride, and either 2.5 umoles phenylalanine and 150 nmoles $6MPH_4$ or 100 nmoles phenylalanine and 15 nmoles BH_4. Incubations were carried out for 15 minutes at 25°. Reactions were quenched with 1 ml 12.5% trichloroacetic acid, and tyrosine measured by the colorimetric reaction with nitrosonaphthol (14). Curve A: (———), no additions; Curve B: (- - - -), 8.6 u$\underline{M}$ BH_4; Curve C: (— — —), 30 ug/ml chymotrypsin; Curve D: (— - — -), 8.6 u$\underline{M}$ BH_4 + 30 ug/ml chymotrypsin.

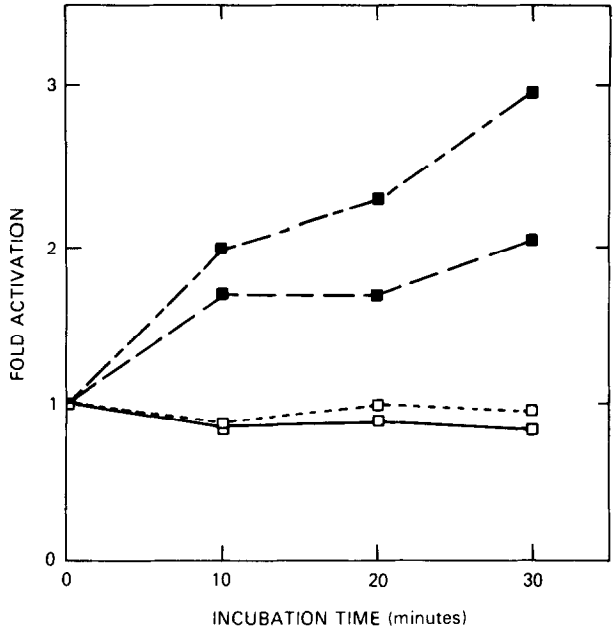

FIGURE 2

EFFECT OF L-PHENYLALANINE ON ACTIVATION OF PAH BY CHYMOTRYPSIN: Phenylalanine hydroxylase (200 ug/ml) was incubated as described in Methods, either in the absence (open squares) or in the presence of chymotrypsin (filled squares). At the indicated times, aliquots were removed for activity measurements. Activity was measured as described in the legend of Figure 1, except that the phenylalanine and pterin were left out of the assay mixture, and were added to start the assay after a 5 minute preincubation at 25°. Curve A: (——), no additions; Curve B: (- - - -), 200 uM L-Phe; Curve C: (— — —), 10 ug/ml chymotrypsin. Curve D: (— - — -), 10 ug/ml chymotrypsin + 200 uM L-phenylalanine.

(Figure 1, curve D). Under these conditions, we find that BH_4 has no effect on the chymotrypsin-catalyzed hydrolysis of N-succinyl-L-phenylalanine-p-nitroanilide. As further confirmation that BH_4 is altering the susceptibility of PAH to proteolysis, enzyme samples incubated under similar conditions were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis. After 30 minutes of incubation with chymotrypsin under these conditions, very little intact hydroxylase subunit (M_r = 50,000) remains. A number of fragments are observed, with M_r ranging from about 25,000 to 40,000. On the other hand, in the presence of BH_4, the intact subunit is still the major species present. In the presence of $6MPH_4$, however, the gel pattern is identical to that observed with chymotrypsin alone.

When chymotrypsin-dependent activation was carried out in the presence of 200 uM L-phenylalanine (an amount sufficient to obtain full activation), a small but

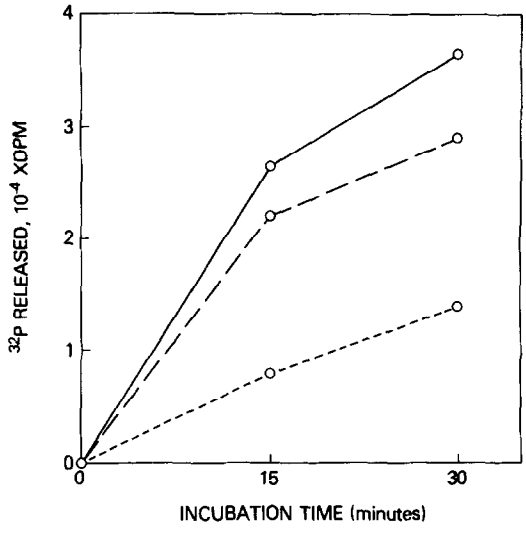

FIGURE 3

THE EFFECT OF BH_4 AND PHENYLALANINE ON THE RELEASE OF TRICHLOROACETIC ACID-SOLUBLE RADIOACTIVITY FROM [^{32}P] PHOSPHORYLATED PAH: [^{32}P] Phosphorylated PAH (200 ug/ml) was incubated as described in Methods. At the indicated times, 50 ul aliquots were removed for analysis. Curve A: (——), 30 ug/ml chymotrypsin; Curve B: (- - -), 30 ug/ml chymotrypsin + 8.6 uM BH_4; Curve C: (— — —), 30 ug/ml chymotrypsin + 200 uM L-phenylalanine. In the absence of chymotrypsin, the release of radioactivity under these conditions is negligible.

reproducible increase in the rate of irreversible activation is observed, as shown in Figure 2, curves C and D. It is important to note that while phenylalanine is a potent activator of PAH, that activation is not expressed in this experiment (Figure 2, Curve B) due to the dilution of the enzyme into the assay mixture, and the 5 minute preincubation prior to the assay.

Previous work in our laboratory has established that PAH can be activated by cAMP-dependent protein kinase-catalyzed phosphorylation, and that the phosphorylation site is released upon proteolytic activation (6). When [^{32}P] phosphorylated PAH is incubated with chymotrypsin in the presence of BH_4, there is a dramatic decrease in the rate of appearance of trichloroacetic acid-soluble radioactivity (Figure 3, curves A and B). However, when phenylalanine was included in these incubations, no stimulation of proteolysis is observed (Figure 3, curves A and C), in contrast to the results of Figure 2. Indeed, under these conditions, the presence of phenylalanine results in a slight inhibition of proteolysis. Since phenylalanine has no effect on the rate of hydrolysis of N-

succinyl-L-phenylalanine-p-nitroanilide under these conditions, this inhibition must be due to phenylalanine binding to PAH. These results suggest that native phosphorylated PAH is a better substrate for proteolysis than either the BH_4-complex or the phenylalanine-activated enzyme, and must, therefore, exhibit a conformation distinct from either of these. This conclusion is supported by our finding that both phenylalanine and BH_4 inhibit the dephosphorylation of PAH by phosphoprotein phosphatase (R. S. Phillips and S. Kaufman, manuscript in preparation). In a separate experiment, when both phenylalanine and BH_4 were incubated together with PAH and chymotrypsin under turnover conditions, the rate of proteolysis was identical to that observed with phenylalanine alone. Thus, the activating effect of phenylalanine is able to completely reverse the inhibitory effect of BH_4.

Taken together, these results are consistent with the conclusion that the activated forms of PAH are more susceptible to proteolysis. This suggests that the activated enzyme must exhibit an unfolded or "open" conformation, in which some portion of the peptide chain is sufficiently exposed to allow protease action, while the deactivated enzyme, with BH_4 bound, must adopt a more compact or globular conformation. These results are also consistent with our original proposal of an internal inhibitory polypeptide (1,4), which is displaced upon activation. An alternative explanation, that the bound tetrahydropterin is "shielding" a normally exposed peptide bond from the protease, can be ruled out by the lack of a protective effect of the synthetic cofactor, $6MPH_4$, even at 150 uM.

The physiological significance of these effects is unclear at present. However, it is interesting that the presence of BH_4 also strongly inhibits the phosphorylation of PAH by cAMP-dependent protein kinase (R. S. Phillips and S. Kaufman, manuscript in preparation). It is possible that the protective effect of BH_4 may have a role in the control of the turnover of PAH protein in vivo. This raises the intriguing possibility that disorders in which BH_4 levels are reduced, as in quinonoid dihydropterin reductase or biopterin biosynthesis-deficient patients (16,17), might result in increased rates of PAH degradation, leading to lower steady-state levels of hydroxylase activity. However, in the case of one biopterin-

deficient patient, in whom BH_4 levels were 10% of normal, levels of PAH activity in a liver biopsy sample were in the normal range (17).

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