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Effect of Alkaline pH on the Activity of Rat Liver Phenylalanine Hydroxylase*

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The pH optimum of rat liver phenylalanine hydroxylase is dependent on the structure of the cofactor employed and on the state of activation of the enzyme. The tetrahydrobiopterin-dependent activity of native phenylalanine hydroxylase has a pH optimum of about 8.5. In contrast, the 6,7-dimethyltetrahydropterin-dependent activity is highest at pH 7.0. Activation of phenylalanine hydroxylase either by preincubation with phenylalanine or by limited proteolysis results in a shift of the pH optimum of the tetrahydrobiopterin-dependent activity to pH 7.0. Activation of the enzyme has no effect on the optimal pH of the 6,7-dimethyltetrahydropterin-dependent activity. The different pH optimum of the tetrahydrobiopterin-dependent activity of native phenylalanine hydroxylase is due to a change in the properties of the enzyme when the pH is increased from pH 7 to 9.5.

Phenylalanine hydroxylase at alkaline pH appears to be in an altered conformation that is very similar to that of the enzyme which has been activated by preincubation with phenylalanine as determined by changes in the intrinsic protein fluorescence spectrum of the enzyme. Furthermore, phenylalanine hydroxylase which has been preincubated at an alkaline pH in the absence of phenylalanine and subsequently assayed at pH 7.0 in the presence of phenylalanine shows an increase in tetrahydrobiopterin-dependent activity similar to that exhibited by the enzyme which has been activated by preincubation with phenylalanine at neutral pH. Activation of the enzyme also occurs when *m*-tyrosine or tryptophan replace phenylalanine in the assay mixture. The predominant cause of the increase in activity of the enzyme immediately following preincubation at alkaline pH appears to be the increase in the rate of activation by the amino acid substrate. However, in the absence of substrate activation, phenylalanine hydroxylase preincubated at alkaline pH displays an approximately 2-fold greater intrinsic activity than the native enzyme.

Phenylalanine hydroxylase shows an absolute requirement for a tetrahydropterin (1). This requirement can be met by the naturally occurring compound tetrahydrobiopterin (BH_4)[1] (2) or by a number of synthetic tetrahydropterins such as 6-methyltetrahydropterin or 6,7-dimethyltetrahydropterin (3). The activity of the hydroxylase from rat liver is markedly influenced by the structure of the tetrahydropterin cofactor used in the assay of the enzyme. Native rat liver phenylalanine hydroxylase is in a low activity form when assayed with BH_4 (4). This low BH_4-dependent activity of the native hydroxylase can, however, be increased by a variety of treatments, including preincubation of the enzyme with its substrate, phenylalanine (5–14), and covalent modification of the enzyme by phosphorylation (15, 16). With both of these modes of activation, the enzyme retains its BH_4-dependent expression of positive cooperativity in response to increasing phenylalanine concentrations. Other modes of activation of the enzyme, such as the incubation with phospholipids (4), limited proteolysis (4, 17), and sulfhydryl modification (18), result in the abolition (desensitization) of the positive homotropic effect of phenylalanine. The activation phenomena described are fully observed only in the presence of BH_4 (4).

Evidence has been presented for the existence of an ionizable group on phenylalanine hydroxylase, with a pK_a of about 8, which is presumed to be involved in the substrate-level activation of the enzyme (9). However, in this earlier study, the hydroxylase was assayed with 6-MPH_4. Rat liver phenylalanine hydroxylase appears to be in both an activated and a desensitized form in the presence of synthetic cofactors such as 6-MPH_4 and $DMPH_4$ (4, 14, 18). In this report, therefore, we have compared the effect of changes in pH on the activity of both native and activated species of phenylalanine hydroxylase with the naturally occurring cofactor, BH_4, and with the synthetic cofactors.

MATERIALS AND METHODS

Rat liver phenylalanine hydroxylase was purified by the procedure of Shiman *et al.* (10). Dihydropteridine reductase from sheep liver was purified through the calcium phosphate gel step (19). Phenylalanine hydroxylase activated by limited proteolysis with chymotrypsin was prepared as previously described (4). L-Phenylalanine, L-tryptophan, glycine, potassium phosphate, and NADH were from Sigma. Catalase was from Boehringer-Mannheim. BisTris propane and 6-MPH_4 were obtained from Behring Diagnostics. (6*R*)-BH_4 was prepared from (6*RS*)-BH_4 (Dr. B. Schiricks, Jona, Switzerland) according to the procedure of Bailey and Ayling (20). Phenyl-Sepharose was purchased from Pharmacia LKB Biotechnology Inc. *m*-Tyrosine was a generous gift from Dr. Sheldon Milstien, Laboratory of Neurochemistry, National Institute of Mental Health.

Initial rates of the phenylalanine hydroxylase reaction were determined in a spectrophotometric assay (14, 21) in which the quinonoid dihydropterin formed during phenylalanine oxidation is reduced to the tetrahydro form with dihydropteridine reductase and NADH.

* A preliminary account of this work has been presented (Parniak, M. A., and Kaufman, S. (1986) in *Chemistry and Biology of Pteridines* (Cooper, B. A., and Whitehead, V. M., eds) pp. 359–362, Walter de Gruyter, Berlin). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BH_4 (tetrahydrobiopterin), 6-[dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropterin]; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; 6-MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; BisTris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane.

Unless otherwise indicated, the assays contained, in a total volume of 1.0 ml, 50 mM BisTris propane (at the appropriate pH), 150 mM potassium chloride, 0.2 mM NADH, catalase and dihydropteridine reductase in excess, and variable amounts of phenylalanine, tetrahydropterin, and phenylalanine hydroxylase. Assays were performed at 25 °C unless otherwise indicated, and the reactions were always initiated by the addition of phenylalanine hydroxylase to an otherwise complete assay mixture equilibrated at 25 °C. All velocities are corrected for the contribution of tetrahydropterin autoxidation in the absence of phenylalanine.

For those assays which involved preincubation of the hydroxylase, the enzyme was incubated in a separate tube at 25 °C for 10 min (unless otherwise specified), and then an aliquot of this sample was added to an otherwise complete assay mixture equilibrated at 25 °C. Phenylalanine hydroxylase stimulator protein (22, 23) had no effect on the observed velocities of the hydroxylase reaction under the conditions used in these studies.

Protein fluorescence emission spectra were obtained with the use of an SLM-Aminco SPF-500C spectrophotofluorometer or a Spex Fluorolog spectrometer. In the former case, the spectra were obtained at ambient temperatures, exciting at 295 nm with the use of 5-nm band passes for both excitation and emission monochrometers. The spectra were corrected for buffer contributions and instrumental factors with the use of the intrinsic correction software facility of the SPF-500C. In the latter case, a 1.25-nm band pass was used for the excitation monochrometer, whereas 2.5-nm band passes were used for the dual emission monochrometers. Spectra were obtained at 25 °C and were corrected for buffer and instrumental factors with the use of the Spex DMIB Spectroscopy Laboratory Coordinator. Emission spectra were obtained by exciting at 280 nm.

RESULTS

The variation of V_{max} as a function of pH was found to be different when native phenylalanine hydroxylase[2] was assayed with BH_4 or with $DMPH_4$. In the latter case, the pH optimum of the pterin-dependent activity was rather broad and was centered around pH 7.0 (Fig. 1*A*). Within the pH range that we have studied, the apparent K_m for phenylalanine in the presence of $DMPH_4$ was unaffected by the pH of the assay and remained about 0.8 mM. No cooperativity with respect to phenylalanine was noted ($n_H = 1$ at all pH values). In contrast, the pH optimum for the BH_4-dependent activity of the native enzyme was approximately 8.5 (Fig. 1*B*). There was a high degree of cooperativity of the BH_4-dependent activity of phenylalanine hydroxylase in response to variations in phenylalanine concentration over the range of pH studied, which remained constant ($n_H = 2.5 \pm 0.1$). However, the concentration of phenylalanine which gave half-maximal velocity ($S_{0.5}$) decreased with increasing pH, from a value of about 0.3 mM at pH 6.8 to approximately 0.08 mM at pH 9.0. A similar decrease in apparent $S_{0.5}$ for phenylalanine has been noted for the enzyme which has undergone substrate-level activation upon preincubation with phenylalanine at pH 6.8, followed by assay of the BH_4-dependent activity at pH 6.8.[3] It is also of interest that the half-maximal concentration of phenylalanine required for the substrate-level activation of phenylalanine hydroxylase at pH 6.8 is about 0.06 mM (9, 12, 14).

It has been reported that there is a greater than 10-fold increase in BH_4-dependent activity when the enzyme is assayed at pH 6.8, following a 10-min preincubation with 1 mM phenylalanine prior to the addition of the pterin cofactor (9, 12). An identical preincubation had no effect on the $DMPH_4$-dependent activity of the hydroxylase (data not shown). However, as the pH values of the preincubation and assay mixtures are raised, the phenylalanine stimulation of the BH_4-dependent rate diminishes (Fig. 2*A*).[4] The relationship between pH and the BH_4-dependent hydroxylase activity now more closely resembles that of the $DMPH_4$-dependent reaction, with the observed maximum velocity decreasing as the pH increases from 7.0 to 8.6 (see Fig. 1*A*). A similar correlation between pH and velocity of the BH_4-dependent activity of phenylalanine hydroxylase has been found with the chymotrypsin-activated enzyme (4), which has a pH optimum at neutrality (data not shown). The proteolyzed hydroxylase is not further activated by preincubation with phenylalanine. From these results, it is apparent that the pH optimum of activated phenylalanine hydroxylase, whether activated by preincubation with phenylalanine, by limited proteolysis, or by exposure to pH 8.5–9.5, is approximately pH 7.0.

The rate of the phenylalanine hydroxylase-catalyzed hydroxylation reaction at pH 6.8 with its natural cofactor (Fig. 3, *top curve*) is small when compared with the rate of the hydroxylase that has been activated by preincubation with phenylalanine (Fig. 3, *bottom curve*). When assayed at pH 6.8, the activity of phenylalanine hydroxylase that had been incubated at pH 9.3 (Fig. 3, *middle curve*) is between that of the control enzyme and the phenylalanine-activated enzyme. This activation due to exposure to pH 9.3 is transient as seen by the upward curvature of the reaction trace at relatively early times after mixing. Nonetheless, this result demonstrates that the enzyme has been changed on being incubated in alkaline solution and that this new species of enzyme retains its activated state during catalysis at pH 6.8 for at least 30 s. The activation due to exposure to alkaline pH increases as the pH of the preincubation mixture increases (Fig. 2*B*). The pH-rate profile of the BH_4-dependent activity of phenylalanine hydroxylase which had been preincubated at pH 9.3 (in the absence of phenylalanine) and then assayed at various pH values was identical to that of the enzyme which had been activated by preincubation with phenylalanine at either pH 6.8 or 9.3 and is thus similar to that of the $DMPH_4$-dependent activity of both the native and the activated enzymes (data not shown).

These results indicate that phenylalanine hydroxylase at alkaline pH is in a form similar to the enzyme which has been activated at neutral pH by preincubation with phenylalanine. The latter species of phenylalanine hydroxylase is in an altered conformation compared to the native enzyme (5, 7, 10). Evidence for a pH-induced conformational change of phenylalanine hydroxylase comes from a study of the fluorescence properties of the enzyme. We have reported that upon incubation of the hydroxylase with phenylalanine, the fluorescence emission spectrum undergoes a red shift with an increase in overall fluorescence intensity (13). This spectral change is consistent with the exposure of the partially buried protein-bound tryptophan residues to the aqueous solvent. Raising the pH of the enzyme from 6.8 to 9.3 yields a similar spectral change (Fig. 4*A*). Furthermore, a comparison of the two difference spectra calculated by substrating the initial fluorescence of the resting enzyme from either phenylalanine-activated species or phenylalanine hydroxylase at pH 9.5 shows that the resulting two difference spectra are similar, although at alkaline pH, a larger change in intensity is observed (Fig. 5). Again, the changes due to the phenylalanine and those due to the alkaline pH do not appear to be additive. As can be seen, the emission spectrum of phenylalanine hydroxylase at pH 9.5 changes only very slightly upon the addition of 1.2 mM phenylalanine (Fig. 4*B*).

These results indicate that preincubation of phenylalanine hydroxylase at alkaline pH activates the enzyme in an analogous manner to that seen during activation by phenylalanine. Either or both of the following mechanisms could account for activation by alkaline pH. Preincubation at alkaline pH could

[2] "Native" phenylalanine hydroxylase refers to the purified rat liver enzyme, not otherwise pretreated.

[3] M. A. Parniack, unpublished results.

[4] Preincubation of the enzyme with 1 mM phenylalanine at pH 8.6 for up to 1 h at 25 °C prior to assaying at the same pH failed to show an increase in the BH_4-dependent activity.

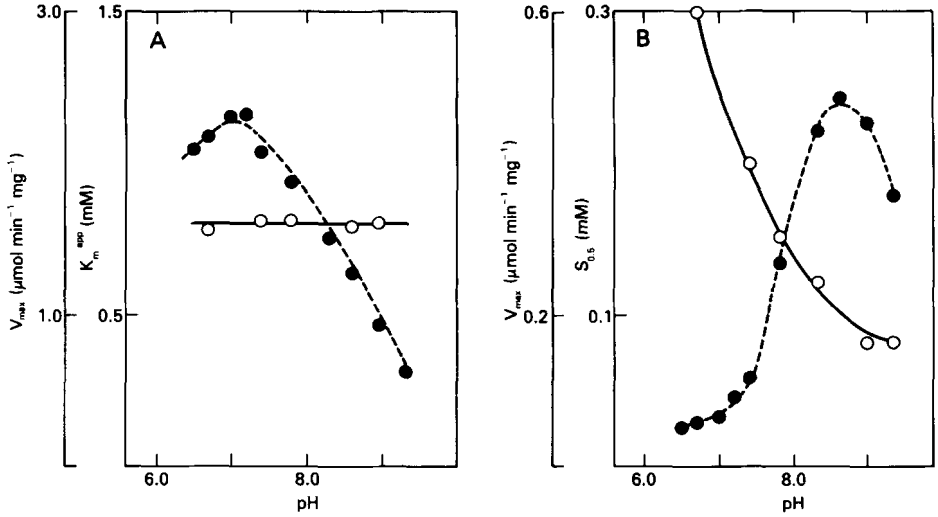

FIG. 1. **Effect of pH on kinetic parameters of phenylalanine hydroxylase with phenylalanine as a variable substrate.** *A*, variation of $DMPH_4$-dependent phenylalanine hydroxylase activity with pH. ●, V_{max}; ○, apparent K_m for phenylalanine. The concentration of $DMPH_4$ in all assays was 180 μM. *B*, variation of BH_4-dependent phenylalanine hydroxylase activity with pH. ●, V_{max}; ○, apparent $S_{0.5}$ for phenylalanine. The concentration of (6*R*)-BH_4 in all assays was 60 μM. The actual pH of the complete reaction mixtures was determined by direct measurement for each pH value studied.

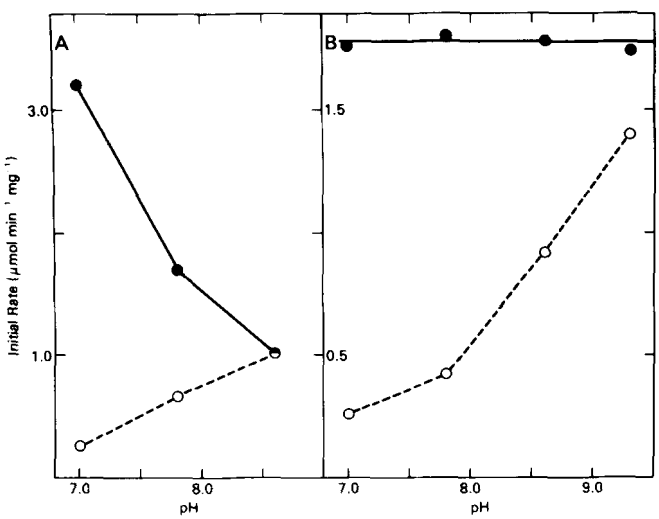

FIG. 2. **Effect of pH on the activation and activity of phenylalanine hydroxylase.** *A*, effect of pH on the BH_4-dependent activity of phenylalanine hydroxylase. Samples were preincubated for 10 min at 25 °C at the specified pH in the absence (○) or presence (●) of 1 mM phenylalanine and then assayed at the same pH in reaction mixtures that contained 1 mM phenylalanine, 41 μM (6*R*)-BH_4, and other components as described under "Materials and Methods." *B*, effect of the pH of the preincubation mixture for phenylalanine hydroxylase at various pH values in the presence (●) or absence (○) of 1 mM phenylalanine. The hydroxylase was preincubated for 10 min at 25 °C at the pH values indicated and then subsequently assayed at pH 7.0 in reaction mixtures containing 1 mM phenylalanine, 30 μM (6*R*)-BH_4, and other components as described under "Materials and Methods." The actual pH of the complete reaction mixture was determined by direct measurement for each pH value studied.

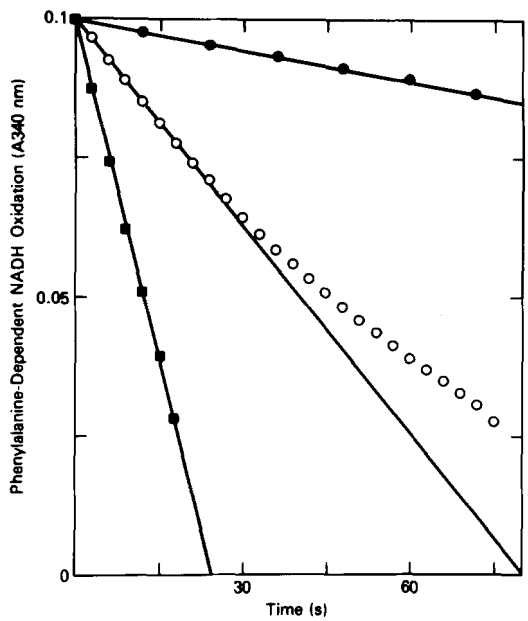

FIG. 3. **Phenylalanine-dependent NADH oxidation by preactivated and native phenylalanine hydroxylase.** The enzyme was preincubated at 25 °C for 3 min in 50 mM potassium phosphate (pH 6.8) *plus* (■) or *minus* (●) 1.2 mM phenylalanine or in 50 mM glycine/KOH (pH 9.3) (○) in the absence of the phenylalanine. Assays were performed at 25 °C in 50 mM potassium phosphate (pH 6.8) in the presence of 40 μM (6*R*)-BH_4 and 1.2 mM phenylalanine.

alter the conformation of the enzyme in such a way that it now has (*a*) enhanced intrinsic hydroxylase activity or (*b*) an enhanced ability to be activated by its substrate. In an attempt to discriminate between these two possibilities, experiments were carried out with the alternative substrates tryptophan and *m*-tyrosine, both substantially poorer activators of phenylalanine hydroxylase than phenylalanine (12, 14). As can be seen in Table I (Experiment 1), at pH 7.0 in the presence of BH_4, *m*-tyrosine appears to be a poor substrate. However, the rate of hydroxylation of *m*-tyrosine is significantly increased in the presence of a synthetic cofactor (6-MPH_4) (Experiment 2) or by preactivating the enzyme with phenylalanine prior to the assay (Experiment 3). (It should be noted that a small part of the measured NADH oxidation observed in Experiment 3 is probably due to the hydroxylation of phenylalanine (24 μM) which was carried over into the final assay mixture with the enzyme following the preincubation. A control for this carry-over is listed (Experiment 4).) *m*-Tyrosine is also a much better substrate after preincubation of the enzyme at pH 9.3 prior to assaying at pH 7.0 with BH_4 (Experiment 5). As anticipated, preincubation of phenylalanine hydroxylase at pH 9.3 had little effect on the 6-MPH_4-dependent rate (Experiment 6). In a parallel experiment with tryptophan used as the substrate, qualitatively similar results were obtained. In confirmation of our previous results (12, 14), at pH 7.0 tryptophan is a much poorer substrate for phenylalanine hydroxylase in the presence of BH_4 relative to 6-MPH_4; and, as previously reported (9), phenylalanine greatly increases the

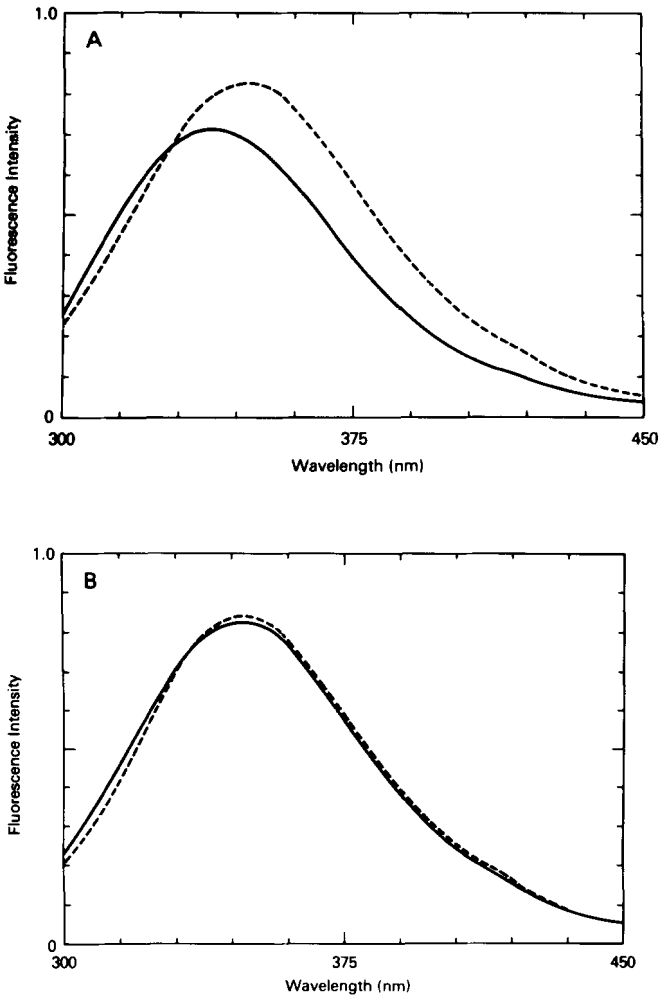

FIG. 4. **Effect of pH on the fluorescence spectrum of phenylalanine hydroxylase in 50 mM BisTris propane and 150 mM potassium chloride.** *A*, comparison of the fluorescence of phenylalanine hydroxylase at pH 6.8 (——) and pH 9.5 (– – –). *B*, phenylalanine hydroxylase was diluted into 50 mM BisTris propane, 150 mM potassium chloride (pH 9.5) (——). Phenylalanine was then added to a final concentration of 1 mM (– – –). These spectra were obtained on the Spex Fluorolog spectrometer as described under "Materials and Methods." The fluorescence intensity is in arbitrary units.

BH_4-dependent reaction. Whereas preincubation of the enzyme at an alkaline pH does not activate the enzyme with tryptophan as the substrate to the same extent as it does with *m*-tyrosine as the substrate, a doubling of the rate of tryptophan hydroxylation is observed (Experiment 11). Again, preincubation of phenylalanine hydroxylase at pH 9.3 has little effect on the 6-MPH_4-dependent rate.

These results demonstrate that preincubation of the hydroxylase at alkaline pH increases its ability to catalyze the hydroxylation of substrates that are relatively poor activators of the enzyme. These results, therefore, are consistent with

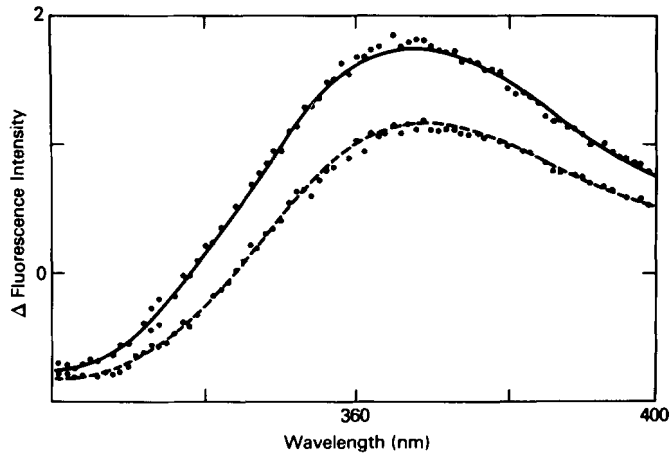

FIG. 5. **Fluorescence difference spectra of alkaline pH-activated phenylalanine hydroxylase and phenylalanine-activated enzyme with the resting enzyme.** Phenylalanine hydroxylase was preincubated for 10 min at ambient temperature (22 °C) in 50 mM BisTris propane and 150 mM potassium chloride at the appropriate pH in the presence or absence of 0.5 mM phenylalanine. The fluorescence emission spectrum was then obtained using an excitation wavelength of 295 nm. Corrections were made for the contributions of buffer and phenylalanine to the observed fluorescence. The difference fluorescence spectra illustrated were generated by subtraction of the appropriate primary spectra as indicated. The *dashed line* is the difference between the spectrum of phenylalanine hydroxylase at pH 7.0 in the presence or absence of phenylalanine. The *solid line* is the difference between the spectrum of phenylalanine hydroxylase at pH 9.5 in the absence of phenylalanine and that of the enzyme at pH 7.0 in the absence of phenylalanine. The fluorescence intensity is in arbitrary units.

TABLE I

Activation of phenylalanine hydroxylase toward alternative amino acids by preincubation at alkaline pH

Phenylalanine hydroxylase was preincubated at the pH indicated for 2 min at 25 °C and assayed at pH 7.0. The final concentrations of the substrates and cofactors were 200 μM *m*-tyrosine, 9.8 mM tryptophan, 19.5 μM (6*R*)-BH_4, and 90 μM 6-MPH_4. Preincubation of the enzyme with phenylalanine was carried out at a concentration of 1.2 mM phenylalanine. Since 20 μl of the preincubation solution was added to the assay mixture, 24 μM phenylalanine was present in the assay. Control samples were therefore run with 24 μM phenylalanine in the assay solution, but without phenylalanine in the preincubation mixture, in order to assess the effect of this concentration of phenylalanine during catalysis.

Exp.	Substrate	Preincubation pH	Preincubation with			Assay with phenylalanine	nmol/min/mg	Activation
			6-MPH_4	BH_4	Phenylalanine			
								-fold
1	*m*-Tyrosine	7.0	–	+	–	–	11.0	1
2	*m*-Tyrosine	7.0	+	–	–	–	140	12.6
3	*m*-Tyrosine	7.0	–	+	+	–	414	37.4
4	*m*-Tyrosine	7.0	–	+	–	+	58	5.7
5	*m*-Tyrosine	9.3	–	+	–	–	146	13.2
6	*m*-Tyrosine	9.3	+	–	–	–	162	14.7
7	Tryptophan	7.0	–	+	–	–	34	1
8	Tryptophan	7.0	+	–	–	–	474	14.1
9	Tryptophan	7.0	–	+	+	–	293	11.6
10	Tryptophan	7.0	–	+	–	+	65	1.9
11	Tryptophan	9.3	–	+	–	–	70	2.1
12	Tryptophan	9.3	+	–	–	–	520	15.4

the conclusion that phenylalanine hydroxylase that has been exposed to alkaline pH has higher intrinsic hydroxylase activity. Nonetheless, the finding that the rate enhancement factor is greater for *m*-tyrosine than for tryptophan leaves open the possibility that a significant fraction of the enhanced activity seen with *m*-tyrosine may also be due to the greater ability of *m*-tyrosine to activate the enzyme at alkaline pH. With respect to this last possibility, it should be noted that the ability of *m*-tyrosine to activate the enzyme would have to be enormously greater at pH 9.3 than at pH 6.8 since it has been reported that at the latter pH, 2.0 mM *m*-tyrosine only activates 3.5-fold (12) compared to the 13.2-fold activation seen with 0.2 mM *m*-tyrosine at pH 9.3 (Table I).

Shiman and Gray (9) have shown that the rate of activation of phenylalanine hydroxylase by phenylalanine is dependent on the pH of the solution, with the unprotonated species undergoing activation at a rate of 5.9 times that of the protonated enzyme (a pK_a of 8.1 for the ionizable group on the enzyme was determined). Despite the fact that these workers also reported that the adjustment of the enzyme to the pH of the assay solution appeared to be instantaneous compared to the rate of activation when the artificial cofactor 6-MPH$_4$ was employed, it is still conceivable that exposure to alkaline pH does not activate the enzyme *per se*, but rather that at alkaline pH, the enzyme is in a conformation that is more rapidly activated by its substrate and that this phenomenon is more noticeable in the presence of BH$_4$. Another characteristic of substrate activation that might be useful in discriminating between substrate-dependent or -independent activations is the large temperature dependence for the activation of phenylalanine hydroxylase by phenylalanine (9). Thus, phenylalanine hydroxylase was assayed with phenylalanine at 5 °C, following a 15-min preincubation at 25 °C at pH 9.3 or at pH 6.8 *plus* or *minus* lysolecithin (an activator of phenylalanine hydroxylase (4)). In order to observe the initial turnover of the enzyme, phenylalanine hydroxylase was maintained in excess of three different substoichiometric concentrations of phenylalanine. Thirty seconds after the reaction was started, aliquots were removed from the samples. As can be seen in Table II, at pH 6.8, the lysolecithin-activated enzyme converted 20% of the phenylalanine to tyrosine. By contrast, at this pH in the absence of lysolecithin, very small amounts of tyrosine were formed. The enzyme preincubated at alkaline pH, however, converted approximately twice the amount of substrate to product as the control enzyme. Analysis of the aliquots removed from these same reaction mixtures 5 min later again showed that lysolecithin-activated enzyme catalyzes the conversion of phenylalanine to tyrosine at a much faster rate than the control enzyme. Although the pH-activated enzyme proceeds at a rate considerably slower than phenylalanine hydroxylase activated by lysolecithin, it does perform the catalysis at a rate approximately twice that of phenylalanine hydroxylase preincubated at pH 6.8. When a similar study was performed at 25 °C, in order to compare more carefully the rate of catalysis of the alkaline pH-activated and control enzymes, the 2-fold difference was again observed (Fig. 6). The plot of the decrease in the percent of radioactivity in phenylalanine with time under both conditions is linear, and the rate of reaction is independent of the concentration of substrate over the 6-fold range examined. Furthermore, the plots are linear until 95% of the initial phenylalanine is depleted.

The above studies were performed with 6-MPH$_4$ as the cofactor because earlier reports demonstrating the large effect of temperature on substrate activation were performed with this synthetic cofactor (9). In view of these earlier studies, it is unlikely that phenylalanine can activate phenylalanine

TABLE II

Reaction of phenylalanine hydroxylase with substoichiometric concentrations of phenylalanine at 5 °C

Phenylalanine hydroxylase was preincubated for 15 min at 25 °C under the conditions specified. The enzyme was then added to the reaction mixtures incubated at 5 °C, and aliquots were removed at the specified times. These aliquots were placed into perchloric acid to stop the reaction immediately. The samples were then centrifuged in a Microfuge, and the clear supernatant was placed onto a thin-layer chromatographic plate as described by Milstien and Kaufman (25). The two spots observed after spraying the plate with ninhydrin (0.25% in ethanol) were then scraped and transferred to scintillation vials; the amount of radioactivity of each vial was then determined. The incubation buffers were 5 mM glycine/KOH, 150 mM potassium chloride (pH 9.5) or 5 mM potassium phosphate, 150 M potassium chloride (pH 6.8). The assay was run at 5 °C and contained 31 μg of catalase, the specified concentrations of the phenylalanine (494.5 mCi/mmol), 300 μM 6-MPH$_4$, and 70 μg of phenylalanine hydroxylase (~13 μM) in 50 mM potassium phosphate (pH 6.8).

Preincubation conditions	Phenylalanine	% Tyrosine	
		30-s aliquot	360-s aliquot
	μM		
pH 6.8	0.21	1.7	6.4
	0.42	1.9	5.1
	1.26	1.5	6.9
pH 9.3	0.21	3.0	13.2
	0.42	3.3	17.6
	1.26	2.8	13.6
pH 6.8 + 2 mM lysolecithin	0.21	22.0	79.0
	0.42	23.3	67.0
	1.26	24.5	79.0

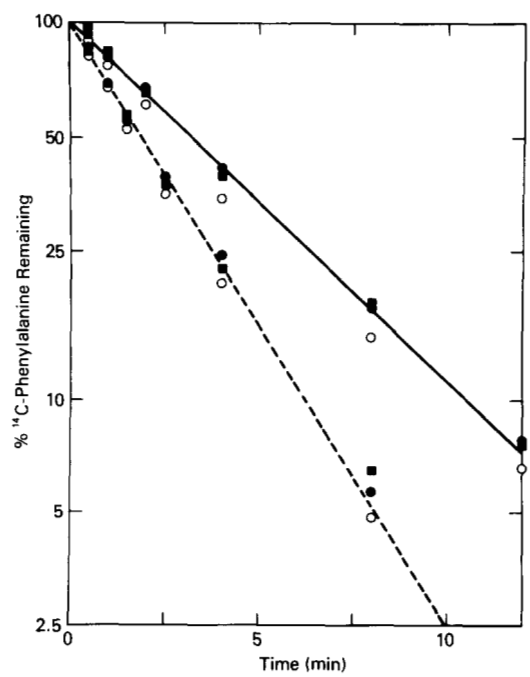

FIG. 6. **Reaction of phenylalanine hydroxylase with substoichiometric concentrations of [^{14}C]phenylalanine.** Phenylalanine hydroxylase was incubated at 25 °C for 15 min in 5 mM glycine/KOH, 150 mM potassium chloride (pH 9.5) (---) or 5 mM potassium phosphate, 150 mM potassium chloride (pH 6.8) (——). Assays were performed at 25 °C in 50 mM potassium phosphate (pH 6.8) using 0.3 mM 6-MPH$_4$, 6.4 μM phenylalanine hydroxylase, and 1.25 (○), 0.42 (■), and 0.21 (●) μM phenylalanine. Aliquots from the reaction vessel were removed at the times shown, and the protein was precipitated with perchloric acid. These samples were then centrifuged, and the resulting supernatant was treated as described for Table II.

hydroxylase at 5 °C within 30 s. Furthermore, the concentration of phenylalanine is very low in our experiments (in some cases, below micromolar); and although no value for the dissociation constant of phenylalanine has been reported at pH 9.3, Shiman and Gray (9) state that 50% of the phenylalanine hydroxylase present is activated at pH 8.0 by 38 μM phenylalanine. Finally, both at 5 and 25 °C, the reaction rate is the same for all three substrate concentrations, a result indicating that the activation is independent of the substrate concentration under the conditions of this experiment. The observed rate of the reaction is dictated by the reactant in excess, which, in this case, is the enzyme. The fact that the reaction appears to describe a first-order equation (Fig. 6) provides evidence that the enzyme is already activated prior to the start of the reaction, which is the first time the enzyme is in contact with phenylalanine. Furthermore, since essentially all of the phenylalanine is converted to tyrosine, there would not appear to be any substrate available to occupy the activation site. In summary, it appears that lysolecithin does indeed activate the enzyme independently of the substrate, a result consistent with those of previous workers (4, 17). Similarly, incubation of the enzyme at alkaline pH also converts the enzyme into an activated species. However, the extent of this activation is far more limited than activation by lysolecithin or by phenylalanine. Rather, it is similar in magnitude to the activation by phosphorylation (15). The remainder of the activation observed when the enzyme is preincubated at alkaline pH (Fig. 3 and Table I) must be due to substrate activation. We conclude, therefore, that the major effect of exposure of phenylalanine hydroxylase to an alkaline buffer is to induce a conformational change in the enzyme which facilitates activation by the substrate. In addition, this altered conformation appears to be intrinsically more active than the resting enzyme.

Since the intrinsic activation of phenylalanine hydroxylase due to preincubation at alkaline pH is small, it can easily be masked by the larger effects caused by substrate activation. To obtain further evidence for intrinsic activation, a poor enzyme activator, tryptophan, and the noninhibitory cofactor, 6-MPH_4, were selected for further study. In the presence of the natural cofactor, phenylalanine hydroxylase is maximally activated by tryptophan at a substrate concentration of 25 mM; concentrations of tryptophan less than 5 mM do not activate (9, 12). With 6-MPH_4 as the cofactor, preincubation of the enzyme at pH 9.3 has little effect on the phenylalanine hydroxylase-dependent rate of oxidation of NADH when high concentrations of tryptophan are included in the assay mixture (Table III). This is consistent with all of our previous findings which indicate that at a sufficiently high concentration of the substrate, activation of the enzyme at either pH 6.8 or 9.3 is due exclusively to substrate activation. However, at lower concentrations of substrate, the ratio of the rate of alkaline-treated to control enzyme increases, finally approximating a 2-fold increase between 1 and 2 mM tryptophan. We suggest that at these low concentrations, tryptophan can no longer activate the enzyme, even in the absence of BH_4, and that the rates observed are the intrinsic rates of the enzyme, with the alkaline-treated enzyme having twice the activity of the control sample.

The final question to answer is whether the extrinsic activation (*i.e.* the enhancement of the activation due to the substrate) of the enzyme caused by the preincubation at alkaline pH is a thermodynamic or a kinetic phenomenon, that is, does the concentration of substrate necessary to activate the enzyme decrease or does the rate of activation of the enzyme by the substrate increase? Preliminary experiments show that the $S_{0.5}$ only decreased by a factor of 2 when the enzyme was preincubated at pH 9.3 and assayed at pH 6.8 (data not shown), which suggests that the effect of preincubation at high pH is predominantly a kinetic one. This result is consistent with the data in Fig. 7, where the kinetic traces of two enzyme assays are shown. These experiments were performed at 12 °C to decrease activation by phenylalanine (which was also kept at a low concentration (100 μM) for the same reason), but were run in the absence of BH_4 so as not to prevent the ultimate activation. As can be seen, the rate in the presence of the control enzyme is characterized by a lag prior to reaching a steady state. In contrast, there is essentially no lag with enzyme that had been preincubated at pH 9.3. The slightly greater steady-state rate for the enzyme exposed to alkaline pH may be indicative of the slightly lower $S_{0.5}$ mentioned above. However, the substantial lag observed

TABLE III

Dependence on tryptophan concentration of the ratio of alkaline pH-activated and unactivated phenylalanine hydroxylase activity

Phenylalanine hydroxylase was preincubated at 25 °C at the pH indicated in the absence of substrate with either 91 μM potassium phosphate (pH 6.8) or 91 μM glycine/NaOH (pH 9.3). All assays contained 50 μg of catalase, 34 μM NADH, 38.2 μg of phenylalanine hydroxylase, an excess of dihydropteridine reductase, and the amount of tryptophan indicated. The assays were performed at 25 °C in the presence of 50 μM 6-MPH_4 and were begun by the addition of preincubated enzyme. A blank rate was substracted from all values listed and was obtained by running the reaction in the absence of tryptophan. Hydroxylase activity was determined by measuring substrate-dependent oxidation of NADH monitored at 340 nm.

Tryptophan	pH 6.8	pH 9.3	Ratio
mM	*μmol/min/mg*[a]	*μmol/min/mg*	
1.0	0.013	0.025	1.9
2.0	0.030	0.060	2.0
4.0	0.14	0.16	1.2
6.0	0.28	0.32	1.1
10.0	0.44	0.45	1.0
17.5	0.50	0.51	1.0

[a] μmol/min/mg, micromoles of NADH oxidized per minute per milligram of phenylalanine hydroxylase.

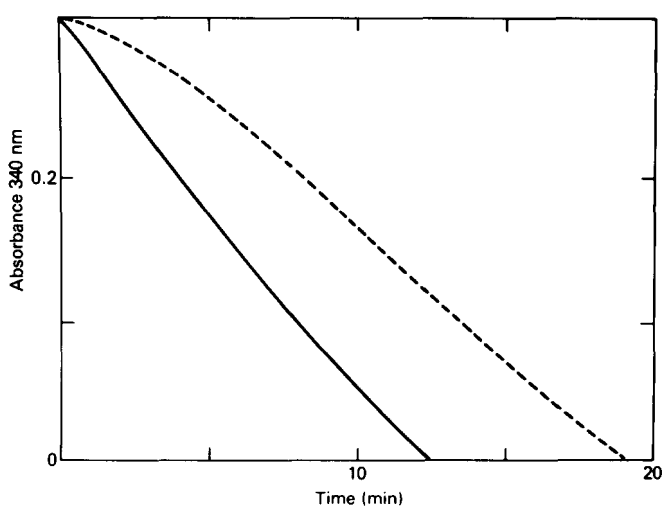

FIG. 7. **Comparison of the approach to steady state of the reaction of phenylalanine and 6-MPH_4 with phenylalanine hydroxylase preincubated at alkaline or neutral pH.** Phenylalanine hydroxylase was preincubated at 25 °C for 2 min in 91 mM potassium phosphate (pH 6.8) (– – –) or 91 mM glycine/KOH (pH 9.3) (——). Assays were performed in 0.1 M potassium phosphate (pH 6.8) at 12°C in the presence of 0.1 mM phenylalanine, 0.05 mM 6-MPH_4, 50 μg of catalase, 128 μM NADH, and an excess of dihydropteridine reductase. The reaction was monitored at 340 nm.

for the reaction with enzyme not preincubated at alkaline pH suggests that most of the substrate-dependent portion of the alkaline pH activation is due to an increase in rate of the activation of the enzyme. This result is consistent with that of Shiman and Gray (9) who found, in a complementary experiment, that phenylalanine hydroxylase incubated and assayed at the same pH was also activated more rapidly by phenylalanine as the pH increased.

DISCUSSION

Native phenylalanine hydroxylase shows low activity at neutral pH when assayed with BH_4. It has been proposed that the enzyme exists as an equilibrium mixture of an inactive or low activity form and a high activity form (9). Preincubation with phenylalanine is believed to shift the equilibrium between these two species in favor of the high activity species, resulting in an apparent activation of the BH_4-dependent activity of the enzyme at neutral pH (9). As discussed under "Results," the pH optimum for the BH_4-dependent activity of the high activity form of phenylalanine hydroxylase is about pH 7.0. We have also observed that the apparent pH optimum for the BH_4-dependent activity of the native (low activity) form of phenylalanine hydroxylase is pH 8.5. However, five pieces of evidence suggest that this apparent increase in pH optimum with the native enzyme may be due to a shift in the equilibrium toward the high activity form upon increasing the pH. First, at pH 8.6, the BH_4-dependent activity of phenylalanine hydroxylase cannot be further increased by preincubation of the enzyme with phenylalanine. Second, when the hydroxylase is preincubated at pH 8.6 in the *absence* of phenylalanine and then assayed at pH 7.0, the enzyme shows an activation in BH_4-dependent activity analogous to that attained by preincubation of the enzyme with phenylalanine. Third, phenylalanine hydroxylase shows greater activity toward substrate analogs following preincubation at pH 9.3. Fourth, phenylalanine hydroxylase that has been preincubated at neutral pH with phenylalanine has the same pH optimum as that of the enzyme that has been preincubated at alkaline pH in the absence of phenylalanine. Finally, the conformation of the enzyme at high pH appears to be similar to that of the high activity form which results from preincubation with phenylalanine at neutral pH. The conclusion that the enzyme is in an altered conformation at high pH comes from our studies of the intrinsic protein fluorescence of phenylalanine hydroxylase. We have reported that the fluorescence emission spectrum of the enzyme at pH 7.0 is altered by the presence of phenylalanine in a concentration-dependent manner that exactly parallels the state of activation of the hydroxylase (13). In this study, we have found that the fluorescence emission spectrum of phenylalanine hydroxylase at pH 9.5 is essentially identical with that of the enzyme at pH 6.8 in the presence of phenylalanine. Furthermore, phenylalanine has only a minor effect on the fluorescence emission spectrum of phenylalanine hydroxylase at pH 9.5. This finding is consistent with our kinetic data (Fig. 2).

It has been postulated that phenylalanine hydroxylase, on binding phenylalanine, undergoes a conformational change that is associated with activation and that the rate of this phenylalanine-induced activation increases with increasing pH (9). Our fluorescence data indicate that phenylalanine hydroxylase at high pH, even in the absence of phenylalanine, may already exist in a conformation similar to that of the phenylalanine-activated enzyme at neutral pH and that perhaps the increased rate of activation of the enzyme by its substrate with increasing pH (9) could be due to the presence of greater concentrations of this conformational state at higher pH values, which in turn could more readily bind phenylalanine at the activator site. In any case, the change in fluorescence of the enzyme upon incubation of phenylalanine hydroxylase at high pH suggests that the conformational change previously detected only during the activation of the enzyme by its substrate or lysolecithin (9, 10, 13) is not sufficient for full activation of the enzyme. Furthermore, this rearrangement of the structure of the enzyme is probably reflected in the high temperature dependence and overall slow rate of activation of the enzyme by phenylalanine. Similarly, we find that the reversibility of the activation following preincubation of the enzyme at high pH values is slow as evidenced by the transient nature of the subsequent increase in activity of phenylalanine hydroxylase at neutral pH (see Fig. 3).

Enzyme preincubated at alkaline pH shows an increase in activity under conditions which would make substrate activation unlikely (*i.e.* with low concentrations of phenylalanine relative to the amount of enzyme and at a low temperature (Table II)). In fact, the rate of the reaction remained the same over a 6-fold range in substrate concentration, consistent with the lack of substrate activation. Since the 2-fold increase in intrinsic activity found under these conditions is less than the 6–10-fold increase we observed when much higher concentrations of substrate were used, it seems likely that the conformational change brought on by preincubation of phenylalanine hydroxylase under alkaline conditions not only allows phenylalanine to activate more readily phenylalanine hydroxylase, but also results in a 2-fold activation of the enzyme. The simplest explanation for these phenomena is that a conformational change due to the increase in pH precedes the substrate-dependent activation step, *i.e.* activation by substrate at alkaline pH is a two-step process.[5]

$$E_u \underset{+H}{\overset{-H}{\rightleftarrows}} E_p \xrightarrow{\text{activator}} E_a$$

In this model, deprotonation of the unactivated enzyme (E_u) results in a primed species (E_p) which has the following properties: 1) a fluorescence spectrum similar to the activated enzyme, E_a; 2) a 2-fold greater intrinsic reaction rate relative to that of the unactivated enzyme, E_u; and 3) an enhanced rate of substrate (*e.g.* phenylalanine, *m*-tyrosine) activation as compared to the protonated form.

Finally, as discussed above, phenylalanine (and to a lesser extent, other substrates) can shift the equilibrium toward the right even at pH values below neutrality.

The variation with pH of the V_{max} of the BH_4-dependent activity of native phenylalanine hydroxylase implicates an ionizable group, with a pK_a of about 8, in the transition between low activity and high activity conformations. This is in agreement with the pK_a estimated for the effect of hydrogen ion concentration on the rate of activation of phenylalanine hydroxylase by preincubation with phenylalanine (9). Preincubation of phenylalanine hydroxylase with phenylalanine at pH 6.8 results in an ultraviolet absorbance difference spectrum characterized by a positive absorbance change at 238 nm (13). The change in the magnitude of the absorbance difference maximum at 238 nm correlates exactly with the extent of activation of the hydroxylase due to preincubation with phenylalanine. The maximum at 238 nm is typical of an ionized sulfhydryl residue (24). We have also previously found (18) that the modification of a single sulfhydryl residue per subunit of phenylalanine hydroxylase results in large increases in BH_4-dependent activity of the enzyme at neutral

[5] The possibility that substrate activation of the hydroxylase, in general, proceeds by a two-step process was considered previously (26), but no evidence was presented to support it.

pH and have proposed that this modification traps the active species of phenylalanine hydroxylase, which has an exposed sulfhydryl moiety. Although the assignment of a single ionizable group as the effector of an alteration in the conformation of a complex macromolecule (such as phenylalanine hydroxylase) upon changing the pH may be an oversimplification, it seems likely that ionization of a sulfhydryl residue plays a role in the activation-deactivation of phenylalanine hydroxylase.

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