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Spectroscopic Investigation of Ligand Interaction with Hepatic Phenylalanine Hydroxylase: Evidence for a Conformational Change Associated with Activation†

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ABSTRACT: We have examined the interaction of phenylalanine hydroxylase with phenylalanine, tetrahydropterin cofactors, and an activating phospholipid, lysophosphatidylcholine. Incubation of native phenylalanine hydroxylase with phenylalanine or lysophosphatidylcholine results in an increase in the fluorescence emission of the enzyme at 360 nm, which closely parallels the increase in tetrahydrobiopterin-dependent activity observed under these conditions. The presence of tetrahydrobiopterin in the absence of phenylalanine results in quenching of the enzyme fluorescence emission; this quenching exhibits a sharp end point at about 1 mol of tetrahydrobiopterin bound/mol of enzyme subunit. The binding of tetrahydrobiopterin under these conditions is unexpectedly tight, with an estimated K_D of 10-20 nM, while in the presence of lysophosphatidylcholine, the K_D is increased to about 25 μM. Quenching experiments with sodium iodide indicate greater exposure of tryptophan residues in the phenylalanine-activated enzyme. The ultraviolet difference spectrum of phenylalanine hydroxylase in the presence of phenylalanine exhibits a peak at 238 nm, which correlates with the fluorescence increase and activation, as well as additional changes in the aromatic region, which do not correlate well with activation. Phenylalanine does not alter the far-ultraviolet circular dichroism spectrum of phenylalanine hydroxylase. In contrast, lysophosphatidylcholine appears to induce a dramatic change in enzyme secondary structure upon activation. These results suggest that activation of phenylalanine hydroxylase results in a conformation change and the exposure of buried tryptophan(s) and possibly a cysteine residue.

Phenylalanine hydroxylase, as the first and rate-limiting step in the hepatic catabolism of phenylalanine (Milstien & Kaufman, 1974), is subjected to strict metabolic control. In the presence of the natural cofactor, (6*R*)-L-*erythro*-tetrahydrobiopterin (BH_4)[1] (Kaufman, 1963), the enzyme only expresses a small fraction of the activity observed with synthetic cofactors such as 6,7-dimethyltetrahydropterin (Kaufman, 1970) and 6-methyltetrahydropterin (6-MPH_4) (Hasegawa & Kaufman, 1982). A number of treatments are known,

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[1] Abbreviations: BH_4, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin; 6MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; phenylalanine hydroxylase, L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating) (EC 1.14.16.1); Pipes, 1,4-piperazinediethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

however, which dramatically increase the BH_4-dependent V_{max}, whereas they have only a modest effect on the V_{max} observed with 6-MPH_4. These treatments include limited proteolysis or the presence of phospholipids (Fisher & Kaufman, 1973), phosphorylation by cAMP-dependent protein kinase (Abita et al., 1976), alkylation with *N*-ethylmaleimide (Parniak & Kaufman, 1981), and preincubation with phenylalanine (Nielsen, 1969; Kaufman, 1970; Tourian, 1971; Ayling & Helfand, 1975; Shiman et al., 1979; Kaufman & Mason, 1982). These results have led to proposals that phenylalanine hydroxylase exists in a low-activity conformation in the native state, which can be converted to a highly activated form by these treatments. Although these proposals have been primarily based on kinetic properties of phenylalanine hydroxylase, they are supported by other data. It has been found (Fisher & Kaufman, 1973), for example, that activation of the hydroxylase by lysophosphatidylcholine is accompanied by the exposure of one sufhydryl group and that this structural alteration and the activation show essentially identical responses to changes in concentration of the phospholipid. In addition, the observation that activated forms of phenylalanine hydroxylase can be adsorbed to hydrophobic supports such as phenyl-Sepharose and can be eluted by removal of activators from the buffer has been presented as evidence for a conformational change upon activation (Shiman et al., 1979). We have recently presented further evidence for conformational mobility in phenylalanine hydroxylase, based on the stimulation of proteolysis in the presence of phenylalanine and protection in the presence of BH_4 (Phillips et al., 1983).

Spectroscopic techniques such as UV difference spectroscopy (Herskovits & Sorenson, 1968), fluorescence emission (Burstein et al., 1973), collisional quenching of emission by solutes (Lehrer, 1971; Eftink & Ghiron, 1976), and far-UV circular dichroism (Chen & Yang, 1971) have proven to be sensitive probes of the conformational states of proteins. In this paper, we have utilized these techniques to characterize the interaction of phenylalanine hydroxylase with phenylalanine, lysophosphatidylcholine, and tetrahydropterin cofactors. The results of these experiments provide unequivocal evidence for multiple conformational states of phenylalanine hydroxylase. In addition, these data demonstrate that phenylalanine and lysophosphatidylcholine produce different activated states.

Experimental Procedures

Materials

Phenylalanine hydroxylase was purified to homogeneity from rat liver on a column of phenyl-Sepharose (Shiman et al., 1979), as previously described (Phillips et al., 1984). Dihydropteridine reductase was purified from sheep liver through the calcium phosphate gel step (Craine et al., 1972). Tetrahydrobiopterin was obtained as a mixture of diastereoisomers with respect to C-6 (Dr. Schircks, Wettswil, Switzerland) and was resolved exactly as described (Ayling & Bailey, 1978). 6-Methylpterin (Storm et al., 1971) was reduced by catalytic hydrogenation with PtO_2 in 1 M HCl. Catalase, L-phenylalanine, NADH, and lysophosphatidylcholine (Type I, from egg yolk) were purchased from Sigma.

Methods

Fluorescence Measurements. Fluorescence measurements were performed on an Aminco-Bowman spectrophotofluorometer equipped with an Aminco ratio photometer. Spectra were obtained with the use of an Aminco X-Y chart recorder. Spectra were corrected for blank emission by subtracting blank values at 5-nm intervals, and the resultant corrected spectra were plotted with the use of the MLAB curve fitting routine on the NIH DEC-10 computer system. All solutions for fluorescence measurements were prepared with glass-distilled deionized water and filtered through 0.22-μm Millipore filters before use. The phenylalanine titrations were performed with the use of two different procedures that gave identical results. In the first procedure, aliquots of phenylalanine stock solution (10 mM) were added sequentially to a single sample of phenylalanine hydroxylase in a fluorescence cuvette; the solutions were equilibrated for 5 min after each addition prior to reading the fluorescence. In the second procedure, a new sample of phenylalanine hydroxylase was used for each point. The initial fluorescence reading was made, then an aliquot of 10 mM phenylalanine was added, and after 5 min of incubation the fluorescence was determined. At this point, an aliquot of 0.1 M phenylalanine was added to bring the final concentration in the sample cuvette to 1 mM and the maximum fluoresence change recorded after 5 min of incubation. The data were then expressed as percentage of the maximum fluorescence change. For BH_4 titrations, freshly prepared solutions of (6*R*)-BH_4 were standardized by a determination of the absorbance at 265 nm in 0.1 N HCl, assuming an $\epsilon_{265} = 1.6 \times 10^4$ L/(mol·cm) (Shiman et al., 1971). Aliquots of the BH_4 stock solution, in 5 mM HCl containing 0.5 mM DTT, were added sequentially to a single sample of phenylalanine hydroxylase in a cuvette, mixed, and read within 1–2 min. The resultant data were corrected for inner filter effects by subtraction of the linear, nonsaturable quenching, which continued at BH_4 concentrations far beyond the equivalence point. In the range of interest, the inner filter effect contributed 10% or less of the total quenching observed. In all of these titrations, it was found that phenylalanine hydroxylase is inactivated if left in the light beam for extended periods, so it was necessary to make the readings as quickly as possible to minimize exposure. Iodide quenching experiments were carried out by variation of KI from 0 to 0.5 M, while KCl was varied such that $[I^-] + [Cl^-] = 0.5$ M. After the initial reading, 10 μL of 0.1 M phenylalanine was added to each sample (1 mL), and after 5 min of incubation, the readings were repeated. K_{sv}(eff) values were calculated as described by Eftink & Ghiron (1976).

UV Difference Spectroscopic Measurements. Difference spectral measurements were made in matched tandem cuvettes at room temperature (23 °C) with a Hewlett-Packard 8450 spectrophotometer. All spectra were determined in 0.05 M potassium phosphate, pH 6.8. A solution–solution base line was first determined and stored, with phenylalanine in a separate chamber from the hydroxylase in each cuvette. The sample cuvette was then mixed and allowed to stand for 10 min at ambient temperature, and the difference spectrum was recorded and stored. Each spectrum was an average of at least 10 scans. The solution–solution base line was subtracted from the observed difference spectrum for each experiment. Upon completion of the experiment, the reference cuvette was mixed and allowed to stand for 10 min at ambient temperature, and the spectrum was determined in order to ensure that the original solution–solution base line was reproduced. The concentrations of phenylalanine indicated in the figure legends are the concentrations subsequent to mixing of the tandem cell.

Circular Dichroism Measurements. Measurements of the circular dichroism of phenylalanine hydroxylase were performed at ambient temperatures in 0.01 M potassium phosphate, pH 6.8, in 1-cm cells with a Jasco SP-500 spectropolarimeter. All spectra were recorded from 210 to 250 nm and were corrected for spectra observed in the absence of phenylalanine hydroxylase.

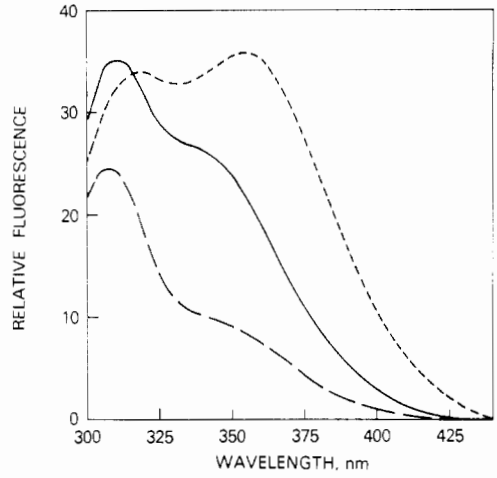

FIGURE 1: Fluorescence emission spectra of phenylalanine hydroxylase (150 μg/mL) excited at 295 nm, in 0.05 M Pipes (pH 6.8)–0.05 M KCl: (solid line) native phenylalanine hydroxylase; (short dashes) phenylalanine hydroxylase after addition of 1 mM L-phenylalanine; (long dases) phenylalanine hydroxylase after addition of 8 μM BH_4. This spectrum is not corrected for the inner filter effect of the tetrahydropterin; however, the inner filter effect only contributes about 10% of the observed quenching at this concentration of pterin. The extent of quenching observed in the presence of BH_4 is somewhat variable but is typically in the range of 40–60% at 340 nm.

Phenylalanine Hydroxylase Activity Measurements. The activity of phenylalanine hydroxylase was determined at 340 nm in a spectrophotometric assay in which the quinoid dihydropterin, formed during the hydroxylation of phenylalanine, is reduced to tetrahydropterin by dihydropteridine reductase and NADH (Kaufman, 1957). Assays were performed at atmospheric O_2 tension and 25 °C and contained, in a total volume of 1.0 mL, 100 μmol of potassium phosphate, pH 6.8, 150 nmol of NADH, 30 nmol of BH_4, 1 μmol of phenylalanine, catalase, and dihydropteridine reductase in excess. Reactions were always initiated by the addition of phenylalanine hydroxylase to an otherwise complete assay mixture equilibrated at 25 °C. Initial rates were determined from the first 30 s of reaction.

For measurements of the activation of phenylalanine hydroxylase by preincubation with phenylalanine, the enzyme was incubated with the indicated concentrations of phenylalanine for 5 min at 25 °C in 0.05 M Pipes (pH 6.8)–0.05 M KCl. Aliquots of these preincubated samples were then added to standard assay mixtures as described above.

Results

Fluorescence Properties of Phenylalanine Hydroxylase. Excitation of native phenylalanine hydroxylase at 280 nm results in a single broad emission peak centered at about 320 nm. Excitation at 295 nm, however, results in a peak at about 315 nm and a shoulder at 330–340 nm (Figure 1, solid line). The difference in these emission spectra is probably due to the "red-edge" effect, in which excitation on the red side of the protein absorption band selectively excites the most deeply buried, and most blue-shifted emitting, tryptophan (Purkey & Galley, 1976; Barboy & Feitelson, 1978). These results suggest that phenylalanine hydroxylase has at least two different classes of tryptophan residues which reside in distinct environments. This conclusion is consistent with amino acid analyses (Fisher et al., 1972; Nakata & Fujisawa, 1980; Shiman, 1980; Døskeland et al., 1982), which have determined that phenylalanine hydroxylase contains between two and four tryptophan residues per subunit of M_r 50 000. We have independently determined the tryptophan content of phenylalanine hydroxylase by a fluorescence method (Pajot, 1976), and we find four tryptophyls per subunit, in excellent agreement with the data of Shiman (1980). The emission spectrum obtained in 6 M guanidine hydrochloride exhibits a single peak at 355 nm, as expected for fully solvent exposed tryptophan (Burstein et al., 1973). Thus, the heterogeneity exhibited in the emission spectrum of the native enzyme is probably due to the conformation of the enzyme, although is possible that it is due to an adventitious small molecule. If the 315-nm emission is due to a contamination, it must fluoresce only when bound to the enzyme, as it is absent from the spectrum in 6 M guanidine. However, if the 315-nm emission is due to tryptophan residues in the protein, the short wavelength of the emission peak suggests that one or more tryptophans is in a very hydrophobic environment (Finazzi-Agro et al., 1970; Burstein et al., 1973). The shoulder in the emission spectrum of phenylalanine hydroxylase, at 330–340 nm, is typical of tryptophan emission in proteins and may represent either a class I (λ_{em} = 330 nm) or a class II residue (λ_{em} = 340 nm) (Burstein et al., 1973). Class I tryptophan residues are buried in the interior of the protein and thus not solvent exposed, while class II residues are partially exposed at the surface of the protein.

Effect of Phenylalanine on Phenylalanine Hydroxylase Fluorescence. When phenylalanine is added to phenylalanine hydroxylase at pH 6.8, the emission spectrum is dramatically altered (Figure 1, short dashes). In the presence of phenylalanine, two distinct peaks, with λ_{max} at about 320 and 355 nm, are observed, and there is a net increase in quantum yield. The red shift of the emission maximum, as well as the increased emission intensity, suggests that upon phenylalanine binding the environment of a tryptophan residue (or residues) has been altered from the interior of the protein to a fully solvent exposed position (Burstein et al., 1973). Since phenylalanine hydroxylase is strongly activated by preincubation with phenylalanine (Nielsen, 1969; Kaufman, 1970; Tourian, 1971; Sullivan et al., 1973; Ayling & Helfand, 1975; Dhondt et al., 1978; Shiman & Gray, 1980), this result provides direct evidence for a conformational change associated with activation of phenylalanine hydroxylase. The presence of 0.5 mM lysophosphatidylcholine, another potent activator of phenylalanine hydroxylase (Fisher & Kaufman, 1973), results in a spectrum very similar to that observed with phenylalanine present (data not shown).

The increase in fluorescence at long wavelength in response to phenylalanine is saturable and exhibits strong positive cooperativity (n_H = 3), with a half-maximal change at 0.06 mM (Figure 2). The increase in fluorescence closely parallels the activation of the enzyme, as shown in Figure 2 (compare squares for fluorescence measurements with open circles for activity measurements).

In contrast, when the emission spectrum of phenylalanine hydroxylase is measured in 0.1 M Tris-HCl, pH 9.2, the spectrum is nearly identical with that of the native enzyme in the presence of phenylalanine at pH 6.8 (data not shown), and little further change is seen upon addition of phenylalanine. This result is consistent with our observation that phenylalanine hydroxylase is activated by preincubation at high pH, when subsequently assayed at pH 6.8 (M. A. Parniak and S. Kaufman, unpublished results), and provides further evidence for a conformational change associated with activation of phenylalanine hydroxylase.

Effect of Tetrahydropterins on Phenylalanine Hydroxylase Fluorescence. When (6*R*)-BH_4, the natural cofactor, is added to solutions of phenylalanine hydroxylase, there is no change

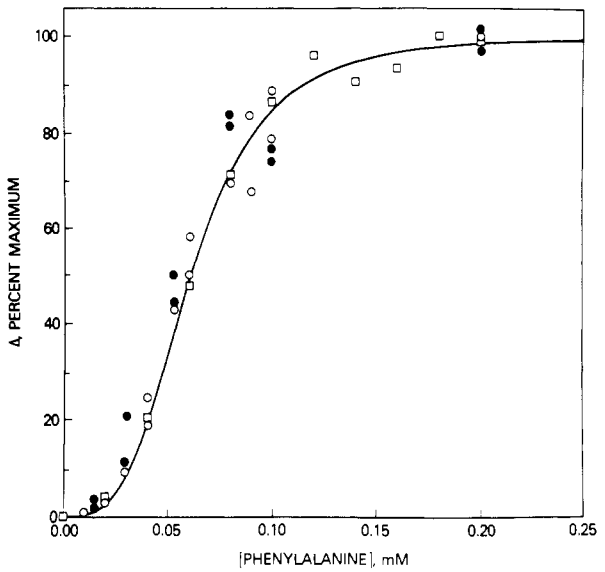

FIGURE 2: Correlation of phenylalanine activation and fluorescence and UV difference spectral changes. Phenylalanine hydroxylase was incubated with the indicated concentrations of phenylalanine for 5 min and assayed with 1 mM phenylalanine with 50 μM BH_4 as the cofactor, as described under Experimental Procedures. Open circles represent the activation data, expressed as percent of maximum change. For fluorescence measurements, phenylalanine was added at the indicated concentration and incubated for 5 min, and the emission at 360 nm was measured, with excitation at 295 nm. Open squares represent the fluorescence titration data, expressed as percent of maximal change. UV difference measurements (filled circles) were carried out as described under Methods and are expressed as percent of maximal change at 238 nm.

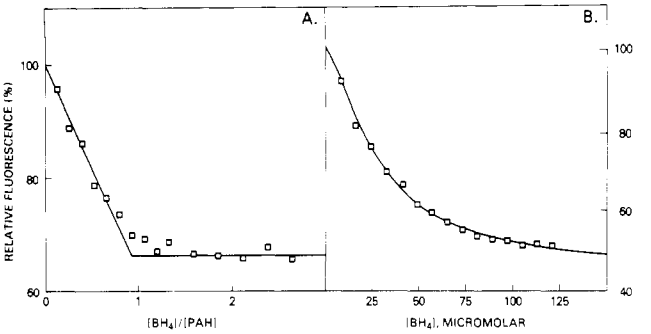

FIGURE 3: Titration of phenylalanine hydroxylase with (6*R*)-BH_4. (A) Native enzyme, 150 μg/mL in 0.05 M Pipes (pH 6.8)–0.05 M KCl–0.5 mM DTT, was titrated with aliquots of freshly prepared, standardized BH_4 solution. The Pipes buffer was deaerated and saturated with nitrogen prior to use and was gently stirred after each addition to avoid aeration. The cell compartment was thermostated to 25 ± 1 °C. Excitation was at 295 nm, with emission read at 340 nm. The titration data are corrected for the inner filter effect due to the absorbance of BH_4 at 295 nm (ϵ = 1.0 × 10^4 M^{-1} cm^{-1}). Mathematical analysis of these data indicates an end point of 0.93 mol of BH_4 bound per subunit of M_r 50000. The K_D, estimated from the deviation around the equivalence point, is approximately 2 × 10^{-8} M. (B) Native phenylalanine hydroxylase, 150 μg/mL, in 0.05 M Pipes, pH 6.8, 0.05 M KCl, 0.5 mM DTT, and 0.5 mM lysophosphatidycholine, was titrated under otherwise identical conditions to that in (A). The quenching is no longer stoichiometric in the presence of lysophosphatidycholine, and the quenching curve exhibits positive cooperativity ($n_H \sim 1.5$), with a half-maximal effect at about 25 μM. PAH is the abbreviation for phenylalanine hydroxylase.

in the peak positions in the emission spectrum, but the intensity is greatly diminished (Figure 1, long dashes). The quenching by BH_4 is most dramatic on the long-wavelength shoulder and results in about 40–60% quenching at 340 nm, when corrected for the inner filter effect, but 20% or less at 320 nm. The quenching by (6*R*)-BH_4 is observed in the presence of stoichiometric amounts of cofactor, and a titration of native phenylalanine hydroxylase with BH_4 exhibits a sharp end point at 0.93 mol of BH_4 bound per subunit of M_r 50000, as shown in Figure 3A. This experiment was performed several times and resulted in end points ranging from 0.8 to 1.2 mol of BH_4 bound per hydroxylase subunit. Thus, these results provide strong support for the existence of one active site per subunit in phenylalanine hydroxylase. While it is difficult to obtain an accurate value of K_D when binding is nearly stoichiometric, we estimate, from the deviation of the points around the equivalence point, that K_D is (1–2) × 10^{-8} M. These results are consistent with other experiments demonstrating protection against proteolysis and inhibition of phosphorylation by cAMP-dependent protein kinase, in the presence of BH_4, where maximum effects were observed with stoichiometric amounts of BH_4 (Phillips et al., 1983; Phillips & Kaufman, 1984). While in some cases the quenching of protein fluorescence has been found to be nonlinear with respect to binding of quenching ligands (Stinson & Holbrook, 1973; Theorell & Tatemoto, 1971), in our case the quenching is apparently linear, as indicated by the equivalence point at close to 1 per subunit.

When BH_4 is added to solutions of phenylalanine hydroxylase in the presence of 0.5 mM lysophosphatidylcholine, quenching is no longer observed in the presence of stoichiometric quantities of BH_4, and much higher concentrations are required in order to obtain the maximum quenching, as shown in Figure 3B. Under these conditions, the binding of BH_4 exhibits weak positive cooperativity, as a Hill plot (not shown) exhibits a slope of about 1.5 with a half-maximal change at about 25 μM. Thus, the affinity of the lysolecithin-activated enzyme for BH_4 is about 1000-fold weaker than that of the native enzyme. Interestingly, when native phenylalanine hydroxylase is titrated with $6MPH_4$, a synthetic cofactor, the quenching data are very similar to that exhibited by the lysophosphatidylcholine-activated enzyme and BH_4, with positive cooperativity (n_H = 1.5) and a half-maximal change at about 50 μM. This difference in the behavior of the two cofactors is probably due to the fact that BH_4 acts both as a cofactor and as a negative effector (Shiman & Gray, 1980), whereas $6MPH_4$ apparently has no ability to act as a negative effector.

The quenching of the enzyme fluorescence by tetrahydropterin binding is most likely a result of Forster energy transfer (Forster, 1948) from the tryptophan residues to the bound pterin. At pH 6.8, the ultraviolet absorption spectrum of BH_4 exhibits a peak at 300 nm, with a tail extending to about 350 nm, and thus shows significant overlap with the enzyme emission spectrum. Using the Forster equation (Forster, 1948), we estimate a critical distance for energy transfer between tryptophan residues in the enzyme and BH_4 of 21.9 Å. Thus, energy transfer can readily account for the quenching observed in the presence of tetrahydropterins. However, an alternative mechanism for the quenching could involve ring stacking of the indole ring of a tryptophan with the cofactor, in a manner similar to that which has been observed with tryptophans and purine bases in nucleic acids (Toulmê & Hêlène, 1977).

Effect of Phenylalanine on Dynamic Quenching of Phenylalanine Hydroxylase Fluorescence. The quenching of protein fluorescence by solutes such as iodide (Lehrer, 1971) and acrylamide (Eftink & Ghiron, 1976) is another technique that can provide valuable information on the exposure of tryptophan residues (Eftink & Ghiron, 1981). We have examined the quenching of phenylalanine hydroxylase fluorescence at 350 nm, with 280-nm excitation, by iodide ion in the absence and in the presence of 1 mM phenylalanine (Figure 4). The Stern–Volmer plots both in the absence and in the presence

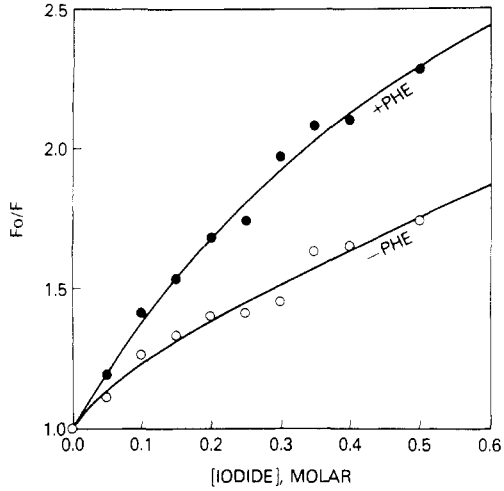

FIGURE 4: Iodide quenching of phenylalanine hydroxylase fluorescence in the absence and presence of phenylalanine. Each sample contained phenylalanine hydroxylase (50 μg/mL) in 0.05 M Pipes, pH 6.8, containing 0.5 M KCl or a mixture of KCl and the indicated concentration of KI such that $[I^-] + [Cl^-] = 0.5$ M. Excitation was at 280 nm, with emission read at 350 nm. After the initial reading, phenylalanine to a final concentration of 1 mM was added to each sample, and the emission was read after 5 min of incubation at 25 °C. The curves are for illustration only. K_{SV}(eff) was calculated by the method of Eftink & Ghiron (1976). K_{SV}(eff) for the native enzyme is 2.45 M^{-1}; in the presence of phenylalanine, K_{SV}(eff) is 4.05 M^{-1}.

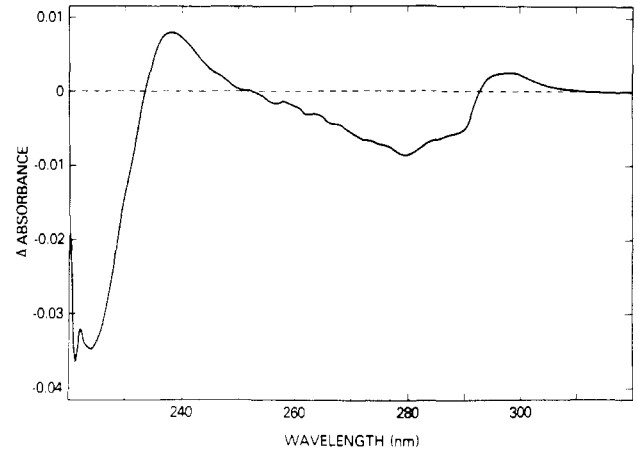

FIGURE 5: Ultraviolet difference spectrum of phenylalanine hydroxylase in the presence of phenylalanine. The spectra were obtained in 0.1 M potassium phosphate, pH 6.8, at 23 °C, as described under Methods, with a final concentration of 200 μg/mL purified phenylalanine hydroxylase and 0.5 mM phenylalanine: (—) difference spectrum observed 10 min after mixing of the sample tandem cell; (- - -) solution–solution base line generated 10 min after mixing of the reference tandem cell subsequent to the generation of the initial difference spectrum.

of phenylalanine are downward curving, indicative of heterogeneity of emission, with large differences in the quenching constant between the emitters (Eftink & Ghiron, 1976, 1981). This result is not surprising, since phenylalanine hydroxylase contains four tryptophyls per subunit. However, in the presence of phenylalanine the extent of quenching is greatly increased, as can be clearly seen from the data in Figure 4. The analysis of quenching data is extremely complex in the case of heterogeneous emission but can be analyzed in terms of an effective quenching constant, K_{SV}(eff) (Eftink & Ghiron, 1976), which is derived from the initial slope of the quenching data. Application of this analysis to the data of Figure 4 results in a K_{SV}(eff) of 2.45 M^{-1} in the absence of phenylalanine and 4.05 M^{-1} in the presence of phenylalanine. This result confirms our conclusion, based on the shift in emission maximum, that incubation of the hydroxylase with phenylalanine results in increased exposure of tryptophan residues upon activation.

Ultraviolet Difference Spectrum Produced by Phenylalanine. The interaction of L-phenylalanine with phenylalanine hydroxylase at pH 6.8 results in a difference spectrum that is characterized by positive absorbance changes at 238 and 298 nm and a negative absorbance change with several minima in the 250–290-nm region (Figure 5). The ultraviolet spectrum of the hydroxylase is perturbed by the addition of L-phenylalanine in a manner that is dependent on L-phenylalanine concentration and is saturable. The maximum at 238 nm is typical of an ionized sulfhydryl residue (Benesch & Benesch, 1955). The change in the magnitude of the absorbance difference at 238 nm is directly proportional to the concentration of phenylalanine (Figure 2, solid circles) and correlates almost exactly with both the increase in fluorescence emission of the enzyme at 360 nm and the increase in BH_4-dependent activity of phenylalanine hydroxylase upon preincubation with phenylalanine. No significant difference spectrum is given by the enzyme in the presence of 0.5 mM D-phenylalanine. In addition, no activation of the BH_4-dependent activity of the hydroxylase is noted upon preincubation of the enzyme with this concentration of D-phenylalanine, although activation has been reported at 28 mM D-phenylalanine (Kaufman & Mason, 1982). In contrast, this same concentration of L-phenylalanine is well above that necessary to allow the maximum expression of both the increase in BH_4-dependent activity and the increase in the absorbance change at 238 nm.

The difference spectral changes at 298 nm and in the 260–280-nm region do not correlate with the increase in BH_4-dependent activity of phenylalanine hydroxylase. These changes in the UV difference spectrum in the aromatic region are dependent on the concentration of phenylalanine added to the enzyme and are saturable but show a hyperbolic response to L-phenylalanine concentration (data not shown). The half-maximal concentration of L-phenylalanine required to effect these changes is about 0.2 mM.

Far-Ultraviolet Circular Dichroism Studies. The far-UV circular dichroism spectrum of native phenylalanine hydroxylase shows two minima, at 208 and 222 nm (data not shown). The magnitude of the minimum at 222 nm is increased in the presence of lysophosphatidylcholine in a saturable manner, which is proportional to both the concentration of lysophosphatidylcholine and the increase in BH_4-dependent activity of phenylalanine hydroxylase (J.-P. Abita, S. Kaufman, and M. A. Parniak, unpublished results). In contrast, the addition of 0.5 mM L-phenylalanine to native phenylalanine hydroxylase does not alter the CD spectrum of the enzyme in this region, even though this concentration of L-phenylalanine is sufficient to allow an approximately 20-fold increase in the BH_4-dependent activity of the enzyme. When 0.2 mM lysophosphatidylcholine is added to phenylalanine hydroxylase that has been incubated with 0.5 mM L-phenylalanine, the circular dichroism spectrum of the enzyme changes in a manner that is identical with that seen in the presence of lysophosphatidylcholine itself. It thus appears that the lysophosphatidylcholine induces a more dramatic change in gross secondary structure of phenylalanine hydroxylase than does the substrate, phenylalanine.

Tetrahydrobiopterin has been observed to reverse the phenylalanine-activated state of the hydroxylase (Ayling & Helfand, 1975) and also to increase the extent of the lag period noted upon initiation of the hydroxylase reaction subsequent to preincubation of phenylalanine hydroxylase with BH_4 (M. A. Parniak, unpublished observations). However, the presence

of BH_4 does not induce any change in the far-UV circular dichroism spectrum of native phenylalanine hydroxylase.

Discussion

The present results provide the first direct physical evidence for multiple conformational states of phenylalanine hydroxylase. The red shift in the fluorescence emission spectrum of the hydroxylase under conditions in which the enzyme is highly activated (high pH or the presence of phenylalanine or lysophosphatidylcholine) demonstrates that buried tryptophan residues become fully exposed upon activation (Burstein et al., 1973). This conclusion finds strong support in the results of our iodide quenching experiments, where we find nearly a doubling in the apparent quenching constant, K_{SV}(eff), in the presence of phenylalanine. Previous studies by Shiman et al. (1979) indicated an increase in hydrophobicity upon activation. In this regard, we note that tryptophan is one of the most hydrophobic amino acids. Taken together, these results provide unequivocal evidence for a conformational change associated with activation of phenylalanine hydroxylase. However, in contrast to the situation with lysophosphatidylcholine (J.-P. Abita, S. Kaufman, and M. A. Parniak, unpublished results), phenylalanine activation does not result in any gross alteration in the secondary structure of the hydroxylase, as determined from the far-UV circular dichroism spectrum. Thus, while the phenylalanine- and lysophosphatidylcholine-activated enzymes are similar (Shiman, 1980), our present results show that this similarity is superficial, since lysophosphatidylcholine results in much more dramatic changes in the structure of the enzyme. Activation by phenylalanine may be a result of movement of a regulatory domain on a hinge, resulting in an opening up of the protein molecule, and exposure of the catalytic site, and a tryptophan residue(s), to the solvent. This conclusion is supported by our recent discovery that the activated enzyme is a better substrate for proteolysis by α-chymotrypsin (Phillips et al., 1983) and for phosphorylation by cAMP-dependent protein kinase (Phillips & Kaufman, 1984).

The short wavelength of the emission maximum (λ = 315 nm) is rather remarkable, as most proteins emit in the 330–350-nm range (Burstein et al., 1973); however, azurin exhibits an emission maximum at 308 nm (Finazzi-Agro et al., 1970), and horse spleen ferritin exhibits a maximum at 315 nm (Longworth, 1971; Stefanini et al., 1976, 1982). These strongly blue shifted emission maxima are generally attributed to tryptophan residues in a highly hydrophobic environment (Finazzi-Agro et al., 1970). A contribution from tyrosine is also possible, as phenylalanine hydroxylase contains approximately 20 tyrosine residues per subunit (Fisher et al., 1972; Nakata & Fujisawa, 1980; Shiman, 1980; Døskeland et al., 1982), but is unlikely with excitation at 295 nm.

The binding of BH_4 to native phenylalanine hydroxylase, monitored by quenching of the enzyme emission, is unexpectedly tight. The K_D that we estimate from the titration data, about $(1–2) \times 10^{-8}$ M, is about 2 orders of magnitude lower than the apparent K_m of 2 μM for the unactivated enzyme (Fisher & Kaufman, 1973). It is possible that this tight binding is related to the role of BH_4 as a negative effector, since BH_4 is able to antagonize most activations of phenylalanine hydroxylase (Shiman, 1980; Phillips et al., 1983; Phillips & Kaufman, 1984). When the hydroxylase is in an activated state, as after preincubation with lysophosphatidylcholine, the affinity of the enzyme for BH_4 is dramatically reduced, a result that is in accord with the increase in K_m for BH_4 that results from activation of the hydroxylase by the phospholipid (Fisher & Kaufman, 1973); in this case, the apparent K_D of about 25 μM is comparable to the apparent K_m of 15 μM under these conditions (Fisher & Kaufman, 1973).

The increase of the difference spectrum observed at 238 nm is also of interest. It is possible that this is due to ionization of a cysteine sulfhydryl group which is exposed upon activation, as ionization of the cysteine sulfhydryl group leads to a strong absorption between 230 and 240 nm (ϵ = 4400 at 230 nm) (Benesch & Benesch, 1955). There is a considerable amount of evidence which implicates a role for a sulfhydryl residue in activation of phenylalanine hydroxylase. We have previously demonstrated that alkylation of a single sulfhydryl by *N*-ethylmaleimide leads to a dramatic activation of phenylalanine hydroxylase (Parniak & Kaufman, 1981). In addition, activation of phenylalanine hydroxylase by lysophosphatidylcholine results in exposure of a single sulfhydryl, as determined by titration with 3,3′-dithiobis(6-nitrobenzoic acid) (Fisher & Kaufman, 1973). Furthermore, phenylalanine hydroxylase is activated by preincubation at high pH, and the pH–activation profile exhibits an apparent pK_a at about pH 8, very close to what would be expected for ionization of a sulfhydryl (M. A. Parniak and S. Kaufman, unpublished results). Additionally, the ΔA_{238} closely parallels the activation of the enzyme and the fluorescence change, all of which exhibit a half-maximal change at about 60 μM phenylalanine. In contrast, the ΔA in the 250–300-nm region shows a response to phenylalanine that is half-maximal at about 200 μM, similar to the direct binding data that we have previously obtained (Parniak & Kaufman, 1981). The positive difference spectrum observed at 298 nm implies a red shift in tryptophan absorption and thus suggests a decrease in tryptophan exposure (Herskovits & Sorenson, 1968). This result may appear to be inconsistent with our results obtained from fluorescence measurements, which imply increased exposure of tryptophan residues. However, since the fluorescence changes and ΔA_{238} correlate with the activation state of the enzyme, whereas the ΔA_{298} does not, our conclusion that activation of phenylalanine hydroxylase results in increased exposure of tryptophan residues is not altered. It is likely, therefore, that the difference spectrum in the aromatic region reflects binding at both the regulatory and active sites. Furthermore, the interpretation of UV difference spectra in the aromatic region is particularly difficult when there is a high tyrosine:tryptophan ratio in the protein (Herskovits & Sorenson, 1968). For phenylalanine hydroxylase, this ratio is about 6.

In conclusion, the data presented in this paper provide direct evidence for conformational adaptability in phenylalanine hydroxylase. Taken together, our data suggest that activation of phenylalanine hydroxylase results in exposure of buried tryptophan residues and possibly a cysteine sulfhydryl group. Confirmation of these conclusions must ultimately await results of further studies utilizing other techniques such as X-ray crystallography.

Added in Proof

Recently, Marota & Shiman (1984) have presented the fluorescence spectrum of phenylalanine hydroxylase. With excitation at 275 nm, they observed emission at 310–315 nm, in excellent agreement with our results. However, they observed an increase in fluorescence intensity upon incubation of the enzyme with tetrahydropterins under aerobic conditions, which they attribute to formation of a reduced enzyme. The reduction of the enzyme was found to be dependent on molecular oxygen; thus, we did not observe these results in our experiments, which were performed in deaerated, N_2-saturated buffer solutions. The changes in the fluorescence spectrum

of phenylalanine hydroxylase in the presence of BH_4 under our conditions are therefore due to binding of the cofactor to the enzyme and not to reduction of the enzyme.

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