The Interaction of Aromatic Amino Acids with Rat Liver Phenylalanine Hydroxylase*

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We have examined the interaction of hepatic phenylalanine hydroxylase with the phenylalanine analogs, tryptophan and the diastereomers of 3-phenylserine (β-hydroxyphenylalanine). Both isomers of phenylserine are substrates for native phenylalanine hydroxylase at pH 6.8 and 25 °C, when activity is measured with the use of the dihydropteridine reductase assay coupled with NADH in the presence of the synthetic cofactor, 6-methyl-5,6,7,8-tetrahydropterin. However, while erythro-phenylserine exhibits simple Michaelis-Menten kinetics (K_m = 1.2 mM, V_max = 1.2 μmol/min·mg) under these conditions, the three isomers exhibit strongly positive cooperativity (S_0.5 = 4.8 mM V_max = 1.4 μmol/min·mg, n_H = 3). Tryptophan also exhibits cooperativity under these conditions (S_0.5 = 5 mM, V_max = 1 μmol/min·mg, n_H = 3). The presence of 1 mM lysolecithin results in a hyperbolic response of phenylalanine hydroxylase to tryptophan (K_m = 4 mM, V_max = 1 μmol/min·mg) and three-phenylserine (K_m = 2 mM, V_max = 1.4 μmol/min·mg). Erythro-Phenylserine is a substrate for native phenylalanine hydroxylase in the presence of the natural cofactor, L-erythro-tetrahydrobiopterin (BH_4) (K_m = 2 mM, V_max = 0.05 μmol/min·mg, n_H = 2). Preincubation of phenylalanine hydroxylase with erythro-phenylserine results in a 26-fold increase in activity upon subsequent assay with BH_4 and erythro-phenylserine, and hyperbolic kinetic plots are observed. In contrast, both three-phenylserine and tryptophan exhibit negligible activity in the presence of BH_4 unless the enzyme has been activated. The product of the reaction of phenylalanine hydroxylase with either isomer of phenylserine was identified as the corresponding p-hydroxyphenylserine by reaction with sodium periodate and nitrosonaphthol. With erythro-phenylserine, the hydroxylation reaction is tightly coupled (i.e. 1 mol of hydroxyphenylserine is formed for every mole of tetrahydropterin cofactor consumed), while with three-phenylserine and tryptophan the reaction is largely uncoupled (i.e. more cofactor consumed than product formed). Erythro-phenylserine is a good activator, when preincubated with phenylalanine hydroxylase (A_0.5 = 0.2 mM), with a potency about one-third that of phenylalanine (A_0.5 = 0.06 mM), while three-phenylserine (A_0.5 = 6 mM) and tryptophan (A_0.5 ~ 10 mM) are very poor activators. Addition of 4 mM tryptophan or three-phenylserine or 0.2 mM erythro-phenylserine to assay mixtures containing BH_4 and phenylalanine results in a dramatic increase in the hydroxylation at low concentrations of phenylalanine. This increase in activity is due entirely to a change from a sigmoid to a hyperbolic kinetic response, without affecting V_max. These results suggest that these compounds are acting as positive heterotropic effectors, presumably by binding to a regulatory site. In contrast, these compounds are inhibitors when activated forms of enzyme are used or when 6-methyl-5,6,7,8-tetrahydropterin is used as the cofactor. Binding studies have shown that native phenylalanine hydroxylase exhibits positive cooperativity in binding phenylalanine, with about 1.5 mol of phenylalanine bound per subunit of M_s = 50,000. In the presence of 4 mM tryptophan, the cooperativity of phenylalanine binding is greatly diminished, and the total binding is reduced to 1 mol/subunit. These results provide strong supporting evidence for a regulatory site on phenylalanine hydroxylase, distinct from the catalytic site. The differences in the properties of the phenylserines may be the result of altered conformations for the two diastereomers.

Phenylalanine hydroxylase (EC 1.14.16.1) catalyzes the tetrahydropterin-dependent oxygenation of L-phenylalanine to yield L-tyrosine. This is the rate-limiting step in the complete catabolism of phenylalanine in mammals (1) and might thus be expected to be subject to metabolic regulation. The hydroxylase can be activated in vitro by a number of processes: partial proteolysis or interaction with certain phospholipids (2), sulfhydryl modification (3), phosphorylation by cAMP-dependent protein kinase (4), and preincubation with the substrate, phenylalanine (5-11). Most of these activations are expressed in the presence of the natural cofactor, BH_4, and are not observed when synthetic tetrahydropterins such as 6MPH, or 6,7-dimethyltetrahydropterin are used. Although some activation by phenylalanine can be observed in the presence of these analogues, it is very transient.

The observation that phenylalanine hydroxylase is activated by its substrate, as well as the sigmoid response of hydroxylase activity to phenylalanine concentration in the presence of BH_4 (2), has led to suggestions of the existence of a regulatory site for phenylalanine, distinct from the catalytic site, on the hydroxylase molecule (5-12). Direct measurements of the binding of phenylalanine to native phenylalanine hydroxylase have provided further support for the existence of distinct sites (3). In order to further define the role of these sites in the expression of activity of the hydroxylase, we have

* A preliminary account of this work has been presented (Phillips, R. S., Parniak, M. A., and Kaufman, S. (1982) Fed. Proc. Am. Soc. Exp. Biol. 41, 5962 (abstr)). 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.

1 The abbreviations used are: BH_4, tetrahydrobiopterin; 6MPH, 6-methyltetrahydropterin; phenylalanine hydroxylase, L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1.
undertaken a search for compounds which can bind selectively to the catalytic site or to the putative regulatory site.

Since several aromatic amino acid analogs of phenylalanine have been reported to serve as substrates and activators for phenylalanine hydroxylase, it seemed likely that within this class of compounds, an amino acid might be found that could bind selectively to one of these sites. Thus, tryptophan, 2, 8, 13, 4-fluorophenylalanine, 14, 4-chlorophenylalanine, 15, 4-bromophenylalanine, 15, and 4-methylphenylalanine 16 can be utilized by the enzyme, with varying efficiencies. In addition, we have recently reported that both methionine and norleucine can be hydroxylated by phenylalanine hydroxylase 17. However, all of these compounds can be considered to retain the alanine side chain intact.

Phenylserine (β-hydroxyphenylalanine) is a phenylalanine analog with a modified alanine side chain. The compound exists in two diastereomeric forms and has been reported to interact with several phenylalanine-sensitive enzymes. For example, phenylserine has been reported to be a competitive inhibitor of phenylalanine ammonia lyase 18, an allosteric inhibitor of the phenylalanine-sensitive 3-deoxy-d-xylo-hexulosonate-7-phosphate synthetase 19, and a substrate for phenylalanyl-tRNA synthetase 20. Unfortunately, only one diastereomer or mixtures of both isomers were used in these studies. In this paper, we report the results of studies of the interaction of the resolved diastereomers with rat liver phenylalanine hydroxylase. These results are compared with data obtained with the common aromatic amino acids phenylalanine hydroxylase 21. In the preparation of large quantities of phenylalanine hydroxylase, from 60 to 100 livers, we have found that it is more convenient to proceed through the ethanol and ammonium hydroxylase hydrolysis of the corresponding N-trifluoroacetyl derivatives with carboxypeptidase A 27. The threo isomers of m-hydroxy- and p-hydroxyphenylserine were prepared by the method of Shaw 28.

The isomers of phenylserine were prepared by the method of Shiman et al. 21. In the preparation of large quantities of phenylalanine hydroxylase, from 60 to 100 livers, we have found that it is more convenient to proceed through the ethanol and ammonium sulfate fractionation of our original procedure 22, followed by a single phenyl-Sepharose column. This procedure routinely yields electrophoretically homogeneous enzyme with specific activity of 7·10 µmol/min·mg in about 40% overall yield. For the experiments reported in Table IV, phenylalanine hydroxylase was activated by reaction with N-ethylmaleimide exactly as described in Ref. 3. Dihydropteridine reductase was purified from sheep liver through the ethanol and ammonium hydroxylase and reduced by catalytic hydrogenation with PtOn in a flow rate of 1 ml min⁻¹, with detection at 280 nm. Analysis of the binding of phenylalanine to phenylalanine hydroxylase was performed by the method of Paulus 35 as previously described 3.

RESULTS

Aromatic Amino Acids As Substrates for Phenylalanine Hydroxylase—As shown in Fig. 1, both diastereomers of phenylserine are able to stimulate the phenylalanine hydroxylase-dependent oxidation of NADH in the dihydropteridine reductase-coupled assay in the presence of the synthetic cofactor 6MPH₄. The relationship between initial rate and concentration of the erythro isomer of phenylserine is hyperbolic (nH = 1), yet strong positive cooperativity (nH = 3) is noted with the threo isomer under similar conditions. The observed Vₘₐₓ values for both isomers were essentially identical and are approximately 20% of that obtained with the normal substrate, phenylalanine. The hydroxylase also exhibits a sigmoidal response to variation of L-tryptophan concentration with 6MPH₄ as cofactor.

When either L-tryptophan or threo-phenylserine are used as substrates for phenylalanine hydroxylase in the presence of l-lysine, hyperbolic kinetics is observed, with little change in Vₘₐₓ (Table I). Thus, l-lysine acts solely as a heterotrophic positive effector with these substrates when 6MPH₄ is used as cofactor. It was previously reported that l-lysine could act as an “activator” with tryptophan as substrate when 6MPH₄ was used as cofactor 11. This observation was based on the use of a presumably subsaturating concentration of tryptophan (3 mM). We have found that the apparent Sₘₐₓ for tryptophan under these conditions is approximately 5 mM (Table I). Thus, the previously described “activation” of the tryptophan-dependent phenylalanine hy-

![Fig. 1. Kinetic response of phenylalanine hydroxylase to the diastereomers of 3-phenylserine. Phenylalanine hydroxylase activity was measured in 0.1 M potassium phosphate, pH 6.8, at 25 °C, by following the oxidation of NADH at 340 nm in an assay coupled with dihydropteridine reductase. All samples contained 160 µM 6MPH₄ as cofactor. Under these conditions, a short lag is noted, and the rates illustrated are steady state values. The lag can be eliminated by preincubation of the hydroxylase with the amino acid, with no apparent effect on the kinetic parameters. □, erythro-3-phenylserine; ○, threo-3-phenylserine.](http://www.jbc.org/)

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droxylase activity with 6MPH, in the presence of lysolecinth can be accounted for solely by an alteration from a sigmoid to a hyperbolic kinetic response.

*Erythro*-phenylserine gives a readily observable initial rate with native phenylalanine hydroxylase in the presence of the natural cofactor, BH$_2$° (Fig. 2). Native phenylalanine hydroxylase exhibits a sigmoid response to variations in concentration of the *erythro* isomer ($n_H = 2$). A 26-fold increase in $V_{max}$ is observed when the native enzyme is preincubated with 10 mM *erythro*-phenylserine prior to subsequent assay with this same isomer. In addition, preincubation of the hydroxylase with the *erythro* isomer results in a hyperbolic response to alterations in the concentration of *erythro*-phenylserine ($n_H = 1$). These data differ from those obtained with phenylalanine, since with this substrate the hydroxylase appears to retain a sigmoid kinetic profile even after activation by preincubation (9, 10, 17). However, we have preliminary evidence which suggests that the observed retention of sigmoid response after preincubation of the enzyme with phenylalanine may be due to rapid loss of the activated state at low phenylalanine concentrations. When very rapid initial rate measurements are made (within 5 s of initiation of the reaction), a considerably diminished sigmoid response to phenylalanine is observed. Below a concentration of about 4 mM neither tryptophan nor *threo*-phenylserine give significant activity with native phenylalanine hydroxylase and the natural cofactor, BH$_2$. Either preincubation of the enzyme with high concentrations (20 mM) of these amino acids (17) or the presence of 1 mM lysolecinth is required to observed readily measurable substrate activity with BH$_2$ as cofactor. The kinetic parameters for the phenylalanine hydroxylase-catalyzed reaction with phenylalanine, tryptophan, and the phenylserines are summarized in Table I.

In order to identify the product of the phenylserine-dependent phenylalanine hydroxylase reaction, changes in the aromatic region of the UV spectrum were followed, in a manner similar to that described in Ref. 21. An increase in absorbance at 275 nm was observed with both *erythro* - and *threo*-phenylserine which suggested that both isomers were being hydroxylated. Subsequent treatment of these reaction mixtures with nitrosonaphthol reagents (33) resulted in a positive color reaction with both isomers. This implies $p'$-hydroxylation, since the nitrosonaphthol reaction is quite specific for $p'$-phenols (33). That the major product of hydroxylation of either phenylserine isomer is the corresponding $4'$-hydroxyphenylserine was confirmed by the use of periodate oxidation (34) of the reaction mixtures. This oxidation resulted in the formation of $p'$-hydroxybenzaldehyde as determined from (a) the large increase in absorbance at 330 nm in 3 M NH$_3$OH and (b) reverse phase high performance liquid chromatography (C$_8$ column; 20% methanol) which showed a peak that co-migrated with authentic standards.

Under certain conditions, phenylalanine hydroxylase exhibits an “uncoupled” reaction with nonphysiological substrates, a reaction in which more tetrahydropterin is oxidized than hydroxylated product formed (13, 36). For example, with tryptophan as substrate, only 1 mol of 5-hydroxytryptophan is produced for approximately every 3 mol of tetrahydropterin consumed (13) (Table II). The hydroxylase reaction is tightly coupled with either 6MPH$_1$, or BH$_2$ as cofactor when either *erythro*-phenylserine or phenylalanine is used as substrate. In contrast, when *threo*-phenylserine is used, the reaction is only loosely coupled (Table II). Of considerable interest is the observation that the reaction with the *threo*-isomer is far more uncoupled when 6MPH$_1$ is used as cofactor than when BH$_2$ is employed. A somewhat similar result has been recently reported for $o$-methylphenylalanine (37). These coupling ratios are not significantly altered by the presence of lysophosphatidylcholine.

**Aromatic Amino Acids As Activators of Phenylalanine Hydroxylase**—Preincubation of phenylalanine hydroxylase with the substrate, phenylalanine, results in a dramatic increase in initial velocity when measured in the presence of BH$_2$ (6, 9). The relationship between the extent of activation and the

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**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of kinetics</th>
<th>$K_m$ (S$H_m$) $\mu$M</th>
<th>$V_{max}$ $\mu$mol min$^{-1}$ mg$^{-1}$</th>
<th>Type of kinetics</th>
<th>$K_m$ (S$H_m$) $\mu$M</th>
<th>$V_{max}$ $\mu$mol min$^{-1}$ mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6MPH$_1$</td>
<td></td>
<td></td>
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<tr>
<td>1-Phe</td>
<td>H</td>
<td>0.5</td>
<td>7.5</td>
<td>H</td>
<td>0.5</td>
<td>7.5</td>
</tr>
<tr>
<td>1-Phe (preincubated)</td>
<td>H</td>
<td>0.5</td>
<td>7.5</td>
<td>H</td>
<td>0.5</td>
<td>7.5</td>
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<tr>
<td>1-Tlp + LPC</td>
<td>S ($n_H = 3$)</td>
<td>5</td>
<td>1</td>
<td>S ($n_H = 3$)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>1-Tlp + LPC</td>
<td>H</td>
<td>4</td>
<td>1</td>
<td>H</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><em>threo</em>-L-Phenylserine</td>
<td>S ($n_H = 3$)</td>
<td>4.8</td>
<td>1.4</td>
<td>S ($n_H = 3$)</td>
<td>4.8</td>
<td>1.4</td>
</tr>
<tr>
<td><em>threo</em>-L-Phenylserine</td>
<td>H</td>
<td>2</td>
<td>1.4</td>
<td>H</td>
<td>2</td>
<td>1.4</td>
</tr>
<tr>
<td><em>erythro</em>-L-Phenylserine</td>
<td>H</td>
<td>1.2</td>
<td>1.2</td>
<td>S ($n_H = 2$)</td>
<td>2</td>
<td>0.05 (DL)$^e$</td>
</tr>
<tr>
<td><em>erythro</em>-DL-Phenylserine</td>
<td>H</td>
<td>2.6</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(preincubated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>erythro</em>-DL-Phenylserine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>+ LPC</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*H, hyperbolic; S, sigmoid.

$^a$ $V_{max}$ values are in $\mu$mol min$^{-1}$ mg$^{-1}$.

$LPC$, lysophosphatidylcholine or "lysolecithin."

$^d$ S.I., substrate inhibition.

$^e$ N.R., "negligible reaction."

$^f$ These results with BH$_2$ are from Ref. 2.

$^g$ These experiments were performed with the racemic amino acid.

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1 The naturally occurring (6R)-isomer of BH$_2$ was used in all studies in this report, as there are significant differences in the cofactor properties of the isolated diastereomers as well as the racemic mixture.
preincubated with the amino acid show a substantial loss of activity.

Assays were carried out in 0.1 M potassium phosphate, pH 6.8, at 25 °C, with 60 mM (6R)-BH₄ as cofactor. The values for Vₒ are calculated from initial rates, since the samples which have been preincubated with the amino acid show a substantial loss of activity with time, whereas the native enzyme shows a continual increase in reaction velocity. For the preincubated series, phenylalanine hydroxylase; the native enzyme on the BH₄-dependent activity of phenylalanine hydroxylase with 

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Fig. 2. Effect of preincubation with erythro-3-phenylserine on the BH₄-dependent activity of phenylalanine hydroxylase. Assays were carried out in 0.1 M potassium phosphate, pH 6.8, at 25 °C, with 60 mM (6R)-BH₄ as cofactor. The values for Vₒ are calculated from initial rates, since the samples which have been preincubated with the amino acid show a substantial loss of activity with time, whereas the native enzyme shows a continual increase in reaction velocity. For the preincubated series, phenylalanine hydroxylase; the native enzyme on the BH₄-dependent activity of phenylalanine hydroxylase.

Concentration of phenylalanine which is used in the preincubation is sigmoid (n_H = 3), with a half-maximal value of 0.06 mM phenylalanine at pH 6.8 and 25 °C. Under these same conditions, both tryptophan (17) and threo-phenylserine are very poor activators, with at least 100-fold higher concentrations required for activation (Table III). In contrast, the erythro isomer is a good activator, approximately 30% as effective as phenylalanine. Thus, there is about a 30-fold difference in the ability of the diastereomers of phenylalanine hydroxylase with 4 mM L-tryptophan to activate phenylalanine hydroxylase. Instead, these compounds act as heterotropic positive effectors (38, 39), presumably by interacting with the enzyme molecule at a site distinct from the catalytic site. Similar results have been reported by Coulson et al. (40) with both p-fluorophenylalanine and tryptophan.

In contrast to the results obtained with the native enzyme, the addition of tryptophan to reaction mixtures which contain phenylalanine and an activated form of phenylalanine hydroxylase results in inhibition of enzyme activity (Table IV). It is of interest that with respect to the effects of tryptophan on the activity of the hydroxylase toward phenylalanine, the enzyme appears to be in an activated state in the presence of 6MPH₄ as cofactor. Thus, the addition of tryptophan to reaction mixtures which contain phenylalanine and 6MPH₄
The inhibition observed with racemic threo-phenylserine is considerably more complex. The threo-diastereomer shows good agreement with its apparent kinetic parameter. The observed inhibition for the racemic compound of about 10 mM.

**DISCUSSION**

Several groups of investigators have proposed that rat liver phenylalanine hydroxylase possesses two distinct sites capable of binding the substrate phenylalanine, one of which is the catalytic site and the other a regulatory or activator site (5–12, 17). These proposals, for the most part, have been based on studies of the kinetic response of the enzyme to phenylalanine. The model has received additional support from the observation that native phenylalanine hydroxylase can bind more than 1 mol of phenylalanine/mol of subunit, whereas enzyme which has been desensitized, so that it no longer expresses regulatory characteristics, binds only 1 mol of phenylalanine/mol of subunit (3). In order to further characterize and clarify the role of the multiple binding sites for phenylalanine on the hydroxylase, we have been screening compounds for their ability to activate the native hydroxylase, as well as to serve as substrates in the hydroxylation reaction. One analog of phenylalanine which we expected would be useful in these studies is 3-phenylserine (β-hydroxyphenylalanine).

Phenylalanine hydroxylase exhibits remarkably different responses to the diastereomers of phenylserine. In most respects, the *erythro* isomer mimics phenylalanine in its interactions with the enzyme, whereas the *threo* isomer possesses quite different properties, similar in some respects to those exhibited by tryptophan. The most dramatic difference between the diastereomers of phenylserine is observed in their relative abilities to activate phenylalanine hydroxylase upon preincubation. The *erythro*-phenylserine is approximately 30-fold more effective in this respect than the *threo* isomer. In contrast, the apparent *Kₐ* values for the two isomers (with 6MPP as cofactor) differ by less than a factor of two. These results are consistent with the idea that the hydroxylase has a regulatory or activator site, distinct from...
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activate phenylalanine hydroxylase is apparently responsible for the difference in the kinetic response to these compounds in the presence of 6MPH₄ as cofactor (Fig. 1). For a simple two-site model, with ordered binding at both a regulatory and a catalytic site required for catalysis (Equation 1), computer simulations which we have carried out with the use of the MLAB curve-fitting system (44) on the DEC-10 computer at the National Institutes of Health demonstrate that positive cooperativity will begin to be observed when \( K_a/K_L < 3 \), while simple hyperbolic kinetics will be obtained when \( K_a/K_L > 3 \).

\[
\frac{S}{K_a} + \frac{S}{K_L} \rightarrow \frac{V_{max}}{K_a} \rightarrow \frac{P}{K_a - K_L}
\]

where PAH is phenylalanine hydroxylase, S is the substrate, phenylalanine, and P is the product, tyrosine. We then examined our data with \( K_a \) values taken from Table I and approximate \( K_L \) values taken from the concentration of amino acid giving half-maximal activation from Table III. Since, for threo-phenylserine, \( K_a/K_L = 0.3 \), it is not surprising that a sigmoid response is observed. For erythro-phenylserine, \( K_a/K_L = 10 \), and thus simple Michaelis-Menten kinetics would be expected and are, indeed, observed. Similarly, when phenylalanine is used as a substrate, with 6MPH₄ as cofactor, \( K_a/K_L = 15 \), and the kinetic response is hyperbolic. However, when BH₄ is used as cofactor, with phenylalanine as substrate, \( K_a/K_L = 1 \) (using the \( K_a \) for lysophosphatidylcholine-activated enzyme as the "intrinsic" \( K_a \) value), sigmoid kinetics is predicted by this model and is observed.

While the "intrinsic" \( K_a \) values (i.e., the \( K_a \) observed with the completely desensitized enzyme) for the phenylserines are similar, both with 6MPH₄ and BH₄ as cofactor (Table I), it is not clear how well the observed \( K_a \) reflects the true binding at the active site, particularly in view of the high degree of uncoupling observed with the threo isomer. The difference in coupling for the two phenylserines suggests that there must be significant differences in the structures of the respective ternary amino acid-tetrahydropterin-O₂ complex, such that a productive arrangement is not obtained with the threo isomer. This again may be due to the different preferred conformations of the two isomers. Furthermore, the strong dependence of the coupling ratio for threo-phenylserine on the structure of the cofactor suggests that there may be differences in the stabilities of these complexes as well. The difference between the isomers of phenylserine is also observed when these compounds are used as effectors rather than activators. L-Phenylalanine is known to exert a positive cooperative effect on native phenylalanine hydroxylase in the presence of the natural cofactor, BH₄. From binding data, this is true even in the absence of tetrahydropterin cofactor (3). A previous report has shown that D-phenylalanine, although not hydroxylated by the enzyme under the conditions of the study, can exert a positive heterotropic effect (10). D-Phenylalanins has also been found to be a potent activator of the enzyme (17).

We have found that L-tryptophan, at concentrations of less than 5 mM, also exerts a positive heterotropic effect on the hydroxylase in the presence of phenylalanine and BH₄. At these same concentrations, L-tryptophan is unable to serve as substrate or to activate the enzyme upon preincubation. These observations suggest that L-tryptophan (and presumably D-phenylalanine (10, 17)) is able to interact with an allosteric site on the hydroxylase that is distinct from the catalytic site. This interaction results in a desensitization of the enzyme so that phenylalanine no longer acts as a heterotropic effector. The direct binding data shown in Fig. 4 provide strong support for this idea. In the absence of D-tryptophan, phenylalanine hydroxylase binds 1.5 mol of phenylalanine/mol of M₄ =

The difference in the ability of the phenylserine isomers to

The catalytic site, the former site having the greater binding specificity. The same conclusion was reached from recent studies of other novel amino acid substrates and activators of the enzyme (17). This preference for the erythro isomer might

stabilities of these complexes as well. The difference between the isomers of phenylserine is also observed when these compounds are used as effectors rather than activators. L-Phenylalanine is known to exert a positive cooperative effect on native phenylalanine hydroxylase in the presence of the natural cofactor, BH₄. From binding data, this is true even in the absence of tetrahydropterin cofactor (3). A previous report has shown that D-phenylalanine, although not hydroxylated by the enzyme under the conditions of the study, can exert a positive heterotropic effect (10). D-Phenylalanins has also been found to be a potent activator of the enzyme (17). We have found that L-tryptophan, at concentrations of less than 5 mM, also exerts a positive heterotropic effect on the hydroxylase in the presence of phenylalanine and BH₄. At these same concentrations, L-tryptophan is unable to serve as substrate or to activate the enzyme upon preincubation. These observations suggest that L-tryptophan (and presumably D-phenylalanine (10, 17)) is able to interact with an allosteric site on the hydroxylase that is distinct from the catalytic site. This interaction results in a desensitization of the enzyme so that phenylalanine no longer acts as a heterotropic effector. The direct binding data shown in Fig. 4 provide strong support for this idea. In the absence of D-tryptophan, phenylalanine hydroxylase binds 1.5 mol of phenylalanine/mol of M₄ =

\[
\text{Scheme 1}
\]

Phenylalanine has been shown by NMR measurements (41) to exist as a mixture of three rotational conformers in solution, depicted as Newman projections in Scheme 1, Structures IA–IC. Structure IA, with the phenyl ring and carboxyl trans, is the lowest energy conformation and represents about 48% of the molecules, while IB, with the phenyl ring and amino trans, contributes about 24%, and IC, with both the amino and carboxyl gauche to the ring, about 28%. In contrast, NMR measurements (42) of proton coupling constants for the phenylserines suggest that the threo isomer exists predominantly as IA, in which the phenyl ring and the carboxyl group are trans (analogous to the phenylalanine conformation IA), while the erythro isomer exists as IIB, with the phenyl and amino group trans (analogous to IB). Thus, one can consider the isomers of phenylserine to be conformationally restricted analogs of phenylalanine. The high specificity of the erythro isomer for activation suggests that the regulatory site preferentially binds phenylalanine in the conformation IB. This would appear to violate the general rule that enzymes bind to the lowest energy conformations of substrates; however, a phenylalanine derivative which is an inhibitor of thermolysin has recently been shown to bind in a high energy conformation (43).

The difference in the ability of the phenylserine isomers to

\[
\begin{align*}
\text{IA (48\%)} & \quad \text{IB (24\%)} & \quad \text{IC (26\%)} \\
\text{IIA (>95\%)} & \quad \text{IIB} & \quad \text{IIIC} \\
\text{IIIA} & \quad \text{IIIB (>95\%)} & \quad \text{IIIC}
\end{align*}
\]

1 We have confirmed the results in Ref. 42 by measurement of proton NMR coupling constants. In addition, we have measured the H-13C coupling constants between the α-proton and the β-carboxyl, which unequivocally establish the above assignment of the conformations of the phenylserines (R. Phillips, V. Labroo, and H. Yeh, unpublished observations).
50,000 subunit and exhibits high cooperativity in binding. This greater than stoichiometric binding of phenylalanine suggests that the native enzyme possesses both a catalytic site and an additional, presumably regulatory, binding site for substrate. In the presence of 5 mM tryptophan, the extent of phenylalanine binding is reduced to about 1 mol of phenylalanine/mol of M₄ = 50,000 subunit, and the cooperativity of binding is markedly reduced. These binding data bear a striking resemblance to those obtained with phenylalanine hydroxylase which has been activated and desensitized by reaction with the sulfhydryl reagent N-ethylmaleimide (3).

These observations appear to contradict a previous report (12) which concluded that tryptophan was unable to bind to the regulatory, or activator, site of the hydroxylase, although it was able to bind to the catalytic site, both as a substrate and as a competitive inhibitor for phenylalanine. These conclusions were based mainly on kinetic data obtained with the synthetic cofactor, 6MPH. We find that phenylalanine hydroxylase appears to be in a desensitized and activated state in the presence of synthetic cofactors such as 6MPH. We also find, in agreement with the previous report (12), that with 6MPH as cofactor, tryptophan can interact with the catalytic site of the enzyme as a competitive inhibitor for phenylalanine. Tryptophan is also able to inhibit the hydroxylation of phenylalanine by the hydroxylase which has been desensitized (and activated) by sulfhydryl modification. Thus, with activated phenylalanine hydroxylase, tryptophan is able to bind to the catalytic site of the enzyme and thereby act as an inhibitor of phenylalanine hydroxylation, or, when tested by itself, it can serve as substrate. However, with the native enzyme, which still possesses a regulatory site, tryptophan is able to function as a positive heterotropic effector for phenylalanine hydroxylase.

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