Hydroxylation of 4-Methylphenylalanine by Rat Liver Phenylalanine Hydroxylase*

Hans-Ulrich Siegmund and Seymour Kaufman

From the Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20892

(Received for publication, April 9, 1990)

Rat liver phenylalanine hydroxylase that has been activated with lyssolecithin catalyzes the hydroxylation of 4-methylphenylalanine in the presence of a pterin cofactor. Two products, 4-hydroxymethylphenylalanine and 3-methyltyrosine, can be detected. The total amount of amino acids hydroxylated is equal to the amount of tetrahydropterin oxidized. Isotopic labeling studies with \(^{15}O_2\) and \(H_2^{18}O\) show that the hydroxyl groups of both products are derived from molecular oxygen and not from water. Results obtained with \(^2H\)-labeled substrates support the conclusion that these products are formed via different mechanistic pathways.

Our previous investigations on substrate analogs, as well as the present results, indicate that a highly reactive oxygen-containing intermediate, such as an enzyme-bound iron-oxo compound, must be the hydroxylating species. Our present results could stimulate further discussion of the possibility that the reaction mechanism for the "NIH-shift" of the methyl group may not involve the spontaneous opening of an epoxide intermediate.

Phenylalanine hydroxylase catalyzes the oxidation of phenylalanine to tyrosine by molecular oxygen with tetrahydrobipterin as its cofactor (1). It has been postulated that the mechanism of the hydroxylation proceeds through an epoxide intermediate that, in a similar way as has been described for many cytochrome-dependent oxygenases, rearranges to the corresponding phenol and may cause an observable "NIH-shift" (2). On the other hand, an ionic intermediate that also leads to the same kind of rearrangement has also been postulated (3, 4). Substituent shifts occur with several 4-substituted phenylalanines, e.g. 4-chlorophenylalanine (5) or 4-methylphenylalanine (6). The latter compound was found to be hydroxylated by both bacterial (Pseudomonas species) and rat liver phenylalanine hydroxylase to two major products, 4-hydroxymethylphenylalanine and 3-methyltyrosine, and one minor one, 3-hydroxy-4-methylphenylalanine (6-p-methyl-tyrosine). For the bacterial enzyme, the ratio of these products was reported to be 15:11:1; for the liver enzyme, the ratio was reported to be similar (6) (see Fig. 1). Whereas the formation of the two isomeric methylated tyrosines is consistent with the understanding of the hydroxylation process, the occurrence of a hydroxymethyl amino acid, which is even the main product, is rather surprising. Later studies carried out with lyssolecithin-activated phenylalanine hydroxylase in this laboratory (7) showed that phenylalanine hydroxylase can also catalyze the hydroxylation of a number of other nonaromatic amino acids such as norleucine. Thus, a closer look at the reaction of 4-methylphenylalanine, in particular, a determination of the source of the oxygen in the newly synthesized 4-hydroxymethylphenylalanine, seemed capable of providing additional information that might contribute to our understanding of hydroxylations catalyzed by phenylalanine hydroxylase.

EXPERIMENTAL PROCEDURES

Reagents were obtained from commercial sources and were of the highest available purity. Tetrahydrobipterin and 6-methyltetrahydroydropterin were purchased from B. Schirks Laboratories, Jona, Switzerland.

Enzymes

Catalase and superoxide dismutase were obtained from Boehringer Mannheim. Phenylalanine hydroxylase was prepared from rat liver by a modification of the procedure (8) of Shimanto et al. (9). Dihydropseudotripterine reductase was purified from sheep liver according to a published procedure (10).

Organic Synthesis

Since, with the exception of phenylalanine and tyrosine, none of the amino acids used in this study was commercially available at the time this work was carried out, they had to be synthesized. A small sample of 4-methylphenylalanine was a gift from Dr. G. Plaut, Temple University, Philadelphia. The amino acid syntheses were carried out following the procedure of O'Donnell et al. (11) with the use of commercially available (Aldrich) \(\alpha\)-chloroacetyl, 1,4-benzenedimethanol, 3-methoxy-4-methylbenzoic acid, and 3-methyl-4-anisaldehyde as starting materials that were reacted with a glycine ester Schiff base to form 4M-Phe, \(^4\text{H}-\text{Phe}, \text{3H}-\text{M-Phe}, \text{and} \text{3M-}\text{Tyr}, \text{respectively.} \text{The last two compounds had to be reduced first by standard methods, and the alcohols obtained were eventually converted to the corresponding benzyl bromides with phosphorus tribromide, 1,4-Benzendimethanol was transformed to the monobromide by a method described in (12), and the remaining hydroxyl group was blocked by 3,4-diiodopropan to obtain the synthon necessary for 4-hydroxymethylphenylalanine. TLC and capillary GC were used to monitor the reactions and purifications. The amino acids obtained were separated from glycine that is formed as a side product from excess Schiff base by crystallization from water or by column chromatography with the use of Sephadex LH-20 and 10 mM HCl in methanol as eluent. The structures and the purities of the DL-amino acids were confirmed by 200 MHz \(^1\text{H} \text{NMR (Varian XL-200), by GC/MS, and by HPLC (see}}

1 The abbreviations used are: 4HM-Phe, 4-hydroxymethylphenylalanine; 4M-Phe, 4-methylphenylalanine; 3HM-Phe, 3-hydroxy-4-methylphenylalanine; 3M-Tyr, 3-methyltyrosine; 6MPha, 6-methyltetrahydropterin; DMPha, 6,7-dimethyltetrahydropterin; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; MSTFA, N-methyl-N-(trimethylsilyl) trifluoroacetamide; TMS, trimethylsilyl; BH4, tetrahydrobiopterin. The following NMR abbreviations are used: s, singlet; d, doublet; m, multiplet.

* 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.

§ To whom correspondence should be sent.
Hydroxylation of 4-Methylphenylalanine

The following procedures were used to analyze mixtures of amino acids formed during enzymatic incubations.

HPLC Analysis and Separation—For this procedure, a reverse phase (RP-18, Supelco, Bellefonte, PA) column was used. The elution of the amino acids was carried out isocratically with 50% methanol in water, buffered with 10 mM ammonium acetate, pH 5.7. The elution was followed with a Waters model 440 UV monitor at 254 nm.

GC Analysis—These analyses were carried out by an extension of the method of Pellizzari et al. (14) with the use of Dowex 50 to separate the amino acids from phosphate and other contaminants. After lyophilization, the sample was taken up in 50 μl of 20% methanol, filtered through a 0.45-μm nylon filter, and finally injected in three portions into the HPLC to separate the amino acids and remove interfering impurities from the enzyme assay. After another lyophilization, the samples were silylated with 25 μl of MSTFA (Aldrich) for 1 h at 60 °C. The chromatograph used was a Shimadzu GC-mini-1 with a 15-m SPB-1 capillary column (inner diameter, 0.25 mm, Supelco, Bellefonte, PA) at a temperature program from 100 to 250 °C, 10 °C/min.

Mass Spectroscopy—These measurements were carried out on an LKB 2091 attached to a Shimadzu GC-9A, using a 25-m SE-54 capillary (inner diameter, 0.25 mm) column, at a temperature program from 100 to 300 °C, 15 °C/min. Electron impact ionization was performed at 70 eV.

Determination of Isotope Incorporation—Incorporation was measured by determination of the mass spectra of the silylated amino acids and comparison with the spectra of the synthetic standards. Generally, a mixture of the disilylated and trisilylated amino acids was separated by the capillary column. Either one of these derivatives was suitable. The highest mass observed was generally M⁺ – 15, generated by a loss of a methyl group. Because of the noticeable natural abundance of heavier silicon (²⁹Si 4.67%, ³⁰Si 3.10%), the calculations for ²⁰O incorporation had to be corrected by subtracting these contributions. Preferentially, fragment ions that partially lost the alanine side chain (and with it two or silicon atoms) were chosen for the calculation. If more than one ion was suitable, the obtained values were averaged.

Amino Acid Quantitations—These determinations were performed on a Waters Associates Fico-Tag gradient HPLC system and a derivatization procedure with phenylisothiocyanate. The enzyme reactions were run as described below (1-ml total volume) and stopped with an equal volume of methanol. 20–100-μl aliquots were taken for the derivatization procedure and processed as recommended by the Waters Fico-Tag manual. Blank reactions with either boiled enzyme or without amino acid substrate but with undeuterated phenylalanine hydroxylase were run to cancel out peaks related to other compounds in the reaction mixture. The detection limit was less than 0.2 nmol/ml for each amino acid.

The amount of 3-methyltyrosine formed was also quantitated with the nitrosonaphthol method (15).

Enzymatic Incubations—The hydroxylations catalyzed by phenylalanine hydroxylase were carried out at 25 °C in a total volume of 1, 2, or 3 ml, as specified. The mixtures contained the following components (unless otherwise stated): 100 mM potassium phosphate buffer, pH 6.8; 0.5 mM lysolecithin; 256 μM NADH; 0.2 mg/ml catalase; 80 μg/ml superoxide dismutase; 10 μg/ml dihydroterephendyl reductase; 1 mM amino acid substrate. The reactions were started by the addition of 6MPPH, to a final concentration of 250 μM.

The enzyme reactions in which ³⁰O₂ was used were carried out in a closed cuvette that could be evacuated. The total inner volume was 22 μl, and the cuvette was filled with 3 ml of liquid volume. The enzyme mixture was evacuated with a vacuum pump until boiling of the mixture just started, flushed with argon, and shaken gently. This procedure was repeated six times. Argon was purged of remaining oxygen by passing it through a freshly prepared alkaline solution of pyrogallol (16). Finally, the cuvette was connected to the vacuum.
pump one more time, ¹⁸O₂ was allowed to enter the system, and the gas phase was mixed with the liquid phase by agitation. The reaction was then started by the addition of the tetrahydropterin from a side arm of the cuvette. Alternatively, to test the efficiency of oxygen removal, the pterin was added first (while under argon), and then labeled oxygen was added after the remaining oxygen from air was used up, i.e., the absorbance at 340 nm became constant.

The reactions were stopped by the addition of 10 ml of 1 N acetic acid, and the resulting mixture was analyzed according to the following procedures: (a) Dowex 50 ion exchange chromatography (14); (b) HPLC with the use of a reverse phase column; the amino acids were collected separately; (c) silylation with 25 μl of MSTFA; (d) analysis by the use of GC/MS.

The enzyme reactions in the presence of H,¹⁸O were carried out in ordinary cuvettes but worked up the same way afterward. The same procedure was used when incubations were carried out with deuterated 4M-Phe and the deuterium content of the hydroxylation products was measured.

RESULTS AND DISCUSSION

Optimization of the Reaction Conditions—As described in their original paper, Daly and Guroff (6) carried out their experiments with purified bacterial phenylalanine hydroxylase and a rather crude rat liver phenylalanine hydroxylase preparation. For our experiments, essentially pure phenylalanine hydroxylase was isolated from rat liver by a combination of two methods, described previously as a hybrid procedure (8), which involves ethanol and ammonium sulfate precipitation steps and two column chromatography steps with the use of phenyl-Sepharose and DEAE-cellulose (9). For reasons that are not clear, when tested under the described incubation conditions our enzyme preparation showed very low activity toward 4M-Phe. In order to get satisfactory reaction rates and product formation, the conditions described originally by Daly and Guroff were modified according to conditions worked out in this laboratory for other nonnatural amino acid substrates. The following modifications were used. (a) Phenylalanine hydroxylase activated by lysolecithin (17) was used in all experiments. (b) 6MPh, a cofactor with higher activity than DMPh, (18), was used. (c) Dihydropteridine reductase was added to regenerate the pterin cofactor. (d) Catalase and superoxide dismutase were added to decrease the concentration of peroxides, thereby minimizing the instability of the hydroxylation system. For certain comparative studies (see below), the natural cofactor, tetrahydrobipterin, was used instead of 6MPh. The reaction conditions are compared in Table I.

The kinetic characteristics of 4M-Phe were compared with those of phenylalanine in the presence of 6MPh. As can be seen in Table II, the $K_{max}$ is about 1.8-fold greater, and $V_{max}$ is about 50% that of phenylalanine.

The reaction is inhibited at higher concentrations (≥0.5 mM) of 6MPh. Thus, the concentration of the pterin used in the $K_v$ determination for the amino acids (and those of the deuterated substrates to be presented later on) was limited to 0.25 mM. The concentration of 4M-Phe was varied between 0.04 and 4 mM.

Analysis and Quantitation of the Products Formed—The enzyme reaction mixtures were analyzed by several methods for the formation of hydroxylated products to compare our results with the finding of Daly and Guroff (6) that bacterial and crude rat liver phenylalanine hydroxylase catalyze the conversion of 4-methylphenylalanine to 4-hydroxymethylphenylalanine, 3-methyltyrosine, and 3-hydroxy-4-methylphenylalanine (Fig. 1).

By several different analytical procedures, 4-hydroxymethylphenylalanine and 3-methyltyrosine were the only two hydroxylated products that were detected when pure lysolecithin-activated rat liver phenylalanine hydroxylase was incubated with 4-methylphenylalanine. The analytical methods used were gas chromatography (of silylated amino acids that were difficult with mass spectrometry), HPLC (with or without derivatization), and the colorimetric determination of 3-methyltyrosine by the nitrosanaphthol method. By isocratic reverse phase HPLC, only the educt and 4-hydroxymethylphenylalanine could be detected as separate peaks; other products were obscured because of interfering peaks caused by other compounds of the reaction mixture.

For the GC and GC/MS analysis, the method of Pellezzi et al. (14) was modified to enhance the sensitivity and reduce the interference by other compounds contained in the enzyme assay. An (isocratic) HPLC separation was introduced after the ion exchange chromatography (see "Experimental Procedures"), and the silylation was carried out with MSTFA instead of BSTFA. However, the derivatizations generally appeared to be not quantitative and usually led to mixtures of bis- and tris-trimethylsilyl amino acids that were difficult to compare with our authentic standards. Thus, this method only proved to be useful for qualitative analysis or in connection with mass spectrometry for the evaluation of labeling experiments. By GC and GC/MS analysis, beside the educt, only two products, 4-hydroxymethylphenylalanine and 3-methyltyrosine, could be detected. However, the silylated 4-hydroxymethylphenylalanine turned out to be very sensitive to moisture and had to be handled cautiously.

Finally, with the use of a gradient HPLC and a derivatiza-
tion procedure (with phenylisothiocyanate) that enhances both the separation and the sensitivity for amino acids, 3-hydroxy-4-methylnphenylalanine, the third (and minor) hydroxylation product found by Daly and Guroff (6), could still not be detected among the products formed by our hydroxylating system. Even when the sensitivity was improved by separating the contaminants of the enzyme reaction mixture in the same way as was done for mass spectroscopy, none of this substance could be detected. In this case, the detection limit was estimated to be about 25 pmol/ml. This negative result was independent of the pterin cofactor (6MPH, or BH$_4$) used.

We therefore conclude that 3-hydroxy-4-methylnphenylalanine is not an obligatory product of the enzymatic hydroxylolation of 4-methylnphenylalanine with rat liver phenylalanine hydroxylase. Although we do not know the reasons that we were unable to detect the formation of any 3-hydroxy-4-methylnphenylalanine with the pure rat liver phenylalanine hydroxylase preparations used in the present work, the explanation is probably related to the fact that the earlier experiments with the rat liver hydroxylase used rather crude fractions of the enzyme.

In an earlier discussion of the mechanism of enzyme-catalyzed hydroxylations of aromatic compounds (2) in which an arene oxide was postulated as the intermediate (see Fig. 3), the formation of both of the ring-hydroxylated species (3-methyltyrosine and 3-hydroxy-4-methylnphenylalanine) and the fact that the nonenzymatic decomposition of p-xylene 1,2-oxide, a synthetic model compound, leads to a similar product pattern and ratio as was reported for the hydroxylation of 4-methylnphenylalanine catalyzed by bacterial phenylalanine hydroxylase was presented as strong evidence supporting this pathway. The 3-hydroxylated product is believed to be formed by the opening of the arene oxide intermediate to the unsubstituted position of the aromatic ring. Thus, our results obtained with the rat liver enzyme (specifically, our failure to detect the formation of any 4-methyl-3-hydroxyphenylalanine) provide some support for the conclusion that the conversion of 4M-Phe to 3-substituted tyrosines involves a direct hydroxyl attack at the para position without the formation of any epoxide intermediate that rearranges spontaneously. These results with 4M-Phe are in accord with results obtained with the natural substrate phenylalanine, in which no m-tyrosine (i.e., 3-hydroxyphenylalanine) was found under conditions in which we could have detected an amount of this compound equal to less than 0.3% of the amount of tyrosine formed. Another possible mechanism suggested for the hydroxylation in the meta position, a 1,2-migration of the OH group after an initial attack in the para position, does not seem to be very likely either. A phenolic hydroxyl group is not one of the favored migrating residues compared with a

![Fig. 3. Proposed mechanisms for the phenylalanine hydroxylase-catalyzed hydroxylation of phenylalanine.](image)

**Table III**

**Amounts of substrate and reaction products detected after the reaction of 4-methylphenylalanine with phenylalanine hydroxylase in the presence of 6MPH.**

The nitrosonaphthol method was used to quantitate the amount of 3-methyltyrosine formed. The measured amounts of this amino acid are consistent with those determined by the HPLC method. Deviations up to 25% between results obtained by the two methods are probably due to the statistical error of the methods. With the use of an ion exchange amino acid analyzer with postcolumn derivatization, the values obtained deviated by 10% or less from the HPLC values. The total volume of each experiment was 1 ml, the incubation time was 17.5 min; 42 µg of phenylalanine hydroxylase was used. Other details are the same as under "Experimental Procedures." The final concentrations of 6MPH in experiments 1, 2, and 3 were, respectively, 250 µM, 100 µM, and 50 µM.

| Substance | Substrate and products | Hydroxylation product ratio$^a$ | Total | NADH consumed | Product ratio$^b$ |
|-----------|------------------------|-------------------------------|-------|---------------|-----------------|
| Exp. 1    |                        |                               |       |               |                 |
| 4HM-Phe   | 176                    | 4.09                          | 219   | 197           | 0.90            |
| 3M-Tyr    | 43                     |                               |       |               |                 |
| 4M-Phe    | 785                    |                               |       |               |                 |
| Exp. 2    |                        |                               |       |               |                 |
| 4HM-Phe   | 133                    | 3.80                          | 158   | 154           | 0.92            |
| 3M-Tyr    | 35                     |                               |       |               |                 |
| 4M-Phe    | 798                    |                               |       |               |                 |
| Exp. 3    |                        |                               |       |               |                 |
| 4HM-Phe   | 101                    | 3.74                          | 128   | 116           | 0.91            |
| 3M-Tyr    | 27                     |                               |       |               |                 |
| 4M-Phe    | 839                    |                               |       |               |                 |

$^a$The hydroxylation product ratio is the ratio of nmol of 4HM-Phe formed divided by the nmol of 3M-Tyr formed.

$^b$Product ratio is the ratio of nmol of NADH consumed divided by the sum of the nmol of hydroxylated products formed.

M. Davis and S. Kaufman, unpublished results.
methyl group. Moreover, the lack of product formation, especially 3,4-dihydroxyphenylalanine, when tyrosine is used as a substrate (19, 20), can be taken as additional evidence against a hydroxyl migration. On the other hand, it can always be postulated that phenylalanine hydroxylase from rat liver has a more "stringent" steric control compared with its bacterial counterpart, thus directing the arene oxide to open to the "natural direction" by catalyzing a hydroxyl migration. At present, we cannot rule out this possibility. It should be noted that the hydroxylation mechanism depicted in Fig. 3b, involving attack by a highly reactive oxygen species at the para position of the substrate to generate a carbonium ion species followed by an NIH-shift migration to form products, is analogous to the mechanism proposed for a bacterial non-heme iron monooxygenase acting on an olefin substrate (21, 22).

The quantitative analyses for some typical assays are shown in Tables III and IV. The results show that the hydroxylation reaction is tightly coupled, i.e. approximately one molecule of 4-methylphenylalanine is hydroxylated for each reduction equivalent consumed. In the case of BH4, the amount of products formed appears to be significantly higher than the amount of NADH consumed. We cannot explain this result unequivocally although an irreversible degradation of the pterin cofactor that is not regenerated after it is oxidized is a possibility.

The ratio of the two detected hydroxylation products (4HM-Phe/3M-Tyr) is higher than that reported by Daly and Guroff (6) for bacterial phenylalanine hydroxylase. It is possible that the use of lysolecithin-activated phenylalanine hydroxylase in the present experiments could explain these differences. It should also be noted that the pterin cofactor used seems to have a significant influence on this ratio. Thus, switching from 6MPH4 to BH4 changes this ratio from about 3.9 to 1.6 (4HM-Phe/3M-Tyr).

Substrate Activity of the Hydroxylation Products—With the use of the spectrophotometric assay for phenylalanine hydroxylase in which substrate-dependent oxidation of NADH is measured, we tested the hydroxylated products formed from 4-methylphenylalanine as possible substrates for the enzyme. We found that the hydroxylation products of 4-methylphenylalanine (including the third possible one, whose formation was not detectable, 3-hydroxy-4-methylphenylalanine) are able to function as substrates of phenylalanine hydroxylase. 3-Hydroxy-4-methylphenylalanine is the most active, and 4-hydroxymethylphenylalanine the least active (see Table V).

Neither one is as good a substrate as their educt, 4-methylphenylalanine, itself. The assay mixtures were analyzed for amino acids in the same way as described for the 4-methylphenylalanine reaction. 3-Methyltyrosine apparently is not hydroxylated further, which is consistent with the results obtained in the totally uncoupled reaction in the presence of tyrosine (19). With 4-hydroxymethylphenylalanine, two new peaks were found. Two different product peaks were found.

**Table IV**

**Amounts of substrate and reaction products detected after the reaction of 4-methylphenylalanine with phenylalanine hydroxylase in the presence of BH4.**

The amount of BH4 used was 100 nmol/ml, the amount of hydroxylase was 42 µg, and the incubation time was 12 min. The other conditions were the same as under "Experimental Procedures."

| Substance | Substrate and products | Total | NADH consumed | Product ratio* |
|-----------|------------------------|-------|---------------|---------------|
|           | nmol                   | Σ nmol| nmol          |               |
| Expt. 1.  |                        |       |               |               |
| 4HM-Phe   | 76                     | 122   | 93            | 0.76          |
| 3M-Tyr    |                        |       |               |               |
| 4M-Phe    | 859                    | 981   |               |               |
| Expt. 2.  |                        |       |               |               |
| 4HM-Phe   | 83                     | 137   | 103           | 0.75          |
| 3M-Tyr    | 54                     |       |               |               |
| 4M-Phe    | 835                    | 972   |               |               |

* The product ratio is the ratio of nmol of NADH consumed divided by the sum of the nmol of hydroxylated products formed.

**Table V**

**Relative substrate activities of the hydroxylation products compared with the natural substrate, phenylalanine.**

Conditions: 1 mM amino acid, 100 µM 6MPH4, (note that these are not Vmax conditions), 1-ml total incubation volume. Hydroxylase activity was measured as the amino acid-dependent oxidation of NADH (see "Experimental Procedures").

| Substrate | V0 | Activity compared with Phe |
|-----------|----|----------------------------|
|           | nmol/min | %               |
| 4HM-Phe   | 0.63     | 0.18            |
| 3M-Tyr    | 3.4      | 0.96            |
| 3M4M-Phe  | 13.0     | 3.7             |
| 4M-Phe    | 44.8     | 12.7            |

**Table VI**

**Incorporation of 18O into the hydroxylation products of 4-methylphenylalanine.**

The NADH consumptions and times marked as *** refer to the reaction with remaining unlabeled oxygen before the addition of the isotopic gas. Where the reactions were carried out in these two steps, experiments 1–3, the percentages of NADH consumption before and after 18O addition are given in parentheses. The degree of incorporation is calculated from the intensities of the ions that lost the alanine side chain, i.e. m/z = 193 and 195 (for 4M-Phe hydroxylation products) or m/z = 179 and 181 (for tyrosine). See "Experimental Procedures" for other details.

| Substrate | NADH oxidized | Time | Product | 18O incorporation |
|-----------|---------------|------|---------|------------------|
|           | nmol %        | min  |         | Actual           |
|           |               |      |         | Corrected        |
| Phe       | 120 (18)      | 4.5  | ***     | 79               |
|           | 640 (84)      | 9    | Tyr     | 89               |
| Phe       | 350 (43)      | 6    | ***     | 56               |
|           | 460 (57)      | 9    | Tyr     | 82               |
| 4M-Phe    | 230 (37)      | 8    | ***     | 82               |
|           | 390 (63)      | 13.5 | 4HM-Phe | 92               |
| 4M-Phe    | 760           | 10.5 | 3M-Tyr | 89               |
| 4M-Phe    | 780           | 11   | 4HM-Phe | 83               |
|           |               |      | 3M-Tyr | 85               |
when 3-hydroxy-4-methylphenylalanine was used as a substrate. One of the latter has the same retention time as 3-methyltyrosine on both amino acid analyzer and GC. Although this peak was also detectable to a minor extent in control reactions using boiled enzyme or when the pterin cofactor was omitted, most of it seems to originate from an enzyme-catalyzed reaction. In the absence of an unequivocal identification of this compound, speculation about the mechanism of this novel reaction would be premature. Neither one of the other "new" peaks (of either substrate) co-chromatographed with any of the available synthetic standards, and no further attempts were made to identify them.

It can be concluded that 4-hydroxymethylphenylalanine is not a precursor of 3-methyltyrosine or vice versa. It should be noted that the substrate activity of either of these amino acids seems to be too low to alter significantly the ratio of products formed from the hydroxylation of 4-methylphenylalanine which is present in large excess (compare the activities shown in Table V). Formation of tyrosine was not observed with any of these compounds, including 4-methylphenylalanine.

Incorporation of Labeled Oxygen into the Hydroxylation Products—It is known for rat liver phenylalanine hydroxylase (23) that the hydroxyl group in newly synthesized tyrosine is derived from molecular oxygen and not from water. Because of the different nature of a benzylic compared with an aromatic carbon, it was possible that this would not be true in the presumably nonelectrophilic hydroxylation that leads to 4-hydroxymethylphenylalanine. Accordingly, the source of the oxygen in the enzymatically synthesized 4-hydroxymethylphenylalanine was determined.

As can be seen from Table VI, both of the hydroxylation products that were detected show incorporation of labeled oxygen into their new hydroxyl groups when they are formed in the presence of $^{18}O_2$.

As a control experiment, the natural reaction, in which phenylalanine was used as the substrate, was carried out under the same conditions. The tyrosine formed was analyzed by the same method. The percentages of $^{18}O$ incorporation are in the same range as those found for the methylated compound and show a similar degree of isotopic enrichment of molecular oxygen. Attempts to remove the unlabeled oxygen more effectively were limited because the enzyme became inactivated when subjected to low pressure too often or too long.

From the comparison of the percentages of $^{18}O$ incorporation and NADH oxidation before the addition of labeled oxygen (values marked with "**"), we conclude that the oxygen of the newly introduced hydroxyl groups are derived exclusively from $O_2$.

To exclude the possibility that some of the oxygen in these products might be derived from water, $H_2^{18}O$ was added to the reaction mixture to get an enrichment of 50%. The analytical procedure and the interpretation of the mass spectrometric results were the same as for the experiments using labeled oxygen gas. The results in Table VII show 2.5% or less incorporation into the two detected products. These results confirm the conclusion that the source of the oxygen in the newly synthesized hydroxylated products is $O_2$. The small incorporation values observed are in the range of the systematic error, caused by noise background in the mass spectrometer, mostly originating from impurities eluted off the capillary column.

Reaction of Deuterated 4-Methylphenylalanines—Two deuterated 4-methylphenylalanines, d5-4-methylphenylalanine and d9-4-methylphenylalanine (see Fig. 2), were synthesized to be used as probes for obtaining further insights into the mechanisms of both the methyl and the aromatic ring hydroxylations.

Both amino acids are substrates for phenylalanine hydroxylase, with only small differences in the apparent kinetic constants. The following values were obtained in the presence of 250 μM 6MPH (1.0 ml final volume; see Table II for other experimental details; $V_{\text{max}}$ values are expressed as μmol min$^{-1}$ mg$^{-1}$): unlabeled 4-M-Phe, $K_m$, 0.9 mM, $V_{\text{max}}$, 2.0; d5-4-M-Phe, $K_m$, 0.4 mM, $V_{\text{max}}$, 1.0; d9-4-M-Phe, $K_m$, 0.8 mM, $V_{\text{max}}$, 1.8.

The small amount hydroxylated products are formed from the deuterated substrates as from the unlabeled substrate, but in a reversed ratio; 3-methyltyrosine is the major product, and 4-hydroxymethylphenylalanine is the minor product formed from both deuterated substrates. As can be seen in Tables III

---

**TABLE VII**

| Substrate | NADH | t (min) | Product | $^{18}O$ incorporation |
|-----------|------|--------|---------|-----------------------|
| 4M-Phe | 219 | 18 | 4HM-Phe | 2.5 |
| 4M-Phe | 236 | 21 | 4HM-Phe | 1.3 |

**TABLE VIII**

| Substance | Substrate and products detected | Hydroxylation product ratio | Total NADH used | Product ratio |
|-----------|---------------------------------|----------------------------|----------------|-------------|
| 4HM-Phe  | 29 | 0.25 | 144 | 0.96 |
| 3M-Tyr  | 115 | 988 | |
| d5-4M-Phe | 27 | 0.28 | 122 | 1.13 |
| d9-4M-Phe | 900 | 1022 | |

*Hydroxylation product ratio is the ratio of nmol of 4HM-Phe formed divided by the nmol of 3M-Tyr formed.

*The product ratio is the ratio of nmol of NADH consumed divided by the sum of the nmol of hydroxylated products formed.

---

**FIG. 4.** Reaction scheme for the hydroxylation of 4-methylphenylalanine.
and VIII, the hydroxylation product ratio (4HM-Phe to 3M-Tyr) changes from 3.9 for the unlabeled compound to 0.25 or 0.28 for the deuterio-5 or deuterio-9, respectively. The stoichiometry of NADH oxidized to hydroxylated products formed from the two deuterated substrates is about the same as that found for the unlabeled substrate, around 1.0.

The alteration of the hydroxylation product ratio necessitated an arithmetic separation of the isotope effects for both reaction pathways. The equations in Fig. 4 show the reaction resolved into the classic enzyme-substrate intermediates with their corresponding rate constants. Assuming that both the hydroxylation product ratio and the $V_{max}$ ratio are determined by the rate constants $k_{2a}$ and $k_{1a}$, the $V_{max}$ ratio can be expressed by

$$R = R \times k_{2a}$$

with

$$R_{1a} = k_{1a} + k_{2a} = (R + 1) \times k_{2a}$$

$$R_{max} = \frac{V_{max,a}}{V_{max,l}} = \frac{R_{1a} + 1}{R_{1a} + 1} = \frac{k_{2a}}{k_{1a}}$$

The isotope effect for 3M-Tyr ($k_{2a}$ ratio) can therefore be obtained by the experimentally determined ratios for $V_{max}$ and the hydroxylation product ratios $R$

$$k_{2a} = \frac{R_{max}}{R_{max,l}} = \frac{V_{max,a}}{V_{max,l}} = \frac{R_{1a} + 1}{R_{1a} + 1} = \frac{k_{2a}}{k_{1a}}$$

The rate constant ratio for 4HM-Phe can be calculated by equation

$$k_{2a} = \frac{R_{1a}}{R_{1a,l}} = \frac{k_{2a}}{k_{1a}}$$

With d5-4M-Phe as the substrate, an isotope effect of 8.0 for the formation of 4-hydroxymethylphenylalanine can be calculated whereas the ring hydroxylation pathway seems to be speeded up (by secondary isotope effects), resulting in an inverse deuterium effect of 0.5. This indicates that the large isotope effect for the methylhydroxylation was masked by both the variation in the reaction path and an inverse secondary isotope effect for the ring hydroxylation. It is known (24) that enzymes that catalyze different pathways show a “metabolic switching” that shifts the reaction away from the deuterated part of the molecule.

With d9-4M-Phe as the substrate, the deuteriation of the aromatic ring results in a further increase of the reaction rate, giving a total deuterium effect of 4.0 for the formation of 4-hydroxymethylphenylalanine and 0.3 for the formation of 3-methylyrosine.

We conclude that when hydroxylation of the methyl group is slowed down by isotope effects, the aromatic ring will react instead, leaving the overall velocity of the reaction close to the value for the unlabeled substrate.

The almost constant ratio of $V_{max}$ to $K_m$ allows the conclusion that once the substrate is bound, it reacts with a high probability (“high commitment to catalysis”) (25). This is consistent with observations that lyssolecithin-activated phenylalanine hydroxylase reacts with a remarkable variety of different substrates (7, 17), indicating the involvement of an oxidizing intermediate with an extraordinary potency, such as an iron-oxo compound (7, 19).

Mass spectrometric studies show that one deuterium from the methyl group is lost in the process of its hydroxylation whereas this group migrates with no loss of deuterium to the 3-position when the ring is hydroxylated (see Table IX). The fact that only one ring deuterium is lost can also be counted as evidence against an initial hydroxyl attack in the 3-position, eventually followed by a hydroxyl migration.

Concluding Remarks—In the present study, the two different hydroxylation products that are formed from 4M-Phe, namely 4HM-Phe and 3M-Tyr, are probably generated by two different reaction pathways. With respect to the mechanism of the ring hydroxylation, the lack of formation of 3-hydroxy-4-methylphenylalanine makes pathway a in Fig. 3 somewhat less likely because, as was pointed out by Kaubisch et al. (2), the spontaneous decomposition of an epoxide should proceed in either way whereas the migration of a methyl group could be the preferred way of pathway b. As mentioned earlier, however, an enzyme-controlled opening of a putative epoxide intermediate could explain the observed selectivity in the group migration. At the minimum, our present results should serve to reopen for further study the question of whether an epoxide is an obligatory intermediate in all hydroxylase-catalyzed ring hydroxylation reactions.

In this regard, it should also be noted that the recent demonstration that rat liver phenylalanine hydroxylase converts L-[2,5-H$_2$]phenylalanine to the corresponding 3,4-epoxide (28) indicates that formation of an epoxide is part of the enzyme’s repertoire. This finding, however, cannot be taken as evidence that an epoxide is a normal intermediate in the conversion of phenylalanine to tyrosine. Rather, it may mean that when the normal reaction pathway is blocked by a structural change in the substrate, the hydroxylase-catalyzed reaction is channeled, as a kind of metabolic switching, into an unusual pathway. It is known, for example, that when no aromatic hydroxylation is possible, as for example with methionine and noreleucine, phenylalanine hydroxylase switches to thioether oxygenation and alkyl oxygenation, respectively (7).

3-Methylyrosine, the “expected” product, seems to be formed according to the proposed mechanism for the hydroxylation of phenylalanine (and some of its 4-substituted derivatives), involving an NIH-shift of the intact methyl group. The lack of a large primary isotope effect, similar to the results with deuterophenylalanine (29), and the migration of the intact methyl group are consistent with this assumption.

By contrast, it is clear from the large primary isotope effect for hydroxylation of the methyl group that this pathway must involve an abstraction of a hydrogen as a rate-limiting step. Thus, it can be concluded that different mechanisms are involved for the phenylalanine hydroxylase-catalyzed aromatic and aliphatic hydroxylations. Our experiments with labeled oxygen show that there cannot be any stable carbenium ion intermediate (e.g. after a hydride abstraction) for this should be able to pick up an OH$^-$ ion from water. Instead, the observed “tight coupling” of hydroxylation and NADH consumption can be taken as evidence for a direct and irreversible attack of the hydroxylating agent. Therefore, our results suggest the presence of a highly reactive intermediate derived...
from molecular oxygen such as the previously postulated iron-oxo compound (7, 19) as part of the hydroxylation complex of phenylalanine hydroxylase. This intermediate is usually directed by the enzyme to the correct position of the substrate. Once activated with lysolecithin, that control is less stringent.

Finally, our finding that the ratio of products formed from the deuterium substituted substrates differs from that of the unlabeled substrate, together with the observation that there is little or no isotope effect in the phenylalanine-catalyzed hydroxylation of ring-directed by the enzyme to the correct position of the substrate.

Acknowledgments—We wish to thank Dr. Peter Backlund for his help with the amino acid analyses and Tysir Jaouni for recording the mass spectra.

REFERENCES

1. Kaufman, S. (1987) Enzymes 18, 217–282
2. Kaufman, S., Daly, J. W., and Jerina, D. M. (1972) Biochemistry 11, 3080–3088
3. Guroff, G., Daly, J. W., Jerina, D. M., Renson, J., Witkop, B., and Udenfriend, S. (1967) Science 157, 1524–1530
4. Lehmann, W. D., and Heinrich, H. C. (1986) Arch. Biochem. Biophys. 250, 180–185
5. Guroff, G., Kondo, K., and Daly, J. (1966) Biochim. Biophys. Res. Commun. 25, 622–628
6. Daly, J., and Guroff, G. (1968) Arch. Biochem. Biophys. 125, 136–141
7. Kaufman, S., and Mason, K. (1982) J. Biol. Chem. 257, 14667–14678
8. Kaufman, S. (1987) Methods Enzymol. 142, 3–17
9. Shiman, R., Gray, D. W., and Pater, A. (1979) J. Biol. Chem. 254, 11300–11306
10. Craine, J. E., Hall, E. S., and Kaufman, S. (1972) J. Biol. Chem. 247, 6082–6091
11. O'Donnell, M. J., Wojciechowski, K., Ghosez, L., Navarro, M., Sainte, F., and Antoine, J.-P. (1984) Synthesis 313–315
12. Kang, S., Kim, W., and Moon, B. (1985) Synthesis 1161–1162
13. Lewis, T. R., and Archer, S. (1951) J. Am. Chem. Soc. 73, 2109–2113
14. Pellizzari, E., Rising, C., Brown, J., Farmer, R. W., and Fabre, L. F. (1971) Anal. Biochem. 44, 312–316
15. Udenfriend, S., and Cooper, J. R. (1952) J. Biol. Chem. 196, 227–233
16. Metzger, H., and Muller, E. (1959) in Methoden der organischen Chemie (Houben-Weyl) Vol. 1/2, p. 332, Georg Thieme Verlag, Stuttgart, Federal Republic of Germany
17. Fisher, D. B., and Kaufman, S. (1973) J. Biol. Chem. 248, 4345–4353
18. Kaufman, S., and Levenberg, B. (1959) J. Biol. Chem. 234, 2683–2688
19. Davis, M. D., and Kaufman, S. (1989) J. Biol. Chem. 264, 8585–8596
20. Fisher, D. B., and Kaufman, S. (1973) J. Biol. Chem. 248, 4300–4304
21. Katopodis, A. G., Wimalasena, K., Lee, J., and May, S. W. (1984) J. Am. Chem. Soc. 106, 7928–7935
22. Colbert, J. E., Katopodis, A. G., and May, S. W. (1990) J. Am. Chem. Soc. 112, 3983–3986
23. Kaufman, S., Bridgers, W. F., Eisenberg, F., and Friedman, S. (1962) Biochem. Biophys. Res. Commun. 9, 497–502
24. Harada, N., Miwa, G. T., Walsh, J. S., and Lu, A. Y. H. (1984) J. Biol. Chem. 259, 3005–3010
25. Walsh, C. (1979) Enzymatic Reaction Mechanisms, p. 120, W. H. Freeman, San Francisco
26. Phillips, R. S., and Kaufman, S. (1984) J. Biol. Chem. 259, 2474–2479
27. Shiman, R., and Jefferson, L. S. (1982) J. Biol. Chem. 257, 839–844
28. Miller, R. J., and Benkovic, S. J. (1988) Biochemistry 27, 3658–3663
29. Abita, J.-P., Parniak, M., and Kaufman, S. (1984) J. Biol. Chem. 259, 14560–14566