Aromatic Amino Acid Hydroxylases

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Regulatory properties of phenylalanine, tyrosine and tryptophan hydroxylases

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I have previously discussed the idea that the three pterin-dependent enzymes, phenylalanine (EC 1.14.16.1), tyrosine (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4), constitute a family of enzymes whose individual members share many properties, both physical and catalytic (Kaufman, 1974). This notion has predictive value since there have been many instances where the discovery of a regulatory property for one of the enzymes, often first with phenylalanine hydroxylase, has led to the expectation that this property will be shared by the others, an expectation that frequently has been realized.

The general characteristics of this group of enzymes were first established from studies carried out with hepatic phenylalanine hydroxylase. The feature which distinguishes them from other enzymes, of course, is that they all require a naturally occurring unconjugated pterin. After showing that the hydroxylating system is complex, consisting of at least two essential enzymes and a non-protein cofactor, we isolated the cofactor from rat liver extracts on the basis of its ability to stimulate the conversion of phenylalanine to tyrosine in the presence of the two enzymes (Kaufman, 1958; Kaufman & Levenson, 1959), and proved that it is the reduced form of the unconjugated pterin, L-phenylalanine hydroxylase catalyses a coupled reaction in which L-phenylalanine is oxidized to L-tyrosine and the BH₄ is oxidized to an extremely unstable quinonoid dihydropterin; molecular oxygen is the electron acceptor and is normally reduced to water (Kaufman, 1964). The second essential enzyme of the system, DHPR, catalyses the reduction of the quinonoid dihydropterin back to the tetrahydro level, utilizing a reduced pyridine nucleotide as the electron donor (Kaufman, 1957; Kaufman & Fisher, 1974). This reaction serves to regenerate the active form of the pterin coenzyme and thus allows the coenzyme to function catalytically. Once the roles for BH₄ and DHPR has been established in the phenylalanine-hydroxylating system, it was shown that they are also essential components of the tyrosine- (Brenneman & Kaufman, 1964; Shiman et al., 1971) and tryptophan- (Friedman et al., 1972) hydroxylating systems.

An example of how our knowledge of the characteristics of these other two hydroxylating systems relies heavily on prior results obtained with the phenylalanine-hydroxylating system is provided by the fact that the oxidized pterin product that is formed during the course of tyrosine and tryptophan hydroxylation has not been rigorously identified as the quinonoid dihydropterin. That there can be little doubt that it is indeed the same product as that formed during phenylalanine hydroxylation is based on the demonstration, mentioned previously, that DHPR is an essential component of the tyrosine- and tryptophan-hydroxylating systems. It is also known that in the presence of BH₄ and DHPR, the stoichiometry of the enzymic conversion of tyrosine to 3,4-dihydroxyphenylalanine and of tryptophan hydroxylating system are phenylalanine hydroxylase and dihydropteridin reductase (DHPR) (EC 1.6.99.7). The reactions catalysed by these two enzymes with BH₄ as the coenzyme are illustrated in Fig. 1. As can be seen, phenylalanine hydroxylase catalyses a coupled reaction in which L-phenylalanine is oxidized to L-tyrosine and the BH₄ is oxidized to an extremely unstable quinonoid dihydropterin; molecular oxygen is the electron acceptor and is normally reduced to water (Kaufman, 1964). The second essential enzyme of the system, DHPR, catalyses the reduction of the quinonoid dihydropterin back to the tetrahydro level, utilizing a reduced pyridine nucleotide as the electron donor (Kaufman, 1957; Kaufman & Fisher, 1974). This reaction serves to regenerate the active form of the pterin coenzyme and thus allows the coenzyme to function catalytically.

Abbreviations used: BH₄, tetrahydrobiopterin; 6-MPH₄, 6-methyltetrahydropterin; DMPH₄, 6,7-dimethyltetrahydropterin; DHPR, dihydropteridine reductase.
to 5-hydroxytryptophan, catalysed by tyrosine hydroxylase (Shiman et al., 1971) and tryptophan hydroxylase (Friedman et al., 1972), respectively, is the same as that described for phenylalanine hydroxylation (Fig. 1). All three hydroxylation reactions conform to the general reaction described by eqn. (1), where RH is the amino acid substrate, ROH is the hydroxylated amino acid product and q-BH2 is quinonoid dihydrobipterin.

\[ \text{RH} + \text{BH}_2 + \text{O}_2 \rightarrow \text{ROH} + q\text{-BH}_2 + \text{H}_2\text{O} \] (1)

Although the idea that these enzymes are members of a family with many common properties has been of value, it should not be pushed too far. Thus, although the general mode of regulation of all three enzymes may be similar, the precise mechanism of the organism for this way, it is clear that the role of phenylalanine hydroxylase is different from that of the other two enzymes. Phenylalanine hydroxylase catalyses the rate-limiting step in a catabolic pathway, the only pathway that leads to the complete oxidation of phenylalanine to CO2 and water (Kaufman, 1971). It would be expected, therefore, that the activity of phenylalanine hydroxylase would be responsive to blood levels of phenylalanine, which, in turn, are determined, in part, by the dietary intake of phenylalanine. The expectation has been realized: there is evidence that elevated levels of phenylalanine activate phenylalanine hydroxylase (Nielsen, 1969; Kaufman, 1970; Tourian, 1971; Aylings & Helfand, 1975; Shiman et al., 1979; Kaufman & Mason, 1982) both directly, by altering the enzyme's conformation, and indirectly, by causing the release of hormones that increase the enzyme's level of phosphorylation.

In contrast to phenylalanine hydroxylase, it is clear that neither tyrosine hydroxylase or tryptophan hydroxylase is involved in the catabolism of their amino acid substrates: their catabolism involves other pathways. Rather, these hydroxylases catalyse steps in neurotransmitter synthesis and one would expect that the enzymes would be particularly responsive to tissue levels or dietary intake of their substrates, but rather to the needs of the organism for neural transmission.

It also seems likely that the way in which activation of these enzymes is expressed may be different because it will depend on the relationship between \( K_m \) values for substrate and coenzyme and tissue levels of these compounds. There are indications that the \( K_m \) values of phenylalanine hydroxylase for both phenylalanine and BH4 are not too far from their concentrations in liver (Kaufman, 1977). In such a situation, only limited degrees of activation of the enzyme could be achieved by mechanisms that led to a decrease in the enzyme's \( K_m \) for substrate or cofactor. It would seem reasonable, therefore, that activation of this enzyme would be expressed mainly by an increase in \( V_{max} \). There is evidence that this is the case.

By contrast, the \( K_m \) values of both tyrosine and tryptophan hydroxylase for BH4 appear to be considerably larger than the brain concentration of this pterin (Kaufman, 1974). In addition, the \( K_m \) value of tryptophan hydroxylase (determined in the presence of BH4) also appears to be larger than normal brain concentrations of this amino acid (Kaufman, 1974). Based on these considerations it would seem likely that activation of both of these hydroxylases would be expressed, in part, by a decrease in \( K_m \) for BH4 and that, for tryptophan hydroxylase, it might also involve a decrease in \( K_m \) for tryptophan. Again, with these enzymes, the results appear to be a consonant with most of these expectations.

I want to review briefly some other aspects of the regulation of phenylalanine hydroxylase and to point out, where data are available, similarities and differences between its behaviour and that of tyrosine hydroxylase and tryptophan hydroxylase.

The first example of regulation of phenylalanine hydroxylase that we discovered is the marked activation of the enzyme by certain phospholipids, such as lysolecinthin (Fisher & Kaufman, 1972, 1973). Many of the characteristics of this type of activation proved to be quite general and established a pattern to which other activations of this enzyme have conformed.

Perhaps the most important characteristic is that full activation, which results in a 20-40-fold increase in activity, is manifest only when the enzyme is assayed in the presence of the natural cofactor, BH4, and not in the presence of synthetic analogues such as 6-MPH4 or DMPH4.

Secondly, the activation is predominantly due to an increase in \( V_{max} \), the \( K_m \) for BH4 being actually somewhat greater, although the \( K_m \) for phenylalanine is decreased by about 50%.

Activation also changes many other catalytic properties of the enzyme. It converts the dependence of initial velocity on phenylalanine concentration from a sigmoid curve to a hyperbolic curve, increases dramatically the inhibition by phenylalanine, and finally, it leads to a dramatic broadening of the substrate specificity of the enzyme (Fisher & Kaufman, 1973; Kaufman & Mason, 1982).

Subsequently, we showed that limited proteolysis of phenylalanine hydroxylase by chymotrypsin, which reduces the molecular mass of the monomer by about 30%, from 50000 to 35000 daltons, activates the enzyme to about the same extent as does lysolecinthin, and produces an enzyme with catalytic properties that are essentially indistinguishable from those of the lysolecinthin-activated enzyme (Fisher & Kaufman, 1973).

After this work, it was shown that both tyrosine hydroxylase and tryptophan hydroxylase from some species are also activated both by phospholipids and by limited proteolysis (Kuczynski, 1973; Lloyd & Kaufman, 1974; Robertson et al., 1977; Hampe et al., 1978a). Although the fact that all three hydroxylases can be activated by these same means is congruent with the idea that they constitute a family of enzymes, the changes in properties of tyrosine and tryptophan hydroxylase that are induced by these treatments differ considerably from the changes induced in phenylalanine hydroxylase.

Starting right from the extent of activation, which is a modest 2-4-fold for tyrosine and tryptophan hydroxylase, most of the characteristics are different from those that describe phenylalanine activation. Thus, the activation of the former two enzymes appears to be independent of which pterin cofactor is used and appears to be manifest predominantly as a decrease in \( K_m \) for the pterin cofactor rather than as an increase in \( V_{max} \).

These early studies on the activation of phenylalanine hydroxylase by phospholipids and by limited proteolysis were useful because they established some of the general characteristics of the activated enzyme. There is no evidence to suggest, however, that these modes of regulation are of any physiological significance. A mechanism of activation of the enzyme that does appear to operate in vivo is that mediated by phosphorylation.

We have shown that pure rat liver phenylalanine hydroxylase can be activated 3-4-fold when it is phosphorylated by ATP in the presence of cyclic AMP-dependent protein kinase (EC 2.7.1.37) (Abita et al., 1976; Milstien et
contains about 0.3 mol of protein-bound phosphate/mole of
Vol. of hydroxylase subunit. Phosphorylation also affects the
electron pattern of the hydroxylase from calcium phosphate
gel-cellulose columns, the fully phosphorylated enzyme
being bound more tightly (Donlon & Kaufman, 1980).

That phosphorylation is probably a physiologically
significant mode of regulation of the hydroxylase is
indicated by the finding that glucagon administration to
rats leads to a prompt increase in phosphorylation of the
enzyme in vitro and to a 3-4 fold increase in the BH-
dependent phenylalanine hydroxylase activity measured in
rat liver extracts (Donlon & Kaufman, 1978). In addition,
the pattern of elution of the hydroxylase during calcium
phosphate gel chromatography provided independent
indirect evidence that the enzyme from glucagon-treated
rats was more highly phosphorylated than the enzyme from
control rats (Donlon & Kaufman, 1980). The half-maximal
stimulation of hydroxylase activity by glucagon was found to
be at an intraperitoneal dose of 270 μg/kg (Donlon &
Kaufman, 1978). When the glucagon was given intravenously,
the half-maximal response was observed at only 15 μg/kg
(H. Wilgus & S. Kaufman, unpublished work). Subse-
quent work showed that the protein-bound phosphate of the
enzyme from glucagon was demonstrated in isolated rat hepatocytes
(Abita et al., 1980; Carr & Pogson, 1981). Our findings that
glucagon increases the extent of phosphorylation of the
hydroxylase has also been confirmed with rat liver hepatocytes (Carr & Pogson, 1981; Garrison & Wagner, 1982).

We have recently found that BH₂ and phenylalanine have
marked and divergent effects on the phosphorylation in vitro
and the concomitant activation of phenylalanine hydroxyl-
ase. BH₂, at physiological concentrations of approx. 9 μM,
inhibits both the activation and phosphorylation of the
enzyme catalysed by cyclic AMP-dependent protein kinase
(Phillips & Kaufman, 1984). The data in Table 1 show that
the inhibition of phosphorylation by BH₂ is relatively
specific for the naturally occurring 6R-isomer of BH₂;
neither 6-MPH or DMPH, is inhibitory. Phenylalanine,
itslself, was found to stimulate the rate of phosphorylation
and the extent of activation to a modest extent. Probably
of greater significance, phenylalanine was found to be very
effective in overcoming the inhibition of phosphorylation
by BH₂ (Phillips & Kaufman, 1984).

These results are consistent with many proposals that
phenylalanine hydroxylase exists as an equilibrium mixture of
active and inactive conformations. As shown in eqn. (2),
where Ei is a conformation which is inactive, Ea is an active
conformation, and Ea-P is the phosphorylated form of the
enzyme, the state of activation of the enzyme may be largely
determined by opposing effects of BH₂ and phenylalanine:

\[
E_i \xrightarrow{(Ph)} E_a \xrightarrow{E_{a-P}} \text{eqn. (2)}
\]

Our data, when taken together with other findings,
indicate that phenylalanine can activate phenylalanine
hydroxylase by three distinct but inter-related mechanisms:
(a) direct activation; (b) by overcoming the inhibition by
BH₂, phenylalanine can increase the rate of phosphoryl-
ation and activation; (c) phenylalanine can increase the
release of glucagon from the pancreas (Güttler et al., 1978),
which can increase the activity of hepatic cyclic AMP-
dependent protein kinase. Therefore it is reasonable to
predict that the extent of phosphorylation and state of activation of
the hydroxylase. It is clear that phenylalanine hydroxylase is
poised so that it can quickly increase its activity when tissue
levels of phenylalanine increase. These seemingly
redundant mechanisms of phenylalanine-mediated activation of
the enzyme would serve to protect the developing brain
from deleterious effects of excess phenylalanine.

Although there is ample evidence that both tyrosine and
tryptophan hydroxylase can be activated by phosphoryl-
ation, there is much less known about the quantitative
relationship between extent of activation and total content of
protein-bound phosphate for these two enzymes than there is
for phenylalanine hydroxylase. It is known, however, that the
kinetic expression of the activation of both these enzymes is
not the same as it is for phenylalanine hydroxylase. As already
mentioned, activation of phenyl-
alanine hydroxylase by phosphorylation is expressed as an
increase in \( V_{\text{max}} \) with little or no change in the \( K_m \)
values for either BH₂ or phenylalanine. By contrast, the expression of
the activation of either rat or bovine striatal tyrosine
hydroxylase by cyclic AMP-dependent phosphorylation
varies with pH: at pH 6, there is a large decrease in \( V_{\text{max}} \), and a
large decrease in the \( K_m \) for the pterin cofactor, whereas at
pH 7 there is a large increase in \( V_{\text{max}} \), and only a small
increase in \( K_m \) for the pterin cofactor (Pollock et al., 1981).

With tryptophan hydroxylase, activation that is mediated
by a Ca²⁺-dependent phosphorylation process has been
reported to be due to a decrease in \( K_m \), for the pterin cofactor
with either no change in the \( K_m \) for tryptophan (Kuhn
et al., 1979); or a decrease in the \( K_m \) for
tryptophan (Hamon et al., 1978a). There is no agreement
as to whether or not this type of activation is also expressed
as an increase in \( V_{\text{max}} \); there have been reports that \( V_{\text{max}} \)
increases (Hamon et al., 1978a; Yamauchi & Fujisawa, 1979) or a decrease in the \( K_m \)
for tryptophan (Hamon et al., 1978a). There is no agreement
as to whether or not this type of activation is also expressed
as an increase in \( V_{\text{max}} \); there have been reports that \( V_{\text{max}} \)
increases (Hamon et al., 1978a; Yamauchi & Fujisawa, 1979); or does not change (Kuhn et al., 1978). There is also
evidence in favour of a cyclic AMP-dependent activation of
tryptophan hydroxylase in rat brainstem slices that appears
to involve a decrease in \( K_m \) for both tryptophan and pterin
cofactor and an increase in \( V_{\text{max}} \) (Boadle-Biber, 1980).

While there is evidently some variability in the findings
with this enzyme, there appears to be general agreement
that activation involves a decrease in \( K_m \) for the pterin
cofactor.

### Table 1. Specificity of the inhibition of phenylalanine hydroxy-
ase phosphorylation by tetrahydropterins

<table>
<thead>
<tr>
<th>Rate of phosphorylation (mol of phosphate/mole of subunit per 10 min)</th>
<th>Relative rate (( V_{\text{max}} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0.096</td>
</tr>
<tr>
<td>8.6 μM (6R)-BH₂</td>
<td>0.024</td>
</tr>
<tr>
<td>8.6 μM (6S)-BH₂</td>
<td>0.060</td>
</tr>
<tr>
<td>150 μM 6-MPH</td>
<td>0.091</td>
</tr>
<tr>
<td>200 μM DMPH</td>
<td>0.096</td>
</tr>
</tbody>
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On the mechanism of action of phenylalanine hydroxylase

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1-Phenylalanine hydroxylase (PAH; phenylalanine 4-mono-oxygenase, EC 1.14.16.1) catalyses the formation of 1-t-tyrosine and phenylalanine and molecular oxygen by utilizing a tetrahydropterin (Kaufman & Fisher, 1974). In the course of this reaction the cofactor is oxidized to the quinonoid is depicted as a non enzymic process, the dehydration of the adduct whether a semiquinone form of the pterin participates in this step. Dithionite can substitute for 6-MPH, in an reduction step to become catalytically active (Marota et al., 1983). Although the dehydration of the adduct to the quinonoid is depicted as a non enzymic process, the PAH stimulator protein has been shown to catalyse this reaction and as such is a 4a-carbinolamine dehydratase (Lazarus et al., 1983).

Scheme 1 omits a crucial additional role for the tetrahydropterin. The hydroxylase undergoes an obligatory reduction step to become catalytically active (Marota & Shiman, 1984; Wall et al., 1984) in which two equivalents of the pterin are oxidized directly to the quinonoid form per mol of tetramericy hydroxylase. It is not known whether a semiquinone form of the pterin participates in this step. Dithionite can substitute for 6-MPH, in an anaerobic reduction step (two equivalents/mol of hydroxylation) generating a catalytically active hydroxylase, but the pterin is required for the hydroxylation event. The addition of one electron per subunit is sufficient to impart tightly

1982b; Sammons et al., 1985) investigations that established the p-endo structure for the quinonoid dihydropterin, allow the following formulation for species found in the PAH catalysed reaction. Although the dehydration of the adduct to the quinonoid is depicted as a non enzymic process, the PAH stimulator protein has been shown to catalyse this reaction and as such is a 4a-carbinolamine dehydratase (Lazarus et al., 1983).

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Abbreviations used: PAH, phenylalanine hydroxylase; 6-MPH, 6-methyltetrahydropterin, p-CIPhe, p-chlorophenylalanine; PH4, tetrahydropterin.