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ACTIVATION OF PHENYLALANINE HYDROXYLASE BY PHENYLALANINE

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SUMMARY

1. Phenylalanine activates phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.3.1), when dithiothreitol is used to regenerate 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine.

2. Phenylalanine hydroxylase activation by phenylalanine follows first order kinetics; the activation rate is time and temperature dependent, and is described by the exponential equation $v_a = v_f + (v_o - v_f)\, e^{-at}$.

3. The kinetic analysis, derived from the effect of phenylalanine on phenylalanine hydroxylase, is consistent with the postulate that the enzyme has a second activating site for phenylalanine distinct from its catalytic site.

4. The sedimentation constant of non-activated phenylalanine hydroxylase is 6.1 and shifts to 8.14 on activation. Activation shifts the equilibrium from a dimer of 110 000 molecular weight to a tetramer of 210 000.

INTRODUCTION

Phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase, EC 1.14.3.1) is likely to be subject to metabolic regulation since it catalyzes the initial degradation of phenylalanine and is the only pathway for the synthesis of tyrosine. A further indication for the metabolic regulation of this enzyme is the observation that tyrosine spares the dietary requirement for phenylalanine[1]. Yet our understanding of the control mechanisms that operate on this step is incomplete. Part of the difficulty in elucidating any biologically significant effect on this extremely complex system is the requirements of the reaction. These include phenylalanine, tetrahydrobiopterin and oxygen in addition to dihydropteridine rereductase, NADPH (NADPH generator, which is $NADP^+$, glucose, glucose dehydrogenase) and catalase[2-4]. BUBLITZ[5] has published a direct assay for rat liver phenylalanine hydroxylase based on the non-enzymatic regeneration of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ("quinonoid" form) by dithiothreitol. Dithiothreitol also protects the enzyme from peroxides formed from aerobic oxida-

tion of tetrahydropteridine. This system eliminates the need for dihydropteridine reductase, NADPH, NADPH generator and catalase, proceeds without substrate inhibition, and thus gives an unambiguous measurement of phenylalanine hydroxylase over a wide range of substrate concentration.

It is the purpose of this paper to describe the activation by phenylalanine of a partially purified rat hepatic phenylalanine hydroxylase which may be of significance in the metabolic regulation of phenylalanine hydroxylase, utilizing a modification of the direct assay of BUBLITZ[5]. The kinetic data and the sedimentation behavior is consistent with the postulate that phenylalanine hydroxylase has a second "activating" site for phenylalanine distinct from its catalytic site. Phenylalanine activation induces a shift in the sedimentation coefficient of the enzyme.

MATERIALS AND METHODS

Phenylalanine hydroxylase was prepared from rat liver through the first $(NH_4)_2SO_4$ step by a modification of the procedure of KAUFMAN[2]. The modification was that an ethanol fraction was collected between 21 and 30% ethanol by volume rather than between 10 and 21%. The yield varied from 25 to 45% of the starting material. The specific activity of the $(NH_4)_2SO_4$ fraction ranged from 0.20 to 0.38 μmole/mg per min at v_{max} for a 75-fold purification from the crude homogenate. This purified fraction has 2–5-fold more activity than a similar fraction by KAUFMAN's methods under the conditions of our standard assay, with 2 μM phenylalanine. None of the preparations used destroyed tyrosine. The concentration of tetrahydropteridine obtained from Calbiochem in individual 10-mg vials was measured by titration with 2,6-dichlorophenol indophenol which was standardized with ascorbate[6].

L-Phenylalanine, L-tyrosine, 4-fluorophenylalanine, L-tryptophan, dithiothreitol, were obtained from Calbiochem. D-Phenylalanine, β-phenyllactate, alanine were obtained from Sigma; 1-nitroso-2-naphthol, 1,2-dichloroethane from Eastman Kodak. Horse liver alcohol dehydrogenase was obtained from C. F. Boehringer. Protein was measured by the biuret[7] or LOWRY[8] methods with lysozyme (Mann) as standard.

Assay for phenylalanine hydroxylase

The standard assay (1.0 ml) contained in μmoles: potassium phosphate, pH 7.0 or 7.2, 100; L-phenylalanine, 8 or as specified for each experiment; tetrahydropteridine, 1; dithiothreitol, 10. The reaction was initiated by addition of the enzyme and incubated for 1 min at 25°.

Phenylalanine activation assay

Two incubations were performed to assay phenylalanine activated enzyme; (a) The first incubation contained all the components, except tetrahydropteridine, and the enzyme was the last addition. The duration of this incubation varied depending on the concentration of phenylalanine, and its volume was 0.9 ml or as indicated. During the first incubation no tyrosine was formed; (b) the second incubation was initiated by tetrahydropteridine which started the hydroxylation reaction. The complete system was incubated for 3 min or as specified, at 25° with

shaking in a water bath. The reaction was stopped by the addition of 1 ml of 30% trichloroacetic acid and diluted to 5 ml. Tyrosine in 2-ml aliquots of deproteinized solution was determined by the nitrosonaphthol method fluorimetrically[9]. Corrections for appropriate zero time controls were routinely made. Tyrosine standard curves were routinely included ranging in concentrations 0.005–0.5 μM.

RESULTS

The initial lag of phenylalanine hydroxylation

Preliminary incubations were carried out to determine the initial rates for the first 5 min and at phenylalanine concentrations varying from 0.1 to 8 μmoles/ml. It immediately became apparent that there was an initial 60–90-sec lag in the velocity of the reaction when time was the variable. The duration of the lag depended on the concentration of phenylalanine.

A similar lag of 7 min in the initial rate of the phenylalanine hydroxylation reaction was first described by KAUFMAN[10]. However, this lag is almost completely eliminated by preincubation of both phenylalanine hydroxylase and dihydropteridine reductase with the NADPH generator system, either aerobically or anaerobically. Thus the suggestion made was that the formation of a reduced cofactor (tetrahydrobiopterin) was necessary for the reaction to proceed, even though the preincubation did not completely eliminate this lag.

Phenylalanine activation of phenylalanine hydroxylase

The first incubation of the various components of the reaction mixture with the enzyme revealed a slight stimulation by phosphate buffer, but a marked stimulation by phenylalanine. The first incubation of the enzyme with dithiothreitol or tetrahydropteridine had no significant effect. And finally, the omission of dithiothreitol from both the first and second incubation rules out the possibility that phenyl-

TABLE I

ACTIVATION OF PHENYLALANINE HYDROXYLASE BY PHENYLALANINE

Components of the full incubation system contained in μmoles: potassium phosphate, pH 7.2, 100; dithiothreitol (DTT), 10; 0.69 mg enzyme; phenylalanine (Phe), 1; tetrahydropteridine (PtH_4), 1. The first incubation was for 10 min at 25° and the volume was 0.7 ml. The enzyme was the last addition during this incubation. The second incubation's final volume was 1 ml, PtH_4 was the last addition except when the first incubation was omitted, then the enzyme was the last addition. The second incubation which initiated the hydroxylation reaction was for 1 min at 25°.

Additions during first incubation	*Additions during second incubation*	*Tyrosine formed (μmoles·mg protein^{-1}·min^{-1})*
None	Phe, buffer, PtH_4, DTT, enzyme	0.0154
Buffer, enzyme	Phe, DTT, PtH_4	0.0226
Buffer, DTT, enzyme	Phe, PtH_4	0.0210
Buffer, DTT, PtH_4, enzyme	Phe	0.0226
Buffer, Phe, enzyme	DTT, PtH_4	0.0703
Buffer, Phe, DTT, enzyme	PtH_4	0.0616
None	Buffer, Phe, PtH_4, enzyme	0.0108
Buffer, Phe, enzyme	PtH_4	0.0508

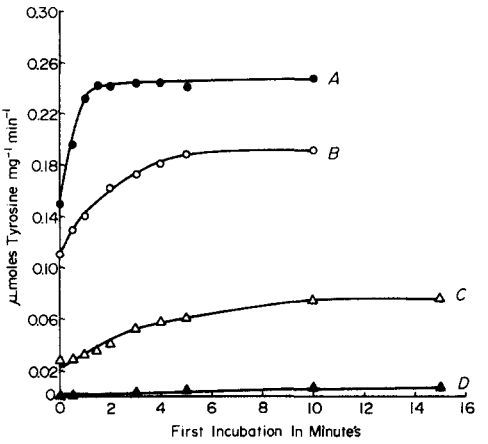

Fig. 1. The effects of various concentrations of phenylalanine during the first incubation on the time course of activation of phenylalanine hydroxylase. Phenylalanine and the full components except tetrahydropteridine were first incubated for the indicated time periods. The addition of 0.32 mg enzyme initiated the first incubation for the indicated time periods. The second incubation for the hydroxylase reaction was started by tetrahydropteridine for 3 min at 25°. Phenylalanine concentrations in μmoles were: Curve A, 8; Curve B, 4; Curve, C, 1; Curve D, 0.1.

alanine activates a dithiothreitol inhibited phenylalanine hydroxylase (Table I).

The degree of activation and the time required to arrive at the fully activated form of the enzyme was directly proportional to phenylalanine concentration for activation during the first incubation phase (Fig. 1). Thus at 0.1 μmole/ml phenylalanine 15 min and at 8 μmoles/ml phenylalanine 90 sec were required for full activation.

Specificity of L-*phenylalanine for activation of phenylalanine hydroxylase*

The specificity of phenylalanine hydroxylase activation was tested by D-phenylalanine, L-tryptophane, β-phenyllactate, alanine. None of these compounds at $1 \cdot 10^{-3}$ M had any effect on phenylalanine hydroxylase. Likewise, Na^+, K^+, Mg^{2+}, Fe^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, NH_4^+, SO_4^{2-}, HCO_3^-, acetate, Cl^- at $1 \cdot 10^{-4}$ M had no effect whatsoever on phenylalanine hydroxylase. GUROFF[11] has reported activation of phenylalanine hydroxylase from Pseudomonas by Fe^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}. 4-Fluorophenylalanine activated phenylalanine hydroxylase. It is also a substrate for phenylalanine hydroxylase, but proceeds at one-sixth the rate of phenylalanine as reported by KAUFMAN[12].

Determination of phenylalanine activation rates of phenylalanine hydroxylase

The activation rate of the non-activated enzyme was determined with respect to the fully activated enzyme, at two concentrations, 1 and 8 μmoles/ml L-phenylalanine. For the former the contribution by phosphate stimulation was also calculated by first incubation with phosphate buffer but without phenylalanine (Figs. 2, 3). Phenylalanine hydroxylase activation by phenylalanine is first order with respect to non-activated enzyme. The final rate achieved never equals to the fully activated rate of the enzyme which has been first incubated and activated with phenylalanine prior to the initiation of the hydroxylation reaction by the second

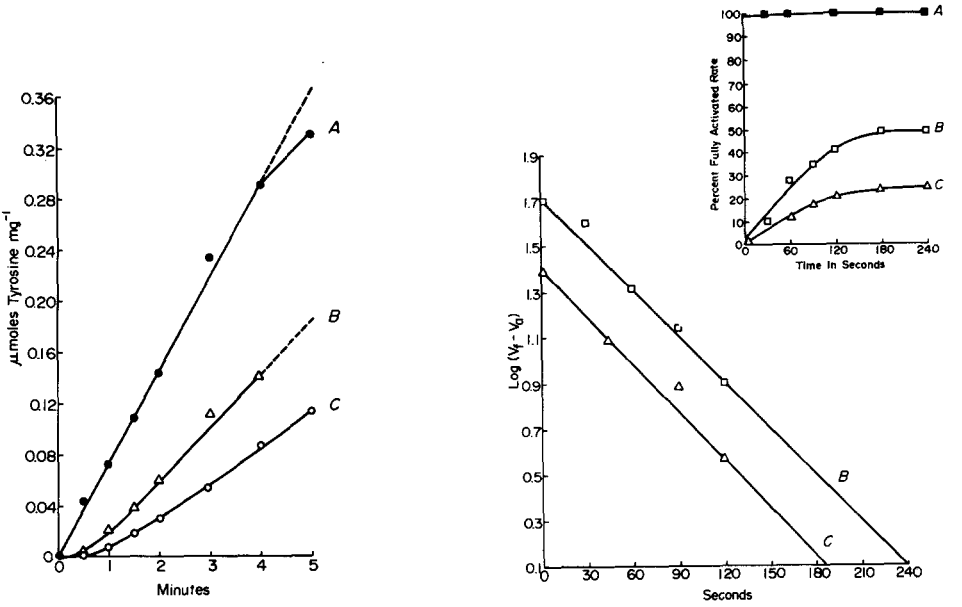

Fig. 2. Activation of phenylalanine hydroxylase by phenylalanine and elimination of the initial lag. The complete incubation system contained in μmoles: potassium phosphate, pH 7, 100; dithiothreitol, 10; tetrahydropteridine, 1; L-phenylalanine, 1. Curves A and B were first incubated for 15 min at 25°, with the complete system except A without tetrahydropteridine, and B without phenylalanine. The first incubation period was started by the addition of 0.32 mg enzyme. The second incubation was started by tetrahydropteridine for A, and phenylalanine for B for the specified time periods at 25°. Curve C, the incubation mixture was not first incubated; the reaction was started by the addition of enzyme.

Fig. 3. Exponential activation of phenylalanine hydroxylase by phenylalanine. The data of Fig. 2 are replotted to calculate the phenylalanine activation rate of phenylalanine hydroxylase (Curve C); the phosphate buffer first incubated enzyme (Curve B); relative to the fully activated enzyme, Curve A. The activation rates can be calculated from the exponential equation $v_a = v_f + (v_o - v_f)\, e^{-at}$ (ref. 13) where v_o is the fully activated rate of the first incubated enzyme, v_a is the activated rate at time t of Curves B and C, v_f is the final equilibrium rate achieved of Curves B and C, and a is a constant. It follows that log $(v_f - v_a)$ *vs.* t is a straight line with a slope $-a/2.3$, from which the rate constant is calculated using the least square method, Curve B, $a = 0.01556$ sec^{-1}; Curve C, $a = 0.01698$ sec^{-1}. Since the difference between these two rate constants is less than 10%, the phosphate buffer probably does not affect the rate constant of activation.

incubation. The rate constant for activation a is 0.01556 sec^{-1} for the phosphate buffer first incubated enzyme and 0.01698 sec^{-1} for the only second incubated enzyme at 1 μmole/ml phenylalanine. Since the difference between these two constants is less than 10%, it would follow that phosphate buffer does not alter the rate constant of phenylalanine activation of phenylalanine hydroxylase. At 8 μmoles/ml phenylalanine a equals 0.02179 sec^{-1}. Similarly, activation rates were determined at 15° and 20° and at 1 μmole phenylalanine. These were $a = 0.0020$ sec^{-1} for the former and 0.00338 sec^{-1} for the latter temperature. The fully activated enzyme has a linear rate for only 3–4 min after which it drops to a new linear rate for 10 min. This drop is not due to substrate or cofactor depletion, since a 10-fold addition of phenylalanine and 2-fold cofactor does not alter this drop. Anaerobic incubation

of the enzyme with phenylalanine in evacuated Thunberg tubes during the first incubation phase does not prevent phenylalanine activation of phenylalanine hydroxylase.

To test the effect of tetrahydropteridine on phenylalanine hydroxylase activation, the enzyme was first incubated in evacuated Thunberg tubes with both tetrahydropteridine and phenylalanine, followed by aerobic incubation to initiate the hydroxylation reaction during the second incubation. The initial rate of hydroxylation is identical to enzyme that has been activated by only phenylalanine, however, the linear phase extends to 7 min then declines very gradually. This experiment suggests that the presence of tetrahydropteridine during the activation of phenylalanine hydroxylase by phenylalanine, does not prevent full activation, and the activated enzyme is stabilized by the presence of cofactor prior to the initiation of the hydroxylation reaction.

The apparent K_m for phenylalanine of the fully activated enzyme at pH 7.0 and 25° is 1.75 ± 0.08 mM for three determinations in duplicate. The K_m is determined from the slope of the Hofstee plot, v *vs.* $v/[S]$ from a least square fit.

Phenylalanine hydroxylase activity as a function of protein concentration for both activated and "activating" enzyme

Phenylalanine hydroxylation activity is a linear function of protein concentration for both the fully activated and the "activating" enzyme as evidenced by the data of Fig. 4. Thus activation of phenylalanine hydroxylase is not dependent on enzyme concentration.

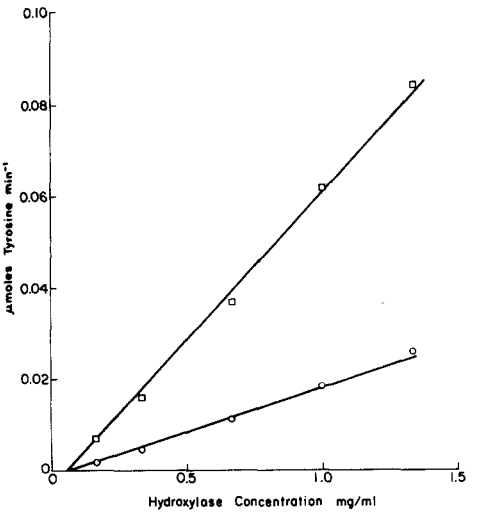

Fig. 4. Phenylalanine hydroxylase activity as a function of enzyme concentration for both activated and "activating" enzyme. The complete incubation system contained in μmoles: potassium phosphate buffer, pH 7.2, 100; dithiothreitol, 10; phenylalanine, 1; and the appropriate concentration of enzyme. The activated group was first incubated with the complete system except tetrahydropteridine for 10 min at 25°. The "activating" enzyme was likewise first incubated with the complete system except phenylalanine and tetrahydropteridine for 10 min at 25°. Tetrahydropteridine was the penultimate addition. The second incubation was initiated by phenylalanine for 1 min at 25°.

The operational separation by kinetic analysis of the "activating" effect of phenylalanine from its effect on fully activated phenylalanine hydroxylase

From the activation experiments it became obvious that the effect of phenylalanine concentration on phenylalanine hydroxylase can be determined under two separate sets of conditions: (a) When the enzyme is fully activated; (b) when the enzyme is "activating". The conditions for the fully activated enzyme are obtained easily by first incubation of the enzyme with the appropriate concentrations of phenylalanine for the required time for full activation followed by initial rate measurements. The conditions for the "activating" enzyme are more difficult to meet since during this period of measurement there may be fully activated molecules of enzyme which result in a mixture of "activating" and "activated" rates. From the activation data, it can be observed that this period is limited to the first minute of

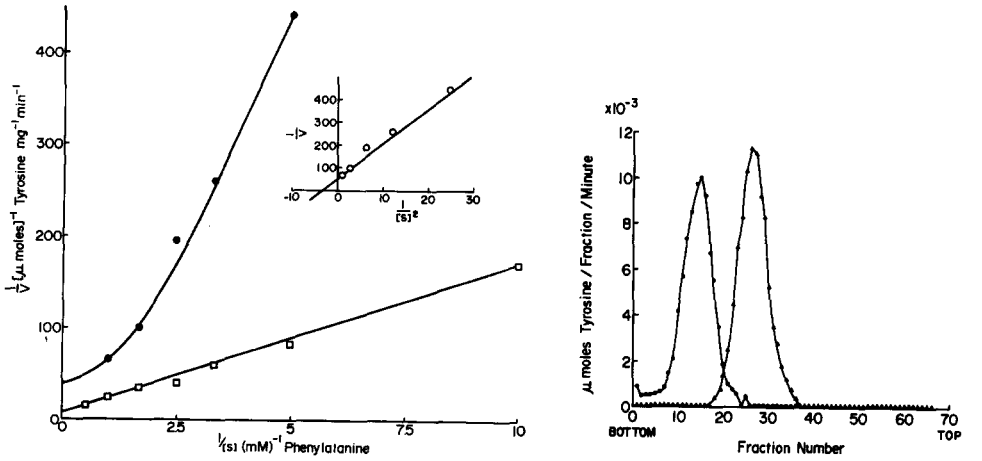

Fig. 5. The effect of phenylalanine concentration on the rate of the hydroxylation reaction of phenylalanine activated and "activating" enzyme by the double reciprocal plot of Lineweaver–Burk. The complete incubation system contained in μmoles: potassium phosphate buffer, pH 7.2, 100; tetrahydropteridine, 1; dithiothreitol, 10; phenylalanine, 0.1 to 2; and 0.69 mg enzyme. For the activated group, the enzyme was first incubated with the complete system except tetrahydropteridine for 15 min at 25°. The second incubation was initiated with tetrahydropteridine for 1 min at 25°. For the "activating" group, the enzyme was first incubated with the complete system except phenylalanine and tetrahydropteridine for 15 min. Tetrahydropteridine was the penultimate addition and the second incubation was initiated with enzyme for 1 min at 25°. The double reciprocal plot of the fully activated enzyme (□—□) is a straight line, and the non-activated but "activating" enzyme (●—●) is a parabola. When the reciprocal of velocity of the "activating" enzyme (○—○) is plotted against the square of the reciprocal of substrate concentration, a straight line ensues.

Fig. 6. Sucrose gradient centrifugation pattern of phenylalanine activated and non-activated phenylalanine hydroxylase. 5–20% continuous sucrose gradients were placed in each of the three buckets of the SW-39 rotor. Gradient 1 also contained $1 \cdot 10^{-3}$ or $8 \cdot 10^{-3}$ M phenylalanine, which was layered by 1 mg, 250 μl of $1 \cdot 10^{-3}$ or $8 \cdot 10^{-3}$ M phenylalanine activated phenylalanine hydroxylase. Gradient 2 was layered with the same amount of non-activated phenylalanine hydroxylase. Alcohol dehydrogenase, 0.5 mg in 250 μl, was layered on Gradient 3. The rotor was run for 22 h at 38 000 rev./min in a Beckman L2-65 Ultracentrifuge at 2°. 5-drop fractions were assayed for phenylalanine hydroxylase from Gradients 1 and 2 after activating the enzyme from the latter tube. The standard incubation mixture contained 8 μmoles phenylalanine and was assayed for 3 min at 25°. Alcohol dehydrogenase from Tube 3 was assayed according to the method of MARTIN AND AMES[18].

the reaction for the lower regions of phenylalanine concentration, *i.e.* 0.1–1 μmole/ml. The effect of phenylalanine concentration was determined on the fully activated and "activating" enzyme at pH 7.2 (since this is the optimum pH under the present conditions of the assay with phosphate buffer). The stimulating effect of the phosphate buffer on phenylalanine hydroxylase, which interferes with the kinetic analysis, was cancelled out by preincubation of both the fully activated and non-activated enzyme. Thus when the reaction was initiated by phenylalanine it was possible to measure the sole effect of phenylalanine concentration on initial rates of the hydroxylation reaction. The fully activated enzyme gives a classical Michaelis–Menten rectangular hyperbola and the double reciprocal plot of $1/v$ *vs.* $1/[S]$ is a straight line for an 80-fold change in phenylalanine concentration (0.1–8 μmoles). The double reciprocal plot for the "activating" enzyme approaches a parabola. When the reciprocal of velocity $1/v$ is plotted against the reciprocal of the square of substrate concentration (0.1–1 μmole phenylalanine), $1/[S]^2$, a straight line ensues (Fig. 5). Such curves seem to be characteristic of certain regulatory proteins, like aspartate transcarbamylase[14], threonine deaminase[15], acetyl-CoA-carboxylase[16] and NAD-specific isocitrate dehydrogenase[17].

The sedimentation behavior of phenylalanine hydroxylase activated by phenylalanine compared to non-activated enzyme

The sedimentation behavior of phenylalanine activated and non-activated phenylalanine hydroxylase was compared in a continuous sucrose gradient by the method of MARTIN AND AMES[18]. There was complete separation of the activated form which sedimented more rapidly, and had an s value of 8.14 compared to the non-activated enzyme which had an s value of 6.1 (Fig. 6). An estimate of the molecular weights calculated from the sedimentation constants by the method of MARTIN AND AMES[18], gives values of 110 000 and 177 000 for each of the forms. KAUFMAN[19] has published data demonstrating that phenylalanine hydroxylase can exist in two major forms, a dimer with molecular weight of 110 000 and a tetramer with molecular weight of 210 000. Our data would indicate that the probable mechanism of phenylalanine activation of phenylalanine hydroxylase involves the shift of equilibrium from a dimer to a tetramer. The present proposal for the direction of shift of equilibrium for the more active form of the enzyme is in variance with KAUFMAN'S[20] postulate.

DISCUSSION

The analysis of the kinetic data of phenylalanine hydroxylase is characteristic of certain regulatory proteins[14–17]. The criteria of precursor activation are: (1) the activating compound should be a precursor; (2) the effect should be specific; (3) the activating effect must be brought about by the precursor at low, physiological concentration, and finally, in order to be an effective control mechanism; (4) the catalytic step should be irrreversible[17]. All these criteria are met in the case of phenylalanine hydroxylase. The activating compound is a precursor for phenyalanine hydroxylation. No other small molecules have been found that activate this enzyme. As has already been shown, phosphate does not change the rate constant of activation. The phosphate buffer stimulating effect has also been noted by KAUFMAN[19]. The phenylalanine activating effect is brought about by low, physiological concentra-

tions. Mammalian tissue concentrations of phenylalanine are in the range of 0.2 mM (ref. 21). And finally, phenylalanine hydroxylation is an irreversible reaction[2]. That certain allosteric processes may be time dependent has already been shown by BARBER AND BRIGHT[22]. Phenylalanine activation of phenylalanine hydroxylase may be an important control mechanism in the metabolism of aromatic amino acids. This is supported by the observation that in the human genetic disease, phenylketonuria, where there is a metabolic block of phenylalanine hydroxylation[23] tissue levels of phenylalanine rise resulting in a multitude of secondary biochemical aberrations[24], and mental retardation. It could be formulated that when tissue phenylalanine levels rise, it is ensured by the activation mechanism that phenylalanine will be rapidly metabolized and put to use by the cells.

The presence of yet another site for phenylalanine distinct from its catalytic site on phenylalanine hydroxylase would predict genetic variants with modification or loss of function of this "activating" site. A clinical variant of phenylketonuria known as "atypical" is a candidate for mutation at the "activating" site. JUSTICE *et al.*[25] have reported phenylalanine hydroxylase levels of around 5% of normals in these patients.

An initial burst of activity in the time course of phenylalanine hydroxylation has also been noted by KAUFMAN[20] with a 400-fold purified enzyme, utilizing the natural cofactor tetrahydrobiopterin. This initial burst of activity is seen only when phenylalanine is added to the hydroxylase prior to tetrahydrobiopterin, and not when the order of addition is reversed. This observation makes it unlikely that phenylalanine activation of phenylalanine hydroxylase is unique to the system where tetrahydropteridine is used instead of the natural cofactor tetrahydrobiopterin, or the activation recorded will disappear when more highly purified fractions are used. The interactions of phenylalanine activation, the binding of tetrahydrobiopterin and oxygen with more highly purified fractions awaits further investigation.

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