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Stability Properties of Activated Tryptophan Hydroxylase from Rat Midbrain

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Abstract: Time courses of the activation-inactivation sequence in rat midbrain tryptophan hydroxylase after preincubation with calcium, ATP + $MgCl_2$, or sulfhydryl reagents and after freezing and thawing suggest that the activated enzyme is more vulnerable to loss of activity. The sequence induced by calcium was prevented by the protease inhibitor leupeptin, and an accelerated decline in activity after activation by ATP + $MgCl_2$ was reduced greatly by increasing levels of tetrahydrobiopterin (BH_4) cofactor. The effects of calcium and ATP + $MgCl_2$ were additive, which suggests independent mechanisms. The findings suggest that time courses of enzyme activation and inactivation processes may offer a useful way to study the influence of a range of effectors on tryptophan hydroxylase function. **Key Words:** Calcium—Enzyme denaturation—Phosphorylating conditions—Proteolysis—Tetrahydrobiopterin—Tryptophan hydroxylase. **Vitto A. and Mandell A. J.** Stability properties of activated tryptophan hydroxylase from rat midbrain. *J. Neurochem.* **37**, 601–607 (1981).

Variable instability of brain tryptophan-5-monooxygenase (TPOH; EC 1.14.16.4), the rate-limiting enzyme in the biosynthesis of brain serotonin, has been a consistent feature of that enzyme's behavior during efforts to purify it and study its regulation (see Mandell, 1978 for a review), and at present no procedure yields enough purified TPOH protein for adequate kinetic characterization (Tong and Kaufman, 1975; Kuhn et al., 1980*b*). In our quest for the sources of the instability, we found, as had been suggested by the work of Hamon et al. (1977), that stabilization and destabilization by endogenous proteases could represent a mechanism for the regulation of TPOH activity in brain (Vitto and Mandell, 1979). Recently the importance of proteolysis and other mechanisms of enzyme inactivation in regulating tissue enzyme levels has been recognized. An enzyme may be inactivated either rapidly or slowly, depending on physiological conditions (Schimke, 1973), and cofactor and ligand effects on its conformation appear to affect vulnerability to the process (Citri, 1973; Levy and McConkey, 1977). In the studies described in this report we set out to examine the stability of TPOH under conditions that initially increase activity: i.e., in the presence of calcium, under phosphorylating conditions, after exposure to sulfhydryl bond-disrupting reagents, and after a period of storage in the frozen state.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (150–200 g, Hilltop Laboratories, Scottdale, Pennsylvania) were decapitated, and midbrains (130 mg), extending rostrally from the pons to the mammillary bodies (Zeman and Innes, 1963), were dissected freehand, pooled, and homogenized in a glass homogenizer with clearance of 0.01 cm in 0.01 M-*N*-2-hydroxyethylpiperazine-*N*′2-ethanesulfonic acid

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Abbreviations used: BH_4, Tetrahydrobiopterin; DPCC, Diphenylcarbamyl chloride; DTT, Dithiothreitol; HEPES, *N*-2-Hydroxyethylpiperazine-*N*′2-ethanesulfonic acid; NADH, β-Nicotinamide adenine dinucleotide, reduced form; NaF, Sodium fluoride; PMSF, Phenylmethyl sulfonyl fluoride; TLCK, *N*-α-*p*-Tosyl-L-lysine chloromethyl ketone HCl; TPCK, L-1-Tosylamide-2-phenylethylchloromethyl ketone; TPOH, Tryptophan-5-monooxygenase.

(HEPES; Calbiochem, La Jolla, California), pH 7.4, containing 0.1 mM-dithiothreitol (DTT; Sigma Chemical Co., St. Louis, Missouri) at one part brain per 0.5 ml buffer. Homogenates were centrifuged at 35,000 × *g* for 20 min, and the resulting supernatant served as the enzyme source after being adjusted to 0.05 M-HEPES, pH 7.4, with an appropriate volume of 0.5 M-HEPES buffer. One milliliter of midbrain supernatant contained an average of 14 mg of protein as determined by A280/A260 (Layne, 1957).

TPOH was assayed fluorometrically by a modification of the method of Friedman et al. (1972) as adapted by Baumgarten et al. (1973). The method measures the accumulation of L-5-hydroxytryptophan and its decarboxylated product, L-5-hydroxytryptamine (serotonin), the two indolealkylamines fluorescing with equal intensity at the pH used. Specific activity is reported as nanomoles of product per milligram of protein per 20 min.

In a final volume of 0.2 ml, the reaction mixture contained the following: 0.02 ml of 1.0 mM-L-tryptophan; 0.02 ml of 1.0 mM-DTT; 0.02 ml of 1.0 mM reduced β-nicotinamide adenine dinucleotide (NADH); 0.02 ml of tetrahydrobiopterin (BH_4); and 0.08 ml midbrain supernatant. BH_4, 1.0 mM, was prepared before each assay by catalytic reduction of biopterin (Regis Chemical Co., Chicago, Illinois) with PtO_2 under hydrogen. After 20 min at 37°C, 0.025 ml cold 50% trichloroacetic acid was added to each reaction mixture. The mixtures, in 10 × 75 mm glass culture tubes, were centrifuged at low speed for 15 min, after which 0.1 ml supernatant was transferred to new culture tubes that contained 0.1 ml of 4.0 M-HCl. Samples were vortexed and read on an Aminco-Bowman spectrophotofluorometer at excitation/emission wavelengths of 300/530 nm and quantified by comparison with known standards of L-5-hydroxytryptophan in unincubated reaction mixtures.

Enzyme inactivation studies were carried out as follows. After the indicated pretreatment, midbrain supernatants were incubated at 37°C. At the times indicated, duplicate aliquots were removed and assayed for TPOH activity. Over time, the averages of the duplicate values reflected the rate of loss in activity, and the inactivation curves are plotted semilogarithmically as percentages of initial activity. The standard convention for plotting inactivation data is the semilogarithmic plot, where first-order loss and any deviation from first-order loss can be observed. We have included initial activity values in all figures, and we urge the reader to keep these values in mind when considering the data. It is easy to forget initial differences in activity when percent of initial activity log plots are used as the ordinate of the inactivation curves.

RESULTS

Figure 1 portrays typical functions of rat midbrain TPOH inactivation under control conditions and in the presence of various concentrations of $CaCl_2$, plotted as percentages of respective initial activities. In the control the decline in activity was multiphasic: a rapid loss of 30–40% within 20 min, gradual loss of 20% more over the next 40 min, and an approach to a stable value of 40–50% of the initial activity after 1 h. Graduated increases in $CaCl_2$ resulted in respectively higher initial activities, from 0.411 to 0.679 nmol/mg protein/20 min. At concentrations of 0.4 and 0.6 mM, $CaCl_2$ retarded the decline in TPOH activity, thus evoking a three-phase sequence of effects: initial activation, a period of increased stability, then more rapid loss of catalytic activity compared with control. Concentrations of 0.8 and 1.0 mM-$CaCl_2$ simply increased the initial activity and facilitated the subsequent loss of activity.

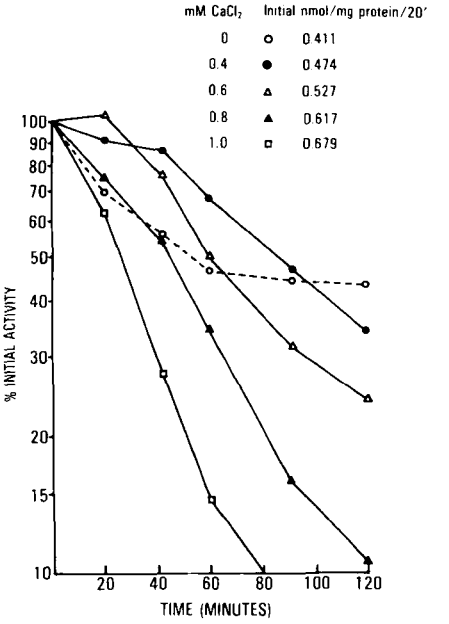

FIG. 1. Typical inactivation functions of rat midbrain TPOH preparations that were incubated at 37°C for increasing periods of time with various concentrations of $CaCl_2$ prior to assay. Duplicate aliquots were removed for assay at the times indicated. Mean values are plotted semilogarithmically as percentages of activities at time zero. At the four concentrations examined, $CaCl_2$ increased initial activity. At 0.4 or 0.6 mM (but not above) $CaCl_2$ induced an intermediate period of stability. As the concentration of $CaCl_2$ was increased, the decline in TPOH activity was accelerated. Each point represents the mean of three experiments assayed in duplicate.

At a concentration of 50 μM, leupeptin (Sigma), which has a relatively wide range of specificities with regard to proteases and their substrates (Aoyagi and Umezawa, 1975; Levy and McConkey, 1977) and is an especially effective inhibitor of calcium-activated proteases (Libby and Goldberg, 1978; Banik et al., 1979; Kameyama and Etlinger, 1979), prevented the entire sequence of effects induced by 0.5 mM-calcium and had no influence on control preparations (Fig. 2). A series of seven other inhibitors of proteolysis, which manifest various degrees of specificity, failed to alter the sequence of calcium effects: phenylmethyl sulfonyl fluoride (PMSF); L-1-tosylamide-2-phenyl-ethyl-

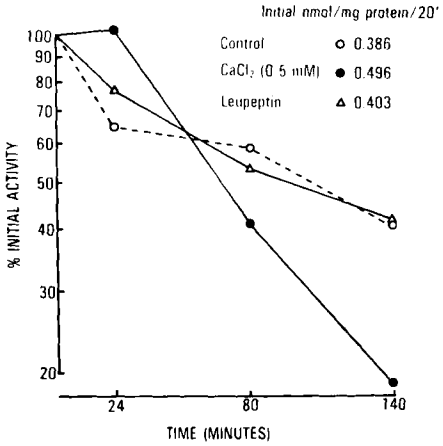

FIG. 2. Same procedure as described in the legend to Fig. 1, with the incubation mixture prior to assay containing 0.5 mM-$CaCl_2$ and 50 μM-leupeptin, a neutral protease inhibitor. Leupeptin prevented all three in the sequence of calcium-evoked effects, but did not alter the time course of the control preparation. Each point represents the mean of two experiments assayed in duplicate.

chloromethyl ketone (TPCK); *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl (TLCK); diphenylcarbamyl chloride (DPCC); benzamidine; aprotinin; and soybean trypsin inhibitor (all from Sigma Chemical Co., St. Louis, Missouri).

Incubation with both 0.5 mM-ATP (Sigma) and 5.0 mM-$MgCl_2$, but not with either one alone, led to a 100% increase in initial TPOH activity (Fig. 3). Under this phosphorylating condition, activity declined rapidly to nearly control levels within 20 min, and thereafter followed the rate of loss of control enzyme preparations. Because a dephosphorylating event has been hypothesized as a mechanism for the reversal of activation induced by phosphorylating conditions (Yamauchi and Fujisawa, 1979*a*), we examined the preparation in the presence of sodium fluoride (NaF), an inhibitor of protein dephosphorylation (Miyamoto et al., 1969), but 10 mM-NaF failed to retard the facilitated inactivation under the phorphosylating condition. Nor was the function influenced when ATP and $MgCl_2$ were added again at 20 min. Likewise, the course of the function was not influenced by either leupeptin or PMSF (data not shown).

Although addition of BH_4 did not significantly alter initial TPOH activation, the subsequent rapid decline in activity was progressively reduced when the effects of phosphorylating conditions were studied in the presence of increasing BH_4 concentrations (Fig. 4). A level of 100 μM-BH_4 led to an inactivation curve approximating that of the control condition. Unlike BH_4, the substrate tryptophan in the preincubation mixture in concentrations up to 150 μM failed to influence the facilitation in decline induced by ATP + $MgCl_2$.

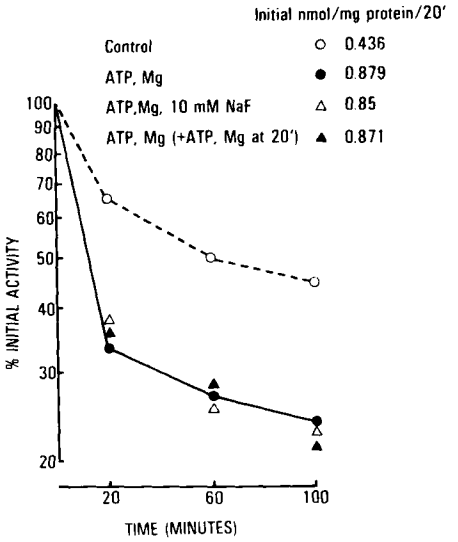

FIG. 3. Time courses of inactivation in midbrain TPOH preparations after activation induced by prior incubation under phosphorylating conditions (0.5 mM-ATP + 5.0 mM-$MgCl_2$). The presence of an inhibitor of dephosphorylation (10 mM-NaF) or the addition of ATP and $MgCl_2$ at 20 min had no effect on the functions, nor did the protease inhibitors PMSF or leupeptin (data not shown). Each point represents the mean of three experiments assayed in duplicate.

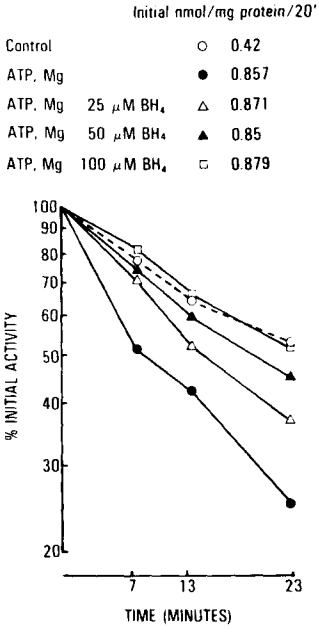

FIG. 4. Effects of the phosphorylating conditions in the presence of increasing concentrations of BH_4. BH_4 did not increase initial activity, but as cofactor concentrations were increased the activity appeared to become more stable. Each point represents the mean of three experiments assayed in duplicate.

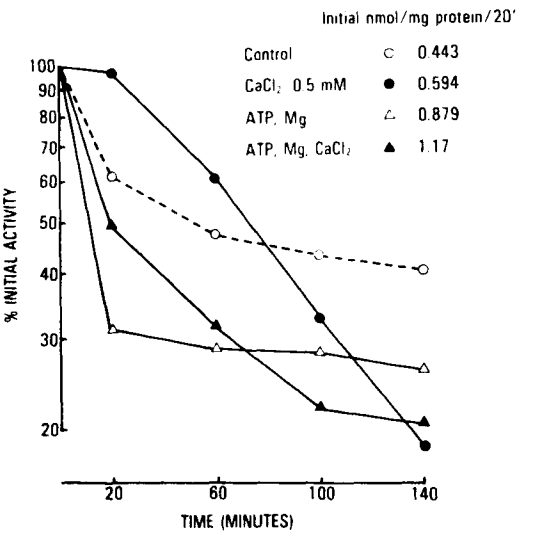

FIG. 5. After dialysis against 20 vol. of 0.05 M-HEPES, pH 7.4, in the presence of 0.5 mM-DTT for 2 h, midbrain supernatant preparations were made 0.5 mM in ATP, 5.0 mM in $MgCl_2$, and/or 0.5 mM in $CaCl_2$. The effects of the combined treatments were essentially additive with respect to activation, whereas ATP-Mg^{2+} effects seemed to predominate during inactivation. Each point represents the mean of two experiments assayed in duplicate. See text.

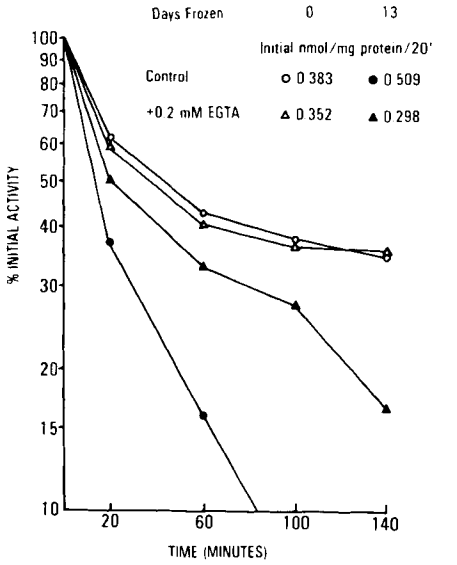

FIG. 6. Enzyme preparations stored at −20°C for 13 days manifested both a 33% initial activation and subsequent facilitated decline in activity when compared with controls. The presence of 200 μM-EGTA partially reduced both the activation and the accelerated decline induced by the cryogenic conditions. Each point represents the mean of two experiments assayed in duplicate.

The combined presence of $CaCl_2$ (0.5 mM) and ATP plus $MgCl_2$ resulted in essentially additive effects on the initial TPOH activity (Fig. 5). Specifically, $CaCl_2$ enhanced control and ATP-Mg^{2+}-treated enzyme preparations by 34 and 33%, respectively. Likewise, ATP plus $MgCl_2$ enhanced control and $CaCl_2$-treated preparations by 98 and 97%, respectively. These results indicate that each type of activation is independent of the other and is not influenced by the activated state of the enzyme. However, with regard to inactivation, the ATP-Mg^{2+} effects seem to predominate insofar as the ATP-Mg^{2+} activity component is rapidly lost regardless of the presence of calcium, whereas the presence of ATP plus $MgCl_2$ seems to stabilize TPOH somewhat against the long-term loss of activity due to calcium.

In other experiments, enzyme preparations that had been stored at −20°C for 13 days manifested 33% activation over controls and a subsequent increase in instability (Fig. 6). Both processes were only partially mitigated when the supernatants were studied in the presence of 200 μM-ethyleneglycol bis(aminoethylether)tetraacetate (EGTA), which suggests that in addition to more available calcium, the physical concomitants of freezing and thawing the preparation may have provoked a sequence of activation followed by increased instability.

In preliminary experiments we examined the effects of yet another potential conformational influence, preincubation with sulfhydryl bond-disrupting reagents: mercaptide formation with *p*-chloromercuribenzoate (0.1 mM) and alkylation with iodoacetamide (0.1 mM). Under conditions described above for the calcium and ATP-Mg^{2+} activation-inactivations, both sulfhydryl reagents led to increases in initial TPOH activity greater than 50% and to first-order decreases that reached zero activity before an hour had elapsed (data not shown).

DISCUSSION

TPOH activity in crude supernatant fractions of rat midbrain homogenates is increased in the presence of added calcium, phosphorylating conditions, and sulfhydryl reagents, and after freezing and thawing. After activation, the rate of TPOH inactivation is facilitated.

At levels of 0.2–5.0 mM, the calcium ion has been reported to decrease the K_m of TPOH for both tryptophan and BH_4 and to increase slightly its V_{max} with regard to tryptophan (Knapp et al., 1975; Boadle-Biber, 1975). In the studies by Hamon et al. (1977), that effect was completely inhibited by EGTA and partially inhibited by the protease inhibitors benzethonium and PMSF. There is evidence for kinetic activation of TPOH by lower, and presumably more physiological, concentrations of calcium in the presence of low levels of BH_4 and tryptophan (Knapp and Mandell, 1979). Moreover, in enzyme preparations without calcium TPOH activ-

ity is low (Boadle-Biber, 1979*b*); in brain slices treated with agents that increase tissue calcium TPOH activity is increased (Boadle-Biber, 1979*a*), as it is also in the presence of calcium-dependent phosphorylating conditions (Hamon et al., 1978; Kuhn et al., 1978; Yamauchi and Fujisawa, 1979*a*). The latter instance seems to require the presence of the calcium-binding protein calmodulin (Yamauchi and Fujisawa, 1979*b;* Kuhn et al., 1980*a*). We have not observed a leupeptin-sensitive effect with less than 0.2 mM-calcium, which suggests the necessity of higher levels of the ion in relevant cellular compartments, possibly achieved by metabolic channeling or brief prediffusional accumulation at calcium influx sites. Another possibility is that proteolytic enzyme activation is not physiologically relevant here despite its regulatory role in other enzyme systems (Goldberg and St. John, 1976; Switzer, 1977; Vitto and Gaertner, 1978; Vitto et al., 1979).

Phosphorylation and/or phosphorylating conditions, well-studied mechanisms of enzyme activation (Greengard, 1978), have been characterized variously as decreasing K_m and increasing V_{max} for both substrate and cofactor (Boadle-Biber, 1980); decreasing K_m and increasing V_{max} for cofactor only (Yamauchi and Fujisawa, 1979*a*); decreasing K_m for both substrate and cofactor (Hamon et al., 1978); and decreasing K_m for cofactor only (Kuhn et al., 1978). *In vitro*, this activation is cAMP-independent, whereas in rat brain slices there may be some cyclic nucleotide dependency, especially when endogenous ATP concentrations are low (Boadle-Biber, 1980). It has been suggested that activation by ATP + $MgCl_2$ is under the control of physiological calcium concentrations (Hamon et al., 1978; Kuhn et al., 1978; Yamauchi and Fujisawa, 1979*a*), but in our hands even dialysis for 2 h against two changes of 20 vol. of 0.05 HEPES buffer containing 0.5 mM-DTT at pH 7.4 failed to sensitize the enzyme preparations to 0.05 mM-$CaCl_2$ in the presence of phosphorylating conditions. It should be noted that the activation we observe under phosphorylating conditions with no calcium is equivalent to the calcium-enhanced activity reported by other workers (Hamon et al., 1978; Kuhn et al., 1978; Yamauchi and Fujisawa, 1979*a*). Dialysis in the above experiment may not have been extensive enough, or, it is possible that nondialyzable (tightly bound or sequestered) calcium in the supernatant preparations is preventing us from observing any low calcium (<0.05 mM) effects. Along these lines, it is conceivable that the initial rapid loss of activity of control preparations (Fig. 1) reflects levels of enzyme in an endogenously activated state. Evidence for the presence of endogenous levels of activated enzyme has been found in rat brain stem slices (Boadle-Biber, 1979*b*).

After a short period of activation, with or without an intermediate period of increased stability, TPOH inactivation was accelerated by preincubation under our four treatment conditions, which suggests that there may be multiple ways to destabilize this enzyme protein. That is, there may be several transconformational paths by which it could pass through a semi-stable state of increased activity en route to inactivation (Lumry and Eyring, 1954; Tanford, 1968; Williams, 1979). The mechanisms underlying these patterns of activation and destabilization and whether such phenomena are reversible or irreversible remain to be determined. Ichiyama et al. (1974), Hori (1975), and more recently Kuhn et al. (1980), have identified oxidative processes that are capable of activating and then inactivating TPOH, yet another circumstance in which activated TPOH is also an unstable molecule, the instability in this case being at least partially reversible when enzyme preparations are exposed to a reducing environment.

Stabilization against denaturing transitions by substrates and ligands might be represented in our experiments by the protection afforded by graded levels of BH_4 against the accelerated decline in TPOH activity after activation by ATP + $MgCl_2$ (Fig. 4). This is consistent with our finding that in the incubation for our TPOH assay (with 100 μM-BH_4 and 100 μM-tryptophan), 1.0 mM-$CaCl_2$ failed to alter the linear rate of product formation for over an hour, whereas under noncatalytic conditions (Fig. 1) that level of calcium evoked an 85% loss in activity within the same time period.

The physiological significance of both these activation/inactivation phenomena and the precise role calcium plays in them must remain speculative at this time. However, enzyme destabilization adds a further nuance to the regulatory potential of TPOH during nerve firing. One could presume that the stability properties of TPOH in cell bodies as examined in this report are similar to those of TPOH at the nerve terminal. An activated but unstable enzyme could effect a limited, self-terminating, pulsed synthesis of neurotransmitter in response to a similar pulsed release brought about by nerve firing and calcium influx. What emerges is the potential for a rapidly responding homeostatic mechanism capable of maintaining constant intraneuronal transmitter levels in response to nerve firing.

Although conclusions from this work must remain indefinite without sufficient purified TPOH, it appears that the progression of activation, stabilization, and inactivation induced by added calcium, and the pattern of activation followed by increased instability induced by phosphorylating, proteolytic, cryogenic, and sulfhydryl bond-disrupting conditions may be general and reflect intrinsic kinetic/conformational properties associated with increasing macromolecular instability (Lumry and Eyring, 1954; Tanford, 1968; Williams, 1979).

We are encouraged to think speculatively about these results because it has been demonstrated that many labile enzymes manifest the same stability properties *in vitro* as *in vivo* (Hopgood and Ballard, 1974; Bond, 1975; Ballard, 1980), tend to be regulatory, and are often stabilized by either substrate or cofactor (Litwack and Rosenfield, 1973; Levy and McConkey, 1977; Vitto and Gaertner, 1978). By comparison, we have found two associated enzymes, aromatic amino acid decarboxylase and quinonoid dihydropterin reductase, to be quite stable and unactivated in the presence of either calcium or phosphorylating conditions (Vitto and Mandell, unpublished observations). Those enzymes are also very resistant to general heat activation. Further, they are not believed to play as critical a rate-limiting role as TPOH in the biosynthesis of serotonin (Mandell, 1978). For these reasons we are encouraged to regard the instability of both control and activated preparations of TPOH as indicating that this enzyme may undergo significant regulation *in vivo* by stabilizing and destabilizing factors. A comparable activation/inactivation sequence following treatment with phosphorylating conditions has recently been reported for rat brain tyrosine hydroxylase activity (Vrana et al., 1981). Also, Lloyd (1979) has reported a phosphatidylinositol-mediated activation/inactivation of tyrosine hydroxylase; in that case, as we have found for TPOH, the catalytic state appeared to stabilize the enzyme. Although much systematic work remains to be done, we believe that time courses of enzyme activation and inactivation processes may offer a useful approach to the conformational regulation of TPOH activity by a range of effectors.

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