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Conformational Influences on Brain Tryptophan Hydroxylase by Submicromolar Calcium: Opposite Effects of Equimolar Lithium

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With 6 Figures

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Summary

Tryptophan hydroxylase from rat midbrain, EGTA-pretreated and dialyzed, manifested allosteric properties with respect to its substrate tryptophan, cofactor tetrahydrobiopterin, and the calcium ion. Kinetic studies suggest two preferred enzyme conformations in the presence of low concentrations of the cosubstrates: a higher affinity form manifesting hyperbolic substrate kinetics, induced by submicromolar (0.4—0.8 μM) calcium *in vitro* and cocaine *in vivo*, and a lower affinity form exaggerating cooperativity with respect to substrate, induced by submicromolar (0.4 to 0.8 μM) lithium *in vitro* and lithium *in vivo*. Lithium's effect on serotonin biosynthesis may be due to its antagonism of the positive effector influence of calcium on tryptophan hydroxylase, either as a negative effector or by blocking the calcium site.

Introduction

Catecholamine biosynthesis in brain has been shown to have the potential for product-feedback inhibitory regulation; catechols of varying structure, in not unrealistic concentrations (*Weiner*, 1970), compete for the pterin cofactor site (*Nagatsu et al.*, 1964; *Udenfriend et al.*, 1965). No similar metabolic regulatory "message" reflecting the activity of the brain's serotonergic biosynthetic system has been as clearly elucidated. A demonstrated inverse relationship between

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serotonin (5-HT) content and biosynthetic rates in brain slices (*Macon et al.*, 1971) has been shown subsequently to involve presynaptic receptor impingement rather than the direct influence of a metabolite (*Hamon et al.*, 1977 b). 5-HT in concentrations higher than 0.1 mM fails to inhibit brain tryptophan hydroxylase (TPH) (*Jequier et al.*, 1969; *Friedman et al.*, 1972). The intermediate product 5-hydroxytryptophan (5-HTP), which is present in brain in significant concentrations only under conditions of aromatic amino acid decarboxylase inhibition (*Carlsson et al.*, 1972), at concentrations between 0.1 and 0.2 mM manifests an inhibitory effect that is non-competitive with respect to tetrahydrobiopterin (PH_4) (*Kaufman*, 1974); this effect is probably similar to inhibition by substrate due to out-of-sequence binding (*Cleland*, 1970) and involves concentrations too high to be realistic.

There are suggestions of a ligand role for calcium with respect to TPH (*Knapp et al.*, 1975; *Boadle-Biber*, 1975, 1978; *Hamon et al.*, 1977 b), the ion increasing the enzyme's affinity for tryptophan substrate. The calcium levels used in earlier studies, however, were as much as three orders of magnitude too high to reflect intraneuronal fluxes of ionized calcium (*Blaustein et al.*, 1978), and the assays made use of the 6-monomethyl pterin analogue, which has been shown to induce atypical kinetic properties (*Kaufmann*, 1974). From a theoretical point of view the results suggested a feed-forward system yoking the increase in intracellular calcium accompanying neuronal discharge (*Baker*, 1972) to an increasing rate of 5-HT biosynthesis rather than an opportunity for regulatory control.

We have been able recently to study brain TPH activity after EGTA treatment and dialysis, using PH_4 cofactor and tryptophan in concentrations from about 10 to 100 μM, and have observed: sigmoidal kinetics with respect to tryptophan at low cofactor concentrations; sigmoidal kinetics with respect to cofactor at low tryptophan concentrations; and sensitivity to the conformational influence of ionized calcium in the submicromolar range, *i.e.* at concentrations corresponding to those of the ion within central nervous system neurons (*Blaustein et al.*, 1978). Such concentrations of calcium (a) increased the affinity of the enzyme for the tryptophan substrate at low substrate concentrations, (b) decreased the relative velocity of enzyme activity at higher substrate concentrations and the substrate concentration at which the maximum velocity occurs, (c) converted the sigmoidal substrate kinetic function to a hyperbolic curve, (d) manifested a homotropic effect, which in conjunction with calcium's positive heterotropic effector capacity with respect to substrate becomes indicative of TPH as an allosteric enzyme

(*Koshland et al.*, 1966), and (e) sensitized the enzyme to a reiterative site-opening effect of increasing substrate concentration, an effect of micromolar levels of calcium analogous to that reported in the depolymerization of microtubular protein into tubulin (*Weisenberg*, 1972).

TPH activity is sensitized to the ligand effect of calcium at low concentrations of tryptophan and PH_4, both of which are variable parameters *in vivo* (*Bullard et al.*, 1978; *Tagliamonte et al.*, 1971), and calcium can be seen as a positive effector because it increases the enzyme's affinity for substrate at low substrate concentrations, as a negative control because it decreases the relative velocity of the enzyme at higher substrate concentrations and the substrate concentration at which the maximum velocity occurs, and as a positive influence sensitizing the enzyme to opening new sets of active sites in response to increasing substrate concentrations within a given range. Thus a multivariate model incorporating these parameters (plus perhaps others not yet elucidated) will be required to characterize the role of calcium in the regulation of 5-HT biosynthesis (*Mandell*, 1978). We report here also that lithium in equimolar concentrations evokes kinetic properties in TPH opposite to those evoked by calcium.

Methods

Midbrains, extending from the pons to the mammillary bodies, from adult male Sprague-Dawley rats (150—200 g, Hilltop Laboratories, Scottsdale, PA) were dissected freehand, pooled, homogenized in 15 vol 1.0 mM Tris-acetate buffer, pH 7.4, in a Thomas glass-Teflon homogenizer with clearance of 0.01 cm and centrifuged at 35,000×g for 20 min, after which all measurable TPH activity was found in the supernatant fraction. In some instances the supernatant fraction was made 100 or 500 μM in EGTA, stirred for 15 min at 5 °C, and dialyzed for 2 hours against 100 vol of 1.0 mM Tris-acetate buffer, pH 7.4, with a change of dialysate at 1 hour. In some instances this preparation was dialyzed for 16 hours. The dialyzed enzyme preparation was centrifuged for 20 min at 35,000×g and the resulting supernate or the undialyzed enzyme preparation was used as the enzyme source. TPH was assayed using a modification of the fluorimetric method of *Friedman et al.* (1972) with dithiothreitol and NADH in place of sheep-liver dihydropterin reductase, which when added to our assay resulted in at most a 15 % augmentation in enzyme activity. Tryptophan was varied from 2.02 to 40.3 nmol and PH_4 was varied from 5.9 to 41.3 nmol in our standard incubation mixture, which contained 12 μmol Tris-acetate buffer, pH 7.4, 0.32 μmol dithiothreitol, and 0.16 μmol reduced β-nicotinamide adenine dinucleotide, in a final vol of 395 μl. Blanks consisted of reaction mixtures in which d-tryptophan replaced l-tryptophan,

and internal standards of authentic 5-HTP were run in the blanks each day. The reaction was stopped at 30 min with the addition of 50 μl of 50 % trichloroacetic acid after which the precipitated protein was removed by centrifugation, and 800 μl HCl was added to 300 μl of the supernate for the determination of fluorescence at 538 nm, with an excitation wavelength of 195 nm, using an Aminco Bowman spectrophotofluorimeter. The amount of 5-HTP formed was calculated from standard fluorescence curves run each day with authentic 5-HTP from 0.03 to 3.0 nmol. Protein determinations were made on each enzyme preparation by the method of *Lowry et al.* (1951).

Simultaneous study of supernates with this assay and the isotopic assay we used previously (*Knapp* and *Mandell,* 1975), now with 10 to 100 μM PH_4 and 10 μM tryptophan, produced agreement of values within 10 %. The kinetic phenomena reported here were seen in at least three repetitive experiments. With phyisological concentrations of reaction participants, the effects, although small, were consistent.

Results

Fig. 1 presents typical substrate and cofactor velocity curves for TPH activity with increasing levels of tryptophan at a saturating concentration of PH_4 and for increasing levels of PH_4 at a saturating concentration of tryptophan. The functions are hyperbolic, compar-

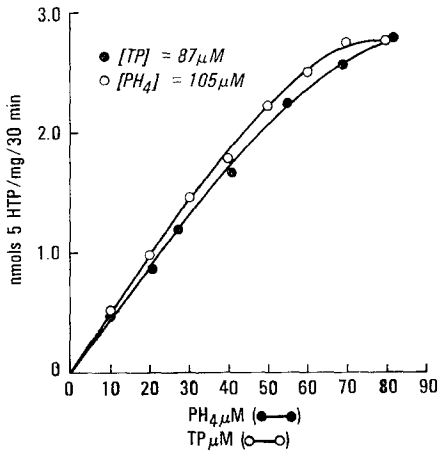

Fig. 1. Increasing substrate at saturating cofactor and increasing cofactor at saturating substrate produce reaction velocity curves that are hyperbolic. At concentrations beyond these curves (not shown), are a sequence of additional hyperbolic kinetic functions, comparable to those seen in Fig. 5. Both tryptophan and PH_4 at concentrations close to saturation phenomena by TPH induce site-opening. See text

able to those reported by *Friedman et al.* (1972), who studied rabbit brainstem enzyme purified ten-fold. In repeated determinations, the affinity constants for both cosubstrates were in the area of 30 μM, and maximum velocity (V_{max}) was achieved with the cosubstrates in the range of 60 to 80 μM. These values are comparable to values obtained by others (*Friedman et al.*, 1972; *Kaufman*, 1974). As will be seen, it was not possible to obtain a true V_{max} because of the site-opening phenomenon that occurs at substrate concentrations in the region where either curve approaches its asymptote, rendering K_m only an approximation as well. Our values relating to V_{max} and K_m, therefore, will be referred to as V_1 and K_1 to acknowledge this indeterminancy and to indicate that the focus is the first curve in a system manifesting reiterative kinetic functions. Values for brain PH_4 and tryptophan concentrations are in the range of values found for their K_1's (*Bullard et al.*, 1978; *Eccleston et al.*, 1965).

The EGTA-treated, dialyzed preparation and the native enzyme preparations studied at low cofactor concentrations exhibit sigmoidal kinetic functions with regard to substrate (control curves, Figs. 2 and 3). Cofactor concentrations in this range (below K_1 for cofactor in Fig. 1) and lower than baseline levels of endogenous PH_4 can be observed following a variety of drug treatments *in vivo* (*Bullard et al.*, in press) and probably elicit the kinetic characteristics of TPH *in vivo* more accurately than studies using saturating levels of cofactor (*Friedman et al.*, 1972). Sigmoidal kinetic functions have been reported for partially purified hepatic TPH under baseline conditions, the substrate cooperativity being sensitive to temperature (*Sullivan et al.*, 1973), and for an enzyme preparation from rat brainstem (*Hamon et al.*, 1976). In our hands, EGTA treatment and dialysis, reducing intrinsic calcium concentration, made substrate cooperativity more marked and consistent at low PH_4 concentrations.

Fig. 2 illustrates three small but consistent effects of submicromolar calcium concentrations in our treated, dialyzed enzyme preparations: an increase in the affinity of the enzyme for tryptophan substrate (decrease in K_1); a decrease in the relative velocity of TPH activity at higher substrate concentrations (decrease in V_1) and a decrease in the substrate concentration at which V_1 occurs; sensitization of the enzyme preparation to open new sets of catalytic sites (reflected by additional kinetic curves within a given substrate range). These three effects are associated with a loss of substrate cooperativity, reflected by the conversion of the substrate function from sigmoidal to hyperbolic form in the initial concentration range and in the reiterative curves beyond it. It appears that tryptophan serves as a ligand altering subunit interactions, as reflected by substrate co-

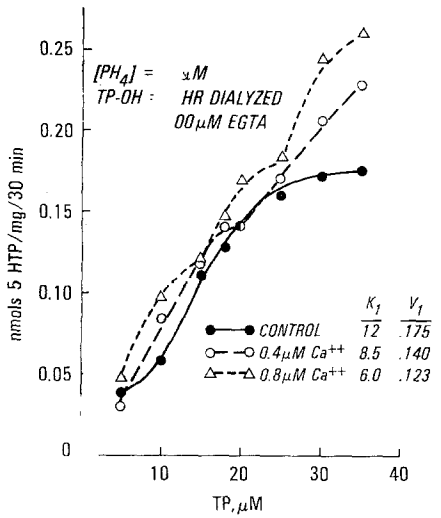

Fig. 2. EGTA-treated and dialyzed TPH preparations studied with increasing tryptophan at low cofactor concentrations consistently manifested substrate cooperativity. $CaCl_2$ at 0.4 and 0.8 μM concentrations produced three small but consistent graded kinetic effects: a decrease in K_1; a decrease in V_1 and the substrate concentration at which V_1 occurs; an increase in sensitivity to the site-opening effect of increasing tryptophan in the region of the inflection point approaching V_1 so that there are additional hyperbolic functions within a given substrate range. These effects are associated with the conversion of the sigmoidal to a hyperbolic kinetic function. The chloride ion, as seen in Fig. 6, is not responsible for these effects

operativity, and effecting the opening of new sets of sites when others are nearing saturation. In earlier studies of calcium "activation", use of the 6-monomethyl pterin analogue and calcium in millimolar concentrations may have confounded both the affinity change and the opening of new catalytic sites (*Knapp* and *Mandell*, 1975; *Boadle-Biber*, 1975; *Hamon et al.*, 1977 a).

Fig. 3 shows curves, similar to those in Fig. 2, from studies of an untreated, undialyzed enzyme preparation which required higher concentrations of calcium to manifest its effects on affinity, velocity, and site opening. The comparability of the pattern of kinetic changes seen in response to graded amounts of calcium by native enzyme (Fig. 3) to that seen with the treated enzyme preparation (Fig. 2) suggests a potentially specific role for calcium in submicromolar concentrations in the regulation of TPH. The affinity constant for extramitochondrial calcium binding in nerve endings is 0.35 μM, and fluxes related to depolarization alter endogenous levels around this value (*Blaustein et al.*, 1978). It is intriguing that native preparations

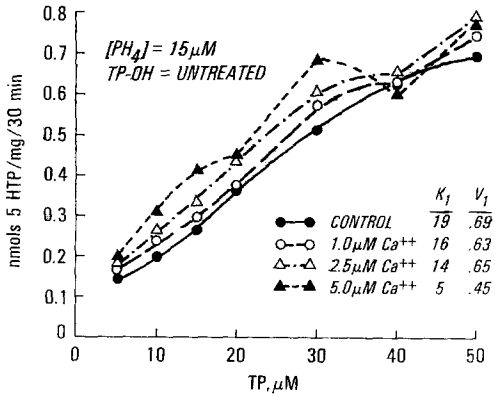

Fig. 3. Native TPH preparations without EGTA pretreatment or dialysis, studied with increasing tryptophan concentrations at low PH_4 levels, manifest control substrate velocity functions less sigmoidal in form than those shown in Fig. 2 and manifest the same three Ca^{++} ligand effects, which here require a ten-fold increase in the concentration of the ion. EGTA pretreatment and dialysis (*cf.* Fig. 2) strengthened the sigmoidicity thus sensitizing the enzyme preparations to the effects of the calcium ion

manifest the same calcium-induced kinetic phenomena as preparations sensitized by calcium-depleting pretreatment, but require higher levels of the ion ligand.

Fig. 4 shows that with low tryptophan concentrations a cofactor/velocity curve manifests the same sigmoidal character as the tryptophan curve does with low PH_4 concentrations. It appears that

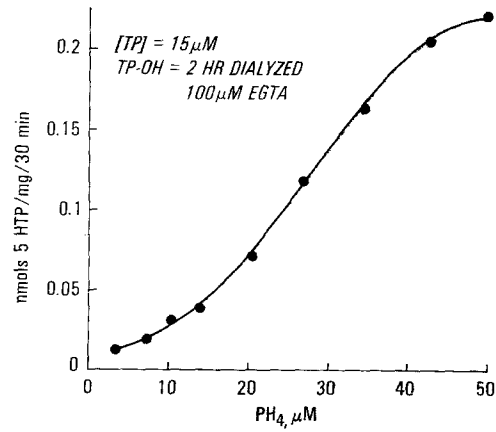

Fig. 4. Dialyzed TPH preparation studied with increasing PH_4 at low tryptophan concentrations manifests a cofactor velocity function that is sigmoidal in form, indicative of cofactor cooperativity. See text

TPH manifests cooperativity with respect to cofactor as well as to substrate. As can be seen in Fig. 2, enzyme sensitivity to conformational influence by calcium is partially a function of cofactor concentration, which itself has homotropic effector properties in the presence of subsaturating tryptophan concentrations (Fig. 4). Similarly, the level of tryptophan differentially sensitizes the enzyme to the calcium effector influence while itself serving a ligand function, as manifested by substrate cooperativity in the presence of subsaturating cofactor concentration. The dual roles of cosubstrate and ligand manifested by both PH_4 and tryptophan with respect to each other and to themselves is characteristic of the activity of allosteric enzymes (*Hammes* and *Wu*, 1971). Fig. 5 is an extended calcium/velocity curve using saturating concentrations of both cofactor and substrate in which cooperativity with respect to calcium is observed. The observation that calcium serves as both heterotropic effector with respect to tryptophan and homotropic effector with respect to itself confirms that brain TPH is an allosteric enzyme (*Monod et al.*, 1963). Note that, as was the case with tryptophan (Figs. 2 and 3), increasing concentrations of calcium lead to the reiterative opening of new active sites as the kinetic function(s) approach the asymptote(s). This is directly analogous to calcium's induction of the depolymerization of microtubular protein (*Weisenberg*, 1972) and might be related to

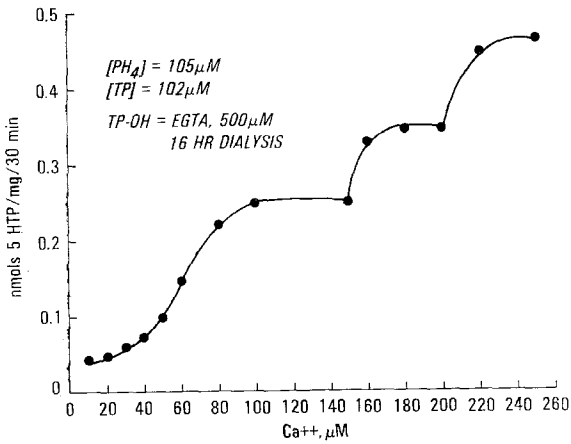

Fig. 5. EGTA-treated and dialyzed TPH preparations were studied with increasing concentrations of $CaCl_2$ at saturating concentrations of tryptophan and PH_4. The ligand manifests homotropic cooperativity which, when taken with its heterotropic effector properties (see Figs. 2 and 3), indicates that TPH is an allosteric protein. Higher levels of calcium, as were the cases with tryptophan and PH_4, evoke the opening of new catalytic sites as the kinetic function approaches the asymptote

the immunocytochemical localization of TPH in neurons in cytoplasmic "free" and microtubular "bound" forms (*Pickel et al.*, 1976).

Fig. 6 illustrates the effects of the lithium ion on the substrate curve of an enzyme preparation that had been dialyzed but not pretreated with EGTA, in the presence of low PH_4 concentration. We used the same concentrations to study the effect of lithium that we had used to examine the effect of calcium (see Fig. 2). Note that whereas calcium converted the substrate curve from sigmoidal to hyperbolic form in association with increased affinity for substrate and induced additional hyperbolic kinetic functions within the same substrate range, equimolar lithium exaggerated the substrate cooperativity and decreased the enzyme's affinity for tryptophan. Because experiments with either calcium or lithium involved the chloride ion, each served the other as a counter-anion control. Extended substrate curves in the presence of these concentrations of lithium (not shown) failed to indicate site-opening phenomena in the region of V_1 or beyond, nor did extended lithium curves comparable to that for calcium (Fig. 5) indicate reiterative opening of new sites. The lack of such an effect, considered with the lithium-induced exaggeration of substrate cooperativity and decrease in enzyme affinity for tryptophan, suggests that the ligand effects of the lithium

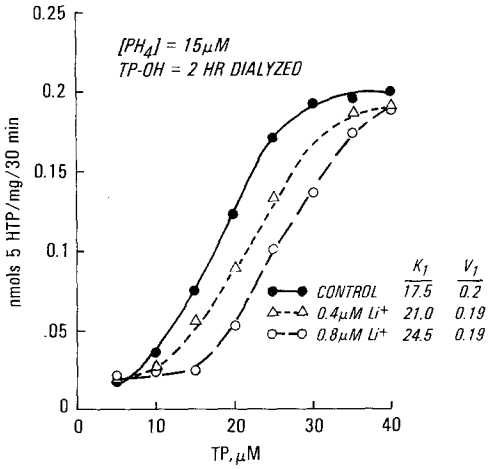

Fig. 6. Dialyzed TPH preparations studied with increasing tryptophan at low cofactor concentrations in the presence of graded amounts of LiCl (0, 0.4 μM, 0.8 μM) demonstrate exaggerated sigmoidal functions (compared to control), associated with an increase in K_1 (decreased substrate affinity) and no change in V_1, but an increase in the level of substrate at which V_1 occurs. In the presence of LiCl there was no reiterative opening of new sites with increasing tryptophan levels. See text

ion are opposite to those of the calcium ion. When lithium chloride is present the maximum velocity (V_1) is reached at higher substrate concentrations, which permits induction of increased 5-HT biosynthesis by lithium at increased brain levels of tryptophan. The sensitivity of TPH activity to such low levels of lithium is remarkable because clinical levels of lithium in red-cell and muscle compartments are about a thousand times greater (*Williams*, 1973). The possibility of lithium and calcium interacting at a specific site is suggested by this demonstration of their opposite effects at equimolar concentrations on TPH kinetic functions. Multivariate kinetic studies of ion competition are in progress to determine whether lithium itself is a negative effector or achieves negative effector influence by blocking calcium binding at a positive effector site.

Discussion

Brain TPH has been described as labile in efforts to account for the difficulties impeding its enrichment, purification, and kinetic characterization (*Grahame-Smith*, 1964; *Lovenberg et al.*, 1967; *Ichiyama et al.*, 1968; *Friedman et al.*, 1972). In the course of our studies requiring enzyme preparation and dialysis, we have observed, as others have, a rather significant loss of activity. However, as indicated, TPH activity could be returned to control levels by the addition of calcium in μM concentrations. Even nearly total loss of observable enzymatic activity after 16-hour dialysis could be reversed almost completely with this ion. It may be that previous work has not taken sufficient cognizance of the potential role of calcium in regulating baseline TPH activity. Moreover, the enzyme's complex allosteric properties may have produced confusing irregular curvilinear functions that were interpreted as reflecting activity loss due to protein denaturation rather than kinetic property changes as a function of conformational alteration.

In this preliminary report we suggest that, in addition to their roles as cosubstrates, tryptophan and PH_4 influence the kinetic properties of TPH and the degree to which its activity can be influenced by the calcium ion, a positive effector. For the enzyme to reach saturation (V_1) 60 to 80 μM of each reactant was required (Figs. 1 and 5). This implies that TPH is a multi-site enzyme with perhaps the same number of binding sites for all three ligands (*Gerhart*, 1964). In concentrations equimolar to those of calcium, the lithium ion, acting like a negative effector (*Monod et al.*, 1963; *Koshland*, 1970) may occupy and block the calcium site, or it may

occupy the site of an unidentified negative effector, for which possibility magnesium must certainly be considered because it has peptide-binding characteristics and physical properties closely resembling those of lithium (*Blout et al.*, 1974).

The multivariate character of the regulatory potential of TPH is emphasized in our work. The enzyme's sensitivity to the effect of calcium is a function of cofactor and tryptophan levels, both alterable parameters in brain (*Bullard et al.*, 1978; *Tagliamonte et al.*, 1971); its sensitivity to the ligand effects of cofactor and tryptophan is a function of the intraneuronal level of calcium, which is dependent upon discharge rate (*Baker*, 1972) and sensitive to pharmacological influences (*Leslie et al.*, 1978).

It is likely, in view of the regulation of similar enzymes, that a variety of such other influences as hormones and pH will prove to be significant in TPH regulation (*Hammes* and *Wu*, 1971). For instance, brain tyrosine hydroxylase manifests allosteric properties and has been shown to have multiple conformational influences including ions, pH, and the redox potential of the enzyme's environment (*Kuczenski* and *Mandell*, 1972; *Kuczenski*, 1975; *Katz et al.*, 1976). Multivariate kinetic studies will be necessary to assess the full range of potential effectors of TPH (*Mandell*, 1978).

At this early stage of the work it is perhaps best to focus on the functional implications of what appear to be the two preferred conformational states of the enzyme: (1) a high substrate-affinity form manifesting hyperbolic substrate kinetics and induced at low cofactor concentrations by submicromolar concentrations of calcium and (2) a lower affinity, native form, found when the preparation is assayed at low cofactor concentrations, manifesting sigmoidal substrate kinetics, and exaggerated by submicromolar concentrations of lithium. Both forms have been observed *in vivo*. Cocaine (*Knapp* and *Mandell*, 1976) and phenylalanine loads (*Neckers et al.*, 1977) induce the high affinity form and are associated with a decrease in brain 5-HT which could be explained as the effect of decreased substrate supply (both drugs block tryptophan access to the intraneuronal environment [*Mandell* and *Knapp*, 1978]), and an associated increase in TPH sensitivity to intraneuronal calcium, which as we have seen induces hyperbolic substrate kinetics and reduces the maximum velocity (V_1) of the enzyme as well as the substrate concentration at which V_1 occurs. The decrease in substrate supply diminishes 5-HT synthesis by both mass action and sensitization of TPH to a conformational alteration by calcium.

Lithium treatment (*Knapp* and *Mandell*, 1975) and tryptophan loads (*Mandell* and *Knapp*, 1978) *in vivo* lead to the low affinity

form and an increase in 5-HT synthesis in brain. In this case, the increase in 5-HT synthesis follows from increased substrate supply to TPH (*Mandell* and *Knapp*, 1977), a decreased TPH sensitivity to calcium, and a primary kinetic effect of lithium as a negative effector (either by blocking the calcium site or acting in another site), so that calcium-induced reduction of V_1 is countered, allowing a greater maximum velocity of 5-HT synthesis *in vivo* at higher levels of substrate even though TPH affinity for substrate is reduced.

We showed that lithium pretreatment antagonizes the effect of cocaine on 5-HT biosynthesis (*Knapp* and *Mandell*, 1976) and we attributed the effect to an antagonism at the level of the neuronal transport of tryptophan; it may be that a parallel antagonism exists between calcium and lithium at the level of the regulation of enzyme conformation. Amphetamine, which decreases brain PH_4 (*Bullard et al.*, 1978) would be predicted to lead to the higher affinity form of the enzyme with hyperbolic substrate kinetics due to the sensitization of TPH to calcium by low cofactor levels, but the drug also increases brain tryptophan three- to four-fold (*Tagliamonte et al.*, 1971) which would desensitize the enzyme to calcium and lead to the lower affinity form of TPH. The net effect of these countervailing conformational influences is the lower affinity enzyme form, comparable to that seen following lithium (*Mandell* and *Knapp*, 1976). Whereas lithium pretreatment prevents the change evoked by cocaine in the substrate affinity of TPH, it pre-empts the one induced by amphetamine (*Mandell* and *Knapp*, 1976).

Williams (1976) has pointed out that most proteins with calcium binding sites have oxygen as the binding ligand in carboxylate or carbonyl centers and that of all the monovalent cations, only lithium could compete with calcium in a system using carboxyl groups as chelators. With greater ease of adjusting its bond lengths, lithium would also be capable of displacing intracellular magnesium, even though an ion cavity of lithium's size would contain magnesium (*Lehn*, 1973). Further work to define these ion/protein interactions as reflected in kinetic and regulatory phenomena will require the purification of TPH, which is in progress now in our laboratory.

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