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Mechanism of inhibition of pineal hydroxyindole-*O*-methyltransferase by pyridoxal 5′-phosphate

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Of possible significance relative to the nature of the active site of the enzyme hydroxyindole-*O*-methyltransferase (HIOMT) was the recent report [1] that pyridoxal 5′-phosphate (PLP) inhibited the HIOMT-catalyzed transmethylation of *N*-acetylserotonin to melatonin. Nir *et al.* [1] proposed that this inhibition by PLP could represent a mechanism by which HIOMT activity was regulated in the pineal gland. In addition, Nir *et al.* [1] suggested that PLP inhibited HIOMT through a mechanism involving PLP competing for the *S*-adenosylmethionine (L-SAM) enzymatic binding site.

Interestingly, other examples of PLP inhibition of L-SAM-dependent methyltransferases or vice versa have been reported. For example, Black [2] reported PLP inhibition of the *O*-methylation of norepinephrine by catechol-*O*-methyltransferase. The mechanism of this inhibition was later clarified by our laboratory [3], when it was shown that PLP and norepinephrine underwent a facile chemical reaction resulting in the formation of a tetrahydroisoquinoline adduct. The removal of the norepinephrine by reaction with PLP and the generation of the tetrahydroisoquinoline, which was shown to be a reversible inhibitor of COMT, accounted for the majority of the inhibitory effects observed with PLP. Similar mechanisms have been reported for the inhibition of PLP-dependent enzymes by catecholamines [4–7].

SAM has also been reported by Trewyn *et al.* [8] to be an inhibitor of tyrosine aminotransferase, which is a PLP-dependent enzyme. The mechanism of this inhibition was not completely characterized, but data were presented which suggested a chemical reaction was occurring between SAM and PLP, perhaps involving Schiff base formation, which resulted in the observed inhibition.

In order to determine if PLP inhibited HIOMT by direct interaction with the enzyme (e.g. Schiff base formation with an ϵ-amino group of a lysine residue at the enzyme active site) or by nonenzymatic reaction with either of the substrates (e.g. L-SAM or *N*-acetylserotonin), a study of the mechanism of PLP inhibition of HIOMT was carried out. The present paper describes the results of this study and a mechanism is proposed to account for the PLP inhibition of the HIOMT-catalyzed transmethylation *in vitro*.

MATERIALS AND METHODS

HIOMT was isolated from bovine pineal glands (Pel-Freez Biologicals) according to the procedure of Jackson and Lovenberg [9] and purified through the DEAE-Sephadex chromatography step, which resulted in a 10-fold purification of the enzyme. For the HIOMT assay, [^{14}C]H$_3$-L-SAM (New England Nuclear, 55.0 mCi/mmole) was stored at −20°F. L-SAM iodide and *N*-acetylserotonin (Sigma) were stored as 0.01 M aqueous stock solutions. Enzyme activity was measured by a previously described radiochemical assay [10]. A normal enzyme assay mixture consisted of the following components (in μmoles): water, so that the final volume was 0.25 ml; *N*-acetylserotonin (0.25); SAM (variable); PLP (variable); 0.55 μCi [^{14}C]H$_3$-L-SAM; phosphate buffer, pH 7.9 (10);

and the enzyme preparation. Assay mixtures were incubated for 30 min at 37° and the reaction was terminated by the addition of 0.10 ml of 0.2 M borate buffer, pH 10.0. The aqueous layer was extracted with 10 ml of toluene–isoamyl alcohol (5:1) and an aliquot (5 ml) of the organic phase was checked for radioactivity. The enzyme activity was corrected using an *N*-acetylserotonin blank.

The chemical reaction between PLP and L-SAM (or SAM analogs) was monitored by following the decrease in absorbance at 388 nm using a Gilford model 240 spectrophotometer. In order to simulate the normal HIOMT assay conditions, these spectrophotometric studies were carried out in 0.1 M phosphate buffer, pH 7.9, at 37°. Plots of the reciprocals of the absorbance changes at 388 nm vs the reciprocals of the L-SAM concentrations (or SAM analogs) were linear. Using the Benesi–Hildebrand's equation, the dissociation constants for the resulting complexes could be calculated [11]. The compounds used in these spectrophotometric studies were obtained from the following sources: L-methionine (Met), L-methionine-*S*-methyl sulfonium iodide (methyl-L-Met) and *S*-adenosyl-L-homocysteine (SAH) (Sigma); *S*-adenosyl-D-methionine (D-SAM), *S*-adenosyl-3-methylthiopropylamine (decarboxylated-SAM), *S*-adenosyl-4-methylthiobutyric acid (deaminated-SAM), *S*-adenosyl-L-*N*-acetylmethionine (N^3-acetyl-L-SAM) and D-methionine-*S*-methyl sulfonium iodide (methyl-D-Met) were prepared by methylation of the corresponding sulfur ethers (e.g. D-SAH, D-methionine, etc.) with methyl iodide according to a procedure described in earlier papers from our laboratory [12, 13].

As a model system, the Schiff base complex formed between PLP and L-methionine-*S*-methyl sulfonium iodide was characterized by reduction with sodium borohydride. The resulting N^3-(4′-deoxy-5′-phosphopyridoxine-4′-yl)-L-methionine-*S*-methyl sulfonium iodide was isolated and identified as follows. A solution of pyridoxal 5′-phosphate (247 mg, 1 m-mole) and L-methionine-*S*-methyl sulfonium iodide (728 mg, 2.5 m-moles) in 20 ml of phosphate buffer (0.1 M, pH 7.9) was incubated at 37° for 60 min. Sodium borohydride (314 mg, 5 m-moles) was then added to the reaction mixture in several portions over a 1-hr period. The reaction mixture was lyophilized after which the resulting powder was dissolved immediately in 5 ml of cold water and filtered. The filtrate was chromatographed on Silica gel plates [9 parts of the solution containing EtOH, H_2O and HOAc (20:2:2) and 4 parts of 0.1 M, pH 7.6, phosphate buffer]. The band corresponding to $R_f = 0.2$ was extracted with 10 ml of cold water. This extract was rechromatographed on a cellulose plate (EtOH:H_2O = 3:2) to remove inorganic salts. The desired reduced adduct was isolated in 26 per cent yield with a m.p. = 198° (dec.). The adduct exhibited an ultraviolet absorption at 325 nm and the following nuclear magnetic resonance spectra was consistent with the assigned structure: (D_2O) δ 1.93 (*s*, 3 H, C_2-H_3), 2.2–2.70 (*m*, 2 H, C_β-H_2), 2.99 (*s*, 6 H, methyl sulfonium), 3.33–4.07 (*m*, 4 H, C_γ-H_2 and C_4-H_2), *ca.* 4.25 (*m*, 1 *H*, C_α-H), 4.97, 4.87 (2*d*, 2 H, C_5-H), 7.77 (*s*, 1 H, C_6-H).

RESULTS AND DISCUSSION

In agreement with the earlier report of Nir *et al.* [1], we have confirmed in our study that PLP has an inhibitory effect on the HIOMT-catalyzed transmethylation of *N*-acetylserotonin to melatonin. In an effort to elucidate the mechanism of this inhibitory effect, we carried out spectrophotometric studies looking for spectral changes upon incubation of PLP with L-SAM or *N*-acetylserotonin under conditions similar to those used in the enzymatic assay. Incubation of solutions of PLP and increasing concentrations of *N*-acetylserotonin produced no significant changes in ultraviolet or visible spectrum of PLP. However, incubation of PLP with increasing concentrations of L-SAM at pH 7.9 (37°) resulted in a decrease in the characteristic PLP absorption at 388 nm with a concomitant red shift. The absorption at 388 nm is attributable to the aldehyde functionality of PLP [14]. With high concentrations of SAM, the PLP spectrum exhibited peaks at 410 nm and 335 nm, characteristic of a classical Schiff base adduct formed between PLP and an amino acid [15]. When structurally related compounds such as D-SAM, decarboxylated SAM, methyl-L-Met, and methyl-D-Met were incubated with PLP, similar spectral changes were observed. Smaller spectral changes were observed, however, when PLP was incubated with structurally related compounds which did not contain a sulfonium group such as SAH or methionine. No spectral changes were observed when PLP was incubated with deaminated SAM or N^3-acetyl SAM, suggesting that the reaction between PLP and L-SAM probably involved the formation of a Schiff base between the aldehyde of PLP and the terminal amino group of L-SAM. In order to obtain firm evidence for the Schiff base formation between PLP and sulfonium-containing amino acids, the adduct formed between PLP and methyl-L-Met in 0.1 M phosphate buffer (pH 7.9) was reduced with sodium borohydride. The reduced adduct, N^3-(4′-deoxy-5′-phosphopyridoxine-4′-yl)-L-methionine *S*-methyl sulfonium iodide, was purified and identified by its nuclear magnetic resonance and ultraviolet-visible spectrums as described in Materials and Methods. The reduced adduct which was isolated had the characteristic ultraviolet absorption maximum at 325 nm [15]. Attempts were also made to reduce the PLP·SAM Schiff base adduct and isolate the resulting product. However, these attempts were unsuccessful, because of the apparent unstable nature of this Schiff base adduct.

In an effort to account for the observation that sulfonium-containing amino acids (e.g. L-SAM and methyl-L-Met) produce a greater spectral change with PLP than amino acids which did not contain a sulfonium group, we determined the dissociation constants for the adducts formed between PLP and amino acids structurally related to L-SAM. As shown in Table 1, the stability of the complexes formed between PLP and sulfonium-containing amino acids (e.g. L-SAM, D-SAM, decarboxylated SAM, methyl-L-Met and methyl-D-Met) was much greater than that of the corresponding nonsulfonium compounds (SAH and methionine). A possible explanation for this finding is that the positive charge on the sulfonium group may contribute to the stability of the Schiff base adduct through intramolecular ionic interactions with the phosphoryl

Table 1. Dissociation constants for Schiff bases formed between PLP and L-SAM analogs*

SAM analogs	Dissociation constants for PLP complex† (μM)
L-SAM	409 ± 13.5
D-SAM	409 ± 6.10
Decarboxylated-SAM	413 ± 23.1
Methyl-L-Met	466 ± 47.2
Methyl-D-Met	456 ± 47.2
L-SAH	703 ± 94.7
L-Methionine	1342 ± 29.6

* Dissociation constants were determined by measuring final absorbance changes at 388 nm when PLP was incubated with increasing concentrations of the SAM analogs. See text for details concerning the experimental procedures.

† Dissociation constants were determined using the Benesi–Hildebrand's equation [11].

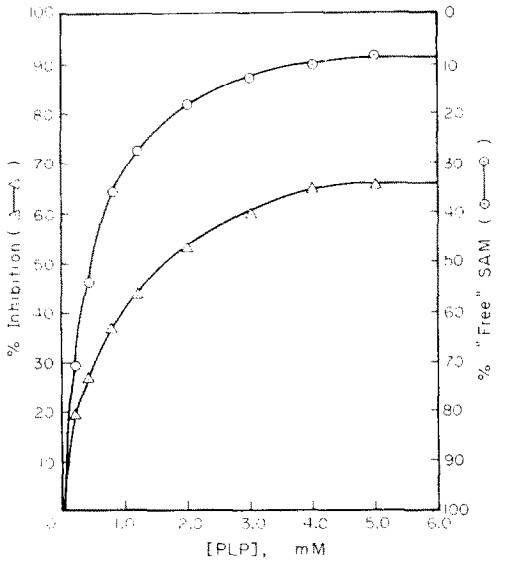


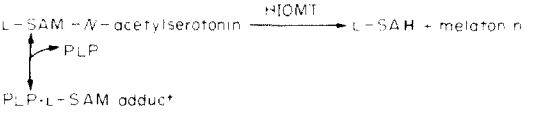
Fig. 1. Relationship between HIOMT inhibition, PLP concentration and the concentration of the PLP·SAM Schiff base adduct. The HIOMT assay conditions are as described in the text. Assay conditions were: SAM concentration, 0.04 mM; [^{14}C]H_3-SAM, 0.05 μCi; *N*-acetylserotonin concentration, 1.0 mM; PLP concentration, variable; phosphate buffer, pH 7.9. Incubations were carried out at 37° for 30 min, and the reaction was stopped with 0.10 ml of 0.2 M borate buffer, pH 10.0. The concentration of "free" L-SAM was calculated from the dissociation constant for the PLP·SAM complex reported in Table 1. Free SAM refers to that amount of L-SAM not in the PLP·SAM Schiff base.

anion on PLP. Spacing filling models of the PLP·methyl-L-Met and PLP·L-SAM Schiff base adducts suggest that this intramolecular interaction is possible.

In earlier work from our laboratory, we reported that the terminal amino group of L-SAM is an absolute requirement for its activity as a methyl donor in the HIOMT-catalyzed reaction. Removal or blocking of this functionality on SAM results in complete loss of methyl donor activity. Therefore, the formation of a PLP·L-SAM Schiff base adduct through the terminal amino group of L-SAM would decrease the "free" L-SAM concentration and produce an apparent inhibition of HIOMT activity.

Further evidence to support this mechanism is shown in Fig. 1, where a comparison is made between the concentration of PLP and the per cent inhibition of HIOMT activity or the concentration of PLP and the per cent of "free" SAM present in the incubation mixture. The data in Fig. 1 show that there is a good correlation between the per cent inhibition of HIOMT activity and the decrease in free SAM. Further evidence in support of this hypothesis comes from the observation that methyl-L-Met is able to protect HIOMT from inhibition by PLP. Methyl-L-Met alone in the incubation mixture has no effect on HIOMT activity, but apparently prevents PLP inhibition of the enzyme by competing with L-SAM for PLP, resulting in a higher "free" concentration of the methyl donor, and therefore less apparent inhibition.

In conclusion, the results of the experiments reported in this paper would support the hypothesis that PLP inhibits HIOMT *in vitro* by decreasing the methyl donor concentration through formation of a Schiff base adduct with L-SAM (Scheme 1). No evidence was obtained for direct interaction between PLP and HIOMT. This same mechanism is probably functioning in the inhibition of the PLP-dependent enzyme tyrosine aminotransferase by L-SAM [8].



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