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Some Properties of Pineal Gland Hydroxyindole-O-Methyltransferase From Black Rhinoceros (*Diceros bicornis*)

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Pineal glands were obtained from two young female black rhinoceri that had died as a result of postcapture trauma during a translocation exercise. Hydroxyindole-O-methyltransferase (HIOMT) from these pineal glands showed a peak activity at pH 8.2, although high activity extended over a fairly wide pH range (7.8–8.4). N-acetylserotonin was the best hydroxyindolic substrate for the enzyme, although other hydroxyindoles were methylated, the relative affinities being similar to values previously reported for bovine HIOMT. Kinetic analyses revealed that black rhinoceros HIOMT was subject to substrate inhibition by both substrates at high concentration; this observation is unlikely to have physiological significance. The catalytic mechanism was found to be ordered Bi-Bi, in which S-adenosylmethionine is the obligatory first substrate to bind to the enzyme, such binding allowing for binding of the hydroxyindolic substrate followed by catalysis, products again leaving the catalytic site in a sequential fashion.

Key words: enzyme kinetics, substrate inhibition, catalytic mechanism

INTRODUCTION

The pineal gland is a complex organ that has been found to influence numerous physiological processes [Ariens-Kappers, 1979; Reiter, 1981] primarily through the actions of melatonin, although other pineal products have been shown to exert hormonal activity [Pevet, 1983; Haldar-Misra and Pevet, 1982]. Since the discovery that the pineal gland contained hydroxyindole-O-methyltransferase (HIOMT), the enzyme responsible for the production of melatonin [Axelrod and Weissbach, 1960], a considerable amount of work has been done in order to understand more fully the catalytic behaviour of HIOMT and the mechanisms involved in the production of melatonin.

Although melatonin is generally accepted as the principal pineal hormone,

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HIOMT does catalyse production of other methoxyindoles [Axelrod and Weissbach, 1960; McIsaac et al., 1965; Balemans et al., 1981], some of which have also been shown to exert physiological activity [Mullen et al., 1979; Pevet, 1983]. The production of these various methoxyindoles by pineal HIOMT appears to be dependent, at least in the rat, on the relative concentrations of the various hydroxyindole precursors and their affinities for the enzyme [Morton and Potgieter, 1982a; Morton, 1986a, 1987]. The catalytic mechanism of HIOMT has been shown to be ordered Bi-Bi and is similar regardless of which hydroxyindolic substrate is being used by the enzyme [Satake and Morton, 1979; Morton, 1986b]. In addition, although species heterogeneity of HIOMT has been shown to exist [Nakane et al., 1983], the catalytic mechanism appears to be the same regardless of species [Morton, 1986b; Morton and Forbes, 1989a]. In this regard, it is interesting to note that, although there does not appear to be a species-related difference in catalytic mechanism, there are substantial differences in certain kinetic parameters with possible physiological consequences [Morton, 1986b; Morton and Forbes, 1989b]. For this reason, it is important to determine the kinetic behaviour of HIOMT from various species in order to understand more fully the complex factors involved in the biochemical regulation of pineal melatonin production; the following discussion represents the first step in this process.

MATERIALS AND METHODS

Materials

S-adenosyl-L-[methyl-14C] methionine (18.5 MBq/mmol) was obtained from Amersham International plc (Bucks, UK); N-acetylserotonin, hydroxytryptophan, hydroxytryptamine HCl, hydroxyindole acetic acid, hydroxytryptophol, and S-adenosylhomocysteine from Sigma (St. Louis, MO), and all other chemicals and solvents were from Merck (Darmstadt, West Germany). Distilled, deionised water was used for all solutions.

Preparation and Assay of HIOMT

Two pineal glands obtained from young female black rhinoceri (*Diceros bicornis*) that had died as a result of postcapture trauma during a translocation exercise (110.4 and 82.7 mg wet weight) were homogenised in distilled water (25 μl/mg wet weight). Equal aliquots of this homogenate (25 μl) were mixed with buffer and substrates, and the final 100 μl reaction mixtures were incubated at 40°C for an hour, reaction being terminated by the addition of 100 μl of 0.2 M borate buffer, pH 10, and radioactive product extracted into 1 ml toluene:isoamyl alcohol (97:3) mixture prior to quantitation by scintillation spectrometry.

Determination of Optimum pH

Equal aliquots of pineal homogenate were incubated as above in 0.05 M phosphate buffer, with pH varying from 6.4–9.2 and containing N-acetylserotonin (100 μM) and S-adenosylmethionine (100 μM).

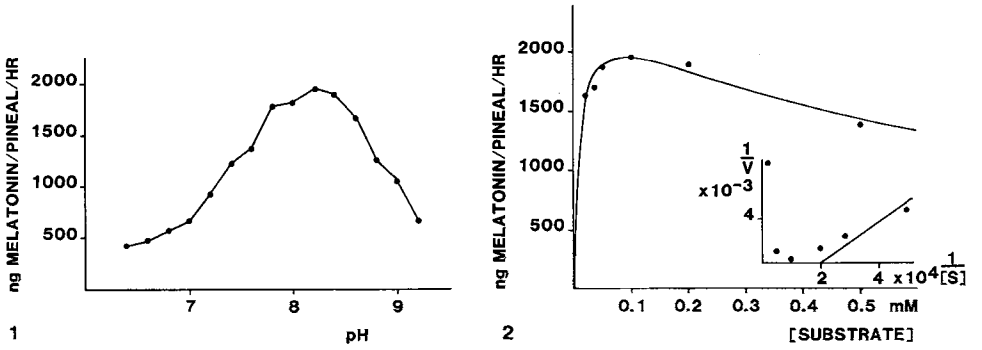


Fig. 1. pH dependence of black rhinoceros pineal HIOMT in 0.05 M phosphate buffer using N-acetylserotonin as the hydroxyindolic substrate.

Fig. 2. Velocity vs. N-acetylserotonin concentration for black rhinoceros pineal HIOMT. Double reciprocal plot illustrates the nonlinearity at high substrate concentrations being indicative of substrate inhibition.

Determination of Substrate Specificity

Equal aliquots of pineal homogenate were incubated as above in 0.05 M phosphate buffer, pH 7.9, containing S-adenosylmethionine (100 μM) and the different hydroxyindoles (100 μM).

Determination of Kinetic Parameters

Equal aliquots of pineal homogenate were incubated as above in 0.05 M phosphate buffer, pH 7.9, containing either S-adenosyl-methionine or N-acetyl-serotonin (100 μM) and varying concentrations of the second substrate (5–500 μM). Where S-adenosylmethionine was the varying concentration, substrate reaction mixtures were incubated in the presence and absence of S-adenosyl-homocysteine (50 and 100 μM).

Data Analysis

Results were obtained in duplicate and expressed as ng melatonin formed/pineal/hr. These values were fitted to the Michaelis-Menten equation by means of computer assisted iterative nonlinear regression analysis.

RESULTS

The pH/activity profile showed a peak at pH 8.2, although high activity was maintained over a wide pH range (7.8–8.4) (Fig. 1). From incubation of HIOMT with various substrates, it was apparent that N-acetylserotonin was the best substrate, followed by hydroxytryptophol (Table 1).

From observation of the kinetic parameters determined (Table 2; Figs. 2–4), it can be seen that black rhinoceros HIOMT is subject to substrate inhibition by both substrates at high concentrations (Table 2; Figs. 2, 3) and that S-adenosylhomocysteine is a competitive inhibitor of the enzyme with respect to S-adenosylmethionine (Fig. 4).

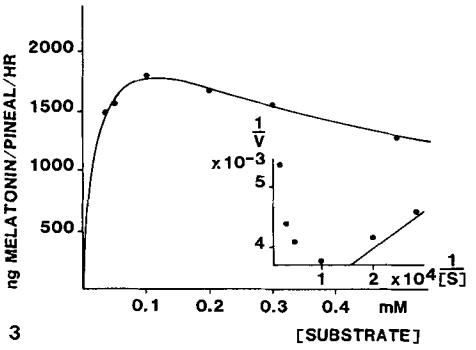
TABLE 1. Relative Activity of Black Rhinocerous Pineal HIOMT When Incubated With Various Hydroxyindolic Substrates (N = 2)

Substrate	Relative activity (%)
N-acetylserotonin	100
Hydroxytryptophol	79
Hydroxytryptamine	13
Hydroxytryptophan	7
Hydroxyindole acetic acid	4

TABLE 2. Michaelis-Menten Kinetic Constants and Inhibitor Constants for Black Rhinoceros Pineal HIOMT Determined for Both Substrates (N = 2)

	Substrate	
Kinetic constant	N-acetylserotonin	S-adenosylmethionine
Vm (ng melatonin/pineal/hr)	2,096 ± 34	2,034 ± 27
Km (μM)	5.7 ± 0.5	13.7 ± 0.7
Ki (μM)	932 ± 121	676 ± 88

[1]Vm, maximum velocity; Km, Michaelis-Menten kinetic constant; Ki, substrate inhibitor constant.



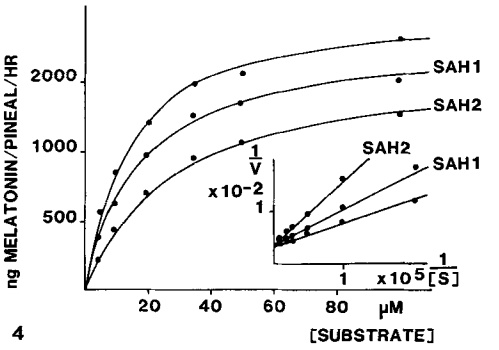


Fig. 3. Velocity vs. S-adenosylmethionine concentration for black Rhinoceros pineal HIOMT. Double reciprocal plot illustrates the nonlinearity at high substrate concentrations being indicative of substrate inhibition.

Fig. 4. Velocity versus S-adenosylmethionine concentration for black rhinoceros pineal HIOMT activity in the presence and absence of S-adenosylhomocysteine. Double reciprocal plots are shown to indicate the competitive nature of the interaction. (SAH1 = 50 μM, SAH2 = 100 μM).

DISCUSSION

Black rhinoceros pineal HIOMT, like the enzyme from other species [Morton and Potgieter, 1982b; Morton and Forbes, 1989a], showed high activity over a fairly wide range with a peak at pH 8.2; as these observations were so similar to those of other species, all subsequent determinations were conducted using the commonly employed pH 7.9 phosphate buffer. The relative activity of HIOMT with various hydroxyindolic substrates (Table 1) was similar to values reported for bovine HIOMT [Morton, 1987], but it differed from values reported for poikilothermic enzyme [Morton and Forbes, 1989a]. This difference in substrate specificity might suggest that methoxyindoles other than melatonin

are more important hormonal mediators in cold-blooded but not in warm-blooded animals; further work is indicated to examine this possibility in greater detail.

Black rhinoceros pineal HIOMT, like the trout enzyme [Morton and Forbes, 1989b], was subject to substrate inhibition (Table 2; Figs. 2, 3), although at concentrations of substrate above normal expected physiological levels. It is likely, therefore, that the observed substrate inhibition has no physiological function as has been alluded to in the trout [Morton and Forbes, 1989b] and can be considered to exert no influence on regulatory processes involved in the production of melatonin and other methoxyindoles by black rhinoceros pineal gland.

S-adenosylhomocysteine, the product resulting from demethylation of S-adenosylmethionine, was found to be a competitive inhibitor of black rhinoceros HIOMT (Fig. 4); on the basis of this product-inhibitory pattern, the catalytic mechanism appears to be ordered Bi-Bi [Fromm, 1975] and can be represented as follows:

where E represents enzyme; AMe S-adenosylmethionine; NAS N-acetyl-serotonin; MTN melatonin; SAH S-adenosylhomocysteine; and E.AMe, E.AMe.NAS, E.SAH.MTN, and E.SAH the enzyme-substrate/product complexes.

In this mechanistic scheme, S-adenosylmethionine is the obligatory first substrate that must bind to HIOMT in order to cause conformational changes that allow N-acetylserotonin to bind at the catalytic site. Once both substrates have bound to the enzyme, methyl transfer then occurs and the products leave, again in a sequential fashion. This catalytic mechanism is the same as has been previously demonstrated for bovine HIOMT [Satake and Morton, 1979; Morton, 1986b] and for trout HIOMT [Morton and Forbes, 1989a] and suggests that, although differences may exist between species HIOMTs in terms of molecular structure [Nakane et al., 1983] and substrate specificity, there is species homogeneity in the mechanism by which the enzyme methylates hydroxyindolic substrates.

ACKNOWLEDGMENTS

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