AFFINITY LABELING OF CATECHOL-O-METHYLTRANSFERASE WITH N-IODOACETYL-3,5-DIMETHOXY-4-HYDROXY-PHENYLETHYLAMINE*

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SUMMARY: Catechol-O-methyltransferase is inactivated rapidly by incubation with N-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine; not by the N-acetyl analogue. Iodoacetate or iodoacetamide produce slight inactivation. Inactivation is first order with respect to enzyme activity. A kinetic analysis suggests the formation of a dissociable enzyme-inhibitor complex prior to inactivation. Substrate, 3,4-dihydroxybenzoate, protects the enzyme from alkylation and loss of activity.

Catechol-O-methyltransferase (COMT)(E.C.2.1.1.6) is an S-adenosyl-methionine (SAM) dependent enzyme which plays an important role in the inactivation of catecholamines (1,2). As part of our continuing studies of this enzyme we have attempted to develop affinity labeling techniques which could be used to elucidate the relationship between the chemical structure and enzymatic function of this enzyme. One of our approaches to affinity labeling this enzyme has been the preparation of a chemically reactive derivative of 3,5-dimethoxy-4-hydroxyphenylethylamine (DMH-PEA) a dead-end inhibitor of COMT (3). This derivative should be capable of reacting with a wide variety of nucleophiles and yield a modified amino acid readily amenable to isolation and identification. Since iodoacetamides are capable of alkylating histidine, cysteine, methionine and lysine residues of proteins (4,5), we have prepared N-iodoacetyl-3,5-dimethoxy-4-hydroxy-phenylethylamine (N-iodoacetyl-DMH-PEA) and found that it inactivates COMT rapidly. We describe here the evidence that this analog serves as an affinity

*The abbreviations used are: SAM, S-adenosyl-L-methionine; COMT, catechol-O-methyltransferase (E.C.2.1.1.6); DHB, 3,4-dihydroxybenzoate; DMH-PEA, 3,5-dimethoxy-4-hydroxyphenylethylamine; N-acetyl-DMH-PEA, N-acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine; N-iodoacetyl-DMH-PEA, N-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine.

labeling reagent of COMT.

Materials and Methods

COMT was purified from rat liver (male, Sprague-Dawley, 180-200 g) according to a previously reported procedure (3,6). The enzyme was purified through the calcium phosphate gel step resulting in a preparation which contained 2.13 mg of protein per milliliter with a specific activity of 58.2 nmol product/mg of protein/min with 3,4-dihydroxybenzoate (DHB) as a substrate. The enzyme activity was determined using SAM-$^{14}CH_3$ (New England Nuclear, 55.0 mCi/mmol) and SAM iodide (Calbiochem) according to a previously described radioassay (6). N-Iodoacetyl-DMH-PEA was prepared in 42% yield by the condensation of DMH-PEA (3) with iodoacetic acid in the presence of N,N-dicyclohexylcarbodiimide. N-Acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (N-acetyl-DMH-PEA) was prepared by direct acetylation of DMH-PEA with acetic anhydride. The structures of the derivatives were confirmed by IR, NMR, U.V. and elemental analysis.

A typical alkylation experiment consisted of the following components (in μmoles) added in this sequence: water, so that final volume was 0.25 ml; magnesium chloride (0.30); N-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (TES, Sigma), pH 7.6 (10); N-iodoacetyl-DMH-PEA (variable) and purified enzyme preparation. The preincubation step was started by the addition of enzyme and incubation was carried out at 37°. In the competition experiments SAM (variable) or DHB (variable) were included during the pre-incubation. After the appropriate preincubation time the samples were assayed by addition of 0.05 μCi of SAM-$^{14}CH_3$, SAM (0.25) and/or DHB (0.50), if they were not already present in the preincubation mixture. The assay mixtures were incubated for 5 min at 37° and the reaction stopped by addition of 0.10 ml of 1.0 N HCl. The assay mixture was extracted with 10 ml of toluene and after centrifugation a 5 ml aliquot of the organic phase was measured for radioactivity. The results were corrected using the appropriate DHB blank. The percent activity remaining at any given time was

calculated relative to zero-time activity. The pseudo-first-order kinetic constants of inhibition, K_{app}, were calculated from the slope of plots of log of percent of activity remaining vs. time (7).

Results and Discussion: The time course for inactivation of COMT by 0.415 mM N-iodoacetyl-DMH-PEA (pH 7.60 and 37°) is presented in Figure 1. The

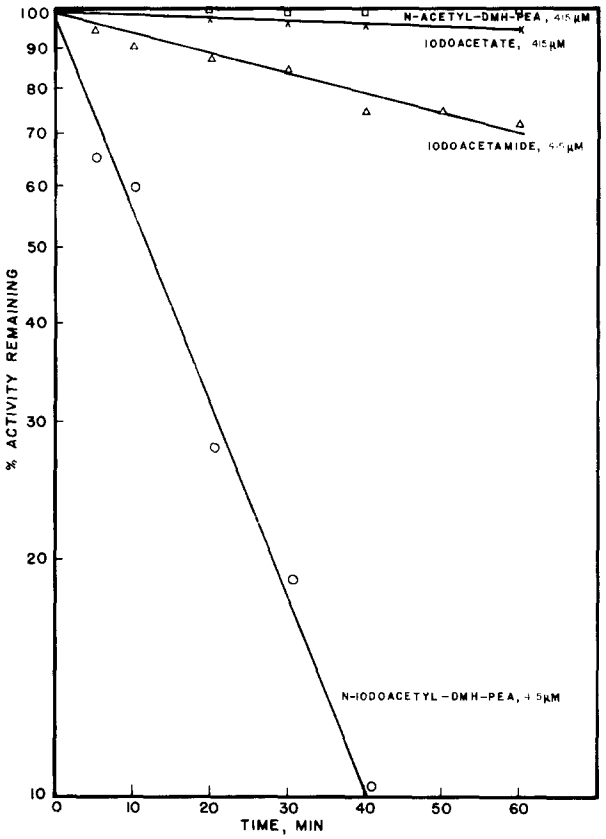

Figure 1: Effect of N-iodoacetyl-DMH-PEA, N-acetyl-DMH-PEA, iodoacetate and iodoacetamide on COMT activity. Purified COMT was preincubated with the inhibitors and the activity of the enzyme was monitored as a function of time as described in Table 1.

reaction is first order with respect to active enzyme remaining with a half-time of 13 min. Incubation for longer periods of time or with higher concentrations of inhibitor results in complete inactivation. The activity

can't be recovered after dialysis or gel filtration on Sephadex G-25 indicating the inhibition is completely irreversible. As shown in Figure 1 iodoacetate has little or no effect on enzyme activity, whereas iodoacetamide results in moderate inactivation of the enzyme with a half-time of approximately 124 min. Similar inactivation of COMT by other nonspecific alkylating reagents has been observed previously (8,9). As would be predicted N-acetyl-DMH-PEA produces no inactivation of COMT. The substantial increase in activity of N-iodoacetyl-DMH-PEA over that of iodoacetate or iodoacetamide is characteristic of an active-site-directed reagent.

In order to provide additional lines of evidence that the irreversible alkylation proceeds via unimolecular reaction within a dissociable complex rather than a non-specific bimolecular reaction, the rate of enzyme inactivation as a function of inhibitor concentration was investigated. Kinetic evidence for the existence of such a rate-limiting step in the irreversible inhibition of cholinesterase (10), trypsin (11) and carboxypeptidase (12) by substrate-like reagents has been demonstrated previously. The model for this type of inactivation is shown in eq 1 and eq 2, where E·I is the reversible complex, E-I the inactive enzyme, K_I the steady-state constant of inactivation and k_2 the first-order rate constant.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E - I \qquad (1)$$

$$K_I = \frac{[E][I]}{[E \cdot I]} \qquad (2)$$

The variation of the rate of COMT inactivation as a function of the concentration of N-iodoacetyl-DMH-PEA (pH 7.60 and 37°) is shown in Figure 2. As predicted the inhibition is pseudo-first-order in enzyme concentration in all cases. By plotting the reciprocal of the pseudo-first-order rate constants ($1/K_{app}$) vs. the reciprocal of the inhibitor concentrations ($1/[I]$) according to eq 3 (7), a steady-state constant of inactivation, $K_I = 1.13 \pm .69$ mM, and a first-order rate constant at saturation, k_2 =

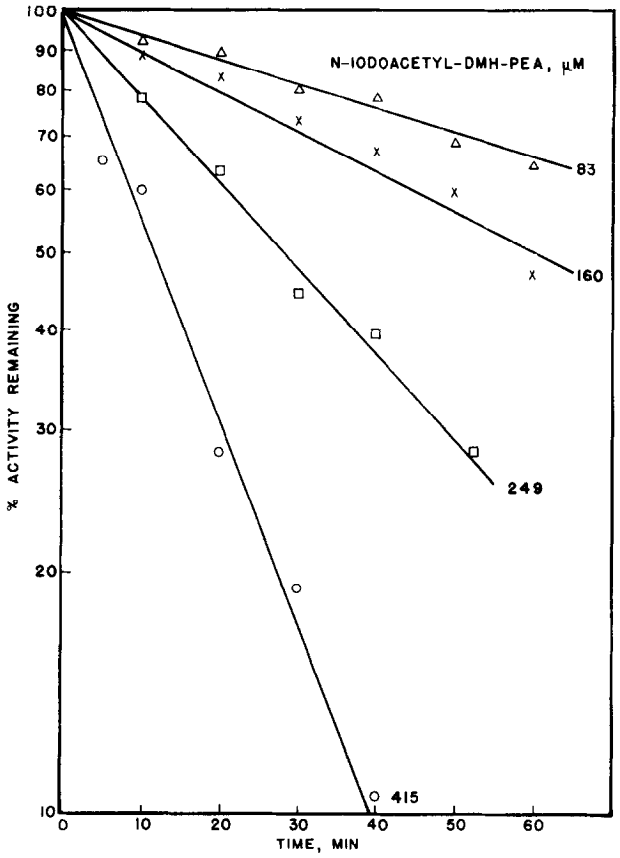

Figure 2: Effect of N-iodoacetyl-DMH-PEA concentration on the rate of inactivation of COMT. Activity remaining after the appropriate preincubation time was determined as outlined in Table 1. The pseudo-first-order rate constants of inactivation, K_{app}, were calculated from the slopes for each concentration of inhibitor.

$0.145 \pm .06$ min^{-1} were calculated (Figure 3). The linearity observed in this reciprocal plot provides evidence for the formation of a dissociable enzyme-inhibitor complex.

$$\frac{1}{K_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \qquad (3)$$

Further evidence to support the formation of a dissociable complex prior to the irreversible inactivation of COMT comes from the competitive experiments shown in Table 1. If the preincubation is performed in the

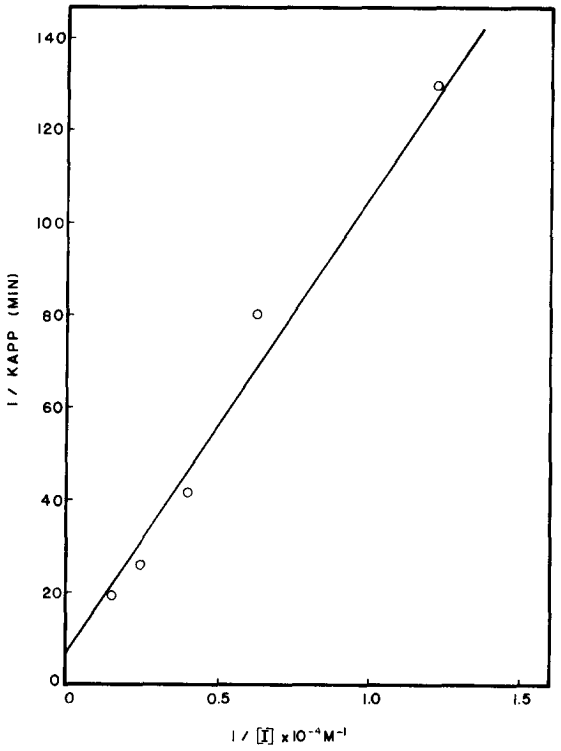

Figure 3: Double reciprocal plot of the pseudo-first-order rate constant of inactivation, K_{app}, vs. N-iodoacetyl-DMH-PEA concentration. k_2 and K_I were calculated from the y intercept and the slope, respectively, using the least-squares method.

Table 1

Substrate Protection of COMT from Inactivation

Additions[a]	Conc.	Residual activity after 60 min, 37°
	mM	%
none	--	6
3,4-Dihydroxybenzoate	2	11
"	4	18
"	8	48

[a]The standard preincubation mixture consisted of N-iodoacetyl-DMH-PEA (0.415 mM); magnesium chloride (1.2 mM); TES buffer pH 7.6 (40.0 mM) and purified enzyme preparation (53 µg) in a total volume of 0.25 ml. The preincubation was carried out for 60 min at 37° after which the samples were assayed as described in the text.

presence of increasing concentrations of DHB, the inactivation of COMT by N-iodoacetyl-DMH-PEA is greatly diminished. The fact that DHB doesn't completely protect COMT from inactivation probably results because this general group of inhibitors exhibit noncompetitive kinetics with respect to the catechol substrate (8). Addition of SAM during the preincubation, however, didn't provide protection from inactivation by this inhibitor.

From the above data, it may be concluded that N-iodoacetyl-DMH-PEA is an effective affinity labeling agent for COMT. Further studies on the isolation and characterization of the modified amino acid residues of COMT are in progress.

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