PURIFICATION OF CATECHOL-O-METHYLTRANSFERASE BY AFFINITY CHROMATOGRAPHY

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SUMMARY: An affinity chromatographic system has been developed to purify catechol-O-methyltransferase. This system consists of a 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose conjugate in which the ligand is coupled through a 30-Å hydrocarbon side chain. Partial purification of the enzyme (approx. 40 fold) can be achieved by passing the 100,000 x G supernatant obtained from homogenized rat liver through a column containing this conjugate and eluting with buffers of increasing ionic strength. Further purification of the enzyme (approx. 900 fold) has been achieved by using this affinity column in conjunction with several of the more classical enzyme purification techniques.

The inactivation of catecholamines and the detoxification of many xenobiotic catechols is dependent upon the enzyme catechol-O-methyltransferase (COMT)(E.C.2.1.1.6). COMT is a soluble, magnesium-requiring enzyme which transfers a methyl group from S-adenosylmethionine (SAM) to a catechol substrate. The enzyme has been isolated from a variety of tissues and in general the majority of the activity is found in the soluble fraction (1,2). Partial purification of soluble COMT from rat liver has been achieved using classical techniques for enzyme purification including differential centrifugation, ammonium sulfate fractionation, calcium phosphate adsorption, gel chromatography and ion exchange chromatography (1,4-11). Since these isolation procedures are time consuming and COMT is relatively labile, substantial losses of enzyme activity are encountered. As part of our continuing studies of this enzyme we have developed a more rapid and efficient method for the purification of COMT using the technique of affinity chromatography (12).

Materials and Methods

Rat livers (male, Sprague-Dawley, 180-200 g) homogenized in isotonic sucrose were used as a source of COMT. COMT activity was determined using

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SAM-$^{14}CH_3$ (New England Nuclear, 55.0 mCi/mmol), SAM chloride (Sigma), and 3,4-dihydroxybenzoic acid (Aldrich) according to a previously described radioassay (1,13). The synthesis of 3,4-dimethoxy-5-hydroxyphenylethylamine was modeled after a general procedure for the preparation of phenylethylamines (14-16). This pathway involved the reaction of 3,4-dimethoxy-5-benzyloxybenzaldehyde (prepared by the reaction of 3,4-dimethoxy-5-hydroxybenzaldehyde [17] with benzyl chloride) and nitromethane to yield 3,4-dimethoxy-5-benzyloxy-β-nitrostyrene. Reduction of the β-nitrostyrene with lithium aluminum hydride and removal of the benzyl ether protecting group by catalytic hydrogenation afforded the desired amine. The structures of the various synthetic compounds were confirmed by IR, NMR, UV and elemental analyses.

Cyanogen bromide activation of agarose (Sepharose 4B, Sigma) and subsequent attachment of the hydrocarbon side chain was performed essentially as described by Lefkowitz, et al. (18). As shown in Figure 1 the spacer arm (∿30 Å) consisted of units of 3,3'-iminobispropylamine and succinate. The ligand, 3,4-dimethoxy-5-hydroxyphenylethylamine, was attached to the succinylated gel using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide(Aldrich). In a typical preparation 100 ml of the succinylated gel was suspended in 100 ml of distilled water. To the suspension was added 3,4-dimethoxy-5-hydroxyphenylethylamine·HCl (1 g, 4.26 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (0.82 g, 4.26 mmol) and the pH was adjusted to 4.8. During the first 3-4 hours of the reaction a pH = 4.8 was maintained using 1.0 N HCl. When the pH stabilized, the reaction mixture was stirred at ambient temperature for 2 days after which the gel was filtered and washed with 4 liters of distilled water followed by 500 ml of 0.005 M phosphate buffer, pH 7.2. To determine the extent of coupling, samples of the substituted agarose were hydrolyzed with 2 N NaOH at 100°. An equivalent amount of the succinylated gel was used as a reference. The ultraviolet absorption of these samples at 285 nm were measured (λ max for 3,4-dimethoxy-5-hydroxyphenylethylamine = 285 nm, ε = 2007), from which the amount of ligand incorporated could be calculated.

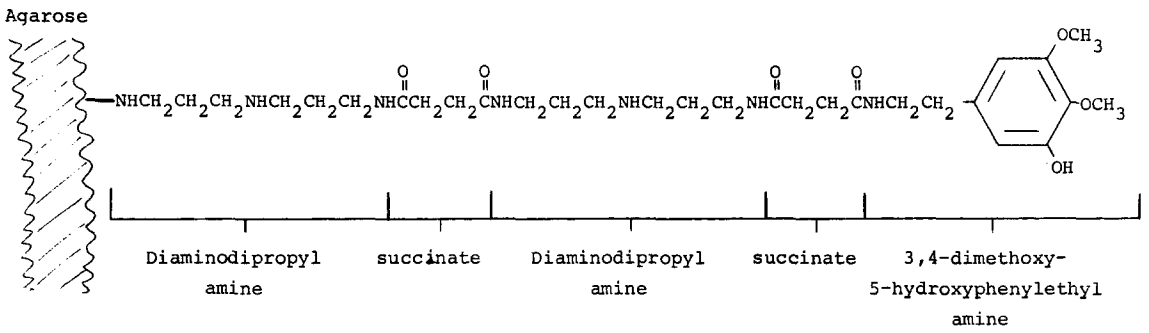

Figure 1: Structure of the 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose conjugate used for affinity chromatography of COMT.

A typical preparation showed an incorporation of 10 μmoles of the ligand per ml of packed volume of the gel.

Protein concentrations were determined according to the method of Lowry, et al. (19). Electrophoresis was carried out using SDS-polyacrylamide gels (10% acrylamide, 0.27% bisacrylamide cross linked, pH 7.0)(20). The gels were fixed and stained by incubation for 2 hours at room temperature in a solution of 0.25% Coomassie blue in 45% methanol-9% acetic acid. Excess dye was removed by extensive washing with methanol-acetic acid solutions.

Results and Discussion

The general approach used in the design of an affinity chromatographic system for COMT, was to covalently link various known dead-end inhibitors of this enzyme to agarose. Ligands of particular interest were 3,4-dimethoxy-5-hydroxyphenylalkylamines and 3,5-dimethoxy-4-hydroxyphenylalkylamines, since they had previously been shown to be potent inhibitors of this transmethylation (7,16,21). The most effective system for purification of COMT was found to be a 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose conjugate, which had the ligand separated from the insoluble matrix by a spacer of approx. 30-Å (Figure 1). The spacer arm utilized in this system is similar to that used by Lefkowitz, et al. (17) to couple norepinephrine in their purification of the β-adrenergic receptor protein.

The ability of the 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose con-

jugate to selectively bind COMT is shown in Figure 2. To this particular affinity column we had applied an aliquot of the 100,000 x G supernatant from rat liver eluting with 5 mM phosphate buffer, pH 7.2. All of the enzyme applied was retained and none was detected in the eluant even after extensive washing. However, the enzyme could be eluted with 100% recovery by washing the column with 60 mM phosphate buffer, pH 7.2. Interaction between the matrix-bound ligand and COMT was apparent from the fact that unsubstituted agarose did not retain this enzyme. The affinity column described above provided a 40-50 fold overall purification of COMT. Numerous variations of the experimental conditions described in Figure 2 were attempted, however no substantial improvement in the extent of purification was observed. The system described in Figure 2 provides a rapid and simple method for partial purification of COMT

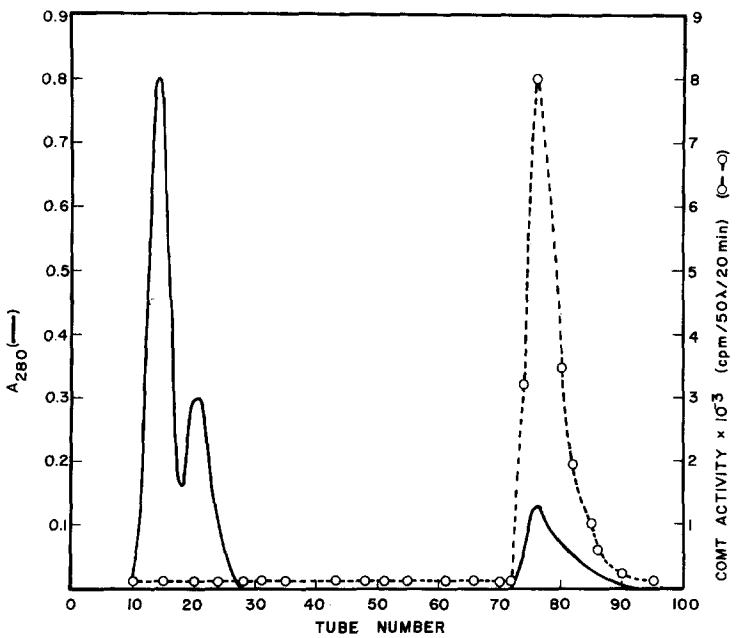

Figure 2: Retention of COMT on a matrix consisting of 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose conjugate and subsequent elution. The supernatant obtained from the 100,000 x G centrifugation of rat liver homogenate, which contained 141 mg of protein, was applied to a 1x20 cm column of the affinity conjugate previously equilibrated with 5 mM phosphate buffer, pH 7.2 ([EDTA] = 0.2 mM, $[Mg^{++}]$ = 0.2 mM). The protein was eluted with the same buffer and 3.0 ml fractions were collected. At tube 66 (arrow) elution of COMT with 60 mM phosphate buffer, pH 7.2 ([EDTA] = 0.2 mM, $[Mg^{++}]$ = 0.2 mM) was begun. COMT activity was determined using the assay described in the text.

Table 1

Purification of Rat Liver COMT

Fraction	Total[a] Protein(mg)	Specific Activity[b] (nmol/mg protein/min)	Purification	%Recovery
Homogenate[c]	252,000	1.04	1	-
100,000 x G[d] Supernatant (S_4)	86,000	2.16	2.1	59
$(NH_4)_2SO_4$[e] Fractionation	6,520	10.5	10.0	26
Sephadex G-25[e] Chromatography	5,831	17.3	16.6	39
$CaHPO_4$[e] Adsorption	1,350	49.6	47.8	26
Affinity[f] Chromatography	5.10	518.0	498.0	55
Sephadex G-100[g] Chromatography	0.47	950.0	913.0	73

[a]Protein concentrations were determined using the Lowry method (19).

[b]Assay conditions: 3,4-dihydroxybenzoate concentration, 2.0 mM; SAM concentration, 1.0 mM; 0.05 µCi of SAM-$^{14}CH_3$; Mg^{++} concentration, 1.2 mM; phosphate buffer, pH 7.60. Specific activity expressed as nmoles of product formed per mg protein per minute.

[c]Rat liver (male, Sprague-Dawley, 180-200 g) was homogenized in 10 mM phosphate buffer, pH 7.0 (0.25 M sucrose).

[d]The crude homogenate was centrifuged at 3,000 x G (S_1), 12,000 x G (S_2), 50,000 x G (S_3) and 100,000 x G (S_4).

[e]These stages of purification were carried out as previously described (7).

[f]The $CaHPO_4$ purified enzyme (97.8 mg) was chromatographyed on a 3,4-dimethoxy-5-hydroxyphenyethylamine-agarose conjugate (2.5 cm x 18 cm) eluting with PO_4 buffer, pH 7.2 ([PO_4] = 5-60 mM, [EDTA] = 0.2 mM, [Mg^{++}] = 0.2 mM) as described in Figure 2.

[g]Part of the enzyme purified by affinity chromatography (1.17 mg) was chromatographed on Sephadex G-100 (1 cm x 60 cm) eluting with PO_4 buffer, pH 7.2 (0.01 M) as described in Figure 3.

resulting in states of purity comparable to previously described procedures (6,7), which are much more time consuming and result in substantial loss of enzyme activity.

Although all of the COMT was bound by this affinity column, it was apparent from SDS-polyacrylamide gel electrophoretic studies that other proteins were also being retained and eluted with 60 mM phosphate buffer, pH 7.2. Therefore, in an effort to obtain more highly purified enzyme, this affinity system was used in conjunction with several other enzyme purification techniques. Shown in Table 1 is the general purification scheme developed for obtaining homogenous, soluble COMT. The initial steps in the purification are modeled after a procedure previously described by Nikodejevic, et al. (7). Several modifications of this procedure have been made, which include homogenization of the rat liver in 10 mM phosphate buffer, pH 7.0 (0.25 M sucrose) rather than isotonic KCl, followed by a more extensive differential centrifugation (See Table 1). The steps involving ammonium sulfate fractionation, Sephadex G-25 chromatography and negative calcium phosphate adsorption are identical to those previously reported (7). Purification of COMT through this calcium phosphate stage results in a 50 fold increase in specific activity.

Chromatography of the calcium phosphate purified enzyme on the 3,4-dimethoxy-5-hydroxyphenylethylamine-agarose conjugate (Figure 1), using conditions similar to those described in Figure 2, resulted in a substantial

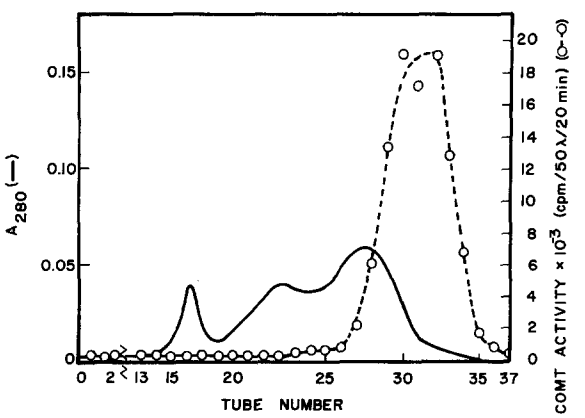

Figure 3: Elution pattern of COMT from Sephadex G-100. A 1.17 mg sample of COMT purified by affinity chromatography (Table 1) was applied to a Sephadex G-100 column (1x55 cm) previously equilibrated with 10 mM phosphate buffer, pH 7.2 and the enzyme was eluted with the same buffer. Fractions of 1.5 ml were collected and immediately checked for COMT activity using the assay described in the text.

increase in the specific activity of COMT. Overall purification of COMT at this stage was about 500 fold as compared to the crude homogenate. SDS-polyacrylamide gel electrophoretic studies of this 500 fold purified enzyme showed one major band with a molecular weight of 23,000 corresponding to COMT (10) and 6-7 minor bands of higher molecular weight. Further purification of this fraction was achieved by chromatography on Sephadex G-100 eluting with 10 mM phosphate buffer, pH 7.2. The Sephadex G-100 elution pattern is shown in Figure 3 where it can be seen that the majority of the COMT is eluted in fractions 27-35. The pooled enzyme eluted from this column represented an overall 913 fold purification as compared to the crude homogenate (Table 1). Electrophoretic studies of this fraction showed one major band with 2-3 minor bands (Figure 4A). The Coomassie blue stained gel was scanned at 550 nm as shown in Figure 4A and from the areas under the curves it can be estimated that COMT represents 60-70% of the protein present. The COMT obtained in this manner is relatively stable and can be stored at -4°C for several weeks without substantial loss of activity. If only the trailing fractions having COMT activity are pooled (Figure 3, fractions 33-35), essentially pure, single band COMT can be isolated. The SDS-polyacrylamide gel for this single band enzyme as well as the 550 nm densitometric scan are shown in Figure 4B. The major peak of 23,000 molecular weight represents more than 95% of the protein present in this fraction. This single band COMT has been found to be extremely unstable and catalytic activity is lost within several hours after isolation. Numerous variations in the experimental techniques used to handle this highly purified enzyme have been attempted, however we have been unsuccessful in increasing its stability. Similar problems have been encountered by other investigators working on the purification of this enzyme (1,4-11). However, since this Sephadex G-100 chromatography step is relatively simple, workable amounts of the single band COMT are being isolated and used in our laboratory for kinetic, immunological and affinity labeling studies.

In conclusion, we have developed an affinity chromatographic system

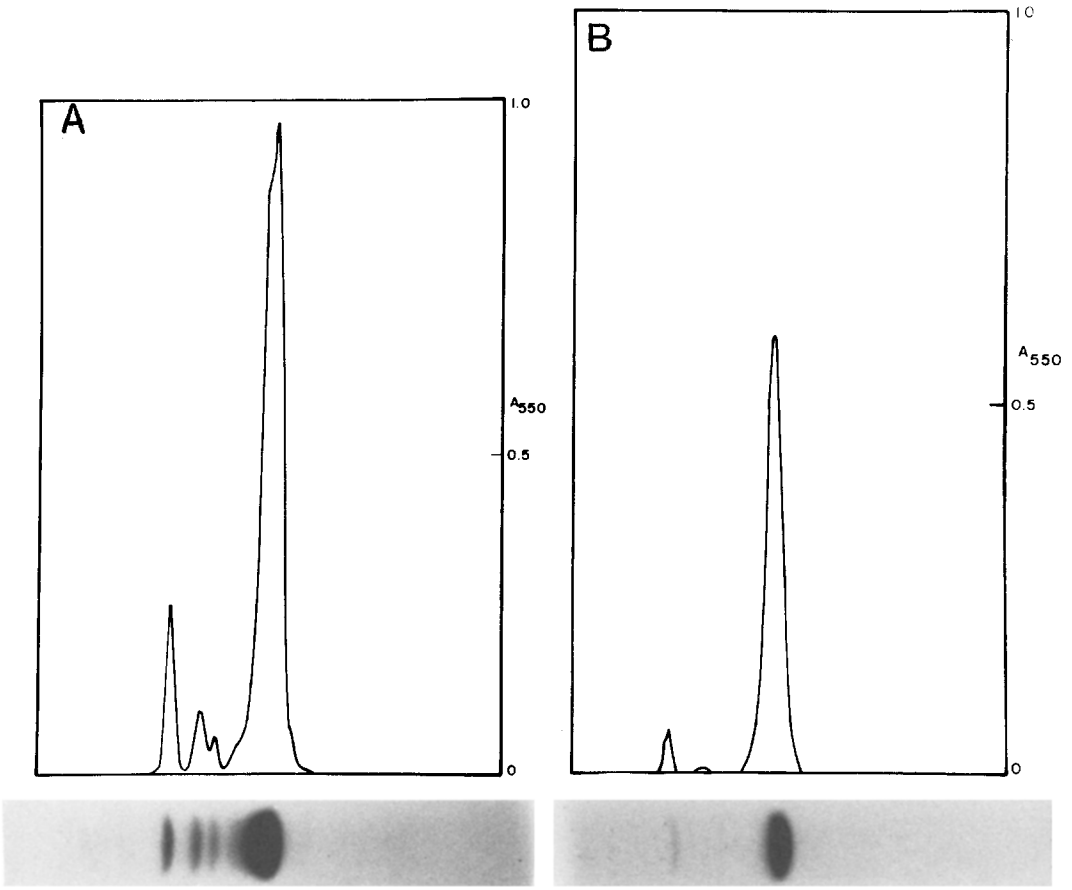

Figure 4: SDS-Polyacrylamide gel electrophoretic patterns and 550 nm densitometric scans of COMT containing fractions isolated from Sephadex G-100 chromatography. Gels were stained with Coomassie blue as outlined in text and densitometric scans were run on a Gilford 240 spectrophotometer equipped with an automatic gel scanner.

(A) The four band enzyme from the pooled fractions containing COMT activity. (Figure 3, fractions 27-35).

(B) Single band enzyme from the trailing fractions of COMT activity. (Figure 3, fractions 33-35).

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which can be used to very rapidly isolate partially purified COMT (approx. 40-50 fold) directly from rat liver homogenates or can be used in conjunction with other enzyme isolation techniques to prepare highly purified enzyme (approx. 900 fold). In either case these systems allow for a more rapid and efficient means of purifying COMT than those procedures previously reported (1,4-11).

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References

1. Axelrod, J. and Tomchik, R., J. Biol. Chem., 233, 490 (1958).
2. Molinoff, P. B. and Axelrod, J., Annu. Rev. Biochem., 40, 465 (1971).
3. Flohe, L, Int. Pharmacopsychiat., 9, 52 (1974).
4. Anderson, F. J. and D'Iorio, A., Biochem. Pharmacol., 17, 1943 (1968).
5. Rock, G. D., Tong, J. H. and D'Iorio, A., Canad. J. Biochem., 48, 1326 (1970).
6. Flohe, L. and Schwabe, K. P., Biochim. Biophys. Acta, 220, 469 (1970).
7. Nikodejevic, B., Senoh, S., Daly, J. W. and Creveling, C. W., J. Pharmacol. Exp. Ther., 174, 83 (1970).
8. Assicot, M. and Bohuon, C., Eur. J. Biochem., 12, 490 (1970).
9. Assicot, M. and Bohuon, C., Biochim., 53, 871 (1971).
10. Bohuon, C. and Assicot, M., in "Frontiers in Catecholamine Research" (Usdin, E. and Snyder, S., eds.) Pergamon Press, New York, p. 107, 1973.
11. Creveling, C. R., Borchardt, R. T. and Isersky, C., in "Frontiers in Catecholamine Research" (Usdin, E. and Snyder, S., eds.) Pergamon Press, New York, p. 117, 1973.
12. Cuatrecases, P., J. Biol. Chem., 245, 3059 (1970).
13. Borchardt, R. T., J. Med. Chem., 16, 377 (1973).
14. Ramirez, F. A. and Burger, A., J. Amer. Chem. Soc., 72, 2781 (1950).
15. Benington, F., Morin, R. D. and Clarke, L. C., J. Amer. Chem. Soc., 76, 5555 (1954).
16. Borchardt, R. T. and Thakker, D., J. Med. Chem., in press.
17. Mauthner, F., Justus Liebigs Ann. Chem., 449, 102 (1926).
18. Lefkowitz, R. J., Haber, E. and O'Hara, D., Proc. Nat. Acad. Sci., 69, 2828 (1972).
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
20. Weber, K. and Osborn, M., J. Biol. Chem., 244, 4406 (1969).
21. Borchardt, R. T. and Thakker, D., Biochem. Biophy. Res. Commun., 54, 1233 (1973).