Purification and partial characterization of rat liver soluble catechol-*O*-methyltransferase

Carola Tilgmann[1,2] and Nisse Kalkkinen[2]

[1]*Orion Corporation, Genetic Engineering Laboratory, Valimotie 7, 00380 Helsinki,* and [2]*Institute of Biotechnology, University of Helsinki, Karvaamokuja 3, 00380 Helsinki, Finland*

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The rat liver soluble catechol-*O*-methyltransferase (EC 2.1.1.6.) has been purified utilizing a combination of conventional chromatography and HPLC. The purified enzyme has a molecular mass of 25 kDa, a pI of 5.1, and exists in two forms which differ in the nature of their intramolecular disulfide bonds. This difference causes these two protein forms to behave differently in reversed phase chromatography.

Methylating enzyme; Catechol-*O*-methyltransferase, soluble

1. INTRODUCTION

Catechol-*O*-methyltransferase (EC 2.1.1.6; COMT) catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine to the hydroxyl group of catechols and catecholamines in the presence of magnesium [1,2]. The enzyme has a physiological role in the inactivation of catecholamine hormones and neurotransmitters as well as in the detoxification of a variety of xenobiotic amines and drugs [3]. COMT enzyme activity has been demonstrated in many mammalian tissues with the highest levels in the liver, kidney, uterus and placenta [4]. *O*-methylation of circulating catecholamines occurs preferentially in the liver, which makes it a potential source for purification of the COMT enzyme [5]. Several reports on the purification and properties of rat liver S-COMT have been published with various results concerning its molecular weight, isoelectric point, subunit structure and stability [1,4–18]. In addition, rat liver COMT has been found in both soluble and membrane bound forms [2–4,6,9,13], of which the soluble enzyme seems to predominate in the rat liver (95–99%) [19]. Ongoing investigations of the COMT enzyme gene, including cloning and sequencing, will soon give more information about the molecular biology of the enzyme [20]. To support these investigations, we have developed a method to obtain pure COMT. The pure enzyme permitted production of specific antibodies for clone detection as well as derivation of peptide sequences to be used for oligonucleotide probing.

Correspondence address: C. Tilgmann, Institute of Biotechnology, University of Helsinki, Karvaamokuja 3, 00380 Helsinki, Finland

Abbreviations: S-COMT, soluble catechol-*O*-methyltransferase; SDS-PAGE, sodium dodecylsulfate; RP, reversed phase; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Purification of rat liver S-COMT*

All purification steps, except RP-chromatography, were performed at +4°C. The enzymatic activity was measured as described [21] by monitoring at 254 nm. Rat livers (50 g) were homogenized in 150 ml of sodium phosphate pH 7.2 and 0.2 mM phenylmethylsulfonylfluoride. The homogenate was first centrifuged at 10000 × *g* for 25 min, then at 30000 × *g* for 25 min, and finally at 100000 × *g* for 1 h. All the pellets were discarded. The pH of the resulting solution was adjusted to 5.1 with acetic acid, and the mixture was kept at 0°C for 30 min and then centrifuged (15000 × *g*, 20 min). The pH of the clear supernatant (200 ml) was adjusted to 7.2 with 1 M sodium hydroxide and the solution mixed with hydroxyapatite (100 ml) (Bio-Gel HT, Bio-Rad). The mixture was stirred for 20 min and centrifuged (15000 × *g*, 20 min). Ammonium sulfate was added (65% saturation at 0°C) and the precipitate collected after centrifugation (30000 × *g*, 20 min). The precipitate was dissolved in 30 ml 20 mM sodium acetate, pH 4.8 and applied (10 ml) on a Bio-Gel P-100 column. The fractions which contained COMT activity were pooled and concentrated by ultrafiltration (Filtron Novacell NC10).

Subsequently, cation-exchange chromatography was performed on a Mono-S (HR 5/5, Pharmacia) column equilibrated with 20 mM sodium acetate, pH 4.8 and eluted with a linear gradient of sodium chloride (0–0.5 M in 20 min). The fractions with COMT activity were pooled and prepared for anion-exchange chromatography by ultrafiltration. Anion-exchange chromatography was performed on a Mono Q (HR 5/5, Pharmacia) column. The active fraction from the Mono-Q column was subjected to RP-chromatography.

2.2. *Alkylation*

Proteins collected manually from RP-columns were dried, alkylated [22] and desalted by RP-chromatography. The eluted proteins were then dried in a vacuum centrifuge.

2.3. *Digestion with trypsin and separation of tryptic peptides*

The proteins were dissolved in 100 μl 0.1 M ammonium bicarbonate and treated with 4% (w/w) TPCK-trypsin (Sigma) for 8 h.

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Resulting peptides were separated by RP-chromatography and the individual peptides collected and dried.

2.4. *Amino acid sequencing*

The peptides or proteins were degraded in a gas/pulsed liquid sequencer [23] after application on polybrene (2 mg) pretreated glass fiber filters. In addition, proteins were analyzed after electrophoretic transfer from SDS-PAGE onto a polyvinylidene difluoride membrane [24].

2.5. *Antibody production*

COMT antibodies were raised by immunizing rabbits in lymph nodes [11]. All blood samples were analyzed for COMT antibodies using Western immunoblotting [25] and Protoblot (Promega) detection.

2.6. *Chromatofocusing and SDS-PAGE*

Chromatofocusing was performed on a Mono-P (HR 5/20, Pharmacia) column equilibrated with 20 mM bis-Tris-HCl, pH 7.2 using a pH gradient from 7 to 4 (Polybuffer 74, Pharmacia). Fractions of 0.5 ml were collected and measured for pH and COMT activity. Analytical SDS-PAGE was according to Laemmli [26].

3. RESULTS AND DISCUSSION

The purification procedure presented in this paper is a combination of conventional chromatography and HPLC.

The first steps include homogenization of the rat liver and centrifugations to clarify the supernatant. The supernatant (140 ml) after the first centrifugation contained 1420 U of COMT activity and 1400 mg of total protein corresponding to a spec. act. of 1.0 U/mg protein. The clarification steps were followed by precipitation and adsorption of unwanted components as well as ammonium sulfate precipitation of the COMT enzyme. It has been reported that the COMT enzyme is labile after ammonium sulfate fractionation [9] but in our hands this method has given a fairly stable enzyme preparation also for longer (several months) storage at −20°C. 900 U of COMT activity could be recovered in the dissolved ammonium sulfate precipitate (9 ml) corresponding to a 69% yield from the first centrifugate (1420 U). The preliminary purification steps were followed by gel filtration. COMT activity was found only in one symmetrical peak clearly separated from most of the other proteins corresponding to an apparent molecular mass of 25 kDa (Fig. 1A). To optimize the ion-exchange chromatography steps the pI of the enzyme was determined by chromatofocusing, in which the enzymatic activity eluted at pH 5.1. The active fraction from the Bio-Gel P-100 column was subjected to cation exchange chromatography, in which the COMT enzymatic activity did not correspond to any visible protein peak (not shown). The COMT active fraction from the Mono-S column was, after changing the buffer, subjected to anion exchange chromatography. The main activity then corresponded to one protein peak (7.56 min), but some minor COMT activity was also found in the later fractions (Fig. 1B). The specific activity of the collected main fraction (Fig. 1B)

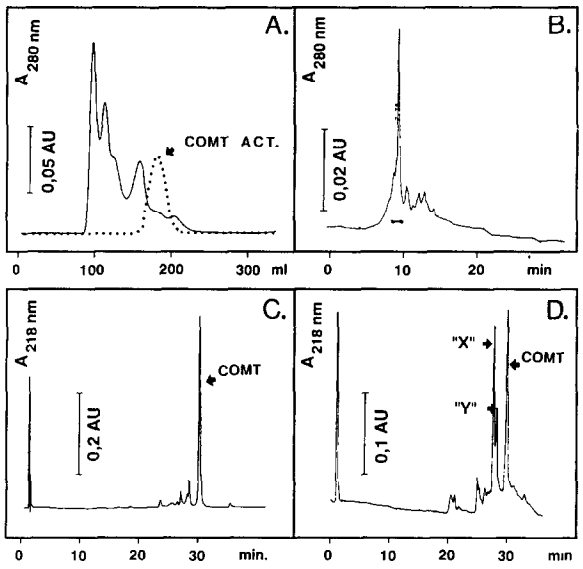

Fig. 1. Chromatograms from different purification steps of COMT. (A) Gel filtration on a Bio-Gel P-100 column (2.5 × 90 cm, 20 mM sodium acetate, pH 4.8). (B) Anion exchange chromatography on a Mono-Q (HR 5/5, Pharmacia) column. A linear gradient of sodium chloride (0–0.65 M in 20 min) in 20 mM triethanolamine acetate pH 7.2 was used for elution. The collected COMT active fraction is indicated with a solid line. (C, D) Reversed phase chromatography of the COMT active anion exchange chromatography fraction from two individual purifications. Chromatography was performed on a TSK TMS 250 (0.46 × 3 cm) column using a linear gradient of acetonitrile (0–100% in 60 min) in 0.1% TFA. Fractions (COMT) and ('X') were collected and used for peptide mapping or to raise antibodies.

varied between 1330 U/mg protein (no inactive 'X' form of COMT present, see below) and about 660 U/mg protein (about 50% of the 'X' form of COMT present). Thus, the highest specific activity obtained corresponded to a 1330-fold purification from the centrifuged liver extract with a total yield of 11% (156 U) of enzyme activity. The amount of COMT polypeptide in the collected Mono Q fraction (Fig. 3B), however, always represented at least 90% of the total protein.

It has been reported that the highly purified COMT enzyme is fairly to extremely labile and loses its activity rapidly under various conditions [14,17,18]. It has also been claimed that the instability of the enzyme is due to the oxidation of the free cysteine -SH groups [17]. We found, however, that the highly pure enzyme, prepared by anion exchange chromatography, was remarkably stable and lost only a few percent of its activity during storage for 48 h at room temperature.

For final purification the main COMT fraction from the Mono-Q column was subjected to RP-chromatography. In all chromatograms, peak 'COMT' was present and its intensity was related to the amount of enzyme activity applied to the RP-column. In SDS-PAGE it gave a single band corresponding to a molecular mass of 25 kDa (Fig. 2A). In some

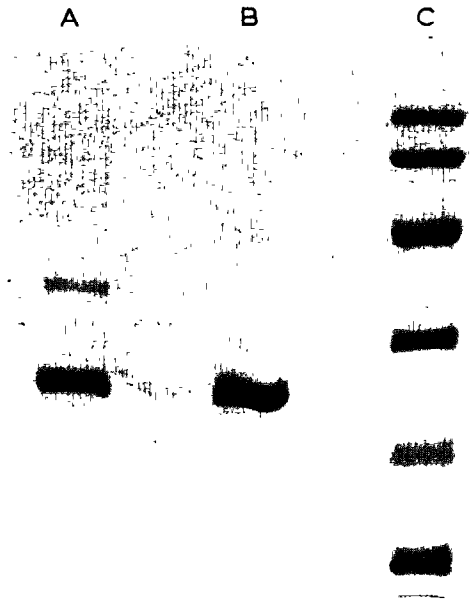

Fig. 2. SDS-PAGE of reversed phase chromatography fractions. (A) 'X' from Fig. 1D with a slight 'Y' contamination. (B) Combined COMT fractions from Fig. 1C, D. (C) A standard mixture containing proteins with molecular masses of 94, 68, 43, 30, 20.1 and 14.4 kDa. Electrophoresis was performed in a 12.5% polyacrylamide gel and the gel stained with Coomassie brilliant blue.

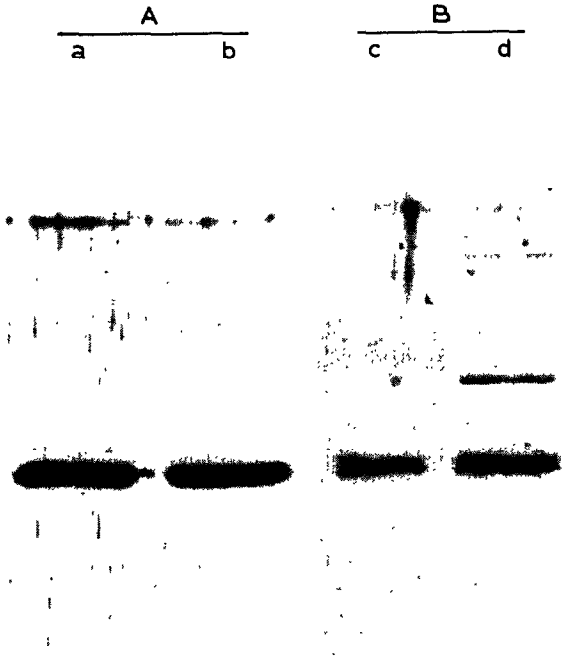

Fig. 3. Western blots of proteins from reversed phase chromatography separated by 12.5% SDS-PAGE. The blotting membrane was treated (A) with anti-COMT-antibodies, (B) with anti-'X'-antibodies. Lanes a and c contain the COMT fraction and lanes b and d the 'X' fraction.

chromatograms, also an additional double peak was present (Fig. 1D) which contained proteins named 'X' and 'Y' having molecular masses of 25 and 37 kDa, respectively (Fig. 2B). The quantity of fraction 'X' (25 kDa) varied from about zero (Fig. 1C) to equal to that of peak 'COMT' (Fig. 1D). To further identify the RP-chromatography fractions, we subjected both 'COMT' and 'X' fractions to direct N-terminal sequence analysis and measured their COMT activity after drying and rehydration into the enzyme assay buffer. COMT activity was seen only in fraction 'COMT' (Fig. 1C and 1D), identifying it as the enzyme. The sequence analyses gave no results, which suggested that the N-terminus of both 'X' and COMT is blocked. Because the identity of 'X' still remained unclear, we raised antibodies both against 'X' (contaminated with a small amount of 'Y') as well as pure COMT.

In Western blotting the COMT as well as 'X' antibodies detected both the purified COMT enzyme and the 25 kDa 'X' protein (Fig. 3C and 3D) which indicates that COMT and 'X' are the same proteins. Alkylation of both the purified COMT and 'X' yielded further evidence. The alkylated COMT and 'X' then eluted identically in RP-chromatography (Fig. 4) and the alkylated 'X' was well separated from the alkylated contaminating 'Y'. The tryptic maps of alkylated COMT and 'X' were similar (Fig. 5). No difference in the primary structure of the peptides from COMT and 'X' could be observed after sequencing peptides corresponding to about 90% (204 amino acids) of the primary structure (data not shown).

Apparently COMT and 'X' have a different conformation due to dissimilar intramolecular disulfide

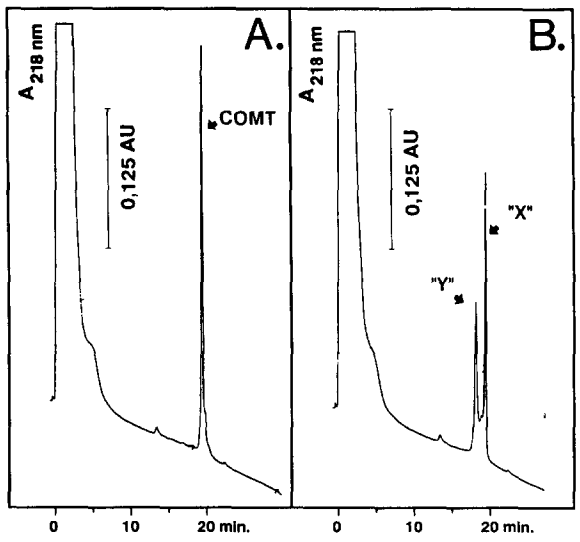

Fig. 4. Desalting of alkylated (4-vinylpyridine) proteins on a 0.46 × 3 cm TSK TMS 250 (C_1) column. A linear gradient of acetonitrile (20–50% in 30 min) in 0.1% TFA was used for elution. (A) Alkylated COMT fractions and (B) alkylated 'X' with 'Y' contamination from reversed phase chromatography. Alkylated COMT and 'X' were collected and used for tryptic mapping.

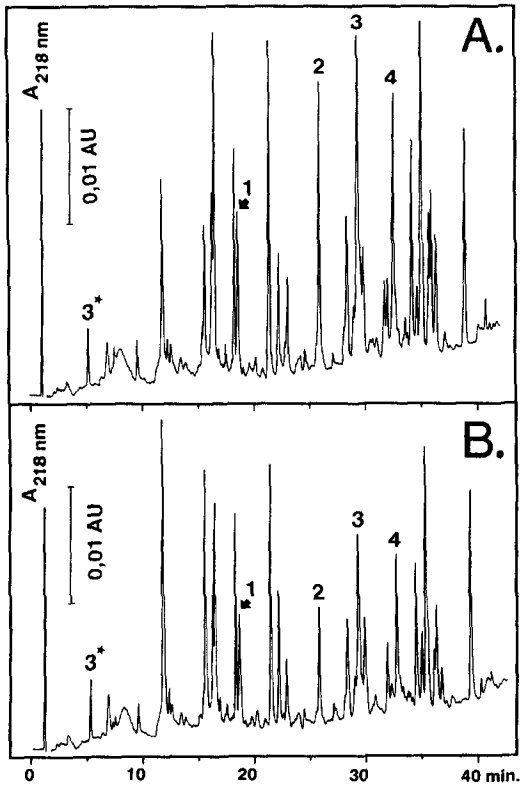

Fig. 5. Tryptic maps from (A) alkylated COMT and (B) alkylated 'X'. Peptides were separated on a 0.46 × 15 cm Vydac 218 TPB5 column using a linear gradient of acetonitrile (0–60% B in 60 min) in 0.1% TFA. Individual peptides were collected and subjected to sequence analysis. The four pyridylethyl-cysteine containing peptides with different primary structures (1–4) are indicated.

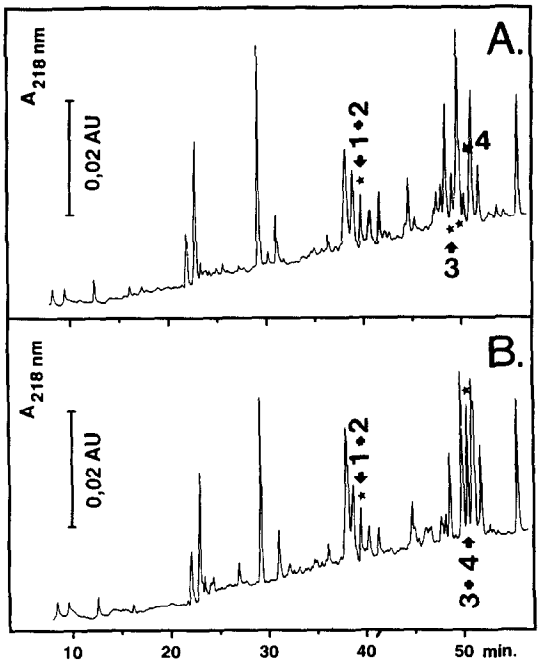

Fig. 6. Tryptic maps from (A) non-alkylated COMT and (B) non-alkylated 'X'. Separation conditions were as in Fig. 5, but an acetonitrile gradient of 0–60% B in 90 min was used for elution. The elution positions of the four cysteine containing peptides (1–4) are indicated.

bonds. Five pyridylethylcysteine containing peptides, (1) Cys-Gly-Leu-Leu-Arg, (2) Gly-Ser-Ser-Ser-Phe-Glu-Cys-Thr-His-Tyr-Ser-Ser-Tyr-Leu-Glu-Tyr-Met-Lys, (3) Tyr-Val-Gln-Gln-Asn-Ala-Lys-Pro-Gly-Asp-Pro-Gln-Ser-Val-Leu-Glu-Ala-Ile-Asp-Thr-Tyr-Cys-Thr-Gln-Lys, (3*) Cys-Thr-Gln-Lys, (4) Gly-Ala-Tyr-Cys-Gly-Tyr-Ser-Ala-Val-Arg were found among the sequenced peptides (Fig. 5). Of these, peptide 3* was a degradation product of peptide 3. The existence of four cysteines in the COMT primary structure has recently been confirmed by nucleotide sequencing [20]. To further examine the suggested differences in disulfide bonds between COMT and 'X', tryptic peptide maps were made from the nonalkylated COMT and 'X' (Fig. 6A and 6B) and all the peptides sequenced. All the four cysteine-containing peptides could be localized in both chromatograms, due to their known primary structure. Cysteine peptides 1 and 2 from both COMT (Fig. 6A) and 'X' (Fig. 6B) coeluted at a retention time of 39.5 min. The peaks collected at this retention time both gave a double sequence corresponding to an equimolar mixture of cysteine peptides 1 and 2. This suggests that peptides 1 and 2 are connected to each other by disulfide bonds in both COMT and 'X'. Similarly, sequence analysis showed that cysteine peptides 3 and 4 from 'X' coelute at a retention time of 50.4 min (Fig. 6B), whereas these peptides from COMT eluted at different positions at slightly earlier retention times (48.5 min and the shoulder at 48.5 min, Fig. 6A). This indicates that cysteine peptides 3 and 4 are interconnected only in 'X' but not in COMT meaning that two free cysteine -SH groups are present in COMT. It has been reported that a free cysteine SH-group at the active center is required for the enzymatic reaction [17]. Thus COMT and 'X' apparently represent the active and inactive form of the enzyme. Whether these two forms are physiologically important, or are caused by different external conditions during the purification, remains at present unclear.

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