Enzymatic Aminopropylation of Certain Secondary Amines

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Two unusual aminopropyl acceptors found in a survey of putrescine binding sites of mammalian spermidine synthase, *N*-methylputrescine (I) and 4-aminomethylpiperidine (II), were examined for their aminopropyl derivatives. Studies under *in vitro* incubation conditions suggested that the aminopropyl derivatives of the secondary amine of I and II, *N*⁴-methylspermidine (Is) and 1-*N*-(3-aminopropyl)-4-aminomethylpiperidine (IIs), and of the primary amine of I and II, *N*⁸-methylspermidine (Ip) and 4-[*N*-(3-aminopropyl)aminomethyl]piperidine (Iip), respectively, were biosynthesized by rat spermidine synthase. Studies on the cell culture system of cultured rat hepatoma (HTC) cells treated with α -difluoromethylornithine, an ornithine decarboxylase inhibitor, clearly showed the presence of Is and Ip when I was administered, and IIs and Iip when II was administered, with no detection of putrescine or spermidine. These results suggested that mammalian spermidine synthase can transfer the aminopropyl moiety of decarboxylated *S*-adenosylmethionine to certain secondary amines in living cells.

Key words aminopropylation; spermidine synthase; *N*-methylputrescine; 4-aminomethylpiperidine

The aminopropyl transfer reaction, catalyzed by spermidine or spermine synthase, occurs in living cells to supply these two substrates using decarboxylated *S*-adenosylmethionine (decarboxy AdoMet) as an aminopropyl donor to putrescine or spermidine, respectively. The substrate specificity of spermidine synthase has been studied using nucleoside derivatives¹⁾ and amine derivatives.²⁾ Sarhan *et al.*^{2a)} tested many putrescine derivatives as aminopropyl acceptors and discussed the structure-activity relationships. Recently, we proposed a model for the putrescine binding site of the spermidine synthase purified from pig liver based on study of a number of potential inhibitors and substrates.^{2b)} The model showed a relatively large hydrophobic cavity adjacent to a negatively charged site with a limited space, to which a protonated amino group of putrescine binds, with another amino group of putrescine being situated in the hydrophobic cavity as a free form to be aminopropylated by decarboxy AdoMet. In the course of the study, we found that two diamino compounds with both primary and secondary amines, *N*-monomethylputrescine and 4-aminomethylpiperidine, were able to accept aminopropyl moiety of decarboxy AdoMet, according to an assay measuring liberated tritium methyl labelled methylthioadenosine, even though the rates were very low. As the relatively large hydrophobic cavity in the putrescine binding site in our model seemed to be able to accept their secondary amino groups, formation of compounds having tertiary amino group was suspected in the enzyme reactions. These considerations led us to identify the aminopropyl derivatives of the two compounds.

MATERIALS AND METHODS

Chemicals Methods of synthesizing authentic samples are briefly described; common methods for alkylation using KF-Celite and removal of protecting phthaloyl and benzyl groups were adopted.³⁾ Sample purity was verified by elemental analyses and NMR. *N*-Methylputrescine (I)

was prepared by alkylation of *N*-monomethylbenzylamine with *N*-(4-bromobutyl)phthalimide followed by deprotection; white crystals of I 2HCl. *N*⁸-Methylspermidine (Ip) was prepared by alkylation of *N,N'*-bis(benzyl)-*N*-methylputrescine with *N*-(3-bromopropyl)phthalimide followed by deprotection; white crystals of Ip 3HCl. *N*⁴-Methylspermidine (Is) was prepared by alkylation of *N*-methyl-*N'*-phthaloyl-1,3-propanediamine with *N*-(4-bromobutyl)phthalimide followed by deprotection; white crystals of Is 3HCl. 4-[*N*-(3-Aminopropyl)aminomethyl]piperidine (Iip) was prepared by alkylation of *N,N'*-bis(benzyl)-4-aminomethylpiperidine with *N*-(3-bromopropyl)phthalimide followed by deprotection; white crystals of Iip 3HCl. 1-(3-Aminopropyl)-4-aminomethylpiperidine (IIs) was prepared by alkylation of 4-phthalimidomethylpiperidine with *N*-(3-bromopropyl)phthalimide followed by deprotection; white crystals of IIs 3HCl. 4-Aminomethylpiperidine (II) was purchased from Aldrich, and used as II 2HCl after recrystallization with aqueous ethanol. The other chemicals used are noted as they appear. They were of the highest purity unless specified otherwise.

Incubation Conditions Final concentrations of ingredients in the *in vitro* incubation mixtures (0.1 ml of total volume per tube) were 0.1 M potassium phosphate buffer (pH 7.4), 5 mM dithiothreitol (from Sigma), 1 mg/ml bovine serum albumin (fraction 5 from Seikagaku Kogyo Co., Ltd.), 5 mM of I or II, 0.4 mM of decarboxy AdoMet prepared by the reported method,⁴⁾ and 2 units of homogeneous spermidine synthase (440 units/mg) from rat liver.⁵⁾ The mixtures were incubated for the indicated number of hours at 37 °C. Incubation was then stopped by adding perchloric acid (final, 0.2 M), and the resulting supernatants were analyzed for reaction products.

Cell Culture Cultured rat hepatoma (HTC) cells (Morris 7288c, obtained from Dainippon Seiyaku Co., Ltd.) were subjected to a suspension culture in a spinner flask at 37 °C in a medium containing 90% (v/v) of Swim's S-77 (50 mM tricine, 2 g/l glucose, 2 mM glutamine, 0.05 mM

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L-cystine and 0.5 g/l NaHCO_3 , all from Sigma), and 10% (v/v) of newborn calf serum (pH 7.6, from Cell Culture Laboratories). After the preculture, HTC cells were washed twice with a medium containing 90% (v/v) Swim's S-77 and 10% (v/v) newborn calf serum (medium A); they were similarly cultured for 2 d initially at 1.5×10^5 cells/ml in a mixture of medium A (98 ml) and a solution (2 ml) containing 50-fold concentrations of final 5 mM α -difluoromethylornithine (DFMO), 1 mM aminoguanidine (from Sigma), and 0.01 mM I or 0.05 mM II (medium B). The cells were then collected, washed and adjusted again to 1.5×10^5 cells/ml with the fresh medium B, and incubation was continued for another 2 d. The cells (0.5 – 2.0×10^6 cells), collected and washed three times with phosphate buffered saline (PBS(–), from Takara Biomedicals Co., Ltd.), were then deproteinized by perchloric acid (final, 0.2 M), and the resulting supernatants were analyzed for polyamines.

Chromatographic Procedures a) Gas Chromatography (GC): Aliquots of sample solutions (supernatants treated with perchloric acid) were subjected to a carboxymethyl cellulose clean-up procedure to collect the triamine fraction.⁶⁾ The dried fractions in tubes with a Teflon screw cap were allowed to react at 100°C in the presence of 0.2 ml each of heptafluorobutyric anhydride and acetonitrile for 30 min. After evaporation of the reagent and solvent in a nitrogen stream, the resulting residues were shaken with 0.5 ml each of 0.5 M sodium carbonate and diethylether to extract heptafluorobutyl (HFB) derivatives of triamines in ether layer.⁷⁾ Aliquots of the ether fractions were injected to a gas chromatograph. Conditions for GC were as follows: Shimadzu Gas Chromatograph GC-9A; column packing, 2% SE-30, 3 mm $\times$ 2 m; carrier gas, He, 40 ml/min; column temperature, programmed from 200°C to 280°C , $8^\circ\text{C}/\text{min}$; detector, hydrogen flame ionization detector (FID). Peak area ratios in an equal molar amount were 1.8 for Ip/Is and 1.6 for IIp/IIs.

b) Gas Chromatography–Mass Spectrometry (GC-MS): Sample preparations and gas chromatographic conditions for GC-MS were the same as those described for GC. The instrument used was a JEOL JMS-DX 300 mass spectrometer. Mass spectra were recorded at ionizing voltage of 70 eV.

c) High Performance Liquid Chromatography (HPLC): Aliquots of sample solutions (supernatants treated with perchloric acid) were directly injected to cation exchange-HPLC for polyamine analysis.⁸⁾ A buffer, 0.28 M sodium citrate buffer (pH 5.5) containing 2.0 M NaCl, was used as

a mobile phase. Peak area ratios in an equal molar amount were 7.6 for Is/Ip and 5.7 for IIs/IIp.

d) Thin Layer Chromatography (TLC): Perchloric acid supernatants obtained after *in vitro* incubation were allowed to react with dansyl (DNS) chloride (from Sigma) in the usual manner,⁹⁾ and DNS derivatives were analyzed on HPTLC pre-coated plate silica gel 60 F 254 (from Merck) with a solvent system of ethylacetate–ethylalcohol–acetic acid (8:1:1). Fluorescence was detected with UV-light (365 nm).

RESULTS

Identification of Reaction Products of I and II under *in Vitro* Incubation Conditions Two probable products were aminopropyl derivatives of primary amine and secondary amine: Ip and Is from I and IIp and IIs from II, respectively (Fig. 1). *In vitro* incubation conditions were set up with reference to standard assay conditions for spermidine synthase activity, but with the modification described in Materials and Methods. These conditions were chosen to obtain as much of the reaction products as possible, since substrate activities of I and II were very weak compared with that of putrescine.^{2b)} The chromatographic properties of the reaction products were compared with those of the authentic Ip, Is, IIp or IIs to identify them. Gas chromatographic analyses of the reaction products strongly suggested the presence of Ip, Is, IIp, and IIs (Fig. 2a, b), which were later identified by GC-MS. Fluorescent TLC using DNS chloride also demonstrated the presence of Is and IIs (Fig. 3).

Administration of I and II to DFMO-Treated HTC Cells The results described above led us to examine whether the aminopropyl derivatives of I or II could be detected in cultured cells. DFMO-treated HTC cells were used, because the culture system is known to deplete putrescine due to the inhibition of ornithine decarboxylase with DFMO and to cause accumulation of a great deal of decarboxy AdoMet in the cells¹⁰⁾; also the conditions were suitable to allow spermidine synthase to utilize I and II effectively to form their aminopropyl derivatives. HTC cells were cultured for 4 d under the conditions described in Materials and Methods. The cells treated with 5 mM of DFMO stopped growing within 3 d. By contrast, the growth curves of the two cultures in medium B containing I or II were similar to those of the control culture in medium A over 4 d. The cells were then harvested and deproteinized, and aliquots of the supernatants were

Fig. 1. Chemical Structures of Aminopropyl Derivatives of I and II

analyzed by HPLC. The chromatogram of the control culture showed a typical peak pattern of putrescine, spermidine and spermine (Fig. 4a). In the culture treated with I and DFMO, peaks corresponding to I, Is (spermidine), and Ip were observed with spermine-like

peaks near t_R 40 min, and no putrescine peak was detected (Fig. 4b). In the culture treated with II and DFMO, peaks corresponding to II and a mixture of IIs and IIp were observed with a trace of a spermine-like peak, and neither putrescine nor spermidine peaks were detected (Fig. 4c). In the culture treated with DFMO alone, only a normal level of spermine peak and a trace of spermidine peak were observed. As Is, Ip, IIs, and IIp seemed to be present, the cells from the two cultures were subjected to GC-MS analyses as described in Materials and Methods. The results conclusively showed the presence of Is, Ip, IIs, and IIp, and the absence of spermidine (Fig. 5a, b), and suggested that the ratio of the produced Is/Ip or IIs/IIP in the cells was 1.1 or 0.18, respectively.

DISCUSSION

These studies using two weak and unusual aminopropyl acceptors, I and II, demonstrated that mammalian spermidine synthase can transfer the aminopropyl moiety of decarboxy AdoMet to certain secondary amines suitable

Fig. 2. Gas Chromatograms of Triamine Fractions after *in Vitro* Incubation with I (a) or II (b)

Is, Ip, IIs, IIp, and I.S. show their HFB derivatives. I.S.: *N*-(3-aminopropyl)-1,5-pentanediamine. X, Y: unknown peaks observed in each blank.

Fig. 3. Thin-Layer Chromatograms of Reaction Products after *in Vitro* Incubation with I or II

Lane A: products from II, lane B: no enzyme (control for lane A), lane C: authentic sample (IIs(DNS)), lane D: products from I, lane E: no enzyme (control for lane D), lane F: authentic sample (Is(DNS)). Compound(DNS) represents the dansyl derivative of the compound.

Fig. 4. HPLC of Polyamines in Cells Cultured for 4 d in (a) Medium A (Control), (b) Medium B (Containing I), or (c) Medium B (Containing II)

Retention time of each compound was 7.5 min for putrescine (Put), 8.2 min for I, 9.8 min for II, 17.8 min for spermidine (Spd) and Is, 19.0 min for Ip, 20.1 min for IIs and IIp, and 38.5 min for spermine (Spm).

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