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ORIGINAL ARTICLE

A conceptual model of the polyamine binding site of N^1-acetylpolyamine oxidase developed from a study of polyamine derivatives

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Abstract We used various polyamine derivatives to study the substrate binding site of N^1-acetylpolyamine oxidase (PAO) that was partially purified from rat liver. The substrate activities of acetylpolyamines indicated the presence of two anionic centers corresponding to the 1,3-diaminopropane (1,3-DAP) structure and a hydrophobic region in addition to the cleavage site of the acetamidopropyl group. Based on the results of the inhibitory activities of 1,3-DAP derivatives, we developed a conceptual model of the polyamine binding site of PAO. We used this model to identify a potent competitive inhibitor, N^1,N^7-dihexyl-1,7-diamino-4-azaheptane, and to develop an affinity column, 1,16-diamino4,13-diazahexadecane–linked Sepharose, which was useful for the purification of PAO.

Keywords Polyamines · Acetylpolyamine oxidase · Diacetylpolyamines · Acetylpolyamines · Affinity column

Introduction

N^1-Acetylpolyamine oxidase (PAO), a constitutive enzyme, catalyzes the oxidative cleavage of natural substrates, N^1-acetylspermidine and N^1-acetylspermine, to liberate 3-acetamidopropanal and form putrescine and spermidine, respectively, in the conversion pathway of polyamine with an inducible enzyme, spermidine/spermine N^1-acetyltransferase (Wallace et al. 2003). PAO is a flavoprotein located in the peroxisomes of animal cells. The PAO purified to homogeneity from rat liver (Hölttä 1977) and the cytoplasm of porcine liver (Tsukada et al. 1988) was used to characterize the enzyme properties. Some PAO inhibitors have been developed and used in studies of PAO (Wang et al. 2005; Federico et al. 2001; Edwards et al. 1990; Bey et al. 1985). The cloning and sequencing of bovine or murine PAO as well as its biochemical and physical properties have been reported in detail (Wu et al. 2003). Recent interest in PAO comes from its possible role in the progression of apoptosis (Chen et al. 2001) and the possible cytotoxicity of 3-aminopropanal (Ha et al. 1997) and hydrogen peroxide (Ivanova et al. 2002) produced by the PAO reaction. The present study was performed to conceptualize the active site of PAO by using various polyamine derivatives. An understanding of the active site of PAO is useful for designing new inhibitors and affinity ligands.

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Materials and methods

Chemicals

Spermidine (34) trihydrochloride, N^1-acetylspermidine (Ac34) dihydrochloride, N^8-acetylspermidine (Ac43) dihydrochloride, *O*-phthaldialdehyde, pargyline, and aminoguanidine were purchased from Sigma (Tokyo, Japan). Boric acid and 2-mercaptoethanol were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Polyoxyethylene lauryl ether and sodium sulfate were purchased from Nacalai Tesque (Tokyo, Japan). The 1,3-diaminopropane (1,3-DAP) dihydrochloride was purchased from Tokyo Chemical Industries (Tokyo, Japan). We prepared the following reagents according to previously described methods (Takao et al. 2007; Samejima et al. 1984; Niitsu and Samejima 1986): N^1-acetyl-1,7-diamino-4-azaheptane

(Ac33) dihydrochloride, N^1-acetyl-1,11-diamino-4,8-diazaundecane (Ac333) trihydrochloride, N^1-acetyl-1,12-diamino-4,8-diazadodecane (Ac334) trihydrochloride, N^1,N^{11}-diacetyl-1,11-diamino-4,8-diazaundecane (DA333) dihydrochloride, N^1,N^{12}-diacetyl-1,12-diamino-4,9-diazadodecane (DA343) dihydrochloride, N^1,N^{14}-diacetyl-1,14-diamino-5,10-diazatetradecane (DA-444) dihydrochloride, N^1,N^{15}-diacetyl-1,15-diamino-4,8,12-triazapentadecane (DA3333) trihydrochloride, N^1,N^{16}-diacetyl-1,16-diamino-4,8,13-triazahexadecane (DA3-343), trihydrochloride, N^1-butyl-1,8-diamino-4azaheptane (Butyl43) trihydrochloride, N^1-hexyl-1,3-diaminopropane (Hexyl3) dihydrochloride, N^1-Acetamidooctyl-1,3-diaminopropane (Ac83) dihydrochloride, N^1-hexyl-1,7-diamino-4-azaheptane (Hexyl33) trihydrochloride, N^1,N^3-didecyl-1,3-diaminopropane (Didecyl3) dihydrochloride, N^1,N^7-dihexyl-1,7-diamino-4-azaheptane (Dihexyl33) trihydrochloride, 1,14-diamino-4,11-diazatetradecane (363) tetrah-ydrochloride, 1,16-diamino-4,13-diazahexadecane (383) tetrahydrochloride, 1,17-diamino-4,14-diazaheptadecane (393) tetrahydrochloride, 1,18-diamino-4,15-diazaoctadecane (3103) tetrahydrochloride, and 1,20-diamino-4,17-diazaicosane (3123) tetrahydrochloride. All other reagents and organic solvents were of commercial analytical grade.

Partial purification of PAO

PAO was partially purified from rat liver as described by Hölttä (Hölttä 1983) with a minor modification. Rat livers were homogenized with four volumes of 0.25 M sucrose-10 mM Tris–HCl buffer (pH 7.4) by using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $600\times g$ for 10 min. The supernatant was centrifuged at $15{,}000\times g$ for 10 min, then the pellet was suspended in 0.1% Triton X-100 resolved in 10 mM Tris–HCl buffer (pH 7.8) containing 0.1 mM EDTA and 0.1 mM DTT (buffer A). The suspension was centrifuged at $25{,}000\times g$ for 30 min. The supernatant was dialyzed against buffer A. Crude extracts were then purified by DEAE-Sephadex chromatography. Extracts were applied to the column equilibrated with buffer A and then eluted with a linear gradient of 0.1–0.4 mM NaCl in buffer A. Active fractions were collected and applied to a hydroxyapatite column that was equilibrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM DTT; fractions were then eluted with a linear gradient of 10–300 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM DTT. Active fractions were concentrated with Amicon Centriflow CF25 (Millipore, Tokyo, Japan), and the concentrated fractions were applied to a Sephacryl S-300 column, gel filtration chromatography, equilibrated and eluted with buffer A containing 50 mM NaCl. Active fractions were concentrated with Amicon Centriflow CF25 for use as the enzyme solution. The final specific activity of the enzyme was 18.2 nmol/min/mg protein.

Assay conditions for PAO activity

The PAO activity was assayed by measuring the amount of Ac34 produced from DA343 with PAO. The standard incubation mixture contained, in a final volume of 90 µl, the enzyme solution, 0.56 mM DA343, 0.56 mM aminoguanidine, 0.036 mM pargyline, 0.02 mM DTT and 1 mM EDTA in 0.15 M borate buffer (pH 9.0). Mixtures in the presence or the absence of inhibitors were incubated for 30 min at 37°C, then the reaction was stopped by the addition of 150 µl of 10% trichloroacetic acid solution. The reaction mixtures were centrifuged and analyzed by ion-exchange HPLC with an OPA-post column system (Shirahata et al. 1993). For kinetics studies, the mixture contained 0–0.56 mM monoacetylpolyamines or diacetylpolyamines, instead of DA343. The K_m and V_{max} values for PAO in the rat liver were estimated by using the Lineweaver-Burk transformation of the Michaelis-Menten kinetic equation. The inhibition studies were performed by measuring the enzyme activities in the presence of the tested compound in the standard incubation mixture. The capability of compounds to inhibit the enzyme activity was expressed as the IC_{50} (the concentration of the compound that inhibited enzyme activity by 50%).

Coupling of 383 to Sepharose

The coupling of 383 to NHS-activated Sepharose 4 Fast Flow (GE Healthcare Science, Tokyo, Japan) was performed according to the manufacturer's directions. The 383-linked gels were stored in 50 mM Tris–HCl at pH 7.1 and 4°C. The amount of 383 bound to the gel was determined by a fluorescent HPLC method, measuring the liberated polyamine after 6 M HCl hydrolysis of the gel at 120°C for 16 h. The capacity measured by this method was 2.2 µmol/ml of gel. The enzyme solution (200 µg protein) was added to the 383-linked Sepharose column (0.5 ml bed volume) equilibrated with buffer A. The column was washed with buffer A containing 0.05, 0.1, 0.2 M NaCl, and the enzyme activity was eluted with buffer A containing 0.2 M NaCl and 0.5, 5 and 12.5 mM DA343. Each 1-ml fraction was collected.

Results and discussion

Substrate activities of acetylpolyamines

To study the PAO recognition site for acetylpolyamines, a series of monoacetyltriamines, monoacetyltetramines,

Table 1 Substrate properties of monoacetylpolyamines and diacetylpolyamines: values are expressed as the mean of duplicate experiments;the apparent K_m values in parentheses were calculated from the sum of the monoacetyltetramine and triamine

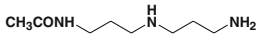
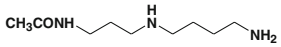
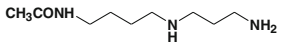
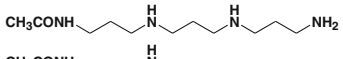
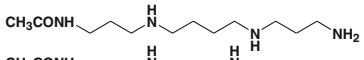
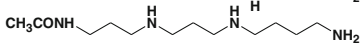
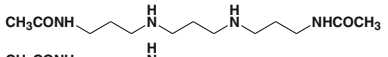
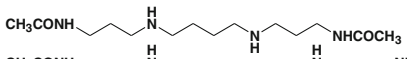
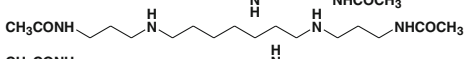
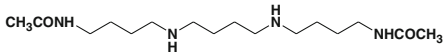
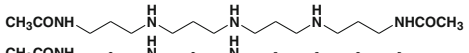
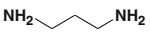
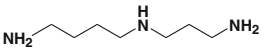
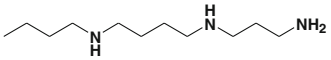
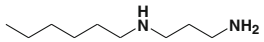
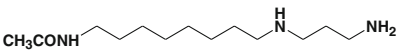
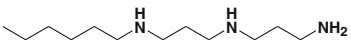
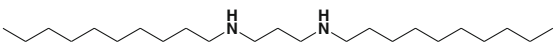
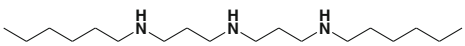
Compound	Structure	Km (μM)	Vmax (nmol/min/mg)
Monoacetyltriamines			
Ac33		69.5	8.5
Ac34		14.8	12.9
Ac43		-	-
Monoacetyltetramines			
Ac333		2.8	26.2
Ac343		1.8	20.9
Ac334		4.1	40.1
Diacetyltetramines			
DA333		25.4	34.8
DA343		19.3	18.2
DA373		-	-
DA444		-	-
Diacetylpentamines			
DA3333		(4.1)	(13.7)
DA3343		(9.0)	(24.0)

Table 2 IC_{50} values of 1,3-DAP derivatives: values are expressed as the mean of duplicate experiments;assay mixture containing each compound was incubated under the standard assay condition

Compound	Structure	IC_{50} (μM)
1,3-DAP		>560
Spermidine		560
Butyl43		100
Hexyl3		33
Ac83		28
Hexyl33		1.3
Didecyl3		0.36
Dihexyl33		0.34

diacetyltetramines and diacetylpentamines with 3, 4 or 7 methylene chain intervals (Table 1) were tested for their substrate activities by using partially purified PAO from rat liver. Each amine product was separated and measured by OPA post-column derivatization ion-exchange HPLC to obtain kinetics data. The product amines were simple for monoacetyltriamines, i.e., 1,3-DAP from Ac33 and putrescine from Ac34, and monoacetyltetramines, i.e., sym-norspermidine from Ac333 and spermidine from Ac343 and Ac334. The apparent K_m values (Table 1) showed that monoacetyltetramines had a higher affinity to PAO than monoacetyltriamines, except for Ac43, which could not act as substrate due to its acetamidobutyl group. These results suggest that PAO recognizes a terminal amino group at a distance equivalent to 8 or 9 methylene chain lengths from the cleavage site. The amine products for diacetyltetramines and diacetylpentamines by PAO using a crude enzyme source were reported previously (Takao et al. 2007). The amine product was Ac34 for DA343 or Ac33 for DA333 under the incubation conditions. The apparent K_m value for DA343 was similar to that of Ac34, suggesting that both compounds have a similar

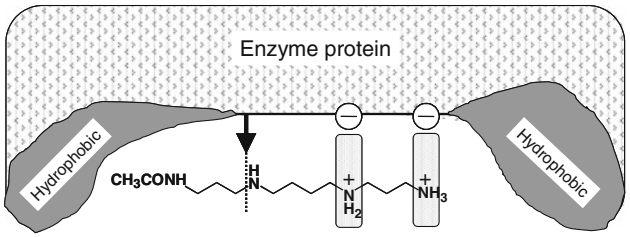


Fig. 1 A conceptual model of the polyamine binding site of PAO

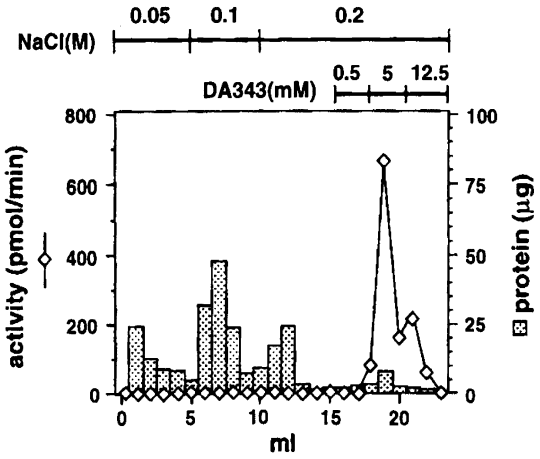


Fig. 2 Elution profile of PAO activity from 383-Sepharose. The crude enzyme solution (200 μg of protein) was added to the affinity column (0.5 ml). The enzyme was eluted from the column with buffer A containing NaCl and DA343

affinity to PAO. The K_m value of DA333 was lower than that of Ac33; this result suggests the presence of a hydrophobic region that could bind the other end-acetamidopropyl group. The DA444 could not act as a substrate due to its acetamidobutyl group. In diacetylpentamines, amine products from DA3333 were Ac333 and a smaller amount of 33 produced by the reaction with the monoacetyltetramine, and the amine products from DA3343 were Ac334 and/or Ac343 and a smaller amount of 34 produced by the reaction with the monoacetyltetramine. Therefore, the apparent K_m values (Table 1) were calculated from the sum of the monoacetyltetramine and triamine produced. The DA3333 had a low K_m value, while DA373, which has a similar molecular size as DA3333, showed no reaction with PAO. This result suggests that the catalytic reaction requires a positive charge at a 3 or 4 methylene chain interval from the cleavage site. Based on these results, we predict that two anionic centers and a hydrophobic region are present in the active site of PAO in addition to the cleavage site of the acetamidopropyl group.

Inhibitory activities of 1,3-DAP derivatives

Based on the assumed presence of two anionic centers corresponding to the two cationic centers of the 1,3-DAP structure, compounds containing the 1,3-DAP structure (Table 2) were tested for their inhibitory activities under the standard assay conditions with DA343 as the substrate. Based on their IC_{50} values, the diamines can be ranked in the following order: 1,3-DAP (highest IC_{50}), Hexyl3, Ac83 and Didecyl3 (lowest IC_{50}). The corresponding ranking for the triamines is: spermidine (highest IC_{50}), Butyl43, Hexyl33 and Dihexyl33 (lowest IC_{50}). These results indicate the presence of a relatively large hydrophobic region on both sides of the two anionic centers in our conceptual polyamine binding site model of PAO (Fig. 1). This model also explains the activity of other compounds reported as substrates or inhibitors for PAO (Vujcic et al. 2003; Bolkenius and Seiler 1981; Wang et al. 2005; Bitonti et al. 1990; Wu et al. 2005). Of the compounds listed in Table 2, Hexyl33, Didecyl3 and Dihexyl33 potently inhibited PAO activity. Didecyl3 had a high hydrophobicity unsuitable for the use as an inhibitor, and Hexyl33 seemed to have very weak substrate activity (data not shown). Hence, Dihexyl33, which has a Ki value of approximately 10 nM (data not shown), could be recommended as a new potent competitive inhibitor for PAO. Dihexyl33 exhibits its inhibitory effect in extremely low concentrations and may be useful in studies of PAO.

Table 3 IC_{50} values of compounds containing 1,3-DAP structure on both sides: values are expressed as the mean of duplicate experiments;assay mixture containing each compound was incubated under the standard assay condition

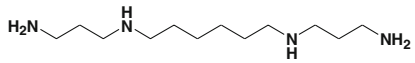
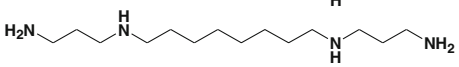
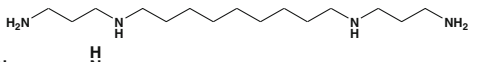
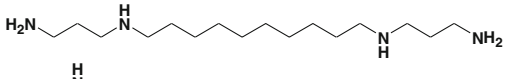
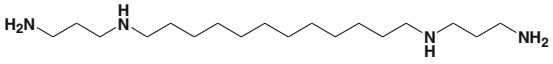
Compound	Structure	IC_{50} (μM)
363		330
383		15
393		2.9
3103		14
3123		42

Table 4 Purification of PAO by 383-Sepharose affinity chromatography

	Total protein (μg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
Gel filtration	191.1	2.9	14.5	100.0	1.0
Affinity	18.0	2.3	127.8	80.0	9.0

Design and usefulness of an affinity ligand for purification of PAO

Based on the results described above, we synthesized a series of tetramines that symmetrically contained the 1,3-DAP structure on both sides of the molecule in order to connect Sepharose through the primary amine on one side. This series of tetramines was tested for their inhibitory activities (Table 3). Of these compounds, 393 had the lowest IC_{50} value. However, the 393-linked Sepharose seemed to bind PAO too tightly to elute its activity with DA343. Therefore, 383-linked Sepharose was chosen for the purification of PAO. A preliminary trial of the corresponding affinity column (Fig. 2) yielded a tenfold increase in the specific activity of the partially purified enzyme solution applied on the column, with an 80% recovery of the enzyme activity (Table 4). We anticipate better results with additional modifications of the capacity of ligand and the conditions of chromatography. The 383-linked Sepharose was expected to bind PAO more tightly than N^8-acetylspermidine–linked Sepharose (Tsukada et al. 1988).

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