Guide Molecule-driven Stereospecific Degradation of α-Methylpolyamines by Polyamine Oxidase

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FAD-dependent polyamine oxidase (PAO; EC 1.5.3.11) is one of the key enzymes in the catabolism of polyamines spermidine and spermine. The natural substrates for the enzyme are N3-acetylsperrmidine, N4-acetylsperrmine, and N3,N4-diacetylsperrmine. Here we report that PAO, which normally metabolizes achiral substrates, oxidized (R)-isomer of 1-amino-8-acetamido-5-azanalone and N3-acetylsperrmidine as efficiently while (S)-1-amino-8-acetamido-5-azanalone was a much less preferred substrate. It has been shown that in the presence of certain aldehydes, the substrate specificity of PAO and the kinetics of the reaction are changed to favor spermine and spermidine as substrates. Therefore, we examined the effect of several aldehydes on the ability of PAO to oxidize different enantiomers of α-methylated polyamines. PAO supplemented with benzaldehyde predominantly catalyzed the cleavage of (R)-isomer of α-methylsperrmidine, whereas in the presence of pyridoxal the (S)-α-methylsperrmidine was preferred. PAO displayed the same stereospecificity with both singly and doubly α-methylated spermine derivatives when supplemented with the same aldehydes. Structurally related ketones proved to be ineffective. This is the first time that the stereospecificity of FAD-dependent oxidase has been successfully regulated by changing the supplementary aldehyde. These findings might facilitate the chemical regulation of stereospecificity of the enzymes.

Polymamines, spermidine (Spd),3 and spermine (Spm) and their precursor, putrescine, are present at millimolar concentrations in mammalian cells (1). They are essential for cell growth, participate in the regulation of cellular metabolism and physiology (2, 3). Their cellular levels are strictly controlled at various levels including the synthesis, degradation, uptake, and excretion (4, 5). Furthermore, the polyamine homeostasis is also regulated by a rapid interconversion (6–8) that in part offers means to supply proper polyamines for each individual process. Spd and Spm appear to be able to substitute for each other to some extent (9, 10). The exact cellular roles of each individual polyamine, however, are not well known (11). One of the approaches to elucidate the impact of each individual polyamine in biological processes is the use of metabolically stable Spd and Spm analogues. Examples of such compounds are MeSpd and bis-α,α’-methylspermine (Me2Spm) that are able to fulfill many of the cellular functions of their native counterparts.

Earlier, we have successfully used racemic α-methyl analogs of polyamines to study the role of each individual polyamine in vivo. Both MeSpd and Me2Spm are capable of preventing acute pancreatitis and restoring early liver regeneration after partial hepatectomy under the condition of severe depletion of the natural polyamines in transgenic rats (12, 13).

The interaction of racemic MeSpd with recombinant human PAO (hPAO) in the presence of benzaldehyde (BA), which allows the enzyme to oxidize nonacetylated Spd, led to the formation of putrescine (13). This prompted us to study the effect of aldehydes on this reaction using recently synthesized isomers of MeSpd and α-methylsperrmine (MeSpm) as well as the three diastereomers of Me2Spm (Fig. 1). In the present paper, the interactions of recombinant hPAO with different enantiomers of AcMeSpd have been studied.

We found that for hPAO, using achiral molecules as native substrates, (S)-AcMeSpd was clearly an inferior substrate in comparison with (R)-AcMeSpd, which itself was as effectively catabolized as AcSpd. Moreover, the supplementation of hPAO with different aldehydes regulated the stereospecificity of this enzyme. Thus, PAO in the presence of BA predominantly oxidized (R)-MeSpd, whereas in the presence of pyridoxal (PL), the (S)–isomer was preferred. In the case of MeSpd and Me2Spm, the stereospecificity of oxidation could also be controlled by different aldehydes.

MATERIALS AND METHODS

Chemicals—Racemic α-methylated polyamine analogs were synthesized as described in Refs. 14 and 15. The two enantiomers of MeSpd were prepared as described in Ref. 16. Syntheses of (R)-AcMeSpd, (S)-AcMeSpd, both isomers of MeSpd, and all diastereomers of Me2Spm were performed using the same (R)– and (S)–N3-(α-nitrophenylsulfonyl)-N3’-(tert-butyloxyacarbonyl)-1,3-diaminobutane as the key chiral intermediates, which, after alkylation with appropriate halides and removal of the protecting groups, afforded the target isomers of α-methylated analogs. Both racemic α-methylated polyamine analogs and their isomers showed purity of >99.5% according to 1H/13C NMR data and HPLC analysis. A preformed adduct of spermine with PL (PL–Spm) was prepared as described in Ref. 17. Aminooxaylputres-
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TABLE 1
The effects of selected aromatic aldehydes on isomer specificity of human polyamine oxidase

| Aldehyde | Racemic MeSpd | (R)-MeSpd | (S)-MeSpd |
|----------|--------------|-----------|-----------|
| BA       | 12.7         | 46.2      | 2.1       |
| OH      | 5.1          | ND        | 14.1      |
| PL       | 11.6         | 14.6      | 8.2       |
| P4CA     | 18.0         | 40.4      | 4.7       |
| OH      | ND           | 0.9       | ND        |
| HO      | 6.7          | 16.1      | 2.0       |
| HO      | 1.2          | 1.7       | 0.7       |

HPLC Analysis and Kinetic Studies—The investigation of the kinetics of hPAO reaction was performed in duplicates at 3–6 different (10–1000 μM) substrate concentrations in the presence of 5 mM BA, pyridine-4-carboxaldehyde (P4CA), or PL. All of the kinetic values with different substrates were determined with Lineweaver-Burk plotting. Some kinetic determinations were also carried out in duplicates at a fixed 1 mM Spm concentration supplemented with 3 or 4 different concentrations of 0.1–1 mM aldehydes (BA, P4CA, or PL). The PAO reactions were carried out in a total volume of 180 μl in different buffers as described separately for each experiment (see legends for the tables and figures) and were allowed to proceed for the indicated time at +37 °C before the addition of 20 μl of 50% (v/v) sulfosalicylic acid containing 100 μM 1,7-diaminoheptane as an internal standard. HPLC with postcolumn phthalaldehyde derivatization was used to determine the concentrations of the polyamines and their methylated analogs essentially as described earlier (22). Chiral HPLC analysis was performed using a Whelk-O1 (R,R) 25 cm × 4.6 mm column; isocratic run 0–75 min with flow rate of 0.55 ml/min, 70% ethanol to separate (R)- and (S)-isomers of MeSpd after dansylation and treatment as described in Ref. 23.

Recombinant hPAO—The enzyme was produced as described earlier (13), omitting the affinity purification step.

RESULTS

Interaction of (R)-AcMeSpd and (S)-AcMeSpd with Recombinant hPAO—An incubation of (R)- and (S)-isomers of AcMeSpd with hPAO in 100 mM glycine-NaOH buffer at pH 9.5 resulted in a predominant oxidation of the (R)-AcMeSpd, whereas (S)-enantomer was much less efficiently catabolized. (R)-AcMeSpd had K_m = 95 μM and k_cat = 9.0 s⁻¹, whereas for (S)-AcMeSpd K_m and k_cat values were 170 μM and 1.2 s⁻¹, respectively. It should be noted that AcSpd, the native substrate of PAO, had K_m = 14 μM and k_cat = 8.5 s⁻¹ under the same conditions.

Initial Screening Studies with hPAO Supplemented with Various Carboxyl Compounds—The effect of seven aromatic aldehydes on the stereoselectivity of hPAO is depicted in Table 1 (for results with 17 tested aldehydes see supplemental Table S1). All reaction mixtures containing racemic, (R)- or (S)-MeSpd were supplemented with a 5 mM concentra-
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TABLE 3
Kinetic characteristics of the degradation of spermidine, racemic, and different isomers of α-methylspermidine by human polyamine oxidase in the presence of different aldehydes

Reactions were carried out at 3–6 different substrate concentrations ranging from 10 to 500 μM in duplicates at +37 °C for 5–30 min in 100 mM glycine-NaOH at pH 9.5 containing 5 mM DTT and 0.05–2 μg recombinant protein. Reactions were stopped and assayed for products with HPLC as described under “Materials and Methods.” NA, not applicable (30-min reaction with 2 μg of recombinant protein and 500 μM substrate resulted in less than 0.5% degradation of the substrate).

|       | k_{cat} | k_{cat}/K_{m} |
|-------|---------|---------------|
|       | μM⁻¹ s⁻¹ | s⁻¹ M⁻¹       |
| AcSpd | 14       | 8.5           |
| Spd   | 9.4      | 0.85          |
| + 5 mM benzaldehyde | 37      | 0.31          |
| + 5 mM P4CA | 25      | 0.49          |
| + 5 mM pyridoxal | 100     | 1.3           |
| Racemic AcMeSpd | 100     | 1.3           |
| Racemic MeSpd | NA      | NA            |
| + 5 mM benzaldehyde | 14      | 0.19          |
| + 5 mM P4CA | 55      | 0.11          |
| + 5 mM pyridoxal | 31      | 0.18          |
| (R)-AcMeSpd | 95      | 9.0           |
| (R)-MeSpd | 20      | 0.68          |
| + 5 mM benzaldehyde | 110     | 0.18          |
| + 5 mM P4CA | 5.9     | 0.01          |
| + 5 mM pyridoxal | 170     | 1.2           |
| (S)-AcMeSpd | 170     | 1.2           |
| (S)-MeSpd | 14      | 0.06          |
| + 5 mM benzaldehyde | 44      | 0.09          |
| + 5 mM P4CA | 5.1     | 0.24          |

The enzyme showed strong preference toward (R)-MeSpd with most of the tested aldehydes, of which BA was the most effective. The structure of the aldehyde was one of the key factors determining the extent of the enhancement. For example, the effect of α-ω-, ω-ω- or ω-ω-α-hydroxybenzaldehydes totally depended on the position of the hydroxyl group (Table 1). Among the tested aldehydes, PL was the only (S)-guiding aldehyde changing the preference of hPAO to (S)-MeSpd. Thus, in the initial screening we were able to identify (S)-guiding, (R)-guiding BA, and P4CA that enhanced the degradation of both isomers of MeSpd. All of the structurally related and tested ketones proved to be inactive at 5 mM concentrations (data not shown, structures of the used ketones are given in supplemental Table S2).

Studies with Liver Extracts Obtained from Wild-type Wistar Rats—To exclude the possibility that hPAO, as a recombinant protein containing His6 tag fragment, would behave differently in comparison with the native enzyme, crude liver extracts were used as the source of PAO to study the effects of guide molecules in vitro. The data in Table 2 clearly show that the reaction rate was increased by the key aldehydes similarly for both recombinant hPAO and rat liver enzyme. Moreover, steering of the isomer specificity was also possible in the crude liver extract.

Guide Molecule-controlled Degradation of Selected Isomer from Racemic MeSpd—Based on the above data, BA and PL were chosen to investigate the oxidation of racemic MeSpd. The reaction mixtures were subjected to chiral HPLC analysis after dansylation of the polyamines. The used conditions (see “Materials and Methods”) did not yield a complete separation of distinct isomers from the reaction mixture (elution times for (S)- and (R)-MeSpd were 49.2 and 52.7 min, respectively). However, the preference for selected isomer by hPAO was clear and in the presence of BA, only traces of (R)-MeSpd were detected after 24 h. In the case of PL, (S)-MeSpd was completely oxidized after 24 h incubation (supplemental Fig. S1).

The Kinetics of hPAO-dependent Oxidation of Different Enantiomers of MeSpd Supplemented with BA, PL, or P4CA—The above described experiment showed that it was possible to enrich racemic MeSpd with the preferred enantiomer by the addition of the proper aldehyde into the substrate mixture of PAO. Therefore, the kinetic parameters of hPAO-dependent oxidations of Spd, racemic, and the two enantiomers of MeSpd were determined in the presence of fixed 5 mM aldehyde concentration (Table 3). Among the tested aldehydes, BA was the most effective with Spd (k_{cat} = 0.85 s⁻¹) although clearly inferior to AcSpd as the substrate. This may partly be due to the reaction of aldehyde with N3-nitrogen, thus reducing the reaction rate. PL treatment provided practically the same affinity for both MeSpd isomers for hPAO, but k_{cat} of 0.24 s⁻¹ for (S)-MeSpd was much higher than k_{cat} of 0.01 s⁻¹ for (R)-MeSpd. P4CA enhanced the degradation of both isomers of MeSpd to about the same extent (Table 3).

Stereospecificity and Kinetics of hPAO-catalyzed Oxidation of Two Enantiomers of MeSpd and Three Diastereomers of Me2Spm Supplemented with the Key Aldehydes—Spm was a rather poor substrate for hPAO and, expectedly, aldehyde supplementation greatly increased its degradation (Table 4). In the case of MeSpd, both Spd and MeSpd were produced by hPAO, whereas only MeSpd was obtained from Me2Spm (Fig. 1). The addition of BA enhanced the reaction rates of all diastereomers of MeSpd but PL clearly enhanced only the degradation of (S,S')-Me2Spm (Table 4). Similarly, an addition of BA enhanced the degradation rates of both isomers of MeSpd. The presence of
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the methyl group did not influence the efficiency of the degradation (i.e., the cleavage of MeSpm took place at both the methylated and the nonmethylated end of the molecule). However, PL-supplementation did not enhance hPAO-mediated oxidation of (R)-MeSpm from the methylated terminus (supplemental Table S3).

Effect of Increasing Aldehyde Concentration on hPAO-dependent Oxidation—The reaction rates of hPAO with Spm as the substrate were enhanced upon increasing BA concentration up to 5 mM in both 100 mM glycine-NaOH and 50 mM borate buffer at high pH (supplemental Table S4). However, 10 mM BA was inhibitory for the enzyme reaction (data not shown). The reaction rate acceleration of hPAO was retarded in the presence of 2 mM or higher PL concentration when Spm or its methylated derivatives were used as the substrate (Table 5; data not shown). Therefore, we determined kinetic values for Spm derivatives with both 1 and 5 mM PL supplementation (Table 4). However, with Spd as the substrate, the reaction rate increased up to 5 mM PL (data not shown). The kinetic values of hPAO-catalyzed oxidation were measured at a constant 1 mM Spm concentration and increasing amount of supplemented aldehydes. In 100 mM glycine-NaOH, \( K_m = 200 \mu M \) and \( k_{\text{cat}} = 5.1 \text{ s}^{-1} \) for PL, \( K_m = 170 \mu M \) and \( k_{\text{cat}} = 6.6 \text{ s}^{-1} \) for BA, and \( K_m = 930 \mu M \) and \( k_{\text{cat}} = 6.6 \text{ s}^{-1} \) values for P4CA were determined. In 50 mM borate buffer, \( K_m = 110 \mu M \) and \( k_{\text{cat}} = 3.7 \text{ s}^{-1} \) for PL and \( K_m = 150 \mu M \), and \( k_{\text{cat}} = 8.4 \text{ s}^{-1} \) for BA were obtained. We also tested MeSO in increasing concentrations as a substitute for water (24), but already 50% Me2SO proved to inhibit the enzyme reaction (data not shown).

Effect of Incubation Order with Substrate, Aldehyde, and hPAO—Both 1 mM Spm andracemic Me2Spm were used as substrates for hPAO (0.3 and 0.6 \( \mu \)g/reaction, respectively) in the presence of 5 mM BA in 100 mM glycine-NaOH at pH 9.5. Two of the three components (substrate, aldehyde, and enzyme) were incubated on ice for 15 min before the addition of the third. The reactions were carried out at +37 °C for 20 min. In all of the combinations with both substrates, no differences in the reaction rates were observed (results not shown).

| Buffer Effect | Preformed Adducts of Spm with PL as Substrates for hPAO—The stock solution of the adduct was prepared by the incubation of 100 mM solution of Spm-base with 200 mM solution of PL-base in methanol in the dark at 20 °C overnight. The obtained adduct was oxidized by hPAO far more effectively than Spm supplemented with the same amount of PL (Table 5).

| Buffer Effect | The stereoselectivity of hPAO toward MeSpd isomers was very strict in 100 mM glycine-NaOH (Table 6) and 100 mM alanine-NaOH buffers at pH 9.5 (results not shown). Glycine and alanine as the key constituents of these buffers have the potency to react with alde-

**FIGURE 1.** Degradation of \( \alpha \)-methylated polyamine analogues by PAO. Methylated polyamines need to be supplemented with aldehydes in order to be degraded by PAO. All reactions should yield corresponding 3-aminobutanal or 3-acetamidobutanal, which are spontaneously degraded. AcMeSpd, acetylated \( \alpha \)-methylsperrmidine.

**TABLE 5** Human polyamine oxidase-catalyzed production of spermidine from spermine-pyridoxal adduct and from spermine supplemented with different amounts of pyridoxal

| 100 mM glycine-NaOH, pH 9.5 | 50 mM borate, pH 9.3 |
|-----------------------------|---------------------|
| nmol Spd | Spm | Spd |
| 1000 \( \mu \)M PL = Spm | 11.95 ± 0.37 | 12.49 ± 0.36 |
| 1000 \( \mu \)M Spm | 1.09 ± 0.03 | 1.07 ± 0.05 |
| + 100 \( \mu \)M pyridoxal | 3.64 ± 0.08 | 3.81 ± 0.04 |
| + 500 \( \mu \)M pyridoxal | 7.92 ± 0.33 | 6.58 ± 0.12 |
| + 1 mM pyridoxal | 9.01 ± 0.27a | 7.13 ± 0.12a |
| + 2 mM pyridoxal | 8.25 ± 0.18a | 6.75 ± 0.29a |

* \( p < 0.001. \)
hydes. Therefore, two other buffer systems were used to exclude any buffer-related effects. Stereoselectivity of hPAO was sustained in all tested buffer systems (Table 6). However, the stringency for enantiomer selection and efficacy of aldehyde-enhanced degradations differed in the studied buffers (Table 6). Moreover, aldehyde-dependent oxidation carried out at pH 7.4 (NaH2PO4 buffer) showed reduced reaction rates, but the stereoselectivity of degradation was retained (data not shown).

**DISCUSSION**

PAO is one of the key enzymes in the catabolism of Spm and Spd. The natural substrates for PAO are N1,N12-diacetylspermine, N1-acetylspermine and AcSpd (27) (Fig. 3), which are formed in cells as the result of acetyl-CoA-dependent acetylation of Spm and Spd by spermidine/spermine N1-acetyltransferase (28). Hence, the substrates and the products of PAO-catalyzed reactions are achiral molecules (29). Recently, we demonstrated that racemic MeSpd and Me2Spm are effective surrogates of the native polyamines (12, 13). MeSpd proved to be metabolically stable as it is not a substrate for spermidine/spermine N1-acetyltransferase-mediated acetylation (13). Therefore, we prepared the corresponding (R)- and (S)-isomers of AcMeSpd (Fig. 1) that are the simplest chiral analogues of AcSpd and tested these compounds as substrates for hPAO. It turned out that the enzyme preferred (R)-AcMeSpd to the corresponding (S)-isomer, as (S)-isomer had a kcat/Km value of less than 10% of the (R)-isomer. This indicates for the first time that PAO has a hidden potency of stereospecificity.

In the presence of certain aldehydes, the substrate specificity of rat liver PAO and the kinetics of the reaction are changed in a way that Spm and Spd are efficiently catabolized (26).
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It is known that an introduction of a guide molecule in the substrate mixture may effect the rate of enzymatic reaction. One of the first examples is the acceleration of the solvolysis of acetyl chymotrypsin in the presence of indole due to its binding at a specific site of the enzyme (34). Hölttä’s (26) finding of the stimulation of PAO activity in the presence of BA (Spm and Spd were used as substrates) is another example, which served as the background of the present work. Another example is the effect of Mg$^{2+}$ ions on the activity of S-adenosylmethionine decarboxylase from Escherichia coli via Mg$^{2+}$-induced conformational changes of the enzyme, which are necessary for catalytic activity (35). In the absence of Mg$^{2+}$ ions, the pyruvate residue, responsible for the decarboxylation, does not react with a powerful and irreversible substrate-like hydroxylamine-containing inhibitor of the enzyme (35).

The mechanism of the change in the substrate specificity of PAO as a result of aldehyde treatment is unknown. It most likely includes an interaction of the aldehyde with the primary amino group(s) of the substrate. PAO has been shown to have potency to effectively oxidize a wide range of aldehydes, including amino acids, ethylenediamine, and trimethylenediamine, is well known and results in the formation of a complex equilibrium mixture of products, which in the case of diamines includes even cyclic aminals (37–39). The latter might be of importance in aldehyde-driven oxidation of α-methylpolyamines by PAO, because the breaking bond is at the γ-position to the chiral center, and the formation of cyclic aminal fixes the chiral center closer to the...
cleft site. The enhanced rate of the oxidase reaction by an increased concentration of PL at constant 1 mM Spm as the substrate in two different buffer systems (Table 5) indicates that glycine does not interfere with the formation of the "substrate adduct" for hPAO. However, with BA, borate buffer was a slightly better reaction medium compared with glycine-NaOH (supplemental Table S4). Furthermore, the order of substrate, aldehyde, and enzyme addition into the reaction mixtures did not have any effect on the reaction rates.

The reaction between an aldehyde and the polyamines may take place either directly in solution or the aldehyde may attack the polyamine-reactants. The reaction rate with 1 mM preformed PL=Spm adduct was higher than the rate with 1 mM Spm supplemented with 2 mM PL (Table 5), and no further enhancement was detected at higher PL concentration (data not shown). Accordingly, it is likely that the aldehyde reacts with a polyamine in solution, and the resultant product(s) serves as the substrate for PAO.

In the next set of experiments, we first saturated hPAO with 1 mM Spm (over 90%, since K_m of Spm is 47 μM) and then added increasing concentrations (from 0.1 to 1 mM) of either BA or PL. At this range of aldehyde concentration, the reaction rate enhanced upon increase of the aldehyde concentration. This could be considered as evidence indicating that the polyamine binds first to PAO and only then reacts with an aldehyde. However, the K_m value of BA=Spm for hPAO was 10 times lower than with Spm (Table 4), which may result in the dissociation of the initial Spm-PAO complex.

Schiff bases were initially suggested by Hölttä (26) as the natural substrates for PAO, and we decided to mimic them with isosteric oximes in order to work with individual compounds and not with the equilibrium mixtures. X-ray study of the complexes of the pyridoxal-5’-phosphate-dependent aspartic aminotransferase with the aminoxy analogues of the substrates showed that the pyridoxal-5’-phosphate-oxime binds at the active center and is a good mimetic of the external aldimine (40). Therefore, we used AOE-PU, AcAOE-PU, and stable BA and acetone oximes (Fig. 2) instead of Spd, AcSpd and BA aldime (40). Therefore, we used AOE-PU, AcAOE-PU, and stable BA aldime (40).

Introduction of the hydrophobic phenyl group, for both BA and AcSpd, resulted in increased binding affinities of BA (23)- and AcSpd (24) oximes in order to work with individual compounds and not with the equilibrium mixtures. X-ray study of the complexes of the pyridoxal-5’-phosphate-dependent aspartic aminotransferase with the aminoxy analogues of the substrates showed that the pyridoxal-5’-phosphate-oxime binds at the active center and is a good mimetic of the external aldimine (40). Therefore, we used AOE-PU, AcAOE-PU, and stable BA aldime (40).

Introduction of oxygen at the -position into the splitting bond of the initial Spm-PAO complex.

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