Pentamines as Substrate for Human Spermine Oxidase

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Received September 18, 2012; accepted December 2, 2012

Substrate activities of various linear polyamines to human spermine oxidase (hSMO) were investigated. The activities were evaluated by monitoring the amount of H2O2 released from sample polyamines by hSMO. H2O2 was measured by a HPLC method that analyzed fluorescent dimers derived from the oxidation of homovanillic acid in the presence of horseradish peroxidase. Six triamines were tested and were found not to be hSMO substrates. Of sixteen tetramines tested, spermine (Spm) was the most active substrate, followed by homospermine and N-butylated Spm. Pentamines showed a characteristic pattern of substrate activity. Of thirteen pentamines tested, 3343 showed higher substrate activity than Spm, and 4343 showed similar activity to Spm. The activities of the other pentamines were as follows: 3443, 4443, 3344, 4334, 4444, and 3334 (in decreasing order). Product amines released from these pentamines by hSMO were then analyzed by HPLC. Triamine was the only observed product, and the amount of triamine was nearly equivalent to that of released H2O2. A marked difference in the pH dependency curves between tetramines and pentamines suggested that hSMO favored reactions with a non-protonated secondary nitrogen at the cleavage site. The Km and Vmax values for Spm and 3343 at pH 7.0 and 9.0 were consistent with the higher substrate activity of 3343 compared to Spm, as well as with the concept of a non-protonated secondary nitrogen at the cleavage site being preferred, and 3343 was well degraded at a physiological pH by hSMO.

Key words spermine oxidase; polyamine; pentamine; hydrogen peroxide; homovanillic acid

The polyamines spermine (Spm), spermidine (34), and their precursor putrescine are important in cell proliferation, differentiation, and survival.1) Recently, there has been increasing interest in polyamine catabolism. Polyamine catabolism is mediated by three enzymes.2) Spermidine/spermine N′-acetyltransferase (SSAT) acetylates Spm and 34 to produce N′-acetylated compounds, which are exported from cells or oxidized by the peroxisomal enzyme N′-acetylpolyamine oxidase (APAO) to yield 34 or putrescine, respectively, with H2O2 and 3-acetamidopropanal. The cysteine enzyme Spm oxidase (SMO) can catalyze the oxidation of Spm directly to 34, bypassing the necessity for acetylation. The human SMO (hSMO) cDNA was first cloned and characterized by Wang et al.,3) and the recombinant SMO protein has been used to elucidate the properties of this enzyme.4–7) The substrate specificity of SMO appears to be limited and distinct from APAO, which catalyzes a number of polyamines and their analogues, some of which are inhibitors of SMO. A few compounds have been reported to exhibit substrate activity for SMO. N′-Ethyl-Spm (Et343) is degraded very efficiently by SMO to produce 34, while N′-Acetyl-Spm (Ac343) is degraded weakly.9) (S,S)-a,o-Dimethyl-Spm served as an excellent substrate for SMO, compared to Spm, based on kcat/Km values.8) These findings using N,N′-diacylated polyamines suggest SMO is capable of oxidizing other polyamine analogues. In this study, the substrate activities of a series of linear polyamines with a terminal primary amine were examined.

MATERIALS AND METHODS

Chemicals Bis(3-aminopropyl)amine (33, norspermidine) and N,N′-bis(3-aminopropyl)-1,3-diaminopropane (333) were purchased from Aldrich (Tokyo, Japan) and their hydrochloride salts were prepared and used after recrystallization from aqueous ethanol. Spm tetrahydrochloride was purchased from Tokyo Chemical Industries (Tokyo, Japan). Spermidine (34) trihydrochloride was purchased from Aldrich. The following compounds were prepared according to previously described methods8–11): Homospermidine (44) trihydrochloride, 1,9-diamino-4-azanorbornane (35) trihydrochloride, 1,10-diamino-4-azadecane (36) trihydrochloride, 1,12-diamino-4-azadodecane (38) trihydrochloride, 1,13-diamino-4,10-diazatetradecene (353) tetrahydrochloride, 1,14-diamino-4,11-diazatetradecene (363) tetrahydrochloride, 1,16-diamino-4,13-diazahexadecane (383) tetrahydrochloride, 1,18-diamino-4,15-diazaoctadecane (3103) tetrahydrochloride, 1,20-diamino-4,17-diazaicosane (3123) tetrahydrochloride, Thermospermine (334) tetrahydrochloride, 1,13-diamino-4,9-diazatetradecene (344, homospermine) tetrahydrochloride, 1,13-diamino-5,9-diazatetradecene (434) tetrahydrochloride, 1,14-diamino-5,10-diazatetradecene (444) tetrahydrochloride, N′-acetyl spermine (Ac343) trihydrochloride, diacetylspermine (DA343) dihydrochloride, N′-butyl spermine (Bu343) tetrahydrochloride, N,N′-diethyl spermine (DiEt343) tetrahydrochloride, N,N′-diethyl-1,14-diamino-5,10-diazatetradecene (DiEt444) tetrahydrochloride, 1,15-diamino-4,8,12-triazapentadecane (3333) pentahydrochloride, 1,16-diamino-4,8,12-triazahexadecane (3334) pentahydrochloride,

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Preparation of hSMO Enzyme Solution The BL21 (DE3) strain of Escherichia coli containing the pET15b/PAOh1/SMO plasmid was cultured. Following isopropyl-β-D-thiogalactopyranoside (IPTG) induction of PAOh1/SMO protein expression, the cells were sonicated in a 10 mM Tris–HCl buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 20 µM dithiothreitol (DTT). After 105000×g centrifugation at 4°C for 1 h, the supernatant was dialyzed against the same buffer. Aliquots were stored at −80°C and used as the hSMO enzyme source. Protein was determined using Coomassie Brilliant Blue and bovine serum albumin for calibration.

Determination of hSMO Activity hSMO activity was assayed by measuring the amount of H₂O₂ generated by the reaction. The standard incubation mixture (final volume, 100 µL) contained the enzyme solution, 0.25 mM Spm, 0.56 mM aminoguanidine, 0.036 mM pargyline and 1 mM EDTA, 0.04 µg horseradish peroxidase, 0.1 mg homovanillic acid in 0.1 M Tris–HCl buffer (pH 9.0). Before the addition of homovanillic acid and Spm, the mixtures were preincubated for 5 min at 37°C to remove endogenous substrates of H₂O₂-producing enzymes, and where used, MDL72527 was added at 0.25 mM. After preincubation, homovanillic acid and Spm were added, the mixtures were incubated (0–120 min) at 37°C and the reaction was stopped by the addition of 100 µL of 20% trichloroacetic acid solution. The reaction mixtures were centrifuged and the resulting fluorescence of homovanillic acid dimer was analyzed by ion-pair reversed phase HPLC. The ion-pair reversed phase HPLC conditions were as follows: column, TOSOH ODS-80TM (4.6 mm ×150 mm); isocratic elution solution, acetonitrile–water (25:75) containing 5 mM tetrabutylammonium bromide and 0.1% trifluoroacetic acid; flow rate, 0.7 mL/min; post-column mixing solution, acetonitrile–water (5:95) containing 0.6% 2-aminoethanol; flow rate, 0.5 mL/min and fluorescence detection, Ex 315 nm and Em 425 nm. Aliquots of sample solutions were injected, and quantitation was performed based on peak heights in comparison with the homovanillic acid dimer standard.

RESULTS AND DISCUSSION

Measurement of hSMO Activity Using an HPLC-Based Method For the measurement of hSMO activity, the H₂O₂ stoichiometrically released by the polyamine-hSMO reaction was monitored. H₂O₂ is usually measured fluorometrically using homovanillic acid. However, methods using fluorescence spectrophotometers often require relatively high amounts of sample and encounter interference by contaminants with other fluorescent substances. In this study, the H₂O₂ released by reactions with homovanillic acid and HRP, and the resulting fluorescent dimer, were separated and determined by HPLC. Temporal changes in the H₂O₂ released by hSMO oxidation of Spm are shown in Fig. 1. Only minimal amounts of activity were observed in the absence of Spm or after preincubation with MDL72527, which inhibited the release of H₂O₂ perfectly. Extracts from E. coli without the pET15b/PAOh1/SMO plasmid had no hSMO-like activity (data not shown). These results demonstrate that this HPLC method is useful for screening potential hSMO substrates.

Substrate Activity of Polyamines for hSMO A series of triamines, tetramines, pentamines, hexamines and their derivatives, with different methylene chain intervals, were tested for their substrate activities for hSMO under the conditions described in Materials and Methods. The results are summarized in Fig. 2. All triamines were found to be poor hSMO substrates, resulting in the release of scarce amounts of H₂O₂. With respect to tetramines, the natural substrate Spm was the most active substrate (100%), followed by homospermine (443) and N′-butylated Spm (Bu343), for which the activity was ≈60%. The other tetramines (434, 353, 444) exhibited low activity (<10%). Ac343 activity was very low compared to Spm, which is consistent with a previous report by Vujcic et al. N′,N′-DiacetylSpm (DiAc343) did not exhibit substrate activity for hSMO. Diethylated tetramines of terminal primary...
amines (DiEt343, DiEt444) also exhibited very low activity, similar to the report by Vujicic et al. In contrast, pentamines, with the exception of 3333, exhibited marked activity: 3343 (>120%), 4343 (100%), 3443 (≈50%), 4443 (≈50%), 4344 and 3344 (≈30%), 4334, 4444 and 3334 (≈20%). Diethylated or diacetylated pentamines of terminal primary amines exhibited very low activity. Of the three hexamines tested, only 34343 exhibited a significant amount of activity (≈50%). In sum—mary, hSMO appeared to recognize pentamines with terminal aminopropyl as well as aminobutyl groups, suggesting an unknown pentamine cleavage reaction catalyzed by hSMO.

Measurement of Amines Released from Pentamines by hSMO  To elucidate the cleavage reaction, product amines were analyzed by OPA-post label ion-exchange HPLC method. Only triamines (no diamines or tetramines) were detected in enzyme reactions with pentamines, and the amounts of the resulting triamines were nearly equal to that of released H2O2. The results are summarized in Fig. 3. A larger amount of 34 was liberated from 3343 than 34 from Spm itself, indicating the production of 34 through oxidative degradation at the central aza and probably an aminopropanal compound corresponding to 33. The amount of 34 liberated from 4343 was similar to that from Spm, indicating degradation at the central aza to produce 34 and probably an aminopropanal and/or an aminobutanal corresponding to 34. The amount of 34 liberated from 3443 was similar to that from Spm, indicating degradation at the central aza to preferentially form 34. The products from 4344 were nearly equal amounts of 34 and 44, and those from 3344 were mainly 44 with a small amount of 33. The similar amounts of 34 from 4343 to 44 from 4444 suggest that hSMO recognized terminal 34 and 44 equally. Products from 3343 were 34 and a small amount of 33. The product from 34343 was a significant amount of 34 only, with triamine release decreasing in the following order: 34 from xx43, 44 from xx44 or 34 from xx34, and 33 from 3344.

Significant Difference in pH Dependency of hSMO Degradation of Tetramines and Pentamines  The effect of pH on H2O2 release by hSMO was examined using tetramines (Spm and 443) and pentamines (3343, 4343, 3443, 4443) (Fig. 4). The two tetramines showed similar pH dependency curves, with the peak H2O2 release at pH 9.0 followed by a marked decrease until pH 7.0. On the other hand, the four pentamines exhibited similar pH dependency curves, with 3343 and 4343 plateauing between pH 7.5 to 9.0 and moderately decreasing over this pH range. The differences in pH dependency curves are potential indicators of the basicity of the nitrogen at the hSMO cleavage site of polyamines. hSMO seemed to favor a non-protonated secondary nitrogen at the catalytic site. Additional information on the activity of hSMO towards Spm and 3343, kinetic data at pH 7.0 and 9.0, is provided in Table 1. Both the $K_m$ and $V_{max}$ values for Spm were markedly different.
at pH 7.0 and 9.0, whereas those for 3343 were largely unchanged. The $K_m$ values for Spm were higher than those for 3343 at both pH levels, and were consistent with 3343 being a better $h$SMO substrate than Spm. At pH 7.0, the ratio of $K_m$ for Spm to that of 3343 was about twice that observed at pH 9.0, suggesting that the affinity of Spm for $h$SMO decreases more rapidly than 3343.

Limited data on pH- and temperature-dependent $h$SMO activity are available. Although $h$SMO activity has been reported to be optimal at pH 8.3, the pH range over which activity is measurable is likely broad. The $h$SMO activity is sensitive to environmental conditions such as temperature, pH, and oxygen concentration.

The cellular distribution of $h$SMO is not well understood. While $h$SMO activity has been observed in both the cytosol and nucleus, the subcellular localization of $h$SMO is complex and dependent on various factors such as cell type, developmental stage, and cellular stress.

The catalytic activity of $h$SMO is influenced by the presence of cofactors such as flavin mononucleotide (FMN) and adenosine triphosphate (ATP). The requirement for these cofactors, along with the presence of specific amino acids, further complicates the understanding of $h$SMO activity.

Table 1. $h$SMO $K_m$ and $V_{max}$ Values for Spm and 3343

<table>
<thead>
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<th>pH</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
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<tr>
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REFERENCES


