RESEARCH COMMUNICATION

Cloning and characterization of multiple human polyamine oxidase splice variants that code for isoenzymes with different biochemical characteristics

Tracy MURRAY-STEWART, Yanlin WANG, Wendy DEVEREUX and Robert A. CASERO, Jr[1]

Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Bunting Blaustein Building, Room 551, 1650 Orleans Street, Baltimore, MD 21231, U.S.A.

The recently cloned and characterized human polyamine oxidase (PAOh1) potentially represents a new class of catabolic enzymes in the mammalian polyamine metabolic pathway capable of the efficient oxidation of polyamines. Here the discovery of three additional human PAO splice variants is reported, and the data support the fact that the human PAO gene codes for at least four isoenzymes, each of which exhibit distinctive biochemical characteristics, suggesting the existence of additional levels of complexity in polyamine catabolism.

Key words: N^1-acetylspermine, isoform, polyamines, spermidine, spermine.

INTRODUCTION

Interest in polyamine catabolism has increased since its role in determining cellular sensitivity to various antitumour polyamine analogues has been acknowledged [1,2]. Until recently, the mammalian polyamine catabolic pathway was thought to consist of two enzymes, namely a rate-limiting spermidine/spermine N^1-acetyltransferase (SSAT) [3] and a polyamine oxidase (PAO) that preferentially catalyses the oxidation of the N^1-acetylpolyamines produced by SSAT activity. This oxidation results in the production of H_2O_2, 3-acetamidopropanal, and putrescine or spermidine (Spd), depending on the starting substrate [4]. H_2O_2 production by increased polyamine catabolism in response to specific polyamine analogues has been shown to result in cytotoxicity by these agents in specific tumour-cell types, and this cytotoxicity can be attenuated through the use of a specific inhibitor [5]. However, studies into the role of polyamine oxidation in mammalian cells have been limited by the lack of any verified mammalian PAO clones. Our recent cloning of the human PAO gene *PAOh1* provided the first mammalian *PAO* clone for study and demonstrated the ability of the gene product to catalyse spermine (Spm) oxidation [6]. By using alternative methods, another group of workers have recently confirmed PAOh1 activity and have shown that, in their system, this enzyme shows a greater specificity for the native polyamine, Spm, as a substrate [7]. These data suggest that this oxidase represents an additional enzyme in polyamine catabolism that preferentially utilizes the polyamines as substrates.

The potential importance of PAOs in anticancer drug response is underscored by the finding that PAO is significantly inducible by antitumour polyamine analogues in a manner similar to SSAT [6], suggesting that its activity may play a direct role in cell death via toxic H_2O_2 production. Additionally, polyamine oxidation has recently been identified as a critical step in the detoxification of one of the antitumour polyamine analogues, and tumour cells that have low to non-detectable levels of polyamine oxidation capacity are significantly more sensitive to the cytotoxic effects of the analogue [8].

Here we report the discovery that the human *PAOh1* gene codes for at least four active isoenzymes that result from alternative splicing of eight exons. The resultant proteins have different biochemical characteristics and substrate specificities, and were identified in a variety of tumour and normal cell types. Because of the potential for cell- or tissue-specific PAO isoenzyme expression levels, the products of the *PAOh1* gene may contribute in unique ways to our understanding of polyamine metabolism and antitumour-drug-sensitivity.

EXPERIMENTAL

PAO splice variant cloning

PAO isoforms were isolated using reverse-transcription (RT-) PCR on total RNA from NCI-H157 non-small-cell lung carcinoma (NSCLC) cells and HEK-293 cells. A human placenta cDNA library was also used as a source for potential splice variants, as previously described [6]. PCR products were ligated into the pCR2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.), sequenced using a PerkinElmer ABI automated DNA sequencer, and analysed by comparison with the previously identified human genomic PAO sequence located in GenBank® (accession no. AL121675). Novel sequences corresponding to alternative splicing were further cloned into the pcDNA3.1 +/− mammalian expression vectors using restriction-enzyme digestion and ligation, and proper insertion was confirmed via restriction analysis. These constructs were designated pPAOh2, pPAOh3, and pPAOh4. All restriction and modification enzymes were purchased from Invitrogen or New England Biolabs (Beverly, MA, U.S.A.).

In vitro transcription and translation

For PAO splice variant protein analysis, each cDNA construct, as well as the pcDNA3.1 empty vector, was linearized using *Nde*I. *In vitro* transcription and translation was subsequently

Abbreviations used: N^1-AcSpm, N^1-acetylspermine; NSCLC, non-small-cell lung carcinoma; (h1)PAO, (human) polyamine oxidase; RT-, reverse transcription; Spd, spermidine; Spm, spermine; SSAT, spermidine/spermine N^1-acetyltransferase.

[1] To whom correspondence should be addressed (e-mail rcasero@jhmi.edu).

Nucleotide sequences for the *PAOh2*, *PAOh3* and *PAOh4* isoforms of the human polyamine oxidase gene (*PAOh*) have been deposited with the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession numbers AY033890, AY033891 and AF519179 respectively.

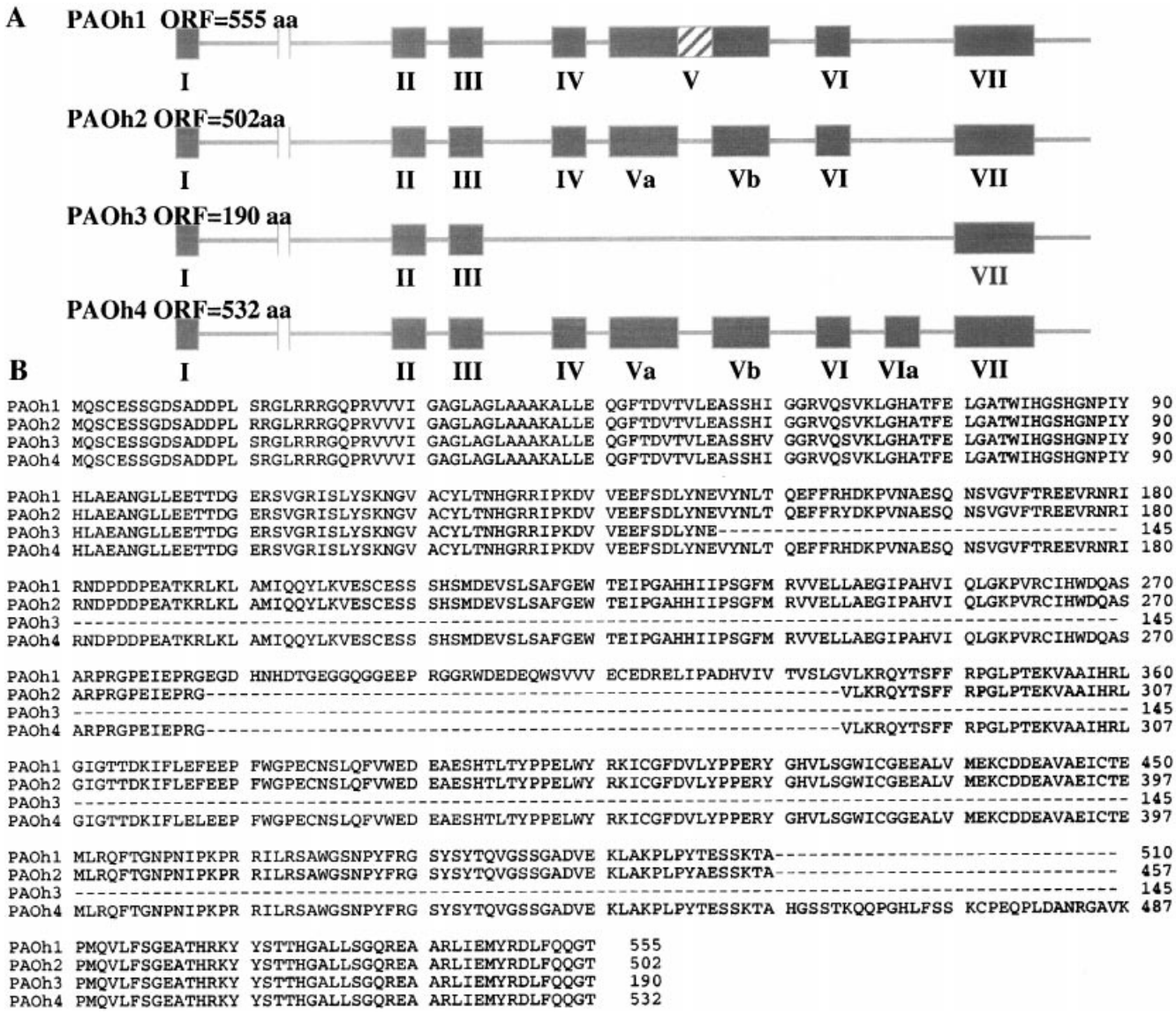

Figure 1 Exon structures of human PAO isoforms (A) and amino acid sequence alignment of the human PAO isoforms (B)

(**A**) The internal exon present in exon V of *PAOh1* can act as an intron and is spliced out of *PAOh2* and *PAOh4*, resulting in exons Va and Vb. *PAOh4* contains a newly identified exon VIa. (**B**) Sequence alignment. Abbreviations: aa, amino acid; ORF, open reading frame.

performed using the Wheat Germ Extract–TnT coupled system from Promega (Madison, WI, U.S.A.) according to the manufacturer's protocol. Reactions were performed in parallel, with one aliquot containing [^{35}S]methionine in the amino acid mixture for subsequent separation and quantification by SDS/PAGE. Labelled reaction products were run on 10 %-(w/v)-BisTris gels (Invitrogen) with 20 mM Mops buffer, pH 7.7, as suggested by the manufacturer. After drying, bands were visualized and quantified using phosphorimage analysis with Image Quant software from Molecular Dynamics (Sunnyvale, CA, U.S.A.).

PAO enzyme activity analysis

TnT products from the reactions using unlabelled substrates were used for PAO enzyme analysis by the method of Suzuki et al [9]. Specifically, 10 μl of the TnT reaction mixture was used for each 600 μl of PAO assay sample (in duplicate, and in the presence of monoamine and diamine oxidase inhibitors, as previously described [6]). Background oxidase activity in the Wheat Germ Extract–TnT reactions was determined for each substrate using the empty pcDNA3.1 vector. These values (always < 0.05 pmol/min) were subtracted from the oxidase activity measured in TnT lysates produced from vectors containing the individual splice variants. To ensure linearity of the reaction (thereby determining the optimal incubation time after substrate addition), time courses were performed for each potential isoenzyme in the presence of 1 mM Spm (Sigma, St. Louis, MO, U.S.A.).

Kinetics of PAO isoenzymes

Apparent kinetics were examined for each isoenzyme using TnT reaction products as described above with increasing concentrations of Spm, Spd (Sigma) or N^1-acetylspermine (N^1-AcSpm;

Fluka). Apparent K_m and V_{max} values were determined using the Lineweaver–Burk transformation of the Michaelis–Menten equation. Values were adjusted for TnT reaction efficiency by SDS/PAGE quantification of a [^{35}S]methionine-labelled aliquot of the TnT reaction mixture normalized for methionine content.

Specific activities of PAO isoforms

Specific activities were determined for each isoform with each substrate at 0.25 mM. Specific activity was calculated on the basis of band intensity resulting from parallel TnT reactions in the presence of [^{35}S]methionine and normalized according to number of methionine residues present in the splice-variant protein sequences. One unit of PAO activity was defined as the ability to produce 1 pmol of H_2O_2/min per unit of protein (where 1 unit of protein corresponds to one band intensity unit, as determined from PhosphorImager analysis).

RESULTS

Upon sequence comparison with our previously identified PAO isoform 1 (GenBank® accession no. AY033889), as well as with the human genomic *PAO* sequence (GenBank® accession no. AL121675), we confirmed the isolation of three additional splice variants, designated *PAOh2*, *PAOh3* and *PAOh4*. Isoforms 1 and 2 were isolated using the cDNA of a human placental library. *PAOh2* was also isolated from the H157 NSCLC cell line, as was *PAOh3*, which was also obtained from HEK-293 mRNA. *PAOh4* was obtained only from HEK-293 mRNA. It should be noted that exon V of *PAOh1* possesses an internal region that can act as an intron, and which is spliced out of exon V of both *PAOh2* and *PAOh4*, resulting in two smaller exons, designated exons Va and Vb. Also, *PAOh4* contains an additional exon, VIa, which is not present in the other three isoforms. *PAOh3* is completely devoid of exons IV–VIa, and possesses an open reading frame of only 190 amino acids, which is less than half that of the other three splice variants (Figures 1A and 1B). Surprisingly, many of the amino acids missing from *PAOh3* correspond to those implicated in FAD cofactor binding in *Zea mays* (maize) PAO [10,11].

Since the stabilities of the various isoforms could differ, it was first necessary to determine the appropriate assay times for each splice variant to ensure measurements were made within the linear range. Time-course data using 1 mM Spm as substrate revealed a linear production of H_2O_2 by all isoforms for approximately 20 min (results not shown). Therefore 10 min was chosen as the period of incubation following substrate addition for further experiments.

Relative kinetic analysis performed with TnT reaction products revealed distinct kinetic parameters and substrate affinities for each splice variant with Spm, Spd and N^1-AcSpm (Figure 2). Apparent K_m and V_{max} values for each isoform with each substrate were calculated from the Lineweaver–Burk transformation, and are presented in Figure 2(A). Importantly, PAOh4 demonstrated the lowest K_m values for each of the substrates (in the nanomolar range), and appeared to have the highest affinity for Spd. In contrast, PAOh1 and PAOh2 had relatively low affinities for the acetylated polyamine (in the high micromolar range). The shortest of the four isoforms, PAOh3, also demonstrated a greater affinity for Spd and Spm than for N^1-AcSpm.

Using saturating concentrations of each substrate as apparent from Figure 2 (0.25 mM), PAO activity assays were carried out using TnT-produced protein that was translated in parallel with an aliquot in the presence of [^{35}S]methionine. This enabled a comparison of specific activities among the splice variants based on the number of methionine residues present in the each splice variant (Figure 3). Normalization for methionine content revealed that the shortest isoform, PAOh3, possessed the highest specific activities for all three substrates. Consistent with the predicted K_m and V_{max} values, Spd and N^1-AcSpm activities were slightly higher than that of Spm. PAOh1 possessed the next highest activities for all substrates, followed by PAOh2 and PAOh4, which demonstrated similar activities, in spite of the much lower K_m values predicted for PAOh4 (Figure 3).

A

	PAOh1		PAOh2		PAOh3		PAOh4	
	V_{MAX}	K_M	V_{MAX}	K_M	V_{MAX}	K_M	V_{MAX}	K_M
Substrate	*(pmol/min)*	*(μM)*	*(pmol/min)*	*(μM)*	*(pmol/min)*	*(μM)*	*(pmol/min)*	*(μM)*
Spm	3.3 +/- 0.4	5.9 +/- 0.1	3.0 +/- 1.7	7.0 +/- 4.2	8.6 +/- 0.2	6.9 +/-4.8	3.4 +/- 1.1	0.029 +/- 0.020
Spd	4.0 +/- 0.0	12.8 +/- 3.1	2.1 +/- 0.9	10.4 +/- 2.9	14.4 +/- 1.5	6.7 +/- 0.4	2.2 +/- 0.1	0.014 +/- 0.006
N^1AcSpm	4.1 +/- 0.1	23.2 +/- 2.6	2.0 +/- 0.1	15.8 +/- 0.4	21.7 +/- 2.1	11.5 +/- 3.7	6.1 +/- 2.4	0.025 +/- 0.012

B

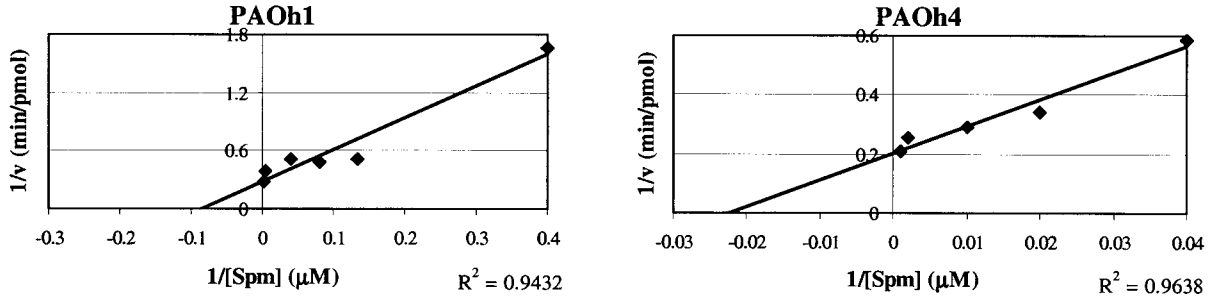

Figure 2 Kinetic properties of human PAO isoforms

(**A**) In this Table all values represent means for at least two experiments, each with all samples prepared and measured in duplicate from the same TnT reaction. V_{max} ('V_{MAX}') and K_m' ('K_M') values were predicted using the Lineweaver–Burk transformation of the Michaelis–Menten equation. V_{max} units are presented as pmol of H_2O_2 generated/min per unit of protein, with protein unit determination via SDS/PAGE analysis of a radiolabelled aliquot of the TnT reaction mixture. (**B**) Shows representative double-reciprocal plots of PAOh1 versus PAOh4 using increasing values of Spm as substrate.

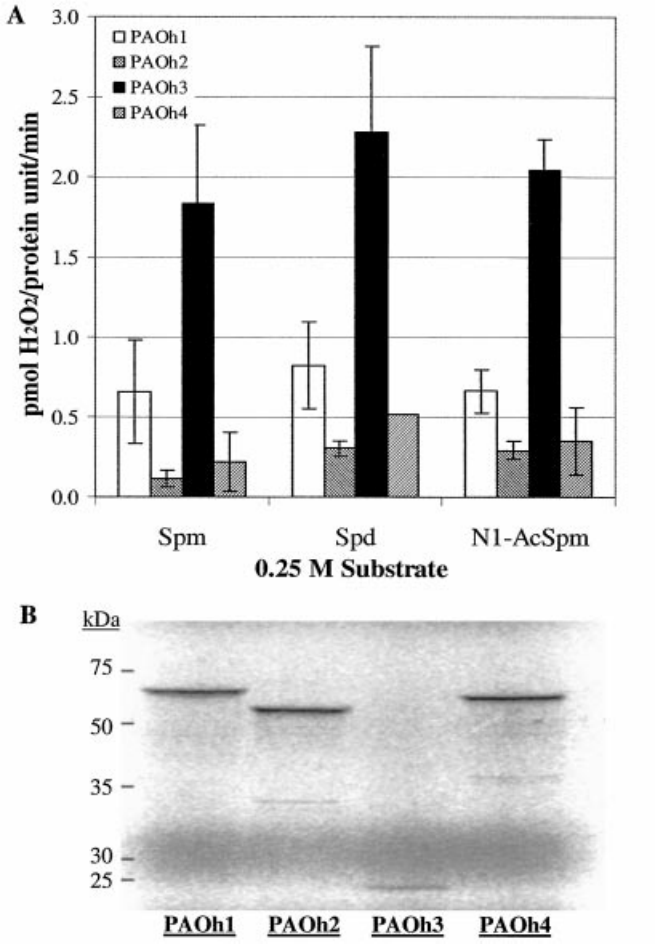

Figure 3 Specific activities of PAO isoforms with various polyamine substrates

Protein produced from parallel TNT reactions in the presence and absence of [^{35}S]methionine was used for PAO activity analysis (**A**) or quantified by SDS/PAGE (**B**). Band intensities in (**B**) were normalized for the methionine content of each isoform and used for determination of specific activity values in (**A**).

DISCUSSION

The catabolism of polyamines has been demonstrated to be associated with the response of specific tumour types to several antitumour polyamine analogues [5,12]. Further, alterations in the enzymes that control polyamine catabolism have been implicated in the progression of neoplastic disease [13]. The mechanisms underlying these observations appear to be associated with oxidation of polyamines concurrent with the induction of SSAT. The results presented here demonstrate that each of the four identified splice variants from the *PAOh1* gene are capable of catalysing the oxidation of multiple substrates, including Spd, Spm and N^1-AcSpm, suggesting that multiple oxidases coded by a single gene may significantly affect polyamine homoeostasis and potential drug response. These results are in contrast with those reported by Vujcic et al. [7], who did not obtain evidence of oxidation of any substrate other than Spm following transfection of cells with a construct homologous with PAOh1. The reasons for this variation may be a result of differences in reaction conditions. Specifically, the enzyme analyses presented here were performed in borate buffer at pH 9.0. The analyses by Vujcic et al. [7] were performed in glycine buffer at pH 9.5. Consistent with their results, when glycine buffer was substituted for borate buffer in the system used here, only Spm was efficiently catalysed by any of the splice variants (results not shown). Another potential basis for the difference in the observations presented here from those of Vujcic et al. [7] may result from the use of a plant system (wheat-germ TNT) to produce our protein. This is necessary, since the mammalian rabbit reticulocyte system contains haem iron that would prevent the precise measurement of H_2O_2 produced [6]. The production of protein in the wheat-germ TNT may result in alternate substrate specificity based on potential differences in protein folding or a difference in cofactors or post-translational modifications between the wheat-germ system and those occurring in the transfected-cell system [14]. These results are, however, consistent with observations with the PAOs of the maize and barley (*Hordeum vulgare*) plants, which are nearly identical in size with the human PAOh1 protein, and which possess a protein domain organization very similar to that of the human protein. Both plant proteins are able to use both Spm and Spd as substrates [15,16]. Although the validation of substrate specificity awaits the availability of purified proteins representing the individual splice variants, the important finding that each of the splice variants codes for active proteins with different kinetic behaviour is both valid and significant.

It should be noted that Vujcic et al. [7] also provided data on two constructs that were referred to as 'splice variants' (GenBank® accession nos. AK025938 and BC000669), neither of which produced oxidase activity in transfected cells. BC000669 corresponds to our *PAOh2* (GenBank® accession no. AY033890) with the exception of one amino acid at position 16. AK025938 possesses an open reading frame that codes for a polypeptide of 389 amino acids that does not correspond to any of the splice variants presented here. However, the open reading frame starts at an AUG codon corresponding to a region within exon 4 of *PAOh1*, and the original clone has no leader sequence associated with it. Consequently, the possibility that this cDNA represents a cloning artifact rather than an actual splice variant must be considered. More importantly, no data are provided to confirm that the transfected cells actually produce any protein from the constructs. Consequently, the studies indicating the lack of PAO activity in cells transfected with these sequences is currently difficult to interpret.

It is clear from our present results that the multiple splice variants are capable of catalysing multiple polyamine substrates in the system as reported. Interestingly, the shortest splice variant, PAOh3, appears to have the highest k_{cat} of all the isoforms, and was the most common variant to be detected by the RT-PCR-based cloning strategy used. It was isolated from the H157 and HEK-293 cells presented here, as well as in subsequent studies using normal human lymphocytes, and from DU145 prostate cancer cells. This prevalence of PAOh3 is quite possibly the result of higher cloning efficiency of the shorter sequence, but the significance that the existence of this isoform does not appear to be cell- or tissue-type-specific should not be overlooked. The K_m values in the micromolar range exhibited by PAOh3, as well as by PAOh1 and PAOh2, for the native polyamines is certainly within the intracellular concentration ranges often predicted, although the free intracellular polyamine concentration is difficult to estimate. PAOh4 exhibits K_m values in the nanomolar range for Spd, Spm and N^1-AcSpm. This result may be significant, since the amount of acetylated polyamines in the cell is generally low, even with a high induction of SSAT activity. However, the ability of N^1-AcSpm to act as a substrate for PAOh4 *in situ* would depend on the concentration of free Spm and Spd, since they, too, are high-affinity substrates for PAOh4. It should be noted

that PAOh4 was only obtained from the human embryonal kidney cell line.

It is not clear that any of the isoenzymes studied here is homologous with the animal PAO reported in the literature prior to our cloning of PAOh1 [4,13]. That PAO has been defined as a peroxisomal enzyme [17,18]. None of the isoenzymes presented here possesses a recognizable peroxisomal signal localization sequence [19]. Consequently, it is possible, as suggested by Vujcic et al. [7], that the isoenzymes coded for by the multiple splice variants of the *PAOh1* gene represent an entirely new family of oxidases.

The results presented here demonstrate that the human PAO gene *PAOh1* codes for multiple isoforms with significant activities that are capable of using multiple polyamine substrates within physiologically relevant K_m values. The relative expression of these various isoforms within the cell and among various cells is currently being investigated. The possibility that the various PAO isoforms may have a direct role in polyamine homoeostasis and, more importantly, in drug response to various anti-tumour polyamine analogues, underscores the critical importance of gaining a better understanding of this interesting group of enzymes.

This research was supported by National Institutes of Health grants CA51085 and CA88843.

REFERENCES

1 Bergeron, R. J., Feng, Y., Weimar, W. R., McManis, J. S., Dimova, H., Porter, C., Raisler, B. and Phanstiel, O. (1997) A comparison of structure–activity relationships between spermidine and spermine analogue antineoplastics. J. Med. Chem. **40**, 1475–1494

2 Casero, Jr, R. A. and Woster, P. M. (2001) Terminally alkylated polyamine analogues as chemotherapeutic agents. J. Med. Chem. **44**, 1–26

3 Casero, Jr, R. A. and Pegg, A. E. (1993) Spermidine/spermine N^1-acetyltransferase – the turning point in polyamine metabolism. FASEB J. **7**, 653–661

4 Seiler, N. (1995) Polyamine oxidase, properties and functions. Prog. Brain Res. **106**, 333–344

5 Ha, H. C., Woster, P. M., Yager, J. D. and Casero, Jr, R. A. (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. Proc. Natl. Acad. Sci. U.S.A. **94**, 11557–11562

6 Wang, Y., Devereux, W., Woster, P. M., Stewart, T. M., Hacker, A. and Casero, Jr, R. A. (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. Cancer Res. **61**, 5370–5373

7 Vujcic, S., Diegelman, P., Bacchi, C. J., Kramer, D. L. and Porter, C. W. (2002) Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. Biochem. J. **367**, 665–675

8 Lawson, K. R., Marek, S., Linehan, J. A., Woster, P. M., Casero, Jr, R. A., Payne, C. M. and Gerner, E. W. (2002) Detoxification of the polyamine analogue N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8-diazaundecane (CHENSpm) by polyamine oxidase. Clin. Cancer Res. **8**, 1241–1247

9 Suzuki, O., Matsumoto, T. and Katsumata, Y. (1984) Determination of polyamine oxidase activities in human tissues. Experientia **40**, 838–839

10 Tavladoraki, P., Schinina, M. E., Cecconi, F., Di Agostino, S., Manera, F., Rea, G., Mariottini, P., Federico, R. and Angelini, R. (1998) Maize polyamine oxidase: primary structure from protein and cDNA sequencing. FEBS Lett. **426**, 62–66

11 Binda, C., Coda, A., Angelini, R., Federico, R., Ascenzi, P. and Mattevi, A. (1999) A 30-angstrom-long U-shaped catalytic tunnel in the crystal structure of polyamine oxidase. Structure Fold. Des. **7**, 265–276

12 Chen, Y., Kramer, D. L., Diegelman, P., Vujcic, S. and Porter, C. W. (2001) Apoptotic signaling in polyamine analogue-treated SK-MEL-28 human melanoma cells. Cancer Res. **61**, 6437–6444

13 Wallace, H. M., Duthie, J., Evans, D. M., Lamond, S., Nicoll, K. M. and Heys, S. D. (2000) Alterations in polyamine catabolic enzymes in human breast cancer tissue. Clin. Cancer Res. **6**, 3657–3661

14 Hartl, F. U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science **295**, 1852–1858

15 Cervelli, M., Cona, A., Angelini, R., Polticelli, F., Federico, R. and Mariottini, P. (2001) A barley polyamine oxidase isoform with distinct structural features and subcellular localization. Eur. J. Biochem. **268**, 3816–3830

16 Federico, R., Alisi, C., Cona, A. and Angelini, R. (1988) Purification of polyamine oxidase from maize seedlings by immunoadsorbent column. Adv. Exp. Med. Biol. **250**, 617–623

17 Van den Munckhof, R. J., Denyn, M., Tigchelaar-Gutter, W., Schipper, R. G., Verhofstad, A. A., Van Noorden, C. J. and Frederiks, W. M. (1995) *In situ* substrate specificity and ultrastructural localization of polyamine oxidase activity in unfixed rat tissues. J. Histochem. Cytochem. **43**, 1155–1162

18 Nishikawa, M., Hagishita, T., Yurimoto, H., Kato, N., Sakai, Y. and Hatanaka, T. (2000) Primary structure and expression of peroxisomal acetylspermidine oxidase in the methylotrophic yeast *Candida boidinii*. FEBS Lett. **476**, 150–154

19 Holroyd, C. and Erdmann, R. (2001) Protein translocation machineries of peroxisomes. FEBS Lett. **501**, 6–10

Received 9 October 2002/23 October 2002; accepted 24 October 2002
Published as BJ Immediate Publication 24 October 2002, DOI 10.1042/BJ20021587