In Situ Substrate Specificity and Ultrastructural Localization of Polyamine Oxidase Activity in Unfixed Rat Tissues

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Received for publication March 17, 1995 and in revised form June 19, 1995; accepted June 24, 1995 (SA3623).

Data concerning the substrate specificity and the exact intracellular localization of the polyamine-catabolizing enzyme polyamine oxidase are conflicting. Biochemical studies have shown that \(N^1\)-acetylation of spermine and spermidine dramatically increases the specificity of these compounds for peroxisomal polyamine oxidase to produce spermidine and putrescine, respectively. On the other hand, polyamine oxidase activity was demonstrated histochemically both in peroxisomes and in cytoplasm of several tissues, using spermidine and/or spermine as substrate. To elucidate the in situ substrate specificity of polyamine oxidase and the localization of its activity, enzyme activity was demonstrated histochemically both in peroxisomes and in cytoplasm of several tissues, using spermidine and/or spermine as substrate. To elucidate the in situ substrate specificity of polyamine oxidase and the localization of its activity, enzyme activity was detected in rat liver, kidney, and duodenum at the light and electron microscopic levels. For this purpose, unfixed cryostat sections were applied to avoid changes in enzyme activity owing to chemical fixation. Spermine, spermidine, their \(N^1\)-acylated forms, and putrescine were used as substrates, and cerium ions as capturing agent for \(H_2O_2\). Control reactions were performed in the absence of substrate or in the presence of substrate and specific oxidase inhibitors. At the light microscopic level, final reaction product specifically generated by polyamine oxidase activity was found exclusively in a granular form in hepatocytes, epithelial cells of proximal tubules of the kidney, and epithelial cells of duodenal villi with \(N^1\)-acetylspermidine or \(N^1\)-acetylspermine as substrates. Final reaction product was not observed in any of the tissues after incubation in the presence of putrescine, spermidine, or spermine. Formation of specific final reaction product was prevented by incubation in the presence of a specific polyamine oxidase inhibitor, but it was not affected by a diamine oxidase inhibitor. Ultrastructural studies revealed that polyamine oxidase activity is localized exclusively to the matrix of peroxisomes of kidney and liver and to microperoxisomes of the duodenum. The localization patterns obtained with unfixed tissues are in agreement with biochemical data. Strong intraperoxisomal, interperoxisomal, and intercellular heterogeneity in polyamine oxidase activity was found in all tissues investigated. (J Histochem Cytochem 43: 1155-1162, 1995)

KEY WORDS: Polyamine oxidase activity; Substrate specificity; Peroxisomes; \(N^1\)-acylation; Unfixed cryostat sections; Cerium salt capture method.

Introduction

The polyamines spermidine (Spd) and spermine (Spm) play an important role in the regulation of growth and development of animal and plant cells (Féray et al., 1994; Tabor and Tabor, 1984; Heby, 1981). Polyamine concentrations and enzymes involved in polyamine metabolism were found to be increased in proliferating cells, whereas induction of polyamine synthesis is related to increased rates of DNA, RNA, and protein synthesis (Jänne and Morris, 1984; Sjoerdasma and Schechter, 1984; Tabor and Tabor, 1984; Pegg and McCann, 1982; Heby, 1981).

A key enzyme in polyamine synthesis is ornithine decarboxylase (Figure 1), which catalyzes the conversion of ornithine into putrescine (Put). Put is converted into Spd and Spd into Spm by Spd synthase and Spm synthase, respectively (Figure 1) (Pegg, 1986; Seiler et al., 1985; Tabor and Tabor, 1984; Heby, 1981). Although the synthesis of polyamines is well understood, discrepancies exist...
in the literature with respect to pathways and intracellular sites of degradation of polyamines. Biochemical studies have shown that polyamines can be oxidized by polyamine oxidase (PAOX) in peroxisomes only (Heby, 1981; Hölttä, 1977). However, oxidation of Spd and Spm in vivo is described to occur only after N\textsuperscript{1}-acetylation by spermidine/spermine N\textsuperscript{1}-acyltransferase (SAT) present in the cytoplasmic matrix (Pegg, 1986; Matsui et al., 1981; Pegg et al., 1981). This would mean that only N\textsuperscript{1}-acetylspermidine (NAcSpd) and N\textsuperscript{1}-acetylspermine (NAcSpm) are true substrates for PAOX, although Spd and Spm may act as substrates under certain artificial conditions (Bey et al., 1985; Bolkenius et al., 1985; Morgan, 1985; Bolkenius and Seiler, 1981). On the other hand, Beard and co-workers (1985) reported in an ultrastructural study that PAOX activity could be detected with non-acetylated Spm as substrate in peroxisomes after chemical fixation of rat liver and kidney. Furthermore, it has been described that polyamines can also be oxidized by other, non-peroxisomal, amine oxidases. For example, diamine oxidase (DAOX), which is localized in the cytoplasmic matrix, or bovine serum amine oxidase, which is present in serum, are believed to be able to oxidize Put, Spd, Spm, NAcSpd, and NAcSpm (Seiler et al., 1985). The discussion on the substrate specificity of PAOX became even more confusing when Robinson et al. (1991) postulated that PAOX and DAOX are one and the same enzyme. Recently, Nakos and Gosssrau (1994) tried to reproduce the ultrastructural findings of Beard et al. (1985) at the light microscopic level. Instead of a granular distribution of the precipitate, they detected final reaction product throughout the cytoplasm of various cell types.

To obtain a clearer insight into the subcellular pathways of polyamine metabolism, we studied the in situ substrate specificity and the intracellular localization of PAOX by incubation of unfixed cryostat sections of rat liver, duodenum, and kidney for PAOX activity. Unfixed cryostat sections were used to avoid alterations of the enzyme by chemical fixation. PAOX activity was detected with the cerium salt capture method (Briggs et al., 1985), which enabled localization of enzyme activity at both the light and the electron microscopic level (Frederiks et al., 1994a; Van den Munckhof et al., 1994a; Van Noorden and Frederiks, 1993; Schellens et al., 1992). For light microscopy, polyvinyl alcohol (PVA) was added to the incubation medium to avoid diffusion of enzyme molecules into the medium. For ultrastructural studies, the semipermeable membrane technique was used (Van den Munckhof et al., 1994a; Schellens et al., 1992). Incubations were performed in the presence of different possible substrates for PAOX and DAOX and several specific inhibitors of these enzymes (Bey et al., 1985; Bolkenius et al., 1985; Schuler, 1952).

**Materials and Methods**

Male Wistar rats weighing 200–250 g were sacrificed under ether anesthesia. Liver, kidney, and duodenum were removed and cut into pieces up to 5 mm\textsuperscript{3}. The tissue was immediately frozen in liquid nitrogen and stored at −80°C. Cryostat sections were cut with a motor-driven Bright cryostat.

The cerium method according to Briggs et al. (1975) to localize oxidase activities was performed in two ways. Substrate specificity of PAOX was studied at the light microscopic level using PVA-containing incubation media, whereas the semipermeable membrane technique was applied for study of the ultrastructural localization of PAOX activity.

**Substrate Specificity.** Unfixed cryostat sections (8 μm thick) of rat liver, kidney, and duodenum were mounted on glass slides and air-dried. The substrate specificity of PAOX was studied by incubating sections for 30–90 min at 37°C with a medium consisting of 300 mM Tris-maleate buffer, pH 8.3, 30 mM CeCl\textsubscript{3}, 100 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}, and 10% PVA (Frederiks et al., 1994a; Nakos and Gossrau, 1994; Kooij et al., 1991). Control reactions were performed by incubating sections in the absence of substrate, in the presence of substrate and 0.1–10 mM 1,4-butanediimine-N,N\textsubscript{bis}[2,3-butanedienyl] (MDL 72527/DA-011), a specific inhibitor of PAOX (Bey et al., 1985; Bolkenius et al., 1985), which was a kind gift of Dr. S. Sarhan and Dr. P. Casara of Marion Merrell Dow Research Institute (Strasbourg, France). Control reactions in the presence of substrate and 0.1–25 mM aminoguanidine, a specific inhibitor of DAOX (Schuler, 1952) were also performed. Moreover, incubations in the presence of 50 mM NAcSpd or NAcSpm were performed on cryostat sections pre-fixed with 2.5% glutaraldehyde in 100 mM Na-cacodylate buffer, pH 7.4, for 5 min at room temperature (RT). After incubation, the electron-dense water-insoluble reaction product, cerium perhydroxide, was visualized in a second-step incubation with a medium containing 1.4 mM 3,3’-diaminobezadine, 100 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}, 42 mM CoCl\textsubscript{2}, and 2.3 μM H\textsubscript{2}O\textsubscript{2} in 50 mM Na-acetate buffer, pH 5.3, for 30 min at RT (Gossrau et al., 1989; Angermüller and Fuhimi, 1988). After rinsing thoroughly in water, the sections were embedded in glycerol jelly.

**Ultrastructural Localization.** For localization of PAOX activity at the ultrastructural level, the semipermeable membrane technique was used (Van den Munckhof et al., 1994a; Van Noorden and Frederiks, 1993; Schellens et al., 1992). Control reactions were performed by incubating sections in the absence of substrate, in the presence of substrate and 0.1–10 mM 1,4-butanediimine-N,N\textsubscript{bis}[2,3-butanedienyl] (MDL 72527/DA-011), a specific inhibitor of PAOX (Bey et al., 1985; Bolkenius et al., 1985), which was a kind gift of Dr. S. Sarhan and Dr. P. Casara of Marion Merrell Dow Research Institute (Strasbourg, France). Control reactions in the presence of substrate and 0.1–25 mM aminoguanidine, a specific inhibitor of DAOX (Schuler, 1952) were also performed. Moreover, incubations in the presence of 50 mM NAcSpd or NAcSpm were performed on cryostat sections pre-fixed with 2.5% glutaraldehyde in 100 mM Na-cacodylate buffer, pH 7.4, for 5 min at room temperature (RT). After incubation, the electron-dense water-insoluble reaction product, cerium perhydroxide, was visualized in a second-step incubation with a medium containing 1.4 mM 3,3’-diaminobezadine, 100 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}, 42 mM CoCl\textsubscript{2}, and 2.3 μM H\textsubscript{2}O\textsubscript{2} in 50 mM Na-acetate buffer, pH 5.3, for 30 min at RT (Gossrau et al., 1989; Angermüller and Fuhimi, 1988). After rinsing thoroughly in water, the sections were embedded in glycerol jelly.

**Figure 1.** Metabolic pathways of polyamines. Specific oxidation of spermidine and spermine takes place via an interconversion pathway by PAOX in peroxisomes after acetylation by cytoplasmic SAT (thick arrows). Nonspecific breakdown of spermine and spermidine may also occur via cytoplasmic DAOX (thin arrows). MAOX, monamine oxidase; DAOX, diamine oxidase; PAOX, polyamine oxidase; CDC, ornithine decarboxylase; OTC, ornithine transcarbamoylase; SAT, spermidine/spermine N\textsuperscript{1}-acyltransferase; Spd-S, spermidine synthase; Spm-S, spermine synthase.
The incubation medium consisted of 300 mM Tris-maleate buffer, pH 8.5, 30 mM CeCl$_3$, 100 mM NaN$_3$ (Van den Munckhof et al., 1994a; Frederiks and Marx, 1993; Schellens et al., 1992; Angermüller and Fahimi, 1988), 5 mM NAcSpd or NAcSpm, and 1% agar noble. Cryostat sections (8 μm thick) were mounted on the semipermeable membrane and incubated for 90 min at 37°C. The specificity of the reactions was investigated by performing incubations in the absence of substrate. After incubation, the gelled incubation media were removed and the inner side of the membrane was cleaned. The piece of semipermeable membrane containing the incubated section was cut out and fixed immediately in a mixture of 1% glutaraldehyde and 4% formaldehyde in 100 mM Na-cacodylate buffer, pH 7.4, for 60 min at RT. Subsequently, sections were post-fixed for 60 min in 1% OsO$_4$ in 100 mM Na-cacodylate buffer, pH 7.4, at 4°C, dehydrated according to standard procedures for electron microscopy, and embedded in LX-112 epoxy resin. Ultra-thin sections were cut on a LKB Ultrotome III, collected on copper grids carrying a Formvar film, and investigated with a Zeiss EM-10c transmission electron microscope. Both unstained ultra-thin sections and sections stained with uranyl acetate and lead citrate were investigated.

Results

Substrate Specificity

The highest amounts of final reaction product were generated when unfixed cryostat sections of rat liver, kidney, and duodenum were incubated for 90 min at 37°C in the presence of 5 mM NAcSpd or NAcSpm (Figures 2D, 2E, 3B, and 3D). Concentrations of sub-

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Figure 2. Light microscopic photographs of PAOX activity as demonstrated in unfixed cryostat sections of rat liver. Incubations were performed (A) in the absence of substrate and in the presence of (B) Spd, (C) Spm, (D) NAcSpd, and (E) NAcSpm. Granular final reaction product is present only after incubations with acetylated polyamines (NAcSpd or NAcSpm). Bars: A-D = 50 μm; E = 25 μm.
substrate higher than 5 mM did not give rise to higher amounts of final reaction product. Final reaction product was exclusively present in a granular form. In liver, the granules were heterogeneously distributed in hepatocytes (Figures 2D and 2E). Some hepatocytes contained hardly any precipitate, whereas various amounts of granules were found in others. No distinct differences were observed between hepatocytes in periportal and pericentral areas. In kidney, epithelial cells of some proximal tubules in the cortex showed large granules at the apical part of epithelial cells. Various epithelial cells did not contain any final reaction product (Figure 1158). Incubations performed in the presence of 1-50 mM Put, Spd, or Spm did not result in formation of any (granular or cytoplasmic) final reaction product (Figures 2B, 2C, 3A, and 3C). Similar results were obtained when incubations were performed in the absence of substrate (Figure 2A). Formation of final reaction product was prevented completely when 0.5 mM MDL 72527, a specific PAOX inhibitor, was added to NAcSpd- or NAcSpm-containing incubation media (data not shown). Addition of up to 25 mM aminoguanidine, a specific inhibitor of DAOX, to substrate-containing media did not affect the amount of final reaction product generated. Pre-fixation of cryostat sections completely prevented formation of final reaction product in all tissues investigated.

Figure 3. Light microscopic photographs of PAOX activity as demonstrated in unfixed cryostat sections of (A, B) rat kidney and (C, D) duodenum. Incubations were performed in the presence of Spd (A, C) and NAcSpd (B, D). Only acetylated Spd (NAcSpd) gives rise to a granular final reaction product (arrows) in rat kidney and duodenum. lpr, lamina propria; muc, mucus; PMN, polymorphonuclear leukocyte. Bars = 25 μm.
**Ultrastructural Localization**

Ultrastructural investigation of cryostat sections of different tissues revealed that PAOX activity was present in (micro)peroxisomes (Figures 4 and 5). In liver parenchymal cells, electron-dense reaction product was present in the matrix of peroxisomes, whereas crystalline cores were free of electron-dense precipitate (Figures 4B and 4C). Different peroxisomes showed various amounts of final reaction product (Figures 4B and 4C). In kidney, final reaction product was distributed homogeneously throughout the peroxisomal matrix in epithelial cells of proximal tubules (Figures 5A and 5B). However, various amounts of reaction product were present in different peroxisomes (Figures 5A and 5B). Microperoxisomes in epithelial cells of duodenal villi showed electron-dense precipitate (Figures 5C and 5D). Reaction product was mainly present at the periphery of microperoxisomes, whereas the centers of the organelles contained less precipitate (Figures 5C and 5D). In all tissues investigated, crystalline deposits were found exclusively in peroxisomes. Other organelles, as well as the cytoplasmic matrix, were completely free of reaction product. Incubations performed in the absence of substrate did not give rise to any electron-dense precipitate in the tissues investigated.

**Discussion**

In this study we have shown that specific NAcSpd- and NAcSpm-dependent PAOX activity is localized solely in peroxisomes of liver, kidney, and duodenum when unfixed cryostat sections are used. The specificity of the reaction was proven by the absence of final reaction product after incubation in the absence of substrate or in the presence of substrate plus a specific PAOX inhibitor. These results are fully in agreement with biochemical findings that NAcSpd- and NAcSpm-oxidizing PAOX is present only in peroxisomes (Fujiwara, 1994; Bey et al., 1985; Bolkenius et al., 1985; Morgan, 1985; Bolkenius and Seiler, 1981; Heby, 1981). Incubations in the presence of up to 50 mM Put, Spd, or Spm did not lead to any formation of final reaction product in granules or cytoplasm, indicating that peroxisomal PAOX specifically oxidizes \( \text{N}^1-\text{acetyl} \)lated polyamines. It shows that peroxisomal PAOX has a very low specificity for Spd and Spm, whereas conversion into NAcSpd and NAcSpm by cytoplasmic SAT leads to increased specificity (Pegg, 1986). Our findings that (a) formation of granular reaction product is prevented completely by addition of a specific PAOX inhibitor (MDL 72527) but not by the DAOX inhibitor aminoguanidine, and (b) PAOX is localized exclusively in peroxisomes, are in con-
Figure 5. Electron micrographs of PAOX activity as demonstrated in unfixed cryostat sections of (A,B) rat kidney and (C,D) duodenum after incubation in the presence of NAcSpm. In kidney, electron-dense final reaction product is homogeneously distributed within peroxisomes (per), but the amount of final reaction product varies strongly in different peroxisomes (A,B). In duodenum, final reaction product is mainly present at the periphery of microperoxisomes (mper) (C,D). mit, mitochondrion. Sections were counterstained with lead citrate and uranyl acetate. Bars: A = 1 μm; B = 200 nm; C = 2 μm; D = 500 nm.
trast with the following data. Beard and co-workers (1985) were able to localize PAOX activity in peroxisomes using Spd as substrate, cytoplasmic DAOX appears to oxidize both acetylated and non-acetylated polyamines (Seiler et al., 1985). DAOX and PAOX are one and the same enzyme (Nakos and Gossrau, 1994), and Spd and Spm are oxidized in the cytoplasm by PAOX (Nakos and Gossrau, 1994). These contradictions between biochemical data and our data on the one hand and histochemical data obtained by others on the other hand may be explained by the fixation procedures used by the latter authors. Since aldehydes exert effects on polyamines (Fujiwara, 1994) and polyamine oxidizing enzymes that are poorly understood (Holtta, 1975), formation of final reaction product might have been affected by chemical fixation. Our attempts to localize PAOX activity in pre-fixed sections using a fixative according to Beard and co-workers (1985) completely prevented formation of final reaction product, which means that the enzyme is very sensitive to aldehyde fixation. For this reason, we have avoided chemical fixation before enzyme incubations but we have ensured precise localization by using a technique that combines the use of PVA with the cerium salt capture method. This procedure has shown to be valid for localization of peroxisomal and cytoplasmic matrix-bound oxidase activities in unfixed cryostat sections at the light microscopic level (Frederiks et al., 1994; Frederiks and Marx, 1993; Frederiks et al., 1993; Patel et al., 1991). Ultrastructural localization of activity of peroxisomal oxidases in unfixed cryostat sections is possible when using the semipermeable membrane technique (Van den Munckhof, 1994a,b; Schellens et al., 1992).

PAOX activity is heterogeneously distributed over (micro)peroxisomes within cells in various tissues (Figures 4 and 5). This heterogeneity has also been demonstrated for other peroxisomal enzyme activities such as for catalase (Veenhuis and Wendelaar Bonga, 1979; Fahimi et al., 1976; Roels et al., 1975; Herzog and Fahimi, 1974), D-amino acid oxidase (Schellens et al., 1992), L-α-hydroxy acid oxidase (Veenhuis and Wendelaar Bonga, 1979; Hand, 1975), and urate oxidase (Van den Munckhof et al., 1994a; Veenhuis and Wendelaar Bonga, 1979; Graham and Karnovsky, 1965). Moreover, cells within one tissue compartment also show distinct differences in PAOX activity (Figures 2 and 3). This heterogeneity was stronger for PAOX than for any other peroxisomal oxidase investigated thus far.

From our results we conclude that physiological breakdown of polyamines occurs exclusively by peroxisomal PAOX. Specific degradation of Spm and Spd occurs via an interconversion pathway in which the polyamines are N1-acetylated first before they are oxidized by peroxisomal PAOX into Spd and Put, respectively (Figure 1). Acetylation of polyamines enhances strongly the oxidation of polyamines by peroxisomal PAOX. Although cytoplasmic DAOX has been described to oxidize polyamines as well (Nakos and Gossrau, 1994; Robinson et al., 1991; Seiler et al., 1985) to form various aldehydes (Figure 1), either the specificity of the enzyme for (non-)acetylated polyamines or its activity is too low to substantiate an important role for DAOX in physiological polyamine turnover. Therefore, terminal degradation of acetylated and non-acetylated polyamines by cytoplasmic DAOX may be only of secondary importance for the regulation of intracellular polyamine levels. To our knowledge, this study is the first histochemical study that is in agreement with extensive biochemical literature (Pegg, 1986; Bey et al., 1985; Bolkenius et al., 1985; Morgan, 1985; Seiler et al., 1985; Tabor and Tabor, 1984; Bolkenius and Seiler, 1981; Heby, 1981), probably because we have used unfixed biological material.

Acknowledgments

We thank Dr S. Sarba and Dr P. Casara of Marion Merrell Dow Research Institute, Strasbourg, France, for kindly supplying the PAOX inhibitor MDL 072327/DA-011. We also thank Dr J.P.M. Schellens for simulating suggestions and editorial criticism. The technical assistance of Ms H. Vreeeling-Sindefelrose and the photographic work by Mr C.E. Grauwemeijer and Mr J. Peeters are gratefully acknowledged.

Literature Cited


Fahimi HD, Gray BA, Herzog V (1976): Cytochemical localization of catalase and peroxidase in sinusoidal cells of rat liver. Lab Invest 34:192


Holtta, 1975

Holtta, 1994

Holtta, 1975

Heby O (1981): Role of polyamines in the control of cell proliferation and differentiation. Differentiation 39:1


Jänne J, Morris DR (1984): Inhibition of S-adenosylmethionine decarboxylase and diamine oxidase activities by analogues of methylglyoxal bis(guanylhydrazone) and their cellular uptake during lymphocyte activation. Biochem J 218:947


Pegg AE (1986): Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234:249


Schuler W (1952): Zur Hemmung der Diaminoxidase (Histaminase). Experientia 8:230


Veenhuis M, Wendelaar Bonga SE (1979): Cytocchemical localization of catalase and several hydrogen peroxide-producing oxidases in the nucleoids and matrix of rat liver peroxisomes. Histochem J 11:561