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SEPARATION OF TWO ISOZYMES OF POLYAMINE OXIDASE FROM MURINE L1210 LEUKEMIA CELLS

Paul R. Libby and Carl W. Porter

Grace Cancer Drug Center,
Roswell Park Memorial Institute,
666 Elm Street, Buffalo, New York 14263

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Polyamine oxidase from L1210 cells has been separated into two isozymes, A and B on the basis of chromatography on hydroxyapatite. The two isozymes also show different elution characteristics from MGBG Sepharose and Sephacryl-300. The molecular weights of A and B isozymes are estimated to be 260,000 and 200,000 daltons, respectively. The two isozymes show different kinetic characteristics with spermidine, spermine, N^1-acetylspermine, and N^1,N^{12}diacetylspermine.

Polyamine oxidase is the intracellular enzyme which converts spermine to spermidine plus aminopropanal, and spermidine to putrescine plus aminopropanal. In the case of N^1-acetyl derivatives of spermidine or spermine, the products are putrescine or spermidine, respectively, plus acetamidopropanal. PAO has been purified to homogeneity from rat liver peroxisomes and characterized by Höllta (1). In addition, Bolkenius and Seiler (2) have partially purified and characterized the enzyme from rat liver cytosol. As part of an on-going study of the relationship of polyamine metabolism to cell prolifera-

Abbreviations: MGBG, methylglyoxal (bisguanylhydrazone); PAO, polyamine oxidase; HEPES, N-2-Hydroxyethylpiperazine-N^1-2-ethanesulfonic acid; DTT, dithiothreitol.

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tion in murine L1210 cells, we have examined the properties of PAO from this cell line. During the course of this examination, it was noted that the enzyme was present in two forms, separable by chromatography on hydroxyapatite. This report presents evidence for the existence of two isozymes of PAO.

Materials and Methods

[^{3}H]-Spermidine and [^{3}H]-Spermine were purchased from New England Nuclear Corp. N^1-Acetylspermine and N^1,N^{12}-diacetylspermine were synthesized by the method of Tabor et al. (3). MGBG-Sepharose was synthesized by the method of Markham et al. (4). Turkey trypsin inhibitor and leupeptin were purchased from Sigma.

Cells. L1210 cells were grown i.p. in the ascites fluid of DBA mice, harvested, washed with 0.85% NH_4Cl, 0.17 M Tris, pH 7.4, to lyse erythrocytes, and stored at -70°C in PBS. Cells were thawed, suspended in buffer A (5 mM HEPES, pH 7.2, 1 mM DTT), and lysed by three freeze-thaw cycles. The supernatant from a centrifugation at 35000 rpm was used as enzyme source.

Enzyme assay: Enzyme extract was incubated with 5 μmoles bicine buffer, pH 9.2, 0.1 μg FAD, 30 nmoles DTT, and 0.1 nmole N^1, N^{12}-diacetylspermine in a final volume of 0.05 ml. The incubation was at 37° for 10 min with continuous shaking. The reaction was stopped by chilling and the addition of 0.05 ml 0.2N HCl. 0.08 mL of the mixture was added to a 0.5-0.6 ml column of Dowex-50-X4-H^+. Acetamidopropanal (or aminopropanal, when spermine or spermidine were substrates) was washed through the column with 1.5 ml 1 N HCl and counted. When spermine or spermidine were substrates, DTT was increased to 0.3 μmoles and benzaldehyde was added to a final concentration of 30 mM or 5 mM, respectively.

Results

L1210 PAO has been partially purified by successive chromatographies on DEAE Sephacel and hydroxyapatite. In early experiments it was noted that approximately 50% activity was lost during the DEAE Sephacel chromatography. Acting on a suggestion by Dr. E. Höllta, the column was washed with a dilute

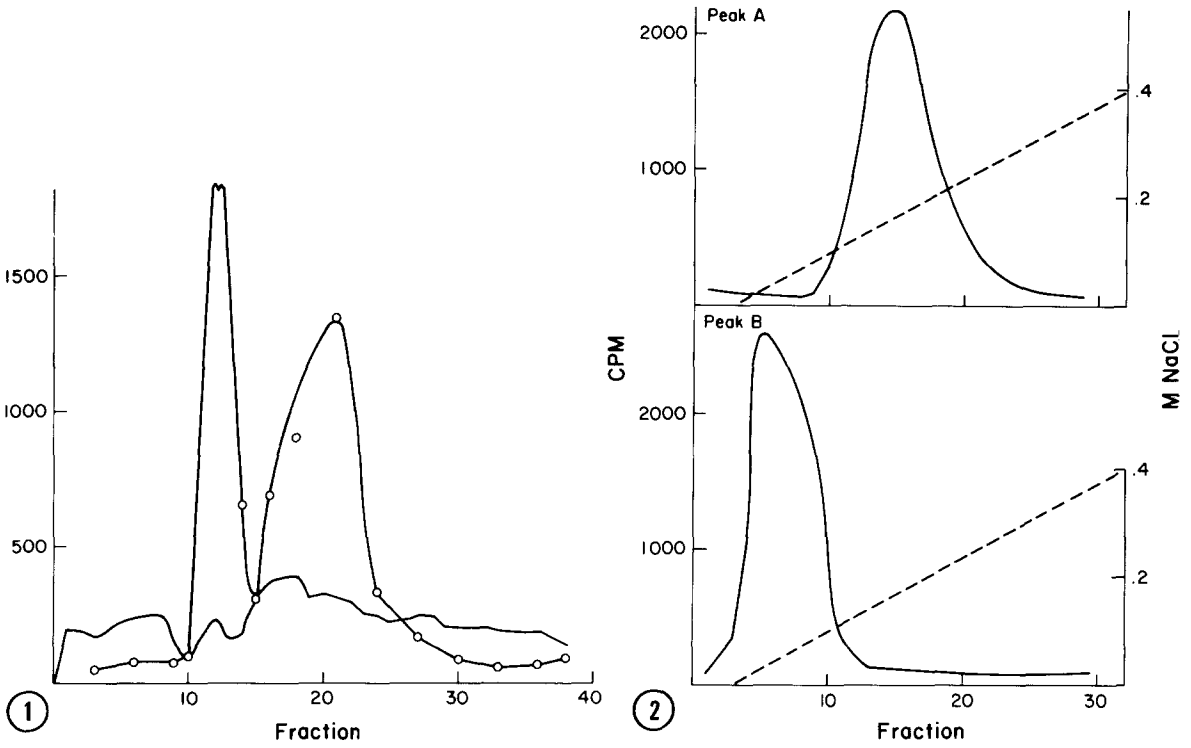


Figure 1. Separation of PAO-A and PAO-B on hydroxyapatite. _____, Protein concentration; o—o, PAO activity.

Figure 2. Chromatography of PAO-A and PAO-B on MGBG-Sepharose. _____, PAO activity; ----, [NaCl].

solution of Non-idet P40 before the cytosolic extract was applied. Under these conditions we found full recovery of PAO at the DEAE Sephacel step. When the pool of material from DEAE was applied to a hydroxyapatite column, and eluted with a gradient of potassium phosphate, two separate peaks (A and B) of PAO activity were observed. Figure 1 shows the results of one such chromatography. Each peak was separately chromatographed on a column of MGBG Sepharose (Figure 2). Isozyme PAO-A was eluted at ca 0.07 M NaCl, and PAO-B was eluted at 0.13 M NaCl. Table 1 summarizes the purification in this particular experiment. PAO was purified 300-fold over the original cytosol and PAO was purified 22-fold.

In order to rule out the possibility that PAO-B was a proteolytic degradation product of PAO-A, an additional preparation was carried out as

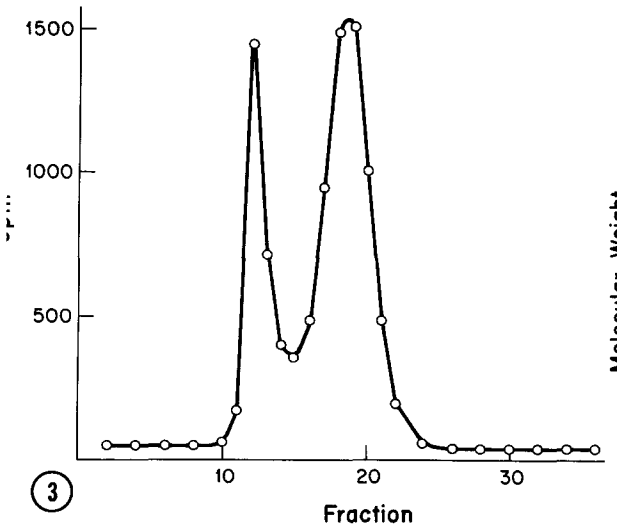
Table 1. Purification of PAO from L1210 Cells

Fraction	Protein (mg)	Activity (pm/min)	Specific Activity (pm/min/mg)	Purification
Cytosol	287.0	27,830	97	1.0
DEAE Pool	41.0	32,140	791	8.2
HTP Pool A	0.98	10,123	10,380	107.0
MGBG Pool A	0.38	11,162	29,750	308.0
HTP Pool B	11.4	20,460	1,789	18.5
MGBG Pool B	6.5	13,730	2,135	22.1

described above with the following modifications: cells were suspended in buffer A which contain 10 μg/ml of turkey trypsin inhibitor and 2 μM leupeptin for lysis by freeze-thaw. In addition, the DEAE Sephacel and hydroxyapatite columns were pre-washed with buffers containing 2 μM leupeptin and elution buffers for both columns contained 2 μM leupeptin. Figure 3 shows the results of the hydroxyapatite chromatography, and it is apparent that the inclusion of protease inhibitors during cell lysis and the subsequent chromatographic procedures has no effect on the separation of the two isozymes.

Further evidence for the presence of two separate isozymes was obtained by chromatography on Sephacryl S-300. Figure 4 shows the plot of Ve/Vo for the two isozymes and several marker proteins. We estimate PAO-A to have a molecular weight of 260,000 and PAO-B, 200,000.

In Table 2 we have summarized the kinetic data for the two isozymes. The K_m values for spermine and its derivatives are quite low for isozyme A; the values being 15.1, 4.7, and 5.1 μM for spermine, N^1-acetylspermine, and N^1,N^{12}-diacetylspermine, respectively. N^1-acetylspermidine also has a rather low K_m value with PAO-A, 35 μM but spermidine is a poor substrate, with a K_m value of 106 μM. PAO-B also shows low K_m values for the acetyl derivatives, with values of 8, 14, and 31.5 for N^1-acetyl spermine,



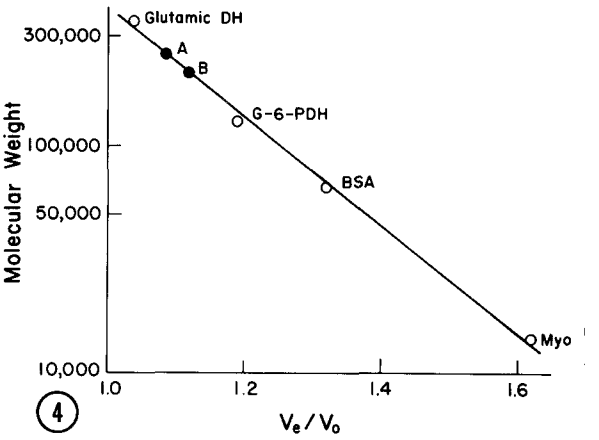


Figure 3. Chromatography of protease inhibitor treated PAO-A and PAO-B on hydroxyapatite in the presence of leupeptin.

Figure 4. Chromatography of PAO-A and PAO-B on Sephacryl S-300. V_e, elution volume; V_o, void volume; Glutamic DH, bovine glutamic dehydrogenase; G-6PDH, yeast glucose-6-phosphate dehydrogenase; BSA, bovine serum albumin; Myo, horse myoglobin.

N^1,N^{12}-diacetylspermine, and N^1-acetyl- spermidine, respectively. The K_m values for spermine and spermidine are 66 and 124 μM, respectively. It may be seen from Table 2 that the Michaelis constants for the two isozymes with various substrates are quite similar, with the exception of the K_m's

Table 2. Kinetic Values for L1210 PAO Isozymes

Substrate	K_m (μ M) PAO-A	PAO-B
Diacetylspermine	5.1	8.0
Acetylspermine	4.7	14.4
Spermine	15.1	66.3
Acetylspermidine	35.2	31.5
Spermidine	106	124

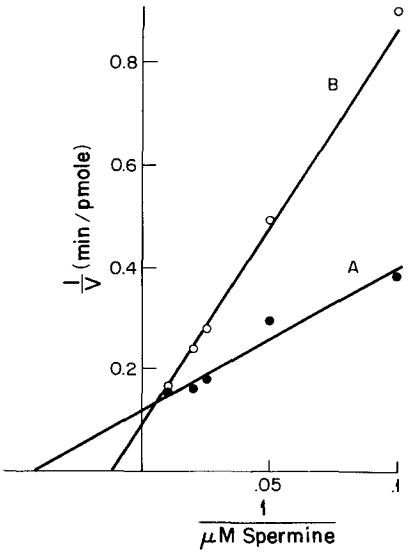


Figure 5. Lineweaver-Burke determination of K_m values for spermine. A, PAO-A; B, PAO-B.

for spermine. Figure 5 shows the Lineweaver-Burke plot for spermine with isozymes A and B.

Discussion

The chromatographic findings reported here provide evidence for the existence of two isozymes of polyamine oxidase in mouse L1210 leukemia cells. While the two isozymes co-elute from DEAE Sephacel, they are separable by chromatography on hydroxyapatite; PAO-A elutes at ca. 0.03 M KPi and PAO-B elutes at ca. 0.1 M KPi (Figure 1). Furthermore, the two isozymes show different elution patterns from MGBG-Sepharose and Sephacryl-300 as shown in Figures 3 and 4, respectively. In addition, the same pattern is found when protease inhibitors are included during purification suggesting that PAO-B is probably not a proteolytic fragment of PAO-A. The results reported here are in agreement with recent findings on PAO of rat liver. We have recently found that rat liver peroxisomal PAO and rat liver cytosolic PAO may be different isozymes. We have shown that rat liver cytosolic PAO is eluted from hydroxyapatite at a lower concentration of phosphate eluant than is the rat

liver peroxisomal PAO (5). By analogy, then, the L1210 PAO-A may be the cytosolic form and the PAO-B may be the peroxisomal form, but as yet we have no direct evidence for this assignment.

Apparently PAO-B is appreciably more hydrophobic than is PAO-A since it is strongly bound to DEAE Sephacel in the absence of non-ionic detergents. However, if the column has been pre-washed with Non-idet P40, PAO-B is eluted by the NaCl wash along with PAO-A.

The kinetic properties of the two enzymes differ significantly from each other and from the rat liver peroxisomal enzyme reported by Hölltä. In the presence of optimal levels of benzaldehyde, the K_m for spermine is 15 μM with isozyme A and 66 μM with isozyme B, as compared to 5 μM with rat liver peroxizomal enzyme (1) Spermidine is a much less effective substrate for the L1210 isozymes, since the K_m values are above 100 μM. By contrast, the rat liver enzyme has a K_m for spermidine of 15 μM in the presence of benzaldehyde (1).

Bolkenius and Seiler (2) have also reported on the kinetics of rat liver polyamine oxidase, using a partially purified cytosolic enzyme preparation. With this preparation, the K_m values for N^1-acetylspermine and N^1,-N^{12}-diacetylspermine are 0.6 and 5 μM, respectively, whereas the K_m values for the two acetylated derivatives are 4.7 and 5.1 μM, respectively, for PAO-A and 14.2 and 8.0 for PAO-B. Suzuki *et al.* (6) have examined the distribution of PAO in human tissues. They have reported that PAO in human liver homogenates has a K_m for N^1-acetylspermine of less than 1.5 μM. Bolkenius and Seiler concluded, on the basis of K_m and V_m values, that acetylated polyamines were the preferred substrates for PAO in rat liver. Recently Shinki *et al.* (7) have demonstrated that spermidine, rather than ornithine, is the major precursor of the increased levels of putrescine found in chick duodenum after treatment with 1α, 25 $(OH)_2$ D_3, and they attribute this finding to the rapid induction of spermidine N^1-acetyltransferase in this tissue.

Our kinetic data, in agreement with the above workers, also suggest that the preferential substrate for PAO, in the production of putrescine from spermidine, is the N^1-acetyl derivative of spermidine. However, the production of spermidine from spermine may not involve acetylspermine since the K_m values for the two substrates, spermine and N^1-acetylspermine are very close to one another with PAO isozyme A. It must also be considered that spermine is present in L1210 cells in millimolar concentrations (8) whereas N^1-acetylspermine has never been detected in these cells (unpublished data). Thus we may conclude, on the basis of kinetic values, that N-acetylation may be of importance is the oxidation of spermidine to putrescine in L1210 cells, but it is less likely to be of importance in the oxidation of spermine to spermidine.

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