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Polyamine-Stimulated Binding of Diamine Oxidase to DNA

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Diamine oxidase is a Cu-containing enzyme which intracellularly participates in the regulation of the levels of putrescine, spermidine and spermine and in this process produces growth inhibitory amino aldehydes and hydrogen peroxide. Extracellularly, the enzyme participates in the inactivation of biogenic amines, notably histamine.

Here we present evidence that in the presence of polyamines, diamine oxidase has the ability to bind DNA and to oxidise DNA-bound polyamines. The enzyme associates with chromosomal DNA since it can be released from human placental DNA by treatment with DNase I and it may be involved in the degradation of DNA. Thus, diamine oxidase may belong to a new class of DNA-binding proteins.

Polyamines are essential to the regulation of cell proliferation and cell differentiation.[1–5] The polyamines putrescine, spermidine and spermine have been shown to influence all levels of nucleic acid function, including replication, transcription and translation.[6–8] Low levels of intracellular polyamines therefore decrease the proliferation of cells and high levels of intracellular polyamines may stimulate cells to a state of hyperproliferation.[9–12]

Cu-containing amine oxidases are a class of enzymes (EC 1.4.3.6) that participate intracellularly in the regulation of polyamine levels through oxidation of putrescine, spermidine and spermine, and extracellularly in the control of the action of biogenic amines through oxidation of these, notably histamine.[13–22]

Since polyamines interact with DNA we decided to investigate whether diamine oxidase (DAO) could use DNA-bound polyamines as substrates, and in this article we show that DAO has the ability to bind single-stranded (ss) DNA in the presence of polyamines and to oxidise DNA-bound polyamines. Moreover, DAO can be liberated from human placental chromosomal DNA by treatment with DNAse I. This property of DAO may point to a role for the enzyme in apoptosis and regulation of growth and differentiation of cells.

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Materials and methods

Chemicals. Disodium hydrogen phosphate, sodium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, ammonium sulfate, calcium lactate, Tween 20, sodium azide, boric acid and 5,5-diethylbarbituric acid were from Merck (Darmstadt, Germany). Divinylsulfonyl-agarose (Minileak), *o*-phenylenediamine substrate tablets (4 mg) and horseradish peroxidase were from Kem-En-Tec (Copenhagen, Denmark). HSA-agarose was from Litex (Glostrup, Denmark). Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Nylon membranes (MAGNA) were from MSI (Massachusetts, USA). Tris, EDTA, putrescine, spermidine, spermine, histamine, amiloride, octylamine, glycerol, glycine, octyl glycopyranoside, human placental chromosomal DNA, calf thymus chromosomal DNA, alkaline phosphatase substrate tablets (10 mg), alkaline phosphatase-conjugated goat immunoglobulins against mouse IgG, protease XXV, RNAse A, DNAse I, tRNA from bovine liver and total RNA from bovine liver were from Sigma (St. Louis, USA). Luminol solution (enhanced chemiluminescence detection reagent 2) and enhanced chemiluminescence hyper-film were from Amersham (Buckinghamshire, UK). Developer (LX 24, X-ray) and fixer (AL 4, X-ray) were from Kodak (New Haven, CT, USA). Eco RI, Hinf I, Pst I, Rsa I and NEBuffer 4 were from BioLabs (Beverly, MA, USA). Bromphenol blue was from Fluka (Buchs, Switzerland). Skimmed milk (powder) was from

Irma (Rødovre, Denmark). NA-agarose and ultrapure dNTP set were from Pharmacia Biotech (Uppsala, Sweden). Ethanol was from Danisco (Aalborg, Denmark). Oligonucleotide primers (PU5, PD9 and PD10) used for PCR reactions were from Clontech (Palo Alto, USA). AmpliTaq DNA polymerase and buffer for the PCR reaction were from Perkin Elmer (Branchburg, USA).

Monoclonal antibody (mAb) against DAO. mAb 40-2 recognising enzymatically active DAO was produced as described.[23]

Synthesis of aminohexyl (divinylsulfonyl) agarose. Divinylsulfonyl agarose (50 ml) was washed twice with 10 mM sodium phosphate, pH 7.2, and once with 0.1 M sodium carbonate, pH 9.0. Then washed divinylsulfonyl agarose (50 ml) was mixed overnight with 50 ml 0.1 M diaminohexane in 0.1 M sodium carbonate, pH 9.0. The mixture was then washed three times with 10 mM sodium phosphate, pH 7.2 before it was poured into a 50 ml column. The washing steps above included suspension in the washing agent and then centrifugation for 15 min at 425*g*.

Purification of DAO. DAO was purified from human placenta by affinity chromatography on aminohexyl (divinylsulfonyl) agarose. A placenta was homogenised with 500 ml 0.1 M sodium phosphate, pH 7.2 and then centrifuged for 30 min at 11 000*g*. The supernatant was then stirred with 400 $g\,l^{-1}$ ammonium sulfate overnight at 4 °C. The resulting precipitate was isolated by 20 min of centrifugation at 12 500*g* and then resuspended in 200 ml water. The suspension was dialysed three times against 2 l 5 mM sodium phosphate, pH 7.2 at 4 °C. Finally the dialysed suspension was passed through a 50 ml aminohexyl (divinylsulfonyl) agarose column, and after the column had been washed with 5 mM sodium phosphate until A_{280} of the effluent was less than 0.01, bound protein was eluted with 10 mM octylamine in 50 mM sodium phosphate, pH 7.2. The eluted fractions were pooled and dialysed as described above.

Agarose gel electrophoresis of proteins. A 1% agarose solution in 50 mM sodium phosphate, pH 7.2, was prepared and heated until the agarose had dissolved. 1% Agarose (30 ml) was used to cast a 10 × 20 cm agarose gel. After 15 min at room temperature and another 30 min at 4 °C the agarose gel was loaded with 2–10 µl samples 1 cm below the middle of the gel, and electrophoresis was performed at 6 $V\,cm^{-1}$ for 2 h. 50 mM Sodium phosphate, pH 7.2 was used as running buffer.

Blotting of agarose gels to nitrocellulose membranes and dotblots. After electrophoresis (80 min) the gel was blotted to a 0.45 µm pore size nitrocellulose membrane. This was performed by placing the membrane, five pieces of Whatman No. 1 paper and 20 pieces of cell-paper on the agarose gel and then applying gravitational pressure for 60 min. The membrane was equilibrated for 5 min in 5 mM sodium phosphate, pH 7.2, and placed for 5 min between two filter papers to remove excess fluid. Dotblots were made by applying 3 µl samples to a 0.45 µm pore size nitrocellulose membrane which was then equilibrated and treated in the same way as above.

Enzyme detection in situ *by enhanced chemiluminescence and DAO detection.* The substrate solution (4 ml) was prepared by mixing 2 ml 5 mM sodium phosphate, pH 7.2, 2 ml luminol solution (enhanced chemiluminescence detection reagent 2), 6.44 mg putrescine (10 mM), and 4 µl 10 $mg\,ml^{-1}$ horseradish peroxidase. The substrate solution was applied to a 10 × 20 cm filter paper placed in a plastic folder and the nitrocellulose membrane was layered on top of the filter paper. The plastic folder was then placed in a film cassette with an enhanced chemiluminescence hyper-film on top and incubated overnight at room temperature. Next morning the film was developed and fixed.

PCR amplification of p53 *cDNA.* Location in human *p53* of:

primer PU5: 13042-13065.
Sequence:
5′-CTCTTCCTGCAGTACTCCCCTGC-3′

primer PD9: 14769-14746.
Sequence:
5′-CCCAAGACTTAGTACCTGAAGGGTG-3′

primer PD10: 17690-17668.
Sequence:
5′-GAGGTCACTCACCTGGAGTGAGC-3′

primer EX4F: 12015-12036.
Sequence:
5′-CTACAGTCCCCCTTGCCGTCCC-3′

The PCR reactions were carried out in a total volume of 20 µl containing template DNA (50 ng), 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatine, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP), 0.4 mM of each primer (EX4F and PD10) and 1 unit of AmpliTaq DNA polymerase. The amplification conditions were 95 °C for 30 s, 55 °C for 2 min and 72 °C for 90 s for a total of 35 cycles. The last cycle was followed by a 7 min extension at 72 °C.

One µl of the above PCR reaction was used in a nested PCR reaction with a new primer set (PU5/PD9). All other conditions were the same as in the first PCR reaction.

A fragment of 1704 bp was visualised on an agarose gel (0.5%), and this DNA fragment was purified from the gel with the Prep-a-Gene kit following the manufacturers instructions (BioRad, Hercules, USA). The purified (5 µl) DNA was run on an agarose gel for quality control.

Eco RI cleavage of human placental DNA. Human chromosomal placental DNA (5 mg) was dissolved in 4.35 ml water and 0.5 ml NEBuffer 4 (×10). The digestion was started by the addition of 150 μl *Eco* RI (20 000 U/ml^{-1}) followed by 24 h incubation at 37 °C. To the digested DNA was then added 3 M sodium acetate (500 μl) and 96% ethanol (15 ml) and the DNA was precipitated overnight at −20 °C. After 30 min centrifugation at 15 000*g* (4 °C) the pellet was washed in 80% ethanol and then in 96% ethanol. The pellet was air-dried and resuspended in 1 ml 5 mM sodium phosphate, pH 7.2.

Small scale restriction enzyme digestion of human placental DNA. Human chromosomal placental ds DNA (70 μl, 1 mg ml^{-1}) was mixed with 10 μl 10× NEBuffer 4, water and 60 Units of *Eco* RI, *Hinf* I, *Pst* I or *Rsa* I in a total volume of 100 μl. The contents of the tubes were briefly mixed and centrifuged. After incubation overnight at 37 °C the digestions were analysed by agarose gel electrophoresis. The samples were then dotted and used for assay of DNA-bound DAO activity.

DNAse, RNAse and protease treatment of DNA. Human chromosomal placental ds DNA or calf thymus chromosomal ds DNA (10 μl, 1 mg ml^{-1}) was mixed with 10 μl DNAse I, RNAse A or protease XXV (2 μg ml^{-1}) in 20 mM Tris, 0.15 M NaCl, and 3 mM $MgCl_2$, pH 7.5. The tubes were briefly agitated and centrifuged. After incubation overnight at 25 °C the samples were stored at −20 °C and then used in dotblots either directly or after denaturation.

Release of DAO from human placental DNA by treatment with DNAse I. A tissue sample from human placenta was homogenised and extracted sequentially with 1 volume of 50 mM sodium phosphate, pH 7.2, 50 mM sodium phosphate, pH 7.2, 1% Triton X-114 and 50 mM sodium phosphate, pH 7.2, 1% Triton X-114, 1 M NaCl. The resulting precipitate was then washed three times with 20 mM Tris, 0.15 M NaCl, and 3 mM $MgCl_2$, pH 7.5, and incubated with 0–100 μg ml^{-1} DNAse I in the same buffer for 2 or 18 h. The samples were then centrifuged and the supernatants analysed by agarose gel electrophoresis, blotted to a nitrocellulose membrane and analysed for DAO content with mAbs. The immuno-stained bands were scanned and quantified as described below (scanning of dotblots and bands).

Denaturation of double-stranded (ds) DNA to ss DNA. DNA dissolved in 5 mM sodium phosphate, pH 7.2, was boiled for 10 min in a waterbath and then quickly cooled on ice for 5 min.

Renaturation of ss DNA to ds DNA. The sample was boiled for 10 min and then slowly cooled over 3 h to room temperature. The sample was stored at −18 °C before use.

Detection of DAO activity bound to immobilised ss DNA. ss DNA (1 mg ml^{-1}) was dotted on a 0.45 μm nitrocellulose membrane and the membrane was blocked for 1 h in 5 mM sodium phosphate, pH 7.2, with 2% skimmed milk. The membrane was washed three times 5 min in 5 mM sodium phosphate, pH 7.2, and incubated 1 h with affinity-purified DAO 1 : 100 in 50 mM sodium phosphate, pH 7.2, or 5 mM sodium phosphate, pH 7.2, with 10 mM polyamine. Bound DAO was then detected by enzyme activity. All incubations other than the blocking step were made in the presence of 0.5% milk powder.

Detection of DAO bound to immobilised tRNA and total RNA. This was performed with the same procedure as for ss DNA but with the use of nylon membranes instead of nitrocellulose membranes because of poor binding of RNA to the latter.

Detection of ss DNA bound to immobilised DAO. DAO was either dotblotted on a nitrocellulose membrane or separated on agarose gel and blotted to a nitrocellulose membrane. The membrane was then blocked for 1 h in 5 mM sodium phosphate, pH 7.2, with 2% skimmed milk and then washed three times over 5 min in 5 mM sodium phosphate, pH 7.2. Then the nitrocellulose membrane was incubated for 1 h with 1.5 μg ml^{-1} ss DNA in 50 mM sodium phosphate, pH 7.2, or 5 mM sodium phosphate, pH 7.2, with 10 mM polyamine and washed three times for 5 min with 10 mM Tris, 0.15 M NaCl, and 0.05% Tween 20, pH 7.5. Finally, bound DNA was detected by 1 h incubation with mAb against ds DNA 1 : 100 in 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20, and 0.5% skimmed milk followed by horseradish peroxidase-conjugated rabbit immunoglobulins against mouse immunoglobulins diluted 1 : 1000 in the same buffer interrupted by a three-times 5 min wash with 10 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20. After another three washes in 10 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20, bound antibodies were visualised by enhanced chemiluminescense.

Analysis of DNA treated with DAO by agarose gel electrophoresis. ss DNA (15 μl, 0.1 mg ml^{-1}) in 5 ml sodium phosphate, pH 7.2, was mixed with 10 μl 3 mM or 3 μM polyamine in 150 mM sodium phosphate, pH 7.2. The degradation was then started by addition of 5 μl affinity-purified DAO (1 mg ml^{-1}) and incubation at 25 °C for 72 h. A 0.7% agarose gel was cast and the samples separated electrophoretically at 65 V for 2 h using a GNA-100 apparatus. 90 mM Tris-borate, 2 mM EDTA, pH 8, was used as running buffer and gel buffer, and 0.5 μg ml^{-1} ethidium bromide was included in the gel and the buffer when visualisation with UV light was desired.

Assay for mAb 40-2 binding (ELISA). A maxisorp plate was coated for 18 h at 4 °C with 50 μl of sample. The

plate was then washed three times for 1 min in 10 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20 and incubated for 1 h with mAb 40-2 (culture supernatant 1 : 25) in 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20, and 1% BSA, pH 7.5 (50 µl per well), and, after another three washes, for 1 h with alkaline phosphatase-conjugated goat immunoglobulins against mouse IgG diluted 1 : 4000 in 50 mM Tris, 0.15 M NaCl, 0.05% Tween 20, and 1% BSA, pH 7.5. Finally, after washing four times for 1 min with 10 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20, alkaline phosphatase substrate solution (1 mg ml^{-1} *p*-nitrophenyl phosphate in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added to the plate (100 µl per well), which was then read on an ELISA reader (SLT, Salzburg, Austria) after approximately 30 min. The reading was done at 405 nm with reference subtraction at 690 nm.

Assay for DAO activity. A 50 µl sample was added to a non-absorbent ELISA plate. Then 50 µL of a substrate solution consisting of 10 mM putrescine, 10 µg ml^{-1} horseradish peroxidase and 1 mg ml^{-1} *o*-phenylenediamine 5 mM sodium phosphate, pH 7.2, were added. After 18 h incubation at 37 °C the reaction was terminated by the addition of 100 µl 1 M sulfuric acid and read on the ELISA-reader at 492 nm with reference subtraction at 690 nm.

Elution of DAO from ss DNA. Human chromosomal placental ss DNA (1 mg ml^{-1}) was dotted (2 µl) on a 0.45 µm nitrocellulose membrane next to dots of affinity purified human placental DAO (2 µl). The nitrocellulose membrane was then blocked with 2% skimmed milk in 5 mM sodium phosphate, pH 7.2 and washed three times for 5 min in 5 mM sodium phosphate, pH 7.2. The nitrocellulose membrane was then incubated with affinity-purified human placental DAO 1 : 100 in 50 mM sodium phosphate, pH 7.2 and then washed three times for 5 min in 5 mM sodium phosphate, pH 7.2. The membrane was cut into strips for each elution assay performed. The elutions were done by washing the membrane strips (each with a dot of DNA and a dot of DAO) for 1 h with different solutions and then finally three times for 5 min with 5 mM sodium phosphate, pH 7.2. Residual DAO bound to DNA and DAO dotted as control were then detected by enzyme activity induced enhanced chemiluminescence.

Scanning of dotblots and bands. Films with activity or immuno-stain detection were photographed on an EagleEye II instrument and converted to TIF-files. TIF-files were then analysed by means of the Mac program NIH-Image v.1.56 using '2D Remove Streaks' background subtraction and circular area reading.

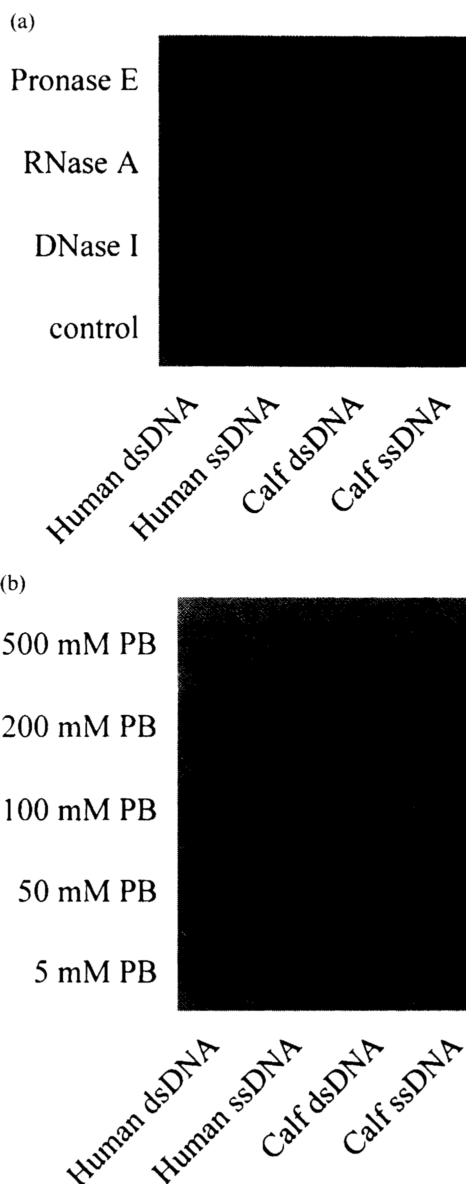

Fig. 1. (a) DNA-bound DAO activity on DNA treated with RNAse, DNAse or protease. 10 µl human chromosomal and calf chromosomal ds DNA and ss DNA (1 mg ml^{-1}) were dotted on a nitrocellulose membrane after 12 h incubation with DNAse I, RNAse A or pronase E (2 µg ml^{-1}) or buffer as control. The DNA dots were then incubated with affinity-purified DAO in the presence of 10 mM putrescine, washed and DNA-bound DAO activity detected by incubation with 10 mM putrescine, 5 mM sodium phosphate, pH 7.2, and horseradish peroxidase (10 µg ml^{-1}) mixed 1 : 1 with luminol solution. (b) Formation of DNA-bound DAO activity on human chromosomal and calf chromosomal DNA at different ionic strengths. Human chromosomal and calf chromosomal ds DNA and ss DNA were dotted on a nitrocellulose membrane and then incubated with DAO in 5–500 mM sodium phosphate pH 7.2. Finally the dots were assayed for DNA-bound DAO activity as in (a).

Results

Fig. 1(a) shows binding of DAO to human and calf chromosomal ss DNA and ds DNA and detection of DNA-bound DAO activity after the DNA had been treated with DNAse I, RNAse A or pronase E. Only the DNAse treatment prevented accumulation of DNA-

bound DAO activity showing that DAO was directly bound to ss DNA and not to RNA or proteins such as histones. Both human and calf ss DNA could bind affinity-purified DAO from human placenta, but the binding to human ss DNA was somewhat stronger than the binding to calf ss DNA. Moreover, weaker binding to ds DNA was also observed. The binding to ss DNA was not diminished when the DNA had been pretreated with one of the following enzymes before denaturation and immobilization: *Eco* RI, *Hinf* I, *Pst* I and *Rsa* I. Weak binding of DAO to tRNA and total RNA from bovine liver was also observed. Amiloride, a compound known to bind and inhibit DAO,[24] effectively prevented the binding of DAO to ss DNA and also inhibited the DNA-bound DAO activity itself.

Fig. 1(b) shows a dotblot where DAO was bound to human and calf ss DNA in 50 mM sodium phosphate pH 7.2, whereas in 500 mM sodium phosphate or in 5 mM sodium phosphate the binding did not take place. Weak binding to ds DNA was seen only in 50 mM sodium phosphate. To investigate the influence of polyamines on the DNA binding of DAO activity, additional experiments were performed. Table 1A represents the results from Fig. 1(b) which showed optimal binding of DAO to DNA in 50 mM sodium phosphate. However, in 5 mM sodium phosphate pH 7.2, prominent binding to ss DNA was seen when putrescine, spermidine, spermine or histamine was added (Table 1B). The four compounds were equally effective in stimulating ss DNA binding of DAO. Other experiments showed that Ca^{2+} ions in the same concentration did not stimulate DNA binding whereas Mg^{2+} and 1,8-diaminooctane had only a minor stimulating effect on DNA binding.

When DAO was bound to ss DNA in 50 mM sodium phosphate, pH 7.2, DNA-bound DAO activity could be detected using polyamines as substrates (Table 1C). Putrescine was a better substrate for DNA-bound DAO than were spermidine, spermine and histamine. An interesting feature of the binding of DAO to DNA was the ability of DNA-bound DAO to oxidise substrates already bound to DNA. This was shown by the fact that DNA-bound DAO activity could be detected without substrate in the detection solution after DAO had been bound to DNA by means of polyamines or by preincubating the dotted DNA with polyamines and then binding DAO in 50 mM sodium phosphate (Table 1D). Even after extensive washing in 5 mM sodium phosphate pH 7.2, polyamines were available for DAO on the DNA. Putrescine and histamine were the best substrates in this respect followed by spermidine and spermine. The only way that DNA-bound DAO activity could be detected without addition of more substrate would be if DAO was capable of moving along the DNA strand, oxidising the polyamines, or if DAO continuously dissociated from and reassociated with the DNA. Prolonged washing in the following buffers: 100 mM sodium phosphate pH 7.2, 10 mM glycine, 10 mM 5,5-diethylbarbituric acid, 10 mM tris, 10 mM EDTA or 1% octylglycopyranoside did not remove DAO from the ss DNA. 500 mM sodium phosphate pH 7.2, 100 mM spermidine and 100 mM spermine were capable of effectively releasing DAO from ss DNA and did not affect the DAO activity itself. Putrescine (100 mM) was not able to release DAO from ss DNA as effectively as spermidine and spermine, and histamine (100 mM) was not able to remove DAO from ss DNA. Tween 20 (1%) released only very small amounts of DAO from ss DNA but inhibited DAO activity itself. Therefore we believe that DAO forms a rather tight complex on DNA which is capable of movement along the DNA strand.

Table 1. The formation and activity of DNA-bound DAO under various conditions. Human and calf chromosomal ds DNA and ss DNA were dotted on a nitrocellulose membrane and incubated with affinity-purified DAO in A) 5–500 mM sodium phosphate, pH 7.2; B, different polyamines (10 mM) in 5 mM sodium phosphate pH 7.2; C, 50 mM sodium phosphate, pH 7.2 and then assayed for DNA-bound DAO activity using different polyamines (10 mM) as substrate; D, different polyamines followed by assay of DNA-bound DAO activity without substrate in the detection solution. DNA-bound DAO activity in A and B was assayed using 10 mM putrescine as the substrate. The DNA-bound DAO activity was detected as in Fig.1 and quantified by videodensitometry ('++++' representing maximum detection, '+' representing lowest detection and '−' no detection).

		Human ds DNA	Human ss DNA	Calf ds DNA	Calf ss DNA
A	500 mM PB	−	−	−	−
	200 mM PB	−	++	−	+
	100 mM PB	−	+++	−	++
	50 mM PB	(+)	++++	(+)	+++
	5 mM PB	−	(+)	−	−
B	Putrescine	+	++++	+	+++
	Spermidine	+	++++	+	+++
	Spermine	+	++++	+	+++
	Histamine	+	++++	+	++
	Control	−	−	−	−
C	Putrescine	−	++++	−	+++
	Spermidine	−	+	−	+
	Spermine	−	+	−	+
	Histamine	−	+	−	−
	Control	−	−	−	−
D	Putrescine	+	++++	+	++++
	Spermidine	−	+++	−	++
	Spermine	−	++	−	+
	Histamine	+	++++	+	++
	Control	−	−	−	−

If DAO activity is accumulated on ss DNA, it should be possible to visualise this by a gelshift assay of the proteins involved. Affinity-purified DAO and human chromosomal ss DNA were incubated for 1 h at 25 °C and then separated by agarose gel electrophoresis, blotted to a nitrocellulose membrane and analysed for DAO activity and binding of mAb 40-2 which recognises enzymatically active DAO [Fig. 2(a)]. In the presence of ss DNA, the enzymatically active protein and the immunoreactivity were detected at the application point

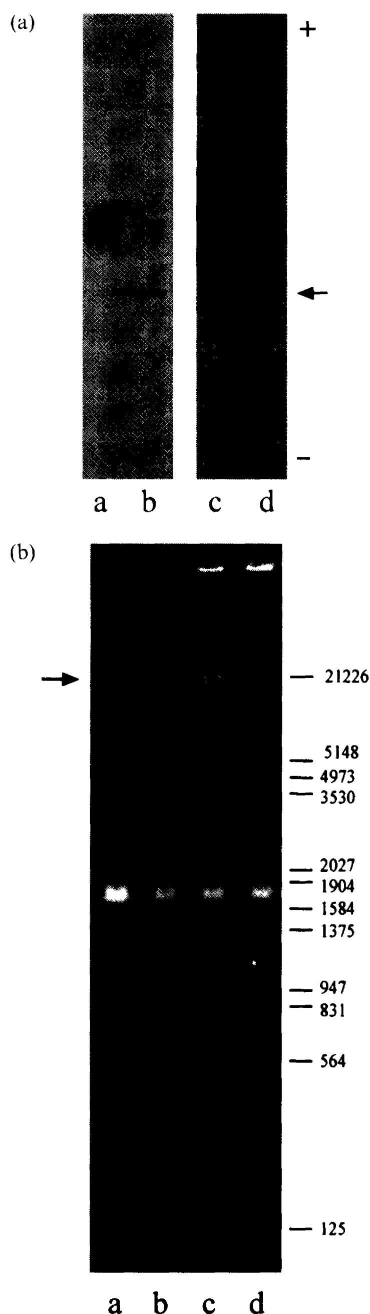

Fig. 2. (a) Identification of DNA-binding DAO activity by agarose gel electrophoresis. Samples of DAO (a, c) and DAO incubated with human chromosomal ss DNA (b, d) were separated by agarose gel electrophoresis and blotted to a nitrocellulose membrane. The nitrocellulose membrane was then analysed for (a, b) DAO activity and (c, d) mAb 40-2 binding. The arrow marks the point of application and +, – mark the anode and cathode. DAO activity and mAb binding were detected as in Fig. 1 and as described. (b) Polyamine stimulated gelshift of *p*53 DNA. *p*53 DNA was incubated with affinity purified DAO alone or in the presence of polyamines and then analysed by agarose gel electrophoresis: a, *p*53 DNA; b, *p*53 + DAO; c, *p*53 + DAO + putrescine; d, *p*53 + DAO + histamine.

strongly indicating the formation of a complex of DNA and active DAO. Other experiments showed that mAb 40-2 could not bind DNA-bound DAO indicating that the epitope for mAb 40-2 is blocked by DNA. This is also the reason for the lack of staining with mAb 40-2 at the application point.

To verify the stimulating effect of polyamines on DNA binding, a gelshift assay was performed using a *p*53 DNA ($\approx$1600 base pairs) fragment as a source of DNA. On addition of putrescine or histamine to the solution of DNA and DAO a protein–DNA complex appeared at approximately 21 000 base pairs [(Fig. 2(b)].

The observed binding of DAO to DNA could theoretically be without any physiological relevance. Therefore it was important to determine whether DAO could be found associated with DNA isolated from human tissue and we therefore performed an experiment to determine whether DAO could be liberated from human chromosomal placental DNA by treatment with DNAse I. Fig. 3 shows an experiment where a human placenta was first extracted with 50 mM sodium phosphate, pH 7.2. The residue from this extraction was then extracted with 50 mM sodium phosphate, pH 7.2, and 1% Triton X-114, and the residue from this extraction further extracted with 50 mM sodium phosphate, pH 7.2, 1% Triton X-114, and 1 M NaCl. The residue from this third extraction, containing cellular DNA and other insoluble cellular constituents, was first washed extensively with DNAse buffer and then treated with DNAse I. The amounts of DAO liberated after 2 h incubation correlated with the amounts of DNAse I used (0–100 $\mu g\ ml^{-1}$). After 18 h incubation with DNAse I, all DNA-bound DAO was liberated from the DNA even with relatively small amounts of DNAse I ($\approx 6\ \mu g\ ml^{-1}$).

Finally, affinity-purified DAO was assayed for DNAse activity on purified chromosomal ss DNA. Slow but pronounced DNAse activity on ss DNA was seen which was inhibited by amiloride (Fig. 4), as was also the DNA-bound DAO activity. The DNAse activity was not dependent on the addition of exogenous putrescine and this we ascribe to the presence of sufficient polyamines in the chromosomal DNA to support the DNAse activity of DAO.

Incubation of ds DNA with DAO and putrescine did not influence the integrity of the DNA, although with prolonged incubation with large amounts of DAO some degradation was observed. With the smaller *p*53 DNA which renatures more easily than chromosomal DNA only very little degradation was observed unless polyamines were added or the amount of DAO was increased tenfold relative to the amount used with chromosomal DNA. The degradation of *p*53 DNA occurred only slowly as with chromosomal ds DNA.

Discussion

The results presented here show that DAO has the ability to bind ss DNA and that DNA-bound DAO activity can

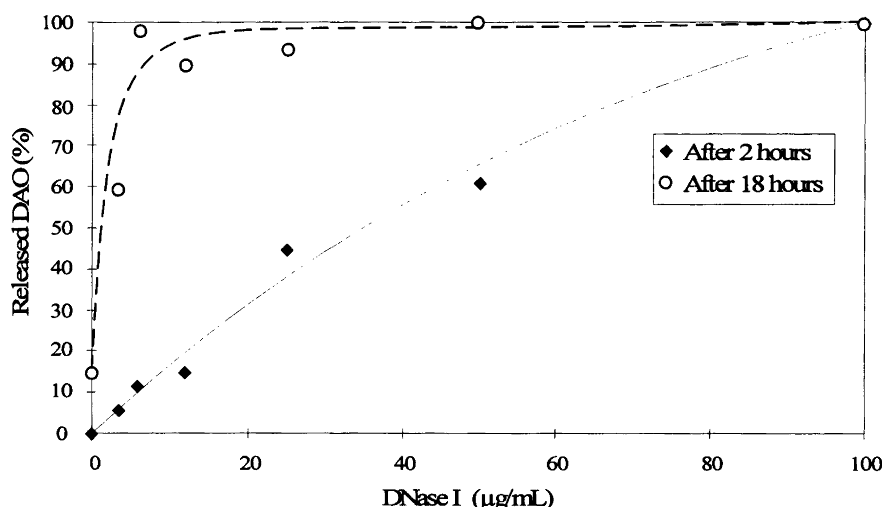

Fig. 3. The release of DAO from human placental DNA by treatment with DNAse I. A tissue sample from human placenta was homogenised in 50 mM sodium phosphate, pH 7.2. After centrifugation (10 000*g*) the precipitate was extracted first with 50 mM sodium phosphate, pH 7.2, and 1% Triton X-114, and then with 50 mM sodium phosphate, pH 7.2, 1% Triton X-114, and 1 M NaCl. The resulting precipitate was then washed three times with 20 mM Tris, 0.15 M NaCl, and 3 mM $MgCl_2$, and incubated with 0–100 µg ml^{-1} DNAse I in the same buffer for 2 or 18 h. The resulting supernatants were separated by agarose gel electrophoresis, blotted to nitrocellulose membrane and detected for DAO with mAb 40-2. The same samples were assayed for DAO activity (results not shown) and the activity found to correlate with reactivity.

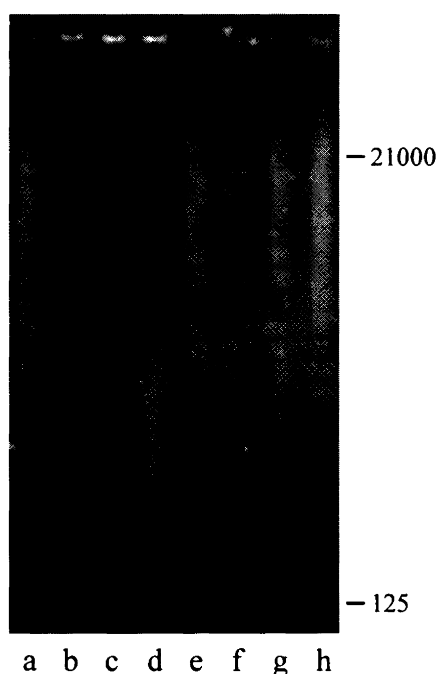

Fig. 4. The influence by DAO on human chromosomal ss DNA analysed by agarose gel electrophoresis. DNA and DAO purified by affinity chromatography and gelfiltration were incubated for 24 h with or without putrescine in combination with amiloride: a, DNA; b, DNA+DAO; c, DNA+DAO+0.1 mM putrescine; d, DNA+DAO+1 mM putrescine; e, DNA+DAO+1 mM amiloride (dissolved in 5 µl dimethyl sulfoxide); f, DNA+DAO+5 µl dimethyl sulfoxide; g, DNA+0.1 mM putrescine+1 mM amiloride; h, DNA+1 mM putrescine+1 mM amiloride.

be observed after incubation of immobilised ss DNA with affinity purified DAO. The weak detection of DAO on ds DNA may be due to the presence of small amounts of ss DNA, a low affinity of DAO for ds DNA, or to sequence-specific binding of DAO to ds DNA, whereas the binding to ss DNA is more general allowing much more DAO to bind. Another explanation could be that DAO binds ds DNA as well as ss DNA but in a way which inhibits DAO activity. The DNA-bound DAO activity is specific because the activity is dependent on the polyamines supplied in the detection solution and is inhibited by amiloride. The reason for the lower activity with spermidine and spermine as substrates is probably due to the fast and efficient binding of these polyamines to DNA[25–27] and their weaker substrate abilities for DAO.[28,29] Another result which showed that DAO must be involved in the DNA binding, was the dependence of the DNA binding on polyamines (Table 1B). The ability of the polyamines to stimulate the DNA binding was much higher than equal amounts of Mg^{2+} and Ca^{2+} which stimulate DNA binding of many other proteins.[30–34] DAO is known to bind amiloride[24] which inhibits DAO activity and amiloride was found to inhibit DNA binding of DAO as well as the DNA-bound DAO activity. This shows that DAO itself is involved in the DNA binding.

The binding of DAO to chromosomal ss DNA could be achieved in 50 mM sodium phosphate, pH 7.2. This is an advantage because it allowed binding of DAO to ss DNA without using a polyamine which could give rise to background activity during detection of DAO activity using various polyamines as substrates. DNA-bound

DAO activity was measurable without added substrate in the detection solution when the DNA binding was performed using a polyamine as binding stimulant or performed in 50 mM sodium phosphate after preincubating the DNA with a polyamine. The most likely explanation for this is that the polyamines are able to bind ss DNA during the incubation with DAO and not only stimulate the DNA binding of DAO itself but also act as DNA-bound substrates for DAO. This shows that DAO is capable of moving along the DNA strand oxidising the polyamines. The four polyamines investigated were all able to act as DNA-bound substrates (Table 1D) but with putrescine and histamine as the best substrates in this respect.

To verify the binding of DAO to ss DNA, affinity-purified DAO was incubated with ss DNA, separated by agarose gel electrophoresis, blotted to nitrocellulose membrane and assayed for DAO activity and reaction with mAb 40-2 (recognizing active DAO). The same pattern was observed for DAO activity and mAb 40-2. The reason for the missing detection of mAb 40-2 at the application point must be that mAb 40-2 recognizes an epitope on DAO blocked by bound DNA. This was also seen when DAO was bound to dotted ss DNA.

The observed binding of DAO to DNA could theoretically be artificial and without any physiological relevance. Therefore it was important to show that DAO could be found associated with DNA and could be liberated by treatment of human chromosomal placental DNA with DNAse I. Preliminary evidence suggests that DAO may bind ss DNA in cooperation with another, not yet identified, protein or protein complex. DAO immobilised on nitrocellulose did not bind ss DNA showing that DAO must be in solution in order to bind ss DNA. This suggests that DAO may bind as a homo- or hetero-dimeric complex to ss DNA. This question will be the subject of further investigations.

The ability of polyamines to bind DNA is well documented[35–42] and it is likely that they facilitate condensation of DNA by forming cation bridges between the negatively charged nucleic acid strings.[43,44] Therefore it is possible that DAO may bind DNA in the presence of polyamines and oxidise the DNA-bound polyamines. During the oxidation of polyamines by DAO, H_2O_2 and amino aldehydes are formed. These compounds are very reactive and toxic to cells and are probably removed by endogenous catalase and aldehyde dehydrogenase under normal growth conditions.[45–48] When these compounds are formed directly on ss DNA, it may be impossible to remove the H_2O_2 and the amino aldehydes immediately. This makes it possible that DNA-bound DAO activity influences the integrity of the ss DNA. Previous results have shown that amino aldehydes from the oxidation of polyamines by DAO in solution have the ability to bind DNA and introduce cross-links into the DNA.[49] Furthermore it has been shown that amino aldehydes formed from polyamines oxidised by extracellular DAO are inhibitors of cell growth[50–52] and that microinjected DAO causes cell growth arrest and induces cellular death in a way resembling apoptosis.[53–55] These and other experiments suggest that DAO may be involved in cell growth regulation, differentiation and apoptosis.[53–57]

In the same way extracellular DAO may participate in defence reactions against intruding micro-organisms by generating hydrogen peroxide and amino aldehydes. Moreover, by binding to foreign DNA and oxidising DNA-bound polyamines, DAO may be an important antimicrobial agent with direct effects on foreign DNA.

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