Expression and Characterization of Recombinant Human Acyloxyacyl Hydrolase, a Leukocyte Enzyme That Deacylates Bacterial Lipopolysaccharides†,‡

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ABSTRACT: The molecular cloning and eukaryotic cell expression of the complementary DNA for human neutrophil acyloxyacyl hydrolase (AOAH) are described. AOAH is a leukocyte enzyme that selectively removes the secondary (acyloxyacyl-linked) fatty acyl chains from the lipid A region of bacterial lipopolysaccharides (endotoxins), thereby detoxifying the molecules. The two disulfide-linked subunits of the enzyme are encoded by a single mRNA. The amino acid sequence of the protein contains a lipase consensus sequence in the large subunit and a region in the small subunit that is similar to the saposins, cofactors for sphingolipid hydrolases. The recombinant enzyme, like native AOAH, hydrolyzes secondary acyl chains from more than one position on the lipopolysaccharide backbone. Acyloxyacyl hydrolase is a novel two-component lipase that, by deacylating lipopolysaccharides, may modulate host inflammatory responses to Gram-negative bacterial invasion.

Acyloxyacyl hydrolase (AOAH)[1] is an enzyme that acts on bacterial lipopolysaccharide (LPS)(Hall & Munford, 1983). LPS, or endotoxin, is found in the cell walls of Gram-negative bacteria and consists of repeating oligosaccharide units linked to an oligosaccharide core, which is coupled to a glycolipid backbone known as lipid A [reviewed in Rietschel et al. (1984)]. Lipid A, which plays a central role in mediating the endotoxic effects of LPS, is a glucosamine disaccharide covalently substituted by phosphate and by N-linked and O-linked hydroxylated fatty acids. Some of the hydroxylated fatty acids are substituted with secondary acyl chains to form acyloxyacyl groups; these groups appear to play an important role in the biological activities of lipid A [reviewed in Takada and Kotani (1989)]. AOAH selectively hydrolyzes the secondary acyl chains from LPS, leaving the hydroxylated acyl chains attached to the glucosamine backbone.

Munford and Hall (1989) described the purification of AOAH from HL-60 human promyelocytes, where it is found principally in intracellular granules. The enzyme is a glycoprotein with a molecular weight of 52 000–60 000; it consists of two subunits of M_r 50 000 and 14 000–20 000, respectively, connected by disulfide bridge(s). Each of the subunits contains N-linked oligosaccharides. The enzyme is present in trace quantities in HL-60 cells: an estimated 0.001% of the cell lysate protein, representing about 2500 molecules per cell (Munford & Hall, 1989).

When compared with mock-treated LPS, enzymatically deacylated LPS (dLPS) shows reduced activities in a number of bioassays, including the dermal Shwartzman reaction, a test of tissue toxicity (Munford & Hall, 1986). dLPS inhibits neutrophil adherence to human vascular endothelial cells induced by LPS (Pohlman et al., 1987), inhibits plasminogen activator inhibitor 1, prostacyclin, and prostaglandin E_2 induction by LPS in endothelial cells (Riedo et al., 1990), and is reduced in its ability to induce the release of TNF in human whole blood ex vivo (Kovach et al., 1990). On the other hand, dLPS retains much of the immunomodulatory activity of LPS when tested in systems such as the murine spleen B cell mitogenicity assay (Munford & Hall, 1986; Erwin et al., 1991). Although the physiological role of AOAH is unclear, these results suggest that the enzyme may play a role in modulating LPS bioactivities or in LPS signal transduction in animals. In order to study the expression, regulation, activities, and physiological roles of AOAH, we have cloned a cDNA molecule encoding the enzyme. This paper describes the structure of DNA obtained from HL-60 and U937 cell mRNA encoding AOAH.

Materials and Methods

Protein Purification and Monoclonal Antibodies. AOAH was purified as described previously (Munford & Hall, 1989). To obtain anti-AOAH antibodies, partially pure AOAH was adsorbed to lentil lectin–Sepharose beads, and the beads were injected intraperitoneally into Balb/c mice. Using standard monoclonal antibody technology (Robertson et al., 1982), splenocytes from the immunized mice were fused with SP/2 cells, and the resulting hybridomas were screened for secretion of anti-AOAH antibodies by testing their ability to deplete AOAH activity from solution (Munford et al., 1990).

Protein Sequence Determination. AOAH, purified to homogeneity from DMSO-treated HL-60 cells, was reduced with 2-mercaptoethanol, subjected to SDS–PAGE, and electroblotted onto aminopropyl-derivatized glass fiber paper as de-

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[1] Abbreviations: AOAH, acyloxyacyl hydrolase; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide; TNF, tumor necrosis factor.

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Table I: Oligonucleotides: Code Numbers and Sequences[a]

code no.	sequence
a	AATTGATAGCGGCCGCA
b	AGCTTGCGGCCGCTATC
c	CTATAGGGAGACCGGAATTCTGTGCTCTGTCAA-GGAT
d	ATCCTTGACAGAGCACAGAATTCCGGTCTCCCT-ATAG
2388	TCAGAATTCGTNYTNGCNAARAT
2295	GATGAATTCACRTCYTTRAANGGNAC
2389	AARYTNGCNATGGARCA
2298	CARAARATIAARITIGCIATGGARCA
2465	ACAGACTGTTCCATAGCTAATTTAATTTTCTGGC-AGAT
2487	GACTCGAGTCGACATCGATCAG$(T)_{17}$
2488	GACTCGAGTCGACATCGATCAG$(C)_{10}$
2631	AGGGAGACCGGAATTCCCATGGAACAGTCTGTG-CCATTCAAAGATGT
2632	GACAGAGCACAGAATTCGACTCGAGTCGACATC-GATCAG
2633	AGGGAGACCGGAATTCGACTCGAGTCGACATCG-ATCAG
2634	GACAGAGCACAGAATTCCCATGGCTAATTTAAT-TTTCTGGCAGATCTTGG
2703	GACAGAGCACAGAATTCGAGCACACAGCATTGC-ACAGTCGT
2704	AGGGAGACCGGAATTCTCCAGCTCTTTGTGTGT-GGCTCTC

[a] R = purine, Y = pyrimidine, N = all four bases, I = inosine.

scribed by Aebersold et al. (1986) or onto poly(vinylidene difluoride) membrane as described by Matsudaira (1987). The 50-kDa subunit of the protein was then excised, and the N-terminal amino acid sequence was determined with an Applied Biosystems (Foster City, CA) Model 470A sequencer using the manufacturer's programming and chemicals.

PCR Amplification of DNA Encoding the N-Terminal Sequence of the AOAH Large Subunit. cDNA was synthesized from 1 μg of poly(A^+) RNA from DMSO-treated HL-60 cells using random primer hexamers (Pharmacia, Piscataway, NJ) according to the Superscript reverse transcriptase protocol (Life Technologies, Inc., Gibco-BRL, Gaithersburg, MD). After removal of the primers by alkaline Sepharose CL-6B chromatography (Pharmacia), DNA was amplified in a 100-μL polymerase chain reaction using the conditions described in the GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT), with the following modifications: 4.2 ng of template cDNA was used with a 4 μM aliquot of each oligonucleotide primer (oligonucleotides 2388 and 2295). All oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and are catalogued in Table I. Incubation in the thermocycler (Perkin Elmer Cetus) was for 3 cycles of 94 °C for 3 min, 30 °C for 2 min, and 72 °C for 4 min; 40 cycles of 94 °C for 1.5 min, 55 °C for 2 min, and 72 °C for 4 min; and 1 cycle cooling to 4 °C. The DNA was digested with *Eco*RI and fractionated on a 4% NuSieve agarose gel (FMC BioProducts, Rockland, ME). The 71 bp DNA was purified from the gel with NA-45 paper (Schleicher & Schuell, Keene, NH) and cloned into λ HG3, a λ vector with a plasmid pGEM-1 (Promega Biotech, Madison, WI) (with a T7 polymerase transcription terminator) linearized at *Hin*dIII and inserted into the *Eco*RI site of λgt11 via *Not*I adaptors [formed by sequences a and b (Table I)] which did not reconstitute the original *Eco*RI site of λgt11 (Hagen, 1989). Phage carrying the insert were identified with radiolabeled degenerate oligonucleotides (2389 and 2298) designed from the AOAH amino acid sequence. DNA was prepared from the plate lysates as described by Helms et al. (1985). Plasmids were obtained from the λ DNA by digestion with *Not*I, dilution, ligation, and transformation into DH5 α cells (Life Technologies, Inc.). Restriction enzymes were obtained from New England BioLabs (Beverly, MA), Life Technologies, Inc., and Boehringer Mannheim (Indianapolis, IN).

Construction and Screening of the cDNA Library. RNA was prepared from DMSO-stimulated HL-60 cells (Munford & Hall, 1989) by guanidine isothiocyanate extraction and CsCl centrifugation (Chirgwin et al., 1979). A random-primed λgt11 cDNA library was prepared from oligo(dT)-selected poly(A^+) RNA by using an Invitrogen Inc. (San Diego, CA) "Lambda Librarian" cDNA kit with the following modification: cDNA of approximately 300 bp or greater was selected, and the unincorporated adaptors were removed by chromatography on a Sepharose 6B-CL column. A plate lysate library was prepared which contained 13.3 million independent isolates with a background of 1%. The actin positivity (Hagen et al., 1988) of the library was 0.34%; 7.2 million clones from the library were screened by using oligonucleotide 2465, and 2 positive clones were identified. The inserts from these clones, 1.1 and 2.1, were subcloned into pVEGT′. pVEGT′ is pGEM-1 (Promega Biotech) with the following elements added: an M13 intergenic region, two T7 RNA polymerase terminators, a poly(A) sequence, and the "Prime" sequence [allows insertion of PCR-generated DNA via long single-strand ends generated by the 3′-exonuclease activity of T4 DNA polymerase in the presence of dATP for the insert DNA and dTTP for the vector DNA (Hagen, 1989)]. The "Prime" sequence is formed by sequences c and d (Table I).

RACE Amplification of 5′ and 3′ AOAH Sequences. For 3′ rapid amplification of cDNA ends (RACE) (Frohman et al., 1988), first-strand cDNA was synthesized from 1 μg of poly(A^+) RNA from the human monocyte cell line U937 and 5 pmol of dT adaptor primer 2487 by using the Superscript reverse transcriptase protocol (Life Technologies, Inc.). The DNA was size-fractionated on a 1% low-melt alkaline agarose gel, and the gel containing the fractionated DNA was cut into 12 0.5-cm fragments, representing DNA from 700 to 7000 nucleotides. The gel fragments were melted at 65 °C and mixed with 400 μL of water. DNA was amplified from these fragments by using the GeneAmp kit conditions except that 1 mM $MgCl_2$ and 50 μM each oligonucleotide primer (2631 and 2632) were used. The amplified DNA was analyzed by Southern blot analysis using an AOAH-specific oligonucleotide probe. DNA from 2100 to 2500 nucleotides in size was gel-purified and used as template for amplification of the AOAH 3′ RACE product.

For 5′ RACE amplification, first-strand cDNA was synthesized from 1 μg of U937 cell poly(A^+) RNA and 5 pmol of dT adaptor primer 2487 in a 10-μL reaction as described above. After synthesis, the RNA was alkaline-hydrolyzed, and the DNA was ultrafiltered with an Amicon (Danvers, MA) Centricon Special YM-100 unit with 50 mM KOH and 0.1 mM EDTA. After ethanol precipitation, the cDNA was G-tailed (Maniatis et al., 1981). Second-strand cDNA was synthesized by using the GeneAmp kit reaction buffer, 50 μM dC adaptor primer 2488, and 50 units/mL *Taq*I polymerase (Perkin Elmer Cetus). The template and primer were annealed at 94 °C for 5 min and at 40 °C for 5 min. After the enzyme was added, the reaction was incubated at 40 °C for 5 min and at 72 °C for 15 min. After ethanol-precipitation, the DNA was fractionated on a 1% low-melt alkaline agarose gel. Gel fragments containing fractionated DNA were obtained and processed as for preparation of the 3′ AOAH RACE template. DNA was amplified from the fragments by using the GeneAmp kit conditions except that 1 mM $MgCl_2$ and 50 μM each oligonucleotide primer (2633 and 2634) were used.

The 3′-exonuclease activity of T4 DNA polymerase was used to prepare the RACE amplified DNA for cloning into pVEGT′. The DNA was suspended in 10 μL of 33 mM Tris–acetate, pH 7.9, 67 mM KOAc, 10 mM MgOAc, 0.1 mg/mL gelatin, 200 μM dATP, 5 mM DTT, and 0.1 units/μL T4 DNA polymerase (Boehringer-Mannheim) and incubated for 1 h at 15 °C, and the reaction was terminated by incubation at 65 °C for 15 min. The DNA was cloned into pVEGT′ which had been digested with *Eco*RI and treated with T4 DNA polymerase in the presence of 200 μM dTTP as described above. The amplified DNA was ligated to the vector and electroporated into *Escherichia coli*. The DNA sequences of the insert DNAs were determined. The full-length coding sequence of AOAH was defined by comparing these sequences with those of partially homologous proteins (Figure 7) and the known amino acid sequence of AOAH, while looking for open reading frames, a translational start methionine, a secretory leader sequence, and a translational termination codon. From the full-length sequence, two PCR primers, 2703 and 2704, were designed and used in PCR reactions to obtain complete clones of DNA encoding AOAH. A full-length corrected wild-type clone was obtained by ligating fragments of these clones into an assembled construct.

DNA Sequencing. cDNA and PCR products encoding AOAH cloned into the Prime vectors (F. Hagen, unpublished results) were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using [α-^{35}S]dATP from DuPont/New England Nuclear (Boston, MA) (Biggin et al., 1983). The protocol of Chen and Seeburg (1985) was used for sequencing double-stranded templates. Sequencing reactions catalyzed by modified T7 DNA polymerase (U.S. Biochemicals, Cleveland, OH) (Tabor & Richardson, 1987) were primed with a universal primer or with oligonucleotides complementary to cDNA or PCR-generated sequences representing AOAH mRNA. All sequences were determined on both strands. All sequences reported here were determined by using more than one clone. This was particularly important for clones originally generated by PCR. In these cases, sequences were determined by using at least three independently derived clones.

Expression of AOAH cDNA and Assay of AOAH Activity. AOAH cDNA was subcloned into the *Eco*RI site of the mammalian cell expression vector pZEM229R (Foster et al., 1991). Isolates representing the correct orientation were chosen. These clones were transfected into BHK570 cells by using calcium phosphate mediated transfection (Waechter & Baserga, 1982) for transient expression (Chen & Okayama, 1988). After incubation for 2 days, the media were collected, and the cells were lysed with detergent (one million cells per 100 μL of PBS, 0.1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride). The samples were incubated for 10 min at room temperature and centrifuged for 10 min at 4 °C, and the cleared lysates were removed and placed on ice. The media and cleared lysate were assayed for AOAH activity with labeled LPS.

E. coli LPS, labeled with [^{3}H]acetate, was treated with native and recombinant AOAH under standard reaction conditions (Erwin & Munford, 1990); in both reactions, less than 15% of the ^{3}H-fatty acids were released from the LPS. The free fatty acids were extracted into chloroform and analyzed by using one-dimensional thin-layer chromatography on Whatman (Maidstone, U.K.) KC18 reverse-phase plates (Schultz & Oroszlan, 1984). ^{3}H-Fatty acids were also acid-hydrolyzed from the same LPS, extracted into chloroform, and run as standards. Acetonitrile/acetic acid (1:1) was the solvent system. The plates were sprayed with En^{3}Hance (Dupont/New England Nuclear) and exposed to X-ray film as previously described (Erwin & Munford, 1990).

Computer Analysis. The DNA sequences representing AOAH mRNA were analyzed on Sun SPARC Station 1 and Compaq 386 computers using Intelligenetics (Mountain View, CA) software and homology search programs by Lipman and Pearson (1985), Altschul and Lipman (1990), Pearson and Lipman (1988), and Brutlag et al. (1990). The sequences were compared to the GenBank (Bilofsky et al., 1986) and EMBL (Hamm & Cameron, 1986) nucleotide sequence databanks, the PIR protein identification resource protein databank (George et al., 1986), and the SWISSPROT protein sequence database (A. Bairoch, Departement de Biochemie Medicale, Centre Medicale Universitaire, 1211 Geneva 4, Switzerland). The significance of matches with database sequences was assessed by the RDF algorithm of Lipman and Pearson (1985).

Results

AOAH, purified from DMSO-treated HL-60 cells, is a glycoprotein (apparent M_r = 52 000–60 000) that has two disulfide-linked subunits (Munford & Hall, 1989). Generating a monoclonal antibody that depleted AOAH activity from solution and identified the large (50 kDa) AOAH subunit by Western blot analysis provided further evidence that the purified protein is AOAH (Munford et al., 1990). The monoclonal antibody was coupled to Sepharose and used to purify AOAH by immunoaffinity chromatography. When the protein purified either by the method of Munford and Hall (1989) or by immunoaffinity chromatography was reduced with 2-mercaptoethanol and analyzed by SDS–PAGE, two bands were observed: one with an apparent molecular weight of 50K (the "large subunit") and one heterogeneous band with an apparent molecular weight of 14–20K (the "small subunit"). The results suggested strongly that the protein is AOAH and that it has two disulfide-linked subunits (Munford et al., 1990).

Cloning AOAH cDNA. Our strategy for cloning the cDNA for AOAH was to use information about the large subunit of the protein, since this subunit runs as a discrete band on SDS–PAGE and transfers electrophoretically to membrane materials. The amino-terminal sequence of this subunit is shown in Figure 1. There were 4 unidentified amino acids in this sequence of 29 amino acids, and many of the amino acids were those with high codon redundancy, so it was difficult to design good hybridization probes from this sequence. We nevertheless attempted to obtain cDNA clones by screening cDNA libraries with degenerate and long "guess-mer" oligonucleotide probes designed from this sequence. When these approaches were unsuccessful, PCR amplification was pursued to obtain the unique DNA sequence encoding the known AOAH amino acid sequence.

Using random-primer hexamers (Pharmacia), we synthesized cDNA from poly(A$^+$) RNA of DMSO-treated HL-60 cells. A degenerate oligonucleotide partially encoding the sequence Val-Leu-Ala-Lys-Ile was synthesized in the sense orientation, and a second degenerate oligonucleotide partially encoding Val-Pro-Phe-Lys-Asp-Val (see Figure 1 for the positions of these sequences) was synthesized in the antisense orientation. These oligonucleotides were used to amplify the 38-nucleotide sequence they flank by the method of Lee et al. (1988). After amplification, the DNA was digested with *Eco*RI and fractionated on a NuSieve gel, and a band containing DNA of approximately 71 bp was purified from the gel and cloned into λ HG3. Positive phage clones were identified by hybridization with radiolabeled degenerate oligonucleotides (2298 and 2389, Table I) that partially encoded

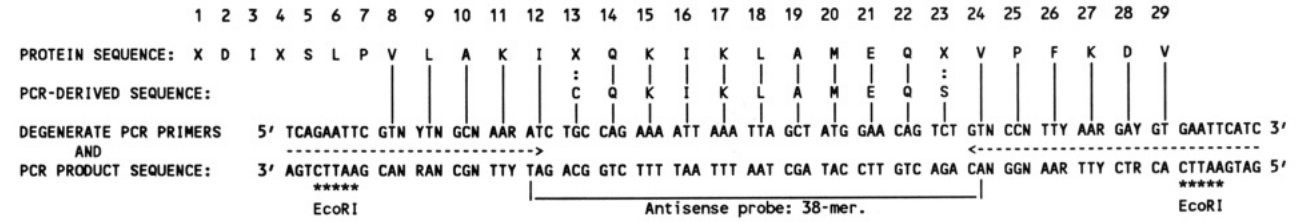

FIGURE 1: Sequences of the amino terminus of the large subunit of AOAH, the degenerate oligonucleotides used to amplify the corresponding cDNA sequence, and the derived PCR product. The first line indicates the position from the amino terminus of the large subunit, which corresponds to positions 157–185 in Figure 4. "Protein sequence" indicates the amino acid sequence determined by protein sequencing, shown using the one-letter code (X indicates undetermined). "PCR-derived sequence" indicates the amino acid sequence which was encoded by the PCR product. "Degenerate PCR primers and PCR product sequence" shows both strands of the nucleotide sequence involved in the amplification reaction, including degenerate oligonucleotides (dashed arrows) containing *Eco*RI sites (asterisks) and the sequence used to design an antisense oligonucleotide probe.

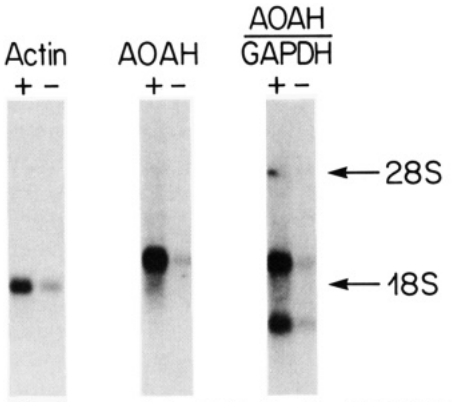

FIGURE 2: Northern analysis of HL-60 cell poly(A^+) RNA using probes for actin, glyceraldehyde-phosphate dehydrogenase (GAPDH), and AOAH (Mathison et al., 1990). Five micrograms of poly(A^+) RNA prepared from uninduced and DMSO-induced HL-60 cells was run in the lanes designated as (−) and (+), respectively; acridine orange staining showed similar amounts of RNA in each lane. The positions of the 18S and 28S ribosomal RNA bands (in total RNA run on the same gel) are indicated. The AOAH probe hybridizes to a band containing mRNA of approximately 2400 bp.

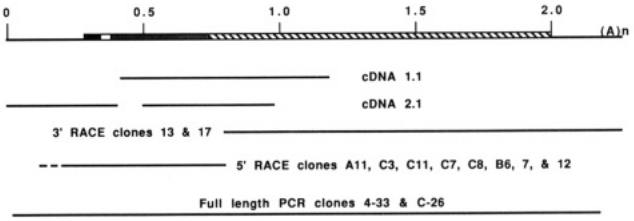

FIGURE 3: Schematic representation of the AOAH PCR and cDNA clones which were obtained and sequenced to provide the consensus sequence of Figure 4. The scale in kilobases is given at the top. The nucleotide sequence encoding the AOAH pre–peptide sequence is indicated by a solid bar, the putative pro-peptide sequence by an open bar, the small subunit by a stippled bar, and the large subunit by a striped bar. The poly(A) tail is indicated by $(A)_n$. The lines indicate the extent of the sequences of the various clones. The break in the cDNA 2.1 line indicates missing sequence apparently due to alternate splicing. The dashed line at the beginning of the 5′ RACE clones indicates that these clones did not share a common 5′ end.

the AOAH amino acid sequence flanked by the PCR primers. The plasmids within λ HG3 that contained the insert DNA were recovered, and the insert DNA was sequenced. Translation of the DNA sequence showed that the deduced amino acid sequence was identical with the AOAH amino acid sequence from Val(8) to Val(29) (Figure 1). This determination also showed that the previously unidentified amino acids at positions 13 and 23 were Cys and Ser, respectively. These results provided a unique 38-nucleotide DNA sequence encoding AOAH amino acids Ile(12) through the first 2 nucleotides of the codon encoding Val(24) (Figure 1). An antisense oligonucleotide probe (2465) was prepared from this sequence and hybridized to HL-60 cell poly(A^+) RNA in Northern blot analysis. A band representing RNA of approximately 2400 nucleotides was observed (Figure 2).

The oligonucleotide derived from the 38-nucleotide sequence was also used as a probe for screening a randomly primed cDNA library constructed from HL-60 cells. Two partial cDNA clones, referred to in Figure 3 as 1.1 and 2.1, were obtained; both clones contained sequence encoding the known amino acid sequence of AOAH, plus additional overlapping sequence, although their sequences diverged at their 5′ ends.

Initial amplification of AOAH DNA using the RACE protocol (Frohman et al., 1988) yielded a smear of AOAH-related DNA as determined by Southern blot analysis. In order to obtain discrete AOAH PCR products, the RACE procedure was modified to include fractionation of the cDNA template by alkaline agarose gel electrophoresis prior to PCR amplification. Analysis of PCR products amplified from DNA of gel fragments by Southern blot analysis showed that the 3′ RACE reaction produced AOAH-specific fragments from a few hundred base pairs to 1500 bp, with two primary size classes of 840 and 1500 bp; the 840 bp product was the most prominent product. Similarly, Southern analysis of the 5′ product showed that AOAH-specific amplified DNA was heterogeneous in size with a maximum size of 800 bp. The sum of the maximum predicted size of 1500 and 800 bp for the RACE products was in agreement with the total predicted size of 2400 nucleotides for the AOAH mRNA, considering that 100–150 nucleotides are contributed by poly(A).

The 5′ and 3′ RACE products were cloned, and their DNA sequences were determined. From the complete AOAH nucleotide sequence, it was possible to design PCR primers 2703 and 2704 to amplify a complete clone encoding the AOAH protein (Figure 4). Template cDNA from the gel fragment which had produced the full-length 3′ RACE product was used for amplification. Two clones, C-26 and 4-33 (Figure 3), derived from separate PCR reactions, were identified for further analysis.

Analysis of the AOAH cDNA. The DNA sequence shown in Figure 4 is a composite derived from the cDNA (1.1 and 2.1) and PCR-generated clones. Excluding the poly(A) tail, the sequence is 2251 nucleotides in length. A number of single-base differences from the consensus sequence shown in Figure 4 were observed in the PCR-generated clones shown in Figure 3. These differences were attributed to incorporation errors by Taq polymerase. The sequence shown in Figure 4 is a consensus based on redundant sequencing of multiple independently derived clones (see Materials and Methods) and is the sequence of a composite, assembled clone, called AOAH-1. There is an open reading frame extending from nucleotide 273 to nucleotide 1997, indicating that the message contains at least 272 nucleotides of 5′-untranslated sequence as well as 251 nucleotides of 3′-untranslated sequence (not

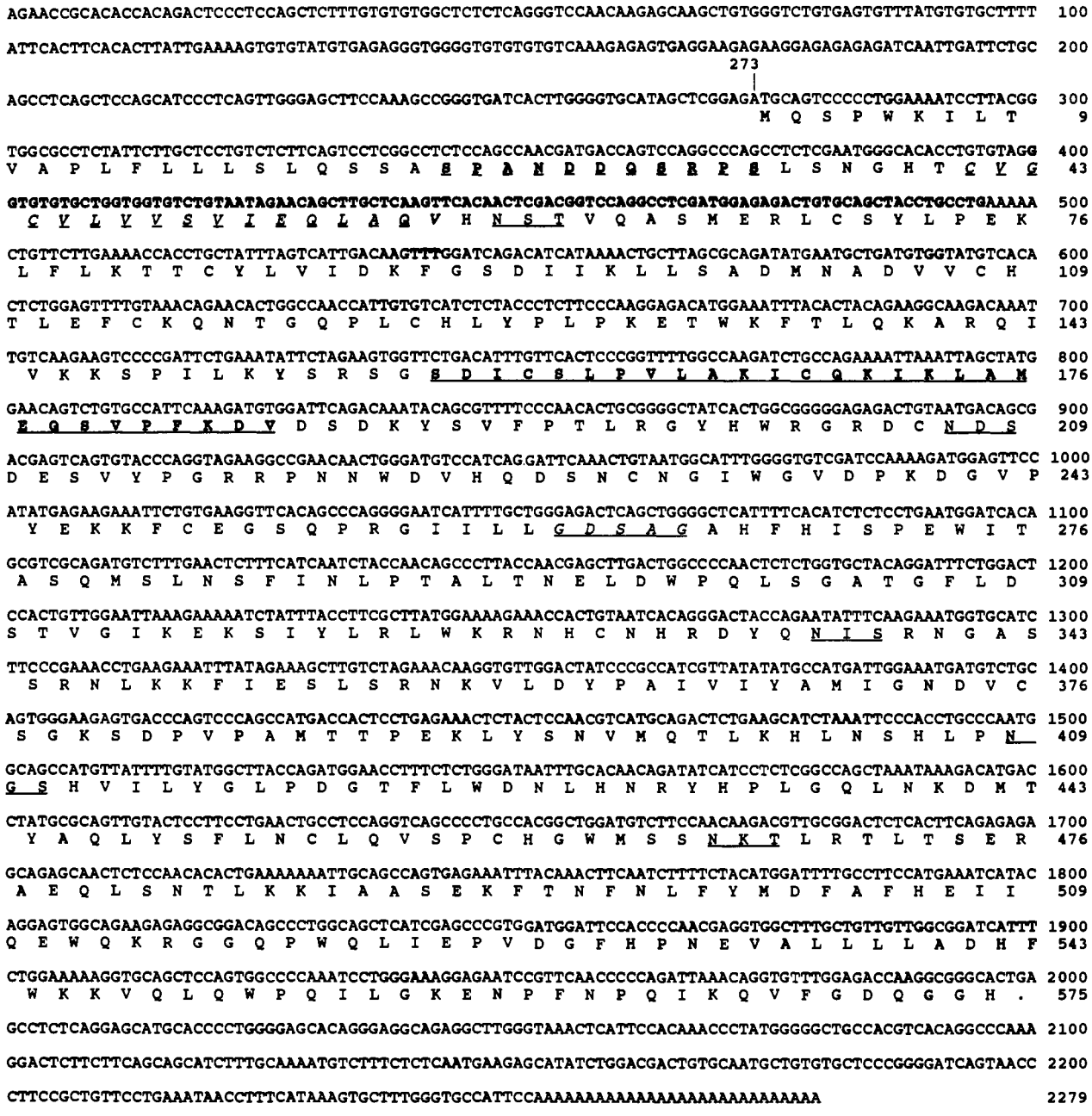

FIGURE 4: Nucleotide sequence of AOAH cDNA and the derived amino acid sequence of AOAH. Nucleotide numbering starts at the 5′ end and is indicated by the numbers at the end of each line of nucleotide sequence. Nucleotides 400–495, absent in an alternatively spliced form of AOAH mRNA, are indicated by bold type. The open reading frame extends from nucleotide 273 (indicated) to 1997. The stop codon is indicated by a period. Translation is shown using the one-letter code directly underneath the second base of each codon. Amino acid numbering extends consecutively from the initiating methionine and is indicated at the end of each line of amino acid sequence. The putative signal peptide extends up to the start of the putative propeptide. This propeptide is indicated by bold type with dashed underlining and extends from amino acid 24 to 34. This is followed by the small subunit which extends from amino acid 35 (arrow) to 156; amino acids 35–49 were determined by automated Edman degradation of the protein. The large subunit extends from amino acid 157 to 575. The location of the amino-terminal sequence of the large subunit determined by automated Edman degradation of the protein (Figure 1) is shown in bold type and solidly underlined. Putative N-linked glycosylation sites are indicated by solid underlining. A hydrophobic stretch of amino acid sequence from position 41 to 57 which may correlate with membrane association is shown in italics with dashed underlining. The G-X-S-X-G motif (Figure 6) is shown in italics with solid underlining. This sequence has been deposited in GenBank (Accession No. M62840).

including the stop codon). The open reading frame of 1725 nucleotides can encode 575 amino acids. In cDNA clone 2.1, nucleotides 400 through 495 are not present. The beginning of this deletion corresponds to the position of an intron in a partial genomic clone (Durnam et al., unpublished results), suggesting that the message encoding AOAH can be alternatively spliced. The beginning of the open reading frame encodes a sequence with the characteristics of a signal peptide (von Heijne, 1983). The signal peptide is predicted to be 23 amino acids in length with a cleavage site between Ala(23) and Ser(24) using the prediction method of von Heijne (1986). The 5′-untranslated sequence contains a stretch of 43 nucleotides (nucleotides 145 to 188) which can generally be described as a GT dinucleotide-rich region followed by a GA dinucleotide-rich region. Such sequences are fairly common; a search of the GenBank database revealed 18 cases of nearly perfect GT dinucleotide repeats immediately followed by GA dinucleotide repeats. Similar regions have been identified as polymorphic microsatellites (Litt et al., 1989; Weber & May, 1989). The 5′-untranslated portion of the mRNA ends with

the sequence CGGAG just upstream of the initiating ATG codon. It is a poor match with the CC(A/G)CC consensus proposed by Kozak (1984). In the 3′-untranslated region, the sequence does not contain an exact match with an AAUAAA polyadenylation sequence, although the sequence AAUAAC occurs 30 nucleotides upstream of the poly(A) tail.

Analysis of the AOAH Protein Sequence. The large subunit (M_r = 50K) was isolated for automated Edman degradation by SDS electrophoresis of the reduced protein followed by electroblotting onto aminopropyl glass paper. Two sequences were identified, initial yields from which measured 16 and 2.5 pmol, respectively. The major component, shown in Figure 1, differed from the minor component in the presence of an additional residue at the N-terminus of the chain, indicating that some N-terminal heterogeneity existed. Comparison of these observed amino acid sequences with the sequence encoded by the cDNA (Figure 4) permitted the N-terminal residues to be identified as Ser(157) and Asp(158), respectively. Since the small subunit migrates as a diffuse zone on SDS electrophoresis (Munford & Hall, 1989), the location of the amino terminus of the small subunit was established by Edman degradation of the 60-kDa species observed by electrophoresis in the absence of reduction. This species was electroblotted onto poly(vinylidene difluoride) paper after electrophoresis and yielded the amino acids derived previously from the large subunit plus additional residues derived from the small subunit, as shown in Figure 4 (amino acids 35–49). The first of the residues from the small subunit was identified as Leu(35) and was recovered in 9 pmol yield. The small subunit may thus be as much as 124 amino acids in length, depending on the mechanism and site of cleavage between the two subunits. While the exact size and degree of homogeneity of the small subunit await determination of its carboxyl terminus, molecular weights of 13.8K and 47.7K can be estimated for unglycosylated small and large subunits, respectively. The latter figure is consistent with the estimate of 48K for the large subunit based on migration in an SDS–polyacrylamide gel after treatment with *N*-glycanase and 2-mercaptoethanol (Munford & Hall, 1989). There are five potential N-glycosylation sites predicted from the sequence, one in the small subunit and four in the large subunit. They occur at amino acid positions 59, 207, 336, 409, and 466. Other sites of potential posttranslational modification include (as in nearly all proteins) a number of Ser and Thr residues which are potential sites for O-linked glycosylation. Specifically, there are 3 sites in the small subunit and 14 sites in the large subunit of AOAH which follow the pattern for O-linked glycosylation suggested by Takahashi et al. (1984). Finally, there are 3 potential phosphorylation sites (Woodget et al., 1986; Kuenzel et al., 1987; Hunter, 1982) in the small subunit and 12 such sites in the large subunit. It is not known if any of these sites is actually modified. The predicted amino acid sequence was also examined for potential membrane-associated sequences by using the algorithms of Rao and Argos (1986), Eisenberg et al. (1984), and Klein et al. (1985). No region scored positive as a membrane-associated helix using the first two methods, yet one region (beside the predicted signal sequence) scored as a potential transmembrane segment by the third algorithm: the region from amino acids 39 to 57 (Figure 4). The biochemical relevance of this prediction for AOAH is not as yet known.

Expression of Recombinant AOAH. The AOAH cDNA was removed from clones C-26 and 4-33 and cloned into the mammalian expression vector pZEM229R (Foster et al., 1991). These clones were transfected into BHK570 cells

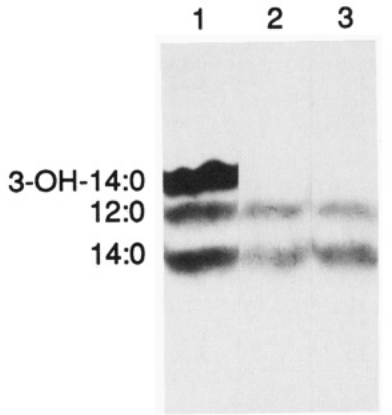

FIGURE 5: rAOAH releases both secondary fatty acids from *E. coli* LPS. Reverse-phase TLC analysis (see Materials and Methods). Lane 1, fatty acids acid-hydrolyzed from LPS. Lane 2, fatty acids released by native AOAH. Lane 3, fatty acids released by rAOAH. Both native and recombinant AOAH release the two secondary fatty acids, 12:0 and 14:0, while leaving 3-OH-14:0 attached to the glucosamine backbone.

(Waechter & Baserga, 1982), and the cells were cultured for transient expression. When assayed for AOAH activity, the conditioned media and all the negative controls released low amounts of ^{3}H-fatty acid from the labeled LPS (150–250 cpm). The C-26 and 4-33 cell lysates released about 500 and 3000 cpm, respectively. The positive AOAH control (from a HL-60 cell lysate) was also about 3000 cpm. These results indicated definite expression of AOAH activity, with clone 4-33 having greater activity than clone C-26, consistent with the presence of fewer alterations from the consensus sequence in the cDNA of clone 4-33. Recombinant AOAH, like native AOAH, released both of the secondary acyl chains from *E. coli* LPS (Figure 5).

Discussion

Mature AOAH consists of two glycosylated polypeptide chains linked by disulfide bond(s). We have determined the amino terminus of each chain and have cloned cDNA encoding AOAH by a combination of classical cDNA and PCR technology. Our results indicate that the heterodimer is synthesized from a single mRNA. Confirmation that our clone encoded AOAH came from transient expression of the cDNA in BHK570 cells.

Native AOAH catalyzes the hydrolysis of secondary acyl chains that are linked to 3-hydroxyacyl chains located at various positions on the diglucosamine backbone of LPS (Erwin & Munford, 1990). Recombinant AOAH also has this substrate range, as shown by its ability to release both laurate (attached to the 3-hydroxymyristoyl residue at 2′) and myristate (at 3′) from *Escherichia coli* LPS (Figure 5). Since these secondary acyl chains are thought to be situated on opposite faces of the lipid A structure (Ray et al., 1984), rAOAH appears to share the native enzyme's ability to recognize acyloxyacyl linkages in different topological contexts within lipid A. This result also provides strong support for the conclusion that native AOAH is a single enzyme.

In order to obtain cDNA representing the low-abundance AOAH mRNA, we made several modifications of standard PCR and RACE protocols. To improve RACE amplification, CAG was added to the 3′ end of the adaptor primer in order to reduce primer dimer formation. G tails instead of A tails were used for the 5′ amplification. Gel fractionation of AOAH-specific DNA was used to obtain discrete RACE products. We used large numbers of PCR cycles to amplify DNA representing the low-abundance AOAH mRNA. In our

```
                    *   *   *
AOAH.   G I I L L G D S A G A H F
  1.    R I C L A G D S A G G N L
  2.    N V H V I G H S L G A H A
  3.    N V H V I G H S L G S H A
  4.    Q V Q L I G H S L G A H V
  5.    H V H L I G Y S L G A H V
  6.    K V H L I G Y S L G A H V
  7.    N V H L L G Y S L G A H A
  8.    N V H L L G Y S L G A H A
  9.    N V H L L G Y S L G A H A
 10.    N V H L L G Y S L G A H A
 11.    Q L H Y V G H S Q G T T I
 12.    K I H Y V G H S Q G T T I
 13.    N I T I F G E S A G G A I
 14.    P V F L I G H S L G C L H
 15.    K V H L V G H S M G G Q T
 16.    P V H F I G H S M G G Q T
 17.    R V N L I G H S Q G A L T
 18.    K V A V T G H S L G G A T
 19.    E H P W W G P S F G E Y Y
```

FIGURE 6: Comparison of the sequences around the G-X-S-X-G motif of AOAH and a number of lipases: (1) rat hormone-sensitive lipase (Holm et al., 1988); (2) human, (3) porcine, and (4) canine pancreatic lipases (Lowe et al., 1990; Bianchetta et al., 1979; Kerfelec et al., 1986); (5) human and (6) rat hepatic lipase (Datta et al., 1988; Komaromy & Schotz, 1987); (7) human, (8) murine, (9) bovine, and (10) guinea pig lipoprotein lipases (Wion et al., 1987; Kirschgessner et al., 1987; Senda et al., 1987; Enerback et al., 1987); (11) human gastric lipase (Bodmer et al., 1987); (12) rat lingual lipase (Docherty et al., 1985); (13) rat lysophospholipase (Han et al., 1987); (14) human phosphatidylcholine–sterol acyltransferase (McLean et al., 1986); (15) *Staphylococcus aureus*, (16) *Staphylococcus hyicus*, (17) *Pseudomonas fragi*, and (18) *Rhizomucor miehei* lipases (Lee & Iandolo, 1986; Gotz et al., 1985; Kugimiya et al., 1986; Boel et al., 1988); and (19) *Pseudomonas aeruginosa* lipophosphodiesterase I (Pritchard & Vasil, 1986). The conserved G-S-G residues are indicated by asterisks.

hands, the RACE amplification product could not be cloned despite various modifications until the "Prime" cloning system (Hagen, 1989) was utilized (see Materials and Methods).

Expression of AOAH appears to involve a number of steps of posttranslational modification, including proteolytic processing, glycosylation, and targeting. While we have shown that the amino terminus of the small subunit is at Leu(35), signal peptide cleavage should occur, using standard prediction methodology (von Heijne, 1986), between amino acids Ala(23) and Ser(24). This suggests that the AOAH precursor contains an 11 amino acid propeptide at amino acids 24 through 34. It is possible that this propeptide is involved with some aspect of targeting. The mature protein is also glycosylated: there are five potential Asn-linked glycosylation sites. One of these sites is in the small subunit (at residue 59); the rest are in the large subunit. It appears that there are two proteolytic processing events in addition to signal peptidase cleavage during expression of mature AOAH. One involves the removal of the putative propeptide by cleavage after Ser(34). The other involves the cleavage between the small and large subunits. This latter cleavage apparently can involve events between Gly(156) and Ser(157) or between Ser(157) and Asp(158).

Analysis of AOAH amino acid and DNA sequences using existing databases revealed only two regions with significant similarity to known proteins; there was no homology to members of a family of proteins that contain lipid A binding sites (Tobias et al., 1988). The region from residues 256 to 268 is compared to similar regions from a number of other lipases in Figure 6. This is a G-X-S-X-G lipase consensus motif, found in all known mammalian and microbial lipases. This region may be associated with interfacial lipid binding (Lowe et al., 1989), although recent reports indicate that the central serine is part of a Asp-His-Ser catalytic triad in human and bacterial triacylglycerol lipases (Winkler et al., 1990; Brady et al., 1990).

The second region of sequence similarity is found in the small subunit of AOAH. It is homologous to a number of polypeptides, including human sphingolipid activator protein (SAP) precursor repeats (prosaposin) (Dewji et al., 1987; O'Brien et al., 1988), rat sulfated glycoprotein 1 (SGP-1) repeats (Collard et al., 1988), pulmonary surfactant-associated protein B (PSP-B) (human, rabbit, rat) (Jacobs et al., 1987; Glasser et al., 1987; Emrie et al., 1989; Xu et al., 1989), and canine pulmonary surfactant protein SP 18 precursor (Hawgood et al., 1987) (Figure 7). The human prosaposin and rat SGP-1 each contain four homologous repeats. In the case of prosaposin, these repeats give rise to different activators, called saposins A–D, through proteolytic processing (O'Brien & Kishimoto, 1991). An estimation of the statistical significance of one of these matches, that between the AOAH small subunit and human saposin C, was obtained by using the RDF algorithm (Lipman & Pearson, 1985). The z values for the small subunit and the saposin C were initial = 8.99 and optimized = 19.71, which indicates a high probability of biological significance of the relationship. The significance of the individual matches is strengthened by the multiple occurrence of similar matches and by the striking conservation of Cys residues.

A comparison of the physiological roles of the structurally similar proteins suggests functional similarity. The first of these biological similarities is substrate. Bacterial lipopolysaccharides, which serve as substrates for AOAH, resemble sphingolipids by having a relatively constant lipid core and a carbohydrate head group of variable size and structure. However, unlike the sphingolipid glycohydrolases, which cleave

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            *  *                             *            *                         *     *
a.      PLPYCWLCRALIKRIQAMIP-KGA----LAVAVAQV-CRVVPLV--AGGICQCLAERYSVILLDTLLGRML-PQLVCRLVLRCSMD
b.      PLPLCWLCRTLLKRIQAMIP-KGV----LAMAVAQV-CHVVPLV--VGGICQCLAERYTVILLEVLLGHVL-PQLVCGLVLRCSSV
c.      PLPFCWLCRTLIKRVQAVIP-KGV----LAVAVSQV-CHVVPLV--VGGICQCLAERYTVLLLDALLGRVV-PQLVCGLVLRCSTA
d.      ADDLCQECQDIVRILTKMTK-EAIFQDMVRKFLEHE-CDVLPLKL-LTPQCHHMLGTYFPVVVDYFQSQIN-PKIICKHLGLCKPG
e1.     KSLPCDICKDVVTAAGDMLK-DNATEEEILVYLEKT-CDWLPKPN-MSASCKEIVDSYLPVILDIIKGEMSRPGEVCSALNLCESL
e2.     KGDVCQDCIQMVTDIQTAVR-TNSTFVQALVEHVKEECDRLGPG--MADICKNYISQYSEIAIQMMMH-MQ-PKEICALVGFCDEV
e3.     SDVYCEVCEFLVKEVTKLID-NNKTEKEILDAFDKM-CSKLPKS--LSEECQEVVDTYGSSILSILLEEVS-PELVCSMLHLCSGT
e4.     DGGFCEVCKKLVGYLDRNLE-KNSTKQEILAALEKG-CSFLPDP--YQKQCDQFVAEYEPVLIEILVEVMD-PSFVCLKIGACPSA
f1.     KSLPCDICKTVVTEAGNLLK-DNATEEEILHYLEKT-CAWIHDSS-LSASCKEVVDSYLPVILDMIKGEMSNPGEVCSALNLCQSL
f2.     NEDVCQDCMKLVTDIQTAVR-TNSSFVQGLVDHVKEDCDRLGPG--VSDICKNYVDQYSEVAVQMMMH-MQ-PKEICVMVGFCDEV
f3.     NVIFCQVCQLVMRKLSELII-NNATEELLIKGLSKA-CSLLPAP--ASTKCQEVLVTFGPSLLDVLMHEVN-PNFLCGVISLCSAN
f4.     NGGFCEVCKKLVIYLEHNLE-KNSTKEEILAALEKG-CSFLPDP--YQKQCDEFVAEYEPLLLEILVEVMD-PSFVCSKIGVCPSA      @
g.    LSNGHTCVGCVLVVSVIEQLAQVHNST---VQASMERL-CSYLPEKLFLKTTCYLVIDKFGSDIIKLLSADMN-ADVVCHTLEFCKQNTGQPLCHLYPLPKETWKFTLQKARQIVKKSPILKYSRSG
                              |
                            glycos
```

FIGURE 7: Comparison of the sequence of (a) human, (b) rabbit, and (c) rat pulmonary surfactant protein B (Jacobs et al., 1987; Xu et al., 1989; Emrie et al., 1989), (d) canine pulmonary surfactant protein SP18 (Hawgood et al., 1987), (e1–4) human prosaposin repeats (O'Brien et al., 1988), and (f1–4) rat SGP-1 repeats (Collard et al., 1988) with (g) the small subunit of AOAH. The aligned asparagines indicated by "glycos" are potential sites of N-linked glycosylation in the prosapsosin and SGP-1 repeats and in AOAH. Positions of conserved cysteine residues are indicated by asterisks. An additional cysteine in the small subunit of AOAH which may be a site of disulfide linkage with the large subunit is indicated by @. Gaps inserted in the alignment are indicated by a dash (–).

carbohydrate residues, AOAH releases fatty acyl chains from LPS. The small subunit of AOAH may modulate AOAH–substrate interactions, perhaps by analogy to the saposins, by increasing the solubility of the lipid A region of LPS; the hydrolytic site would be in the large AOAH subunit. Pulmonary surfactant protein B, though lacking in enzymatic activity, resembles AOAH and the saposins by acting at a lipid–water interface. The second functional similarity is that the saposin activity and the enzymatic activity of sphingolipid hydrolases are, like the analogous activities of AOAH, in different polypeptide chains. However, the sphingolipid hydrolases and the saposins are products of separate genes. In the case of AOAH, the two activities are encoded by a single mRNA, and the two chains are covalently linked by a disulfide bond(s). The novel structure of AOAH also suggests that, unlike the sphingolipid hydrolases, AOAH activity may not require additional cofactors.

Each human saposin contains an N-linked glycosylation site which aligns with the N-linked glycosylation site in AOAH. While this glycosylation site is also conserved in rat SGP-1, which is secreted from rat Sertoli cells, it is possible that this site in human saposins is involved with mannose 6-phosphate targeting to lysosomes [a mutation in the saposin B glycosylation site causes an activator-deficient metachromatic leukodystrophy (Kretz et al., 1990)]. The apparently alternatively spliced portion of the AOAH message encodes the region of the small subunit which includes the N-linked glycosylation site. If the alternative splicing phenomenon is physiologically relevant, perhaps the two forms of AOAH differ in their localization within the cell or potential for secretion, in addition to possibly more generalized effects, such as affinity for substrate. The pattern of disulfide bond formation in the small subunit of AOAH is suggested by a comparison with its homologues. Each of the human saposins contains potential amphipathic helices separated by helix breakers to give a cylindrical hydrophobic domain probably stabilized by disulfide bridges (O'Brien et al., 1988). If the AOAH small subunit follows a similar pattern, we predict that six of the seven Cys residues in the small subunit are involved in intrachain disulfide bonding, that only one Cys in the small subunit is disulfide-bonded to the large subunit, and that this residue is Cys(123). The large subunit also has an odd number of Cys residues (nine), eight of which could then be involved in interchain disulfide bonding.

AOAH is an enzyme that removes secondary (acyloxyacyl-linked) acyl chains from the LPS moieties of a variety of bacteria, including several human pathogens (Erwin & Munford, 1990). In each case, acyloxyacyl hydrolysis greatly reduces the toxicity of the LPS. Found principally in phagocytic cells, the front line of host defense against invading bacteria, the enzyme may constitute a host mechanism for detoxifying LPS. It is also possible that the enzyme plays a role in the LPS signal transduction pathway or that it carries out other enzymatic activities. Cloning and expressing the cDNA for the enzyme is a major step toward testing these alternatives, as it should now be possible to obtain large quantities of recombinant AOAH, to determine the impact of administering AOAH on the responses of animals to Gram-negative bacterial infection, and to analyze the effects of under- and overproduction of the enzyme on the ability of cells and animals to interact with LPS.

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