Ether lipid biosynthesis: isolation and molecular characterization of human dihydroxyacetonephosphate acyltransferase

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Abstract In this paper we describe isolation and molecular characterization of human dihydroxyacetonephosphate acyltransferase (DAP-AT). The enzyme was extracted from rabbit Harderian gland peroxisomes and isolated as a trimeric complex by sucrose density gradient centrifugation. From peptide sequences matching EST-clones were obtained which allowed cloning and sequencing of the cDNA from a human cDNA library. The nucleotide-derived amino acid sequence revealed a protein consisting of 680 amino acid residues of molecular mass 77 187 containing a C-terminal type 1 peroxisomal targeting signal. Monospecific antibodies raised against this polypeptide efficiently immunoprecipitated DAP-AT activity from solubilized peroxisomal preparations, thus demonstrating that the cloned cDNA codes for DAP-AT.

Key words: Ether lipid biosynthesis; Plasmalogen; Dihydroxyacetonephosphate acyltransferase; Alkyldihydroxyacetonephosphate synthase

1. Introduction

Among the glycerophospholipids, ether phospholipids (EPLs) including plasmalogens (PMs) represent a special subclass distinguished by their ether and vinylether bond on C-1 of the glycerol moiety, respectively. Although biochemists have paid much attention to the ester-bonded glycerophospholipids, EPLs and PMs are much less explored. Except platelet activating factor (PAF) [1–4], which has been implicated in processes involving immune complexes, endotoxins and reperfusion, the physiological function of EPLs and PMs are not well understood. Jones and Hajra [5] were the first to show that dihydroxyacetonephosphate acyltransferase (DAP-AT), the key enzyme in EPL biosynthesis, is exclusively localized within peroxisomes. To date, it is firmly established that the first three enzymes of EPL biosynthesis, DAP-AT, alkyl-DAP synthase (ADAP-S) and most of acyl/alkyl-DAP-reductase (ADAP-R) are localized to peroxisomes, while parts of ADAP-R as well as the enzymes required to complete EPL and PM biosynthesis are all found within the endoplasmic reticulum (ER) [6–8]. ADAP-S which was recently cloned and sequenced [9] is synthesized with a peroxisomal targeting signal type 2 (PTS2) known to be sufficient to target a protein to peroxisomes [10].

PMs are strongly deficient in patients suffering from generalized peroxisomal disorders, such as the cerebro-hepato-renal (Zellweger) syndrome [11,12], in which functional peroxisomes are absent. This points not only to an important role of peroxisomes in EPL and PM biosynthesis but also to essential functions of EPLs and PMs during ontogenesis. Within the last years various functions were attributed to PMs. The surface charge and the particular geometry of these lipids reduce membrane fluidity [13] and influence the temperature of phase transition [14] and structure of phospholipids [15]. A potential role of PMs in protecting cells against oxidative stress has been suggested by Zoeller et al. [16]. A similar function PMs may exert in serum lipoproteins in which they occur in low amounts [17,18]. Another interesting aspect of PMs is their putative role in signal transduction. In several tissues PMs have been demonstrated to predominantly contain at their sn-2 position arachidonic acid which is released upon proper stimulation [19,20]. Thus, PMs may function as membrane-localized stores of arachidonic acid. Consistent with this idea is the presence of a Ca^{2+}-independent PM-selective phospholipase A_2 which has been identified in cytosolic fractions of the canine myocardium [21–23] as well as in neural membranes [24] suggesting that the breakdown of PMs is a receptor-mediated process delivering potential second messengers, such as arachidonic acid and eicosanoids.

In the present paper we describe morphology and isolation of peroxisomes of the Harderian gland of the rabbit, one of the richest sources for DAP-AT [25–27], isolation of the active enzyme from these peroxisomes as well as cloning and sequencing of its cDNA from a human brain cDNA library. Biochemical as well as immunological studies suggest that DAP-AT and ADAP-S form a stable complex within peroxisomes that catalyzes ether bond formation. The molecular characterization of DAP-AT may offer great possibilities for future studies dealing with the physiological function of EPLs and PMs.

2. Material and methods

Harderian glands of male and female rabbits were obtained from a local breeder. The red portion of 100–120 glands (60 g wet weight of tissue) were homogenized in 0.25 M sucrose, 1 mM EDTA, 10 mM glycylglycine and peroxisomes isolated by Nycodenz density gradient centrifugation of the light mitochondrial fraction [28,29]. DAP-AT was extracted from these peroxisomes using the CHAPS-based solubilization buffer (500 μl/mg of protein) described by Webber and Hajra [30]. The samples were sonicated (Branson Sonifier B-30, Branson, Danbury, CT, USA) at 30% duty and output 3 for 3×20 s on ice before pelleting the CHAPS-insoluble membrane residue by high speed centrifugation. Rate sedimentation of CHAPS-extracted DAP-AT activity was performed by layering the solubilized enzyme on top of a continuous 15–45% w/w sucrose gradient containing 0.2% CHAPS and sedimenting DAP-AT activity at 150 000×*g* for 15 h

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in a RPV 50 vertical rotor (Hitachi, Tokio, Japan). The DAP-AT peak fractions were diluted and further purified by Mono Q anion exchange chromatography eluting DAP-AT activity by a linear 15–500 mM NaCl gradient.

The polypeptides of the pooled DAP-AT peak fractions eluted from the Mono Q column were separated by SDS-PAGE (2.7% C, 7% T). Three major components exhibiting molecular masses of 76, 72 and 69 kD were visualized. By tryptic digestion [31] the following peptide sequences were obtained: GISDPLSVFEQTEAAAR, EYVDPNNIFGNR, WNGWGYNDEK (76 kD); KWDEFEDILEE, NSVLNSEEIHYVVR, AIQEHPVVLLPSHR, EVFDTYLVPISISY, KILSENFGSIH (72 kD); ILSENFGSIH (69 kD). The 76 kD peptide sequences identified the polypeptide as being ADAP-S and the ILSENFGSIH sequence demonstrated that the 72 and 69 kD polypeptides are basically identical. According to the peptide sequences obtained from the 72 kD polypeptide the following expressed sequence tag- (EST-) clones were identified: AA316351, T77274, AA402300, AA478758 and AA425273. Except AA316351 and T77274, the clones contained overlapping sequences and clones AA316351 and AA425273 contained the 5′- and 3′-end of the cDNA, respectively. The peptide sequences obtained from the rabbit enzyme matched the corresponding human sequences of the EST-clones by 100%, suggesting a high homology of the rabbit and human primary structure. The lacking sequence between AA316351 and T77274 was amplified by PCR using a human brain cDNA library (Stratagen, Heidelberg, Germany) as a template. Oligonucleotide probes of the 5′- and 3′-ends of the identified cDNA were synthesized in order to screen the human brain cDNA library. Sequencing (PRISM Ready Reaction Cycle sequencing kit with AmpliTaq FS, PE Applied Biosystems and ABI 373 sequencing machine) of the obtained cDNA demonstrated identity to that assembled from the EST-clones.

Catalase and succinate dehydrogenase activities were assayed as described [28,32]. DAP-AT activity was determined as in [30] except total assay volume was reduced to 60 μl and the radioactive acyl-DAP collected on Whatman 3 mm filters was washed three times with decreasing concentrations of trichloroacetic acid (10, 5 and 1%) each for 5 min at 4°C.

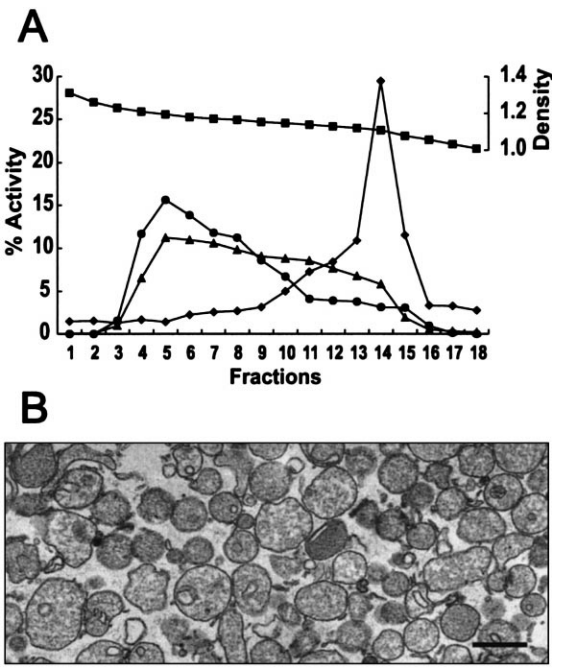

Fig. 1. Isolation of purified peroxisomes of the rabbit Harderian gland. A: The light mitochondrial fraction of the glandular tissue was separated by Nycodenz density gradient centrifugation. The gradient distribution of mitochondria and peroxisomes is demonstrated by the marker enzymes succinate dehydrogenase (■) and catalase (●), respectively. Distribution of DAP-AT activity is shown by (▲). B: Transmission electron micrograph of isolated Harderian gland peroxisomes pooled from fractions 4–6 of the Nycodenz gradient demonstrating the high state of purification. Bar = 0.5 μm.

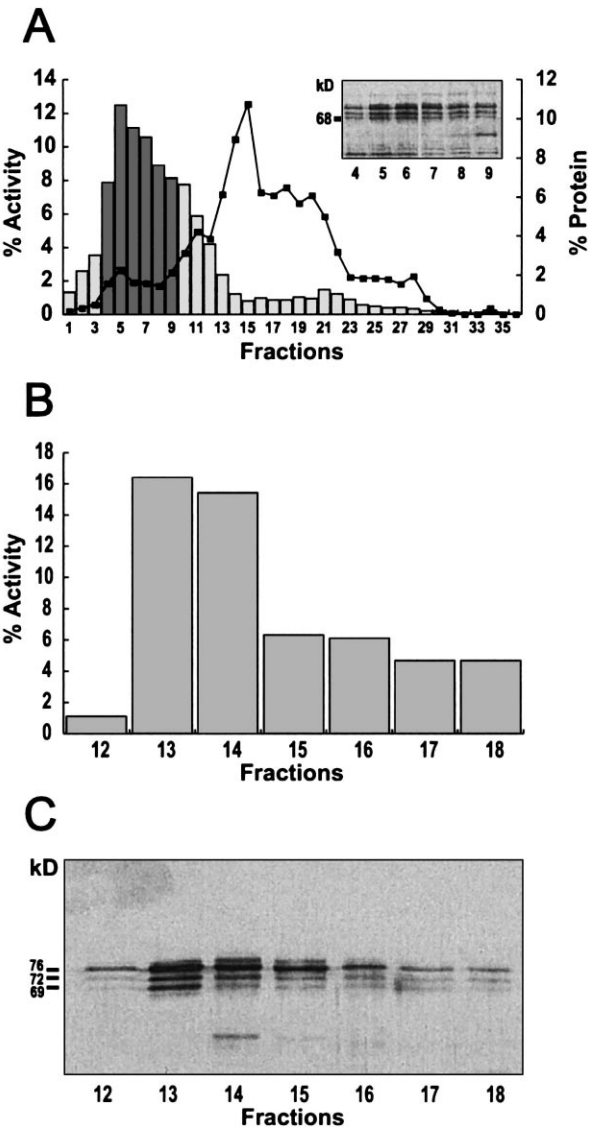

Fig. 2. Sedimentation of DAP-AT activity extracted from Harderian gland peroxisomes following sucrose density gradient centrifugation in the presence of 0.2% CHAPS in the gradient solution. A: Distribution of DAP-AT activity and protein (■). The polypeptide composition of DAP-AT peak fractions 4–9 is visualized by SDS-PAGE and silver staining (inset). DAP-AT peak fractions from the gradient (fractions 4–6) were pooled and separated by anion exchange chromatography. The elution profile of DAP-AT activity (B) is compared with the polypeptide composition of the eluted fractions after SDS-PAGE and silver staining (C). Note that DAP-AT activity coelutes exactly with the 72 kD polypeptide.

Monospecific anti-DAP-AT and anti-ADAP-S antisera were raised in rabbits by injection of their C-terminal peptides, C-EMLGCKKPIGKPAT and C-KSVKEYVDPNNIFGNRNLL, respectively, (1 mg/rabbit) coupled to keyhole limpets hemocyanin [33]. Animals were boostered once or twice at three week intervals with half the amount of antigen. IgGs of the obtained antisera as well as control (preimmune) IgGs were coupled to CH-Sepharose 4B (2 mg IgGs/ml of gel) according to the manufacturer's recommendations (Pharmacia, Freiburg, Germany). Immunoprecipitation was carried out by incubating the CHAPS-extracted peroxisomal supernatant (1 μg of protein/25 μl) with 25 μl packed IgG-Sepharose for 30 min at 37°C. Experiments demonstrating the specificity of the immunoprecipitation reaction were performed by preincubating DAP-AT IgG-Sepharose

with 25 μg of DAP-AT tail peptide for 1 h at 4°C. DAP-AT activity bound to IgG-Sepharose was then determined as described above.

Isolated peroxisomes were processed for electron microscopy as described [28]. For ultrastructural immunolocalization of DAP-AT rat liver tissue was fixed in situ with 0.25% glutaraldehyde in 0.1 M Pipes-NaOH, pH 7.4, containing 2% sucrose. Tissue slices were stained with tannic acid (0.25% in Pipes-NaOH, pH 7.4) and uranyl acetate (1% in 0.05 M sodium maleate, pH 6) prior to embedding in LR-White.

3. Results

3.1. Isolation of DAP-AT

We used the Harderian gland of the rabbit as a rich source of DAP-AT activity to isolate a highly purified peroxisomal fraction (Fig. 1) which served as starting material for the isolation of the enzyme. Specific activity of DAP-AT in these peroxisomes was determined to be 1.8 mU/mg of protein which is about 500 times that in rat liver peroxisomes. The subsequent use of different chromatographic steps [30,34], (i) gel filtration (Superdex 200), (ii) anion exchange (Mono Q), (iii) hydroxylapatite, (iv) hydrophobic interaction (Phenylsuperose) and (v) chromatofocusing, allowed us to isolate DAP-AT as a homogeneous 72 kD polypeptide (results not shown). The amount of protein obtained, however, was to low to initiate peptide sequencing. Therefore, we decided to enrich the enzyme prior to chromatography by sucrose density gradient centrifugation. The analysis of this gradient is shown in Fig. 2A. Surprisingly, DAP-AT activity sedimented as a heterooligomeric complex with an apparent mass of about 210 kD that essentially consisted of three polypeptides of 76, 72, and 69 kD (inset in Fig. 2A). Separation of the DAP-AT peak fractions from the sucrose gradient by anion exchange chromatography revealed that the 72 kD polypeptide exactly coeluted with DAP-AT activity (Fig. 2B, C). After tryptic digestion of the three polypeptides we obtained peptide sequences which revealed that (i) the 76 kD species is identical with ADAP-S [9] and (ii) the 72 and 69 kD species share peptide sequences, suggesting that they are basically identical polypeptides.

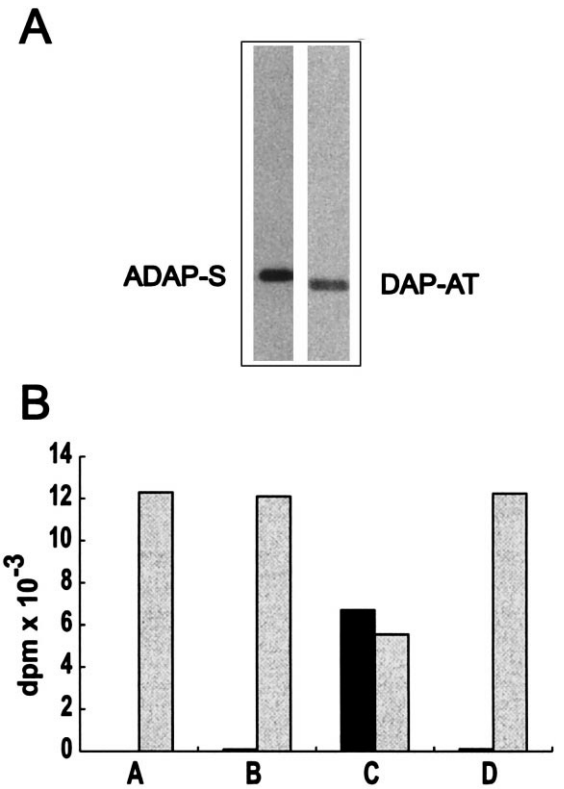

Fig. 3. Immunoprecipitation of DAP-AT activity with anti-DAP-AT antiserum. A: Monospecificity of anti-ADAP-S and anti-DAP-AT antisera, as demonstrated by SDS-PAGE and immunoblotting of Harderian gland peroxisomes (6 μg of protein). B: Immunoprecipitation of DAP-AT activity from Harderian gland peroxisomal CHAPS-extracts (15 μg of protein/25 μl of gel) using anti-DAP-AT IgGs immobilized to Sepharose 4B (lane C). DAP-AT activity immunoprecipitated by the antiserum is represented by black columns, whereas total DAP-AT activity as well as activity remaining in the supernate is shown in grey. Preincubation of anti-DAP-AT IgGs with DAP-AT tail peptide used for immunization strongly inhibited immunoprecipitation by anti-DAP-AT antiserum (lane D). IgGs of control serum (lane B) immobilized to Sepharose at the same concentration as anti-DAP-AT IgGs were unable to immunoprecipitate DAP-AT activty.

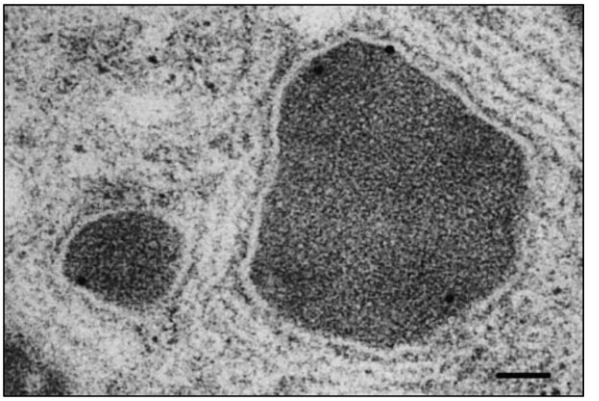

Fig. 4. Intraperoxisomal localization of DAP-AT by ultrastructural immunogold labeling. LR-White embedded rat liver sections were stained with anti-DAP-AT antiserum followed by 14 nm gold-labeled protein A. The gold particles are exclusively located to the inner aspect of the peroxisomal membrane. Bar = 0.1 μm.

3.2. Molecular characterization of DAP-AT

Based on the peptide sequences we screened cDNA databases and identified a large number of EST-clones from different species. Five clones of human species seemed to cover nearly the entire cDNA sequence. There were overlapping sequences between clones 2 and 3, 3 and 4, and 4 and 5. The missing sequence between clones 1 and 2 was amplified by PCR. The DAP-AT cDNA sequence put together from the EST-clones was confirmed by sequencing the cDNA obtained by screening a human brain cDNA library (Scheme 1). The sequence information is available at the EMBL data library under the accession number AJ002190 (locus; HSJ002190). From the nucleotide-derived amino acid sequence we noted that DAP-AT consists of 680 amino acid residues exhibiting a molecular mass of 77 187 kD. At the C-terminus DAP-AT contains a PTS1 represented by the tripeptide AKL which has also been found in various other peroxisomal matrix proteins [10,35]. Between amino acid residues 165–187, 382–395 and 482–498 there are extended hydrophobic domains which possibly mediate interaction of DAP-AT with the peroxisomal membrane.

C-terminal peptides of DAP-AT and ADAP-S were synthe-

Nucleotide sequence and and the predicted amino acid sequence of human DAP-AT

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  -157  gaattcggcacgagccgggatcctgtgtagcggctgcagagggtgccgccgccctaggcg   -98

   -97  aagtagggccgtcctgagcgaaagaaccgcccccagcaggagcaccaccacggcttagca   -38

   -37  aagaatcccagaccccgcccgggaaggcagccgcaccatggagtcttccagttcatctaa    22
aa    1                                        M  E  S  S  S  S  S  N    20

    23  ctcttatttctccgttggcccaaccagtcccagcgctgtcgtgctcctctactcgaagga    82
aa   21   S  Y  F  S  V  G  P  T  S  P  S  A  V  V  L  L  Y  S  K  E    40

    83  gctcaaaaagtgggatgagtttgaagatattttagaagagaggaggcatgtcagtgactt    142
aa   41   L  K  K  W  D  E  F  E  D  I  L  E  E  R  R  H  V  S  D  L    60

   143  gaaatttgcaatgaaatgctacacacctcttgtctataagggaattactccatgtaaacc    202
aa   61   K  F  A  M  K  C  Y  T  P  L  V  Y  K  G  I  T  P  C  K  P    80

   203  aattgatattaaatgtagtgttctcaattctgaggagattcattatgtcattaaacagct    262
aa   81   I  D  I  K  C  S  V  L  N  S  E  E  I  H  Y  V  I  K  Q  L   100

   263  ttccaaggaatcccttcaatctgtggatgtcctccgagaggaagtgagtgagatcttaga    322
aa  101   S  K  E  S  L  Q  S  V  D  V  L  R  E  E  V  S  E  I  L  D   120

   323  tgaaatgagtcacaaactgcgtcttggagccattcggttttgtgccttcaccctgagcaa    382
aa  121   E  M  S  H  K  L  R  L  G  A  I  R  F  C  A  F  T  L  S  K   140

   383  agtatttaaacaaattttctcgaaggtgtgtgtaaatgaagaaggtattcagaaactaca    442
aa  141   V  F  K  Q  I  F  S  K  V  C  V  N  E  E  G  I  Q  K  L  Q   160

   443  aagagccatccaggagcatcctgttgttctgctgcctagtcatcgaagttacattgactt    502
aa  161   R  A  I  Q  E  H  P  V  V  L  L  P  S  H  R  S  Y  I  D  F   180

   503  cctcatgttgtcttttcttctatacaattatgatttgcctgtgccagttatagcagcagg    562
aa  181   L  M  L  S  F  L  L  Y  N  Y  D  L  P  V  P  V  I  A  A  G   200

   563  aatggacttcctgggaatgaaaatggttggtgagctgctacgaatgtcgggtgccttttt    622
aa  201   M  D  F  L  G  M  K  M  V  G  E  L  L  R  M  S  G  A  F  F   220

   623  catgcggcgtacctttggtggcaataaactctactgggctgtattctctgaatatgtaaa    682
aa  221   M  R  R  T  F  G  G  N  K  L  Y  W  A  V  F  S  E  Y  V  K   240

   683  aactatgttacggaatggttatgctcctgttgaatttttcctcgaagggacaagaagccg    742
aa  241   T  M  L  R  N  G  Y  A  P  V  E  F  F  L  E  G  T  R  S  R   260

   743  ctctgccaagacattgactcctaaatttggtcttctgaatattgtgatggagccattttt    802
aa  261   S  A  K  T  L  T  P  K  F  G  L  L  N  I  V  M  E  P  F  F   280

   803  taaaagagaagtttttgatacctaccttgtcccaattagtatcagttatgataagatctt    862
aa  281   K  R  E  V  F  D  T  Y  L  V  P  I  S  I  S  Y  D  K  I  L   300

   863  ggaagaaactctttatgtgtatgagcttctaggggttcctaaaccaaaagagtctacaac    922
aa  301   E  E  T  L  Y  V  Y  E  L  L  G  V  P  K  P  K  E  S  T  T   320
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Scheme 1.

sized to raise antibodies in rabbits. In the C-terminal DAP-AT peptide used for immunization the last three amino acids (PTS1) were omitted in order to avoid cross-reactivity with other PTS1 containing polypeptides. Similar to ADAP-S, primary structure of DAP-AT seems to be highly conserved within mammals. Human and mouse cDNAs, for example, are about 80% homologous to each other. Compared with the human sequence, the last 14 C-terminal amino acids of mouse DAP-AT only differ at position 670 (K/T). Monospecificity of these anti-DAP-AT and anti-ADAP-S antisera for the Harderian gland peroxisomal antigens is depicted in Fig. 3A. Subsequently, the antibodies were used to immunoprecipitate DAP-AT from Harderian gland peroxisomal CHAPS-extracts. As shown in Fig. 3B, anti-DAP-AT IgGs immobi-

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   923  tgggttgctgaaagccagaaagattctctctgaaaattttggaagcatccatgtgtactt   982
aa 321   G  L  L  K  A  R  K  I  L  S  E  N  F  G  S  I  H  V  Y  F   340

   983  tggagatcctgtgtcacttcgatctttggcagctgggaggatgagtcggagctcatataa  1042
aa 341   G  D  P  V  S  L  R  S  L  A  A  G  R  M  S  R  S  S  Y  N   360

  1043  cttggttccaagatacattcctcagaaacagtctgaggacatgcatgcctttgtcactga  1102
aa 361   L  V  P  R  Y  I  P  Q  K  Q  S  E  D  M  H  A  F  V  T  E   380

  1103  agttgcctacaaaatggagcttctgcaaattgaaaacatggttttgagcccctggaccct  1162
aa 381   V  A  Y  K  M  E  L  L  Q  I  E  N  M  V  L  S  P  W  T  L   400

  1163  aatagttgctgttctgcttcagaaccggccatccatggactttgatgctctggtggaaaa  1222
aa 401   I  V  A  V  L  L  Q  N  R  P  S  M  D  F  D  A  L  V  E  K   420

  1223  gactttatggctaaaaggcttaacccaggcatttggagggtttctcatttggcctgataa  1282
aa 421   T  L  W  L  K  G  L  T  Q  A  F  G  G  F  L  I  W  P  D  N   440

  1283  taaacctgctgaagaagttgtcccggccagcattcttctgcattccaacattgccagcct  1342
aa 441   K  P  A  E  E  V  V  P  A  S  I  L  L  H  S  N  I  A  S  L   460

  1343  tgtcaaagaccaggtgattctgaaagtggactccggagactcggaagtggtcgatgggct  1402
aa 461   V  K  D  Q  V  I  L  K  V  D  S  G  D  S  E  V  V  D  G  L   480

  1403  tatgctccagcacatcactctcctcatgtgctcagcttataggaaccagctgctcaacat  1462
aa 481   M  L  Q  H  I  T  L  L  M  C  S  A  Y  R  N  Q  L  L  N  I   500

  1463  ttttgtgcgcccatccttagtagcagtagcattgcagatgacaccagggttcaggaaaga  1522
aa 501   F  V  R  P  S  L  V  A  V  A  L  Q  M  T  P  G  F  R  K  E   520

  1523  ggatgtctacagttgctttcgcttcctacgtgatgtttttgcagatgagttcatcttcct  1582
aa 521   D  V  Y  S  C  F  R  F  L  R  D  V  F  A  D  E  F  I  F  L   540

  1583  tccaggaaacacactaaaggactttgaagaaggctgttacctgctttgtaaaagtgaagc  1642
aa 541   P  G  N  T  L  K  D  F  E  E  G  C  Y  L  L  C  K  S  E  A   560

  1643  catacaagtgactacgaaagacatcctagttacagagaaaggaaatactgtgttagaatt  1702
aa 561   I  Q  V  T  T  K  D  I  L  V  T  E  K  G  N  T  V  L  E  F   580

  1703  tttagtaggactctttaaaccttttgtggaaagctatcagataatttgcaagtacctttt  1762
aa 581   L  V  G  L  F  K  P  F  V  E  S  Y  Q  I  I  C  K  Y  L  L   600

  1763  gagtgaagaagaggaccacttcagtgaggaacagtacttggctgcagtcagaaaattcac  1822
aa 601   S  E  E  E  D  H  F  S  E  E  Q  Y  L  A  A  V  R  K  F  T   620

  1823  aagtcagcttctcgatcaaggtacctctcaatgttatgatgtattatcttctgatgtgca  1882
aa 621   S  Q  L  L  D  Q  G  T  S  Q  C  Y  D  V  L  S  S  D  V  Q   640

  1883  gaaaaacgccttagcagcctgtgtgaggctcggagtagtggagaagaagaagataaataa  1942
aa 641   K  N  A  L  A  A  C  V  R  L  G  V  V  E  K  K  K  I  N  N   660

  1943  taactgtatatttaatgtgaatgaacctgccacaaccaaattagaagaaatgcttggttg  2002
aa 661   N  C  I  F  N  V  N  E  P  A  T  T  K  L  E  E  M  L  G  C   680

  2003  taagacaccaataggaaaaccagccactgcaaaactttaataatcaacaaatagttatgg  2062
aa 681   K  T  P  I  G  K  P  A  T  A  K  L  *                        700
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Scheme 1 (*continued*).

lized to Sepharose beads are able to specifically precipitate DAP-AT, whereas immobilized IgGs of control serum failed to immunoprecipitate the enzyme. Incubation of anti-DAP-AT IgGs with DAP-AT C-terminal peptide prior to immunoprecipitation completely abolished binding of DAP-AT to the Sepharose-bound IgGs, demonstrating the specificity of the immunoprecipitation reaction.

3.3. Intraperoxisomal localization of DAP-AT

Biochemical studies on isolated rat liver peroxisomes indicate DAP-AT and ADAP-S to be localized to the inner aspect of the peroxisomal membrane [36,37]. Although in intact tissue several matrix enzymes, e.g. acyl CoA oxidase or bifunctional protein are clearly distributed within the matrix, in isolated peroxisomes they appear to strongly associate with

the membrane. For that reason we analyzed the intraorganellar localization of DAP-AT by ultrastructural immunocytochemistry. Fig. 4 demonstrates that in rat liver the enzyme is exclusively localized to the lumenal side of the peroxisomal membrane. This observation is in agreement with biochemical findings that demonstrate complete extraction of the enzyme by carbonate (not shown).

4. Discussion

During the initial state of our studies on the isolation of DAP-AT we followed the procedures of Webber and Hajra [30] and Ofman and Wanders [34]. These authors provide valuable data on the isolation and purification of DAP-AT from guinea pig liver and human placenta, including choice of detergent for enzyme extraction and the observation that the solubilized enzyme has an altered pH-optimum. As starting material for the isolation of peroxisomes we chose the Harderian gland of the rabbit which, like mammalian sebaceous glands, produces large amounts of EPLs [25,27] serving eye protection or sebaceous or pheromonal purposes.

Sucrose gradient centrifugation of CHAPS-extracted peroxisomes proved to be a favorable step for the enrichment of DAP-AT, since the enzyme obviously is solubilized as a trimeric complex which is completely separated by centrifugation from the vast majority of other peroxisomal proteins increasing the specific activity of DAP-AT about 5-fold. The gradient distribution of the 72 kD polypeptide exactly followed the distribution of DAP-AT activity. Separation of DAP-AT peak fractions from the sucrose gradient by anion exchange chromatography again revealed that this polypeptide exactly co-eluted with DAP-AT activity, providing strong indication for their identity. The 69 kD polypeptide, also present in the complex, most likely is a modified form of DAP-AT, presumably generated by posttranslational modification which still has to be characterized.

The cDNA sequence obtained from the identified EST-clones as well as clones of the cDNA library screen coded for an unknown polypeptide. The C-terminal PTS1 [10,35] distinguishes the polypeptide as a peroxisomal one and suggests that the C-terminal AKL sequence mediates peroxisomal targeting of DAP-AT. Screening of protein databases revealed homologies of human DAP-AT with glycerol-3-phosphate acyltransferase (GP-AT) of wide species variety, including rat, mouse, *E. coli* and *Haemophilus influenzae*. The homologies are restricted to distinct domains of about 35–80 amino acid residues in length showing up to 78% similarities and up to 45% identities. Other homologous proteins include hypothetical 69 and 118 kD polypeptides of *Mycobacterium tuberculosis* and *Saccharomyces cerevisiae*, respectively.

The striking partial homologies of DAP-AT and GP-AT strongly underline that the cloned cDNA indeed codes for DAP-AT. Both enzymes catalyze the transfer of acyl residues from CoA to a glycerol derivative, thus homologous domains within these polypeptides were to be expected. Additional evidence that the cloned cDNA codes for DAP-AT is derived from immunoprecipitation of DAP-AT activity using immobilized IgGs of a monospecific peptide antiserum.

The primary structure of DAP-AT reveals several hydrophobic domains which may explain the membrane-bound character of the enzyme. Membrane association of the enzyme was already noted previously by the observations that the enzyme is not solubilized by sonication and only partially digested by exogenously added protease [36]. The present studies including ultrastructural immunocytochemistry confirm this view by demonstrating localization of DAP-AT to the inner aspect of the rat liver peroxisomal membrane. Since EL biosynthesis is initiated exclusively within peroxisomes, and subsequent steps required for its completion are localized to the ER, EL intermediates have to be transported from peroxisomes to the ER. Thus, it is tempting to speculate that the EL biosynthetic apparatus, including DAP-AT and ADAP-S, as well as an ADAP/alkyl-GP transporter build up a functional unit for the export of EL precursors. This transporter still needs to be identified.

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