Molecular and biochemical aspects of carnitine biosynthesis

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Chapter 4

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Molecular and biochemical characterisation of rat 4-N-trimethylaminobutyraldehyde dehydrogenase and evidence for the involvement of human ALDH9 in carnitine biosynthesis

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SUMMARY

The penultimate step in carnitine biosynthesis is mediated by 4-N-trimethylaminobutyraldehyde dehydrogenase (EC 1.2.1.47), a cytosolic NAD^+-dependent aldehyde dehydrogenase that converts 4-N-trimethylaminobutyraldehyde into 4-N-butyrobetaine. This enzyme was purified from rat liver and two internal peptide fragments were sequenced by Edman degradation. The peptide sequences were used to search the EST data base, which led to the identification of a rat cDNA containing an open reading frame of 1485 basepairs encoding a polypeptide of 494 amino acids with a calculated molecular mass of 55 kDa. Expression of the coding sequence in *Escherichia coli* confirmed that the cDNA encodes 4-N-trimethylaminobutyraldehyde dehydrogenase. The previously identified human aldehyde dehydrogenase 9 (EC 1.2.1.19) has 92% identity with rat 4-N-trimethylaminobutyraldehyde dehydrogenase and has been reported to convert substrates, which resemble 4-N-trimethylaminobutyraldehyde. When aldehyde dehydrogenase 9 was expressed in *Escherichia coli*, it exhibited high 4-N-trimethylaminobutyraldehyde dehydrogenase activity. Furthermore, comparison of the enzymatic characteristics of the heterologously expressed human and rat dehydrogenases with those of purified rat liver 4-N-trimethylaminobutyraldehyde dehydrogenase revealed that the three enzymes have highly similar substrate specificities. In addition, the highest V_{max}/K_m values were obtained with 4-N-trimethylaminobutyraldehyde as substrate. This indicates that human aldehyde dehydrogenase 9 is the 4-N-trimethylaminobutyraldehyde dehydrogenase, which functions in carnitine biosynthesis.

INTRODUCTION

Carnitine (3-hydroxy-4-N-trimethylaminobutyrate) is a vital compound, which plays an indispensable role in the transport of activated fatty acids across the inner mitochondrial membrane into the matrix, where β-oxidation takes place (1,2). Furthermore, carnitine is involved in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO_2 and H_2O in the Krebs cycle (3,4). Apart from the dietary intake of carnitine, most eukaryotes are able to synthesise this compound from 6-N-trimethyllysine (5-7). The 6-N-trimethyllysine is generated by the hydrolysis of proteins containing lysines that are trimethylated at their ε-amino group by a protein-dependent methyltransferase using S-adenosylmethionine as a methyl donor. In the carnitine biosynthetic pathway, 6-N-trimethyllysine is first hydroxylated at the 3-position by 6-N-trimethyllysine, 2-oxoglutarate dioxygenase, after which the resulting 3-hydroxy-6-N-trimethyllysine is cleaved by a specific aldolase into 4-N-trimethylaminobutyraldehyde (TMABA) and glycine (6,8). Subsequently, TMABA is oxidised by 4-N-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH) to form 4-N-trimethylaminobutyrate

(4-N-butyrobetaine) (9). In the last step, 4-N-butyrobetaine is hydroxylated at the 3-position by a second dioxygenase, 4-N-butyrobetaine, 2-oxoglutarate dioxygenase, yielding L-carnitine (5,10,7). In rat and mouse, 4-N-butyrobetaine, 2-oxoglutarate dioxygenase is exclusively localised in the liver, whereas in man, the enzyme is present in kidney, liver and brain. Although most tissues are capable of converting 6-N-trimethyllysine into 4-N-butyrobetaine, liver and kidney are the main sites of carnitine biosynthesis in all animals (11,10,12-14).

Kaufman and Broquist were the first to demonstrate that TMABA is an intermediate in the carnitine biosynthesis of *Neurospora crassa* using isotope labelling experiments and they suggested that an aldehyde dehydrogenase mediates its conversion to 4-N-butyrobetaine (6). Perfusion experiments showed that TMABA is readily absorbed by rat liver and converted to carnitine via 4-N-butyrobetaine, demonstrating the conservation of the dehydrogenation-step in higher eukaryotes (15). Subsequently, Rebouche and Engel showed that TMABA-DH activity was present in the cytosolic fraction of human liver, kidney, brain, heart and muscle homogenates (12). In the same year, Hulse and Henderson purified a cytosolic NAD^+-dependent aldehyde dehydrogenase from bovine liver showing maximum activity with TMABA, converting it into 4-N-butyrobetaine (9).

Except for the human 4-N-butyrobetaine, 2-oxoglutarate dioxygenase, which has recently been identified in our laboratory (16,17), none of the enzymes of the carnitine biosynthetic route have been characterised at the molecular level. We therefore purified the aldehyde dehydrogenase responsible for the conversion of TMABA to 4-N-butyrobetaine from rat liver and determined part of its amino acid sequence. Using this sequence information we identified the cDNAs encoding TMABA-DH from rat, human and mouse. Finally, we expressed the cDNAs in *E. coli* and compared the substrate specificities of the recombinant enzymes with those of the purified rat liver TMABA-DH.

EXPERIMENTAL PROCEDURES

Materials

4-Aminobutyraldehyde diethylacetal, 1,8-bis(dimethylamino)naphatalene, methyliodide, 2,4-dinitrophenylhydrazine, methanal, ethanal, propanal, butanal, pentanal, hexanal, heptanal, octanal and betaine aldehyde chloride were from Sigma (St. Louis, MO). NAD^+ and NADH were from Roche Molecular Biochemicals (Basel, Switzerland). Hexadecanal and octadecanal were synthesised as described earlier (18). SP-Sepharose fast flow and Red-Sepharose CL-6B were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden) and Hydroxylapatite CHT-II from Biorad (Hercules, CA). All other reagents were of analytical grade. The pMAL-C2X vector was purchased from New England Biolabs (Herts, United Kingdom).

Synthesis of 4-N-trimethylaminobutyraldehyde

4-Aminobutyraldehyde diethylacetal was trimethylated in ethylacetate using methyliodide in the presence of 1,8-bis(dimethylamino)naphatalene (proton sponge). The iodide salt of 4-N-trimethylaminobutyraldehyde diethylacetal precipitated together with the protonated proton sponge. This precipitate was subsequently dissolved in distilled water by heating the mixture in a boiling water bath. After slow cooling to room temperature, only the protonated proton sponge crystallised while 4-N-trimethylaminobutyraldehyde diethylacetal remained in solution. After removal of the proton sponge by filtration, the process was repeated five times in smaller volumes of distilled water to completely remove

the remainder of the proton sponge. Hydrolysis of the resulting acetal in 0.1 M HCl for 30 min at room temperature gave TMABA. Water and HCl were evaporated in a rotavapor and the TMABA was taken up in distilled water. In solution, TMABA was stable for at least three months at −20°C.

TMABA-DH assay

TMABA-DH activity was determined either spectrophotometrically or fluorometrically at 37°C by monitoring the formation of NADH using a centrifugal analyser (COBAS FARA; Roche, Basel, Switzerland). The assay mixture used in both methods contained 0.1 M sodium pyrophosphate buffer at pH 9.0, 0.5 mM NAD^+ and the enzyme sample in a final volume of 250 µl. The reaction was started by adding TMABA to a final concentration of 100 µM, unless otherwise indicated. In the spectrophotometric assay the increase in absorbance at 340 nm was measured and the activity calculated using 6220 M^{-1} cm^{-1} as the molar extinction coefficient of NADH. In the fluorometric method NADH formation was detected by measuring the fluorescence at 450 nm after excitation at 340 nm. Standard solutions of NADH were used for calibration.

Purification of TMABA-DH

Livers were taken from Wistar rats and homogenised by five strokes of a Teflon pestle in a Potter-Elvehjem glass homogeniser at 500 rpm in a 5 mM MOPS buffer, pH 6.0, containing 0.25 M sucrose, and 2 mM EDTA. The crude homogenate was centrifuged for 10 min at 800 × *g* at 4°C to remove nuclei and whole cells. The resulting post nuclear supernatant was centrifuged for 3 hours at 20.000 × *g* at 4°C to obtain the cytosolic fraction. The cytosolic fraction was applied to an SP-Sepharose fast flow column (Ø =2.8 cm, h=10 cm), which was pre-equilibrated with a 10 mM MES buffer, pH 6.0, containing 200 g/l glycerol and 1 mM dithiothreitol (DTT). Bound proteins were eluted with a linear gradient from 0 to 100 mM NaCl in the same buffer. Fractions containing high TMABA-DH activity were pooled and loaded onto a Red-Sepharose CL-6B column (Ø =0.8 cm, h=7.5 cm), which was pre-equilibrated with a 10 mM MES buffer, pH 6.0, containing 200 g/l glycerol, 1 mM DTT and 25 mM NaCl. Bound proteins were eluted with a linear gradient from 25 to 500 mM NaCl in the same buffer. Fractions containing TMABA-DH activity were pooled and dialysed against a 10 mM MES buffer, pH 6.0, containing 200 g/l glycerol, 1 mM DTT and 20 mM potassium phosphate. This dialysate was loaded onto an Econo-Pac Hydroxylapatite CHT-II column (Ø =1 cm, h=5 cm) equilibrated with the same buffer. Bound proteins were eluted with a linear gradient from 25 to 250 mM potassium phosphate. Fractions were tested for TMABA-DH activity and analysed by sodium dodecylsulphate acrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. SDS-PAGE and silver staining were performed as described by Laemmli (19) and Rabilloud *et al.* (20), respectively. Protein concentrations were determined by the method of Bradford (21), using bovine serum albumin as standard.

Protein digestion, Western blotting and automated Edman degradation

10 µg of the purified TMABA-DH was digested for one hour at 37°C with 0.05 µg of endoprotease Glu-C (Roche Molecular Biochemicals, Basel, Switzerland) in a 50 mM Tris-HCl buffer, pH 8.0, containing 0.01% SDS. Protein fragments were resolved on a 15% SDS-PAGE gel and a Multiphor II Nova Blot electrophoretic transfer unit (Pharmacia Biotech, Uppsala, Sweden) was used to transfer proteins onto a polyvinylidene di-fluoride (PVDF) sequencing membrane (Millipore, Bedford, MA) as described by the manufacturer of the transfer unit.

Proteins were visualised with Coomassie Brilliant Blue. N-terminal amino acid sequencing was performed using a Procise 494 protein sequencer.

Cloning, expression and purification of the rat TMABA-DH and ALDH9 in E. coli

The complete open reading frame (ORF) of TMABA-DH was amplified by the polymerase chain reaction (PCR) from rat liver cDNA using Advantage cDNA polymerase (Clontech, Palo Alto, CA) and the following primers: an *EcoRI*-tagged forward primer 5'-tatagaattcATGAGCACTGGCACCTTCG-3' and a *SalI*-tagged reverse primer 5'-tatagtcgacTTTTCAAAARGCWGAYTCCAC-3'. The degenerate nature of the second primer also allowed the amplification of the ALDH9 ORF from human liver cDNA using the same primer set. The PCR products were cloned downstream of the IPTG-inducible P_{TAC} promoter into the *EcoRI* and *SalI* sites of the bacterial expression vector pMAL-C2X, to express the TMABA-DH and ALDH9 as a fusion protein with maltose binding protein (MBP). The ORFs were sequenced to exclude sequence errors introduced by PCR after which the constructs were transformed to the *E. coli* strain BL21. Transformed cells were grown on LB-medium to an OD_{600} of 0.7 and IPTG was added to a final concentration of 1 mM to induce expression of the fusion protein. After two hours, cells were pelleted and lysed in 1/10 of the culture volume in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 200 g/l glycerol and 1 mM DTT by sonicating 2 times for 15 seconds at 8 W. The bacterial lysate was centrifuged for 10 min at 14.000 × *g* and the pellet was discarded. Fusion proteins were purified from the supernatant following the specifications of the manufacturer of the expression system (New England Biolabs) and stored at -80°C in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 200 g/l glycerol, 1 mM DTT and 3 mg/ml bovine serum albumin.

Characterisation of TMABA-DH

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) for TMABA and several other aldehydes were determined for the purified rat liver enzyme and the purified recombinant fusion proteins using the assay described above. The concentration of TMABA was determined with 2,4-dinitrophenylhydrazine as described by Ariga *et al.* (22). 4-Aminobutyraldehyde was freshly prepared from 4-aminobutyraldehyde diethylacetal as described by Kurys *et al.* (23). Its concentration was determined with *o*-aminobenzaldehyde as reported by Jakoby *et al.* (24). Because of the instability of 4-aminobutyraldehyde at alkaline pH, activity measurements with this compound as substrate were performed at pH 7.4 using a 0.1 M sodium phosphate buffer. For the determination of the K_m of NAD^+ and $NADP^+$, TMABA was used at a fixed concentration of 100 µM.

RESULTS

Purification of TMABA-DH from rat liver

In initial experiments high TMABA-DH activity (~3 nmol/min.mg) could be measured in crude rat liver homogenates. Subsequent measurement of TMABA-DH activity in subcellular fractions of rat liver showed that the activity was only present in the cytosolic fraction (results not shown). Therefore, rat liver cytosol was used as source of enzyme for the purification of TMABA-DH using liquid chromatography. An overview of the purification scheme is given in TABLE I. TMABA-DH activity was completely retained by all columns used and eluted as a single peak during all purification steps. Samples obtained after each purification step were analysed by SDS-PAGE followed by silver-staining (FIG. 1).

A single protein band with an apparent molecular mass of 55 kDa was observed after the last purification step. The purified enzyme was highly unstable, except when stored at -80°C in the presence of 1 mM DTT and 200 g/l glycerol. Even after several months of storage, no loss of activity could be measured.

TABLE I
Purification of TMABA-DH from rat liver

Purification step	Protein	Specific activity	Activity	Yield	Purification
	mg	*nmol/min.mg*	*nmol/min*	*%*	*-fold*
Post nuclear supernatant	1760	3.9	6867	100	-
20.000 × g supernatant	751	5.6	4206	61	1.4
SP-Sepharose fast flow	8.3	288	2390	35	74
Red Sepharose CL-6B	1.1	483	531	8	123
Hydroxylapatite CHT-II	0.6	772	463	7	198

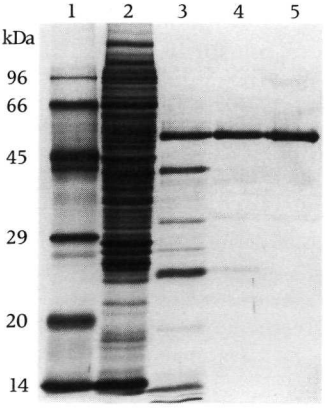

FIG. 1: Overview of TMABA-DH purification. Protein samples of the various purification steps were analysed by 12% SDS-PAGE followed by silver staining. Lane 1: molecular mass marker, lane 2: 20.000 × *g* rat liver supernatant, and pooled fractions of: lane 3: SP-Sepharose, lane 4: Red-Sepharose, lane 5: Hydroxylapatite CHT-II.

Identification of the cDNA encoding TMABA-DH

Attempts to directly sequence the protein by Edman degradation failed, suggesting that the N-terminus of TMABA-DH is blocked. Therefore, the purified enzyme was subjected to digestion with the endoprotease Glu-C to generate peptides with a free N-terminus. Peptide fragments were separated on SDS-PAGE, blotted onto a PVDF sequencing membrane and visualised by Coomassie Brilliant Blue staining. Two peptide fragments were N-terminally sequenced which resulted in the following sequences: E x I N N G K S I F E A and E A R L D V D T S (where x denotes an amino acid which could not be identified unambiguously). When the Swissprot data base was screened with these sequences, the only homology found was with the human aldehyde dehydrogenase 9 (ALDH9, EC 1.2.1.19, Swiss-Prot P49189). Subsequent searches in the EST (Expressed Sequence Tag) data base identified several rat, mouse, and human EST clones with high homology to the peptide sequences. The homologous human ESTs all corresponded to the ALDH9 cDNA (GenBank Accession Number: U34252) (25,26). Based on the EST data, primers were selected to amplify the ORFs from rat and mouse liver cDNA. The rat and mouse amplicons both contained an ORF of 1485 basepairs, coding for a polypeptide of 494 amino acids with a predicted molecular mass of 55 kDa (GenBank Accession Numbers: AF170918 and AF170919, respectively). The translated rat and mouse ORFs both have 92% positional identity with the human ALDH9

protein. The rat and mouse proteins are also highly homologous and share 96% positional identity. The two peptides obtained by Edman degradation were found to overlap, resulting in the following sequence: E x I N N G K S I F E A R L D V D T S. This sequence is identical to a 19 amino acid stretch in the N-terminal region of the rat sequence (95-114), demonstrating that the rat cDNA encodes TMABA-DH.

Expression of the rat ORF as MBP fusion protein in E. coli

The entire coding sequence of the rat cDNA was cloned into the pMAL-C2X expression vector and expressed in *E. coli* as a fusion protein with MBP. The fusion protein was purified from the *E. coli* lysate by affinity chromatography and TMABA-DH activity was measured. The purified fusion protein exhibited high TMABA-DH activity, which confirmed that the cDNA encodes TMABA-DH.

Expression of the ALDH9-MBP fusion protein in E. coli

Human ALDH9 has high homology with the identified rat TMABA-DH, and has been reported to dehydrogenate 4-aminobutyraldehyde and betaine aldehyde (23,27), which are compounds with considerable structural resemblance to TMABA (FIG. 2). Therefore, the ALDH9 ORF was cloned in the pMAL-C2X vector to express ALDH9 as an MBP fusion protein. Sequencing of the ALDH9 ORF revealed three additional nucleotides in a GC-rich stretch of the previously reported ALDH9 cDNA, altering the predicted amino acid sequence. This information can be accessed through the GenBank data base, GenBank Accession Number AF172093. The MBP-ALDH9 fusion protein was affinity-purified from *E. coli* lysate to determine whether ALDH9 was also active towards TMABA. The fusion protein exhibited high TMABA-DH activity, which indicates that ALDH9 is the human orthologue of rat TMABA-DH.

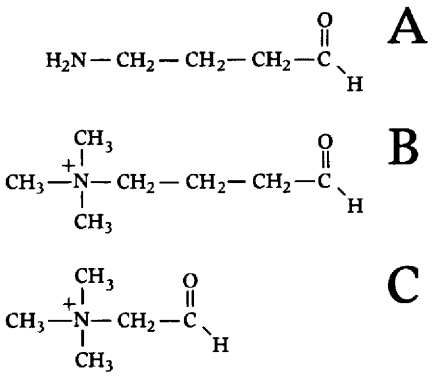

FIG. 2: Structure of (A) 4-aminobutyraldehyde, (B) TMABA and (C) betaine aldehyde.

Characterisation of the purified rat liver TMABA-DH and comparison with MBP-fusion proteins

To investigate if the purified rat liver TMABA-DH and the rat MBP-TMABA-DH could also handle the substrates reported for ALDH9, and to further characterise the substrate specificity of the three enzymes, their kinetic properties were determined. TABLE II shows the kinetic parameters of the purified rat liver TMABA-DH, rat MBP-TMABA-DH and MBP-ALDH9 with NAD^+, $NADP^+$, TMABA, 4-aminobutyraldehyde, betaine aldehyde and a range of aliphatic aldehydes as substrates. The K_m and relative V_{max} values of the purified

MBP-fusion proteins for the different substrates show a similar profile as the purified rat liver TMABA-DH. NAD^+ was by far the preferred oxidant for all substrates, although $NADP^+$ could also be used. The three enzymes have the lowest K_m for TMABA in combination with a high V_{max} value. As a consequence, the V_{max}/K_m ratio is highest for TMABA when compared to the other substrates. The presence of a free amino group in 4-aminobutyraldehyde instead of the trimethylated amino group in TMABA results in considerably lower efficiency. Betaine aldehyde, the carbon backbone of which is two atoms shorter than TMABA but which contains the trimethylated amino group, is readily oxidised to betaine as reflected in the high V_{max} values. The three enzymes have a high K_m for betaine aldehyde, however, which results in a substantially lower efficiency when compared to TMABA. For the aliphatic aldehydes in the C_2-C_8 range, the decrease in the K_m values is accompanied by a steady increase of V_{max}, showing that the efficiency of the enzymes is higher when the chain length of the aliphatic aldehyde increases. The efficiency of the enzymes with the longer aldehydes, hexadecanal and octadecanal, is very low if not undetectable.

DISCUSSION

In order to identify the enzymes of the carnitine biosynthetic pathway at the molecular level we previously purified rat liver 4-N-butyrobetaine, 2-oxoglutarate dioxygenase, the last enzyme in carnitine biosynthesis, and used protein sequence data in combination with the EST data base to identify the corresponding human cDNA (16). In this study the same approach was used to identify TMABA-DH, which mediates the penultimate step in carnitine biosynthesis. The enzyme was purified from rat liver to apparent homogeneity and used for peptide sequencing. The resulting peptide sequences were subsequently used to search the EST data base and two ORFs were identified from rat and mouse, encoding proteins with high homology to the previously reported human ALDH9. The following observations demonstrated that the identified rat cDNA truly encodes TMABA-DH. Firstly, the peptide sequence obtained by sequencing of the purified rat TMABA-DH exactly matched a 19 amino acid stretch in the translated coding region of the rat cDNA. Secondly, the cDNA encodes a protein with a calculated molecular mass of 55 kDa, which is in accordance with the apparent molecular mass of the purified rat liver TMABA-DH. Thirdly, heterologously expressed rat cDNA exhibited high TMABA-DH activity. Finally, the kinetic properties of the recombinant rat MBP fusion protein are highly similar to those of TMABA-DH purified from rat liver.

Although we did not express the mouse ORF in *E. coli*, it has 96% positional identity with the rat TMABA-DH and therefore most likely represents the mouse orthologue of rat TMABA-DH.

The rat TMABA-DH has high positional identity (92%) with human ALDH9. ALDH9 is a cytosolic NAD^+-dependent dehydrogenase belonging to the human aldehyde dehydrogenase gene family (28). It has been extensively investigated because of its proposed function in the alternative synthesis of the inhibitory neurotransmitter 4-aminobutyric acid (GABA) (29-31,26). In this pathway, diamine oxidase oxidatively deaminates putrescine (1,4-diaminobutane) to 4-aminobutyraldehyde, which is subsequently oxidised to GABA by ALDH9. The majority of the GABA in rat adrenal gland is produced via this alternative pathway, whereas the GABA in brain is predominantly synthesised from glutamate by glutamate decarboxylase (32). Both the physiological importance of the conversion of putrescine to GABA and the function of the latter outside the central nervous system is not well understood and remains to be established.

TABLE II

Substrate specificity of purified TMABA-DH and heterologusly expressed enzymes

Enzyme assays were performed as described in "Experimental Procedures" and results are the mean of 3 independent measurements. V_{max} is expressed as percentage of the maximal velocity measured with TMABA. In the measurements with $NAD(P)^+$ 100 µM TMABA was used as substrate. Measurements with 4-aminobutyraldehyde were performed in a 0.1 M sodium phosphate buffer, pH 7.4. The V_{max} values for this substrate are based on measurements with 100 µM TMABA as substrate at the same pH.

Substrate		rat liver TMABA-DH			rat-MBP-TMABA-DH			MBP-ALDH9		
		K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
		µM	%		*µM*	%		*µM*	%	
NAD^+		28	-	-	49	-	-	68	-	-
$NADP^+$		1630	-	-	4230	-	-	4695	-	-
TMABA		1.4	100	71.40	2.6	100	38.46	4.8	100	20.83
4-Aminobutyraldehyde		24	20	0.83	31	34	1.10	28	20	0.71
Betaine aldehyde		123	226	1.84	118	250	2.12	182	129	0.71
Acetaldehyde	(C_2)	102	7	0.07	115	16	0.14	175	20	0.11
Propionaldehyde	(C_3)	16	8	0.50	34	21	0.62	48	11	0.23
Butyraldehyde	(C_4)	13	15	1.15	12	39	3.25	13	16	1.23
Pentanal	(C_5)	10	16	1.60	11	36	3.27	10	21	2.10
Hexanal	(C_6)	9	18	2.00	8	47	5.88	8	21	2.63
Heptanal	(C_7)	7	18	2.57	4	66	16.50	7	20	2.86
Octanal	(C_8)	6	28	4.67	7	52	7.43	7	29	4.14
Hexadecanal	(C_{16})	9	2	0.23	n.d.	n.d.	-	n.d.	n.d.	-
Octadecanal	(C_{18})	n.d.[a]	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-

[a] n.d. = not detectable

More recently, ALDH9 has also been implicated in the synthesis of betaine. Betaine can serve as a methyldonor in the biosynthesis of methionine and has also been proposed to be involved in the regulation of the osmolarity in the kidney during antidiuresis (33-35). For the synthesis of betaine, choline is oxidised by choline dehydrogenase to betaine aldehyde, which is subsequently converted to betaine by ALDH9 (27,36). In human tissues, betaine aldehyde dehydrogenase activity is predominantly found in liver, adrenal gland and kidney. Northern blot analysis has shown the presence of the ALDH9 mRNA in liver, kidney, skeletal muscle, heart, brain, pancreas, lung and placenta (31,26,36).

The high homology with rat TMABA-DH and the structural resemblance of the substrates of ALDH9 with TMABA prompted us to study whether human ALDH9 is in fact the human TMABA-DH. The finding that the recombinant human MBP-ALDH9 fusion protein exhibits high TMABA-DH activity suggests that human ALDH9 is, indeed, the human TMABA-DH. Since ALDH9 has been reported to oxidise betaine aldehyde and 4-aminobutyraldehyde, the kinetic properties of the two recombinant MBP fusion proteins and the purified rat liver TMABA-DH were also determined for these, and other substrates. Like the ALDH9 MBP fusion protein, both rat liver TMABA-DH and the rat TMABA-DH MBP fusion protein oxidised 4-aminobutyraldehyde and betaine aldehyde. However, when considering both affinity and maximal velocity, TMABA is clearly the best substrate for all three enzymes.

Recently, the three-dimensional structure of cod liver ALDH9 has been determined at 2.1 Å resolution by X-ray crystallography (37). This protein has 70% positional identity with the human ALDH9 and is considered to be the cod orthologue of human ALDH9. The structural information revealed that the active site of cod ALDH9 is capable to handle larger aldehydes than betaine aldehyde, which is in accordance with our results which reveal that the three forms of ALDH9 studied in this paper show the highest V_{max}/K_m ratio for straight-chain aldehydes with a length of 7/8 carbon atoms. The preference for longer aldehydes also explains the relatively low K_m values for TMABA and 4-aminobutyraldehyde opposed to the high K_m value for betaine aldehyde. The high V_{max} value of the enzymes for betaine aldehyde is difficult to explain on the basis of the data presented here. Additional research is needed to understand this phenomenon.

Further investigation of the active site of cod ALDH9 showed that there is no negatively charged residue in the substrate pocket that interacts with the trimethylated amino-group of betaine aldehyde. Instead, a hydrophobic interaction has been proposed between a tryptophan residue and the trimethylamino group of betaine aldehyde (37). If the nature of the interaction with the amino-group of the substrate is hydrophobic instead of electrostatic, the reason for a higher K_m value for 4-aminobutyraldehyde than for TMABA could be that the positively charged amino group in 4-aminobutyraldehyde is shielded by three methyl groups in TMABA.

The high activity of the heterologously expressed human ALDH9 with TMABA and the highly similar substrate specificity of ALDH9 and rat TMABA-DH strongly suggest that the human ALDH9 is the human TMABA-DH. This is supported by the presence of high betaine aldehyde dehydrogenase activity in human kidney and liver and the ALDH9 mRNA in tissues that contain high TMABA-DH activity (12,26,36).

Although our data do not exclude an additional function of ALDH9 in GABA and/or betaine synthesis, the results presented in this paper indicate that ALDH9 is the predominant, if not exclusive aldehyde dehydrogenase which functions in carnitine biosynthesis.

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