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PURIFICATION AND CHARACTERIZATION OF A HUMAN LIVER ARYLACETAMIDE DEACETYLASE

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Arylacetamide deacetylation is an important enzyme activity in the metabolic activation of arylamine substrates to ultimate carcinogens, best described as a carboxylesterase/amidase type of reaction. A 7-fold variation in the Vmax of 2-acetylaminofluorene deacetylation in 24 human livers was observed. An acetylaminofluorene deacetylase was purified 90 fold from human liver microsomes by PEG-fractionation, anion exchange and hydrophobic interaction chromatography. The purified 45kD protein showed no amino acid sequence homology to other carboxylesterases, neither in its N-terminus nor in tryptic peptides. Antibodies raised against the deacetylase recognized the protein with high specificity. This report thus describes the first arylacetamide deacetylase in human liver.

1. INTRODUCTION

One of the best studied examples of a genetic variation of a drug metabolizing enzyme is the polymorphism of acetyl-CoA dependent N-acetyltransferase (NAT, EC 2.3.1.5), a cytosolic enzyme primarily responsible for the biotransformation of clinically important arylamine and hydrazine drugs and carcinogens [1]. The molecular mechanisms leading to a division of human populations into "slow" and "rapid" acetylators have been studied in this and other laboratories [2,3]. Although the products of enzymatic N-acetylation usually represent the endpoint of a metabolic detoxication pathway [4], some acetylated arylamides may be deacetylated and

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Abbrevations used: AAF: 2-acetylaminofluorene, AF: 2-aminofluorene, DAC: deacetylase, PEG: polyethylenegycol, PMSF: phenylmethylsulfonylfluoride, BHT: butylated hydroxylated toluene, CHAPS: 3-(3-Cholamidopropyl)dimethylammonio-1-propane sulfonate, TFA: trifluoroacetic acid, BPNPP: bis-p-nitrophenyl phosphate, NAT: N-acetyltransferase, DMSO: dimethylsulfoxide, DEAE-: diethylaminoethyl-, DTT: dithiothreitol, EDTA: ethylenediaminetetraacetic acid, AcN: acetonitrile, HPLC: high performance liquid chromatography, SDS: sodium dodecylsulfate, PAGE: polyacrylamide gel electrophoresis, KDL: kidney donor liver, PNPA: p-nitrophenylacetate.

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converted back to the former NAT substrates, thus influencing half life and toxicity of a given arylamine compound. The hydrolytic reaction of arylamide deacetylation is catalyzed by esterases, an enzyme family with broad and overlapping substrate specificity [5], which is contributing to the biotransformation of xenobiotics [6]. Of particular interest is the role of deacetylation in the metabolic activation of arylamine carcinogens [7]. Inhibition of this activity by organophosphates results in a drastic reduction of mutagenicity [8] and carcinogenicity [9], suggesting that deacetylation is mediated by a carboxylesterase type of reaction.

As an initial study towards the characterization of human liver arylacetamide deacetylation, we report on the purification of this enzyme activity from human liver, based on a sensitive HPLC assay for the deacetylation of 2-acetylaminofluorene.

2. MATERIALS AND METHODS

2.1. N-Arylacetamide deacetylase assay

For enzyme purification and characterization, 2-acetylaminofluorene deacetylation was assayed as follows: 2-acetylaminofluorene (AAF, final concentration 400 µM) in 90 µl 0.1M KPi pH 7.4, containing the appropriately diluted enzyme source, was incubated at 37°C for 30 min. Incubations were stopped by the addition of 10 µl 15% $HClO_4$. Reversed phase HPLC analysis was a modification of a previously published method [10]: After mixing and precipitating the protein by centrifugation, the tubes were loaded directly into an autosampler (Gilson model 231), programmed to inject 50 µl of each supernatant onto a column packed with Nucleosil 5-C18 (Macherey-Nagel, FGR). Samples were eluted at 2.0 ml/min with a mobile phase containing 20 mM $NaClO_4$ (pH 2.5) and acetonitrile (Lichrosolv, Merck, FRG) (70 : 30, w/w), and the compounds were detected by UV absorbance at 260 nm. Under these conditions, the retention times of AAF and its deacetylated metabolite 2-aminofluorene (AF) were 7.3 and 1.93 min, respectively. The sensitivity of the assay (injection volume 50 µl, signal to noise ratio 3 : 1) was 5 pmoles.

2.2. Enzyme purification

Human livers were obtained from kidney transplant donors (KDL), immediately shock frozen in liquid nitrogen and stored at -80°C. 25 - 100 g tissue was thawed and homogenized in TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) containing 0.1 mM PMSF, 0.02 mM BHT, 0.01 mM leupeptin. Microsomes were prepared by differential centrifugation and resuspended in 0.1 M KPi pH 7.4. After solubilization with an equal volume of 20 mM CHAPS in 0.1 M KPi pH 8.0, containing 10% glycerol, 1 mM EDTA, 0.1 mM DTT, the supernatant was fractionated with a 50% PEG 6000 solution. The 6-19% pellet was dissolved in 10 mM KPi pH 8.0, 10 % glycerol, 0.05% Triton X-100 (Serva, FRG), 1 mM EDTA, 0.1 mM DTT (buffer A) and applied to a DEAE-Sephacel column (2.6 ID x 12.5 cm, Pharmacia) equilibrated with buffer A at 30 ml/min. The flow-through containing the enzyme activity was applied to an octylsepharose CL 4B column (1.6 ID x 12.5 cm, Pharmacia), equilibrated with buffer A. The column was washed with 4 volumes of buffer A at 12 ml/h, and eluted with 10 mM KPi pH 8.0, 10% glycerol, 0.5% Triton X-100, 20% ethyleneglycol, 1 mM EDTA, 0.1 mM DTT (buffer B) at 4.2 ml/h. Active fractions were pooled and stored at -80°C.

2.3. N-terminal amino acid sequencing
Aliqouts of the octylsepharose pool were concentrated by ultrafiltration and further purified using an Aquapore RP-butyl column (3.2 mm ID x 15 mm, Brownlee Labs) with a linear gradient from 0% to 60% AcN in 0.1% TFA. Pure fractions were sequenced on a pulsed liquid phase sequencer A477 connected to a 120A PTH-amino acid analyzer (Applied Biosystems).

2.4. Internal sequences of tryptic peptide fragments
An aliquot of the octylsepharose-pool was further purified by preparative SDS-PAGE. After Coomassie Blue staining, the protein band corresponding to DAC was excised, electroeluted as in [11], digested with trypsin and peptides separated by HPLC [12]. Selected peptides were sequenced as described in 2.3.

2.5. Antibody production and immunoreaction on Western blots
Preparative SDS-PAGE [13] was used to further separate the proteins, the 45 kD band was visualized [14], excised and processed as described in [15]. Antigen preparations with complete Freund's adjuvans were injected intradermally into a rabbit. Boost injections followed 5, 9, 13 and 17 weeks later.
Electrophoretic transfer of SDS-PAGE separated proteins onto nitrocellulose [17], immunoreaction with rabbit antiserum and detection by autoradiography using ^{125}I-protein A were performed using standard protocols.

2.6. Enzymatic assays
Kinetic constants were determined by varying the AAF concentrations from 1 μM to 400 μM. Enzyme dilutions and incubation times were adjusted to ensure no more than 15% substrate conversion. Km and Vmax were obtained from linear regression according to Hofstee [17]. Protein content was determined with a dye binding assay in the presence of detergent, using bovine serum albumin as standard [18]. Hydrolysis of p-nitrophenylacetate was performed essentialy as described in [19].

3. RESULTS AND DISCUSSION

Lack of detailed information on human arylacetamide deacetylation prompted us to study this enzyme activity in human liver with the main goal of characterizing the responsible enzyme(s) and to prepare antibody and DNA probes in order to determine the influence of DAC on the clinical and toxicological consequences of polymorphic N-acetylation.

Of a series of acetylated products of NAT (sulfamethazine, p-aminobenzoic acid, 2-aminofluorene, procainamide, p-aminosalicylic acid) which were tested as possible candidates for deacetylation, only the model carcinogen 2-acetylaminofluorene (AAF) was a substrate for DAC, indicating i) that N-acetylation in fact is a final metabolic step in the detoxication for a majority of arylamine compounds and ii) that DAC plays an important role in the metabolic activation process of arylamine carcinogens. Purification and characterization of the enzyme(s) was therefore based on the newly developed and highly sensitive HPLC assay measuring the deacetylation of AAF.

On a purely mechanistic basis, the AAF-DAC activity is best described as an ester/amide hydrolysis type of reaction. The Spearman rank correlation coefficient of r= 0.90 in 24 adult human liver samples between AAF-DAC and the hydrolysis of PNPA, a prototype substrate for carboxylesterases [20], provided evidence for a relation between AAF-DAC and carboxylesterases. AAF-DAC was inhibited non competitively by the organophosphate BPNPP but was not inhibited by increasing concentrations of PMSF. The latter observation is in contrast to other carboxylesterases which were reported to be sensitive towards both inhibitors [21].

The degree of purity of DAC obtainable by two consecutive chromatographic steps depended mainly on the dimensions of the octylsepharose column and on the amount of DEAE-sephacel eluate applied to it. These two steps resulted in enzyme preparations ranging from 10 - 90 % purity. However, it was possible to consistently identify a 45 kD protein as AAF-DAC. The staining intensity of this band correlated exactly with the activity of the corresponding fractions. Since most of the so far characterized carboxylesterases have molecular weights of about 60 kD [22], this indicated another particularity of AAF-DAC. Table 1 summarizes a typical purification resulting in a 87 fold purification and a recovery of 4.2 % . The purity of this fraction was estimated to be more than 85 % as judged by silver-staining after SDS-PAGE. The DEAE-Sephacel anion exchanger did not retain AAF-DAC at a pH of 8.0. This

TABLE 1. Purification of arylacetamide deacetylase from human liver microsomes

PURIFICATION STEP	PROTEIN		ACTIVITY		PURIFICATION	RECOVERY
	mg total	mg/ml	nmoles/min/mg	nmoles total	fold	%
Human liver microsomes	236.3	8.75	11.5	2718	1	100
Solubilized microsomes	144.8	2.84	17.3	2499	1.5	92
PEG 6% supernatant	122.27	2.15	19.3	2412	1.7	88
PEG 19% pellet	59.5	1.19	29.8	1775	2.6	65
DEAE-Sephacel Anion Exchange	2.2	0.02	602.8	1326	59.0	49
Octylsepharose Hydrophobic Interaction	0.12	0.012	999.9	115	87	4.2

was in strong contrast to most other carboxylesterases which have pIs between pH 4.7 and 6.5 [22]. The break-through fractions from the anion exchanger could be loaded directly onto the hydrophobic interaction column. The behaviour of AAF-DAC on octylsepharose columns strongly suggested that AAF-DAC is a highly hydrophobic species, an observation which was further supported by the requirement of relative high concentrations of detergent and ethyleneglycol for the elution of the enzyme activity. The different steps of a representative purification are depicted in figure 1.

For N-terminal sequence determination of AAF-DAC, a desalting step on a Aquapore RP C4 column with gradient elution allowed the recovery of the enzyme in a volatile, detergent and salt free buffer, which was essential for successfull sequencing. The most striking result of analyzing the sequence of this DAC is the lack of homology to distinct carboxylesterases of 5 different species as shown in figure 2 [23-25]. It also revealed homology to signal sequences for protein insertion into the endoplasmic reticulum [26,27], consistant with its subcellular localization.

For tryptic digestion and antibody production, an upscaled purification procedure yielded an enzyme preparation of only 10% purity. After SDS-PAGE, the protein bands were visualized, excised and electroeluted with 24 % efficiency. After tryptic digestion

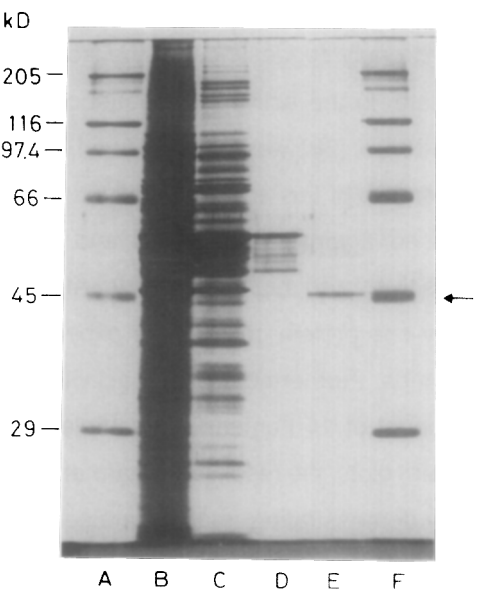

Figure 1. Silver stained SDS PAGE gel showing the different steps of AAF-DAC purification procedure. Lanes A and F: Molecular weight standards, Lane B: human liver microsomes, Lane C: Solubilized and PEG fractionated microsomes, Lane D: Pooled fraction after DEAE anion exchange chromatography, Lane E: Pooled and concentrated fraction after octylsepharose chromatography. The arrow indicates the 45kD DAC.

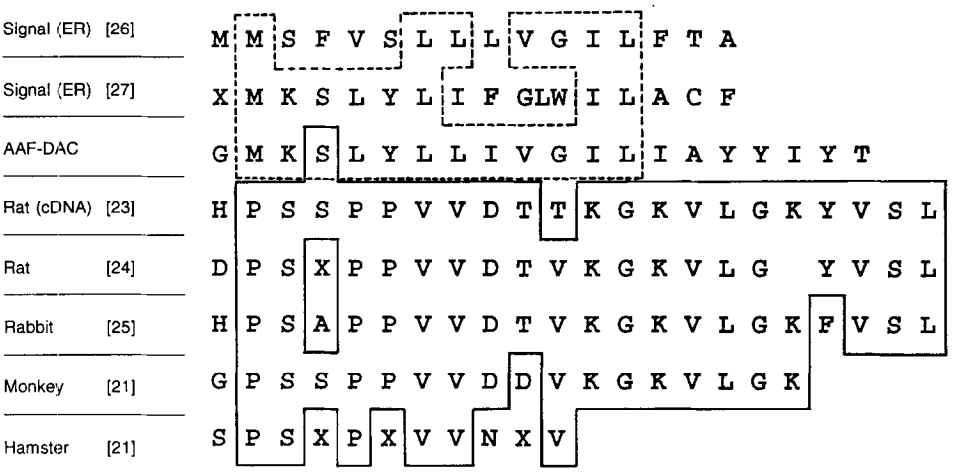

Figure 2. N-terminal amino acid sequence of AAF-DAC. As comparison the amino termini of carboxylesterases of rat [23,24], rabbit [25], hamster and monkey [21] isozymes and two signal sequences for protein insertion into the ER [26,27].

of approximately 1 nmol AAF-DAC, 15 of the approximately 30 peaks separated by HPLC were isolated and sequenced with recoveries between 8 and 46 % . A total of 119 amino acids including the N-terminus were determined, representing approximately 30 % of the complete amino acid sequence for DAC. Two observations are of interest: i) no homology with already known protein sequences in the databank at the EMBL were found, strongly suggesting that the isolated human AAF-DAC has not been sequenced to date. ii) the active site sequence motif commonly found in serine hydrolases -G-X-S-X-G- [28] was not observed, leaving the question open whether AAF-DAC is a member of this enzyme family.

The rabbit antiserum raised against the excised and denatured 45 kD antigen displayed both high sensitivity and high specificity. At a dilution of 1:1000, the antiserum recognized only one protein band of the expected size on Western blots from human liver microsomes. Furthermore, when activities ranging from 1.0 to 7.0 nmoles AF/min/mg of a panel of 14 human liver samples were compared with the signal intensities on Western blots, the resulting Spearman rank correlation coefficient was r = 0.93, convincingly demonstrating that the antiserum specifically recognized AAF-DAC. In conclusion, we have purified a 45 kD human liver arylacetamide deacetylase. Although the AAF-DAC activity is best described by carboxylesterases/amidases, we were not able to conclusively determine the relation between AAF-DAC and this group of enzymes. Cloning of the corresponding genes is presently in progress and will enable us to finally characterize the enzyme at the DNA level, to elucidate the role of arylacetamide deacetylation in the metabolic

activation of arylamine carcinogens and to determine its influence on polymorphic N-acetylation.

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