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Evidence for two closely related isozymes of arylamine *N*-acetyltransferase in human liver

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Acetyl CoA-dependent arylamine *N*-acetyltransferase (EC 2.3.1.5) is the target of a genetic polymorphism in the metabolism of drugs and carcinogens. *N*-Acetyltransferase was purified 1000-fold from cytosol of human liver and its identity was verified by amino acid sequence homology of two of its tryptic peptides with published rabbit and chicken *N*-acetyltransferase sequences. Enzyme activity correlated with the presence of two proteins, NAT-1 and NAT-2, with indistinguishable molecular masses (31 kDa). NAT-1 and NAT-2 could be separated by anion-exchange chromatography and were functionally distinguished by their different apparent affinities for the acceptor amine sulfamethazine (SMZ). Antibodies raised against NAT-1 were able to recognize both isozymes on Western blots.

Genetic polymorphism; Arylamine *N*-acetyltransferase; Purification; (Human liver)

1. INTRODUCTION

One of the first genetic polymorphisms found to affect the biotransformation of drugs and xenobiotics in man was that leading to interindividual variation in the *N*-acetylation of primary arylamine and hydrazine substrates [1,2], catalysed by the acetyl-CoA-dependent arylamine *N*-acetyltransferase (EC 2.3.1.5) present in highest levels in liver cytosol [3]. Although the clinical and toxicological consequences of the human acetylation polymorphism have been studied in considerable detail [4,5], as yet only limited data are available concerning its molecular basis. The present understanding of the mechanisms underlying polymorphic drug acetylation in human populations therefore relies largely upon extrapolation from animal model systems [5,6].

In the rabbit, a genetically determined polymorphism in liver *N*-acetyltransferase activity closely parallels that seen in man. Kinetic and immunological experiments [7,8] suggest the presence of a structurally altered *N*-acetyltransferase protein in slow acetylator rabbits with altered kinetic properties. A 33 kDa protein with *N*-acetyltransferase activity has recently been purified to homogeneity from liver of rapid acetylator rabbits [9]. However, it has not yet been possible to purify and to compare the proposed variant form from slow acetylator liver. An acetylation polymorphism is also observed in hamster populations, but the pattern of substrates affected by the genetic defect is essentially the inverse of that observed in man and rabbit: arylamines which are able to discriminate rapid and slow acetylators in the latter two species are unable to do so in hamsters. Hamster liver contains two distinct *N*-acetyltransferase enzymes, and the evidence so far suggests that variation in the liver content of only one of these forms is responsible for the observed in vivo phenotypic variations [10]. Purification of these genetically variable and constant isozymes has not yet been reported.

As a first step towards a direct understanding of the molecular basis of the human acetylation polymorphism, we have used a sensitive new

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HPLC assay for partial purification and characterization of arylamine *N*-acetyltransferase from cytosol of human liver.

2. MATERIALS AND METHODS

2.1. N-*Acetyltransferase assay*

Incubation conditions were essentially as described [11], using a final acetyl-CoA cofactor concentration of 100 μM. For enzyme purification, sulfamethazine (SMZ) was used as acceptor amine at 200 μM. The HPLC assay measuring AcSMZ formation was developed as a modification of a published procedure [12]. Reactions (90 μl volume) were stopped by addition of 10 μl of 15% $HClO_4$, samples were mixed and centrifuged to precipitate protein and tubes were loaded directly into an autosampler (Gilson model 231) programmed to inject 50 μl of each supernatant onto a reversed-phase column packed with Nucleosil 5-C18 (Machery-Nagel, FRG). Samples were eluted at 2.0 ml/min with a mobile phase consisting of 20 mM $NaClO_4$ (pH 2.5) and acetonitrile (88.5:11.5, w/w), and compounds were detected by UV absorbance at 254 nm. Under these conditions the retention times of SMZ and its metabolite AcSMZ were 3.0 and 4.2 min, respectively. AcSMZ for assay calibration was synthesized according to [13] and its purity was verified by HPLC. The sensitivity of the assay (injection volume, 50 μl; signal-to-noise ratio, 3:1) for the measurement of AcSMZ was 8 pmol, representing detection of 10% conversion of 1 μM SMZ or 0.01% conversion of 1 mM SMZ. Coefficients of variation (n = 3) averaged 1.4% for quantities of AcSMZ between 0.08 and 1.5 nmol.

2.2. *Enzyme purification*

Procedures were adapted from those reported for rabbit [9] and pigeon [14] liver *N*-acetyltransferases. Human liver [100–250 g, obtained from kidney transplant donors (KDL) and stored at −80°C after shock-freezing in liquid N_2] was thawed and homogenized in TEDK buffer (10 mM triethanolamine-HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) and cytosol was prepared by differential centrifugation. Solid ammonium sulfate was added to 55% saturation and the precipitated protein was redissolved and dialysed vs TEDK. The pH of the dialysate was adjusted to 5.5 with HCl, the sample was centrifuged, the precipitated protein was discarded and the supernatant was readjusted to pH 7.0 for application to a column (5 × 30 cm) of DEAE-Sephacel (Pharmacia) equilibrated with TEDK. The column was washed and then developed with a linear 2 l gradient from 50 to 200 mM KCl in the same buffer, active fractions (10 ml) pooled, concentrated by ultrafiltration and applied to a Sephacryl S-200 (Pharmacia) column (2.5 × 70 cm). Fractions (3 ml) with activity were pooled and concentrated, then passed through a Sephadex G-25 column equilibrated with TDKP buffer (TEDK without EDTA, and containing 10 mM phosphate) and applied to a hydroxyapatite column (Bio-Gel HTP, 1.6 × 15 cm) equilibrated with TDKP. Enzyme activity was eluted with a linear gradient from 10 to 300 mM phosphate in the same buffer, active fractions (2 ml) concentrated by ultrafiltration, dialysed vs TED buffer and applied to a column (1 × 6 cm) of CoA-Sepharose (Pharmacia) equilibrated with TED buffer. The column was developed with a linear gradient from 0 to 50 mM KCl in the same buffer. Fractions (1 ml) with activity were pooled, concentrated by ultrafiltration and either used directly for preparative SDS-PAGE [15] and analytical-scale chromatographic procedures, or stabilized with glycerol (to 10%) and bovine serum albumin (2 mg/ml) for activity measurements.

2.3. *Amino acid sequencing of* N-*acetyltransferase tryptic peptides*

The first peak of *N*-acetyltransferase activity eluting from a DEAE-Sephacel anion-exchange column, designated NAT-1 (fig.1) was pooled separately and further purified as described above. An aliquot of the NAT-1 activity pool was subjected to preparative SDS-PAGE (10% gel), protein in the excised 31 kDa band was digested with trypsin and peptides were separated by HPLC [16]. Selected peptides were sequenced using a gas-phase sequencer (model 470A, Applied Biosystems) and the phenylthiohydantoin derivatives determined using a reversed-phase HPLC system as in [17].

2.4. *Antibody production and immunoreaction on Western blots*

Another portion of the NAT-1 activity pool was run on a second SDS gel, excised 31 kDa gel band pieces being homogenized in complete Freund's adjuvant and injected directly into a rabbit. Further boosts with identical antigen preparations followed 4 and 6 weeks later. Electrophoretic transfer of SDS-PAGE-separated proteins onto nitrocellulose [18], immunoreaction with rabbit antiserum and detection by autoradiography using ^{125}I-protein A were performed using standard protocols.

2.5. *Enzyme kinetics*

Velocities of AcSMZ production were determined for a range of SMZ concentrations from 1 μM to 1 mM, with incubation times and enzyme dilutions adjusted to ensure no more than 15% substrate conversion. Kinetic parameters (K_m and V_{max}) were determined by linear regression after transformation of the data according to Hofstee [19]. Protein was assayed with a dye-binding method [20].

3. RESULTS AND DISCUSSION

A full understanding of the underlying molecular causes of genetic polymorphisms in drug-metabolizing enzymes requires the direct study of the human liver proteins taking part in the affected reactions [21,22]. Using a sensitive new HPLC assay, we undertook the purification of *N*-acetyltransferase with the goal of developing antibodies and DNA probes that could be used to compare genetically rapid and slow acetylator individuals with respect to enzyme content and function, levels of gene transcription and ultimately the exact nature of the gene mutations involved.

A total of 6 preparative-scale purifications were performed using two human livers (KDL 21, KDL

23) chosen from a pool of 35 livers on the basis of high cytosolic *N*-acetyltransferase activity. Chromatographic procedures were used in a variety of sequences, resulting in between 830 and 2400-fold purification with recovery of 0.3–48% of the original cytosolic activity. Table 1 summarizes a typical purification sequence. *N*-Acetyltransferase was a very minor component even of high-activity liver cytosol: after greater than 1000-fold purification, analytical SDS-PAGE showed that such preparations were still no more than about 10% pure. Moreover, after reaching this stage of purity, enzyme activity was rapidly lost unless high concentrations of glycerol and albumin were added for stabilization.

An important observation from the enzyme purifications was the presence of two separate peaks of enzyme activity, which we designated NAT-1 and NAT-2, after anion-exchange chromatography on DEAE-Sephacel (fig.1). The same two activity peaks were seen in a total of 12 independent anion-exchange column runs using extracts from four different human livers, and also when column fractions were analysed for enzyme activity using *p*-aminobenzoic acid, *p*-aminosalicylic acid, 5-aminosalicylic acid, procainamide, sulfamerazine, sulfadiazine or sulfapyridine as acceptor amine substrate. Omission of the ammonium sulfate or pH-shift precipitation steps did not alter this pattern, and when either peak was collected separately and reapplied to a second anion-exchange column it eluted again as a single peak in the expected position. During purifications where NAT-1 and NAT-2 were separated at the DEAE-Sephacel step, the activities showed identical behavior on subsequent size-exclusion columns and only partial resolution by chromatography on hydroxyapatite columns. Chromatofocussing of the separated activities [pH gradient 7–4 with Polybuffer (Pharmacia)] gave apparent p*I* values of 4.80 and 4.75 for NAT-1 and NAT-2, respectively, with very low recovery of both activities.

The low liver content and instability of native *N*-acetyltransferase prevented its complete purification in reasonable quantity for physicochemical and functional analysis and for development of antibody probes recognizing the enzymatically active protein. However, analytical SDS-PAGE of column eluate fractions during purification of NAT-1 showed that the silver-staining intensity of a protein band at 31 kDa correlated with *N*-acetyltransferase activity through all purification steps, regardless of their order. The 31 kDa protein was therefore purified in its denatured form by preparative SDS-PAGE and subjected to tryptic digestion and peptide microsequencing. Two tryptic peptides (P55, P50) showed 76 and 67% homology, respectively, with the amino acid sequences of tryptic peptides from purified rabbit

Table 1

Purification of *N*-acetyltransferase from human liver

Purification step	Protein		Activity		Purification (-fold)	Recovery (%)
	mg total	mg/ml	mU/mg	U total		
105000 × *g* supernatant	3921	11.6	0.582	2282	1.0	100
Ammonium sulfate precipitation	2684	22.0	0.853	2289	1.5	100
pH-shift precipitation	2079	18.0	1.01	2100	1.7	92
DEAE-Sephacel anion exchange	221	35.6	11.1	2453	19.1	107
Sephacryl S-200 gel filtration	20.5	0.5	70.3	1441	121	63
Hydroxyapatite	3.7	3.1	427	1580	734	69
CoA-Sepharose	0.77	0.35	551	424	947	19

Purification was from 100 g human liver KDL 21. In the purification above, the two peaks of activity eluting from the DEAE-Sephacel column (fig.1) were pooled together for the subsequent column procedures

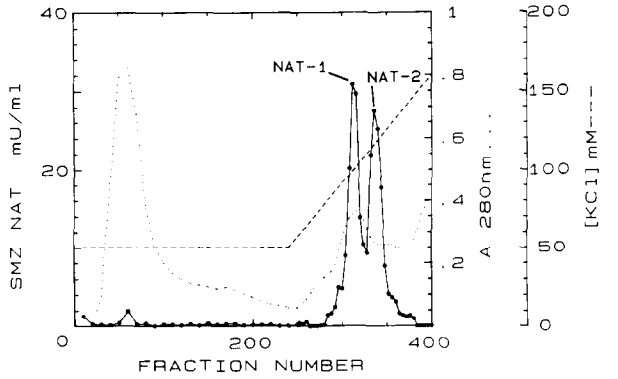

Fig.1. DEAE-Sephacel anion-exchange chromatography of *N*-acetyltransferase activity in extracts from human liver cytosol.

liver *N*-acetyltransferase [9] (fig.2). The latter were verified by comparison with the deduced sequence arising from a full-length rabbit liver *N*-acetyltransferase cDNA which we have recently isolated and functionally expressed in a COS-1 cell transient expression system (submitted). Significant homology was also observed with regions of the deduced amino acid sequence from a cDNA encoding chicken liver *N*-acetyltransferase [23]. Thus, the identity of the SDS-PAGE-purified 31 kDa protein as a human *N*-acetyltransferase was established. Attempts at amino-terminal sequencing of the intact protein using a glass fiber blotting method [24] were unsuccessful, suggesting N-terminal blockage of human *N*-acetyltransferase as has already been observed for the rabbit [9] and chicken [25] proteins.

Peak fractions of NAT-1 and NAT-2 from human liver KDL 21 differed in their affinity for SMZ at a fixed (100 μM) concentration of acetyl-CoA (fig.3). NAT-1 had a lower apparent affinity (K_m = 118 μM) than NAT-2 (62 μM) in the liver tested. When the acetyl-CoA cofactor concentration was varied at a constant (100 μM) level of acceptor amine, K_m values for cofactor binding were 80 and 130 μM, suggesting that the functional difference between NAT-1 and NAT-2 may relate to the efficiency of formation of the covalent acetyl-enzyme complex in the first half-reaction [26].

A rabbit antiserum raised against the SDS-denatured NAT-1 31 kDa antigen recognized bands on Western blots from both NAT-1 and NAT-2 anion-exchange peaks at an identical molecular mass (fig.3). The serum also specifically

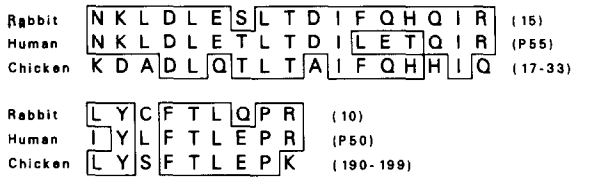

Fig.2. Comparison of tryptic peptide sequences from human NAT-1 with rabbit and chicken liver *N*-acetyltransferases. Numbers in parentheses identify rabbit peptides according to [9] and chicken sequences taken from the deduced amino acid sequence reported in [23].

reacted with purified rabbit liver *N*-acetyltransferase at a higher molecular mass (33 kDa) on Western blots (not shown). These results verify the antigenic relatedness of these three proteins and the higher molecular mass of the rabbit enzyme [9]. The antiserum was unable to inhibit or immunoprecipitate native *N*-acetyltransferase activity in either species.

Taken together, our observations suggest that arylamine *N*-acetyltransferase activity in human liver cytosol is associated with the presence of two proteins with very similar but distinct structural and functional properties. We do not know whether these isozymes are products of separate

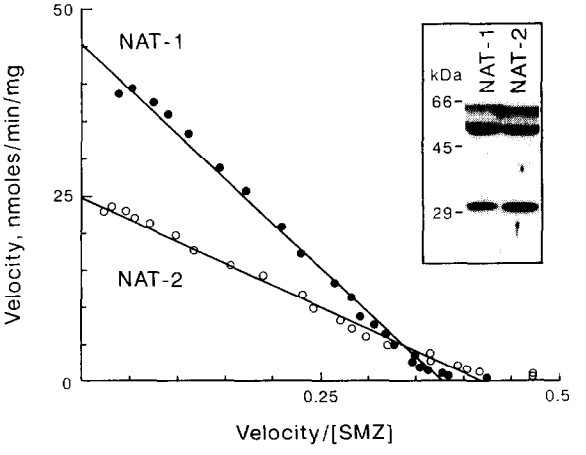

Fig.3. Kinetic analysis (Eadie-Hofstee plot) of *N*-acetyltransferase activities in NAT-1 and NAT-2 anion-exchange chromatography peaks using SMZ as acceptor substrate. Inset: immunoreaction on Western blots of NAT-1 and NAT-2 activity pools using a rabbit antiserum raised against SDS-denatured NAT-1 isolated from human liver. Apparent immunoreactivity at 53 kDa is due to direct binding of ^{125}I-protein A to human IgG heavy chains present in the impure enzyme preparations.

genes or whether one arises from the other via post-transcriptional or post-translational processing, but we have reason to believe that the two proteins are not allelic variants at a single heterozygous gene locus. We have recently isolated *N*-acetyltransferase clones from a λEMBL3 genomic library constructed using DNA obtained from a defined heterozygous rapid acetylator individual. Ultimately, definitive answers to the above questions will arise from a direct characterization of the gene(s) involved. These studies are currently in progress.

Of equal importance, in light of the differing models proposed from the results of the animal studies discussed above, is the question of how the quantity and/or activity of these two proteins relates to the functional expression of the rapid and slow acetylation phenotypes observed in human populations. The results of kinetic and immunological experiments designed to address this issue will be presented elsewhere.

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