Biochemical Pharmacology, Vol. 53, pp. 11–16, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00592-8

A Fragment Consisting of the First 204 Amino-Terminal Amino Acids of Human Arylamine *N*-Acetyltransferase One (NAT1) and the First Transacetylation Step of Catalysis

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ABSTRACT. Human arylamine *N*-acetyltransferase 1 (NAT1) has 290 amino acids and acetylates arylamines from acetyl coenzyme A. The acetyl group forms a thiolester with Cys 68 in the enzyme, and the acetyl group is then transferred to the arylamine. When NAT1 is expressed using the pGEX vector, the glutathione *S*-transferase (GST)–NAT1 fusion protein catalyses the acetylation of the NAT1 substrate *p*-aminobenzoic acid from acetyl CoA. Neither GST alone, nor a fusion protein of GST with the N-terminal 204 amino acids of NAT, catalyses the acetylation of *p*-aminobenzoic acid from acetyl CoA. Using [^{3}H]acetyl CoA as substrate, it is shown that the full-length NAT1 and the N-terminal 204 amino acids of NAT1 each form an acetylated intermediate on reaction with acetyl CoA. *Copyright © 1996 Elsevier Science Inc.*, BIOCHEM PHARMACOL 53;1:11–16, 1997.

KEY WORDS. arylamine; acetyltransferase; expression; NAT; GST fusion protein; purification

Arylamine NATs† that catalyse the *N*-acetylation of hydrazines and arylamine drugs and carcinogens have been described in many organisms, including insects [1], birds, and mammals (reviewed in [2]). The enzymes participate in detoxification of exogenous arylamines and in activation of hydroxylamines (reviewed in [3, 4]). In humans, there are two polymorphic NATs: NAT1 [5] and NAT2 [6]. The allelic variants of NAT2 conferring "slow" acetylation have been found at increased frequency in bladder cancer patients [7]. The NAT family members share a high degree of sequence identity, with over 60% identity between human NAT1 and chick NAT [2, 8]. A further family member of bacterial origin has been described: the *N*-hydroxyarylamine O-acetyltransferase of *Salmonella typhimurium* [9]. The sequence of the bacterial enzyme shows 25–33% identity with the eucaryotic NATs across the N-terminal 170 amino acids and catalyses activation reactions of hydroxylamines similar to those catalyzed by the human enzymes [10].

Pigeon liver NAT was the first NAT shown to have an essential sulphydryl group, and kinetic studies led to the first evidence of a ping pong Bi-Bi mechanism, with a cysteine thio-acetyl ester intermediate [11]. This is the generally accepted mechanism for NAT catalysis [11, 12]. The only conserved cysteine residue across all family members corresponds to residue 69 in the *S. typhimurium* enzyme and to the conserved Cys 68 in other NATs [2]. The importance of this cysteine has been confirmed by site-directed mutagenesis [9, 13]. In 1971, it was suggested that a general base was involved in catalysis [14], and the completely conserved Arg residue (corresponding to Arg 64 in human NATs) is a likely candidate [9, 15].

Human NAT1 and NAT2 are 87% identical in amino acid sequence [8, 16]. Although the human enzymes do have common arylamine substrates [3], they also have unique activities. Only NAT1 is able to catalyse the *N*-acetylation of the folate breakdown product *p*-aminobenzoylglutamate [17], which may be the endogenous substrate of human NAT1 [18], an enzyme expressed in a very wide range of tissues, including red blood cells [19] and monocytes [20]. *p*-aba is also a NAT1-specific substrate. The NAT2 isozyme appears to be involved primarily in metabolism of exogenous arylamines and hydrazines and is expressed in liver and intestinal epithelium. Sulphamethazine is a substrate specific for human NAT2. Comparison of the sequences of human NAT1 and human NAT2 shows that the amino acid differences are clustered in the C-terminal portion of the molecule, suggesting that this region of the molecule is responsible for amine recognition. To determine if this is so, a truncated version of NAT1 has been expressed and its activities have been investigated. It has been determined whether the truncated NAT1 is able to catalyse acetylation of arylamines and whether the trun-

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† *Abbreviations:* NAT, *N*-acetyltransferase; NAT1, human arylamine *N*-acetyltransferase 1; NAT2, human arylamine *N*-acetyltransferase 2; GST, glutathione *S*-transferase; IPTG, isopropyl-thio-β-D-galactoside; *p*-aba, *para*-aminobenzoate.

Received 20 May 1996; accepted 1 August 1996.

cated NAT1 is able to catalyse the formation of an acetylated enzyme intermediate.

MATERIALS AND METHODS

Enzyme Assays

NAT1 activity was determined by a spectrophotometric assay with *p*-aminobenzoate or sulphamethazine as substrate, as previously described [18]. Where necessary, samples to be assayed were diluted with 20 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing 5 mM dithiothreitol.

Protein quantification was carried out by a dye-binding method (Bio-Rad) using bovine serum albumin as standard.

Cloning and Expression of NAT1

DNA (1 μg) from a plasmid construct in pET5a containing NAT1 [18] was amplified by PCR using 2.5 U Pfu polymerase (Stratagene) and 50 pmol of each primer in a total volume of 100 μl. Thirty-five cycles of amplification were carried out as follows: 94°C, 5 min; followed by 35 cycles of annealing, which was at 54°C for 5 min in cycle 1 followed by 1 min for all other cycles; extension, 75°C, 1 min; and denaturation, 94°C, 30 sec. The primers 5′-AGGAGATGGATCCATGGACATTGAAG-3′ (sense) and 5′-CTTCAAGAATTCTAAATAGTAAAA-3′ (antisense) were for the whole coding region of NAT1. The DNA coding for the first 204 amino acids of NAT1 was amplified using the same sense primer and 5′-CTGCAGGTATGAATTCTAAGACTC-3′ as the antisense primer, incorporating a stop codon. Mismatches (shown in italics) introduce restriction sites for *Eco*RI and *Bam*HI (underlined). These constructs, termed NF290 and NF204, respectively, were each cloned into the pGEX-2T vector (Pharmacia, St. Albans, U.K.). The pGEX-2T vector alone was used as a control for the expression of GST. Transformants were screened for the presence of the inserts by PCR with NAT1-specific primers as above or as previously described [18], and the sequences of the inserts were determined as before [21] and were confirmed to correspond to NAT1*4 [2].

Escherichia coli NM554 cells containing one of the pGEX-2T constructs (encoding NF290, NF204, or GST alone) were cultured at 37°C for 2 hr, induced (0.1 mM IPTG, 4 hr, 37°C), and harvested by centrifugation (2500 × *g*, 4°C, 20 min). Cells from 200 ml of culture were resuspended (4°C) in 10 ml of 2.7 mM KCl, 137 mM NaCl, 10 mM sodium phosphate, pH 7.4, containing 100 μg/ml lysozyme (5 min). After addition of DNAse (5 min, 4°C), an aliquot (50 μl) was removed for determination of NAT1 activity; debris was removed by centrifugation (12,000 × *g*, 10 min, 4°C). The supernatant, which is referred to as bacterial cell lysate, contains soluble proteins. Insoluble proteins remain in the pellet.

Purification of Expressed NAT1

Glutathione agarose beads (200 μl of a 50% [v/v] slurry in 10 mM sodium phosphate, pH 7.5) were tumbled (20°C, 30 min) with the bacterial lysate supernatant and were then sedimented (500 × *g*, 5 min, 4°C) and washed three times with 1 ml of 10 mM sodium phosphate, pH 7.5, and 145 mM NaCl. Protein was eluted by incubating the beads three times (5 min, 4°C) with 200 μl of 10 mM glutathione in 50 mM Tris (pH 8.0). Proteins were then each dialysed into 20 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing 1 mM dithiothreitol; absorbance was monitored at 280 nm. Samples were concentrated with an Amicon 10 microconcentrator.

Expression and purification of the fusion protein of GST and NAT1 (NF290) was detected by NAT1 enzymic activity with *p*-aba as substrate [18]. GST and the fusion protein of GST with the NAT1 fragment (NF204) had no detectable activity with *p*-aba as substrate. SDS-PAGE analysis was carried out on all bacterial cell fractions and at each stage of purification of all expressed proteins.

Acetyl CoA Binding

[2-^{3}H]acetyl-enzyme intermediates were isolated by the method of Andres [22]. Incubations to determine the formation of acetylated intermediates were carried out as follows: [2-^{3}H]acetyl CoA (Amersham, Berkhampsted, U.K.) (50 μl, 125 nmol, 50 mCi/mmol) was incubated (20°C, 5 min) with affinity-purified NF290 (0.32 mg protein; approx. 5.4 nmol NF290), NF204 (0.75 mg protein; approx. 15 nmol NF204), or GST (0.4 mg protein; approx. 15 nmol GST) in 1 mM dithiothreitol, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, in a total volume of 100 μl. The reaction was terminated by the addition of 100 μl of SDS (1% w/v), and the mixture was chromatographed on a Sephadex G-50 column (1 × 46 cm, equilibrated in 10 mM NaH_2PO_4/NaOH, pH 6.8, 0.1% [w/v] SDS). A sample (0.7 ml) of each 1-ml fraction was added to 10 ml of ICN EcoLite (+) scintillant, and radioactivity was detected in a Beckman LS 6000IC scintillation counter. An external standard prepared in precisely the same way as the experimental samples was used to determine the tritium counting efficiency. The number of molecules of tritiated acetate bound in the protein peak was determined from the counting efficiency and the radioactivity associated with the fractions containing protein. The concentration of the purified expressed protein in each sample was estimated from Coomassie blue staining of SDS-PAGE. The molarity of the expressed proteins in the purified protein samples was calculated using molecular weights of 26 kDa for GST, 50 kDa for NF204, and 59.5 kDa for NF290. The proportion of molecules of protein with tritiated acetate bound was calculated by comparison of the molarity of bound acetate and the molarity of the protein sample.

RESULTS

Cloning and Expression

Full-length NAT1 has been cloned as a GST fusion protein using the p-GEX vector. The activity that was obtained in

the bacterial cell lysate supernatant was calculated to correspond to 12–15 μmol *p*-aba acetylated/min/50 ml bacterial culture, which corresponds very closely to the yield of NAT1 expressed from other expression vectors, including pET5a with no fusion partner [18]. The GST–NAT1 fusion protein was recovered in the bacterial cell lysate supernatant and was fully soluble. Less than 2% of the NAT1 activity was found associated with the bacterial cell pellet. No activity was found with sulphamethazine as substrate in either the bacterial lysate supernatant fraction or in the pellet.

The GST–NAT1 fusion protein could be affinity purified readily on glutathione-Sepharose, although there was some contamination with a protein of a similar molecular weight to GST (Figs. 1, 2b). Proteolysis of the GST–NAT1 fusion protein with thrombin resulted in a loss of activity that corresponded to the digestion of NAT1 into small fragments, as observed by SDS-PAGE (not shown). Therefore, the GST–NAT1 fusion protein was used without removal of GST for subsequent experiments.

A GST fusion protein was also expressed consisting of the first 204 amino acid residues of NAT1. The truncated NAT1 fusion protein was expressed in the lysate at a concentrations similar to the full-length NAT1 fusion protein and, like the full-length fusion protein, was soluble and was recovered in the supernatant fraction of the bacterial cell lysate. The shorter fusion protein could also be purified by affinity purification on glutathione-Sepharose (Fig. 1). The fusion protein, which consisted of only the first 204 residues of NAT1, was, like GST alone, unable to catalyse the acetylation of *p*-aminobenzoic acid either in the bacterial cell lysate or in the purified state.

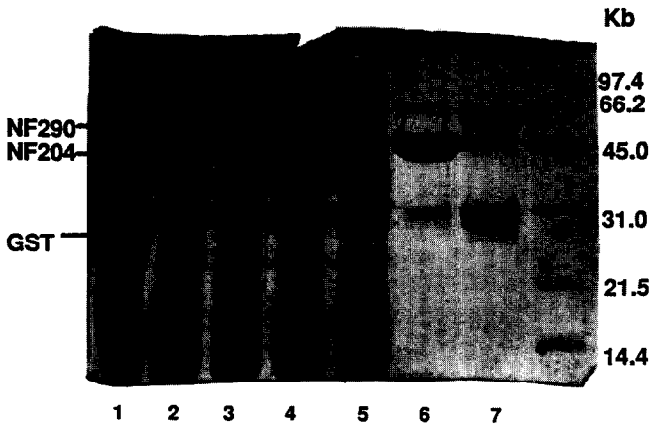

FIG. 1. Expression of GST fusion proteins of human NAT1 in *E. coli*. SDS-PAGE (12%) of total cell lysates (10 μl) (tracks 1–3) from *E. coli* containing the vector pGEX alone (track 1), the pGEX construct with NF204 (track 2), or the pGEX construct with NF290 (track 3). The lysate material that was not retained by glutathione-Sepharose from cells transfected with NF204 (track 4) and NF290 (track 5) is shown. The corresponding proteins were eluted with glutathione from glutathione-Sepharose. The protein concentration of the NF204 eluate (track 6, 10 μl) is 1.1 mg/ml, and the protein concentration of NF290 (track 7, 10 μl) is 0.4 mg/ml. The sizes of the molecular weight markers and the migration positions of expressed proteins are indicated. The gel is stained with Coomassie blue.

Acetyl CoA Binding

When [2-^{3}H]acetyl CoA was incubated with the full-length NAT1 protein fused to GST (NF290), radioactivity corresponding to the acetyl group was found to be associated with the full-length fusion protein after denaturation in 0.5% SDS and gel filtration (Fig. 2a, b). When GST alone was treated in the same way, no radioactivity was bound to the GST protein. The shortened NAT1 fusion protein with GST (NF204) behaved like the full-length NAT1 fusion protein. The polypeptide consisting of the first 204 amino acids of NAT1 bound radioactivity corresponding to the acetyl group of [^{3}H]acetyl CoA (Fig. 2). The radioactivity remained bound after denaturation of the protein in 0.5% (w/v) SDS and removal of unbound ligand by gel filtration on a Sephadex G-50 column in the presence of 0.1% (w/v) SDS.

The only protein bands that were eluted from the gel filtration column in the presence of 0.1% SDS were the fusion protein and a band corresponding to contaminant GST alone. Because GST alone did not bind the [^{3}H]acetyl group, it can be concluded that the acetyl group is bound to both the full-length and the truncated fusion proteins and remains bound to each of these fusion proteins under denaturing conditions. No proteins were eluted from the gel filtration column in the fractions corresponding to the unreacted acetyl CoA. In a separate experiment (not shown), free acetate was found to elute at fraction 24, well resolved from acetyl CoA and the expressed proteins.

DISCUSSION

N-acetyltransferase, when expressed as a fusion protein with GST, is able to catalyse the acetylation of *p*-aba. Therefore, the NAT1 moiety within the fusion protein is folded in a catalytically active form that shows the same specificity as NAT1 itself in that it catalyses the acetylation of *p*-aba but does not acetylate sulphamethazine.

A fusion protein consisting of GST and the first 204 amino acids of NAT1 has been expressed and is also soluble. This is in contrast to results in other studies that have been carried out, where up to 100 of the first amino acids of NAT1 have been expressed as GST fusion proteins. These fusion proteins have resulted in the formation of insoluble aggregates of expressed protein (J. Sinclair, unpublished).

The truncated fusion protein, which consists of the first 204 amino acids, although soluble, does not catalyse acetylation of *p*-aba. Whilst it could be argued that the truncated molecule is incorrectly folded, this seems unlikely, because the fusion protein consisting of the first 204 amino acids (NF204) is soluble and is recovered in the bacterial lysate supernatant fraction. NF204 is also able to catalyse the first step of the reaction with acetyl CoA as substrate. The

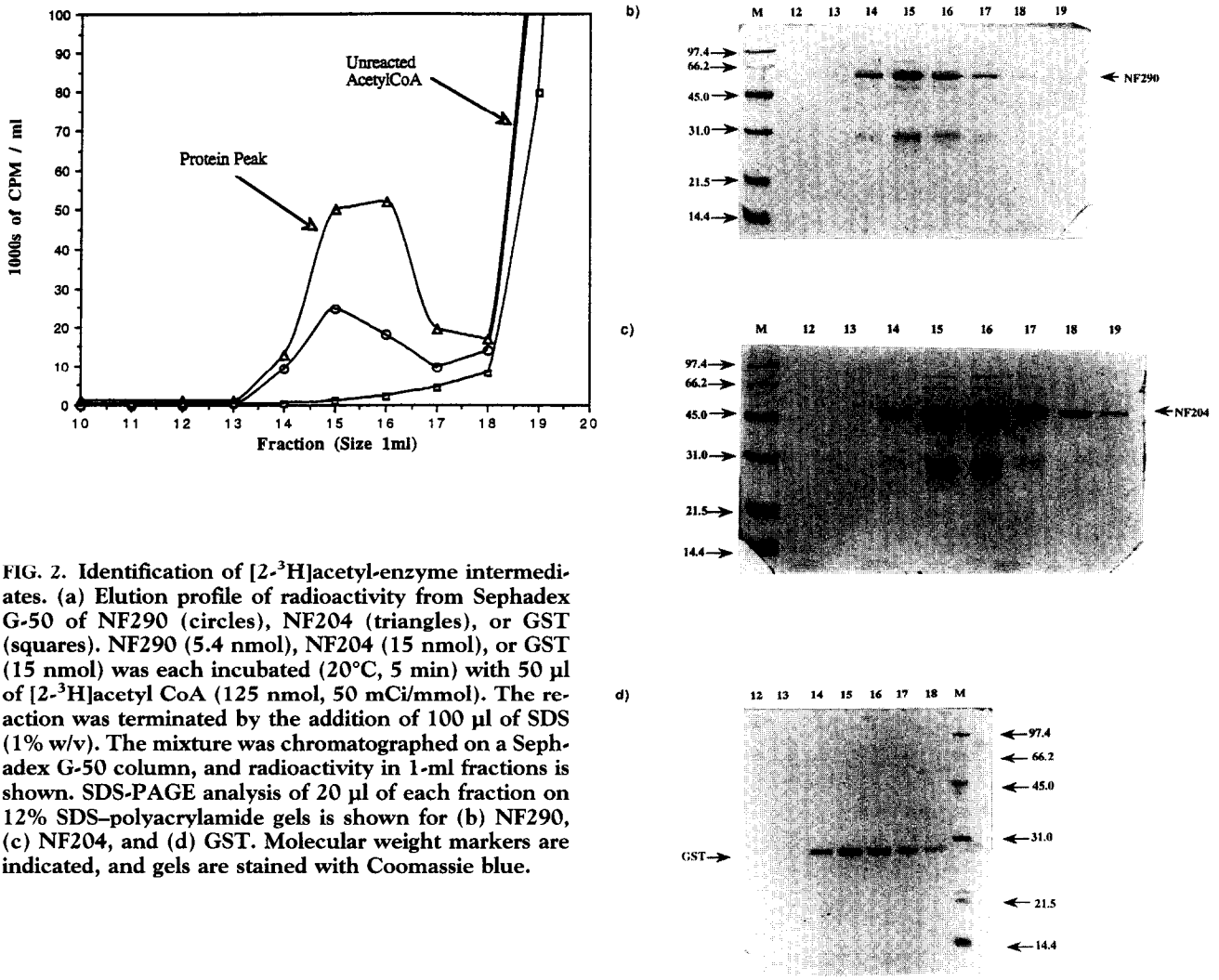

FIG. 2. Identification of [2-^{3}H]acetyl-enzyme intermediates. (a) Elution profile of radioactivity from Sephadex G-50 of NF290 (circles), NF204 (triangles), or GST (squares). NF290 (5.4 nmol), NF204 (15 nmol), or GST (15 nmol) was each incubated (20°C, 5 min) with 50 µl of [2-^{3}H]acetyl CoA (125 nmol, 50 mCi/mmol). The reaction was terminated by the addition of 100 µl of SDS (1% w/v). The mixture was chromatographed on a Sephadex G-50 column, and radioactivity in 1-ml fractions is shown. SDS-PAGE analysis of 20 µl of each fraction on 12% SDS–polyacrylamide gels is shown for (b) NF290, (c) NF204, and (d) GST. Molecular weight markers are indicated, and gels are stained with Coomassie blue.

proportion of molecules of the full-length and truncated fusion proteins that had an acetyl group bound were similar to each other in the experiments described in Fig. 2. Approximately 50% of all NF290 and 30% of all NF204 molecules had a bound acetyl group. These results are comparable to a similar previous study using NAT purified from livers of rapid acetylator rabbits [22]. In these previous studies, approximately 30% of the protein molecules were found to form an acetylated intermediate [22]; it was demonstrated by protein sequence analysis [22] that an acetylated enzyme intermediate is formed with the active site cysteine, and the active site cysteine corresponds in the homologous human NAT enzyme to residue 68 [13]. By comparison, in the present studies, it is likely that the acetyl group is bound to Cys 68 in the NAT1 moieties of the fusion proteins.

The first 204 residues of NAT1 are able to catalyse the first step in the acetylation reaction and can form an acetylated intermediate. The first 204 amino acids of NAT1 are therefore sufficient to bind acetyl CoA and to catalyse the transfer of the acetyl group to the protein in a form that is resistant to denaturation and migrates on gel filtration with the intact protein rather than with the smaller coenzyme or free acetate. Because the control protein, GST, does not bind radioactivity, nonspecific binding to the GST moiety of the fusion proteins can be discounted. The C-terminal portion of the NAT1 molecule appears to be unnecessary for acetyl CoA recognition and formation of the acetylated intermediate, and the high level of conservation in the N-terminal region of all known NATs [2] is indicative of an invariant coenzyme binding domain [23]. However, the C-terminal region of the NAT1 molecule appears to be essential for the second step of the reaction, namely the transfer of the acetyl group to the acceptor arylamine.

Further evidence for the C-terminus having a role in arylamine recognition is the clustering of sequence differences in the NAT enzymes in the C-terminal region [9], which accounts for the arylamine substrate specificity. Rabbit NAT1 and human NAT1 are both specific for acetylation of *p*-aba and rabbit NAT2, and human NAT2 are both specific for sulphamethazine. The two rabbit enzymes differ at only 13 amino acid positions [24], and at only two of these positions are the same mutations observed in the

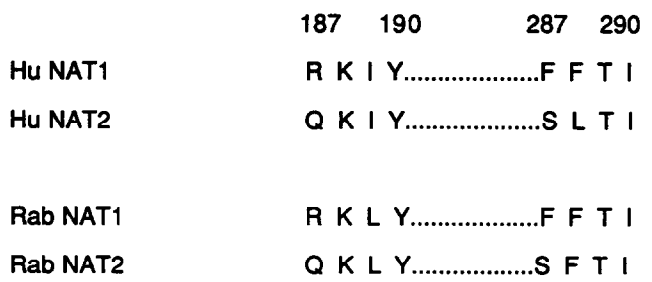

FIG. 3. Protein sequence comparison of human and rabbit NAT1 and NAT2. Numbering is from the first amino acid residue of each enzyme, and no gaps have been introduced. Human sequences are the NAT1*4 and NAT2*4 isozymes, and the rabbit sequences are the NAT1*2 and NAT2*2 isozymes [2, 24], all of which are encoded by the wild-type alleles.

human counterparts (Fig. 3). The positions at which there are the same amino acid substitutions in rabbit and human NAT1 compared with rabbit and human NAT2 are at positions 187 and 287. The NAT1 variants have R at 187 and F at 287, whilst the NAT2 variants have Q at 187 and S at 287, suggesting that these residues in the C-terminal region of the molecule are involved in determining the specificity of arylamine binding. The fragment of NAT1 (NF204) that has been expressed has R at 187, but this alone is insufficient to allow the fragment to catalyse the transfer of the acetyl group from the enzyme intermediate to the arylamine. The C-terminal portion, and in particular residue 287, may be extremely important for arylamine binding and conferring substrate specificity. Site-directed mutagenesis of residues 187 and 287 would be likely to resolve these questions. Preliminary studies [25] using combinations of inhibitors of acetyl CoA and arylamine binding have demonstrated that the two binding sites interact closely, even in the unacetylated molecule. Knowledge of the three-dimensional structure of NAT is required before the interaction can be understood.

We are very grateful to Alison Ward and Lesley Stanley for helpful discussion and to Aileen Watson for technical assistance. We thank the Wellcome Trust for generous financial support and the MRC for a studentship (J. S.).

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