0026-895X/04/6501-220–226$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:220–226, 2004

Vol. 65, No. 1
2860/1119550
Printed in U.S.A.

Human Acetyl CoA:Arylamine *N*-Acetyltransferase Variants Generated by Random Mutagenesis

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Received July 30, 2003; accepted October 8, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Acetyl CoA:arylamine *N*-acetyltransferase (NAT) enzymes catalyze the *N*-acetylation of aromatic amines and the *O*-acetylation of aryl hydroxylamines, reactions that govern the disposition and toxicity of many drugs and carcinogens. The human NAT genes and enzymes NAT1 and NAT2 are highly polymorphic and constitute one of the best studied examples of the genetic control of drug metabolism. Naturally occurring human NAT variants provide limited insight into the relationship between NAT amino acid sequence and enzyme activity. We have shown previously that the expression of recombinant NAT2 in bacterial tester strains results in greatly enhanced sensitivity to mutagenic nitroaromatic compounds (which are reduced to aryl hydroxylamines by bacterial enzymes). We hypothesized that random mutagenesis combined with rapid screening could be used to identify functionally significant amino acid residues in NAT enzymes. Pools of NAT2 variants were generated by polymerase chain reaction-mediated random mutagenesis of the complete coding sequence. Reversion induced by a NAT-dependent mutagen, 3-methyl-2-nitroimidazo[4,5-*f*]quinoline, was used as the basis for screening these pools to identify variants with altered enzyme activity. Eighteen variants were characterized by quantitative mutagenicity assays and enzyme kinetic measurements. This approach can provide new insight into the biochemistry of enzymes involved in the metabolic activation of mutagens.

N-Acetylation is a major route of xenobiotic biotransformation. Enzymes (*N*-acetyltransferases; NATs, EC 2.3.1.5) that catalyze *N*-acetylation of arylamines and hydrazines are found in organisms ranging from bacteria to humans (NAT1, NAT2) (Sim et al., 2003). The human NAT2 polymorphism represents one of the earliest identified pharmacogenetic variations in humans (Hein, 2000). Humans are divided about equally between "fast acetylators" and "slow acetylators". Acetylator status contributes to interindividual variation in drug response. For example, after treatment with isoniazid, NAT2 slow acetylators excrete larger amounts of the parent drug, relative to its acetylated metabolite, and face greatly increased risk of drug-induced neuropathy or hepatotoxicity (Ohno et al., 2000). Acetylator status has also been implicated in susceptibility to numerous diseases, including cancer (Brockmoller et al., 1998).

NAT2 knockout (Cornish et al., 2003) and NAT1-NAT2 double knockout (Sugamori et al., 2003) mice have recently been constructed; the absence of any phenotypic abnormalities shows that these enzymes are not essential for the development in that species. However, the mouse expresses three NAT enzymes (Estrada-Rodgers et al., 1998) rather than two, so the species may not be an appropriate model for human NAT pharmacology.

Although persons may be classified into NAT2 slow and rapid phenotypes, a continuous distribution exists, with a range of activities within both classes (Leff et al., 1999). To date, 29 variant human *NAT2* alleles have been identified, consisting of one or more of 13 single nucleotide polymorphisms (see www.louisville.edu/medschool/pharmacology/NAT.html). NAT2 phenotype is manifested in a number of ways, including alterations in protein expression, stability, and enzyme activity (Blum et al., 1991; Hein et al., 1994). Decreased expression of NAT2 protein bearing the amino acid substitution I114T, resulting from the T-to-C transition found in *NAT2*5* alleles, is probably the most common cause of the NAT2 slow-acetylator phenotype (Leff et al., 1999; Fretland et al., 2001).

Many *N*-Aryl compounds, including nitropolycyclic aromatic hydrocarbons and aromatic or heterocyclic amines, are

J.E.S. was the recipient of an Ontario Graduate Scholarship.

ABBREVIATIONS: NAT, *N*-acetyltransferase; NAT2, acetyl CoA:arylamine *N*-acetyltransferase; AF, 2-aminofluorene; DAVERAMA, detection of active variant enzymes by reversion assay/mutagen activation; LB, Luria broth; DMAB, *p*-dimethylaminobenzaldehyde; IPTG, isopropyl-β-D-thiogalactopyranoside; ML, minimal lactose; NF, 2-nitrofluorene; nitro-IQ, 3-methyl-2-nitroimidazo[4,5-*f*]quinoline; P450, cytochrome P450; PCR, polymerase chain reaction; ORF, open reading frame.

mutagens and carcinogens. Aryl hydroxylamines can be formed by the reduction of nitro compounds or by P450-catalyzed oxidation of amines (Guengerich, 2002). Acetyl CoA-dependent *O*-acetylation of such hydroxylamines (Hein et al., 1993) yields *N*-acetoxy esters, which undergo spontaneous heterolysis, generating DNA-reactive nitrenium ions (Parks et al., 2001). This bioactivation step is catalyzed by *O*-acetyltransferase enzymes (EC 2.3.1.118); in fact, most NAT enzymes also possess *O*-acetyltransferase activity.

We previously constructed a NAT knockout *Escherichia coli* strain that provides a clean background for the expression of recombinant human NAT (Josephy et al., 2002). This strain also carries a *lacZ* frameshift allele suitable for mutagen detection; *lacZ* revertants are selected by growth on lactose minimal medium. Expression of recombinant human NAT2 greatly increases the mutagenicity of nitroaromatic compounds and arylamines in this bacterial strain (Josephy et al., 2002).

The structure and mechanism of NAT enzymes are under study in several laboratories. A conserved cysteine residue at the active site of NAT enzymes accepts the acetyl group of CoASAc to form an acyl-enzyme intermediate and then transfers it to the acceptor substrate in a two-step substituted-enzyme ("ping-pong") mechanism. This mechanism was first demonstrated by chemical modification studies; site-directed mutagenesis identified the cysteine residue in NAT2 as Cys68 (Dupret and Grant, 1992). Other regions of the protein that contribute to substrate selectivity and protein stability have been identified through the construction of NAT1/NAT2 chimeras (Dupret et al., 1994), site-directed mutagenesis experiments (Delomenie et al., 1997), and analyses of naturally occurring allelic variants (Fretland et al., 2001).

Studies of the *Salmonella typhimurium* NAT revealed that the C terminus of the protein is necessary to prevent hydrolysis of CoASAc occurring in the absence of aromatic amine substrate (Mushtaq et al., 2002). Recently, the three-dimensional structures of two bacterial NAT enzymes (*S. typhimurium* and *Mycobacterium smegmatis*) have been determined (Pompeo et al., 2002). These structures revealed that a catalytic triad similar to that found in cysteine proteases is conserved throughout the NAT superfamily. In NAT2, the triad is composed of Cys68-His107-Asp122. Homology models have been constructed for the highly conserved N-terminal catalytic domain (residues 34–131) of human NAT1 and NAT2 (Rodrigues-Lima et al., 2001; Rodrigues-Lima and Dupret, 2002).

The high-throughput detection of active variant enzymes by reversion assay/mutagen activation (DAVERAMA) strategy was developed by our laboratory for screening libraries of variant enzymes that differ in their ability to bioactivate mutagens (Josephy, 2002). This assay relies on a bacterial mutagenicity assay protocol in which revertant colonies are assayed by plating an array of small drops of bacterial culture on a mutagen-containing Petri dish; each drop is a sample of a clone from the library. After growth of the plates, small revertant "microcolonies" can be observed against the background lawn of each culture spot: the dish resembles a microarray of "Ames test" plates. The DAVERAMA technique was first used to identify P450 1A2 variants with novel catalytic properties after random mutagenesis of six putative substrate-recognition sequences (Parikh et al., 1999).

Several studies of site-directed NAT2 variants have been published previously (Dupret and Grant, 1992; Delomenie et al., 1997; Goodfellow et al., 2000). However, application of site-directed mutagenesis requires a prejudgment of the residues and substitutions that are likely to affect enzyme activity. In contrast, the DAVERAMA approach should be applicable to random libraries of variants throughout the coding sequence. Rapid screening allows us to focus on variants with altered activities. Perhaps more importantly, we can readily eliminate variants that have unchanged activities or are totally inactive (e.g., not expressed or completely misfolded). We have now screened almost 300 variants from a NAT2 random library; 18 variants with altered catalytic properties have been identified and characterized.

Materials and Methods

Chemicals. 3-Methyl-2-nitroimidazo[4,5-*f*]quinoline (nitro-IQ) was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). 2-Aminofluorene (AF) and 2-nitrofluorene (NF), CoASAc (lithium salt), isopropyl-β-D-thiogalactopyranoside (IPTG), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical (St. Louis, MO). *p*-Dimethylaminobenzaldehyde, of analytical grade, was purchased from BDH, Inc. (Toronto, Ontario, Canada).

Bacterial Strains. The NAT-deficient *lacZ* tester strain JM106 *uvrA* F′ (CC109) *nhoA*::*kan*^{R} has been described previously (Josephy et al., 2002); we have now designated this strain DJ2002. *E. coli* strain DH10B- and DH5α-competent cells were purchased from Invitrogen (Carlsbad, CA).

NAT2 Variant Library Construction. Plasmid pNAT2 (Dupret and Grant, 1992) is a derivative of the vector pKEN2 (Dr. G.L. Verdine, Harvard University, unpublished data) bearing the NAT2 ORF. Plasmid pNAT2 DNA was purified from *E. coli* DH5α pNAT2 cultures using a Plasmid Midi Kit (QIAGEN, Valencia, CA) and diluted in water to a final concentration of 1 ng/μl.

Oligonucleotides were synthesized (50-μmol scale) by Invitrogen. Primers were designed to amplify the *NAT2* ORF and the adjacent multicloning sites; as a result, unique restriction sites existed within the PCR product at the 5′ (EcoRI) and 3′ (HindIII) ends: NAT2 forward, GGCGAATTGACATTGTGAGCGGATAACA; NAT2 reverse, TTCTCTCATCCGCCAAAACAGCCAAGC. Primer pKK5′ (GGATAACAATTTCACACAGG) was used for DNA sequencing.

Plasmid DNA was mixed with Diversify (BD Biosciences Clontech, Palo Alto, CA) dNTPs (each, 0.2 mM), Titanium *Taq,* reaction buffer, and primers (each 0.2 mM) to a total volume of 50 μl and subjected to PCR under mutagenesis conditions 1 or 2, as outlined in the manufacturer's protocol. The buffer was supplemented with the following: for mutagenesis condition 1 (1–2 mutations per kb), dGTP (40 mM final concentration); for mutagenesis condition 2 (2–3 mutations per kb), dGTP (40 mM) plus $MnSO_4$ (160 mM). The PCR products were recovered by agarose (1%) gel electrophoresis and purification with the Ultra Clean 15 Kit (Mo Bio Laboratories, Inc., Carlsbad, CA).

The mutagenized NAT2 ORF was excised by digestion with EcoRI and HindIII, gel-purified, and ligated into the similarly digested vector pKK223–3 (Amersham Biosciences Inc., Baie d'Urfé, PQ, Canada). Ligation products were recovered in ultra-electrocompetent *E. coli* DH10B cells; transformants were pooled and were used to prepare plasmid libraries (Nucleospin Plus Kit; BD Biosciences Clontech). Some individual transformants, chosen at random, were analyzed by restriction analysis (to verify the ligations) and by DNA sequencing (to test the extent of PCR mutagenesis). A plasmid expressing wild-type NAT2, for use as a positive control, was constructed by nonmutagenic PCR and ligation into plasmid pKK223–3, as described above.

High-Throughput Mutagenicity Assay (DAVERAMA) Protocol. Initial screening of presumptive NAT2 variants was done with

the use of the DAVERAMA method (Josephy, 2002). The pooled DNA of the NAT2 variant library in plasmid pKK223–3 was transformed into *E. coli* strain DJ2002, and the transformants were replated at high dilution onto minimal glucose medium supplemented with thiamine and ampicillin (100 μg/ml). Individual colonies picked from these plates were inoculated into the wells of a 96-well ClusterTube rack (Fisher Scientific Co., Toronto, ON, Canada) filled with LB expression medium (LB + 100 μg/ml ampicillin + 1 mM IPTG; 500 μl per well). Positive (wild-type NAT2) and negative (no NAT2 plasmid; grown without ampicillin) control strains were also cultured in each ClusterTube rack.

Test plates for the mutagenicity assay were prepared by combining mutagen (in dimethyl sulfoxide; final volume 10 μl) with phosphate buffer (0.1 M, pH 7.4 + 60 mM KCl, 500 μl) in 5-ml snap-cap tubes (Sarstedt, Montreal, PQ, Canada). Top agar, 2 ml, supplemented with nutrient broth, 0.8 ml, was added to each tube, and the mixture was overlaid onto minimal lactose (ML) plates.

ClusterTube cultures (96-well) were grown (37°C at 175 rpm for 24 h) to OD_{600} 0.0.8. A multichannel pipettor was then used to transfer aliquots (5 μl) of each culture to the mutagenicity assay (ML + nitro-IQ) test plates and onto master plates (minimal glucose + ampicillin, 50 μg/ml + thiamine). After incubation (48 h at 37°C), revertant microcolonies were counted manually, with the aid of a magnifier. Each determination was done in triplicate; values were averaged and used to construct nitro-IQ dose-response curves. This screening procedure was used to find clones that showed levels of mutagen bioactivation intermediate between those of the simultaneously measured positive and negative controls.

Standard *lacZ* Mutagenicity Assay Protocol. Clones identified by DAVERAMA screening were retested using the standard ("full-size") *lacZ* mutagenicity assay (Josephy, 2000) on 100-mm Petri dishes to obtain more accurate dose-response data. Cultures were grown to OD_{600}. 0.8 in LB medium + IPTG + ampicillin (2.5 ml; 37°C at 250 rpm shaking for 8 h). Mutagen (nitro-IQ) and buffer were combined as described above, and an aliquot of cell suspension (100 μl) was added to each tube. Tubes were incubated at 37°C for 30 min, top agar (2 ml) was added, and the mixture was overlaid on ML plates. Plates were incubated at 37°C for 48 h, and colonies were counted with the aid of a video analysis system. Dose-response curves were constructed for each clone. Some clones that passed the first stage of screening were discarded at this stage because their responses were judged to be similar to that of the wild-type control. Eighteen variants with altered phenotypic properties were further characterized by analyzing their responses to a different mutagen, NF, using the standard *lacZ* assay.

Plasmid DNA was prepared from clones of interest, and the entire NAT2 ORF was sequenced (Molecular Supercenter, University of Guelph, Guelph, ON, Canada). Each mutation was also confirmed by sequencing the complementary strand.

NAT Enzyme Assays. Extracts were prepared from overnight bacterial cultures (40 ml, A_{600} = 1.0). Cells were harvested by centrifugation (4,000*g* for 10 min), resuspended in sonication buffer (66 mM NaH_2PO_4, pH 7.2 + 1 mM EDTA + 2 mM dithiothreitol + 50 μM phenylmethylsulfonyl fluoride), transferred to 2-ml flat-bottomed microtubes, and sonicated. The crude extracts were centrifuged (12,000*g* for 10 min at 4°C), and the resulting supernatants were immediately assayed for NAT activity. Total extract protein was determined using the Bradford assay.

N-Acetyltransferase activities (substrate, AF) were measured by a 96-well plate DMAB colorimetric assay. Assay premixes (total volume, 294 μl) contained crude cell extract (diluted, if required; 23 μl); sonication buffer (250 μl); and AF (4 mM in dimethyl sulfoxide; 21 μl). The 96-well plate was chilled on ice, and aliquots of the assay premix (20 μl) were added to each of four wells per time point per sample. To initiate the reaction, acetyl CoA (6 μl, 10 mM, dissolved in water immediately before assay) was added to three of the wells; distilled water (6 μl) was added to the fourth (control). Final substrate concentrations were 0.22 mM AF and 2.3 mM acetyl CoA. The plate was then transferred to a 37°C water bath for incubation. To terminate the reaction, the plate was placed on ice, and trichloroacetic acid (10% w/v; 15 μl) was added to each well. DMAB solution (300 μl; equal volumes of 1% DMAB in ethanol and 1 M sodium acetate-HCl buffer, pH 1.4) was added to each well, and the color was allowed to develop for a minimum of 5 min at room temperature. Absorbance was read at 450 nm. *N*-Acetylation–specific activity is expressed as nmol substrate acetylated per min per mg total protein.

For the wild-type enzyme and selected variants, these assays were carried out over a range of AF concentrations (typically 28–500 μM) to allow determination of Michaelis-Menten kinetic parameters. K_m and V_{max} (± S.E.) values were determined by nonlinear regression analysis of direct plots (SigmaPlot; SPSS Inc., Chicago, IL). Goodness of fit was tested by examining linear fits to Wolf-Hanes plots; r^2 values were greater than 0.94.

Results

Screening Procedure. The goal of this research was to identify NAT2 variants with altered catalytic properties. Figure 1 illustrates the DAVERAMA screening procedure and the subsequent characterization of enzyme variants. Two pools, representing different degrees of random mutagenesis, were analyzed (PCR1 and PCR2). In trial 1, we screened two 96-well plates, one from PCR1 and one from PCR2. In trial 2, we screened one additional 96-well plate from PCR2. Data for each variant subjected to the high-throughput DAVERAMA screening were compiled into dose-response bar charts (Fig. 2) for identification of variants with responses intermediate to the positive and negative controls. Construction of dose-response curves also allowed for the identification of clones

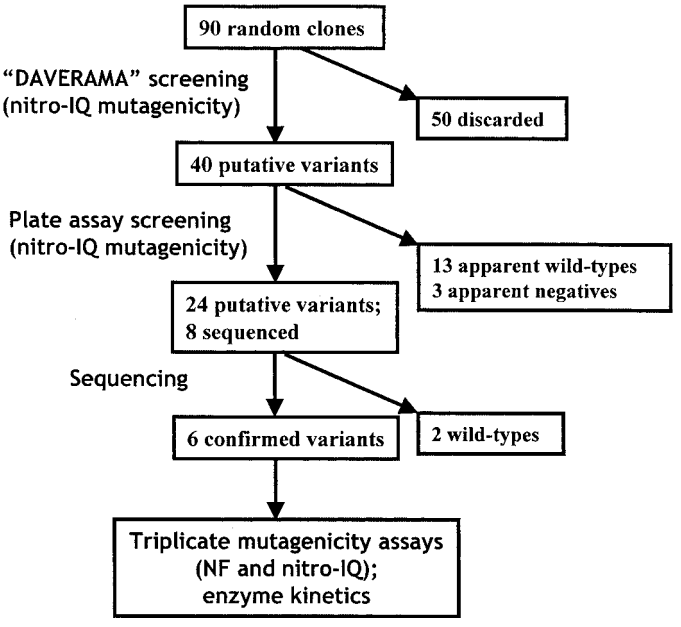

Fig. 1. Flowchart of screening experiments. The DAVERAMA screening procedure is illustrated, using the third trial (see Fig. 2) as an example. In the first screening step (using 5-μl aliquots from each culture well), 40 apparent variants were retained and 50 were discarded on the basis of either apparently wild type, apparently negative, or erratic dose responses. In the second step, standard (full-size plate) *lacZ* assay dose responses were measured; 16 of the clones were discarded on the basis of these results. Of the 24 putative variants retained, eight were selected randomly for sequencing; six of these showed single amino acid substitutions, and two were apparently wild type. An additional four and eight single amino acid substitution variants were obtained from the first and second screening trials, respectively, giving a total of 18 variants. The mutations identified in these 18 variants are presented in Fig. 3.

with unusual responses, e.g., an abnormally high background reversion rate; such clones were excluded from further study. When the milder mutagenesis condition (PCR1) was used, a greater proportion of the variants displayed mutagen bioactivation properties that resembled the wild-type enzyme (Fig. 2). Therefore, in trial 2, we screened clones from the more heavily mutagenized library (PCR2).

The second stage of screening used the standard *lacZ* assay (one plate per dose per variant), with nitro-IQ as the mutagen. Variants were eliminated at this stage if their activity was not clearly different from that of the wild-type NAT2. DNA sequencing was undertaken on all remaining variants.

Complete NAT2 ORF sequences were obtained for 18 variants. In each case, a single amino acid change had occurred. (Three variants had an additional silent base-pair substitution.) The positions of the mutations were distributed throughout the NAT2 ORF (Fig. 3).

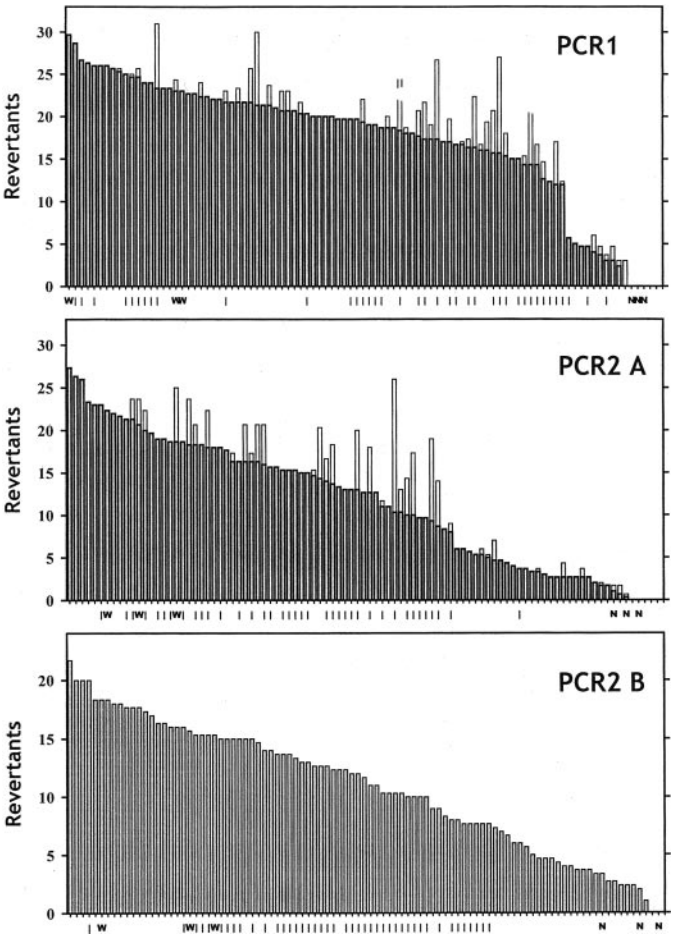

Fig. 2. Screening of NAT2 variants: mutagenicity data. Three trials were performed, each using 90 clones, chosen randomly from pools PCR1 (trial 1) and PCR2 (trials 2 and 3). The bar charts show the mutagenic response of each clone to nitro-IQ. W, wild-type clones (positive controls); N, negative control clones (background strain only, no NAT2 expression); a vertical bar indicates a clone that was selected for further analysis. Trials 1 and 2: the doses used were 0 (data not shown), 10 (dark bars), and 100 (light bars) pmol/plate. The complete data set was sorted according to the response at 10 pmol and is presented in descending order from left to right. In fact, responses at 100 pmol were, in most cases, lower than at 10 pmol, because of the onset of toxicity; therefore, only a few of the corresponding light bars are seen; the other bars are hidden by the 10 pmol bars. To avoid this toxicity, doses were adjusted downward for trial 3; the doses used were 0, 1 (data not shown), and 10 pmol/plate; note change of ordinate scale.

Characterization of NAT2 Variants

Mutagenicity Assays. For each of the 18 confirmed variants, the standard nitro-IQ *lacZ* assay was repeated in triplicate (three plates per dose). These data were compared with results obtained in the DAVERAMA screen to determine whether the high-throughput method gives an accurate assessment of the impact of the mutation on activity. The rank order of the variants, in terms of their responses at the highest nitro-IQ dose (100 pmol), agreed well between the two assays (data not shown).

The responses of the variants to a second mutagen, NF, were also measured in a standard *lacZ* assay. From these data sets, the mutagenic potencies of nitro-IQ and NF in each variant were calculated as revertants per nmol. Figure 4 shows the correlation between the potencies of the two mutagens.

Enzyme Assays. Enzyme activities (AF *N*-acetylation) for each variant were measured in crude cell extracts under identical conditions. Relative activities are expressed as a percentage of wild-type in Fig. 5. Activities varied greatly among the variant enzymes and correlated with mutagen bioactivation data obtained through the high-throughput and standard *lacZ* assays (see *Discussion*). Catalytic parameters K_M (for AF) and V_{max} were estimated for the wild-type NAT2 enzyme and for five variants (Fig. 6). An SDS-polyacrylamide gel electrophoresis immunoblot of these six cell extracts, probed with a polyclonal rabbit antibody to recombinant human NAT2 (Muckel et al., 2002) (generously provided by Dr. Hansruedi Glatt, German Institute for Nutritional Research, Potsdam, Germany), showed very similar levels of expression of immunoreactive NAT2 protein in each case (data not shown).

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     *::: *  *  .*   .   . * *  **::::* :::::*:*** .:***. : *  *    :.::*
1    MDIEAYFERI GYKNSRNKLD LETLTDILEH QIRAVPFENL NMHCGQAMEL GLEAIFDHIV
           #                                        H    #          V

     :::*****::  * ** *.*    *::.::** . .         *:  : **:: :*.: .: **
61   RRNRGGWCLQ VNQLLYWALT TIGFQTTMLG GYFYIPPVNK YSTGMVHLLL QVTIDGRNYI
                    R                          L      -

     **..   : * :*:*:** ** **:**.* :*   : *:   ** *:: :*:: : .:.     ..
121  VDAGSGSSSQ MWQPLELISG KDQPQVPCIF CLTEERGIWY LDQIRREQYI TNKEFLNSHL
                                  A                                I

     :  .  :::*  *:*:** :* :*     : *  :  * :  . *:*:*:* :* .  *:*  ::
181  LPKKKHQKIY LFTLEPRTIE DFESMNTYLQ TSPTSSFITT SFCSLQTPEG VYCLVGFILT
     A- N         R  A    L V S                  # R             T

        :.**::  **:*:  : : **:* .*:   * : *  ::* .        :
241  YRKFNYKDNT DLVEFKTLTE EEVEEVLKNI FKISLGRNLV PKPGDGSLTI 290
       I      G              A
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Fig. 3. Sequence changes of the variant NAT2 enzymes. The complete amino acid sequence of human NAT2 (wild-type allele, NAT2*4) is shown. Symbols above the sequence display the results of homology analysis of available vertebrate NAT2 sequences, aligned by the program ClustalW (http://www.ebi.ac.uk/clustalw/). ⋆, residues identical in all sequences; :, conserved substitutions; •, semiconserved substitutions. The positions at which mutations were recovered in this study are shown in boldface type, with the mutant residue shown below. (A dash indicates a silent mutation recovered in tandem with a second nonsilent mutation.) Previous studies have shown that the NAT2 enzyme active site incorporates a catalytic triad C-H-D, similar to that of the cysteine proteases; these three residues are double-underlined. The published model of the NAT catalytic-core structure (Rodrigues-Lima and Dupret, 2002) includes amino acids 34–131. #, positions at which site-directed mutants have been shown, in other studies, to affect protein stability. Sequences aligned: *Homo sapiens* (human), *Gallus gallus* (chicken), *Mesocricetus auratus* (golden hamster), *Mus musculus* (mouse), *Oryctolagus cuniculus* (rabbit), and *Rattus norvegicus* (rat); NAT1 and NAT2 in each species, except for mouse: NAT1, NAT2, and NAT3.

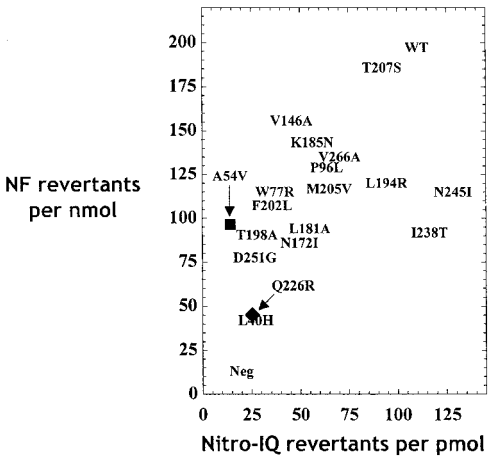

Fig. 4. Comparison of mutagenicities of nitro-IQ and NF for the NAT2 variants. Each of the 18 NAT2 variants was tested for mutagenic response to nitro-IQ and NF in a standard (full-plate) *lacZ* assay. Each variant was tested in three independent triplicate experiments with each mutagen at zero dose and at doses of 0.1, 1, 10, and 100 pmol/plate nitro-IQ; and 0.1, 1, 10, and 100 nmol/plate NF; complete dose-response data sets are not shown. Wild-type and negative (no NAT2 plasmid) control strains were also tested. Mutagenic potencies were calculated from the 10 pmol (nitro-IQ) and 10 nmol doses (NF). (Using lower or higher doses as the basis for the potency calculation did not greatly change the results.) The position of variant Q226R, which overlaps variant L40H, is represented by ♦ for legibility.

Discussion

Human NAT2 polymorphisms are common and may have important pharmacological/ toxicological implications. A thorough understanding of the structure-function relationships of the NAT enzyme would allow the prediction of the effects of mutations (amino acid substitutions), whether natural or engineered, on enzyme activity. In contrast to site-directed mutagenesis, random mutagenesis generates large numbers of variants in a single trial, and no prejudgment need be made with regard to the functionally important sites in a protein. However, random mutagenesis is only applicable when rapid selection or screening procedures are available to identify variants with altered properties. The most powerful strategies are selective. For example, Loeb and colleagues (Christians et al., 1997) isolated novel sequence variants of the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase, from their property of conferring resistance to the toxic alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. However, such an approach is only possible when the enzyme under examination has cytoprotective properties. In some cases, enzymes convert substrates into products that can be detected colorimetrically (Nakamura et al., 2001; Sakamoto et al., 2001). We have developed the DAVERAMA method, a screening approach that can be applied to any enzyme that catalyzes the metabolic activation of a mutagen (Josephy, 2002). In our first application of this method (Parikh et al., 1999), we screened pools of variants of P450 1A2 constructed by random mutagenesis of short stretches of primary sequence postulated to determine substrate specificity. This success prompted us to test whether the screening method is sufficiently robust to allow the identification of variants from a much broader pool, generated by random mutagenesis of an entire open reading frame. In this work, we have successfully applied our screening technique to variant pools constructed by random mutagenesis of NAT2.

In preliminary studies, we used the pNAT2 expression plasmid (Dupret and Grant, 1992). However, technical obstacles were encountered. Very few unique restriction sites are available in that vector, and two of these (EcoRI and XhoI)

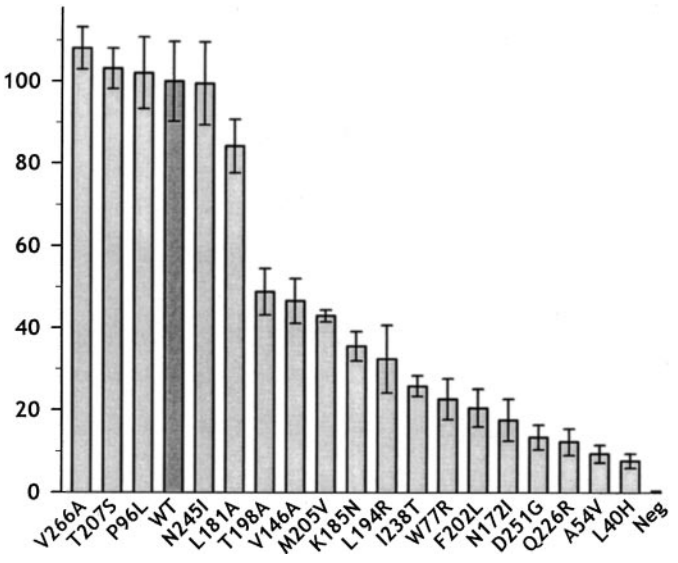

Fig. 5. Relative enzyme activities of NAT2 variants. AF *N*-acetylation activities (AF concentration = 0.22 mM) were measured for extracts from the variant strains; activity (per mg of crude extract protein) is normalized to 100% for the wild type. Data are means ± S.E. of triplicate determinations. Values for variant T198A and all variants to its right are statistically different from the wild type ($p < 0.05$), as determined by a Student's *t* test.

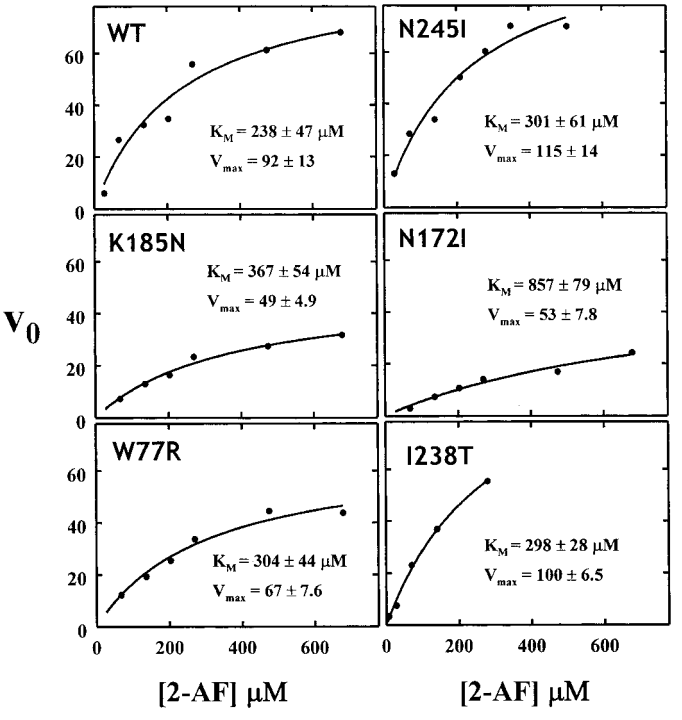

Fig. 6. Kinetic data for selected NAT2 variants. AF *N*-acetylation kinetics were measured for six of the variant NAT2 enzymes. Data are means of triplicate determinations. Michaelis-Menten parameters V_{max} and K_M were determined by direct curve-fitting to the Michaelis-Menten equation (as described under *Materials and Methods*). Ordinate scale represents v_0 in units of nmol/min/mg protein. V_{max} values are given in the same units.

were compromised by "star" activities at sites within or adjacent to the ORF (data not shown). The pKK223–3 expression vector used here proved more suitable for the required subcloning procedures.

The mutagenic PCR system worked effectively to generate libraries of NAT2 variants. The level of mutagenesis clearly increased when the PCR conditions were modified, in accordance with the manufacturer's instructions, to promote mutagenesis (compare PCR1 and PCR2 pools, Fig. 2). Taken from these instructions, we expected to achieve a level of mutagenesis of one mutation per kilobase under condition 1. In fact, from the DNA sequencing results, we found this level was achieved, approximately, under condition 2. According to the manufacturer, the mutational bias (ratio of transitions to transversions) under condition PCR1 should be 0.9. We observed values of 1 and 1.3 under conditions 1 and 2, respectively. Of the 20 mutations identified (18 nonsilent and 2 silent), 19 occurred at template A or T residues, one at a template C, and none at a template G. This strong bias toward mutagenesis at A·T base pairs was expected, as taken from the manufacturer's specifications.

The results obtained by the high-throughput screening approach were borne out by subsequent standard plate assays for mutagenicity. Most of the variation from wild-type mutagenicity was toward lower activity (Fig. 2). A few variants showed greater mutagenicity in the initial screen, but none was dramatically greater.

By comparing responses to nitro-IQ versus NF (Fig. 4), one may identify variants exhibiting substrate-specific differences in mutagen bioactivation. On this scatter plot, most of the variants fall roughly along a band stretching between the negative control and wild type. Points along this band correspond to variants for which the ratio of responses to the two test mutagens is equal to that for the wild-type enzyme. However, a few variants lie further from this band. For example, the mutagenic response of variant N245I seems to be shifted toward nitro-IQ. These variants may have altered specificities for the arylamine substrate; further analysis of the enzymes will be required to determine whether this is the case.

The amino acid substitutions recovered in the 18 variants were distributed throughout the NAT2 primary sequence (Fig. 3). Charge-change substitutions comprised 6 of the 18 variants. The apparent bias (14/18) toward the second half of the sequence is probably an artifact of the screening procedure. Initial characterization was performed by a single sequencing run from the C-terminal end, which usually yielded less than a full-length readable sequence. Only mutants identified by this initial sequencing were selected for further characterization.

The identified variants may be analyzed in the context of the interspecies variability of NAT sequences (Fig. 3). Thirteen vertebrate NAT sequences (from five mammals and the chicken) available on public databases were aligned. These 13 sequences have a high degree of identity or homology. Of 290 positions, 85 (29%) are conserved in all 13 vertebrate NAT sequences. Five (28%) of the 18 variants obtained in this study occurred at these conserved positions. This result indicates that our screening procedure did not disfavor variants at highly conserved sites, possibly because we tended to select variants with distinctly lower mutagenic responses than the wild type. Despite the weaker homology between vertebrate and bacterial NATs, Sinclair et al. (2000) identified 11 positions (including the catalytic triad) that are conserved among all known NAT sequences (both vertebrate and bacterial). None of the variants obtained in our study occurred at one of these sites. However, the size of our study is too small to ascribe significance to this observation. Several of the variants occurred at sites that show considerable variability among the vertebrate NAT sequences. In a few cases, the variants corresponded to naturally occurring substitutions: T207S residue is S in mouse NAT1; I238T residue is T in human NAT1.

Four variants are within the catalytic domain of NAT2 (residues 34–131) modeled in a recent publication (Rodrigues-Lima et al., 2001). None of these four residues interacts directly with the catalytic triad, taken from the model. However, residue Leu40 is adjacent to residue Glu38. A salt bridge between conserved residues Glu38 and Arg64 is believed to be structurally important (Delomenie et al., 1997). The low activity of variant L40H, a nonconservative substitution, may result from disruption of this stabilizing interaction, but structural characterization of the variant and wild-type enzymes will be needed to test such hypotheses.

N-Acetylation kinetic measurements (substrate, AF) were made for six of the NAT2 variants (Fig. 6) and were chosen from across the range of activities. Although the NAT2 enzymes were not purified, immunoblotting indicates that the expression levels of the wild-type and variant enzymes were very similar, so V_{max} values should be reflective of relative specific activities. The apparent K_m value of the wild-type enzyme was 238 μM. Comparisons with literature values (Hein et al., 1994) are problematic because, for a ping-pong kinetic mechanism, the apparent K_m for one substrate depends on the concentration of the other substrate. Relative to the wild-type enzyme, four of the five variants tested had slightly increased K_m values; one, N172I, had substantially increased K_m and decreased V_{max}, accounting for its position near the low-activity end of the variants shown in Fig. 5. (We have not measured K_m values for acetyl CoA, which might also be altered.)

In this article, we have shown that the DAVERAMA screening method can rapidly identify NAT2 variants with altered (usually lowered) catalytic activities. These altered enzymes have properties that are different, but not drastically different, from those of the wild-type enzyme, in terms of expression and enzyme kinetic parameters. The dynamic range of the screening method is limited so that variants with very low activity are hard to distinguish from ones with zero activity. We avoided choosing colonies with extremely weak mutagenic responses, reasoning that "dead" enzymes are less informative than ones with more subtly altered activities. However, in further studies, we will attempt to extend the analysis to low-activity variants.

The limited series of variants identified in the present study already suffices to show that residues located along most of the length of the NAT2 primary sequence contribute to enzyme activity. Scaling up these studies should allow us to isolate hundreds, rather than dozens, of variants. At that scale, regions of the primary sequence with higher or lower densities of "sensitive" residues (sites at which variants have markedly altered activities) may become apparent. We are confident that the approach described here can provide a new window on the relationship of amino acid sequence to enzy-

matic activity of any enzyme that bioactivates mutagenic chemicals.

Acknowledgments

We thank the National Sciences and Engineering Research Council of Canada for research funding. Sherri Robins (a National Sciences and Engineering Research Council of Canada Undergraduate Summer Research Assistant), provided excellent assistance with the screening experiments. We also thank Dr. David Hein for helpful discussions.

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