Study of the role of the highly conserved residues Arg⁹ and Arg⁶⁴ in the catalytic function of human *N*-acetyltransferases NAT1 and NAT2 by site-directed mutagenesis

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The arylamine *N*-acetyltransferases (NATs) NAT1 and NAT2 are responsible for the biotransformation of many arylamine and hydroxylamine xenobiotics. It has been proposed that NATs may act through a cysteine-linked acetyl-enzyme intermediate in a general base catalysis involving a highly conserved arginine residue such as Arg⁶⁴. To investigate this possibility, we used site-directed mutagenesis and expression of recombinant human NAT1 and NAT2 in *Escherichia coli*. Sequence comparison with NATs from other species indicated that Arg⁹ and Arg⁶⁴ are the only invariant basic residues. Either mutation of the presumed catalytic Cys⁶⁸ residue or the simultaneous mutation of Arg⁹ and Arg⁶⁴ to Ala produced proteins with undetectable enzyme

activity. NAT1 or NAT2 singly substituted at Arg⁹ or Arg⁶⁴ with Ala, Met, Gln or Lys exhibited unaltered K_m values for arylamine acceptor substrates, but a marked loss of activity and stability. Finally, double replacement of Arg⁹/Arg⁶⁴ with lysine in NAT1 altered the K_m for arylamine substrates (decreased by 8–14-fold) and for acetyl-CoA (elevated 5-fold), and modified the pH-dependence of activity. Thus, through their positively charged side chains, Arg⁹ and Arg⁶⁴ seem to contribute to the conformational stability of NAT1 and NAT2 rather than acting as general base catalysts. Our results also support a mechanism in which Arg⁹ and Arg⁶⁴ are involved in substrate binding and transition-state stabilization of NAT1.

INTRODUCTION

The human acetyl-CoA:arylamine *N*-acetyltransferases (NATs; EC 2.3.1.5) NAT1 and NAT2 catalyse the transfer of an acetyl group from acetyl-CoA to the nitrogen or oxygen atom of hydrazines, arylamines and hydroxylamines, thus playing important roles in both the detoxification and potential metabolic activation of numerous drugs and xenobiotics. In humans and other mammals, genetically based inter-individual variation in NAT2 protein content is the basis of the well known isoniazid acetylation polymorphism, resulting in significant toxicological implications [1]. Although NAT1 expression is independent of the classically defined acetylation polymorphism, it also displays marked inter-individual variations [2], which may be a source of pharmacological susceptibility. Molecular cloning and sequencing of NATs from different vertebrate species has revealed a high degree of identity at both the nucleotide and amino acid levels, suggesting common functional enzymic features [3]. The *N*-acetylation reaction is likely to involve a simple two-step substituted enzyme (Ping Pong) kinetic mechanism [4]. Previous studies have suggested the existence of a single thiol in the active site, which participates in the formation of a catalytic intermediate acetylcysteinyl enzyme [5].

To facilitate investigations of the structure–function relationships of NAT1 and NAT2, we previously developed an expression system for the production of recombinant human NAT1 and NAT2 in *Escherichia coli*. This system has already enabled us to identify the highly conserved Cys⁶⁸ residue as being essential for the catalytic function of human NAT2 [6], and to map regions critical for NAT stability and substrate specificity [7]. The

analogous Cys⁶⁹ residue in the *O*-acetyltransferase (OAT) protein of *Salmonella typhimurium* was also shown to be essential for both OAT and NAT activities [8]. Apart from the essential role of the putative active thiol group in Cys⁶⁸, other residues could also be critical, most notably in stabilization of the transition state of the acetylation reaction. Thus further use of site-directed mutagenesis may elucidate the contributions of other amino acids to the functional properties of both NAT1 and NAT2. The goal of the present study was to clarify the involvement of highly conserved arginine residues in the catalytic activity of human NAT1 and NAT2. A previous kinetic study of pigeon liver NAT had shown an increase in reactivity of substituted anilines with increasing basicity, suggesting the involvement of a general base catalysis in the enzyme reaction [9]. A basic residue could be involved in abstraction of a proton from the active thiol, as well as from the hydroxyamino or amino group of the acceptor [10]. More recently, use of the arginine-modifying agent phenylglyoxal suggested the existence of an essential Arg residue for the activity of NAT1 and NAT2 from hamster liver [11]. Furthermore, in OAT of *S. typhimurium*, the substitution of Arg⁶⁵ (corresponding to Arg⁶⁴ in eukaryotes) to Ala resulted in an enzyme with no detectable activity. Therefore this single Arg may be involved in a general base catalysis: an adjacent guanidinium group could cause an electrostatic destabilization and thereby a decrease in its apparent pK_a [8]. Based on these findings, a general mechanism was postulated for *N*-acetyltransferase catalysis, whereby an essential Arg residue withdraws a proton from the Cys⁶⁸ -SH group and thus allows it to bind the acetyl moiety of acetyl-CoA and generate an acetylated enzyme intermediate prior to acetylation of the acceptor substrate [12].

Abbreviations used: NAT, arylamine *N*-acetyltransferase; OAT, *O*-acetyltransferase; SMZ, sulphamethazine; PAS, *p*-aminosalicylic acid; 2-AF, 2-aminofluorene; PG, phenylglyoxal hydrate; Gu-HCl, guanidine hydrochloride.

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Based on primary sequences, only two basic residues are conserved among NAT enzymes from all species so far studied. These strictly invariant residues are Arg⁹ and Arg⁶⁴ in vertebrates, corresponding to Arg¹¹ and Arg⁶⁵ respectively in *S. typhimurium*. To test whether these residues could play critical roles in the activity of human NATs, we have substituted them with Ala, Lys, Met or Gln and expressed the mutated recombinant proteins in *E. coli*. The choice of substitutions was dictated by the following considerations. The use of alanine is expected to avoid possible secondary steric effects that might occur with larger side chains, and this residue does not impose considerable constraints on three-dimensional structures. Nonetheless, an Ala substitution is also a drastic, non-conservative change. Methionine maintains some of the steric and hydrophobic features of Arg, despite its lack of charge and polarity. The near isosteric substitution of Arg with Gln conserves some of the hydrophobic properties of the side chain, combined with a polar extremity similar to that of Arg. The replacement of Arg with Lys maintains the charge of the residue (regarding its predictable pK_a). Here we provide experimental evidence regarding the role played by Arg⁹ and Arg⁶⁴ in human NAT activities. We have also examined the potential involvement of adjacent guanidinium groups of arginines in the catalytic mechanism of *N*-acetyltransferases, as proposed previously by others [8].

MATERIALS AND METHODS

Bacterial strains and reagents

The phagemid expression vector pKEN2 and the *E. coli* host strain XA90 (F'*lacI*^{q1}) were kindly provided by Dr. G. L. Verdine (Department of Chemistry, Harvard University, Cambridge, MA, U.S.A.). The previously described [13] *E. coli* host strain RZ1032 (*dut*⁻ *ung*⁻) was kindly provided by Dr. H. Bedouelle (CNRS URA 1129, Institut Pasteur, Paris, France). Recombinant NAT1 and NAT2 coding sequences were mutated and expressed from wild-type plasmids p-NAT1 and p-NAT2 respectively [6]. The arylamine acceptor substrates sulphamethazine (SMZ), *p*-aminosalicylic acid (PAS) and 2-aminofluorene (2-AF), the acetyl donors acetyl-CoA and D,L-acetylcarnitine, and carnitine acetyltransferase were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Sequence alignments

The NAT amino acid sequences were inferred from the DNA sequences available through the GenBank®/EMBL data base. The mouse NAT1 and NAT2 sequences were derived from the NBRF® data base. A computerized alignment algorithm based on Waterman's method [14] was used. To optimize the alignments, a gap was eliminated between residues 35 and 36 in the eukaryotic sequences and a gap was introduced between residues 14 and 15 in the *Salmonella* sequence.

Enzyme assays and protein detection

The expression of recombinant wild-type and mutant NAT1 and NAT2 proteins was induced in XA90 strains harbouring pKEN2-derived phagemid constructs, and lysates of these bacteria were used for *in vitro* acetylation of PAS, SMZ or 2-AF as described [6]. The apparent kinetic parameters were measured using 100 μ M acetyl-CoA, whereas the true K_m and V_{max} values were determined using five acetyl-CoA concentrations within a 50–1000 μ M range, as reported [15]. The acetylated products of NAT activity were quantified using reverse-phase (C18 column; Beckman) HPLC (Shimadzu Corp.) with UV detection [6]. For products without

Table 1 Sequences of oligonucleotides used for site-directed mutagenesis

Sequence accession numbers are X17059 for NAT1 and X14672 for NAT2 (from Genbank). Each underlined codon generates the amino acid substitution as indicated in the oligonucleotide designation. The codon underlined in oligonucleotide N1PC5 generates a Phe⁷ → Leu change.

Oligonucleotide	Sequence	Positions of hybridization to NAT coding region
N1PC5	5'-TGAAGCATATCTTGAAGAAT-3'	9–29
NAT1R9A	5'-ATATCTTGAAGCAATTGGCTA-3'	15–35
NAT1R9K	5'-ATATCTTGAAGCAATTGGCTA-3'	15–35
NAT1R9M	5'-ATATCTTGAAGCAATTGGCTA-3'	15–35
NAT1C44A	5'-TAACATCCATGCTGGGGATGC-3'	120–140
NAT1R61A	5'-TCAAGTTGTGGCAAGAAATCG-3'	171–191
NAT1R62A	5'-AGTTGTGAGAGCAATCGGGG-3'	174–194
NAT1R61A/R62A	5'-GATCAAGTTGTGGCAGCAATCGGGG-3'	169–194
NAT1R64A	5'-GAGAAGAAATGCGGGTGGATG-3'	180–200
NAT1R64K	5'-GAGAAGAAATGCGGGTGGATG-3'	180–200
NAT1R64M	5'-GAGAAGAAATGCGGGTGGATG-3'	180–200
NAT1R64Q	5'-GAGAAGAAATGCGGGTGGATG-3'	180–200
NAT1C68A	5'-GGGTGGATGGGCTCTCCAGGT-3'	192–212
NAT1C223A	5'-TAAATCATTTGCTTCTTCA-3'	657–677
NAT2R9A	5'-ATATTTTGAAGCAATTGGCTA-3'	15–35
NAT2R9K	5'-ATATTTTGAAGCAATTGGCTA-3'	15–35
NAT2R9M	5'-ATATTTTGAAGCAATTGGCTA-3'	15–35
NAT2C44A	5'-TAACATGCAATGCTGGGCAAGC-3'	120–140
NAT2R61A	5'-TCACATTGTAGCAAGAAACCG-3'	171–191
NAT2R62A	5'-CATTGTAAGAGCAAACCGGGG-3'	174–194
NAT2R61A/R62A	5'-GATCACATTGTAGCAGCAAACCGGGG-3'	169–194
NAT2R64A	5'-AAGAAGAAACGCGGGTGGTG-3'	180–200
NAT2R64K	5'-AAGAAGAAACGCGGGTGGTG-3'	180–200
NAT2R64M	5'-AAGAAGAAACGCGGGTGGTG-3'	180–200
NAT2R64Q	5'-AAGAAGAAACGCGGGTGGTG-3'	180–200
NAT2C68A	5'-GGGTGGTGGGCTCTCCAGGT-3'	192–212
NAT2C223A	5'-CACATCATTTGCTTCTTCA-3'	657–677

purified standards, i.e. *N*-acetyl-SMZ and *N*-acetyl-PAS, the concentrations were estimated by comparing the rate of increase of the peak eluting at 7 or 10 min with the decrease in the peak area of the corresponding substrate at a known concentration [15]. Steady-state kinetic parameters were measured after verifying that rates of product formation (initial velocity) were constant throughout the reaction. Kinetic studies were performed using 0.1–10 K_m ranges of arylamine substrate concentrations with maximum conversion rates of 10–20 %, obtained if necessary by diluting the lysate in BSA-containing buffer to ensure homogeneous protein concentrations (1 mg/ml) in the enzyme preparations. Protein contents in bacterial lysates were determined by the Bradford assay [16] using a BSA standard. Immunodetection of recombinant proteins in *E. coli* lysates was carried out using a polyclonal rabbit antiserum raised against purified NAT2, as previously described [6]. The antigenic protein was obtained by expression of a glutathione-S-transferase-tagged NAT2 fusion protein in *E. coli* (Pharmacia Biotech), followed by proteolytic cleavage of the tag and purification of NAT2 by SDS/PAGE. Antisera were preabsorbed against an excess of lysate from the parent bacterial strain to reduce non-specific background immunoreactivity.

Inactivation of NATs by phenylglyoxal

Undiluted lysate from recombinant *E. coli* was preincubated at 37 °C with various concentrations of phenylglyoxal hydrate (PG) (Sigma), and aliquots were withdrawn at indicated times for the enzyme assays described above. The acetylation activity with SMZ was assessed in 3 min assays performed without the D,L-

Table 2 Comparison of apparent kinetic constants for wild-type and mutant NATs

N-Acetylation activities were measured with SMZ or PAS as substrate using lysates from recombinant *E. coli* strains. Kinetic constants estimated from two or more separate experiments are given as means $\pm$ S.D. $V_{\max}/K_m$ values are also given as a percentage of the corresponding wild-type value (in parentheses). Statistical comparisons were performed using Student's *t* test (*****P* < 0.001; ****P* < 0.01; ***P* < 0.02; **P* < 0.05). ND, not determined.

Mutant	PAS			SMZ		
	K_m (μ M)	$V_{\max}$ (nmol/min per mg of protein)	$V_{\max}/K_m$	K_m (μ M)	$V_{\max}$ (nmol/min per mg of protein)	$V_{\max}/K_m$
NAT1						
Wild type	9.4 $\pm$ 1.2	1810 $\pm$ 75	192 (100%)	5600 $\pm$ 1000	123 $\pm$ 19	2.2 $\times 10^{-2}$ (100%)
C44A	9.2 $\pm$ 0.5	147 $\pm$ 1.9****	16 (8.3%)	2260 $\pm$ 470	4.9 $\pm$ 0.6**	2.2 $\times 10^{-3}$ (10%)
C223A	9.4 $\pm$ 2.8	1380 $\pm$ 180	147 (77%)	4500 $\pm$ 820	9.1 $\pm$ 1.1**	2.0 $\times 10^{-3}$ (9.2%)
C68A	ND	< 0.01	—	ND	< 0.01	—
R9A	7.7 $\pm$ 1.8	12 $\pm$ 0.7****	1.5 (0.8%)	2120 $\pm$ 760	0.75 $\pm$ 0.17****	3.6 $\times 10^{-4}$ (1.6%)
R9K	7.8 $\pm$ 2.3	64 $\pm$ 5.9****	8.2 (4.3%)	4520 $\pm$ 870	16 $\pm$ 2.0*	3.6 $\times 10^{-3}$ (16%)
R9M	3.8 $\pm$ 1.1	3.6 $\pm$ 0.2****	0.9 (0.5%)	1130 $\pm$ 180	0.16 $\pm$ 0.01***	1.4 $\times 10^{-4}$ (0.7%)
R64A	9.1 $\pm$ 0.9	2.7 $\pm$ 0.1****	0.3 (0.2%)	3010 $\pm$ 1360	0.43 $\pm$ 0.14***	1.4 $\times 10^{-4}$ (0.7%)
R64K	11 $\pm$ 1.9	14 $\pm$ 0.7****	1.3 (0.7%)	7270 $\pm$ 2130	16 $\pm$ 3.9*	2.2 $\times 10^{-3}$ (10%)
R64M	11 $\pm$ 1.4	2.1 $\pm$ 0.1****	0.2 (0.1%)	4100 $\pm$ 1330	0.28 $\pm$ 0.08***	6.9 $\times 10^{-5}$ (0.3%)
R64Q	18 $\pm$ 3.7	19 $\pm$ 1.1****	1.1 (0.6%)	4840 $\pm$ 1160	12 $\pm$ 0.27**	2.4 $\times 10^{-3}$ (11%)
R61A	8.7 $\pm$ 2.0	2580 $\pm$ 140***	297 (155%)	3640 $\pm$ 850	209 $\pm$ 14	5.8 $\times 10^{-2}$ (263%)
R62A	11 $\pm$ 1.9	2180 $\pm$ 110	209 (109%)	3920 $\pm$ 1410	228 $\pm$ 26	5.8 $\times 10^{-2}$ (266%)
R61A/R62A	7.1 $\pm$ 2.1	290 $\pm$ 18****	40 (21%)	4660 $\pm$ 480	41 $\pm$ 3.5	8.7 $\times 10^{-3}$ (40%)
R9A/R64A	ND	< 0.01	—	ND	< 0.01	—
R9A/R64K	ND	0.05	—	ND	< 0.01	—
R9K/R64A	ND	0.03	—	ND	< 0.01	—
NAT2						
Wild type	7590 $\pm$ 1350	3.9 $\pm$ 0.14	5.2 $\times 10^{-4}$ (100%)	110 $\pm$ 6.0	3.8 $\pm$ 0.07	3.5 $\times 10^{-2}$ (100%)
C44A	2510 $\pm$ 1060	0.41 $\pm$ 0.04****	1.7 $\times 10^{-4}$ (32%)	122 $\pm$ 15	2.16 $\pm$ 0.09****	1.8 $\times 10^{-2}$ (51%)
C223A	4080 $\pm$ 510	1.5 $\pm$ 0.06****	3.7 $\times 10^{-4}$ (71%)	71 $\pm$ 14	3.3 $\pm$ 0.17	4.7 $\times 10^{-2}$ (134%)
C68A	ND	< 0.01	—	ND	< 0.01	—
R9A	5040 $\pm$ 950	(0.04 $\pm$ 3.2) $\times 10^{-3}$ ****	6.9 $\times 10^{-6}$ (1.3%)	79 $\pm$ 22	(0.05 $\pm$ 3.2) $\times 10^{-3}$ ****	6.1 $\times 10^{-4}$ (1.8%)
R9K	3760 $\pm$ 1030	0.99 $\pm$ 0.10****	2.6 $\times 10^{-4}$ (51%)	96 $\pm$ 42	3.7 $\pm$ 0.43	3.8 $\times 10^{-2}$ (110%)
R9M	5030 $\pm$ 1090	(0.04 $\pm$ 3.1) $\times 10^{-3}$ ****	7.0 $\times 10^{-6}$ (1.4%)	51 $\pm$ 18	(0.02 $\pm$ 2.0) $\times 10^{-3}$ ****	3.9 $\times 10^{-4}$ (1.1%)
R64A	4540 $\pm$ 730	0.28 $\pm$ 0.02****	6.2 $\times 10^{-5}$ (12%)	113 $\pm$ 31	0.24 $\pm$ 0.02****	2.1 $\times 10^{-3}$ (6.1%)
R64K	7130 $\pm$ 1350	0.83 $\pm$ 0.06****	1.2 $\times 10^{-4}$ (23%)	214 $\pm$ 22***	3.6 $\pm$ 0.21	1.7 $\times 10^{-2}$ (48%)
R64M	6850 $\pm$ 920	0.57 $\pm$ 0.03****	8.4 $\times 10^{-5}$ (16%)	168 $\pm$ 24	0.25 $\pm$ 0.02****	1.5 $\times 10^{-3}$ (4.2%)
R64Q	3980 $\pm$ 860	0.13 $\pm$ 0.01****	3.2 $\times 10^{-5}$ (6.2%)	138 $\pm$ 19	1.20 $\pm$ 0.05****	8.7 $\times 10^{-3}$ (25%)
R61A	6550 $\pm$ 2400	0.97 $\pm$ 0.15****	1.5 $\times 10^{-4}$ (29%)	108 $\pm$ 31	1.53 $\pm$ 0.10****	1.4 $\times 10^{-2}$ (40%)
R62A	5250 $\pm$ 1900	0.74 $\pm$ 0.10****	1.4 $\times 10^{-4}$ (27%)	119 $\pm$ 10	2.12 $\pm$ 0.05****	1.8 $\times 10^{-2}$ (51%)
R61A/R62A	7380 $\pm$ 880	0.27 $\pm$ 0.01****	3.6 $\times 10^{-5}$ (7.0%)	106 $\pm$ 23	1.42 $\pm$ 0.09****	1.3 $\times 10^{-2}$ (38%)
R9A/R64A	ND	< 0.01	—	ND	< 0.01	—
R9A/R64K	ND	0.02	—	ND	< 0.01	—
R9K/R64A	ND	< 0.01	—	ND	< 0.01	—

change in enzyme activity compared with control experiments. IC_{50} values of Gu-HCl were calculated by regression analysis of the linear portion of the graph of residual enzyme activity against Gu-HCl concentration. *In vitro* stabilities of recombinant enzymes at 37 °C were estimated as previously described [6].

Determination of kinetic parameters

Kinetic constants were obtained from at least two separate experiments. The data were plotted using the Eadie–Hofstee representation and the corresponding plots were linear. Apparent K_m and $V_{\max}$ values ($\pm$ S.E.) were then estimated by fitting initial-rate data to a general Michaelis–Menten equation using a computer-assisted non-linear iterative fitting analysis (Sigma Plot; Jandel Scientific, Erkrath, Germany) according to the Marquardt–Levenberg algorithm. The S.E. values for $V_{\max}$ were in all cases less than 17 % of the mean and for K_m were generally less than 18 %. Reported errors are the S.D.s calculated from the estimates provided by this program. Apparent and true parameter estimates and their S.D.s were averaged by calculating weighted mean and S.D. values [17]. The true kinetic constants were

calculated using a non-linear fitting procedure and a previously reported two-substrate model [18]. In all cases, statistical significance was determined using Student's *t* test.

RESULTS

Sequence identity among NATs from different species

To identify conserved basic residues as targets for site-directed mutagenesis, a comparison was made of the deduced amino acid sequences of 15 NATs from seven different species. GenBank®/EMBL or NBRF® (for mouse NAT1 and NAT2) accession numbers are in parentheses: human NAT1 (X17059) and NAT2 (X14672), rabbit NAT1 (X53765) and NAT2 (X53767), rat NAT1 (U17260) and NAT2 (U17261), mouse NAT1 (A61267), NAT2 (B61267) and NAT3 (X72959), hamster NAT1 (U05271) and NAT2 (U03467), chicken NATL (J03737), p-NAT-3 (X16020) and p-NAT-10 (X16021), and *Salmonella typhimurium* OAT (D90301). There were 24 strictly invariant residues (bold italic in Figure 1), including two arginines (Arg⁹ and Arg⁶⁴) and one cysteine (Cys⁶⁸). Also, a pair of basic residues (Arg, Lys or

Table 3 Comparison of stability parameters for wild-type and mutant NATs

N-Acetylation activities were measured in bacterial extracts with the indicated concentrations of SMZ or PAS. *In vitro* stability was measured in two or three (wild-type) or one or more (mutants NATs) separate experiments performed at 37 °C, with or without Gu-HCl. The data presented are mean values in the case of repeated experiments. ND, not determined.

NAT1	Half-life at 37 °C (min)		IC ₅₀ of Gu-HCl (mM)	
	PAS (10 μ M)	SMZ (500 μ M)	PAS (10 μ M)	SMZ (500 μ M)
Wild type	105	268	94	245
C44A	ND	44	ND	155
C223A	ND	426	ND	221
R9A	3.0	< 15	57	71
R9K	8.3	3.9	47	148
R9M	3.0	< 15	50	61
R64A	2.9	< 15	51	102
R64K	6.1	< 15	60	242
R64M	3.9	< 15	44	130
R64Q	4.7	< 15	89	124
R9K/R64K	2.9	ND	47	ND
R61A	118	ND	137	ND
R62A	123	ND	146	ND
R61A/R62A	9.4	ND	112	ND

NAT2	Half-life at 37 °C (min)		IC ₅₀ of Gu-HCl (mM)	
	SMZ (100 μ M)	SMZ (500 μ M)	SMZ (100 μ M)	SMZ (500 μ M)
Wild type	800	970	557	568
C44A	ND	1040	ND	423
C223A	ND	1490	ND	521
R9A	2.5	ND	249	277
R9K	597	ND	548	585
R9M	2.6	< 15	189	224
R64A	121	ND	334	329
R64K	138	ND	286	318
R64M	203	ND	340	306
R64Q	110	ND	227	ND
R9K/R64K	98	ND	353	ND
R61A	695	ND	462	ND
R62A	652	ND	442	ND
R61A/R62A	423	ND	454	ND

His; boxed in Figure 1) was conserved at positions 61–62 in vertebrates, corresponding to Arg⁶¹-Arg⁶² in humans.

Effect of substitution of Cys⁶⁸ on the activity of human NATs

We have previously shown that mutation of Cys⁶⁸ to Gly (C68G) in human NAT2 completely eliminates its catalytic activity [6]. We further examined the effect of mutating this highly conserved Cys residue in both NAT1 and NAT2 by constructing Cys → Ala mutants, which are expected to have more structural stability than proteins containing Gly substitutions [6]. Substitution of Cys⁶⁸ with Ala inactivated both NAT1 and NAT2 for the acetylation of PAS, SMZ (Table 2) and 2-AF (results not shown). The bacterial strains containing the mutated constructs provided no greater acetylation activity than *E. coli* harbouring the non-recombinant vector alone (i.e. up to 0.004 % and 0.2 % of wild-type NAT1 and NAT2 activities respectively). Two other Cys residues of NAT1 and NAT2 that are fully conserved in vertebrate species, namely Cys⁴⁴ and Cys²²³, were also substituted with Ala. The resulting proteins retained their catalytic activity, for which we estimated kinetic constants (Table 2), thermal stability and resistance to Gu-HCl denaturation (Table 3). The

Figure 2 Time-dependent inactivation of wild-type NATs with PG

Undiluted bacterial lysates containing recombinant NAT1 or NAT2 were preincubated for 0.5–2.5 min or 2–25 min respectively at 37 °C with PG at 3.5 (●), 4 (○), 5 (▲), 6 (△), 7.5 (■), 10 (□) or 12 (×) mM (final concentration). After each preincubation period, the residual *N*-acetylation activity was measured in 3 min assays against SMZ at 2 mM (NAT1) or 0.1 mM (NAT2). Results are the means of three separate experiments. Insets show double-logarithmic plots of the apparent first-order inactivation rate (k_{obs}) against molar PG concentration. Values of 1.7 ($r = 0.99$) and 2.2 ($r = 0.99$) for the reaction order with respect to PG were calculated from the slopes for NAT1 and NAT2 respectively. The initial reaction rates without PG were 33.9 ± 0.19 ($n = 5$) and 4.82 ± 0.98 ($n = 5$) nmol/min per mg of lysate protein for NAT1 and NAT2 respectively.

apparent K_m values for acceptor substrates were not altered for these proteins, but the C44A mutants exhibited significant decreases in V_{max} as well as loss of stability.

Inactivation of NATs by PG

In mild conditions (pH 7–8 and 25 °C), PG reacts with the guanidinium group of accessible Arg side chains in proteins to give a stable single product [19]. We incubated bacterial lysates containing recombinant NAT enzymes with 3.5–12 mM PG, which corresponds to a 14–48-fold molar excess of the modifying agent over the estimated content of Arg residues in bacterial lysates [20]. The inactivation of NAT was time- and PG-concentration-dependent (Figure 2). Apparent pseudo-first-order

Table 4 Effect of double mutation R9K/R64K on apparent and true kinetic parameters of NAT1 and NAT2

N-Acetylation activities were measured in lysates from recombinant *E. coli*. Apparent kinetic parameters were determined for three arylamine acceptors (SMZ, PAS and 2-AF) in the presence of acetyl-CoA (100 μ M) as the donor substrate, and for acetyl-CoA in the presence of 2-AF [concentration of $10 \times K_m(\text{app})$] as the acceptor substrate. True K_m values were measured using 2-AF and acetyl-CoA as the acceptor and donor respectively, and were calculated for each of the two substrates. Two or more separate experiments were carried out. True $V_{\max}$ values were measured in four separate experiments by varying the concentration of both 2-AF and acetyl-CoA. Apparent and true $V_{\max}/K_m$ ratios are also given as a percentage of the value for wild-type NAT (in parentheses; see Table 2). Statistical comparisons were performed by Student's *t* test (**** $P < 0.001$; *** $P < 0.01$; ** $P < 0.02$; * $P < 0.05$).

Mutant	Substrate	$K_m(\text{app})$ (μ M)	$V_{\max}(\text{app})$ (nmol/min per mg of protein)	$V_{\max}(\text{app})/K_m(\text{app})$	Substrate	$K_m(\text{true})$ (μ M)	$V_{\max}(\text{true})$ (nmol/min per mg of protein)	$V_{\max}(\text{true})/K_m(\text{true})$
NAT1								
Wild type†	2-AF	18 $\pm$ 2.8	870 $\pm$ 39	49 (100%)	2-AF	300 $\pm$ 65	6650 $\pm$ 410	22 (100%)
	Acetyl-CoA	460 $\pm$ 58	3670 $\pm$ 250	8.05 (100%)	Acetyl-CoA	400 $\pm$ 31		17 (100%)
	2-AF	2.28 $\pm$ 0.57****	(0.05 $\pm$ 3.3) $\times 10^{-3}$ ****	2.3×10^{-2} (0.05%)	2-AF	29 $\pm$ 5.0**		7.2×10^{-2} (0.33%)
	SMZ	390 $\pm$ 62*	(0.02 $\pm$ 1.0) $\times 10^{-3}$ ****	6.6×10^{-5} (0.30%)‡	Acetyl-CoA	2170 $\pm$ 410**		9.7×10^{-4} (0.01%)
	PAS	1.22 $\pm$ 0.21***	(0.10 $\pm$ 6.1) $\times 10^{-3}$ ****	8.2×10^{-2} (0.04%)‡				
R9K/R64K	Acetyl-CoA	800 $\pm$ 71***	0.58 $\pm$ 0.03****	7.3×10^{-4} (0.01%)			2.11 $\pm$ 0.29****	
NAT2								
Wild type†	2-AF	2.25 $\pm$ 0.12	2.34 $\pm$ 0.04	1.04 (100%)	2-AF	30 $\pm$ 2.8	12 $\pm$ 0.3	0.38 (100%)
	Acetyl-CoA	610 $\pm$ 10	21 $\pm$ 0.2	0.03 (100%)	Acetyl-CoA	720 $\pm$ 180		0.02 (100%)
R9K/R64K	2-AF	3.49 $\pm$ 0.38	0.39 $\pm$ 0.02****	0.11 (11%)	2-AF	23 $\pm$ 11	3.35 $\pm$ 0.40****	0.14 (37%)
	SMZ	214 $\pm$ 27*	1.27 $\pm$ 0.06****	6.0×10^{-3} (17%)‡	Acetyl-CoA	970 $\pm$ 160		3.5×10^{-3} (21%)
	PAS	6940 $\pm$ 740	(0.04 $\pm$ 2.4) $\times 10^{-3}$ ****	6.2×10^{-6} (1.2%)‡				
	Acetyl-CoA	670 $\pm$ 45	4.70 $\pm$ 0.17****	7.1×10^{-3} (21%)				

† $V_{\max}(\text{app})$ and $K_m(\text{app})$ values for SMZ and PAS are given in Table 2.

‡ $V_{\max}(\text{app})/K_m(\text{app})$ values are expressed as a percentage of the corresponding wild-type values given in Table 2.

inactivation constants (k_{obs}) were obtained at each concentration of PG by linear regression of plots of the logarithm of percentage of control activity against time [21]. Secondary plots of $\ln(k_{\text{obs}})$ against $\ln(\text{PG concentration})$ were linear, and had slopes of 1.7 and 2.2 for NAT1 and NAT2 respectively (Figure 2). Thus the inhibition reaction probably had a similar order with respect to PG for both NATs. It is unlikely that PG would react in a detectable manner with the acetyl-CoA regenerating system, since similar results were observed when these auxiliary reactants were present in the enzyme assays (results not shown). Because 2 molecules of PG usually condense with a single guanidinium group [19], it is estimated that a single functional Arg residue reacts with the modifying agent in both NATs.

Comparison of the kinetic properties of arginine-substituted NAT1 and NAT2 enzymes

To determine the relative roles of highly conserved Arg residues in catalysis by NAT, recombinant enzymes bearing either single or double substitutions at Arg⁹ and/or Arg⁶⁴ were compared in steady-state kinetic studies. More or less conservative single substitutions at Arg⁹ or Arg⁶⁴ did not appreciably affect apparent K_m values, but caused significant changes in apparent $V_{\max}$ values for arylamine substrates (Table 2). The $V_{\max}$ was dramatically reduced by single Arg $\rightarrow$ Ala or Arg $\rightarrow$ Met mutations at positions 9 and 64, causing a marked loss of catalytic efficiency as estimated by the $V_{\max}/K_m$ ratios for PAS and SMZ; these were reduced 60–1000-fold for NAT1 and 4–90-fold for NAT2. The single Arg $\rightarrow$ Lys substitutions resulted in smaller decreases in $V_{\max}/K_m$, i.e. 6–150-fold for NAT1 and up to 4-fold for NAT2 (Table 2). The double R9A/R64A mutants of NAT1 and NAT2 showed no significant activity against PAS, SMZ (Table 2) or 2-AF (results not shown), as observed for the Cys⁶⁸ $\rightarrow$ Ala mutants. The R9A/R64K and R9K/R64A mutants showed no or weakly detectable activity (Table 2). Apparent K_m values for arylamine substrates were significantly altered for the double R9K/R64K mutants (Table 4). For NAT1 R9K/R64K, there was a 7.7–14.4-

fold decrease in apparent K_m values for the three arylamine substrates, and for NAT2 R9K/R64K there was a 2-fold increase for SMZ only. Also, the NAT1 R9K/R64K mutant had a 5.4-fold higher true K_m for acetyl-CoA than the wild-type enzyme. The double mutant R9K/R64K of NAT1 also had drastically reduced (300–2300-fold) $V_{\max}/K_m$ values due to a low $V_{\max}$ (6–36-fold lower than those of single Arg $\rightarrow$ Met mutants), whereas the same double mutant retained up to 17% of NAT2 catalytic efficiency (Table 4). The basic pair Arg⁶¹/Arg⁶² was also targeted by alanine substitutions (Table 2). Enzymes bearing single substitutions (R61A or R62A) or double substitutions (R61A/R62A) retained considerable catalytic activity, with no marked alteration of the $V_{\max}/K_m$ values.

Effects of arginine substitutions on NAT stability

To investigate further the relative importance of conserved Arg residues, we measured the acetylation activity of wild-type and Arg-substituted enzymes under conditions of spontaneous destabilization at 37 °C, or with the denaturing agent Gu-HCl. Except for NAT1, the double substitution R61A/R62A affected neither the *in vitro* stability nor the resistance to Gu-HCl (Table 3). The intrinsic stability of NAT1 R61A/R62A was 10-fold lower than that of the wild type. Alternatively, single substitutions to Ala, Met or Gln at Arg⁹ or Arg⁶⁴ lowered the intrinsic *in vitro* stability (10–300-fold) and resistance to Gu-HCl (1.5–4.0-fold) of both NAT1 and NAT2 (Table 3). Replacement of Arg⁹ with Lys did not alter either the intrinsic stability of NAT2 or its resistance to Gu-HCl. The same substitution produced a decrease in NAT1 stability, although less than for the other substitutions tested. The stability parameters of the NAT1 R9K/R64K and NAT2 R9K/R64K mutants were similar to those of R9K- or R64K-substituted NAT1 and R64K-substituted NAT2 respectively.

Effects of arginine substitutions on pH-dependence of NAT activity

To investigate a possible involvement of Arg⁹ and Arg⁶⁴ in the

Figure 3 Effect of pH on apparent kinetic parameters of wild-type and Arg⁹ and/or Arg⁶⁴ mutant NATs

The K_m (μ M) and V_{max} (nmol/min per mg of lysate protein) values were measured in kinetic studies using five different concentrations of PAS (3, 10, 50, 75 and 100 μ M for NAT1, and 0.4, 2, 5, 10 and 20 mM for NAT2) in 15 min assays at various pH values. NAT activity was measured in bacterial lysates containing wild-type (●), R9K/R64K (○), R9A (■) or R64A (□) NAT1 or NAT2 enzymes. The mean ionic strength was held at 50 mM.

mechanisms of NAT-catalysed reactions, apparent kinetic constants were determined with PAS for wild-type and single or double Arg⁹- and Arg⁶⁴-substituted NATs as a function of pH. The logarithm of the V_{max} or K_m was plotted against pH for each enzyme (Figure 3). The pH-dependence profiles of the K_m and V_{max} of single Arg → Ala mutants were similar to those of the related wild-type enzyme, NAT1 or NAT2. The double R9A/

R64A mutant proteins had no detectable activity at any pH between 5.5 and 9.7 (results not shown). However, the double mutant NAT1 R9K/R64K displayed a loss of pH-dependence for the apparent K_m but not for V_{max} (Figure 3). The same double mutation in the NAT2 protein resulted in pH-dependence profiles similar to those described above for single mutants.

Comparative expression levels of mutant and wild-type NAT proteins

Immunoblot analysis of bacterial lysates showed that inactive Cys⁶⁸- or Arg⁹/Arg⁶⁴-substituted recombinant proteins were generated in similar amounts to the active mutant and wild-type NATs (Figure 4). Similar expression levels were observed for all other mutants (results not shown).

DISCUSSION

Two Arg residues and one Cys are fully conserved in all currently known NATs from sources ranging from prokaryotes (*Salmonella typhimurium*) to human (Figure 1). We investigated the roles of these residues using a site-directed mutagenesis strategy and an *E. coli* expression system. Previous reports have suggested the participation of a single active thiol in acetyl transfer within the NAT active site [5], namely Cys⁶⁸ for human NAT2 [6] and the corresponding Cys⁶⁹ for *S. typhimurium* OAT [8]. In the present study, replacement of Cys⁶⁸ with alanine produced immunoreactive NAT1 protein devoid of any detectable activity towards SMZ, PAS or 2-AF as acceptor substrates. In contrast, the same substitution at Cys⁴⁴ and Cys²²³ gave similar quantities of immunoreactive NAT1 which were still catalytically active. Thus Cys⁶⁸ or its counterpart is likely to participate in the catalytic mechanism of acetate transfer from acetyl-CoA to the acceptor amine substrate in the active site of all known NATs.

It has been suggested that NATs act through a general base catalysis [9], involving a basic residue that contributes to the transition state by abstracting a proton from the active thiol and acceptor amine [10]. An Arg residue could play such a role [11]. Here we have shown that the inhibition of wild-type human NAT1 and NAT2 activities by a large excess of the Arg-modifying agent PG followed a 2:1 stoichiometry, suggesting that one essential Arg residue is accessible to the inhibitor in both isoenzymes (Figure 2). Identical findings have been reported for purified hamster NAT isoenzymes [11]. However, due to steric restrictions, other essential Arg residues may react at different rates with PG, rendering them difficult to identify. Results from studies using chemical markers are sometimes difficult to interpret in enzyme catalysis studies; the notion that the modifying agents interact specifically with residues directly involved in catalysis need not necessarily be true for all the systems studied. Such agents could inactivate the enzyme by interacting with other residues, causing conformational changes and/or steric hindrance. This guided our choice to use site-directed mutagenesis in the systematic search for essential arginine residues.

We evaluated whether the highly conserved residues Arg⁹ and Arg⁶⁴ are critical for the enzymic function of human NAT. Their separate substitution with Ala (R9A and R64A mutants) produced drastic decreases in apparent catalytic efficiency, as estimated by a 90% decrease in the V_{max}/K_m ratio with both SMZ and PAS. Conversely, the mutations resulted in only small changes in the apparent K_m ; the latter values were, within experimental error, identical to that of the wild type. Such a lack of alteration in K_m (a rough estimate of the affinity of an enzyme for its substrates) would suggest that separate substitutions of

Figure 4 Detection of immunoreactive wild-type and mutant NATs

Proteins in *E. coli* lysates (10 μ g/lane) containing recombinant NAT1 (A) or NAT2 (B) polypeptides were separated by SDS/15%-PAGE, transferred to a PVDF membrane and hybridized with rabbit antiserum raised against a tagged NAT2 fusion protein. Bacteria harbouring the non-recombinant phagemid vector alone (pKEN2) generated the lower band, which is common to all samples. The arrow points to the NAT-specific band. The positions of size marker proteins are indicated on the left.

Arg⁹ or Arg⁶⁴ are not sufficient to alter amine substrate binding. However, the substantial decrease in $V_{\max}$ could be related to the alteration in intrinsic NAT stability at 37 °C. Thus both of the fully conserved Arg⁹ and Arg⁶⁴ residues seem to be essential to ensure the stability of functionally active NAT1 and NAT2 enzymes.

To verify that the observed properties of Arg-substituted enzymes were probably due to a lack of the conserved Arg rather than to the introduction of an Ala residue, we performed more conservative replacements of Arg⁹ or Arg⁶⁴ to alternatively mimic the putative positive charge (Lys), polarity (Gln) or hydrophobicity and steric features (Met) of the initial Arg residue. Substitution of Arg with Gln or Met reduced the *in vitro* activity and stability to a similar extent as with Ala. This is consistent with the correlation between the slow acetylator phenotype *in vivo* and an Arg⁶⁴ → Gln substitution known as a polymorphic site in the NAT2* locus [22,23]. Indeed, the NAT2 mutant designed in our study is identical to the naturally occurring NAT2*14A (G¹⁹¹ → A) allelic variant. Thus we confirm here that this allele encodes a markedly unstable protein with reduced catalytic activity *in vitro* [24]. Overall, lysine appeared to be the least disruptive substitution of Arg⁹ or Arg⁶⁴, since it resulted in minimal although variable effects on enzyme activity and stability. The notably similar decreases in $V_{\max}$ and loss of intrinsic stability after the replacement of Arg⁹ or Arg⁶⁴ (Tables 2 and 3) suggested that most of these substitutions (except for NAT2 R9K) had destabilizing effects upon NAT1 and NAT2. Also, there was an increased sensitivity of these mutants to denaturation by Gu-HCl. This compound preferentially increases the flexibility of protein regions with a high degree of freedom, such as catalytic sites [25]. Thus these Arg substitutions may alter the conformational stability within the active site of the mutant NATs.

To investigate further the role of the conserved residues Arg⁹ and Arg⁶⁴ in NAT function, we mutated both Arg residues into Ala and/or Lys. The double NAT1 and NAT2 R9A/R64A mutants were devoid of detectable activity, as observed for C68A mutants, and inactivation was also observed with double R9K/R64A and R9A/R64K mutants. Thus the presence of at least one conserved Arg at position 9 or 64 is probably crucial for maintaining the configuration of the active site of NATs. The properties of R9K/R64K mutant enzymes, which possess conservative substitutions, further defined the roles of Arg⁹ and Arg⁶⁴. Marked differences were apparent between the NAT1 and NAT2 double mutants (Table 4). The apparent K_m was decreased significantly with the three arylamine substrates for NAT1 mutants, whereas it was increased significantly (SMZ) or not changed (2-AF, PAS) in the case of NAT2. This distinction may be due to the substitution of Arg with a more hydrophobic Lys residue and may reflect some differences in substrate-binding properties between NAT1 and NAT2; namely, Arg⁹ and Arg⁶⁴ in NAT1 could contribute to hydrophobic interactions with the arylamine substrate. Also, a significantly higher true K_m for acetyl-CoA of the NAT1 double mutant (R9K/R64K) compared with the wild-type protein suggested that Arg⁹/Arg⁶⁴ interacts specifically with acetyl-CoA. Therefore we suggest that both of these positively charged Arg residues in NAT1 might interact with a common anionic group, which could be the terminal phosphates of CoA/acetyl-CoA, and thus contribute to the stabilization of the transition state by charge neutralization. The lack of pH-dependence of the apparent K_m of NAT1 R9K/R64K (Figure 3) could be at least partly explained by an alteration in these ionic bonds, and thereby Arg⁹ and Arg⁶⁴ could both be responsible for the pH-dependence of NAT1. The direct involvement of a conserved Arg residue in binding interactions

with the 3'-phosphate of the substrate CoA/acetyl-CoA has been reported for various enzymes, such as rat choline acetyltransferase [26]. This model is less probable for NAT2, since the double mutation (R9K/R64K) did not modify either the true K_m for acetyl-CoA or the pH-dependence of the enzyme, but cannot be disproved by the design of our studies. Nonetheless, Arg⁹ and Arg⁶⁴ may contribute to the active-site integrity of NAT2 without direct interaction with the acetyl donor. Moreover, our results confirm the hypothesized stabilizing effect of Arg⁶⁵ [8], but do not support a subsequent model in which Arg⁶⁵ in the *S. typhimurium* protein or its human counterpart Arg⁶⁴ is involved in the NAT catalytic mechanism as a general base catalyst [8,12]. If a single basic residue is involved in such a mechanism, it could be another conserved basic residue, such as His¹⁰⁷. Experiments are in progress to clarify this issue.

Finally, we tested the hypothesis of the synergistic action of two adjacent Arg residues leading to proton destabilization, thereby allowing one of these amino acids to act as a proton acceptor [12]. The double substitution of Arg⁶¹/Arg⁶² into an Ala pair did not produce a significant change in kinetic parameters, suggesting that the putative basic residue involved in general base catalysis is not part of this conserved basic amino acid pair. However, this putative basic pair could involve amino acid chains that are adjacent in the ternary structure but not in the primary structure of the protein.

In conclusion, the data presented here show that three amino acids (Cys⁶⁸, Arg⁹ and Arg⁶⁴) are critical for the enzymic function of human NATs. The role of Arg⁹ and Arg⁶⁴ is likely to be structural, rather than being linked directly to proton abstraction during the catalytic process. In view of the absolute conservation of these residues in all known NATs, these results can probably be extended to all arylamine-metabolizing NAT species. Of course, the availability of the three-dimensional structure of the enzyme would be likely to permit the identification of potential active-site residues that could be verified by site-directed mutagenesis.

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