Recombinant rat and hamster \( N \)-acyetyltransferases-1 and -2: relative rates of \( N \)-acetylation of arylamines and \( N,O \)-acyltransfer with arylhydroxamic acids

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Introduction

Genes for the 290 amino acid, 33–35 kDa cytosolic acetyltransferases (\( \text{NAT}1^* \) and \( \text{NAT}2^* \)) from rat and hamster were cloned and expressed in \textit{Escherichia coli}. Active clones were selected by a simple visual test for their ability to decolorize 4-aminooazobenzene in bacterial medium by acetylation. These recombinant acetyltransferases were analyzed for: (i) \( N \)-acetyltransferase, which was assayed by the rate of acetyl coenzyme A-dependent \( N \)-acetylation of 2-aminofluorene (2-AF) or 4-aminooazobenzene (AAB); (ii) arylhydroxamic acid acyltransferase, assayed by \( N,O \)-acyltransfer with \( N \)-hydroxy-\( N \)-acyetyl-2-aminooazobenzene. Both NAT2s showed first order increases in \( N \)-acetylation rates with increasing 2-AF or AAB concentrations between 5 and 100 \( \mu \)M, with apparent \( K_m \) values of 22–32 and 62–138 \( \mu \)M respectively. Although under the same conditions the \( N \)-acetylation rates for the two NAT1s declined by >50%, below 5 \( \mu \)M 2-AF or AAB, the NAT rate data fit Michaelis–Menten kinetics, and the apparent \( K_m \) values were 0.2–0.9 \( \mu \)M. For \( N,O \)-acyltransferase, the apparent \( K_m \) values of the NAT1s were ~6 \( \mu \)M, while the \( K_m \) values of the NAT2s were ~20–70-fold higher. SDS–PAGE/Western blot analysis of the recombinant acetyltransferases gave apparent relative molecular weights (MW\(_r\)) of ~31 kDa for both NAT1s and rat NAT2 and ~29 kDa for hamster NAT2. Comparable MW\(_r\) values were observed for native hamster liver NAT1 and NAT2 and for rat NAT1 under the same conditions. Although we did not detect NAT2-like activity in rat liver cytosol previously, the present data show that the rat NAT2\( ^* \) gene does code for a functional acetyltransferase, with properties similar to those of hamster liver NAT2. The data also indicate that at low substrate concentrations, NAT1 would apparently play the predominant role in vivo in \( N \)-acetylation and \( N,O \)-acyltransfer of aromatic amine derivatives, including their metabolic activation to DNA-reactive agents.

Materials and methods

**Chemicals and other reagents**

Bacto-tryptone, Bacto-yeast extract and Bacto-agar were purchased from DIFCO (Detroit, MI). Isopropyl \( \beta \)-D-thiogalactoside (IPTG), ampicillin and other reagent grade chemicals were from Sigma Chemical Co. (St Louis, MO). 4-Aminooazobenzene (AAB) and 2-AF were from Aldrich Chemical Co. (Milwaukee, WI). Molecular biology grade phenol and electrophoresis grade agarose were from BRL (Gaithersberg, MD). NuSieve agarose was from FMC Bioproducts.
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AAB color in 6-8 h and complete decolorization of AAB typically occurred after an overnight incubation. The pKEN2 vector alone gave no detectable activity. NAT-positive clones from each species were tested for activity by the visual whole-cell AAB acetylation assay in 96-well plates. Approximately 2 dozen ampicillin-resistant clones of each of the rat and hamster NATs were screened for N-acetyltransferase activity. Bacterial cells were centrifuged for 10 min at 1800 g at 5°C, washed once and resuspended in NaP4H buffer (50 mM sodium pyrophosphate, 2 mM dithiothreitol, pH 7.4), sonicated (29), then centrifuged for 5 min at 13 000 g to remove insoluble debris (90% fraction). The S105 fraction was the supernatant after centrifugation at 10 000 g for 1 h at 5°C.

**Results**

**Screening of pKEN2/NAT* clones for N-acetylation activity**

**NAT1** and **NAT2** genes were selectively PCR amplified from rat and hamster genomic DNAs using gene-specific primers (17). As estimated by agarose-ethidium bromide gel electrophoresis (26), the PCR products were typically 890 bp (data not shown). This is the size expected for the coding region of the acetyltransferase genes, which are intronless and contain 290 codons in mammals (3,5,7,8). The PCR products were re-amplified with p5' and 2'3' primers: rat NAT1*, p5' and r1/p3'; rat NAT2*, p5' and 2p3'; hamster NAT1*, p5' and h1/p3'; hamster NAT2*, p5' and 2/p3'. The PCR-amplified DNAs were then restricted with EcoRI and BamHI and recovered by ethanol precipitation (23).

**Preparation of bacterial expression vectors for recombinant NATs**

The IPTG-inducible bacterial expression vector pKEN2 and *Escherichia coli* XA900F'lacP' (25) cells were obtained from Dr G.L. Verdine (Department of Chemistry, Harvard University). This expression system was also used by Dupret and Grant (24) to express the human **NAT1** and **NAT2** genes in *X. coli* X900 cells. pKEN2 plasmid DNA was purified from bacterial cells by an alkaline lysis method (20). In preparation for cloning, the pKEN2 vector was cut with EcoRI and BamHI, dephosphorylated with calf intestinal alkaline phosphatase and then methylated with neutralized phenol and chloroform/isoamyl alcohol (24-1). The DNA was precipitated by addition of ammonium acetate and isopropanol to final concentrations of 1 M and 50% respectively. The DNA was recovered by centrifugation and dissolved in TE buffer (5 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). This pKEN2 vector was then ligated with an equimolar amount of EcoRI/BamH1-restricted **NAT1** or **NAT2** gene DNA with T4 DNA ligase under conditions recommended by the supplier (BRL), giving pKEN2/NAT* vectors.

**Screening of NAT* clones for N-acetyltransferase activity**

*Escherichia coli* XA900F'lacP' was transformed with plasmid DNA as described by Hanahan (25), then plated on agar dishes supplemented with LB medium (10 g Bacto-tryptone, 10 g sodium chloride and 5 g Bacto-yeast extract per liter, pH 7.5) plus ampicillin (125 g/ml) and incubated overnight at 37°C. Ampicillin-resistant colonies were scored visually for NAT activity. Berberine hydrochloride was used as a positive control for NAT activity. The remaining colonies were then transferred to individual wells in 96-well plates containing 100 ml aliquots of LB (26) supplemented with ampicillin (125 g/ml), IPTG (1 mM) and AAB (0.5 mM), then grown overnight at 37°C at 250 r.p.m. The culture medium of clones expressing active NAT was decolorized due to N-acetylation of AAB (bright yellow) to N-acetylaminoazobenzene (colorless in LB medium). Addition to the medium of an equal volume of 50% ethanol/20% trichloroacetic acid (27) at the end of the incubation period causes a more prominent color change in NAT-positive cultures: from bright red-orange (unmodified AAB) to colorless (N-acetylaminoazobenzene), leaving only the background color of LB medium.

**Protein determination**

Protein concentrations were estimated by the Bradford dye binding assay (28) using BioRad Laboratories (Hercules, CA) protein assay reagents and bovine serum albumin as the standard.

**Western blot analysis**

Western analysis of recombinant NAT proteins in bacterial sonicates was performed as described previously for tissue extracts (14), except that the samples were solubilized with 1% Triton X-100 to release membrane-bound proteins prior to diluting with SDS-PAGE buffer. Briefly, the indicated aliquots of bacterial sonic extracts were stored at -70°C and analyzed by Western blotting by the method of Zabrowski et al. (6). Membrane-bound proteins were solubilized with 0.5% sodium dodecyl sulfate, 100 g/ml proteinase K, 1% Triton X-100, 1 mM DTT, 0.1 mM EDTA, pH 8.0). The protein concentrations of the NATs were estimated by comparison with protein size markers (BioRad Laboratories, Hercules, CA). Bacterial sonicates were prepared as follows. Cultures were incubated at 37°C for 6-9 h at 250 r.p.m. in LB medium plus ampicillin (125 g/ml) and IPTG (1 mM) to induce acetyltransferase expression. Bacterial cells were centrifuged for 10 min at 1800 g at 5°C, washed once and resuspended in NaP4H buffer (50 mM sodium pyrophosphate, 2 mM dithiothreitol, pH 7.4), sonicated (29), then centrifuged for 5 min at 13 000 g to remove insoluble debris (90% fraction). The S105 fraction was the supernatant after centrifugation at 10 000 g for 1 h at 5°C.
Table I. Kinetic parameters and relative activities of recombinant rat and hamster acetyltransferases

<table>
<thead>
<tr>
<th></th>
<th>Rat NAT1</th>
<th>Rat NAT2</th>
<th>Hamster NAT1</th>
<th>Hamster NAT2</th>
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<tbody>
<tr>
<td>N-O-acetyltransfera&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<td></td>
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<tr>
<td>Apparent K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>5±3.1</td>
<td>356±171</td>
<td>6.8±1.2</td>
<td>134±104</td>
</tr>
<tr>
<td>Apparent V&lt;sub&gt;max&lt;/sub&gt; (nmol/min/mg)</td>
<td>3.8±1.9</td>
<td>1.29±0.96</td>
<td>1.3±0.23</td>
<td>4.02±1.55</td>
</tr>
<tr>
<td>AAB N-acetylation&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Apparent K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>0.64±0.023</td>
<td>138±34</td>
<td>0.89±0.094</td>
<td>62.1±17.5</td>
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<tr>
<td>Apparent V&lt;sub&gt;max&lt;/sub&gt; (nmol/min/mg)</td>
<td>4.18±0.29</td>
<td>59.7±5</td>
<td>43.4±1.12</td>
<td>142±52.4</td>
</tr>
<tr>
<td>AAB/2-AF N-acetylation&lt;sup&gt;0&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Apparent K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>0.18±0.013</td>
<td>32.2±14.3</td>
<td>0.39±0.054</td>
<td>21.5±9.2</td>
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<tr>
<td>Apparent V&lt;sub&gt;max&lt;/sub&gt; (nmol/min/mg)</td>
<td>47±0.26</td>
<td>833±707</td>
<td>53±0.93</td>
<td>1020±573</td>
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<tr>
<td>Ratio of activities&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AAB/N-acetylation/N-O-acetyltransfer</td>
<td>11</td>
<td>46</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>AAB/2-AF N-acetylation/N-O-acetyltransfer</td>
<td>12</td>
<td>650</td>
<td>41</td>
<td>250</td>
</tr>
<tr>
<td>AAB/2-AF N-acetylation/AAB N-acetylation</td>
<td>1.1</td>
<td>14</td>
<td>1.2</td>
<td>7.2</td>
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</table>

<sup>a</sup>Apparent K<sub>m</sub> and apparent V<sub>max</sub> values (± SD) were calculated using the Eadie–Hofstee method. The values shown are the means of three replicate experiments, where the data for each experiment were calculated from five or six enzyme dilutions giving a linear regression R value of ≥0.9.

<sup>b</sup>Ratios of the apparent V<sub>max</sub> values.

Table II. Acetyl donor preferences of recombinant rat and hamster acetyltransferases

<table>
<thead>
<tr>
<th></th>
<th>N-Acetylation (nmol/min/mg)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>With N-OH-AABP&lt;sup&gt;e&lt;/sup&gt;</th>
<th>With AcCoA&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Ratio of activities (AcCoA/N-OH-AABP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat NAT1</td>
<td>97.1±23</td>
<td>11±2.2</td>
<td>0.11</td>
<td></td>
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<tr>
<td>Rat NAT2</td>
<td>24.9±4.8</td>
<td>75±19</td>
<td>3.0</td>
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<tr>
<td>Hamster NAT1</td>
<td>83.6±18</td>
<td>16±4.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Hamster NAT2</td>
<td>36.2±8.7</td>
<td>113±27</td>
<td>3.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>d</sup>Rate of acetylation of AAB (0.3 mM) ± SD; Booth assay conditions (27).

<sup>e</sup>Acetyl donor 1 mM N-OH-AABP.

<sup>f</sup>Acetyl donor 1 mM AcCoA.

The relative 2-AF N-acetylation and N,O-acetyltransferase activities are given in Table I for the bacterially expressed rat and hamster NAT1s and NAT2s. The N-acetylation reaction velocities of both NAT2s showed a first order dependence on increasing concentrations of the substrates 2-AF and AAB between 5 and 100 μM (where the AcCoA concentration was 0.4 mM), giving apparent K<sub>m</sub> values of 22–32 and 62–138 μM respectively (Table I). Under the same conditions, the rates of N-acetylation for the two NAT1s declined from 50 to 80% (data not shown). However, N-acetylation by the NAT1s at acceptor concentrations of 1–5 μM followed Michaelis–Menten kinetics and these data gave K<sub>m</sub> values of 0.2–0.9 μM for 2-AF and AAB (Table I).

There were marked isozyme-specific differences in the ratios of N-acetylation of 2-AF versus AAB (Table I), which were ~1 for the two NAT1s and >7 for the two NAT2s. There were also isozyme-specific differences in the acetyl group donor preference (Table II) at the substrate concentrations used in the Booth transacetylation assay (acetyl donor 1 mM N-OH-AABP; acceptor 0.3 mM AAB). The ratio of AAB N-acetylation (acetyl donor 1 mM AcCoA, acceptor 0.3 mM AAB) to Booth transacetylation activity was ≈0.2 for the NAT1s, but ~3 for the NAT2s. In addition, the AAB N-acetylation rates for the NAT2s were ~7-fold greater than for the NAT1s. Similar data were obtained for other independent clones of the NAT1s and NAT2s (data not shown). These data indicate that AcCoA is a far more efficient acetyl donor for the NAT2s than are the N-acetyl arylhydroxamic acids under these conditions.

Discussion

We have reported here that NAT1<sup>*</sup> and NAT2<sup>*</sup> genes from both rat and hamster were cloned into the IPTG-inducible expression system. 발간된 연구로 보면, 이들의 특성은 다음과 같습니다. rat NAT1<sup>*</sup> (31; accession no. U17260), rat NAT2<sup>*</sup> (31; accession no. U17261), hamster NAT1<sup>*</sup> (5; accession no. X54142) and hamster NAT2<sup>*</sup> (31; accession no. L24912). [The NAT<sup>*</sup> coding sequences of the Wistar rat acetyltransferase cDNAs (7) are the same as those in CD and Fischer rats (31; R.F.Jones, data not shown).]
of N-acetylation of AAB with AcCoA relative to N-OH-AABP higher than those for the NATls. Also, the ratios of the rates apparent m significant N,O-acyltransferase activity. However, their by acetylation is primarily attributable to NAT1, both in vivo mark- edly. Although the bioactivation of arylamine derivatives at ~32 kDa (33) under similar conditions. E.coli enzymes on Western blots (5,32). When expressed in mammalian cells. Recombinant hamster NAT1 and NAT2 isozymes in CHO cells by Nagata et al. (32) showed that the rate of O-acetylation of 2-N-hydroxyamino-6-methylpyridi [1,2-a]3.2'-d]imidazole was ~20-fold greater for NAT1 than NAT2. Conversely, 2- AF NAT activity was ~4-fold greater for NAT2 in that study. Although we and others have shown by Northern analysis (7,22), RT-PCR analysis (22) and in situ hybridization (13) that both NATs are expressed in many if not all rat tissues, NAT1* transcripts predominate and native rat NAT2-like activity has not been demonstrated in vivo (14). Therefore, the demonstration here and by Ebisawa et al. (7) that the recombinant rat NAT2 is enzymatically active raises questions about the role of this rat acetyltransferase in metabolizing aromatic amines. Depending on the relative levels of the two NAT isoymes, the net result of their combined activities in vivo may be to give relatively constant rates of N-acetylation over a broad substrate range, although NAT activity for some substrates might be modulated in vivo (7). The data also indicate that at low micromolar arylamine concentrations, NAT1 would play the predominant role in bioactivation of arylamine derivatives in rat liver and other tissues.

In summary, we have shown that bacterially expressed rat and hamster NAT1 and NAT2 have very similar isozyme-specific physical and kinetic properties and these are analogous to those reported for their native counterparts in liver cytosol. Both rat and hamster NAT1s efficiently catalyzed N,O-acyltransfer activity implicated in metabolic activation of amines, but showed marked substrate inhibition for N-acetylation reactions. Although the NAT2s also have significant N,O- acetyltransfer capability, their $K_m$ values for arylhydroxamic acids are 1–2 orders of magnitude higher. These recombinant acetyltransferases should be useful for further structure–function studies of arylamine biotransformations in vitro and for determining the biological consequences of their expression in vivo.

Acknowledgements
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References

vector pKEN2 and expressed in E.coli XA90. A convenient 96-well plate assay for AAB N-acetylation activity is described for direct visual identification of clones producing active acetyltransferases. Expression of appropriately sized recombinant NAT proteins was confirmed by Western blot analysis and kinetic parameters were determined for their N-acetyltransferase and N,O-acyltransfer activities.

Western blot analysis indicated that although hamster and rat NAT1 and NAT2 have calculated molecular weights of 33.4–33.9 kDa, the MWs of their bacterially expressed recombinants as estimated by SDS–PAGE were significantly smaller: 31 kDa for hamster and rat NAT1s and 29 kDa for hamster NAT2. Comparable mobilities were seen previously for the native acetyltransferases in rat and hamster liver cytosols under the same conditions (14,17). In the present experiments, the recombinant rat NAT2 appeared to have approximately the same MW, as the NAT1s. These shifts to faster electrophoretic mobility are not likely to be a consequence of post-translational modifications, since such modifications differ in bacteria and mammalian cells. Recombinant hamster NAT1 and NAT2 expressed in COS-1 cells showed analogous mobility differences on Western blots (5,32). When expressed in E.coli with an N-terminal ‘flag’ extension, hamster NAT1 migrated at ~32 kDa (33) under similar conditions.

The kinetic parameters for the two NAT isoymes differed markedly. Although the bioactivation of arylamine derivatives in vivo by acetylation is primarily attributable to NAT1, both recombinant rat and hamster NAT2s were found to have significant N,O-acyltransferase activity. However, their apparent $K_m$ values for N-OH-AAF were ~20- to 70-fold higher than those for the NAT1s. Also, the ratios of the rates of $N$-acetylation of AAB with AcCoA relative to N-OH-AABP as donor were up to 30-fold higher for NAT2 than NAT1. The rates of 2-AF and AAB $N$-acetylation by both NAT isoymes followed simple first order Michaelis–Menten kinetics, but at markedly different acceptor substrate concentration ranges: at 5–100 μM for the NAT2s and at <5 μM for the NAT1s (with rate declines of up to 80% between 5 and 100 μM). Both $N$- and O-acetyltransferase reactions have been reported to operate through a ping-pong Bi Bi mechanism, in which the acetyl group is transferred from AcCoA to a cysteinyl residue in NAT, then to the arylamine or hydrazine substrates (34). Further work will be needed to determine the mechanism and biological implications of the decline in N-acetylation rates by the NAT1s with acceptor substrate concentrations >5 μM. For rat pineal NAT1 expressed in CHO cells, Ebisawa et al. (7) reported a similar decline in NAT activity with increasing 2-AF concentrations. Ferguson et al. (35) reported an apparent $K_m$ of ~100 μM for 2-AF NAT for bacterially expressed NAT1s from rapid or slow acetylator hamsters. A direct comparison of recombinant hamster NAT1 and NAT2 isozymes in CHO cells by Nagata et al. (32) showed that the rate of O-acetylation of 2-N-hydroxyamino-6-methylpyridi [1,2-a]3.2'-d]imidazole was ~20-fold greater for NAT1 than NAT2. Conversely, 2-AF NAT activity was ~4-fold greater for NAT2 in that study.
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