N-Hydroxyarylamine O-Acetyltransferase of Salmonella typhimurium: Proposal for a Common Catalytic Mechanism of Arylamine Acetyltransferase Enzymes

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Acetyl-CoA:N-hydroxyarylamine O-acetyltransferase is an enzyme involved in the metabolic activation of N-hydroxyarylamines derived from mutagenic and carcinogenic aromatic amines and nitroarenes. The O-acetyltransferase gene of Salmonella typhimurium has been cloned, and new Ames tester substrains highly sensitive to mutagenic aromatic amines and nitroarenes have been established in our laboratory. The nucleotide sequence of the O-acetyltransferase gene was determined. There was an open reading frame of 843 nucleotides coding for a protein with a calculated molecular weight of 32,177, which was close to the molecular weight of the O-acetyltransferase protein determined by using the maxicell technique. Only the residue of Cys69 in O-acetyltransferase of S. typhimurium and its corresponding residue (Cys69) in N-acetyltransferase of higher organisms were conserved in all acetyltransferase enzymes sequenced so far. The amino acid sequence Arg-Gly-Gly-X-Cys, including the Cys69, was highly conserved. A mutant O-acetyltransferase of S. typhimurium, which contained Ala69 instead of Cys69, no longer showed the activities of O- and N-acetyltransferase. These results suggest that the Cys69 of S. typhimurium and the corresponding cysteine residues of the higher organisms are essential for the enzyme activities as an acetyl-CoA binding site. We propose a new catalytic model of acetyltransferase for S. typhimurium and the higher organisms. — Environ Health Perspect 102(Suppl 6):83–89 (1994)

Key words: O-acetyltransferase, N-acetyltransferase, arylamine, Salmonella typhimurium, cloning, mutagenicity, sequencing, catalytic model

Introduction

Aromatic amines and nitroarenes, which have been used in many fields and are widely distributed in the environment, are known as environmental hazardous compounds because of their carcinogenicity and mutagenicity (1–4). Neither aromatic amines nor nitroarenes interact with DNA per se, but they require metabolic conversion for their mutagenesis (1–4). Several activating enzymes, such as arylhydroxamic acid N,O-acetyltransferase (5), and sulfotransferase (6), have been identified as enzymes responsible for exerting the carcinogenicity of aromatic amines and nitroarenes. Acetyl-CoA:N-hydroxyarylamine O-acetyltransferase (OAT) also plays an important role in the metabolic activation of N-hydroxyarylamines that are formed by oxidation of aromatic amines or by reduction of nitroarenes (7–9). Acetyl-CoA:arylamine N-acetyltransferases (EC.2.3.1.5) (NAT) of human (9), hamster (8,10,11), and mouse (12) have OAT activity. Acetylator genotypes of human (13,14), rabbit (15), and hamster (13,16) strongly influence not only NAT activity but also OAT activity. It is suggested, therefore, that the mammalian OAT is probably the same enzyme as NAT, which plays a major role in the metabolism of drugs and endogenous substances that possess an amine or hydrazine group (17,18). Both reactions of N- and O-acetylation probably proceed through a common intermediate (i.e., an acetyl-cysteinyl-enzyme) (12,19–21). cDNA and/or genomic clones of NATs have been isolated from humans (22–27), rabbits (28–31), hamsters (32), mice (33), and chickens (34–36). Acetyltransferase activity is demonstrated not only in the higher organisms but also in bacteria (37). From Salmonella typhimurium Ames tester strain TA98, an acetyltransferase enzyme has been partially purified and characterized as OAT (38). The OAT is absent in strain TA98/1,8-DNP6 (38) and plays a key role in the mutagenicity of aromatic amines and nitroarenes (4,39,40).

In this article, we describe the cloning of the S. typhimurium OAT gene (41). We established new Ames tester substrains highly sensitive to mutagenic aromatic amines and nitroarenes using the cloned gene (42). Substrate specificity and inhibition analysis suggested that the S. typhimurium OAT is a counterpart of NAT of higher organisms. Sequence similarity of both enzymes at the amino acid level suggested that the Cys69 of the S. typhimurium OAT and the corresponding cysteine residues of the NAT of higher organisms are essential for the enzyme activities as an acetyl-CoA binding site (43). We also proposed a common catalytic mechanism of acetyltransferase for S. typhimurium and higher organisms.

Cloning of the S. typhimurium OAT Gene

First, we constructed a gene library of S. typhimurium TA1538 into vector plasmid pBR322 and then selected rearranged plas-
mids that confer resistance to the killing effect of 2-nitrofluorene on the OAT deficient strain TA1538/1,8-DNP. One of these plasmids, pYG122 (Figure IA), had a molecular size of 11.65 kb, composed of the insert DNA (7.3 kb) and the vector plasmid pBR322. The pYG122-transformed strain YG1007 (= TA1538/1,8-DNP(pYG122)) showed more than 10 times higher mutagenic sensitivity to 1,8-dinitropyrene, 2-aminofluorene, and Glu-P-1 than did the conventional strain TA1538 with vector plasmid pBR322. YG1007 had about 50 times higher N-hydroxy-Glu-P-1 OAT activity and isonitroazid NAT activity than TA1538(pBR322). Thus, we concluded that pYG122 had the OAT gene.

In order to investigate the region necessary for a functional OAT, we constructed deletion derivatives of pYG122 and found that a 1.35-kb fragment spanning from the EcoRV site (6.3 kb) to the BamHI site (7.65 kb) is necessary for OAT activity.

However, levels of the activity on the transformants depended on organization of the 1.35-kb insert and vectors (Figure IB). For example, the strain having pYG219 showed more than 10 times higher enzyme activity than did the strain having pYG218. The plasmids pYG218 and pYG219 have the same 1.35-kb DNA fragment in opposite directions. From this result, we suggested that the direction of transcription and translation of the OAT gene is from the EcoRV site to the BamHI site and the promoter for the OAT gene resides outside of the 1.35-kb fragment.

Establishment of New Strains Highly Sensitive to Mutagenic Aromatic Amines and Nitroarenes

A plasmid pYG219, the ampicillin resistance gene of which has been disrupted and can be introduced into Ames tester strains TA98 and TA100, conferred high activity of OAT (Figure IB) and high sensitivity to 2-nitrofluorene on the strain TA1538/1,8-DNP. Thus we introduced pYG219 into TA98 and TA100, and resulting transformants YG1024 (=TA98(pYG219)) and YG1029 (=TA100(pYG219)) were checked for their mutagenic sensitivity to typical N-hydroxyarylamines, aromatic amines, and nitroarenes. YG1024 or YG1029 showed 3 to 63 times higher sensitivity to all N-hydroxyarylamines, aromatic amines and nitroarenes tested, except for 4-nitroquinoline 1-oxide (Table 1), than did the conventional strains YG1020 (=TA98(pBR322-Ap')) or YG1025 (=TA100(pBR322-Ap')), respectively. This result indicates that the new strains YG1024 and YG1029 permit the efficient detection of the mutagenicity of environmental aromatic amines and nitroarenes. These strains have been widely used for the efficient detection of mutagenic chemicals (44-53) and complex mixtures (54-58).

Nucleotide Sequence of the S. typhimurium OAT Gene

We sequenced the cloned DNA from the PstI site (5.55 kb) to the BamHI site (7.65 kb), the region that is suggested to carry the OAT gene (Figure 1). The result is shown in Figure 2. This sequence contains an open reading frame of 843 bp from nucleotides 853 to 1695 that potentially encodes a protein of 281 amino acids with a calculated molecular weight of 32,177. A possible ribosome-binding site and a possible transcriptional terminator were found upstream and at the end, respectively, of the open reading frame. Possible -35 and -10 sequences were found outside of the

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**Table 1. Mutagenic sensitivity of YG1024 and YG1029 to typical N-hydroxyarylamines, aromatic amines, and nitro-aromatic compounds.**

| Chemical                   | S9 mix | YG1020<sup>a</sup>   | YG1024<sup>b</sup>   | YG1025<sup>c</sup>   | YG1029<sup>d</sup> |
|---------------------------|--------|----------------------|----------------------|----------------------|----------------------|
| N-Hydroxy-2-amino fluorene| +      | 2,080                | 50,700               | 82                   | 5,170                |
| N-Hydroxy-Glu-P-1         | -      | 7,900                | 69,400               | 84                   | 3,000                |
| 2-Amino fluorene          | +      | 63                   | 241                  | 20                   | 551                  |
| 2-Amino anthracene        | +      | 8,090                | 144,000              | 430                  | 5,970                |
| 2-Nitrofluorene           | +      | 351                  | 6,060                | 292                  | 7,450                |
| 1-Nitro pyrene            | +      | 47                   | 1430                 | 28                   | 855                  |
| 1,8-Dinitro pyrene        | +      | 373                  | 3040                 | 74                   | 875                  |
| 2-Nitro napthalene        | +      | 231,000              | 4,780,000            | 21,900               | 500,000              |
| 4-Nitroquinoline N-oxide  | +      | 2.9                  | 12.9                 | 11.2                 | 36.5                 |

<sup>a</sup>Salmonella mutagenicity assays were carried out according to the method of Ames et al. combined with a preincubation procedure for 20 min at 37°C. The mutagenicity test was performed with four to eight doses. The number of induced His<sup>+</sup> revertants per nmole was calculated at every dose; the highest value for each chemical and strain is indicated.<sup>b</sup>-<sup>d</sup> The values indicate S9 mix used for the metabolic activation. - indicates the test was performed in the absence of S9 mix. <sup>e</sup>YG1020 is TA98 containing pBR322-Ap.<sup>f</sup>YG1024 is TA98 containing pYG219. <sup>i</sup>YG1025 is TA100 containing pYG219. <sup>l</sup>YG1029 is TA100 containing pYG219. The plasmid pBR322-Ap<sup>l</sup> is the same as pBR322 but its bla gene is inactivated by deletion between the first Dral site and the third Dral site of pBR322.

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**Figure 1.** (A) Restriction map of pYG122. The EcoRI restriction site derived from pBR322 was assigned the map position of 0 kilobase (kb) in the plasmid. Numbers represent the distance (kb) from the EcoRI restriction site. An open box represents the 7.3-kb DNA fragment of S. typhimurium TA1538, which was inserted into BamHI site of pBR322. Arrows indicate the transcription direction of the genes. Abbreviations: Ap, ampicillin resistance gene; E, EcoRI; H, HindIII; V, EcoRV; P, PstI; B, BamHI; PvuII. (B) Involvement of promoters of vector sequence in the activities of S. typhimurium OAT produced from the 1.35-kb fragment. The plasmids were introduced into S. typhimurium TA1538/1,8-DNP (pYG122, pYG213, pYG218, or pYG219) or E. coli XL1-Blue (pYG221) and N-hydroxy-Glu-P-1 OAT activity of cytosol fraction of the transformants was measured. Numbers represent the activity of OAT (nmole/min/mg protein).
Table 2. Properties of the S. typhimurium OAT and NATs of higher organisms.

|                      | Human<sup>a</sup> | Hamster<sup>b</sup> |
|----------------------|-------------------|----------------------|
|                      | M     | P     | M     | P     |
| Molecular weight<sup>c</sup> | 32,177 | 33,787 | 33,541 | 33,499 |
| Cellular distribution | C     | C     | C     | C     |
| O-Acetylation of      |       |       |       |       |
| N-Hydroxy-Glu-P-1     | +     | +     | +     | +     |
| N-Hydroxy-2-aminofluorene | +   | +     | +     | +     |
| N-Acetylation of       |       |       |       |       |
| Isoniazid             | +     | –     | +     | +     |
| 2-Aminofluorene       | +     | –     | +     | +     |
| p-Aminobenzoic acid   | 4<sup>w</sup> | +     | –     | +<sup>w</sup> | 4<sup>w</sup> |
| N,N-Diacetyltransfer to|       |       |       |       |
| 4-Aminobenzoic acid   | –     | +     | –     | +     |
| N,O-Diacetyltransfer to|       |       |       |       |
| N-Hydroxy-2-aminofluorene | 4<sup>w</sup> | +     | +     | +<sup>w</sup> | 4<sup>w</sup> |
| Inhibition by         |       |       |       |       |
| N-Ethylmaleimide      | +     | +     | +     | +     |
| Paraaxon              | –     | +     | –     | +     |

Abbreviations: NAT, N-acetyltransferase; M, monomorphic NAT; P, polymorphic NAT; C, cytosol fraction. <sup>a</sup>Data from Minchin et al. (9), Deguchi et al. (22), and Ohnuki and Deguchi (23). <sup>b</sup>Data from Trinidad et al. (8), Ozawa et al. (10), Abu-Zeid et al. (32), and Cheon and Hanna (63). <sup>c</sup>Molecular weight was calculated from deduced amino acid sequences. <sup>w</sup>Weak. General characteristics of NAT (59); NAT is cytosolic and is widely distributed among tissues, with highest activities occurring in liver and intestinal tract. Most animal species have the NAT. N-Ethylmaleimide is a potent inhibitor of the NAT. Paraaxon does not inhibit the NAT activity.

Figure 2. Nucleotide and deduced amino acid sequences of the S. typhimurium OAT gene. Possible -35 and -10 regions and Shine-Dalgarno (S.D.) sequence are underlined. Possible transcriptional terminator is indicated by arrows and asterisks. The highly conserved amino acid sequence of R-G-G-X-C is marked with a double line. Cys<sup>69</sup> is marked with a circle. The 1.35-kb DNA fragment that was boxed was subcloned into pBR322 for construction of sensitive strains and pBluescript KS+ for DNA sequencing and for expression. The CCC sequence enclosed with a small box indicates the site where a -1 frameshift mutation was found in O-acetyltransferase deficient strain TA1538/1.8-DNP.

1.35-kb region. The rearranged plasmids, which deleted DNA corresponding to some C-terminal or N-terminal region of the open reading frame, did not confer any activity of acetyltransferase. This result suggests that the open reading frame is the coding region of the OAT of S. typhimurium. A protein with an approximate molecular mass of 33 kDa on SDS-polyacrylamide gel electrophoresis was identified in maxicells harboring pYG213 or pYG219, both of which have the 1.35-kb fragment. A frameshift mutation was found in the open reading frame of the OAT-deficient strain TA1538/1.8-DNP by PCR and sequencing techniques. The mutation is one G:C pair deletion from three consecutive G:C pairs of nucleotides 1442 to 1444 in Figure 2 and creates a new termination site at codon 204. From these results, we concluded that the open reading frame shown in Figure 2 is the coding region of the OAT.

The S. typhimurium OAT is a Counterpart of NAT of Higher Organisms

Because the OAT is present in cytosol, we have prepared the cytosol fraction of crude lysate of the cells harboring pYG221 and subjected it to enzyme assays and inhibition experiments. Table 2 shows the properties of the OAT of S. typhimurium and NATs of higher organisms for comparison. Besides N-hydroxy-Glu-P-1 OAT activity, the S. typhimurium OAT showed isoniazid and 2-aminofluorene NAT activities. On the other hand, many NATs of higher organisms also show NAT and OAT activities. The molecular weight of the S. typhimurium OAT was almost the same as that of the NATs. The sulphydryl-blocking agents, which inhibit NAT of higher organisms (59), strongly inhibited the isoniazid NAT activity of the S. typhimurium OAT. Paraaxon, an inhibitor of N,O-acetyltransferase and deactylase (60), did not inhibit the activity of the S. typhimurium OAT. From these observations, we suggested that the S. typhimurium OAT, the gene of which we cloned and sequenced, is a counterpart of NAT of higher organisms.

The S. typhimurium OAT and the NAT of Higher Organisms Share Similarity at the Amino Acid Level

Since the OAT of S. typhimurium is functionally similar to NAT of higher organisms, we compared the amino acid sequences. The NATs of human, rabbit, hamster, mouse, and chicken were at least 45%
homologous at the amino acid level to each other. The N-terminal region of the S. typhimurium OAT, containing about 170 amino acids, showed 25 to 33% similarity to those of the NATs of higher organisms at the amino acid level (Figure 3). The remaining C-terminal region of the OAT had few similarities with the corresponding region of the NATs, although some similarities were observed at the nucleotide level.

The Cys$^{69}$ Residue of the S. typhimurium OAT and the Cys$^{68}$ Residue of the NAT of Higher Organisms Are Essential for the Enzyme Activities

The S. typhimurium OAT is functionally similar to NAT of higher organisms and both enzymes share similarity at the amino acid level. These observations suggest that a common catalytic mechanism might exist among them. Both enzymes need a cysteine residue for exerting their activities. Structure-activity studies on pigeon liver NAT (19) and rabbit liver NAT (20) suggest that a cysteine residue reacts with acetyl-CoA and an activated acetyl-cysteinyl intermediate is formed. Thus, we suggest that a conserved cysteine residue among their enzymes plays an important role in the catalytic mechanism as an acetyl-CoA-binding site. The similarity at the amino acid level between the S. typhimurium OAT and the NATs of higher organisms was much less than that of the NATs among the higher organisms. Hence, it was easy to focus on the highly conserved regions. Among the NATs of higher organisms, three cysteine residues are conserved completely. However, only one cysteine residue (Cys$^{69}$ for the OAT, Cys$^{68}$ for the NATs) was conserved between the S. typhimurium OAT and the NATs of higher organisms (Figure 3). The amino acid sequence of R-G-G-X-C including Cys$^{68}$ of the OAT or Cys$^{69}$ of the NATs was highly conserved. The results of site-directed mutagenesis experiments indicated that a mutant OAT of S. typhimurium, which contained Ala$^{69}$ instead of Cys$^{69}$, did not show any NAT and OAT activities (data not shown).

Thus, we suggest that the Cys residue of the OAT of S. typhimurium is essential for the enzyme activities as an acetyl-CoA-binding site. It was plausible that the Cys residue of the NATs of higher organisms is also essential for the enzyme activities because it was the only conserved residue among all enzymes. Recently, Dupret and Grant (61) reported that among three cysteine residues that were highly conserved among NATs of higher organisms, Cys$^{68}$ was essential for the enzyme activities of human liver polymorphic NAT. This observation supports our hypothesis that there is a common catalytic mechanism among acetyltransferase enzymes.

Proposed Mechanism of Enzymatic Reactions of Acetyltransferase Enzymes

Riddle and Jencks (62) showed that a general base is involved in the catalysis of pigeon liver NAT. Andres et al. (19) investigated the kinetics of NAT from pigeon liver and proposed that a basic residue is involved in a general base catalysis by attracting the proton of the cysteine residue of an acetyl-CoA-binding site. Recently, Cheon and Hanna (63) reported that an arginine residue is essential for the activity of hamster liver monomorphic and polymorphic NATs. One arginine residue, Arg$^{65}$ in the S. typhimurium OAT and Arg$^{64}$ in the NATs of higher organisms, was highly...
The proton of cysteinyl group of Cys residues is attracted by Arg residue, which is deprotonated even in neutral pH conditions, because the two adjacent guanidino groups of Arg and Arg provide mutual electrostatic destabilization (64). The activated Cys residue accepts an acetyl group from acetyl-CoA, resulting in an acetyl-cysteinyl-enzyme intermediate. Finally, this acetyl moiety is transferred to the oxygen atom of an N-hydroxyarylamine. In the transfer reaction of the acetyl group to the hydroxyamino group, the deprotonated Arg (or Arg) again serves as a general base. We propose that the principle of this reaction mechanism is applicable to any acetyltransfer reactions in both the S. typhimurium OAT and the NATs of higher organisms. The S. typhimurium OAT acts as an NAT when the acetyl moiety of the acetyl-cysteinyl intermediate is transferred to the nitrogen atom of an aromatic amine instead of the oxygen atom of an N-hydroxyarylamine. It is also proposed, with regard to mammalian NAT and OAT, that N- and O-acetyl transfer involves a common acetylated enzyme intermediate (12, 21). Cys residues of the NATs of human, rabbit, hamster, mouse, and chicken could bind with acetyl-CoA, as for Cys of the S. typhimurium OAT, whereas one of the basic amino acids, (e.g., Arg) may be an activator (Figure 4B). The acetyl moiety of the acetyl-Cys intermediate would be transferred to the nitrogen atom (N-acetylation) or the oxygen atom (O-acetylation) of an arylamine or N-hydroxyarylamine, respectively. An arylhydroxamic acid also may act as the acyl-donor instead of acetyl- CoA in the model, and the resulting activated acyl moiety would be transferred to the nitrogen atom of an arylamine (N,N-acetylation), or the oxygen atom of an N-hydroxyarylamine (inter and/or intramolecular N,O-acetylation). The S. typhimurium OAT has low but measurable N,O-acetylation transferase activity (Igarashi et al., unpublished result). One possible reason why the S. typhimurium OAT has a low N,O-acetylation transferase activity is that an arylhydroxamic acid could not easily fit in the active site where the Cys probably resides. However, we must point out that certain N,O-acetyltransferases have different properties from the OAT and NAT; some of the enzymes are located in microsomes and are sensitive to paraoxon (60). The catalytic mechanism of these enzymes is probably different from that proposed in Figure 4A.

**Future Perspective**

We proposed a catalytic model for the *S. typhimurium* OAT and suggested that this model is applicable for NAT of higher organisms. To examine this model, biochemical and structural analyses of the purified enzyme are important. Hence we have been purifying the *S. typhimurium* OAT from *Escherichia coli* cells harboring plasmid pYG221, the cells which overproduced the OAT (Figure 1B). Analyses of enzymatic properties of the OAT, such as demonstration of the acetyl-Cys-enzyme intermediate by using purified enzyme, are currently being undertaken in our laboratory. For X-ray diffraction analysis, we are trying to crystallize the purified OAT.

Knowledge about acetyltransferase at the molecular level is rapidly growing. The OAT and NAT are involved in the metabolism of drugs and toxic chemicals. We hope that the new knowledge will also lead to the design of new pharmaceuticals and clinical kits, with possible human health applications.

**Conclusions**

We have cloned the gene of *S. typhimurium* OAT and established new strains highly sensitive to mutagenic aromatic amines and nitroarenes. It is suggested that the Cys of the *S. typhimurium* OAT and the corresponding cysteine residues of NAT of higher organisms are essential for the enzyme activities as an acetyl-CoA binding site. We also propose a new catalytic mechanism of acetyltransferase for *S. typhimurium* and higher organisms.

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