Involvement of Cys\(^{69}\) Residue in the Catalytic Mechanism of N-Hydroxyarylamine O-Acetyltransferase of Salmonella typhimurium

SEQUENCE SIMILARITY AT THE AMINO ACID LEVEL SUGGESTS A COMMON CATALYTIC MECHANISM OF ACETYLTRANSFERASE FOR S. TYPHIMURIUM AND HIGHER ORGANISMS*

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Acetyl-coenzyme A:N-hydroxyarylamine O-acetyltransferase is ubiquitous in species ranging from bacteria to mammals and is involved in the metabolic activation of N-hydroxy-arylamine derivatives derived from mutagenic and carcinogenic aromatic amines and nitroarenes. The nucleotide sequence of the gene that encodes O-acetyltransferase of Salmonella typhimurium was determined, and its deduced amino acid sequence was compared with those of arylamine N-acetyltransferases (EC 2.3.1.5) of higher organisms. The gene of S. typhimurium encoded a protein with a calculated molecular weight of 32,177. Chromosome DNA of S. typhimurium TA1538/1,8-DNP\(^6\), an O-acetyltransferase-deficient strain, had a \(-1\) frameshift mutation of CCC to CC at the coding region. To date, 11 genes encoding N-acetyltransferase of Salmonella typhimurium have been cloned from human, rabbit, hamster, and chicken. The N-terminal region of O-acetyltransferase of S. typhimurium with about 170 amino acids showed 25–33% homology with the corresponding region of N-acetyltransferases of the higher organisms. Of the 5 cysteine residues of O-acetyltransferase of S. typhimurium, Cys\(^{69}\) was the only residue that was conserved in all N-acetyltransferases of the higher organisms. The amino acid sequence of Arg-Gly-Gly-X-Cys, including the Cys\(^{69}\), was highly conserved. The mutant O-acetyltransferase of S. typhimurium, which contained Ala\(^{69}\) instead of Cys\(^{69}\), no longer showed the activities of O- and N-acetyltransferase. These results suggest that the Cys\(^{69}\) of S. typhimurium and its corresponding cysteine residues of the higher organisms are essential for the enzyme activities as acetyl-coenzyme A-binding sites.

CoASe-dependent N-hydroxyarylamine O-acetyltransferase is an enzyme involved in the metabolic activation of mutagenic and carcinogenic N-hydroxyarylamines (1–3). The enzyme catalyzes the transfer of the acetyl group from CoASe to the oxygen atom of the hydroxyl group of N-hydroxy compounds, which are formed either by oxidation of aromatic amines or by reduction of nitroarenes (1, 4). Both aromatic amines and nitroarenes are classes of mutagens and carcinogens in the environment (5–7). The enzymatic acetylation of N-hydroxy compounds yields highly reactive N-acetoxyarylamine derivatives, which spontaneously hydrolyze to form DNA adducts (8). Thus, the O-acetyltransferase plays an important role in the metabolic activation of aromatic amines and nitroarenes.

In higher organisms, O-acetyltransferase activities are found mainly in liver cytosol fractions of a number of mammals, including humans. The implications of O-acetyltransferase in arylamine carcinogenesis have been discussed (9–12). The O-acetyltransferase also catalyzes the transfer of the acetyl group from CoASe to the amino group of aromatic amines (13, 14). It is suggested, therefore, that the mammalian O-acetyltransferase is probably the same enzyme as CoASe-dependent arylamine N-acetyltransferase (EC 2.3.1.5), which plays a major role in the metabolism of drugs and endogenous substances that possess an amine or hydrazine group (1, 15). Both reactions of N- and O-acetylation probably proceed through a common intermediate, i.e., an acetyl-cysteinyl-enzyme (1, 16, 17). The N-acetyltransferase in humans and other mammals shows polymorphism with phenotypes that are classified as “slow” or “fast” acetylators (18, 19). CDNA and genomic clones of N-acetyltransferases have been isolated from humans (20–25), rabbit (26–29), hamster (30), and chicken (31–33). Although the information about primary structure of N-acetyltransferase is accumulating, the exact catalytic mechanism of the enzyme has not yet thoroughly been clarified nor has the CoASe-binding site been identified.

Interestingly, the O-acetyltransferase activities are demonstrated not only in the higher organisms but also in bacteria (34). In fact, O-acetyltransferase has been shown to play a key role in the sensitivity of Ames Salmonella tester strains to the mutagenicity of aromatic amines and nitroarenes: Salmonella typhimurium TA98/1,8-DNP\(_6\), an O-acetyltransferase-deficient strain, is resistant to both the killing and mutagenic effects of aromatic amines and nitroarenes (35, 36), whereas S. typhimurium YG1024, an O-acetyltransferase-overproducing strain, is extremely sensitive to the effects (37). The latter strain was constructed in this laboratory by cloning the O-acetyltransferase gene of S. typhimurium TA1538 and introducing the multi-copy-number plasmid carrying the gene.
into an Ames tester strain TA98 (37, 38). The resulting strain, YG1024, shows about 100 times higher O-acetyltransferase activity than does TA98 and has widely been used for the efficient detection of mutagenic aromatic amines and nitroaromatics (39-42).

Since O-acetyltransferase of *S. typhimurium* is apparently similar to N-acetyltransferase of the higher organisms, we postulated that the enzymes might share similarity at the amino acid level. The comparison of their sequences might highlight the highly conserved amino acids which probably play important roles in the enzyme activities. Thus, we determined the nucleotide sequence of the O-acetyltransferase gene of *S. typhimurium* and compared the deduced amino acid sequence with those of the N-acetyltransferase of the higher organisms. The results together with those of site-directed mutagenesis suggested that the Cys\(^\text{61}\) of *S. typhimurium* and the corresponding cysteine residues of the higher organisms are essential for the enzyme activities as CoASAc-binding sites. We also proposed a common catalytic mechanism of acetyltransferase for *S. typhimurium* and the higher organisms.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—Bacterial strains and plasmids used in this study are listed in Table I. A gene library of *S. typhimurium* TA1538 was constructed using a pBR322 vector as described previously (43). Plasmid pYG122 carrying the O-acetyltransferase gene of *S. typhimurium* TA1538 was isolated from the gene library by screening the plasmid which can make TA1538/1,8-DNP strain hypersensitive to the cell-killing and mutagenic effects of 2-nitrofluorene (37, 38). A plasmid pBluescript KS+ was purchased from Stratagene, La Jolla, CA.

DNA Sequencing of the O-Acetyltransferase Gene of *S. typhimurium*—Sets of deletion derivatives of pYG221 and pYG224 were constructed using the exonuclease III-mung bean nuclease digestion protocol from Stratagene. Nucleotide sequence of both strands of 1.35-kilobase pairs (kb) and 1.05-kb insert DNA of pYG221 and pYG224, respectively, was determined by the dideoxy-chain termination technique (44) using Sequenase sequencing kit (U.S. Biochemical Corp.) or 7-deaza sequencing kit with Klenow fragment (Takara Shuzo Co., Kyoto, Japan). Analysis of sequencing data was carried out using SDC-Genetyx software (SDC Software Development Co., Tokyo, Japan).

**Labeling of Plasmid-coded Proteins in Maxicells**—The maximall method was used to label the proteins encoded by the plasmid pYG213 in *Escherichia coli* strain CS603 (recA1, uraA6, phr-1) with [\(^{35}\text{S}\)]methionine (45). Samples were run on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and visualized by fluorography.

**Polymerase Chain Reaction (PCR)—Oligonucleotide primers were synthesized using DNA synthesizer model 381A (Applied Biosystem Co.). The 5'- and 3'-oligonucleotide primers used were 24-mer oligonucleotides that corresponded to nucleotides 677-700 and nucleotides 1813-1836, respectively, of the DNA shown in Fig. 2. The genes encoding O-acetyltransferase of *S. typhimurium* TA1538 and of TA1538/1,8-DNP were amplified using GeneAmp DNA amplification kit (Perkin-Elmer Cetus Co.). Briefly, chromosomal DNA (0.1 mg), the 5'-oligonucleotide primer (100 pmol), the 3'-oligonucleotide primer (1 pmol), four dNTPs (20 nmol), and Taq DNA polymerase (2.5 units) were contained in a reaction mixture (100 Ωl) (46). When the nucleotide sequences of complementary strands were determined, the concentrations of the 5'- and 3'-oligonucleotide primers were reversed. Amplification was carried out using Astec Program Temp Control System PC-500 (Sci-Media Co., Tokyo, Japan). Amplification was repeated 30 times, and each cycle consisted of dissociation step (94 C, 45 s), annealing step (50 C, 90 s), and polymerization step (72 C, 3 min). The amplified samples were removed from excessive primers by centrifugation using Amicon centrifoc (W. R. Grace Co., Danvers, MA) (47). The entire region of mutant O-acetyltransferase gene of *S. typhimurium* TA1538/1,8-DNP was compared with those of the wild-type gene of TA1538. To confirm the mutational change, the parts of complementary strands carrying the mutation were amplified and their nucleotide sequences were compared with those of the N-acetyltransferase of the higher organisms.

**TABLE I**

**Bacterial strains and plasmids**

| Strains or plasmid | Description | Source |
|--------------------|-------------|--------|
| TA1538             | hisD3052    | B. N. Ames*<sup>a</sup> |
| TA1538/1,8-DNP     | As TA1538, but is deficient in O-acetyltransferase | M. Watanabe<sup>a</sup> |
| E. coli            |             | Stratagene* |
| XL1-Blue           | recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, F'proAB+, lacZAM15, TnlO<sup>b</sup> | |
| Plasmids           |             |        |
| pYG122             | As pBR322, but has a 7.3-kb fragment of TA1538 chromosome DNA at its BamHI site | M. Watanabe<sup>a</sup> |
| pYG213             | As pYG122, but has a deletion between EcoRV site (0.2 kb) and EcoRV site (6.3 kb) | M. Watanabe<sup>a</sup> |
| pYG219             | As pBR322, but has a 1.35-kb EcoRV (6.3 kb)-BamHI (7.65 kb) fragment of pYG221 at its Scal site | M. Watanabe<sup>a</sup> |
| pYG221             | As pBluescript KS+, but its EcoRV-BamHI region is replaced by a 1.35-kb EcoRV (6.3 kb)-BamHI (7.65 kb) fragment of pYG122 | This study |
| pYG224             | As pBluescript KS+, but its PspI-HindIII region is replaced by a 1.05-kb PspI (5.56 kb)-HindIII (6.6 kb) fragment of pYG122 | This study |

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pared between the mutant and the wild type.

**Enzyme Assays**—XL1-Blue cells harboring pYG221 plasmid were grown overnight at 37 °C in LB broth supplemented with 50 μg/ml of ampicillin and 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation and disruption by sonication as described (37, 38). N- and O-Acetyltransferase activities were determined using the crude extract (10,000 x g supernatant) or the cytosol fraction (100,000 x g supernatant) of the disrupted cells. Isomiazid and 2-amino fluorescence N-acetyltransferase activities were determined by the colorimetric method as described previously (37). p-Aminobenzoic acid N-acetyltransferase activities were determined by the method of Weber and King (48). O-Acetyltransferase activities were determined by the previously described fluorescent method using 2-hydroxyamino-6-methylpyridine-[1,2-α;2'-d]-imidazole (N-hydroxy-Glu-P-1) as a substrate (37, 49). Final concentrations of the substrates in reaction mixture were as follows: isomiazid, 11.1 mM; 2-amino fluoresceine, 0.22 mM; p-aminobenzoic acid, 0.044 mM; N-hydroxy-Glu-P-1, 0.02 mM. The protein concentration was determined by the method of Lowry et al. (50) using bovine serum albumin (Boehringer Mannheim No. 711454) as a standard.

**Site-directed Mutagenesis**—Site-directed mutagenesis of the O-acetyltransferase gene of *S. typhimurium* was carried out with modifications of the method of Kunkel (51) using the Muta-Gen in vitro kit (Bio-Rad). Single-stranded DNA containing uracil was prepared from *E. coli* strain C2326 (dut, ung) (52) harboring pYG221. Mutagenic oligonucleotides used were 5'-CAGTATCCACCGGCCTCGG-GCATAAAGCGTATCCACCGCG-3' for the mutation of Cys65 to Ala65 and 5'TTCAGTTC-CAAAACGGCTATCCACCGGCC-3' for the mutation of Cys40 to Ala40, respectively. After one of the oligonucleotides was annealed with the single-stranded DNA of pYG221, a complementary strand was polymerized with Sequenase version 2.0 (U. S. Biochemical Co.) followed by sealing the product with T4 DNA ligase (Bio-Rad). The mutagenized plasmids were transferred into *E. coli* strain XL1-Blue. Mutants were verified by sequencing the entire coding region of O-acetyltransferase gene of *S. typhimurium*.

**RESULTS**

**Nucleotide Sequence of the Gene That Encodes O-Acetyltransferase of *S. typhimurium***—In a previous paper, we have reported that the O-acetyltransferase gene is probably located in the 1.35-kb DNA region spanning from the map position of 6.3 kb (the fifth EcoRV site) to that of 7.65 kb (the BamHI site) of the insert DNA of pYG122 (Fig. 1). This assumption was based on the result that *S. typhimurium* harboring pYG213 carrying the 1.35-kb DNA alone shows a high level of O-acetyltransferase activity (37). Thus, we subcloned the 1.35-kb DNA fragment into plBluescript KS+ vector yielding pYG221 plasmid for DNA sequencing. We also subcloned the 1.05-kb DNA, which overlaps the 1.35-kb DNA by 0.3 kb, spanning from the map position of 5.55 kb (the second PstI site) to that of 6.6 kb (the second HindIII site) into the vector yielding pYG224, since some regulatory sequences for the O-acetyltransferase gene might reside in this region. We then constructed sets of deletion derivatives of the subcloned plasmids and determined the nucleotide sequence of both the 1.35-kb and the 1.05-kb DNA fragments. The total nucleotide sequence of 2,134 base pairs (bp) determined in this work is shown in Fig. 2. This sequence contains an open reading frame of 843 bp from nucleotides 853–1,695. The open reading frame was located within the 1.35-kb region and 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 also found upstream of the open reading frame. The potential -35 and -10 sequences were present outside of the 1.35-kb DNA region.

**Identification of the Gene Product of the Cloned DNA**—To verify that the open reading frame shown in Fig. 2 is the coding region of the O-acetyltransferase gene of *S. typhimurium*, we have constructed 3'- or 5'-deletions of the 1.35-kb DNA and measured the isomiazid N-acetyltransferase activities of *E. coli* or *S. typhimurium* strains harboring plasmids carrying such deleted DNA (Fig. 3). The *E. coli* strain harboring pYG221-K2, which had a deletion of about 310 bp from the BamHI site in Fig. 3, showed the same level of enzyme activity as did the strain harboring pYG221 carrying the entire 1.35-kb DNA. The *E. coli* strains harboring plasmids whose insert DNA was digested more than 550 bp from the BamHI site no longer showed the enzyme activity. The putative C-terminal end of the O-acetyltransferase is located at about 440 bp from the BamHI site. On the other hand, when the 1.35-kb DNA was deleted about 320 bp from the EcoRV site in Fig. 3, the *S. typhimurium* strain harboring the plasmid carrying this 5'-deleted DNA did not show the activity. The putative N-terminal end of the O-acetyltransferase is located at about 60 bp from the EcoRV site.

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**FIG. 1.** a, a partial restriction map of pYG122 plasmid. The EcoRI site derived from pBR322 was assigned the map position of 0 kb in the plasmid (37). 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. A thick arrow indicates the coding region and the direction of transcription of O-acetyltransferase gene of *S. typhimurium*. Hatched boxes represent the 1.05- and 1.35-kb DNA fragments which were subcloned into plBluescript KS+ for DNA sequencing. E, EcoRI; P, PstI; V, EcoRV; H, HindIII; B, BamHI. b, sequence strategy of the O-acetyltransferase. Arrows indicate the direction and extent of the sequence determination.

**FIG. 2.** Nucleotide and deduced amino acid sequences of the O-acetyltransferase gene of *S. typhimurium*. One-letter amino acid notation was used. Possible -35 and -10 regions and Shine-Dalgalno (S.D.) sequence are underlined. Possible transcriptional terminator is indicated by arrows and asterisks. The highly conserved amino acid sequence of Arg-Gly-Gly-X-Cys is marked with a double line. Cys* is marked with a circle. The 1.35-kb DNA fragment that is boxed was subcloned into plBluescript KS+ for DNA sequencing and transcriptional analysis. The CCC sequence enclosed with a small box indicates the site where the 5′-frameshift mutation was found in TA1538/1.8-DNP strain.
plasmid. pYG221 and its deletion derivatives were introduced into E. coli XL1-Blue, and the resulting transformants were used for enzyme assay of isoniazid N-acetyltransferase activity. pYG221 and pYG223 were introduced into S. typhimurium TA1538/1,8-DNP and resulting transformants were used for the enzyme assay. The arrow indicates the coding region and the direction of transcription of the O-acetyltransferase gene. Numbers represent the nucleotide numbers shown in Fig. 2. V, EcoRV; H, HindIII; B, BamHI; P, PstI.

Fig. 3. Deletion derivatives of pYG221 and pYG213 plasmids. The hatched box represents the 1.35-kb DNA fragment carrying the O-acetyltransferase gene of S. typhimurium. Both pYG221 and pYG213 plasmids carry this 1.35-kb DNA fragment. Plasmids pYG221-K2, -K3, -K4, -K5, and -K7 and plasmid pYG223 are deletion derivatives of pYG221 and pYG213, respectively. The insert of pYG223 deletes 320 bp (EcoRV-HindIII) of the 1.35-kb fragment. Bars represent the insert DNA which is carried by each deletion plasmid. pYG221 and its deletion derivatives were introduced into E. coli XL1-Blue, and the resulting transformants were used for enzyme assay of isoniazid N-acetyltransferase activity. pYG221 and pYG223 were introduced into S. typhimurium TA1538/1,8-DNP and resulting transformants were used for the enzyme assay. The arrow indicates the coding region and the direction of transcription of the O-acetyltransferase gene. Numbers represent the nucleotide numbers shown in Fig. 2. V, EcoRV; H, HindIII; B, BamHI; P, PstI.

identify gene products of the cloned DNA, the proteins synthesized in maxicells harboring pYG213 or pYG219 were labeled with [35S]methionine and subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography (Fig. 4). Plasmid pYG213 produced a protein with an approximate molecular mass of 33 kDa along with β-lactamase. Plasmid pYG219 also produced the 33-kDa protein along with the product of tetracycline resistance gene. The value, 33 kDa, was close to the molecular mass deduced from the nucleotide sequence. From the results, together with those of the deletion analysis shown in Fig. 3, we concluded that the open reading frame shown in Fig. 2 is the coding region of O-acetyltransferase.

O-Acetyltransferase-deficient Salmonella Strain Has a Frameshift Mutation at the Coding Region—Since the O-acetyltransferase gene is cloned by screening the gene that can complement the deficiency of S. typhimurium TA1538/1,8-DNP, it seemed possible that the TA1538/1,8-DNP strain has mutation(s) at the O-acetyltransferase gene. To examine this possibility, the O-acetyltransferase gene of TA1538/1,8-DNP was amplified by PCR and its nucleotide sequence was compared with that of the gene amplified from TA1538. We found that one G:C pair from a DNA repeat of three G:C pairs at the coding region was deleted from the gene of TA1538/1,8-DNP. The exact location of the mutation is nucleotides 1,442–1,444, which correspond to Ser197 and His198 of the O-acetyltransferase (Fig. 5). This frameshift created a new termination site at codon 204.

Characterization of O-Acetyltransferase of S. typhimurium—To characterize the O-acetyltransferase encoded by the cloned DNA, we have prepared the cytosol fraction of crude lysate of the cells harboring pYG221. This fraction was subjected to enzyme assays and inhibition experiments (Table II). Besides, N-hydroxy-Glu-P-1 O-acetyltransferase activity, the cytosol fraction showed isoniazid and 2-aminofluorene N-acetyltransferase activities. However, it exhibited a very low level of p-aminobenzoic acid N-acetyltransferase activity. The apparent $K_m$ value for CoASAc was 10 μM when N-hydroxy-Glu-P-1 was used as a substrate. The unit of enzyme activities is nanomoles/min/mg of protein. The percent of the remaining isoniazid N-acetyltransferase activities is presented. The concentration of dithiothreitol was 20 μM. The concentrations of the inhibitors used are as follows: N-ethylmaleimide, 0.1 mM; iodoacetamide, 1.0 mM; paraoxon, 0.1 mM.

Table II

| Characteristic                      | Cytosol fraction |
|------------------------------------|-----------------|
| Cellular distribution              |                 |
| Molecular weight                   |                 |
| Calculated from nucleotide sequence| 32,177          |
| Deduced from SDS-polyacrylamide gel| 33,000          |
| Electrophoresis                    |                 |
| $K_m$ value for CoASAc             | 10 μM           |
| O-Acetylation of                   |                 |
| N-hydroxy-Glu-P-1                  |                 |
| N-Acetylation                      |                 |
| Isoniazid                          | 8,720           |
| 2-Aminofluorene                    | 517             |
| p-Aminobenzoic acid                | 1.3             |
| Inhibition by                      |                 |
| N-Ethylmaleimide                   | 0%              |
| Iodoacetamide                      | 0%              |
| Paraoxon                           | 88%             |

$^a$ N-Hydroxy-Glu-P-1 was used as a substrate.
$^b$ The unit of enzyme activities is nanomoles/min/mg of protein.
$^c$ Percent of the remaining isoniazid N-acetyltransferase activity is presented. The concentration of dithiothreitol was 20 μM. The concentrations of the inhibitors used are as follows: N-ethylmaleimide, 0.1 mM; iodoacetamide, 1.0 mM; paraoxon, 0.1 mM.

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O-Acetyltransferase of S. typhimurium and N-Acetyltransferase of Higher Organisms Share Similarity at the Amino Acid Level—Since O-acetyltransferase of S. typhimurium is functionally similar to N-acetyltransferase of higher organisms, we examined the possibility that they might share similarities at the amino acid level. The N-terminal region of O-acetyltransferase of S. typhimurium containing about 170 amino acids showed 25–33% similarity to those of the N-acetyltransferases of human, rabbit, hamster, and chicken at the amino acid level (Fig. 6). The remaining C-terminal region had few similarities with the corresponding region of the N-acetyltransferases at the amino acid level, although weak similarities were observed at the nucleotide level.

Since the activities of both O-acetyltransferase of S. typhimurium and N-acetyltransferases of the higher organisms are inhibited by sulfhydryl-blocking agents, it is suggested that a cysteine residue is involved in a catalytic mechanism as an CoASAc-binding site (1). O-Acetyltransferase of S. typhimurium had 5 cysteine residues at codons 14, 34, 69, 173, and 206 (Fig. 6). Of the 5 residues, Cys69 is the only one that is conserved in all N-acetyltransferases of the higher organisms. The amino acid sequence of Arg-Gly-Gly-X-Cys, including the Cys69, is highly conserved. To act as an CoASAc-binding site, the cysteine residue must be activated, e.g. by a basic amino acid (17). Recently, it was reported that an arginine residue is essential for the activity of N-acetyltransferase from hamster liver (55). Interestingly, Arg69 of O-acetyltransferase of S. typhimurium is highly conserved in the N-acetyltransferases. We hypothesized, therefore, that the Arg69 functions as a base to accept a proton from the Cys69, and the resulting activated Cys69 acts as an CoASAc-binding site.

**Fig. 6.** Similarities in amino acid sequences between O-acetyltransferase of S. typhimurium and N-acetyltransferases of human, rabbit, hamster, and chicken. The sequence in the top line represents the amino acid sequence shown in Fig. 2. Abbreviations used and reference papers of the sequences are as follows: S. typhimurium (S.T.), this study; human liver polymorphic (Human-M), Ref. 21; human liver polymorphic rapid (Human-R), Refs. 21 and 24; human liver polymorphic slow (Human-S1, -S2, -S3), Ref. 21, 22, 25; rabbit liver polymorphic (Rabbit-P), Refs. 26, 27, 28; rabbit liver polymorphic (Rabbit-M), Ref. 30; chicken liver (Chicken-L), Ref. 31; chicken pineal gland (Chicken-PG1) and -PG2, Ref. 33. The deduced amino acid sequences of human liver polymorphic and rabbit monomorphic N-acetyltransferases are also reported by Blum et al. (23) and Saasaki et al. (26), respectively, but their sequences are different from those shown in this figure at three amino acids, i.e. Thr171 to Arg, Thr174 to Arg, Gin178 to Glu of the human monomorphic enzyme and Arg173 to Thr of the rabbit monomorphic enzyme, respectively. The amino acid residues that are conserved between O-acetyltransferase of S. typhimurium and all the N-acetyltransferases of higher organisms are boxed. The cysteine residues of O-acetyltransferase of S. typhimurium are indicated with asterisks.
The Cys60 Residue Is Essential for the Enzyme Activities—
To examine the hypothesis, we changed either the Cys60 or Arg65 residue of O-acetyltransferase of S. typhimurium by site-directed mutagenesis of pYG221 plasmid. We then measured isoniazid N-acetyltransferase activity and N-hydroxy-Glu-P-1 O-acetyltransferase activity of the crude extract of XL1-Blue cells harboring the mutant plasmid with either the Cys60 or the Arg65 replaced with Ala. Neither extract showed detectable levels of the N- and O-acetyltransferase activities regardless of the induction of IPTG. Even without the induction of IPTG, the extract of cells harboring the wild-type pYG221 plasmid showed 1,010 nmol/min/mg protein of N-acetyltransferase activity and 328 nmol/min/mg protein of O-acetyltransferase activity. The XL1-Blue cells harboring pYG221 overproduced the O-acetyltransferase when they were grown in the presence of IPTG (Fig. 7, lane 2). The extract of cells harboring the mutant plasmid carrying the change of Arg65 to Ala also showed an intense band on the gel when they were grown in the presence of IPTG (Fig. 7, lane 6). Mobility and intensity of the band was almost the same as those of the wild-type O-acetyltransferase. From these results, we suggest that the Cys60 is essential for the enzyme activities and also that the change of Cys60 to Ala diminished the enzyme activities without generating the gross conformational changes that would influence the stability and the mobility of the enzyme on an SDS-polyacrylamide gel. On the contrary, the extract of cells harboring the mutant plasmid carrying the change of Arg65 to Ala did not show the intense band corresponding to the mutant protein on the gel even when they were grown in the presence of IPTG (Fig. 7, lane 4). No specific bands were observed when the product, which was produced and labeled in maxicells harboring the mutant plasmid, was examined by SDS-polyacrylamide gel electrophoresis (data not shown). Thus, we suggest that unlike the case of Cys60 to Ala, the change of Arg65 to Ala probably destabilized the protein, thereby diminishing the enzyme activities.

![O-Acetyltransferase of S. typhimurium](http://www.jbc.org/)

DISCUSSION

We have determined the nucleotide sequence of 2.1-kb DNA which carries the O-acetyltransferase gene of S. typhimurium and identified the gene product using the maxicell technique. DNA sequence analysis indicated that the 2.1-kb DNA contained an open reading frame which potentially encodes a protein of a calculated molecular weight of 32,177 (Fig. 2). The cells harboring the plasmids which delete part of the DNA corresponding to the open reading frame did not show a detectable level of isoniazid N-acetyltransferase activity (Fig. 3). The calculated molecular weight was consistent with that of the product estimated by the maxicell experiments (Fig. 4). Thus, we conclude that the open reading frame shown in Fig. 2 is the coding region of O-acetyltransferase of S. typhimurium. Interestingly, S. typhimurium TA1538/1,8-DNP, an O-acetyltransferase-deficient strain, had a +1 frameshift mutation in this coding region (Fig. 5). This observation further supports our conclusion that the open reading frame shown in Fig. 2 is the coding region of O-acetyltransferase and also indicates that a TA1538/1,8-DNP strain lacks the O-acetyltransferase that is encoded by our cloned DNA. It is reasonable that the strain had a frameshift mutation at a cluster of G:C pairs, because the original strain, TA98/1,8-DNPm, is isolated by mutagenizing TA98 cells with 1,8-dinitrotyrene, a potent frameshift mutagen (35, 36).

O-Acetyltransferase encoded by the cloned plasmid was overexpressed in E. coli cells, and some biochemical properties.
of the enzyme were characterized using the cytosol fraction of the cell lysate (Table II). The high level of expression of the enzyme was probably due to gene dosage effects of the gene on multi-copy-number plasmids and to a strong lac promoter, which was induced by adding IPTG in the experiments. The enzyme showed N-acetyltransferase activities along with the O-acetyltransferase activity. Both N- and O-acetyltransferase activities were inhibited by sulfhydryl-blocking agents, suggesting that a cysteine residue is involved in a catalytic mechanism. The apparent K<sub>a</sub> value of CoASAc was 10 μM. These characteristics are consistent with those of the enzyme reported by Saito et al. (48) who have partially purified O-acetyltransferase of <i>S. typhimurium</i> TA98 without using gene cloning techniques. They reported, however, that the apparent molecular weight of O-acetyltransferase of <i>S. typhimurium</i> TA88 was 48,000, instead of 32,000, based upon the results of Sephadex G-150 column chromatography. This observation suggests the possibility that their O-acetyltransferase is different from that encoded by our cloned plasmid. Our recent results indicated that the O-acetyltransferase encoded by our cloned DNA migrated much slower on a Sephadex G-100 column than standard proteins. The estimated molecular weight was 48,000 judged by the chromatography. Thus, we suggest that the O-acetyltransferase encoded by our cloned DNA is the same as that partially purified by Saito et al. and Andres et al. (56) reported that N-acetyltransferase of pigeon liver forms a dimer during catalysis. The O-acetyltransferase of <i>S. typhimurium</i> may form a dimer even without CoASAc.

To date, 11 genes encoding arylamine N-acetyltransferase have been isolated from humans (20–25), rabbit (26–29), hamster (30), and chicken (31–33). They show more than 69% similarity at the amino acid level if the cDNAs of chicken are omitted from the comparison. The calculated molecular weight of these enzymes is 32,914–33,923, which is close to that of N-acetyltransferase of <i>S. typhimurium</i>. N-Acetyltransferases from hamster and mouse have been demonstrated to show O-acetyltransferase activities (15, 14). Structure-activity studies in rabbit liver and pigeon liver suggested that cysteine residue plays an important role in the catalytic mechanism as an CoASAc-binding site (16, 17). These observations prompted us to suggest that they might share a similarity with O-acetyltransferase of <i>S. typhimurium</i> at the amino acid level and a common catalytic mechanism might exist among them. The results indicated that the N-terminal region of O-acetyltransferase of <i>S. typhimurium</i> showed a 25–33% similarity to the corresponding region of N-acetyltransferase of the higher organisms (Fig. 6). This low level of similarity was very helpful in highlighting the highly conserved amino acids. Of the 5 cysteine residues in O-acetyltransferase of <i>S. typhimurium</i>, Cys<sup>60</sup> was the only residue that was conserved in all N-acetyltransferases of the higher organisms. The amino acid sequence of Arg-Gly-Gly-X-Cys including Cys<sup>60</sup> was highly conserved. The results of site-directed mutagenesis experiments indicated that the mutant O-acetyltransferase of <i>S. typhimurium</i>, which contained Ala<sup>60</sup> instead of Cys<sup>60</sup>, did not show any N- and O-acetyltransferase activities. Thus, we suggest that the Cys<sup>60</sup> of <i>S. typhimurium</i> is essential for the enzyme activity even in CoASAc-binding site.

Andres et al. (17) investigated the kinetics of N-acetyltransferase 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 CoASAc-binding site. Recently, Cheon and Hanna (55) reported that an arginine is essential for the activity of N-acetyltransferase from hamster liver. Interestingly, Arg<sup>60</sup> of O-acetyltransferase of <i>S. typhimurium</i> was conserved in all N-acetyltransferases of the higher organisms. Based on our results and the results of others, we postulate the following reaction mechanism for O-acetyltransferase of <i>S. typhimurium</i> (Fig. 8a): Arg<sup>60</sup> (or Arg<sup>62</sup>) gives up its proton and thus loses its cationic charge even in neutral pH conditions, because two adjacent ε-amino groups provide mutual electrostatic destabilization (57). The deprotonated Arg<sup>60</sup> (or Arg<sup>62</sup>) in turn, attracts a proton from the cysteiny1 group of Cys<sup>60</sup>. The activated Cys<sup>60</sup> residue accepts an acetyl group from CoASAc, resulting in an acetyl-cysteinyl-enzyme intermediate. Finally, this acetyl moiety would be transferred to the oxygen atom (O-acetylation) or the nitrogen atom (N-acetylation) of N-hydroxyarylamine or arylamine, respectively. In the transfer reaction of the acetyl group to the hydroxyamino and amino groups, the deprotonated Arg<sup>60</sup> (or Arg<sup>62</sup>) again serves as a general base and attracts a proton from the hydroxyamino or amino groups of the acceptors. This model is applicable to N-acetyltransferase of higher organisms because of the similarity of their enzymatic properties and amino acid sequences (Fig. 8b): Cys<sup>60</sup> residues of N-acetyltransferase of humans, rabbit, hamster, and chicken could be CoASAc-binding sites, whereas one of the conserved basic amino acids may be activators.

Unlike the N-terminal region of O-acetyltransferase of <i>S. typhimurium</i>, the remaining C-terminal region containing about 110 amino acids showed little or no similarity to the corresponding region of N-acetyltransferase of the higher organisms at the amino acid level (Fig. 6). Deletion analysis indicated that the enzyme activity disappears when C-terminal 39 amino acids of the O-acetyltransferase are truncated (Fig. 3). This result suggests that the C-terminal region of O-acetyltransferase of <i>S. typhimurium</i> also plays an important role in the enzyme activity. O-Acetyltransferase of <i>S. typhimurium</i> has no N.O-acetyltransferase activity (2). The K<sub>a</sub> value for CoASAc is much lower than that of N-acetyltransferase of higher organisms (14). Thus, it might be interesting to determine if the amino acid sequences in the C-terminal regions are involved in the differences of biochemical properties.

In this paper, we proposed a catalytic model for O-acetyltransferase of <i>S. typhimurium</i> and suggested that this model is applicable to a catalytic mechanism of arylamine N-acetyltransferase of higher organisms. To examine this model, structural analyses of the purified enzyme using the methods of x-ray diffraction and nuclear magnetic resonance are important. Purification of O-acetyltransferase of <i>S. typhimurium</i> has been hampered by the availability of only small quantities of the enzyme in bacterial cells. The low yield of the enzyme may be due to a weak similarity of the promoter sequences of the O-acetyltransferase gene to the consensus promoter sequence (Fig. 2). The <i>E. coli</i> cells harboring pYG221 plasmid, however, overproduced the O-acetyltransferase when they were grown in the presence of IPTG (Fig. 7). This overproducing system would allow us to purify a quantity of the enzyme that would be sufficient for the structural analyses. This work is currently being undertaken in our laboratory.

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