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Role of Disulfide Bond of Arylsulfate Sulfotransferase in the Catalytic Activity

Ae-Ran Kwon and Eung-Chil Choi

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Korea

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Bacterial arylsulfate sulfotransferase (ASST) catalyzes the transfer of sulfate group from a phenyl sulfate ester to a phenolic acceptor. The promoter region and the transcription start sites of *Enterobacter amnigenus astA* have been determined by primer extension analysis. Northern blot analysis resolved two mRNA species with lengths of 3.3 and 2.0 kb, which correspond to the distances between the transcriptional initiation sites and the two inverted repeat sequences (IRSs). By length, the 3.3 kb RNA could comprise the three-gene (*astA* with *dsbA* and *dsbB*) operon. ASST has three highly conserved cysteine residues. Reducing and non-reducing SDS-PAGE and activity staining showed that disulfide bond is needed for the activity of the enzyme. To identify the cysteine residues responsible for the disulfide bond formation, a series of Cys to Ser mutants has been constructed and the enzymatic activity was measured. Based on the results, we assumed that the first cysteine (Cys349) might be involved in disulfide bond mainly with the second cysteine (Cys445) and result in active conformation.

Key words: Arylsulfate sulfotransferase, *Enterobacter amnigenus*, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Disulfide bond, Site-directed mutagenesis, DsbA, DsbB

INTRODUCTION

Sulfoconjugation is a major metabolic route for the detoxification of phenolic compounds in animals (Dodgson and Tudball, 1960). It is catalyzed by phenol sulfotransferase (PST, EC 2.8.2.1), an enzyme that has been purified from several mammalian organs, including liver, lung, brain, kidney, epithelial cells, and erythrocytes (Roy, 1981; Sekura *et al.*, 1981). Kim *et al.* isolated the arylsulfate sulfotransferase-producing bacteria, *Eubacterium* A-44 (Kim *et al.*, 1986), *Klebsiella* K-36 (Kim *et al.*, 1992), and *Haemophilus* K-12 (Lee *et al.*, 1995), from human, rat, and mouse intestinal flora, respectively. Kim *et al.* suggested that, in addition to liver and extrahepatic tissues, intestinal flora have a role in the detoxification of phenolic compounds by sulfoconjugation (Kim and Kobashi, 1986). These bacterial arylsulfate sulfotransferases (ASSTs, EC 2.8.2.22), which catalyze the transfer of a sulfate group from a phenyl sulfate ester to a phenolic acceptor, are quite different from the mammalian PSTs, which exclusively transfer a sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a phenolic acceptor (Kim *et al.*, 1991; Kim *et al.*, 1994).

Recently we cloned an *astA* gene from *Enterobacter amnigenus* AR-37, sequenced the gene, characterized its product (Kwon *et al.*, 1999), and identified the active site tyrosine residue (Kwon *et al.*, 2001). The *E. amnigenus* ASST is a monomeric protein with a molecular mass of 64 kDa. *N*-terminal amino acid sequence analysis of the recombinant ASST from *Escherichia coli* NM522 showed that it is subject to N-terminal processing. This leader sequence does share significant homology with conserved regions common in signal peptide (von Heijne and Abrahmsen, 1989), and localization experiments showed that the enzyme is present in the periplasmic space (Kwon *et al.*, 2001). The enzyme has three cysteine residues, which are conserved in four ASST homologues; ASST of *Salmonella typhimurium* (87% identical) (Kang *et al.*, 2001), ASST of *Klebsiella* K-36 (81% identical) (Baek *et al.*, 1996), arylsulfatase of *Campylobacter jejeuni* (39% identical) (Yao *et al.*, 1996), and ASST of *Citrobacter freundii* MB4-8242 (85% identical) (Kang *et al.*, 2001).

Correspondence to: Eung-Chil Choi, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, San 56-1, Shillim-Dong, Kwanak-Gu, Seoul 151-742, Korea
Tel: 82-2-880-7874, Fax: 82-2-886-5802
E-mail: ecchoi@snu.ac.kr

Moreover, all these *astA* genes are located side by side with *dsbA* and/or *dsbB* genes, which are key players in disulfide bond formation in the periplasm. Here we present the possibility of operonic arrangement of the *astA* gene cluster, which is *astA*, *dsbA*, and *dsbB*, by transcriptional analysis. It is thus reasonable to expect that there might be intramolecular disulfide bridge, which is important for the correct conformation of the biologically active structure. Therefore, we have shown that disulfide bond is needed for the activity of the enzyme by reducing and non-reducing SDS-PAGE, and activity staining. We also have undertaken site-directed mutagenesis of cysteine residues of the enzyme, and examined the properties of the resulting mutant forms to study the roles of disulfide bond in the reactivity of ASST.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

E. coli CJ236 (F', dut$^-$, ung$^-$), *E. coli* BMH 71-18 *mutS* (*ung*$^+$, *mutS*$^-$), and *E. coli* MV1184 (*ung*$^+$, *mutS*$^+$) were used as hosts for site-directed mutagenesis. *E. coli* NM522 was used for expression. The strains were grown on Luria-Bertani (LB) media containing ampicillin (100 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), tetracycline (10 μg mL^{-1}), or streptomycin (30 μg mL^{-1}), when appropriate. The pEAST72 plasmid (Kwon *et al.*, 1999) encoding the *astA* gene from *E. amnigenus* AR-37 was used as the template for site-directed mutagenesis. Plasmid DNA was prepared using a Qiaprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany).

Northern blot analysis

Total cellular RNA was prepared from log-phase culture of *E. coli* NM522 harboring plasmid pEAST72 using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The probe was a *Bam*HI restriction fragment of the pEAST72 plasmid [nt 937 to 1471] radiolabelled with [α-^{32}P]dATP (specific activity, 3000 Ci/mmol; NEN Life Science Products) by the random oligonucleotide method as previously described (Feinberg and Vogelstein, 1983). Northern hybridization was performed using NorthernMax as described by the manufacturer (Ambion Inc., Austin, Tex.), with a 0.24-9.5 kb RNA ladder (Gibco-BRL, Gaithersberg, Md.) as molecular mass marker.

Primer extension analysis

To determine the 5' end of *astA* mRNA, primer extension analysis was accomplished as previously described (Reeder and Schleif, 1993) with avian myeloblastosis virus (AMV) reverse transcriptase (Promega). Total cellular RNA was prepared from log-phase culture of *E. coli* NM522 harboring plasmid pEAST72 using RNeasy Mini Kit (Quiagen GmbH) in accordance with the manufacturer's instructions and used as templates. Synthetic oligonucleotide (5'-TGCAAATAAGCCTAACGCGAGCGC-3', complementary to nt 267 to 290) was adopted as a primer. The reaction products were analyzed in a 6% denaturing polyacrylamide sequencing gel in parallel with the corresponding DNA sequencing samples resulting from employing the same oligonucleotide primer and plasmid pEAST72 as the template.

Site-directed mutagenesis

Cys to Ser substitution mutations of ASST were introduced into plasmid pEAST72 singly or in combination by the procedures described by Kunkel (Kunkel, 1985; Kunkel *et al.*,1987), using the Mutan-K kit (Takara, Japan). Synthetic oligonucleotides used as mutagenic primers were as follows: 5'-GGCGCCGTCTCCGTTAACGTCG-3' for Cys-349 to Ser mutation, 5'-CCTCTTACCTCCAATGAAAAC-3' for Cys-445 to Ser mutation, and 5'-GGCAAGTCTGAAAATACCGAC-3' for Cys-451 to Ser mutation (codons for Ser are underlined). All the mutations were confirmed by DNA sequencing with the dideoxy chain termination method (Sanger, *et al.*, 1977) using the OccuPower DNA sequencing kit (Bioneer, Korea). The plasmid DNA incorporating the desired mutation was transformed into competent *E. coli* NM522 cells, as described (Sambrook *et al.*, 1989).

Polyacrylamide gel electrophoresis and activity staining

ASST protein was purified from *E. coli* NM522, harboring the plasmid pEAST72 as previously described (Kwon *et al.*, 1999). For sample preparations, protein sample was mixed with the same volume of 2X Laemmli sample buffer with or without 1% of 2-mercaptoethanol (2-ME) for reduced or non-reduced sample. Two sets of reduced and non-reduced samples were loaded in parallel. SDS-PAGE was performed on 4-20% gradient gel (Laemmli 1970). When SDS-PAGE was finished, the gel was cut into two parts. One was fixed with 12.5% trichloroacetic acid and then stained for proteins with Coomassie Brilliant Blue R-250 dye (Neuhoff *et al.*, 1988). The other part was immersed and shaken for 10 min twice in 25% v/v isopropanol in 10 mM Tris-HCl buffer (pH 8.0) to remove SDS, and then finally equilibrated for renaturation in 50 mM Tris-HCl buffer (pH 8.0) for 15 min before activity staining. The gels was soaked in the substrate solution (3 mL of 4-methylumbelliferylsulfate (MUS) + 29 mL of 30 mM phenol + 21 mL of 0.1 M Tris-HCl (pH 8.0)) with gentle shaking for 30 min at 37°C and then the band with ASST activity was identified by UV fluorescence (320 nm).

ASST activity assays

ASST activity was measured using two different spectroscopic assays, as previously described (Kwon *et al.*, 2001). The chromogenic substrate *p*-nitrophenyl sulfate (PNS) was used in a colorimetric assay and the fluorogenic substrate MUS was used in a fluorometric assay.

RESULTS AND DISCUSSION

Transcriptional analysis

The position of the 5' end of the *astA* transcript was investigated by primer extension analysis. It was found that *astA* transcription is initiated from two sites, designated P1 and P2, located 164 and 35 nt upstream of the putative ATG initiation codon (Fig. 1). Analysis of the nucleotide sequence in the close range upstream of these transcription start sites reveals a putative promoter area with considerable homology to the *E. coli* consensus promoter (Harley and Reynolds, 1987). These results suggest that these two 5' ends are the transcriptional start points of the *astA* gene.

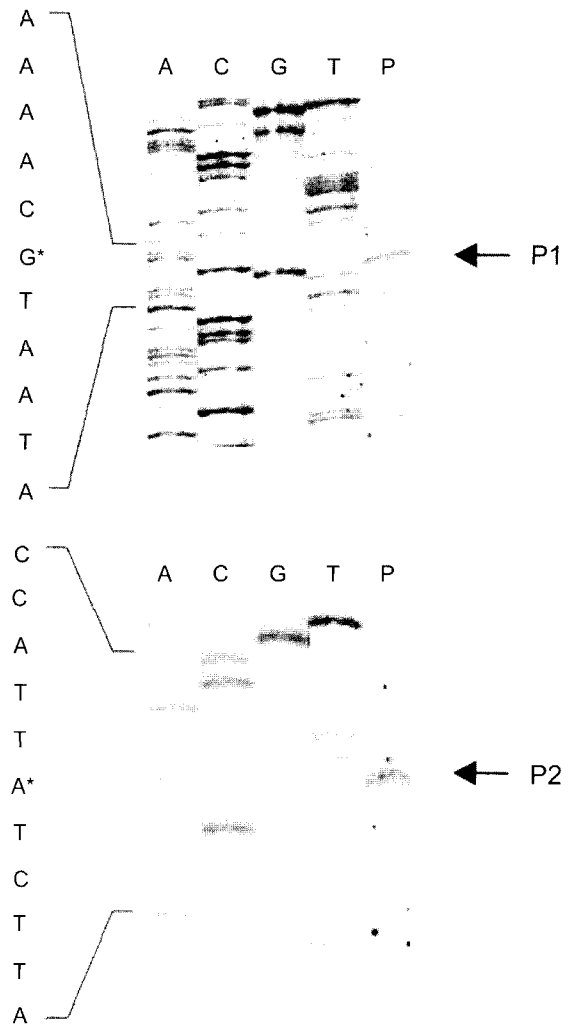

Fig. 1. Primer extension mapping of the transcription start sites of the *astA* gene: The nucleotide sequence of the complementary strand of each promoter region is indicated to the left. The nucleotide designated +1 for each promoter is indicated with an asterisk. Lanes: A, C, G, and T, DNA sequencing ladders; P, Primer extension products.

Northern analysis was performed with an internal fragment of *astA* as probe which hybridized to two mRNA species with lengths of 3.3 and 2.0 kb (Fig. 2). It was estimated that the 3' ends of each transcript would be in the vicinity of the two IRSs (IRS1 and IRS2), that is located in the downstream of *astA* and *dsbB* gene (Kwon *et al.* 1999). The distances between the transcriptional initiation site (+1) of the *astA* and these two IRSs correspond to the sizes of two RNA transcripts. And moreover, upstream of the *dsbA* and *dsbB* genes no obvious promoter sequence can be distinguished. By length, the 2.0 kb RNA corresponds to the *astA* transcript and the 3.3 kb RNA could comprise the three-gene operon (Fig. 3). The present findings support the possibility of the operonic arrangements of *astA* gene cluster.

Roles of disulfide bond in the activity of ASST

In general, the mobility of a protein in SDS-PAGE depends mainly on its molecular weight. But it also

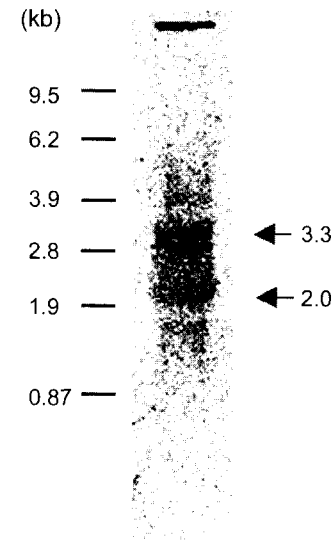

Fig. 2. Northern hybridization analysis of *astA* gene: Two mRNA transcripts with lengths of 3.3 and 2.0 kb were indicated with arrows.

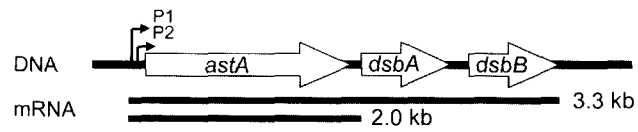

Fig. 3. Schematic diagram showing the operonic arrangements of *astA* gene cluster: The two predicted transcriptional start sites are indicated by P1 and P2. Two mRNA transcripts with lengths of 3.3 and 2.0 kb were indicated. The direction of transcription is shown by arrows.

depends on its conformation. The more compact protein migrates faster. Therefore, disulfide bonded oxidized form and reduced one can be distinguished on reducing and non-reducing gels. Purified ASST was analyzed on SDS-PAGE under reducing and non-reducing conditions. Oxidized form which might have a disulfide bond migrated slightly more than reduced one (Fig. 4(A)) and the size corresponds to a monomer. This suggests that there is one intramolecular disulfide bond in the ASST and coincides with the fact that the purified ASST was found to be monomer by gel filtration chromatography (Kwon *et al.*, 1999).

To elucidate the effect of disulfide bond on the enzymatic activity, ASST activity staining was performed on a reducing and non-reducing SDS-PAGE gel by using MUS as a fluorogenic donor substrate and phenol as an acceptor substrate. Fluorogenic reaction product, 4-methylumbellyferone (MU) was detected only on an oxidized form, not a reduced one (Fig. 4(B)). This result suggests that intamolecular disulfide bond results in a conformation that is suitable for active structure and function.

ASST activity of the Cys to Ser mutants

E. amnigenus ASST has three cysteines, which are well conserved in the majority of ASSTs. To identify the cysteine residues which are involved in disulfide bond formation, the *astA* gene was subjected to site-directed mutagenesis to construct a series of 7 mutants in which a Cys codon was converted into a Ser codon singly or in combination to minimize the conformational changes. For the sake of convenience, these mutant forms of ASST are designated by three-letter notations, in which residues 349, 445, and 451 are indicated by C (Cys) or S (Ser) in this order, respectively. *E. coli* NM522 strains expressing the mutant ASSTs or wild-type ASST were grown, and periplasmic fractions were isolated. Concentrations of total protein were determined and used as a measure for the amount of ASST. Wild-type and all mutants were well expressed and the expression levels of them were comparable (data not shown). ASST mutant activities were compared to that of the wild-type enzyme by a colorimetric and a more sensitive fluorometric assay (Table I). Among single mutation, C349S mutation (SCC) had the greatest effect which barely showed any activity, while C445S mutant (CSC) had 10-20% activity of that of wild type and C451S (CCS) showed comparable activity to wild type. No activity was detected in the multiple mutants (SSC, SCS, CSS, and SSS) which could not form any intramolecular disulfide bond, as expected. Thus it is likely that the first cysteine (Cys349) may be involved in disulfide bond mainly with the second cysteine (Cys445) and result in active conformation. Alternatively the nearby third cysteine (Cys551) might mimic the second cysteine.

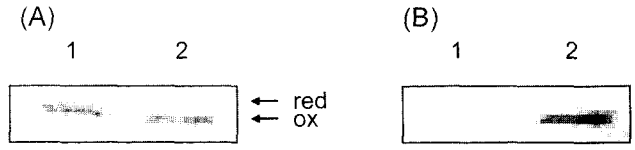

Fig. 4. Roles of the intramolecular disulfide bond on the ASST activity: (A) SDS-PAGE gels showing disulfide bond in the ASST. The positions of the oxidized (ox) and reduced (red) forms are indicated by arrows. (B) ASST activity staining. After SDS-PAGE was finished, the gel was renatured and the enzyme activity was examined by fluorogenic product, MU underer UV (320 nm). Lane 1, reducing condition (with 2-ME), lane 2, non-reducing condition (without 2-ME).

In the cytoplasm, thioredoxin reductase and other factors seem to prevent disulfide bond formation on protein. In the periplasm, the Dsb system facilitates disulfide bond formation (Bardwell *et al.*, 1991; Missiakas *et al.*, 1993). It has been established that a periplasmic factor DsbA directly catalyzes oxidation of cysteines (Bardwell *et al.*, 1991). The membrane-bound factor DsbB is believed to reoxidize the reduced form of DsbA to allow it to function catalytically (Missiakas *et al.*, 1993).

ASST is located in periplasmic space (Kwon *et al.*, 2001) and Bardwell *et al.* revealed the importance of disulfide bond formation in the protein-folding pathway for alkaline phosphatase, OmpA, and β-lactamase *in vivo* (Bardwell *et al.*, 1991). Likewise, it is thus reasonable to expect that DsbA and DsbB are necessary for ASST to do disulfide bond formation and ensure proper folding in periplasmic space.

Our current study shows that it is assumed that the *astA* gene has an operonic arrangement with *dsbA* and *dsbB* genes, and the formation of intramolecular disulfide bond is a crucial event that triggers the correct folding of ASST. Previously, we proposed the kinetic mechanism through a sulfoenzyme intermediate in which Tyr123 is an active site residue (Kwon *et al.*, 2001). The three-dimensional structure of ASST is necessary to provide further insight into the

Table I. Activities of wild-type and mutant ASSTs[a]

Mutation	Activity in colorimetric assay (U)	Relative activity (%)	Activity in fluorometric assay (U)	Relative activity (%)
WT (CCC)	84.8	100	1641	100
SCC	4.3	5.1	ND[b]	-
CSC	20.1	23.7	187	11.4
CCS	79.5	93.8	1289	78.5
SSC	5.7	6.7	ND	-
SCS	6.9	8.2	ND	-
CSS	6.0	7.1	ND	-
SSS	6.1	7.2	ND	-

[a]All data are representatives of three measurements. Standard deviations were within 20%.
[b]No detectable increase in fluorescence.

roles of these amino acids in not only the formation of disulfide bond but also catalytic mechanism.

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