Involvement of Aromatic Amino Acids in Phenylmercury Transport by MerT Protein

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To investigate the role of aromatic amino acids of MerT protein in phenylmercury transport, two *merT* variants (pMRTm1P and pMRTm2P) with specific site-directed mutations were constructed and examined their effects on $C_6H_5Hg^+$-transport. Substitution of Phe-36 and Trp-40 residues located on the periplasmic loop of MerT in turn with Ala and Val, respectively, did not affect the Hg^{2+}-uptake, but caused a significant reduction in the $C_6H_5Hg^+$-uptake by bacterial cells with intact *merT* gene. Introduction of specific mutations changing Phe-108, 114, 115 to Ala, and Tyr-110, 116 to Ser in the C-terminal region of the third transmembrane of MerT also caused large reduction in the uptake of $C_6H_5Hg^+$, but had no effect on the Hg^{2+}-uptake. In addition, both mutations caused a significant reduction in the hypersensitivity to $C_6H_5Hg^+$, but without affecting the Hg^{2+}-hypersensitive phenotype. Together these results suggest that the aromatic amino acids of MerT protein may play an important role in the transport of $C_6H_5Hg^+$ across the cell membrane.

Key words —— phenylmercury transport, aromatic amino acid, hypersensitivity, hyperuptake, MerT

INTRODUCTION

Bacterial resistance to mercurials is often mediated by a number of proteins encoded by mercury-resistance (*mer*) operon. The *merT* and *merP* genes on both broad-spectrum and narrow-spectrum *mer* operons have been implicated in Hg^{2+}-transport across the cytoplasmic membrane and been shown to be essential for bacterial resistance to Hg^{2+}.[1–4] Hg^{2+} is thought to transport into the cytoplasm by a series of exchange reactions between paired cysteine thiols in MerP and MerT. Morby *et al*. recently reported that the vicinal cysteine pair (Cys24 and Cys25) on the first transmembrane region of MerT is essential for Hg^{2+}-transport into bacterial cells.[5] However, there is no direct experimental evidence concerning bacterial transport of organomercury until recently. It seems reasonable to expect that transport of organomercury into bacterial cells is necessary for the cytoplasmic organomercurial lyase to act upon it. Recently, we demonstrated for the first time that the *merT* gene of *Pseudomonas* K-62 plasmid pMR26 that confers bacterial resistance both inorganic and organic mercury, is also involved in the $C_6H_5Hg^+$-transport,[6,7] but do not participate in the transport of CH_3Hg^+.[8] In addition, we found that the vicinal cysteine pair (Cys24 and Cys25) of MerT is not essential for transport of $C_6H_5Hg^+$ through the cytoplasmic membrane, but indeed is involved in the Hg^{2+}-transport as reported previously.[7] However, we had not uncovered which amino acids on the MerT protein are essential for the transport function.

In the present study, we constructed two mutants with site-directed mutagenesis, one in the periplasmic loop between the first and second transmembrane region (pMRTm1P), and the other in the C-terminal region of the third transmembrane (pMRTm2P) of MerT, to investigate which amino acids are important for $C_6H_5Hg^+$-transport. Here we reported that substitution of aromatic amino acids located in both the periplasmic loop and C-terminal region of MerT resulted in significant abolition of $C_6H_5Hg^+$-transport suggesting that aromatic amino acids in the MerT protein may play an important role in the $C_6H_5Hg^+$-transport.

MATERIALS AND METHODS

Bacterial Strain, Plasmids and Growth Conditions —— *Escherichia coli* (*E. coli*) XL1-Blue carrying pKF19K, a cloning vector was grown at 37°C in Luria-Bertani (LB) medium and used for routine plasmid propagation.[9] Plasmid pMRTP containing *merR*-o/p-*merT*-*merP* from *Pseudomonas* K-62 was used as the starting point for mutagenesis.[7]

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Site-Directed Mutagenesis of *merT* ——— The oligonucleotide-directed dual amber-long and accurate (ODA-LA) PCR method was used for the unique site-directed mutagenesis in *merT*.[10] Two PCR primers, OK881 (5′-CTG GTC GCC CTG GGC GCA TCC GGT GCT GTG ATC GGC AAC CTG AC-3′) and OK1098 (5′-TGG TCG CGC TTG GAG CAC CCT CTG TCG TTC CAG CTG CCT CTT AAC CAG GAG TTC-3′) were used for construction of *merT* variants, pMRTm1P and pMRTm2P, respectively. The OK881 primer was used to convert phenylalanine at position 36 and tryptophan at position 40 of MerT to alanine and valine, respectively. The OK1098 primer was used to convert phenylalanine at position 108, 114, 115 and tyrosine at position 110, 116 of MerT to alanine and serine, respectively. Plasmids with the desired mutation were sequenced in their entirety by the dideoxy sequencing method to make sure no other mutations had been introduced inadvertently.

Mercury Resistance Assay ——— Resistance of bacteria to Hg^{2+} and $C_6H_5Hg^+$ was determined on Petri dishes as described previously.[8] The zone of inhibition of growth around the disk was measured after incubation at 37°C for 16 hr.

Mercury Uptake Assay ——— The mid-exponential phase *E. coli* cells were suspended in LB medium containing 16 μM Hg^{2+} or 24 μM $C_6H_5Hg^+$. After incubation at 37°C for 10 min, the amount of total mercury in the cells was measured by atomic absorption spectrometry using atomic mercury analyzes RA-2A (Nippon Instruments, Japan) after the samples were digested with concentrated nitric acid.[11]

RESULTS AND DISCUSSION

The precise role of *merT* in the transport of Hg^{2+} has been studied in greatest detail. Now interest has turned to bacterial transport of organomercurials into the cells. Organomercurial lyase, an enzyme required for bacterial resistance to organomercury is a cytoplasmic enzyme. Therefore, transport of organomercury into cytoplasm is necessary for the enzyme to act upon it. Recently, we reported that MerT is required for bacterial transport of $C_6H_5Hg^+$,[6,7] but Cys24 and Cys25 of MerT which have been shown to be essential for Hg^{2+}-transport,[5] is not required for bacterial transport of $C_6H_5Hg^+$.[7] However, what exchange reactions occurred for phenylmercury transport across the cell membrane mediated by MerT have not been defined.

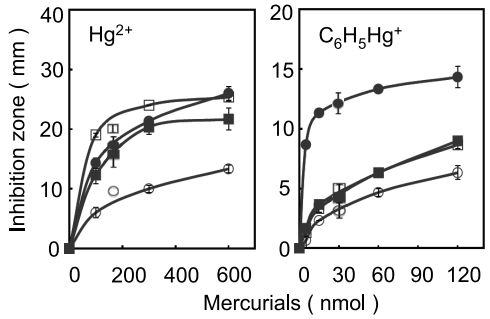

Fig. 1. Mercurial Resistance

Resistance of *E. coli* XL1-Blue with pMRTP (●), pMRTm1P (□), pMRTm2P (■) and pKF19K (○) to Hg^{2+} and $C_6H_5Hg^+$ was determined on petri dishes as described in Materials and Methods. All values are the means of triplicate determinations from three experiments.

In this study, we constructed two MerT variants, pMRTm1P and pMRTm2P, to investigate the role of aromatic amino acids on MerT protein in the transport of $C_6H_5Hg^+$. In agreement with our previous results,[7] plasmid pMRTP that contains intact *merT* gene, conferred bacteria hypersensitivity to Hg^{2+} and $C_6H_5Hg^+$ compared with its isogenic cells carrying the cloning vector pKF19K (Fig. 1). Specific point mutation of two (Phe36 and Tyr40) and five (Phe108, 114, 115 and Tyr110, 116) aromatic amino acids, located in the periplasmic loop and C-terminal region of MerT, respectively, to aliphatic amino acids (Phe to Ala, Try to Val and Tyr to Ser) caused almost complete loss of the $C_6H_5Hg^+$-hypersensitive phenotype, but did not lose the Hg^{2+}-hypersensitivity (Fig. 1). These results suggest that the aromatic amino acids of MerT may play an important role in the expression of hypersensitive phenotype to $C_6H_5Hg^+$ probably via hyperuptake of $C_6H_5Hg^+$ in the cells mediated by *merT* gene. Determination of hypersensitivity to mercury in the absence of mercuric reductase activity has often been used as a method for evaluation of the activity of mercury transport, but this method is not exactly proportional. We then directly examined the uptake of mercurials by the cell with pMRTm1P and pMRTm2P.

As shown in Fig. 2, bacteria with pMRTP took up appreciably more Hg^{2+} and $C_6H_5Hg^+$ than its isogenic strain with pKF19K. Mutation of both aromatic amino acid residues in the periplasmic loop (pMRTm1P) and C-terminal region (pMRTm2P) of MerT, to aliphatic amino acids markedly abolishes the $C_6H_5Hg^+$-uptake, but without significantly affects Hg^{2+}-uptake (Fig. 2). These results suggest that the aromatic amino acid residues in the periplasmic loop

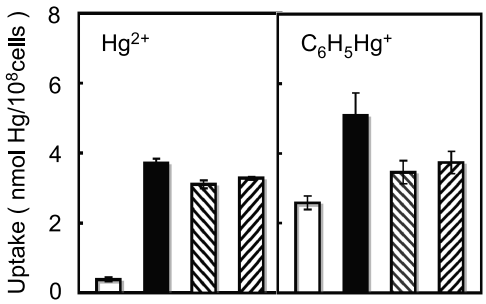

Fig. 2. Bacterial Uptake of Hg^{2+} and $C_6H_5Hg^+$

E. coli XL1-Blue with pMRTP (■), pMRTm1P (▧), pMRTm2P (▨) and pKF19K (□) were grown, prepared, and assayed as described in Materials and Methods. All values are the means of triplicate determinations from three experiments.

and C-terminal region of MerT play an important role in the transport of $C_6H_5Hg^+$ into the bacterial cells.

The MerT protein was predicted to be an inner membrane protein[12] and suggested to have three membrane spanning regions.[13] It is note worthy that among the 17 aromatic amino acids in the MerT molecule, 13 residues are localized in the inner membrane which may play a critical role in the transport of $C_6H_5Hg^+$ into the cells. The movement of $C_6H_5Hg^+$ across the cell membrane may be via interaction of phenylmercury with aromatic amino acids in MerT protein. However, at present, we do not have enough information to warrant further discussion that what exchange reactions are occurred for phenylmercury traversing cytoplasmic membrane mediated by MerT. Clearly further work is needed to understand the role of MerT in the transport of phenylmercury.

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