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Kinetic Properties of Wild-type and C117D Mutant UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase (MurA) from *Haemophilus influenzae*

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In this study, the kinetic properties of wild-type and C117D mutant *H. influenzae* MurA (Hi MurA), which catalyzes the first reaction in the biosynthetic pathway of the cell wall, were characterized. Purified recombinant Hi MurA was active at pH values ranging from pH 5.5 to pH 10, and its K_m (UNAG), K_m (PEP), and k_{cat} values were measured to be 31 μM, 24 μM, and 210 min^{-1}, respectively. Hi MurA activity was effectively inhibited by fosfomycin with an IC_{50} value of 60 μM. Hi MurA contains a cysteine residue (C117) at the loop region near the PEP binding, whereas MurA from fosfomycin resistant *Mycobaterium tuberculosis* or *Chlamydia trachomatis* contain an aspartate residue instead of the cysteine at the corresponding site. Aspartate substitution of Cys117 in Hi MurA shifted its optimum pH from 7.8 to 6.0. In addition, the K_m values for UNAG and PEP were increased to 160 μM and 150 μM, respectively, and the k_{cat} value was significantly reduced to 41 min^{-1}. Furthermore, the C117D mutant form of Hi MurA was not inhibited by 1 mM fosfomycin. These results indicate that the Cys117 of Hi MurA is the binding site of fosfomycin and plays an important role in the fast turnover of the catalytic reaction.

Key Words : *Haemophilus influenzae*, MurA, Cell wall synthesis, Cys117

Introduction

Peptidoglycan layers of bacterial cell walls protect bacteria from osmotic pressure, and *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid are the major components of peptidoglycans. The first step in the biosynthesis of peptidoglycan is catalyzed by UDP-NAG enolpyruvyl transferase (MurA), which transfers the enolpyruvyl group of phosphoenolpyruvate (PEP) to the 3'-hydroxyl group of UDP-*N*-acetylglucosamine (UNAG).[1] The *murA* gene is conserved in gram-positive as well as gram-negative bacteria but has no mammalian homolog. MurA is indispensible to cell growth since deletion of the MurA gene leads to a lethal phenotype in *Escherichia coli* or *Streptococcus pneumoniae*.[2,3] The fact that MurA is required for bacterial growth and that no homologue was found in mammalian cells suggests that MurA may be an attractive target for the development of antibiotics.

The amino acid sequence of MurAs from *Escherichia coli*, *Enterobacter cloacae* and *Haemophilus influenzae* are highly homologous,[4] and the crystal structure of MurAs from *E. cloacae*,[5] *E. coli*,[6] and *H. influenzae*[4] have a common structural feature; they all contain two domains with an active site located at the cleft of the two domains. The active site of these MurAs is covered with a loop sequence that contains a highly conserved cysteine residue. In the absence of ligand, *E. cloacae* MurA is in an open conformation where the active site is not covered by the loop sequence.[5] In contrast, MurAs from *E. coli* and *E. cloacae* have a closed conformation when they are bound to the product analogue, substrate or inhibitors.[6-8] In this closed conformation, the active site of MurA is covered by the loop sequence and the conserved cysteine is located in close proximity to the substrate, suggesting that this conserved cysteine may play a critical role in the catalytic reaction. In addition, it was previously shown that substituting C115 of *E. coli* MurA to an alanine or serine completely abolished its catalytic activity.[9] Furthermore, fosfomycin, which is a broad spectrum antibiotic produced by *Streptomyces*,[10] effectively inhibited MurA by alkylation at the cysteine residue in the conserved loop.[11] The sulfhydryl group of this cysteine is assumed to be involved in the formation of the enzyme-PEP adduct[9] or in the process of product release.[8]

Unlike MurAs from *H. influenzae*, *E. coli*, or *E. cloacae*, MurAs from pathogeneic bacteria such as *Chlamydia trachomatis* or *Mycobacterium tuberculosis* contain an aspartate residue in the conserved loop rather than a cysteine, and these MurAs are resistant to fosfomycin.[9,12,13] Furthermore, a recombinant *E. coli* strain where the MurA gene was replaced with the *C. trachomatis* MurA gene was resistance to fosfomycin,[12] suggesting that the aspartate substitution is highly important to fosfomycin resistance. The structure or kinetic properties of MurAs from *C. trachomatis* or *M. tuberculosis* have not yet been reported due to the difficulties associated with expressing recombinant proteins, even though inhibitors of these MurAs would be valuable for the development of antibiotics against *C. trachomatis* or *M. tuberculosis*. When the Cys115 of *E. coli* MurA was substituted with an aspartate, its optimal pH was altered, the turnover rate at neutral pH was reduced and the mutant *E. coli* strain was resistance to fosfomycin.[9] However, no conclusion could be made as to whether the effect of this aspartate substitution was general or only specific to *E. coli* MurA. Thus, the effects of the aspartate substitution on other

MurA enzymes need to be assessed in order to better understand the role of the aspartate or cysteine residue in the enzyme reaction.

In this study, we characterized Hi MurA as well as a mutant form of C117D Hi MurA in which the cysteine residue was substituted with an aspartate. The cysteine substitution lowered the affinity of Hi MurA to the substrate and its turnover rate. In addition, C117D Hi MurA was resistant to fosfomycin.

Materials and Methods

Materials and Plasmid Preparation. Fosfomycin was purchased from Sigma (St. Luis, MO, USA). Phosphoenolpyruvate and UDP-*N*-acetylglucosamine were also obtained from Sigma. Malachite green and ammonium molybdate were purchased from Sigma. The Cys117 codon (TGT) of Hi MurA was changed to the Asp codon (GAT) by site-directed mutagenesis. The substitution was introduced into pMurA, an expression vector of wild-type Hi MurA[14] with a quick-change kit (Stratagene, USA) to generate pMurA(C117D) using 5'-primer (GTATCATTACCTGGAGGTGATTCTATTGGGGCTAGACCTG) and 3'-primer (CAGGTCTAGCCCCAATAGAATCACCTCCAGGTAATGATAC). The sequence of pMurA(C117D) was confirmed by DNA sequencing.

Expression and Purification of *H. influenzae* MurA. The wild-type and C117D mutant of Hi MurA were expressed and purified as previously described.[14] Briefly, the expression vector [pMurA or pMurA(C117D)] was transformed into *E. coli* Rosetta2(DE3). The transformed cells were grown at 37 °C in LB media containing 0.1 mg/mL ampicillin and 50 μg/mL chloramphenicol, and the expression of recombinant proteins were induced by adding 0.5 mM IPTG when the optical density of the cell culture reached 0.9. The expressed proteins were purified from the cell lysate using Ni-NTA affinity column and size exclusion chromatography.

Assay. The activity of MurA was measured as previously described.[15] Briefly, 20 μL of 100 nM Hi MurA in 100 mM Tris-HCl, pH 7.8, was mixed with 20 μL of substrate solution containing 200 μM phosphoenolpyruvate (PEP) and 400 μM UDP-*N*-acetylglucosamine (UNAG) and incubated for 30 min at 25 °C. To measure MurA activity at different pH values, a poly buffer containing 100 mM citrate, MES, Tris, and Glycine adjusted to a particular pH using concentrated HCl or NaOH was used. The reaction was terminated by adding 200 μL malachite green reagent containing 0.045% malachite green and 4.2% ammonium molybdate in 4 N HCl. The amount of phosphate released from the cleavage of PEP was estimated by measuring the optical density at 620 nm.

Fluorescence Measurements. The conformational change of C117D Hi MurA was examined by measuring the increase in fluorescence of 8-anilino-1-naphthalene sulfonate (ANS) bound to MurA protein. The emission spectrum of 50 μM ANS mixed with 1.5 μM MurA and 1 mM UNAG in 25 mM poly buffer, pH 6.0, was measured at an excitation wavelength of 366 nm using a Fluorescence spectrometer FS-2 (Sinco, Japan).

Results and Discussion

Aspartate Substitution of C117 Alters pH Profile and Reduces Enzymatic Efficiency of Hi MurA. The wild-type and C117D mutant of Hi MurA were expressed in *E. coli* and purified to homogeneity using Ni-NTA and size-exclusion chromatography (Fig. 1) as previously described.[14] The optimum activity of purified wild-type and C117D Hi MurA was examined at different pH values using poly-buffer. The wild-type Hi MurA was active at pH 6.0-9.5 with an optimum pH of 7.8, and its activity gradually decreased when the pH was below 5.5 or above 10 (Fig. 2). The pH profile of wild-type Hi MurA was comparable to MurA from *E. coli*,[9] which has a sequence identity of more than 70% with wild-type Hi MurA.[4] However, the pH profile of the C117D Hi MurA was significantly different from wild-type. The optimum pH was shifted from 7.8 to 6.0 (Fig. 2). In addition, the C117D Hi MurA was active over a narrower pH range than wild-type Hi MurA, and inactivated below pH 5.0 and above pH 8.0. This change of pH-dependent activity profile of C117D Hi MurA is resulted from the aspartate substitution. The protonated form of cysteine in the active site loop of wild-type E. coli MurA was proposed to serve as general acid.[9] The reduced optimum pH value for the C117D Hi MurA suggested the protonated Asp117 of the mutant is required for the catalytic activity since the pKa value of aspatate is lower than cysteine.

The specific activity of wild-type and C117D was measured as 4,700 ± 580 (unit/mg protein) and 910 ± 130 (unit/mg protein) at pH 7.8 and 6.0, respectively. The K_m and k_{cat} values of wild-type and C117D Hi MurA were also determined at pH 7.8 and 6.0, respectively, and these kinetic parameters are listed in Table 1. The K_m values for the UNAG

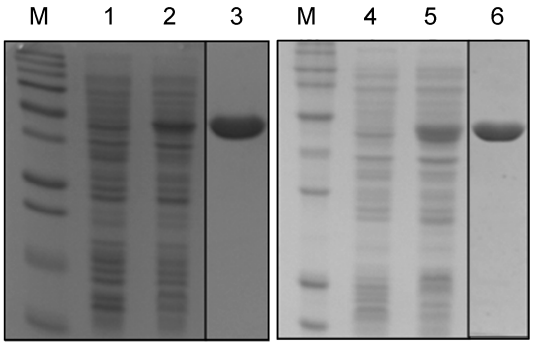

Figure 1. Expression and purification of wild-type and C117D Hi MurA. The expression of Hi MurA in *E. coli*, and homogeneity of purified MurA were analyzed by 15% SDS-PAGE: lane M, molecular weight markers, lane 1 and 4, crude extract of *E. coli* Rosetta2(DE3) expressing wild-type and C117D Hi MurA, respectively, before the induction with 1 mM IPTG, lane 2 and 5, crude extract of Rosetta2(DE3) expressing wild-type and C117D Hi MurA, respectively, after induction, lane 3 and 6, purified wild-type and C117D Hi MurA after size-exclusion chromatography, respectively.

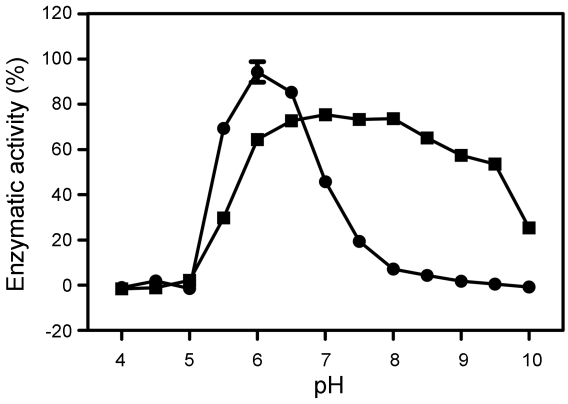

Figure 2. The pH dependency of the activity of wild-type and C117D Hi MurA. The activities of purified wild-type Hi MurA(■) and C117D Hi MurA(●) (100 nM and 500nM, respectively) were measured at pH 4.0-10 in poly-buffer solution (each 100 mM of citric acid, Mes, Tris, and glycine solution adjusted with NaOH or HCl) containing 0.2 mM of PEP and 0.4 mM of UNAG or 0.4 mM of PEP and 0.8 mM of UNAG in wild-type and C117D MurA, respectively. After 30 minutes for wild-type or 1 hour for C117D of incubation at room temperature, the amount of phosphate generated from the reaction was measured using the malachite green reagent as described in the Materials and Methods. All experiments were performed in triplicate.

substrate were obtained from the initial rate at different UNAG concentrations and 1.0 mM of PEP for both wild-type and C117D Hi MurA. The K_m(UNAG) value of wild-type Hi MurA was measured to be 31 μM, which was about two fold higher than the K_m value of *E. coli* MurA. Whereas, the K_m(UNAG) value of the C117D Hi MurA was measured to be 160 μM, which was more than 5-fold higher than that of wild-type. The K_m(PEP) value of wild-type and C117D Hi MurA were also measured to be 24 μM and 150 μM, respectively, at different PEP concentrations and 5 mM of UNAG. The K_m(PEP) value was about 60-fold higher than the K_m value of *E. coli* MurA, indicating that wild-type Hi MurA has a lower affinity for PEP compared to *E. coli* MurA. Also, the K_m(PEP) value of the C117D mutant was more than 6-fold higher than that of wild-type. The increased K_m(UNAG) and K_m(PEP) value of the C117D mutant Hi MurA suggested that the C117D substitution in Hi MurA significantly reduced the affinity of PEP and UNAG. Furthermore, this substitution reduced the efficiency of the wild-type enzyme (k_{cat}/K_m) by up to 20-fold for *E. coli* MurA or 33-fold for Hi MurA (Table 1). These properties of C117D Hi MurA suggest that MurAs containing an aspartate residue at the conserved loop region such as MurAs from *M. tuberculosis* or *C. trachomatis* would have a much lower efficiency than *E. coli* MurA.

Aspartate Substitution of Cys117 Abolished Fosfomycin Sensitivity of MurA. Fosfomycin is an antibiotic that covalently modifies Cys115 of *E. coli* MurA (equivalent of Cys117 of Hi MurA) and inactivates MurA.[10,11] However, fosfomycin failed to suppress the growth of *M. tuberculosis* or *C. trachomatis*.[12,13] The fosfomysin resistance of these bacteria is probably due to the resistance of MurA, since MurA of *M. tuberculosis* or *C. trachomatis* contains an aspartate residue at the active site loop instead of cysteine. Therefore, we tested whether C117D substitution affected the sensitivity of Hi MurA to fosfomycin. The apparent IC_{50} value of fosfomycin was obtained by measuring the residual activity of MurA after pre-incubation of MurA for 20 min with fosfomycin.[16] Wild-type Hi MurA was effectively inhibited by fosfomycin. More than 90% of Hi MurA was inactivated in the presence of 200 μM of fosfomycin (Fig. 3). It should be noted that the IC_{50} value of Hi MurA for fosfomycin was higher than the IC_{50} value (8.8 μM) of *E. coli* MurA (11). Since Hi MurA has a lower affinity for PEP (Table 1) than *E. coli* MurA and fosfomycin is an analogue of PEP, the higher IC_{50} value of Hi MurA compared to

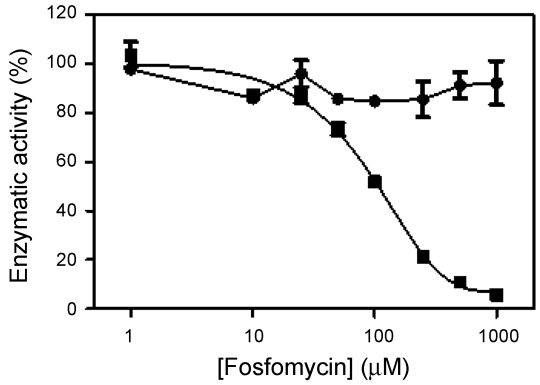

Figure 3. Concentration dependent inhibition of wild-type and C117D Hi MurA by fosfomycin. Various concentrations of fosfomycin were incubated with wild-type Hi MurA (■) and C117D Hi MurA (●) for 20 minutes at room temperature, and then the residual activity of MurA was measured. The final concentrations of enzyme, PEP, and UNAG were 100 nM, 0.1 mM and 0.2 mM for wild-type Hi MurA, and 500 nM, 0.2 mM and 0.4 mM for C117D Hi MurA, respectively. All experiments were performed in triplicate.

Table 1. Kinetic parameters of wild-type and C117D Hi MurA

	pH	K_m(PEP) (μM)	K_m(UNAG) (μM)	k_{cat}(min^{-1})	k_{cat}/K_m(PEP) (μM^{-1} min^{-1})	Specific activity[c] (unit/mg protein)
Hi MurA	7.8	24	31	2.1×10^2	8.9	4.7×10^3
C117D Hi MurA	6.0	150	160	41	0.27	9.1×10^2
Ec MurA[a]	8.0	0.40	15	2.3×10^2	6.0×10^2	5.1×10^3
C115D Ec MurA[b]	6.0	22	21	6.6×10^2	30	1.5×10^4

Data for wild-type[a] and C115D[b] MurA from *E. coli* were from Marquardt *et al.* (11) and Kim *et al.* (9), respectively. [c]One unit was defined as the activity of enzyme that can convert 1 nmol of PEP per minute.

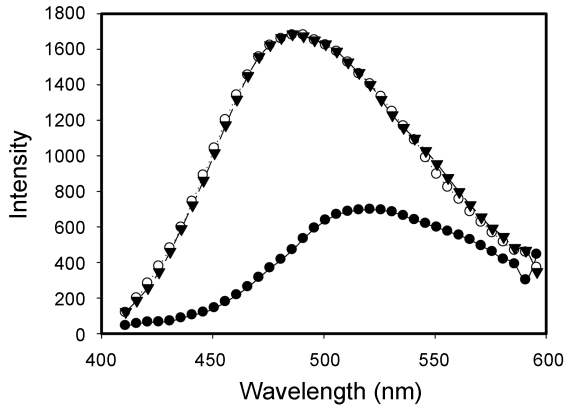

Figure 4. Specific interaction between wild-type C117D Hi MurA and ANS. The fluorescence spectra of free ANS (closed circle) or the ANS bound to C117D Hi MurA (1.5 μM) in the absence (open circle) or presence of 1 mM UNAG (▼) were measured between 400 and 600 nm at excitation wavelength of 366 nm.

E. coli MurA was probably due to a lower affinity of fosfomycin to Hi MurA than *E. coli* MurA. In contrast, the activity of the C117D Hi MurA was not affected by fosfomycin up to 1,000 μM (Fig. 3). This result is consistent with the fosfomycin resistance properties of the C115D *E. coli* MurA mutant (9), and indicates that the Cys117 of Hi MurA is the alkylation site of fosfomycin.

Open Conformation of Hi MurA is Induced by C117D Substitution. It has been shown that extrinsic fluorophore ANS specifically bind to MurA, and the fluorescence of ANS is enhanced upon binding to Hi MurA or *E. cloacae* MurA.[14,17] Particularly, ANS can bind near Pro-112 region of the open conformation of MurA but not the UNAG-bound or closed conformation.[14,18] The increased fluorescence of ANS upon specific binding to MurA in the open conformation was exploited to examine the effect of the C117D substitution on the conformation of Hi MurA. ANS showed fluorescence spectrum with a peak maximum of 520 nm (Fig. 4, closed circle). When the substrate free form of C117D Hi MurA was mixed with ANS, the fluorescence intensity of ANS was significantly enhanced and the peak maximum was shifted from 520 nm to 470 nm compared to the spectrum of free ANS (Fig. 4, open circle). This enhanced fluorescence intensity was comparable to the fluorescence enhancement of ANS bound to the open conformation of Hi MurA.[14] In the presence of 1 mM of UNAG, however, the fluorescence intensity of ANS was almost unchanged (Fig. 4. triangle). Whereas, the fluorescence of ANS bound to wild-type Hi MurA significantly reduced in the presence of UNAG.[14] These results suggested that C117D Hi MurA was in the open conformation even in the presence of 1 mM of UNAG, and C117D substitution stabilized the open conformation of MurA. The effect of C117D substation on the conformation of C117D Hi MurA was consistent with the lowered affinity to UNAG and the increased K_m(UNAG) value of C117D Hi MurA.

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