Evidence That the Fosfomycin Target Cys\textsuperscript{115} in UDP-N-acetylglucosamine Enolpyruvyl Transferase (MurA)
Is Essential for Product Release*  

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MurA (UDP-N-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7) is an essential enzyme in the biosynthesis of the peptidoglycan layer of the bacterial cell. It provides an attractive template for the design of novel antibiotic drugs and is the target of the naturally occurring antibiotic fosfomycin, which covalently attaches to Cys\textsuperscript{115} in the active site of the enzyme. Mutations of Cys\textsuperscript{115} to Asp exist in pathogens such as Mycobacteria or Chlamydia rendering these organisms resistant to fosfomycin. Thus, there is a need for the elucidation of the role of this cysteine in the MurA reaction. We determined the x-ray structure of the C115S mutant of Enterobacter cloacae MurA, which was crystallized in the presence of the substrates of MurA. The structure depicts the product state of the enzyme with enolpyruvyl-UDP-N-acetylglucosamine and inorganic phosphate trapped in the active site. Kinetic analysis revealed that the Cys-to-Ser mutation results in an enzyme that appears to perform a single turnover of the reaction. Opposing the common view of Cys\textsuperscript{115} as a key residue in the chemical reaction of enolpyruvyl transfer, we now conclude that the wild-type cysteine is essential for product release only. On the basis of a detailed comparison of the product state with the intermediate state and an unliganded state of MurA, we propose that dissociation of the products is an ordered event with inorganic phosphate leaving first. Phosphate departure appears to trigger a suite of conformational changes, which finally leads to opening of the two-domain structure of MurA and the release of the second product enolpyruvyl-UDP-N-acetylglucosamine.

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\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Structure & Function & Significance \\
\hline
UDP-N-acetylglucosamine & Enzymatic activity & Essential for peptidoglycan synthesis \\
\hline
MurA & Active site & Inhibitory target for antibiotics \\
\hline
\end{tabular}
\caption{Summary of key enzymes involved in peptidoglycan synthesis.}
\end{table}

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**References**

1. The abbreviations used are: PEP, phosphoenolpyruvate; THI, tetrahedral intermediate; UNAG, UDP-N-acetylglucosamine; EP-UNAG, enolpyruvyl-UDP-N-acetylglucosamine; MES, 4-morpholineethanesulfonic acid.

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transfer toward and from PEP during catalysis (24). Such a dual role for Cys\textsuperscript{115} as an acid-base catalyst in the addition-elimination reaction has recently been questioned by crystallographic analysis of the D305A mutant of MurA crystallized in the presence of UNAG and PEP (12). This structure revealed the genuine tetrahedral reaction intermediate of the enzyme, which is S-configured at the C2 of the attached PEP molecule. The S configuration was also found for the genuine tetrahedral intermediate of the AroA reaction using the corresponding mutant enzyme D313A. Bartlett and co-workers (25, 26) deduced the same S configuration of the intermediate of the AroA reaction by chemical studies. However, the stereochemical course of the enolpyruvyl transfer reactions is a matter of debate, partly because of the lack of understanding the role of Cys\textsuperscript{115} in MurA catalysis and because an active site residue such as Asp\textsuperscript{305} (C1) (26) or through the action of an enzyme residue such as Asp\textsuperscript{305} (C2) (12). Either mechanism would result in the formation of the vinyl ether product, EP-UNAG, and Pi (P\textsubscript{i}).

**Experimental Procedures**

PEP (potassium salt) and UNAG were purchased from Sigma. Protein concentration was determined using the Pierce Coomassie reagent with bovine serum albumin as standard. The mutation C115S was introduced into wild-type \textit{E. coli} MurA using a Kwik Change mutagenesis kit from Stratagene and appropriate primers. The pET-Vector 9d (Novagen) containing the open reading frame of the wild-type enzyme was used as template for the point mutation of MurA. C115S-MurA was overexpressed in a STBL2-DE3 \textit{E. coli} cell. The overexpressed protein was purified as described (21).

**Crystallography**—The mutant enzyme was concentrated to \textasciitilde100 mg/ml using Centricon 30 devices (Amicon) at 4 °C. C115S-MurA was crystallized at 19 °C from 10 mM MES, pH 6.4, 10% (w/v) polyethylene glycol 20,000 in the presence of 5 mM UNAG and 5 mM PEP. Diffraction data were recorded from a single flash frozen crystal of MurA (Protein Data Bank code 1EJC) (28) as search model. Solvent molecules and residues 111–124 were omitted in the translational and rotational searches. Diffracted data were limited to low resolution reflections 20.0–6.0 Å in the cross-rotation and translation search. From the 30 highest peaks of the cross-rotation function, eight solutions could be successfully translated into the asymmetric unit of the crystal. Exploiting the point group symmetry, the eight independent solutions were rearranged in the asymmetric unit. This rearrangement revealed that the asymmetric unit of the crystal contains two tetramers of MurA molecules. Rigid body refinement of the asymmetric octamer, with the two globular domains of each of the eight MurA molecules refined independently, lowered the initial \(R\text{free}\) from 50.3 to 36.1%. Refinement was performed using data to the highest resolution with no \(a\) cut-off applied. Most of
the protein regions were constrained exploiting the noncrystallographic symmetry between the eight molecules, except for the active site and intermolecular contact regions. Solvent molecules were added to the model at chemically reasonable positions. The ligands were modeled according to the clear electron density map. Residue 67 of each of the eight molecules was modeled as iso-aspartate (30). The data collection and refinement statistics are summarized in Table I. Figs. 3, 4 (bottom), and 7 (a–c) were drawn with Molscript (31) and Raster3D (32), the top of Fig. 4 was drawn with Bobscript (33) and Raster3D, and Fig. 7 (d–f) was drawn with GRASP (34).

**Kinetic Analysis**—The catalytic activity of *E. cloacae* C115S MurA was tested at 20 °C with a Shimadzu 1650PC spectrophotometer. To measure C115S MurA activity, we made use of two assays; one uses MurB as a coupling enzyme to detect the product enolpyruvyl-UNAG (EP-UNAG). We have recently used the MurB coupled assay for a reevaluation of the mode of action of fosfomycin on *E. cloacae* MurA (17). The coupled reaction starts with MurA converting UNAG and PEP to UNAG-EP, which is then reduced to UDP-N-acetylmuramic acid by MurB, using one equivalent of NADPH. To compensate for the diaphorase activity of MurB, a glucose oxidase/glucose system was exploited. This resulted in a stable base line prior to initiation of the MurA reaction. The second assay, the Lanzetta assay, was used to detect the diaphorase activity of MurB, a glucose oxidase/glucose system was exploited. To test whether the trapped products could be detected after denaturation of the enzyme-ligand complex. This was accomplished by incubating a mixture containing reaction buffer and 40 mg/ml C115S MurA (0.9 mM) in a final volume of 400 μl at room temperature for 10 min. The reaction was initiated by the addition of 5 mM PEP and allowed to proceed for 5 min. To stop the reaction and denature the mutant enzyme a final concentration of 6 M urea or 5% trichloroacetic acid was added and incubated at 4 °C for 16 h. Alternatively, the reaction was heated at 100 °C for 10 min. To test for the product UNAG-EP, the coupled assay with varied concentrations of the denaturation mixture (0.4–2.9 mg/0.01–0.07 mM) was employed. A stable reading was established prior to the start of the reaction by the addition of 40 μM of MurB, and the decrease in NADPH absorbance was recorded. Control experiments were conducted throughout with C115S MurA treated in the same way but omitting PEP during the reaction time.

**RESULTS AND DISCUSSION**

**Enzyme-Products Complex**—Under the crystallization conditions used, C115S-MurA oligomerizes to densely packed tetramers (Fig. 3), of which two constitute the asymmetric unit of the crystal. X-ray analysis to 2.3 Å resolution revealed the products of the enzymatic reaction, EP-UNAG and P, trapped in the active site of each of the eight crystallographically independent C115S-MurA molecules (Fig. 4). The products uniformly occupy all MurA molecules of the crystal. Refined at unit occupancy, and the mean temperature factor of the atoms

![Role of Cys\textsuperscript{115} in MurA](http://www.jbc.org/doi/figure/3759)

**FIG. 3. Oligomerization of C115S-MurA molecules to tetramers (stereo representation).** The active site in each of the molecules is marked by a space-filling model of the products of the enzymatic reaction. The loop regions Pro\textsuperscript{112}, Pro\textsuperscript{121} in interface between individual MurA molecules are highlighted yellow. A full second tetramer (only partially drawn in transparent gray) completes the packing of the asymmetric unit of the crystal.
of the products lies with 17.0 Å² yet below the mean temperature factor of the protein of 23.9 Å².

This liganded C115S-MurA, which in the following is denoted as MurA:P1P2, exhibits the closed enzyme conformation observed previously for MurA (9, 12, 24). Its overall fold is virtually identical with that of the tetrahedral reaction intermediate state of the enzyme (MurA:THI; Protein Data Bank entry 1Q3G) (12). The positions of equivalent pairs of the 419 C/C/H9251 atoms of MurA:P1P2 and of MurA:THI deviate in average by only 0.23 Å with a maximum discrepancy of 1.7 Å at the C terminus of the polypeptide chain.

Detailed comparison of MurA:THI and MurA:P1P2 suggests that the enzyme cleaves the covalent bond between C-2 and the enol oxygen atom of the PEP moiety without macroscopic changes in the active-site residues of the enzyme (Fig. 4).

Residues that are in polar or hydrophobic interaction with the UNAG moiety of the tetrahedral adduct as well as the charged residues Lys1152, Arg1201, Asp3053, Arg3314, Arg3715, and Arg3976, which are responsible for PEP binding (12, 15), adopt in MurA:P1P2 the same conformation as in MurA:THI. Even the Pro112–Pro121 loop and especially residue 115 retain their conformation in the transition from the intermediate state to the product state. Although the loop lies in the interface of MurA molecules in the noncrystallographic tetramer (Fig. 3), packing interactions only marginally involve residues of the loop; two hydrogen bonds are formed between the main chain of the loop in residues Leu111 and Gly114 to the elongated side chains of Arg340 and Glu337, respectively, from a neighboring molecule.

**Single Turnover Catalysis**—With serine substituted for the wild-type cysteine in position 115, the enzyme is apparently still able to catalyze the enolpyruvyl transfer reaction but in a single turnover only, because both reaction products, EP-UNAG and Pi, are trapped in the active site. Indeed, this mutant enzyme showed no catalytic activity using either the MurB coupled assay (detecting EP-UNAG) or the Lanzetta assay (detecting Pi), which is in agreement with the structure data. EP-UNAG produced by the mutant enzyme was assayed for the presence of EP-UNAG using MurB. Linear regression yielded a slope of 0.93 ± 0.02.

**Fig. 4. Interaction of MurA with its products**. Top stereo pair, electron density of the trapped products in the active site. The density is derived from a 2\(F^\text{o}\) – \(F^\text{c}\) Fourier synthesis to a resolution of 2.3 Å and is contoured at 1 \(\sigma\). The overlaid structure model is color-coded according to atom types: carbon atoms of the products are shown in green, nitrogen atoms are in blue, oxygen atoms are in red, and phosphorus atoms are in magenta. Bottom stereo pair, the trapped products are shown in green; the surrounding enzyme residues are in gray (hydrophobic) or pale violet (polar/charged); the solvent structure, including a glycerol molecule from the cryo buffer, is shown in transparent cyan. Dashed lines represent polar or charged interactions. The view is from EP-UNAG toward the 10-residue loop Pro112-Pro121.

**Fig. 5. Single turnover catalysis by C115S MurA**. The mutant enzyme was allowed to react with saturating UNAG and PEP concentrations and subsequently denatured with 6 M urea. Aliquots of the denaturation mixture were assayed for the presence of EP-UNAG using MurB. Linear regression yielded a slope of 0.93 ± 0.02.
Role of Cys^{115} in MurA

Proposed Role of Cys^{115} in the MurA Reaction—The present data provide evidence that Cys^{115} is crucial for product release. The data also imply that this residue is not involved in the addition step of enolpyruvyl transfer. It is possible, however, that the onset of the reaction is perturbed as a result of the mutation to Ser. Because we have detected the enolpyruvyl-product after a 5-min reaction time, long enough to build up products by an even remotely active enzyme, we cannot rule out other roles that Cys^{115} might exert during catalysis. However, two additional findings prompted us to reconsider the function of Cys^{115}. First, a residue such as Cys^{115} is not present in AroA. Although it has been suggested that Glu^{341}, a strictly conserved AroA residue, might be the catalytic counterpart to Cys^{115} in MurA (15, 26, 37), the location of these two residues in their respective active sites is different (12). Secondly, whereas Glu^{341} in AroA is in short hydrogen-bonding distance to the PEP-moiet of the tetrahedral intermediate, the only apparent interaction that Cys^{115} exerts in the MurA-THI structure is a long range bond (distance = 3.7 Å) to NH_1 of the guanidinium group of Arg^{397} (12). Given the nucleophilic nature of Cys^{115} when reacting with fosfomycin, this interaction is likely to be electrostatic. In the MurA-products complex the distance between the Ser^{115} hydroxyl group and NH_1 of Arg^{397} is 3.5 Å (Fig. 6). Modeling a cysteine side chain into the Ser^{115} mutant enzyme may be sufficient to keep the enzyme in its closed form and prevent product release. On the other hand, aspartate as in position 115 of Mycobacterium MurA could substitute for the proposed Cys^{115} function (Fig. 6). The side chain of aspartate is longer and flexible enough to bring one of its carboxyl oxygen atoms in close salt bridge distance to NH_1 of Arg^{397}. This is reflected in the observation that C115D-MurA from E. coli retains enzymatic activity (20).

Together with Lys^{292} and Arg^{387}, the guanidinium group of Arg^{397} coordinates the phosphate moiety of the tetrahedral intermediate, and after formation of the products, the phosphate ion (Figs. 4 and 6 and Table II). The charged side chain groups form hydrogen bonds or salt bridges to the phosphate oxygen atoms, depending on the protonation state of the phosphate ion. These interactions are principally identical with the coordination of the phosphate group in the tetrahedral intermediate. After cleavage of the covalent bond between the enolpyruvyl and phosphate moieties of the tetrahedral intermediate, the phosphate ion moves 0.6 Å away from its former location and slightly rotates (Fig. 4). Detachment of the phosphate group has been suggested to be either self-catalyzed (syn-elimination) (26) or through the action of a strictly conserved aspartic residue, Asp^{365} MurA/Asp^{113} AroA (anti-elimination) (12). Although the Pro^{112}–Pro^{121} loop in the closed enzyme state shields most of the active site from solvent, the phosphate site is situated in a solvent accessible cavity (Fig. 7). This cavity is narrowed toward the surface by the side chain of Arg^{397}. Upon opening of the enzyme, the side chain of Arg^{397} would swing toward the hinge region into a position parallel to the side chain of Lys^{48} (16), whereby the guanidinium group of Arg^{397} is displaced by about 9 Å (30). Such a shift of Arg^{397} would leave a free exit route for the phosphate ion from the closed enzyme (Fig. 7, b and c). This is different for EP-UNAG. From the active site geometry in the product state, it becomes obvious that EP-UNAG cannot dissociate from MurA as long as the enzyme and the loop are closed. The only direct interaction between the loop and EP-UNAG is a hydrogen bond from the side chain of Arg^{387} to an oxygen atom of the di-phosphate moiety (Fig. 4), but the loop sterically blocks dissociation of this product (Fig. 7).

From this scenario it is conceivable that product release is an ordered event, where the phosphate ion has to leave before the Pro^{112}–Pro^{121} loop opens to release the second product EP-UNAG. The shift of Arg^{387} that accompanies the dissociation of the phosphate ion aligns the positively charged side chain of Arg^{397} parallel to that of Lys^{48}. This would result in repulsive forces in the hinge region between the bottom and top domains of the enzyme, which might initiate the opening of the cleft. Comparison of MurA:P_{23} with the unliganded state of the enzyme (Protein Data Bank entry 1EJC) (28) reveals that the widening of the cleft dislocates the o-carbon atom of Asp^{123} by 3.0 Å from its position in the product state. By this shift the side chain of Asp^{123} would lose its interaction with the uridine moiety of EP-UNAG and would have enough space to rotate about 180° around its ψ angle. Such rotation would swap the positions of the side chain of Asp^{123} and the last turn of the loop-anchoring helix a2 of subdomain IIc of MurA (Fig. 7, b and c). It has been reported previously that the opening of the last turn of helix a2 is a main feature in the transition from the

![Fig. 6. Coordination of the phosphate ion in the product state of MurA (stereo representation). Dashed lines designate hydrogen bonds. In position 115 a modeled cysteine (as in wild-type MurA from E. cloacae) and aspartate (as in MurA from Mycobacterium) are superimposed to the serine from the MurA:P_{23} structure. The respective resulting hydrogen-bonding distances to Arg^{397} are shown in magenta.](http://www.jbc.org/content/3761/4/13312韶)
winded conformation of unliganded MurA observed in 1EJC into the U-shaped loop of liganded MurA (30). We propose that transposition of the last turn of the loop-anchoring helix and Asp123 is the key event for winding up the loop and eventually releasing EP-UNAG.

Conclusions—Because both products are locked in the active site if residue 115 is a serine, the mutation of the wild-type cysteine apparently impedes the initial trigger for a suite of conformational changes in the enzyme that finally leads to the dissociation of Pi and EP-UNAG. This finding, together with the kinetically detected single turnover, questions the previously suggested dominant role of the wild-type cysteine as proton donor in the course of product formation (24). The function of Cys115 might rather be to keep binding of the phosphate group weak enough to allow dissociation of the ion from the enzyme as soon as the covalent bond to the enolpyruvyl moiety is lost. Such a weakening of the coordination of the phosphate ion in the product state of the enolpyruvyl transfer reaction could probably be realized by any acceptor group in hydrogen-bonding distance to NH1 of Arg120. In the mechanistically similar enzyme AroA, which is the only other known enolpyruvyl transferase besides MurA, Arg124 corresponds in position and function to Arg120 of MurA (12, 15). In this enzyme, NH1 of Arg124 exerts a bifurcated hydrogen bond to the phosphate group.

Role of Cys\textsuperscript{115} in MurA

Fig. 7. Proposed structural course of product release from MurA. The view is approximately along the presumable exit route of the phosphate ion. a–c, schematic representation of the active site of MurA. The loop Pro\textsuperscript{112}–Pro\textsuperscript{121} is shown in turquoise, the loop-anchoring structure elements (β-strand b2 and helix a2 of subdomain IIC) are in yellow, EP-UNAG is in green, and the phosphate ion is in orange. Residues involved in phosphate binding and presumably in product release are shown as ball-and-stick forms. The molecular surfaces (d–f) of the structures a–c were calculated with GRASP (32) and displayed in transparent turquoise for the Pro\textsuperscript{112}–Pro\textsuperscript{121} loop region, transparent blue for the side chain of Arg\textsuperscript{397}, and transparent gray for the rest of the MurA molecule. The phosphate ion and EP-UNAG are drawn in solid red and green, respectively. a and d, product state observed in MurA:P1P2. b and e, as above, but with Arg\textsuperscript{397} oriented as in unliganded, open MurA (Protein Data Bank entry 1EJC) (28) and with a modeled cysteine instead of serine in position 115. c and f, open MurA (Protein Data Bank entry 1EJC) (28). EP-UNAG is modeled (transparent green) in the same position relative to the bottom domain as above.
group of the tetrahedral intermediate and to the carbonyl oxygen atom of the main chain at Asn94. The fact that AroA does not possess a cysteine in the vicinity of Arg124 but still catalyzes a comparable reaction as MurA corroborates our findings that Cys115 of MurA is just one of several possible alternatives to facilitate product dissociation in enolpyruvyl transferases.

The proposed ordered mechanism for product release is in contradiction to the previous suggestion that MurA would only open under substrate depletion (30). The structure of the trapped product state shows that the Pro112–Pro121 loop remains in the same conformation like during catalysis. As long as the phosphate ion is bound to the active site, there apparently is no exit route for the large EP-UNAG molecule from the closed enzyme state. Thus, MurA has to close and reopen with every catalytic turnover. Binding of UNAG to MurA initiates the catalytic cycle by triggering enzyme closure (17, 18). Subsequently PEP would enter the closed enzyme on the same route that the phosphate ion later uses to exit and docks to its newly formed binding site. After the enolpyruvyl transfer is completed, the produced phosphate ion leaves the active site. It thereby triggers opening of the two-domain structure of MurA and winding up of the Pro112–Pro121 loop, which in turn allows the dissociation of the second product EP-UNAG.

Although details of the conformational changes required for product release remain speculative, the presented structure expands the data base of x-ray structures that characterizes MurA in different states of its catalytic cycle. The presented structure depicts the moment right before the macro-conformational change from the liganded closed form to an open form of MurA in different states of its catalytic cycle. The presented structure depicts the moment right before the macro-conformational change from the liganded closed form to an open form of MurA sets in and hence contributes to the elucidation of the induced fit mechanism. Understanding the details of domain movement interwoven with the function of MurA would lay the groundwork for an alternate strategy for structure-based drug design. Identification of the prerequisites of the induced fit mechanism may provide new templates for the design of novel antibacterial agents that do not target the active site but block closure of the enzyme and formation of the catalytic center.

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