A Novel Inhibitor That Suspends the Induced Fit Mechanism of UDP-N-acetylglucosamine Enolpyruvyl Transferase (MurA)*§

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MurA (UDP-N-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7) catalyzes the first committed step in the synthesis of the bacterial cell wall. It is the target of the naturally occurring, broad-spectrum antibiotic fosfomycin. Fosfomycin, an epoxide, is a relatively poor drug because an ever-increasing number of bacteria have developed resistance to fosfomycin. Thus, there is a critical need for the development of novel drugs that target MurA by a different molecular mode of action. We have identified a new scaffold of potent MurA inhibitors, derivatives of 5-sulfonyloxy-anthranilic acid, using high-throughput screening. T6361 and T6362 are competitive inhibitors of MurA with respect to the first substrate, UDP-N-acetylglucosamine (UNAG), with a Kₐ of 16 µM. The crystal structure of the MurA:T6361 complex at 2.6 Å resolution, together with fluorescence data, revealed that the inhibitor targets a loop, Pro¹¹² to Pro²¹, that is crucial for the structural changes of the enzyme during catalysis. Thus, this new class of MurA inhibitors is not active site-directed but instead obstructs the transition from the open (unliganded) to the closed (UNAG-ligated) enzyme form. The results provide evidence for the existence of a MurA:UNAG collision complex that may be specifically targeted by small molecules different from ground-state analogs of the enzymatic reaction.

Survival of bacteria depends on the activity of the enzyme MurA (UDP-N-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7) (1, 2). MurA catalyzes the first committed step in the biosynthesis of the bacterial cell wall (3, 4). Because this pathway is absent from mammals, MurA is an attractive target for the development of novel antibacterial agents (5, 6).

The reaction catalyzed by MurA proceeds through the chemically unusual transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP)¹ to UDP-N-acetylglucosamine (UNAG) (Fig. 1). Unlike most PEP-dependent enzymes, which use PEP as a phosphoryl donor through cleavage of the high-energy P-O bond, in this reaction the C-O bond of PEP is cleaved to transfer the enolpyruvyl moiety to a second substrate. The only other enzyme known to catalyze a similar reaction is 5-enolpyruvyl shikimate-3-phosphate synthase (EC 2.5.1.19), also known as EPSPS or AraA. 5-Enolpyruvyl shikimate-3-phosphate synthase is the sixth enzyme in the shikimate pathway toward the synthesis of aromatic amino acids in microorganisms and plants (7, 8). Both enzymes exist in an open, substrate-free state and a closed, liganded state, indicating that their reactions follow an induced-fit mechanism (9–13).

Widespread antibiotic resistance in many common bacterial pathogens has prompted extensive research toward the development of novel antibiotics that act by new inhibitory mechanisms. The enzymes involved in bacterial cell wall biosynthesis are ideal targets for the design of new inhibitors. The only known antibacterial drug targeting MurA is fosfomycin, the active ingredient of Monurol™ (14). This epoxide forms a covalent adduct with a cysteine residue, Cys¹¹⁵ (numbering according to Escherichia coli MurA). Cys¹¹⁵ is located in a solvent-exposed loop that undergoes a large conformational change upon UNAG binding; this in turn allows inactivation by fosfomycin to occur.

There are three ways in which pathogenic bacteria can develop resistance to fosfomycin: (i) the resistance of Mycobacterium tuberculosis and Chlamydia to fosfomycin has been primarily attributed to the lack of Cys¹¹⁵ (15–17). Sequencing alignments of the murA gene from other organisms suggest that the enzymes from Actinomycetales, Actinomyces, Nocardia, Streptomyces, and Borrelia also have a Cys to Asp change and are therefore suspected to be resistant to fosfomycin, too. (ii) A second mechanism of resistance is due to chromosome-encoded changes in the organisms that result in a decrease in transport of fosfomycin into the cell (18). (iii) The plasmid-encoded fosfomycin resistance protein (FosA) is a glutathione S-transferase that inactivates fosfomycin by forming an adduct of glutathione with the epoxide (19, 20).

A small number of novel inhibitors of MurA were discovered recently by various high-throughput screening efforts in the pharmaceutical industry. Procter & Gamble compound PGE-553826 was reported to have an IC₅₀ value of 38 µM (21). Bristol-Myers Squibb Co. identified four compounds from an array of target-specific screening strains that inhibit MurA with IC₅₀ values between 1.4 and 6.2 µM (22). R. W. Johnson...
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EXPERIMENTAL PROCEDURES

UNAG and PEP (potassium salt) were purchased from Sigma. Solvents and reagents for chemical syntheses were purchased from Aldrich, Fluka, and Fisher Scientific and used without further purification. FMOC-N-Me-L-Asp (OTBU)-OH and FMOC-N-Me-L-Glu (OTBU)-OH were from Bachem; WANG resin (theoretical load, 1.11 mmol/g) was from Novabiochem. Pierce Coomassie Blue reagent with bovine serum albumin as a standard was used to determine protein concentrations.

Enzyme Preparation—The plasmid containing Enterobacter cloacae MurA (pET 9d from Novagen) was transformed into BL21 DE3 E. coli competent cells. Overexpression was carried out using standard procedures at 20 °C using a Shimadzu 1650PC spectrophotometer. MurA was purified at 4 °C with an ÄKTA fast protein liquid chromatography system (Amersham Biosciences) essentially as described previously (24). MurA at 20 °C using a Shimadzu 1650PC spectrophotometer. The amount of inorganic phosphate produced in the reaction, using the Lanzetta reagent (25), was added, thereby stopping the reaction. Color development was stopped after 5 min by addition of 100 μl of 34% (w/v) sodium citrate. Change in optical density was measured at 660 nm, and the amount of inorganic phosphate was determined by comparison to phosphate standards. The specific activity of MurA using this assay was calculated to 43.5 Å² (average B value = 23.6 Å²). The amount of inorganic phosphate was determined by comparison to phosphate standards. The specific activity of MurA using this assay was calculated to 43.5 Å² (average B value = 23.6 Å²).

For determination of the Km(obs) value, enzyme activities at increasing UNAG, PEP, and inhibitor concentrations were recorded, and the data were fit to the Michaelis-Menten equation

\[ v = \frac{V_{\text{max}} \times [S]}{K_{\text{m(obs)}} + [S]} \]  (Eq. 1)

where v is the initial velocity, \( V_{\text{max}} \) is the maximum velocity, \( K_{\text{m(obs)}} \) is the observed Michaelis constant, and [S] is the substrate concentration. The Km value for T6362 was determined by linear regression of the replot of the \( K_{\text{m(obs)}} \) values versus the concentration of the inhibitor [I],

\[ K_{\text{m(obs)}} = \left( \frac{K_{n}}{K_{I}} \right) \times [I] + K_{n} \]  (Eq. 2)

where \( K_{n} \) is the true Michaelis constant and \( K_{I} \) is the inhibition constant.

Assaying MurA pre-incubated with the inhibitor prior to the addition of substrates accounted for possible time-dependent inhibition.
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ANS Fluorescence Experiments—The dynamics of MurA were monitored by fluorescence using the extrinsic probe ANS essentially as described previously (11, 24). Experiments were conducted at 20 °C with a Quanta Master spectrophotometer (Photon Technology International, Brunswick, NJ). The buffer used for all measurements was 50 mM sodium/potassium phosphate (pH 6.9) with 1 mM dithiothreitol. Fluorescence of ANS was excited at 366 nm, and emission spectra were recorded between 400 and 600 nm. The ANS concentration was 100 μM, the MurA concentration was 140 μg/ml (3 μM), and those of UNAG, PEP, or T6362 were varied as indicated in Figs. 4 and 5. To determine the dissociation constant of T6362, the quenched fraction (1 – $F_b$/$F_a$) was plotted as a function of ligand concentration (Fig. 5), where $F_a$ and $F_b$ are the maxima of the ANS spectra in the presence and absence of ligand, respectively. Data were fit to

$$Y_{\text{max}} = Y_{\text{max}} \times \frac{[L]}{K_0 + [L]}$$

(Eq. 3)

where $Y_{\text{max}}$ is the fluorescence quench at infinite ligand concentration ([L]), and $K_0$ is the dissociation constant.

X-ray Crystallographic Analysis—MurA was concentrated to 100 mg/ml using Centricron 30 devices (Amicon) at 4 °C for crystallization. Crystals were grown at 19 °C in hanging droplets from 5% polyethylene glycol 8000, 5% polyethylene glycol 1000, 50 mM calcium acetate, 25 mM MES (pH 6.1), and 4 mM T6361. Diffraction data were recorded at −180 °C using the rotation method on a single flash-frozen crystal of MurA liganded with T6361 (detector, Mar image plate; x-rays, CuKα, focused by mirror optics; generator, Rigaku RU300 (Molecular Structure Corp., The Woodlands, TX)). Indexing, data reduction, and scaling were performed with XDS (26). The crystal lattice was indexed as space group P2$_1$2$_1$2; with unit cell dimensions $a = 84.1$ Å, $b = 134.6$ Å, and $c = 175.0$ Å. A complete data set was collected from 16 to 2.6 Å resolution. Whereas the high-resolution data (5 to 2.6 Å) are completely consistent with the indexed space group, the low-resolution data (<5 Å) showed surprisingly bad self-consistency as reflected in the relatively high overall $R_{\text{merge/mom}}$ values (Table I) and the Wilson statistics (see supplementary material). However, the successful structure solution and refinement confirmed the chosen space group and unit cell.

For phasing and refinement, the program package CNS (27) was employed; model building was performed with O (28). The structure was solved by molecular replacement, using unliganded MurA (Protein Data Bank code 1EJC, Ref. 34), stripped of solvent, as search model. Diffraction data were limited to low-resolution reflections 16.0 to 6.0 Å in the cross-rotation and translation search. From the 20 highest peaks of the cross-rotation function, one MurA molecule was placed into the asymmetric unit, and an automated translation search located a second molecule. With the generated “dimer” as search model in cross-rotation and translation searches, we could locate two other MurA molecules in the asymmetric unit. The finding of four tightly packed molecules in the asymmetric unit is a crystallization artifact; the enzyme is known to function as a monomer. Ten rounds of minimization, simulated annealing (2500 K starting temperature), and restrained individual B-factor refinement were carried out using data to highest resolution with no σ cut-off applied. Most of the protein regions were restrained exploiting the non-crystallographic symmetry between the four molecules, except for the inhibitor binding 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 (Fig. 6). Residue 67 of each of the four MurA molecules was modeled as iso-aspartate (34). The protein model showed good stereochemistry with 90.6% of the 4*149 residues having φ/ψ values in the most favored regions. Only two residues, Cys$^{115}$ of chain B and Val$^{225}$ of chain G, have values in the disallowed regions. The Ramachandran plot is shown in the supplementary material. Data collection and refinement statistics are summarized in Table I. Figs. 6–8 were drawn using the final coordinates of MurA (chain identifier A) with Molscript and Raster3D (29, 30); Fig. 6 (top) was drawn with Bobscript (31) and Raster3D.

RESULTS AND DISCUSSION

Inhibition Kinetics and Dynamics—By high-throughput screening we have discovered novel potent inhibitors of MurA. T6361 and T6362 are derivatives of 5-sulfonoxy-anthranilic acid that inhibit MurA with IC$_{50}$ values in the low micromolar range. Steady-state inhibition kinetics and the dynamics of MurA were studied in detail with T6362. Inhibition is competitive with respect to UNAG ($K_i = 16$ μM) (Fig. 3). The influence of T6362 on the kinetics of PEP utilization was not determined because the $K_m$ of PEP is <1 μM, making kinetic evaluation with the assay employed herein unreliable. Incubation of MurA with inhibitor prior to assay did not result in time-dependent inhibition, suggesting that it is a purely reversible inhibitor of the forward reaction of the E. cloacae MurA (data not shown). Inhibition by T6361 was competitive with respect to UNAG, too (data not shown).

Next, we asked whether T6362 affects the global structural changes known to occur in MurA, i.e. the induced fit mechanism, a hallmark of the MurA reaction (11, 32). Using the extrinsic fluorophore ANS, we can monitor parts of the structural changes that occur during the binding of substrates (11, 24). The ANS-binding site in MurA is known in atomic detail (32). It is solvent-exposed in the open enzyme state, located at the interface of the loop hosting Cys$^{115}$ and the side chain of Arg$^{91}$, the latter being a strictly conserved residue. The binding of high affinity ligands, such as UNAG, destroys the ANS-binding site due to substantial conformational changes of the loop; this results in a quenching of the MurA-ANS fluorescence spectra. A series of fluorescence experiments revealed that T6362 induces structural changes in MurA similar to those observed for UNAG. The data indicate competitive binding of T6362 and UNAG to the open form of the enzyme (Fig. 4, A–D). The dissociation constant of MurA-T6362 interaction was de-
The second substrate, PEP, did not exert any effect on the MurA inhibitor complex (Fig. 4, E and F), unlike the action of PEP on the MurA-UNAG complex (11). However, upon displacement of T6362 with UNAG, the PEP-binding site becomes available again. This indicates that the enzyme either is closed (as in the MurA-UNAG-Pep complex) with some moiety of the inhibitor overlapping with the PEP-binding site, or that the enzyme-T6362 complex remains in the open state, where no PEP-binding site exists. Thus, kinetic and fluorescence data corroborate each other, in that UNAG and T6362 target the same binding site. However, the inhibitor-induced alterations are different from those induced by UNAG because PEP cannot interact with the enzyme-inhibitor complex. The data suggested that this inhibitor class binds to the open MurA form, exerting its inhibitory action by obstructing the structural changes of the enzyme needed for catalysis. We addressed this issue by determining the structure of the MurA-T6361 complex.

**MurA-Inhibitor Complex—E. cloacae** MurA crystallized with four molecules of the MurA-T6361 complex in the asymmetric unit, and the structure was determined at 2.6 Å resolution. T6361 binds to the open form of MurA in a solvent-exposed region, spanning the loop Pro112-Pro121, which hosts the fosfomycin target Cys115 (40), and residues of the interdomain section (Figs. 6–8). The electron density of T6361 is well defined in all four MurA molecules, except for part of its carboxylate side chain, which is solvent-exposed and does not interact with enzyme residues. All residues interacting with T6361 belong to the N-terminal (upper) globular domain of MurA (Fig. 6).

T6361 appears to bind to the enzyme primarily through hydrophobic forces (criterion: distance $\leq 4.0 \pm 0.4 \AA$; the two naphthalene rings interact with Arg91/Pro121/Val122/Ile94/Pro112 and Asn23/Trp95/Val163, respectively. The benzene ring interacts with Trp95 and Arg91. The side chains of Lys22, Arg91, and His125 are involved in hydrogen bonding interactions with T6361 oxygen atoms. In addition, two water molecules appear to bridge T6361 oxygen atoms and the main chain amides of Arg91, Ala92, and Gly164.

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**Fig. 4. Interaction of T6362 with MurA as observed by ANS-mediated MurA fluorescence.** Fluorescence emission spectra are displayed; excitation was at 366 nm. A, quenching of MurA fluorescence by addition of 0.25 mM UNAG, which is due to the conformational changes of the loop hosting Cys115 (see Fig. 8), B, addition of 0.1 mM T6362 results in a similar quench as observed in A; additional UNAG has no effect on the spectrum. C and D show that decreasing T6362 concentrations result in lower quenching potency; here additional UNAG does affect the MurA-T6362 spectra. A–D suggest that T6362 induces similar conformational changes in MurA as does UNAG and that UNAG can displace T6362. E, addition of PEP to free MurA only slightly affects MurA fluorescence; upon addition of low UNAG concentrations, a large quench is observed as a result of “complete enzyme closure.” This effect is independent of the order of substrate addition (11). F, when the MurA-T6362 complex is incubated with PEP, no quenching is observed; only addition of UNAG leads to the additional structural changes as observed in E. E and F suggest that the binding site of T6362 overlaps with the PEP-binding site. Upon displacement of T6362 with UNAG, the PEP-binding site becomes available again.

**Fig. 5. Determination of the dissociation constant of T6362 by ANS fluorescence.** T6362-induced fluorescence quenching is concentration-dependent. The quenching effect as a function of T6362 concentration reveals a saturation curve (inset). Data were fit to Eq. 3, yielding a $K_d$ of $13 \pm 2.7 \mu M$, which is in the range of the $K_i$ of 16–27 μM (Fig. 3).
The mode of binding of T6361 to the loop is similar to that observed for the interaction of MurA with ANS, notably, the simultaneous existence of hydrophobic and electrostatic forces with Arg91 (32). Whereas the hydrophobic interaction of the one naphthalene moiety with Arg91 and a proline (Pro112) is also realized in the MurA/ANS structure, the hydrophobic interaction capacity of the other naphthalene with Asn23 was unexpected (Fig. 6). Like ANS, T6361 induces a conformational change in the Pro112-Pro121 loop (Figs. 7 and 8). Thus, T6361-dependent quenching of ANS fluorescence may be interpreted as competition for binding to Arg91. Arg91 is not involved in substrate binding once the active site has been created (33), but it is possibly involved in the open-closed transition of the enzyme (32). Thus, T6361 might prevent Arg91 from forming a collision complex with UNAG and conducting its role in the induced fit mechanism. The other strictly conserved residue interacting with T6361 is Lys22. The precise role of Lys22 in catalysis is not understood and is a matter of debate (33, 35–37). In the ligand-free state of MurA, Lys22 electrostatically interacts with Asp49 (10). This salt bridge is disrupted upon the transition of the enzyme to the closed state, and Lys22 instead interacts with the UNAG and PEP moieties of the genuine tetrahedral intermediate (33). Thus, Lys22 may have a dual function in the enzymatic reaction, the triggering of the open-closed transition upon substrate binding, followed by its participation in catalysis. Apparently, T6361 obstructs the side chain conformational changes of Lys22 that occur during the reaction with substrates.

In essence, T6361 appears to block the induced fit mechanism of the enzyme, and the enzyme remains in its open state (Figs. 7 and 8). As a result, interaction of PEP with the MurA-T6361 complex does not occur (Fig. 5) because the inhib-
Arg91 undergoes drastic conformational changes as a result of inhibitors is that the loop and the side chains of Lys22 and present an attractive starting point for the design of small inhibitors blocks the formation of the PEP-binding site, which is unique for each of these enzyme-ligand interactions.

**CONCLUSIONS**

The molecular mode of action of this new class of inhibitors on MurA provides valuable insights with respect to the structural prerequisites for the induced fit mechanism in catalysis. T6361 appears to inhibit the structural changes leading to the closed MurA state, primarily through its interaction with Lys22 and Arg91, and the loop around Cys115. Because T6361 binding to MurA is competitive with UNAG yet not active site-directed, its binding site may represent part of the collision complex between MurA and UNAG that must exist in the open enzyme state. Therefore, T6361 bound to MurA may present an attractive starting point for the design of small molecules specifically targeting the conformational shift. However, a major obstacle in the rational design of such inhibitors is that the loop and the side chains of Lys22 and Arg91 undergo drastic conformational changes as a result of different enzyme-ligand interactions. Such structural alterations cannot be predicted by commonly employed computational methods, thus reinforcing the need for the continual structure elucidation of various MurA inhibitor complexes for future rational drug design approaches.

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