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Lyme Disease Enolpyruvyl-UDP-GlcNAc Synthase: Fosfomycin-Resistant MurA from *Borrelia burgdorferi*, a Fosfomycin-Sensitive Mutant, and the Catalytic Role of the Active Site Asp

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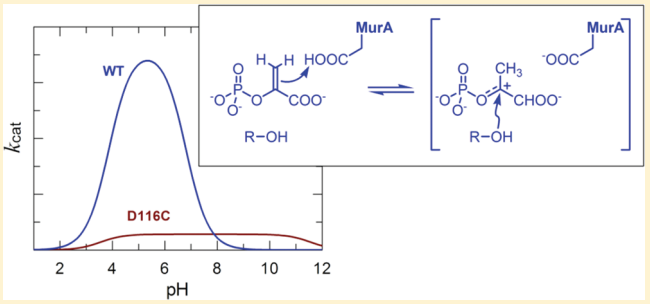
ABSTRACT: MurAs (enolpyruvyl-UDP-GlcNAc synthases) from pathogenic bacteria such as *Borrelia burgdorferi* (Lyme disease) and tuberculosis are fosfomycin resistant because an Asp-for-Cys substitution prevents them from being alkylated by this epoxide antibiotic. Previous attempts to characterize naturally Asp-containing MurAs have resulted in no protein or no activity. We have expressed and characterized His-tagged Lyme disease MurA (Bb_MurA$_{H6}$). The protein was most soluble at high salt concentrations but maximally active around physiological ionic strength. The steady-state kinetic parameters at pH 7 were k_{cat} = 1.07 ± 0.03 s^{-1}, $K_{M,PEP}$ = 89 ± 12 μM, and $K_{M,UDP\text{-}GlcNAc}$ = 45 ± 7 μM. Mutating the active site Asp to Cys, D116C, caused a 21-fold decrease in k_{cat} and rendered the enzyme fosfomycin sensitive. The pH profile of k_{cat} was bell-shaped and centered around pH 5.3 for Bb_MurA$_{H6}$, with pK_{a1} = 3.8 ± 0.2 and pK_{a2} = 7.4 ± 0.2. There was little change in pK_{a1} with the D116C mutant, 3.5 ± 0.3, but pK_{a2} shifted to >11. This demonstrated that the pK_{a2} of 7.4 was due to D116, almost 3 pH units above an unperturbed carboxylate, and that it must be protonated for activity. This supports D116's proposed role as a general acid/base catalyst. As fosfomycin does not react with simple thiols, nor most protein thiols, the reactivity of D116C with fosfomycin, combined with the strongly perturbed pK_{a2} for D116, strongly implies an unusual active site environment and a chemical role in catalysis for Asp/Cys. There is also good evidence for C115 having a role in product release. Both roles may be operative for both Asp- and Cys-containing MurAs.



MurA (enolpyruvyl-UDP-GlcNAc synthase) catalyzes the first committed step in peptidoglycan biosynthesis and is the target of the antibiotic fosfomycin.[1−3] It transfers the carboxyvinyl group from phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine (UDP-GlcNAc), forming enolpyruvyl-UDP-GlcNAc (EP-UDP-GlcNAc). The only other known carboxyvinyl transferase is AroA (EPSP synthase), target of the herbicide glyphosate.[4,5]

Fosfomycin alkylates an active site Cys residue in susceptible MurAs; however, Asp-containing MurAs are fosfomycin resistant, including those from *Mycobacterium tuberculosis*,[6,7] *Chlamydia trachomatis* (chlamydia),[8] *Chlamydia pneumoniae*, *Treponema pallidum* (syphilis), and *Borrelia burgdorferi* (Lyme disease).[9] Lyme disease is the most common vector-borne disease in North America; it is transmitted from ticks to birds, humans, and other mammals.

Cys-containing MurAs have been characterized in detail.[7,10−22] In *Escherichia coli* MurA (Ec_MurA), C115[a] is essential for activity; k_{cat} decreased >2000-fold in the C115A and C115S mutants.[7,11] The C115D mutant had activity,[7] showing that Asp could partially replace Cys. Cys was proposed to be a general acid/base catalyst in protonating/deprotonating C3,[14] though that role was brought into question when evidence for its role in product release was reported.[19]

No natively Asp-containing MurA has previously been characterized. *C. trachomatis* MurA conferred fosfomycin resistance when expressed in *E. Coli* but could not be purified.[8] *M. tuberculosis* MurA expressed in *E. Coli* had no catalytic activity.[6] We were similarly unable to obtain active tuberculosis MurA from *E. Coli* under a variety of conditions. Tuberculosis MurA was transiently expressed in *Mycobacterium smegmatis*, but expression was unstable and eventually lost. The D117C mutant expressed in *M. smegmatis* rendered cell extracts fosfomycin-sensitive.

MurA is a proven antimicrobial target, and the murein pathway continues to be an important target for antibiotic development,[23−26] but the recalcitrance of natively Asp-containing MurAs has blocked detailed mechanistic studies and antibiotic development. We have now expressed, purified,

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and characterized active His-tagged *B. burgdorferi* MurA (Bb_MurA_{H6}).

EXPERIMENTAL PROCEDURES

Cloning. The Bb_MurA sequence was cloned from *B. burgdorferi* strain B31 genomic DNA (American Type Culture Collection) and inserted into the pET23a vector (Novagen) between the *Nde*I and *Bam*HI restriction sites. Because the start codon in the genome sequence is ambiguous, both potential start codons were tested. The "long form" was identical to the coding region in GenBank Gene ID 1195318, starting at nucleotide position 490599. A C-terminal His tag was created by converting the stop codon to Gln, giving an added sequence of QDPNSSSVDKLAAALEHHHHHH. The "short form" was 15 amino acids shorter at the N-terminus, starting from the Met codon at the same position as other MurA sequences (see Figure 1). The data in Figures 2−5 were generated with the short form Bb_MurA_{H6}.

Expression and Purification. A 50 mL overnight culture of *E. Coli* BL21* DE3 cells containing the Rosetta plasmid (Novagen) in 50 mL of lysogeny broth (LB) plus 50 μg/mL ampicillin and 20 μg/mL chloramphenicol was inoculated into two 1 L cultures and grown until $OD_{600} \approx 0.5$; then protein expression was induced with 1 mM IPTG for 5 h. Expression at 18 °C did not improve the protein yield. Cells were harvested by centrifugation at 5000*g* for 20 min, resuspended in 14 mL of wash buffer (50 mM Na·HEPES, pH 7.0, 150 mM NaCl, 20 mM imidazole), and stored at −20 °C. Before cell lysis, 200 μM phenylmethanesulfonyl fluoride, 50 μL of protease inhibitor cocktail (Sigma), and 100 μg/mL DNase I (Sigma) were added. Cells were lysed by two passages through a homogenizer at 10000 psi at 4 °C, followed by centrifugation at 16000*g* for 10 min. Bb_MurA was purified by affinity chromatography using Ni^{2+}-charged Chelating Sepharose (2 mL column volume; GE Healthcare). Cell lysate was loaded onto a column that was stripped with EDTA and recharged with $NiSO_4$ before each use and washed with 5 mL of wash buffer (20 mM imidazole, 50 mM Na·HEPES, pH 7.0, 150 mM NaCl) at 1 mL/min; then washing was continued for 12−18 h at 0.1 mL/min. After intermediate washes at 1 mL/min with imidazole increased to 100 and 200 mM, it was eluted with imidazole increased to 500 mM. Eluted Bb_MurA_{H6} was exchanged into storage buffer (50 mM Na·HEPES, pH 7.0, 650 mM NaCl, 1 mM DTT), flash frozen in dry ice/ethanol, and stored at −75 °C. Purity of the eluted protein, as assessed by SDS−PAGE, was >95%. Protein concentration was determined from A_{280}, using a value of $\varepsilon_{280} = 1.46 \times 10^4\ M^{-1}\ cm^{-1}$, as determined by the method of Edelhoch.[27]

Rate Assays. Initial velocities were measured in a 96-well plate format by detecting phosphate product formation with the Malachite Green/ammonium molybdate assay.[28,29] For steady-state kinetic determinations, rate assays were conducted at 37 °C in 50 mM Na·HEPES, pH 7.0, 150 mM NaCl, 1 mM DTT, and 80 nM to 1 μM enzyme. Under these conditions, catalytic activity was stable for up to 3 h. Selwyn's test was used to detect time-dependent enzyme inactivation;[30] for reactions run at different enzyme concentrations, plots of [product] vs [enzyme] × time will be superimposable unless there is time-dependent enzyme inactivation.

Steady-State Kinetic Constants. Initial velocities were measured at high, fixed [UDP-GlcNAc] and varying [PEP] and then with high, fixed [PEP] and varying [UDP-GlcNAc]. The fixed concentrations were 1.6 mM for Bb_MurA_{H6}, and 0.8 mM for Bb_MurA_{H6}(D116C). Rates were fitted to eq 1:[29,31,32]

$$\frac{v_0}{[E]_0} = \frac{\dfrac{k_{cat}[\text{UDP-GlcNAc}][\text{PEP}]}{K_{M,\,\text{UDP-GlcNAc}}K_{M,\,\text{PEP}}}}{1 + \dfrac{[\text{UDP-GlcNAc}]}{K_{M,\,\text{UDP-GlcNAc}}} + \dfrac{[\text{PEP}]}{K_{M,\,\text{PEP}}} + \dfrac{[\text{UDP-GlcNAc}][\text{PEP}]}{K_{M,\,\text{UDP-GlcNAc}}K_{M,\,\text{PEP}}}} \quad (1)$$

Equation 1 assumes a random sequential mechanism for two substrates, as used for the homologous enzyme AroA.[29]

$(k_{cat}/K_M)_{\text{UDP-GlcNAc}}$ was determined by direct fitting to eq 2:[29]

$$\frac{v_0}{[E]_0} = \frac{\left(\dfrac{k_{cat}}{K_M}\right)_{\text{UDP-GlcNAc}}\dfrac{[\text{UDP-GlcNAc}][\text{PEP}]}{K_{M,\,\text{PEP}}}}{1 + \dfrac{[\text{UDP-GlcNAc}]}{K_{M,\,\text{UDP-GlcNAc}}} + \dfrac{[\text{PEP}]}{K_{M,\,\text{PEP}}} + \dfrac{[\text{UDP-GlcNAc}][\text{PEP}]}{K_{M,\,\text{UDP-GlcNAc}}K_{M,\,\text{PEP}}}} \quad (2)$$

and $(k_{cat}/K_M)_{PEP}$ by suitable modification of eq 2.

pH Dependence. The pH dependence of k_{cat} was probed by running reactions with saturating substrate concentrations, i.e., 1.6 mM each of UDP-GlcNAc and PEP, at 37 °C in 150 mM NaCl, 1 mM DTT, and 50 mM buffer: glycine (pH 2 to 3, 10, 11), potassium acetate (pH 4 to 5), K·MES (pH 5 to 7.6), or Tris·HCl (pH 6.6 to 8.6). As the ionic strength due to NaCl was near Bb_MurA's optimum (see Figure 2), small variations arising from buffer ionization would not significantly affect v_0. Rates were fitted to eq 3:

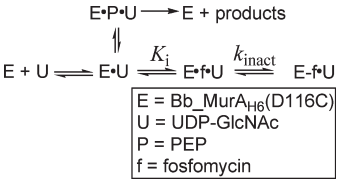
$$\frac{v_0}{[E]_0} = \frac{k_{cat,\,max} \times 10^{pH\,-\,pK_{a1}} \times 10^{pK_{a2}\,-\,pH}}{(10^{pH\,-\,pK_{a1}} + 1)(10^{pK_{a2}\,-\,pH} + 1)} \quad (3)$$

where $k_{cat,max}$ is the maximal value of k_{cat}, pK_{a1} is for the acid, ascending limb, and pK_{a2} is for the basic, descending limb. Multiple replicates of the Bb_MurA_{H6} pH profile were performed before the problem of low k_{cat} values was solved by overnight washing during purification (see Results). Data from that preparation were normalized from $k_{cat,apparent} = 0.28\ s^{-1}$ at pH 7 to the true value of $k_{cat} = 1.07\ s^{-1}$ (see Figure 4A, data points with error bars). A single replicate of the pH profile with fully active Bb_MurA_{H6} (points without error bars) confirmed the validity of the normalization.

Fosfomycin Inhibition. The dissociation constant, K_i, was determined by combining 1 μM Bb_MurA_{H6}(D116C) and 1 mM UDP-GlcNAc and then adding 1 mM PEP and 0−10 mM fosfomycin simultaneously to start the reaction. Phosphate production was measured at as short times as possible to minimize the amount of covalent inhibition.

Fosfomycin binding is uncompetitive with respect to UDP-GlcNAc and competitive with respect to PEP (Scheme 1). For

Scheme 1



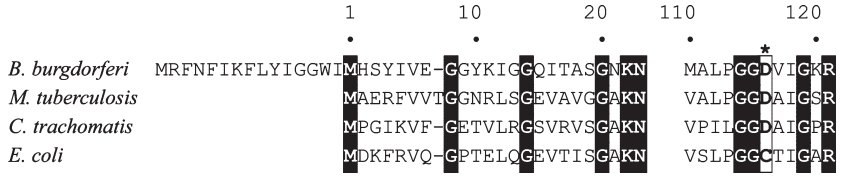


Figure 1. Amino acid sequences of *B. burgdorferi* MurA, other representative Asp-containing MurAs, and *E. Coli* MurA. The N-terminal region and the region around active site Asp/Cys residue are shown. Numbering is according to Bb_MurA. GenBank accession numbers, in order, are NP_212606, X96711, AAD32216, and BAA78108.

uncompetitive inhibition, $K_{i,apparent} = K_{i,true}$ when [substrate] $\gg K_M$.[31] As [UDP-GlcNAc] was 1 mM ($48 \times K_{M,UDP\text{-}GlcNAc}$), it could be neglected, and only PEP was included in fitting to the competitive inhibition equation (eq 4) to K_i:

$$v_0 = \frac{V_{max}[PEP]}{K_{M,PEP}\left(1 + \frac{[fosfomycin]}{K_i}\right) + [PEP]} \quad (4)$$

The alkylation rate, k_{inact}, was determined by preincubating 2 μM Bb_MurA with 2 mM UDP-GlcNAc and 0.2 mM fosfomycin for defined times, then adding an equal volume of 2 mM PEP, and measuring the residual activity. Under these preincubation conditions, the enzyme was saturated with both UDP-GlcNAc and fosfomycin, so k_{inact} could be calculated from the first-order decrease in residual activity as a function of preincubation time, eq 5:

$$v_{0,residual} = V_{max} \exp(-k_{inact}t) + c \quad (5)$$

The constant term, c, accounted for the residual activity, ~9%, present after extended preincubation with fosfomycin.

RESULTS

Start Codon. The apparent Bb_MurA start codon at position 490599 in the *B. burgdorferi* genome sequence[33] gave a predicted amino acid sequence starting 15 amino acids before most MurAs (Figure 1). Another Met residue aligned with the start of most MurA sequences, making the Bb_MurA start codon ambiguous. The long form had activity but appeared less stable in our hands (data not shown). As the short form was sufficient for activity and was more stable, it was used for the rest of this study. The true start codon is not yet known.

Protein Production. Low yields of purified Bb_MurA, ~0.3 mg/L of culture, necessitated adding a C-terminal His tag for purification. The His tag may have affected the activity, but C-terminally His-tagged *Pseudomonas aeruginosa* MurA[34] and Ec_MurA (data not shown) are fully active, and k_{cat} for Bb_MurA$_{H6}$ was close to Ec_MurA, implying that its catalytic activity was not significantly affected. Early Bb_MurA$_{H6}$ preparations had low k_{cat} values, some <0.01 s^{-1}, though K_M values did not change. This low activity was eventually resolved by washing the protein overnight while bound on the Ni^{2+} affinity column. This increased the apparent k_{cat} values and implied that a noncovalent ligand was being eluted upon extended washing. The ligand identity is not known, but purified recombinant Ec_MurA was previously found to contain bound UDP-*N*-acetylmuramic acid, the next product in peptidoglycan biosynthesis, and a potent Ec_MurA inhibitor.[18]

Salt and Temperature Dependence of Activity. Bb_MurA$_{H6}$ was soluble to ~15 μM (0.7 mg/mL) at 650 mM NaCl and was much less soluble at lower ionic strength. Its maximum activity was at physiological ionic strength, $I = 0.15$ (Figure 2). The optimum temperature was 37 °C (data not shown), with time-dependent inactivation at ≥42 °C, as detected by Selwyn's test.[30] Borrelia spirochetes are viable below 30 °C, temperatures encountered in their tick vectors, but have decreased survival times at higher temperatures[35] and cannot synthesize proteins at 42 °C.[36]

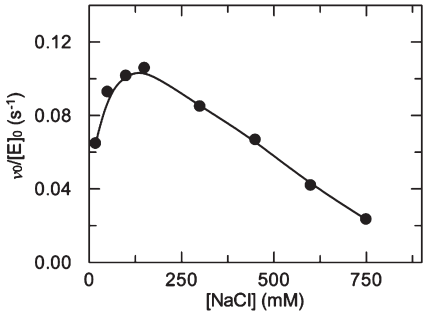


Figure 2. The specific activity ($v_0/[E]_0$) of Bb_MurA$_{H6}$ as a function of ionic strength. Assay conditions were 50 mM Na·HEPES, pH 7.0, and 1 mM DTT, at 37 °C, with NaCl concentrations varied.

Table 1. Steady-State Kinetic Parameters for Bb_MurA$_{H6}$ and Its D116C Mutant[a]

	Bb_MurA$_{H6}$	Bb_MurA$_{H6}$(D116C)
k_{cat} (s^{-1})	1.07 ± 0.03	0.050 ± 0.002
$(k_{cat}/K_M)_{PEP}$ ($M^{-1}\cdot s^{-1}$)	(1.2 ± 0.1) × 10^4	(4 ± 1) × 10^3
$(k_{cat}/K_M)_{UDP\text{-}GlcNAc}$ ($M^{-1}\cdot s^{-1}$)	(2.4 ± 0.3) × 10^4	(2.4 ± 0.5) × 10^3
$K_{M,PEP}$ (μM)	89 ± 12	12 ± 4
$K_{M,UDP\text{-}GlcNAc}$ (μM)	45 ± 7	21 ± 5
pK_{a1}	3.8 ± 0.2	3.5 ± 0.3
pK_{a2}	7.4 ± 0.2	>11[b]

[a] Initial velocities, $v_0/[E]_0$, at pH 7.0 were fitted to eq 1 to determine the values of k_{cat}, $K_{M,PEP}$, and $K_{M,UDP\text{-}GlcNAc}$. $(k_{cat}/K_M)_{PEP}$ and $(k_{cat}/K_M)_{UDP\text{-}GlcNAc}$ were found by fitting to eq 2. pK_{a1} and pK_{a2} were determined by fitting rates to eq 3. [b] The fitted value of pK_{a2} was 11.6 ± 0.4; however, the highest pH value at which k_{cat} was measured was pH 11, so the fitted value is not considered reliable and is simply recorded as >11.

Steady-State Kinetic Parameters. The steady-state kinetic parameters for Bb_MurA$_{H6}$ and Bb_MurA$_{H6}$(D116C) were determined (Table 1, Figure 3). Initial velocities were routinely measured by following phosphate formation,[28,29] but EP-UDP-GlcNAc synthesis was confirmed by anion-exchange chromatography, as described previously[16] (data not shown).

pH Dependence. The pH dependence of k_{cat} was investigated (Table 1, Figure 4). Substrate concentrations were saturating, so the rates reflected k_{cat}. A limited pH profile with

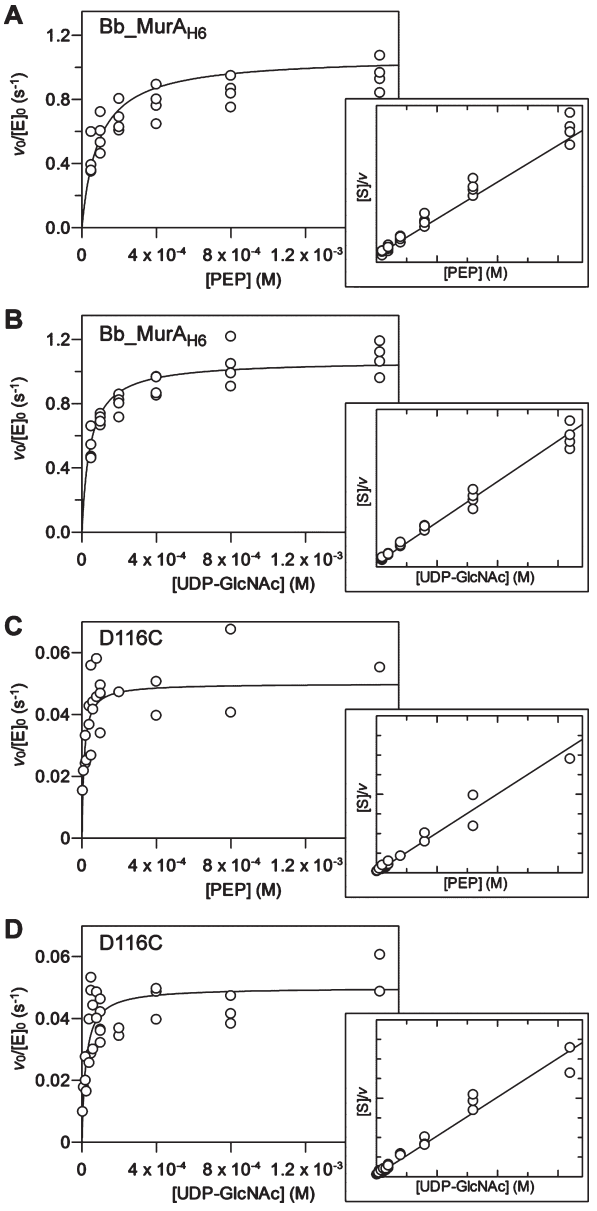


Figure 3. The steady-state kinetic parameters for Bb_MurA$_{H6}$ with substrates (A) PEP and (B) UDP-GlcNAc and for the mutant Bb_MurA$_{H6}$(D116C) with (C) PEP and (D) UDP-GlcNAc. The reactions were run at pH 7. The $v_0/[E]_0$ vs [S] data which were fitted to eqs 1 and 2 are shown in the main panels. Hanes plots ($[S]/v_0$ vs [S]) are shown in the insets.

Bb_MurA$_{H6}$ at low substrate concentrations, i.e., under k_{cat}/K_M conditions, gave a similar bell-shaped curve (data not shown). The previously reported pH profile for wild-type Ec_MurA was essentially flat around neutral pH, while k_{cat} for Ec_MurA-(C115D) decreased from pH 5.5 to pH 9.0.[7]

Reaction of the D116C Mutant with Fosfomycin. Bb_MurA$_{H6}$(D116C) was covalently inhibited by fosfomycin. The concentration dependence without preincubation gave a dissociation constant, K_i = 5.7 ± 0.4 μM (Figure 5A). The fosfomycin concentration required for 50% inhibition was much greater than K_i because the concentration of the competitive substrate, PEP, was much greater than $K_{M,PEP}$. The time dependence at saturating inhibitor concentration gave k_{inact} = 0.021 ± 0.003 s^{-1}, the rate of C116 alkylation (Figure 5B). There was ~9% residual activity, corresponding to 0.3% of wild-type activity, that did not disappear with extended preincubation times. The residual activity was confirmed by following EP-UDP-GlcNAc production by HPLC. This may have been

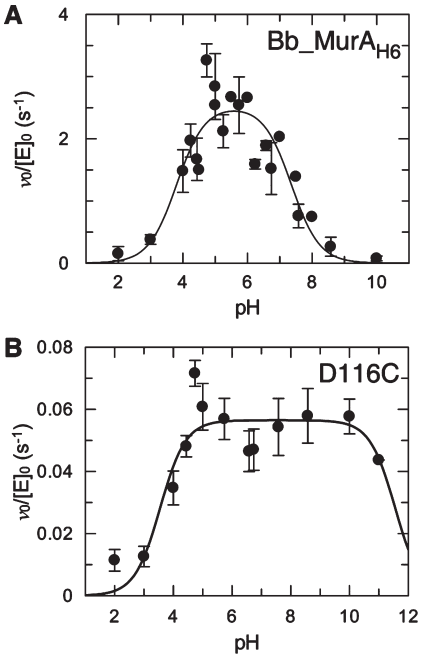


Figure 4. The pH profile of k_{cat} for (A) Bb_MurA$_{H6}$ and (B) Bb_MurA$_{H6}$(D116C).

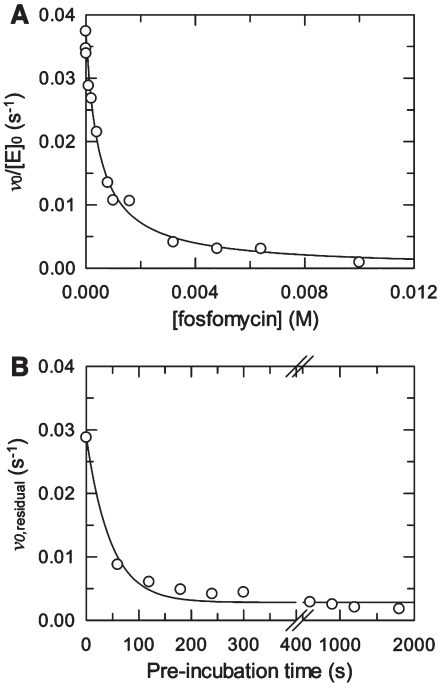


Figure 5. Inhibition parameters for Bb_MurA$_{H6}$(D116C) alkylation by fosfomycin. (A) K_i determination; v_0 vs [fosfomycin] data were fitted to eq 4. (B) k_{inact} determination; $v_{0,residual}$ vs time data were fitted to eq 5. The t = 0 sample contained 200 μM fosfomycin, accounting for the decreased $v_{0,residual}$ value relative to (A).

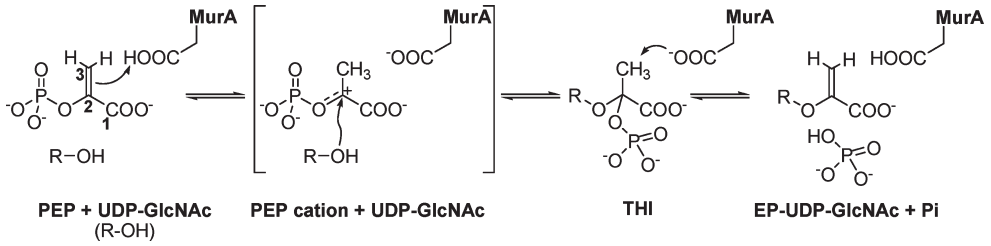


Figure 6. The general acid/base catalytic residue, putative PEP cation intermediate, and the tetrahedral intermediate of the MurA-catalyzed reaction. The active site D116 residue is proposed to be the general acid/base catalytic residue.

genuine activity of the alkylated enzyme, though it may also have been an artifact from, for example, oxidation of the C116 thiol rendering it alkylation resistant. Cys oxidization to the sulfenic, sulfinic, or sulfonic acids would give residues with similar shapes and acidities to Asp. Bb_MurA$_{H6}$ was noncovalently inhibited by fosfomycin, with inhibition being relieved by dialysis.

DISCUSSION

MurA is a proven antimicrobial target, but only the Cys-containing enzymes are susceptible to fosfomycin. Tuberculosis, chlamydia, syphilis, and Lyme disease are all caused by bacteria that are naturally Asp-containing and therefore fosfomycin resistant. Developing new inhibitors against these enzymes requires that their activities be characterized in detail, but Asp-containing MurAs have proven refractory to study.

Kinetic Constants. At pH 7, k_{cat} was 1.07 s^{-1} for Bb_MurA$_{H6}$. This was slightly lower than Ec_MurA's, 3.8 s^{-1},[7] though pH 7 was close to Ec_MurA's optimal pH, while Bb_MurA$_{H6}$'s optimal pH was nearer pH 5 (see below). $K_{M,UDP\text{-}GlcNAc}$, 45 μM, was similar to Ec_MurA's, at 15 μM.[7] $K_{M,PEP}$ was 89 μM, while the value for Ec_MurA is difficult to determine but reported to be in the range of 0.2−4 μM.[7,37,38]

The decrease in k_{cat} for the D116C mutant was 21-fold at pH 7. This is a fraction of MurA's overall catalytic enhancement, >10^9-fold,[39] and demonstrates that Cys was largely able to replace Asp. The K_M values decreased slightly, so that the overall effect on k_{cat}/K_M was only a 3-fold for PEP and 10-fold for UDP-GlcNAc. The reciprocal mutation in Ec_MurA, C115D, caused a change in k_{cat} that ranged from a 10-fold increase at pH 6 to a 10-fold decrease at pH 9.[7]

Fosfomycin Inhibition. Cys-containing MurAs attack the epoxide functional group of fosfomycin, becoming alkylated and irreversibly inhibited. Like Ec_MurA,[1,40] Bb_MurA$_{H6}$-(D116C) only reacted with fosfomycin in the presence of UDP-GlcNAc. The inhibition constants, k_{inact} = 0.021 s^{-1} and K_i = 5.7 μM, were similar to Ec_MurA, where k_{inact} = 0.12 s^{-1} and K_i = 8.6 μM.[40] Thus, k_{inact} was only 5-fold slower in the D116C mutant than Ec_MurA, even though k_{cat} was 76-fold lower (3.8 vs 0.05 s^{-1}).

Fosfomycin does not normally react with simple thiols, nor with most protein Cys residues.[1] Under forcing conditions it is eventually hydrolyzed to the diol, rather than being alkylated. Both *Haemophilus influenzae* MurA and Ec_MurA react with fosfomycin only at C115, not at other free Cys residues.[40,41] The fast alkylation of the D116C mutant illustrated that C116 is not a normal protein thiol. Rather, like Ec_MurA, the active site environment modifies its reactivity by increasing the Cys thiol's nucleophilicity and/or increasing the electrophilicity of the relatively inert epoxide functional group.

pH Dependence. The pH profile of k_{cat} for Bb_MurA$_{H6}$ was centered around pH 5.3, surprisingly low for an organism that is not known to have an unusual intracellular pH. pK_{a1} was similar to an unperturbed carboxylate pK_a and did not change significantly in the D116C mutant, i.e., from 3.5 to 3.8.

The dramatic shift in pK_{a2} in the D116C mutant (Figure 3) demonstrated that pK_{a2} = 7.4 reflected the D116 side chain and that it must be protonated in the rate-limiting step. The unperturbed pK_a for an Asp side chain is 3.4 pH units lower, at 4.0.[42] Such large perturbations in pK_a occur when they are needed to bring catalytic amino acids into the appropriate protonation state at physiological pH. pK_{a2} for the D116C mutant was >11, more than 2 pH units above the unperturbed pK_a of 8.3−9.1 for a Cys thiol.[43,44] It appears that the active site environment of Bb_MurA is poised to perturb the pK_a of whatever residue is present at position 116. The pK_a value for *Enterobacter cloacae* MurA (Enc_MurA) C115 was unperturbed, at 8.3, based on its reactivity with iodoacetamide.[45] This would be expected for natively Cys-containing MurAs, as an unperturbed Cys side chain would already be in the correct, thiol, protonation state.

The source of Bb_MurA(D116)'s high pK_a is not known. Aligning Bb_MurA against the Ec_MurA−fosfomycin·UDPGlcNAc structure[46,47] showed that two potentially cationic residues close to Ec_MurA C115, H394 (5 Å) and H334 (11 Å), are neutral in Bb_MurA, Q403 and F356, respectively. This could potentially raise D116's pK_a, though protein pK_a's are difficult to predict, and it is not clear that these two changes would be sufficient to cause the observed pK_a perturbation.

Asp116 in Catalysis. Two roles have been proposed for C115, and because of its positional homology, Bb_MurA D116 may play the same roles. As each role occurs in different parts of the catalytic cycle, it is possible that both proposals are correct.

The more recent proposed function for C115 was that it is involved in product release.[19] The Enc_MurA(C115S) mutant catalyzes only a single turnover because it appears to be unable to release the products, EP-UDP-GlcNAc and phosphate. The crystal structure revealed a "closed" conformation in the MurA·product complex, while the (Cys-containing) *Aquifex aeolicus* MurA·product crystal structure[48] formed a "staged" conformation, one of the steps in product release.[21] Thus, the active site Cys appears to be required for product release, though its specific role is unknown. The evidence for a role in product release caused the authors to question whether C115 acts as a general acid/base catalyst.

C115 was previously proposed to act as a general acid catalyst to protonate C3 of PEP (Figure 6).[14] General acid catalysis is essential for enolpyruvyl reactivity; we showed that the enolpyruvyl group of EPSP, AroA's product, is completely unreactive to nucleophilic attack without C3 protonation, even under extreme

conditions (1 M KOH, 90 °C, 16 days).[39] The enolpyruvyl group of PEP should also be unreactive without prior protonation, as observed under basic, albeit less harsh, conditions.[49] The homologous enzyme AroA can catalyze exchange of solvent protons into C3 of PEP,[50] showing that it can protonate C3 in spite of its low pK_a value, <−4.[51] There is also good evidence that Ec_MurA protonates C3 to form cationic intermediates during both formation and breakdown of the tetrahedral intermediate[52] (Figure 6). The rapid formation of the phospholactoyl side product between Ec_MurA(C115) and PEP, with $k_{cat}/K_M \sim 10^8$ $M^{-1}\cdot s^{-1}$, demonstrates that C115, which is located on a flexible loop that closes over the active site, is close to bound PEP during catalysis.[12,47] Returning to Bb_MurA, the pK_{a2} of D116, 7.4, demonstrates that the enzyme strongly perturbs its environment to ensure a significant proportion of the correct protonation state for activity. This provides strong support for its role as a general acid catalyst.

D116 is expected to play a similar role to C115 in catalysis. Mutations in either direction (Asp-to-Cys in Bb_MurA$_{H6}$, or Cys-to-Asp in Ec_MurA) result in active enzymes, with k_{cat} decreasing 21-fold in Bb_MurA$_{H6}$. This is a small fraction of MurA's overall catalytic enhancement, >10^9-fold, compared with the noncatalyzed reaction.[39]

The relatively high activity of the Ec_MurA(C115D) mutant demonstrates that, even in mutant context, the Asp side chain permitted product release[7] and presumably would be capable of playing the same role in Bb_MurA.

Implications for Inhibitor Design. Fosfomycin can sometimes be very effective, able to treat urinary tract infections in a single dose.[53] Nevertheless, its spectrum is limited by the need for active transport into bacterial cells[1] and by the fact that it only inhibits Cys-containing MurAs. It reacts because of the Cys residue's unusual nucleophilicity, but this nucleophilicity is not intrinsic to its catalytic role.

The present work strongly supports a general acid/base catalytic role for D116 and, by extension, supports the same role for C115, which had been called into question. If D116 and C115 play the same roles in their respective enzymes, that raises the possibility of designing broad spectrum inhibitors that effectively inhibit both classes of MurA. After protonating C3, the thiolate or carboxylate forms of C115/D116 could further stabilize the putative cationic intermediates electrostatically, as we have proposed for the corresponding residues in AroA.[29] Cationic intermediate mimics could be inhibitors, though, aside from glyphosate, amine-based inhibitors have so far met with limited success.[54−56] Given the fact that D116's carboxylate form, with pK_{a2} = 7.4, will dominate under physiological conditions, a cationic inhibitor could be even more effective against Asp-containing than Cys-containing MurAs, where pK_a ∼8.3.[45]

It is not as clear how to design inhibitors to exploit C115/D116's proposed role in product release. Beyond observing that the Enc_MurA(C115S) mutant cannot release products[19] or form the staged conformation,[21] it is not clear how C115 promotes product release. Nonetheless, in some circumstances, substrate and inhibitor release is exceedingly slow,[12,21] and at least one inhibitor blocks conformational transition, albeit from open to closed conformers.[57]

CONCLUSIONS

Natively Asp-containing MurAs have resisted characterization since the 1990s. *B. burgdorferi* MurA is the first to be prepared in active form and characterized. Its steady-state kinetic constants at pH 7 were similar to *E. Coli* MurA's, but the pH optimum was around pH 5.3. Mutation of the active site Asp residue to Cys (D116C) decreased k_{cat} 21-fold at pH 7. The mutation shifted the basic limb of the pH profile from 7.4 to >11, demonstrating that the D116 side chain must be protonated for the enzyme to be active. This supports its proposed general acid/base catalytic role, and it may also have a role in product release. The D116C mutation made the enzyme susceptible to covalent inhibition by the antibiotic fosfomycin. Given fosfomycin's unreactivity with simple thiols, this demonstrated that the active site environment modifies the reactivity of the Cys side chain and/or the inhibitor to allow alkylation to occur.

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Notes

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ABBREVIATIONS

AroA, EPSP synthase; Bb_MurA, *Borrelia burgdorferi* MurA; Bb_MurA$_{H6}$, Bb_MurA bearing a C-terminal His_6 tag; Ec_MurA, *Escherichia coli* MurA; Enc_MurA, *Enterobacter cloacae* MurA; EPSP, enolpyruvylshikimate 3-phosphate; EP-UDP-GlcNAc, enolpyruvyl-UDP-GlcNAc; PEP, phosphoenolpyruvate; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

ADDITIONAL NOTE

[a]The sequence numbers of the active site Cys/Asp residues are as follows: Bb_MurA, D116; Ec_MurA and Enc_MurA, C115; *M. tuberculosis* MurA, D117.

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