Characterization of a Cys115 to Asp Substitution in the *Escherichia coli* Cell Wall Biosynthetic Enzyme UDP-GlcNAc Enolpyruvyl Transferase (MurA) That Confers Resistance to Inactivation by the Antibiotic Fosfomycin†

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ABSTRACT: The antibiotic fosfomycin inhibits bacterial cell wall biosynthesis by inactivation of UDP-GlcNAc enolpyruvyl transferase (MurA). Prior work has established that Cys115 of *Escherichia coli* and *Enterobacter cloacae* MurA is the active site nucleophile alkylated by fosfomycin and implicated this residue in the formation of a covalent phospholactyl−enzyme adduct derived from the substrate, phosphoenolpyruvate (PEP). On the basis of sequencing information from a putative MurA homolog from *Mycobacterium tuberculosis*, we generated a C115D mutant of *E. coli* MurA that was highly active but fully resistant to time-dependent inhibition by fosfomycin. Fosfomycin still bound to the active site of C115D MurA, as established by the observed reversible competitive inhibition vs PEP. In contrast to the broad pH-independent behavior of wild-type (WT) MurA, C115D mutant activity titrated across the pH range examined (pH 5.5−9) with an apparent pK_a ∼6, with k_{cat}^{C115D} ranging from ∼10k_{cat}^{WT} at pH 5.5 to <0.1k_{cat}^{WT} at pH 9.0. $K_m(PEP)^{C115D}$ was relatively constant in the pH range examined and increased ∼100-fold relative to $K_m(PEP)^{WT}$. A fosfomycin-resistant C115E mutant with ∼1% activity of the C115D mutant was found to follow a pH dependence similar to that observed for C115D MurA. The contrasting pH dependences of WT and C115D MurA were also observed in the reaction with the pseudosubstrate, (*Z*)-3-fluorophosphoenolpyruvate, strongly suggesting a role for Cys/Asp115 as the general acid in the protonation of C-3 of PEP during MurA-catalyzed enol ether transfer. The difference in nucleophilicity between the carboxylate side chains of Asp115 and Glu115 and the thiolate group of Cys115 suggests that covalent enzyme adduct formation is not required for catalytic turnover and, furthermore, provides a chemical rationale for the resistance of the C115D and C115E mutants to fosfomycin inactivation.

Enzymes of the bacterial peptidoglycan biosynthetic pathway have long attracted attention as targets of antibiotic action. Fosfomycin [(1*R*,2*S*)-1,2-epoxypropylphosphonic acid], an antibiotic produced by certain strains of *Streptomyces* (Hendlin et al., 1969; Christensen et al., 1969), acts at the first committed step in the assembly of the peptidoglycan layer of the bacterial cell wall by inactivation of the enzyme UDP-GlcNAc[1] enolpyruvyl transferase (MurA)[2] (Kahan et al., 1974). MurA catalyzes the enol ether transfer from phosphoenolpyruvate (PEP) to the 3′-OH of UDP-GlcNAc, generating the enol ether precursor of *N*-acetylmuramic acid. Recent studies have established Cys115 as the site of inactivating alkylation by fosfomycin and have implicated this residue in the formation of a covalent phospholactyl−enzyme adduct derived from PEP (Wanke & Amrhein, 1993; Marquardt et al., 1994; Brown et al., 1994) (Scheme 1, routes a and b).

An addition−elimination mechanism for MurA (Scheme 1, route c), analogous to the reaction pathway of the related enolpyruvyl transferase, 5-enolpyruvylshikimate-3-phosphate synthase (Bondinell et al., 1971; Anderson et al., 1988a,b), has emerged from a combination of rapid-quench kinetic studies of the MurA reaction with the native substrate PEP (Marquardt et al., 1993; Brown et al., 1994) and the kinetic characterization of the MurA reaction with the pseudosubstrate (*Z*)-3-fluorophosphoenolpyruvate (FPEP) (Kim et al., 1994, 1995a). Implicit in the mechanism of Scheme 1 is that formation of a covalent intermediate is not required for catalytic turnover, but the inactivity of C115S and C115A mutants of MurA (Marquardt, 1993; Wanke & Amrhein, 1993) is suggestive of a critical role for Cys115 other than that of enzyme nucleophile.

In this paper we have addressed the function of Cys115 using site-directed mutagenesis. We report the generation

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[1] Abbreviations: UDP-GlcNAc, uridine diphospho-*N*-acetyl-D-glucosamine; PEP, phosphoenolpyruvate; EP-UDP-GlcNAc, enolpyruvyl UDP-GlcNAc; FPEP, (*Z*)-3-fluorophosphoenolpyruvate; FT, 3′-*O*-(3-fluoro-2-phospholactyl)-UDP-GlcNAc); Bis-Tris, bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; WT, wild-type; HPLC, high-performance liquid chromatography; LC/ESI/MS, liquid chromatography electrospray-ionization mass spectrometry.

[2] Formerly designated MurZ; see Brown et al. (1995) for a discussion of the change in nomenclature.

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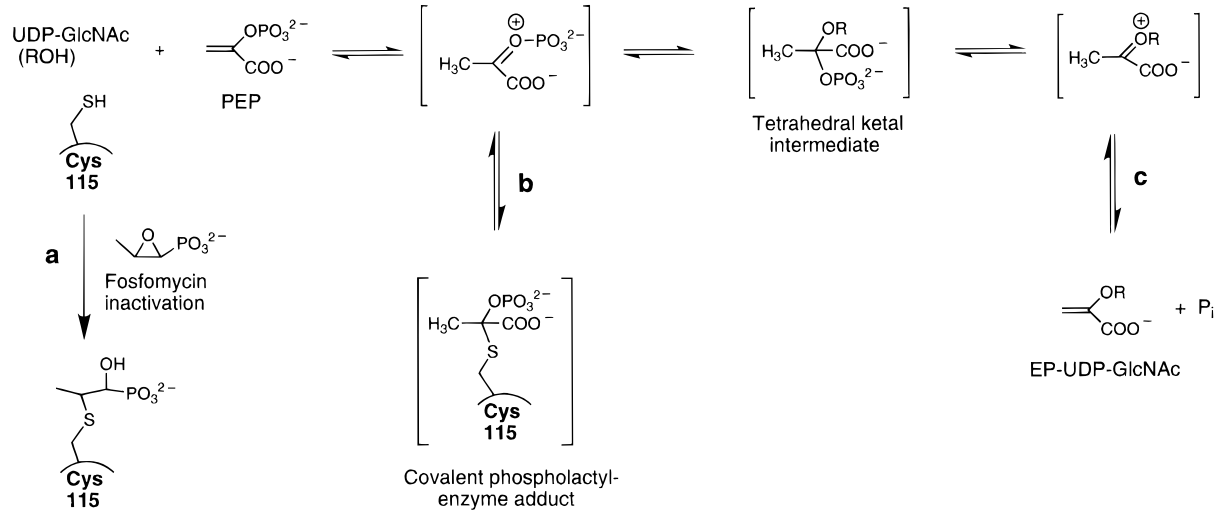
Scheme 1: Reaction Pathway of MurA: (a) Fosfomycin Inactivation, (b) Covalent Phospholactyl−Enzyme Adduct Formation, and (c) Catalytic Turnover by an Addition−Elimination Mechanism



and characterization of an Cys115-to-Asp mutant (C115D) of *Escherichia coli* MurA that is highly active but fully resistant to inactivation by fosfomycin. We were directed toward this substitution by the sequence of a gene from *Mycobacterium tuberculosis* with ~40% identity to the *E. coli murA* gene, which revealed the substitution of Asp in the position corresponding to Cys115 of the *E. coli* sequence (K.E.K. and K.D., unpublished data). This unusual amino acid substitution at the enzyme active site has provided the basis for an investigation of the role of Cys115 in the mechanism of MurA.

EXPERIMENTAL PROCEDURES

General Materials and Methods. UDP-GlcNAc, PEP, and fosfomycin were obtained from Sigma. [^{14}C]UDP-GlcNAc (309 mCi/mmol) was obtained from Amersham. (*Z*)-FPEP was the generous gift of Prof. Ronald Somerville (Purdue University). Wild-type (WT) MurA and all Cys115 mutant enzymes were overproduced and purified as described previously for wild-type MurA (Brown et al., 1994), except no urea denaturation−renaturation step was performed following the final Blue A dye-affinity column. WT and C115D MurA were analyzed for the presence of copurifying (covalently associated) PEP by the addition of a stoichiometric amount of [^{14}C]UDP-GlcNAc as described previously (Brown et al., 1994). Anion-exchange HPLC using a Mono-Q column (5/5) (Pharmacia) and in-line radioisotope detection was performed as described previously (Brown et al., 1994).

Construction of Site-Directed Mutants. The construction of plasmids p22.1 (C115S) and p23.1 (C115A) mutants has been described previously (Marquardt, 1993). Plasmid p5.16 (C115D) was constructed by standard methods using the polymerase chain reaction to introduce an *Xma*I site at nucleotide position 334−339 of the *E. coli murA* gene through silent mutations and the codon change $TGT_{Cys} \rightarrow GAT_{Asp}$ at nucleotide positions 343−345. Plasmids p32.1 (C115E) and p33.4 (C115N) were constructed by replacing nucleotides 334−427 of p5.16 (*Xma*I/*Bst*XI digest) with a synthetic oligonucleotide incorporating the $GAT_{Asp} \rightarrow GAG_{Glu}$ and $GAT_{Asp} \rightarrow AAT_{Asn}$ changes at position 343−345 in the respective constructs.

Kinetic Measurements. Kinetic parameters for the C115D mutant were determined using the MurB coupled assay (Benson et al., 1993). Purified MurB was kindly provided by Timothy Benson (Harvard Medical School). The assay buffer consisted of acetic acid (100 mM), Bis-Tris (50 mM), and Tris (50 mM) [adjusted to the indicated pH (between 5.5 and 9.0) by the addition of concentrated HCl or NaOH], KCl (20 mM), and NADPH (0.2 mM), to which MurB (0.1 units) and MurA (0.01−0.05 units, depending on pH and on the particular mutant being assayed) were added in a final volume of 1.0 mL, with UDP-GlcNAc and PEP concentrations as indicated. The k_{cat} values for WT and C115D enzymes were determined at pH 5.5−9.0 with [UDP-GlcNAc] = [PEP] = 1 mM. For measurements of k_{cat}^{C115E}, [UDP-GlcNAc] = 1 mM and [PEP] = 10 mM. K_m(UDP-GlcNAc)^{C115D} (at pH 6.0, 7.0, and 8.0) was determined by fixing [PEP] = 1 mM and varying [UDP-GlcNAc] = 12.5−200 μM. K_m(PEP)^{C115D} (at pH 6.0, 7.0, and 8.0) was determined by fixing [UDP-GlcNAc] = 1 mM and varying [PEP] = 12.5−200 μM. K_i(fosfomycin)^{C115D} (at pH 6.0 and 8.0) was determined by fixing [UDP-GlcNAc] = 1 mM and varying [fosfomycin] = 0, 2, 4, 6 mM and [PEP] = 50−2000 μM. K_i(FPEP)^{C115D} (at pH 6.0 and 8.0) was determined by fixing [UDP-GlcNAc] = 1 mM and varying [FPEP] = 0, 100, 200, 300 μM and [PEP] = 50−2000 μM.

Time-dependent inhibition was assayed by preincubating MurA (5 μM, WT or C115D; 200 μM, C115E) with UDP-GlcNAc (1 mM) and either fosfomycin (10 mM for C115D or C115E assay; 100 μM for WT inactivation) or (*Z*)-FPEP (1 mM) in the acetic acid/Bis-Tris/Tris buffer at the indicated pH. At periodic intervals activity was measured by diluting a 50 μL aliquot into 1.0 mL of MurB assay buffer with [UDP-GlcNAc] = [PEP] = 1 mM at pH 7.0 for WT and C115D enzymes or with [UDP-GlcNAc] = 1 mM and [PEP] = 10 mM at pH 6.0 for C115E MurA.

^{14}C-Based HPLC Assay for Reaction of C115D MurA with (Z)-FPEP. The forward reaction was followed by incubating C115D (40 μM), [^{14}C]UDP-GlcNAc (16 μM), and FPEP (200 μM) at pH 6.0 (acetic acid/Bis-Tris/Tris buffer). The reverse reaction was followed by incubating C115D (40 μM) with [^{14}C]3′-(3-fluoro-2-phospholactyl)-UDP-GlcNAc (FT) [isolated as described previously (Kim et al., 1995a)] (2 μM) and excess unlabeled UDP-GlcNAc (1 mM) at pH 6.0 (acetic acid/Bis-Tris/Tris buffer). Aliquots of each reaction mixture were quenched in 0.2 N KOH and analyzed by anion-exchange HPLC at periodic intervals.

Table 1: Kinetic Parameters of C115D MurA

	pH	k_{cat} (s^{-1})	K_m (UDP-GlcNAc) (μM)	K_m (PEP) (μM)	k_{cat}/K_m (PEP) (μM^{-1} s^{-1})	K_i (fosfomycin) (μM)	k_{inact} (fosfomycin) (s^{-1})
C115D	6.0	11	21	22	0.5	2000	$<10^{-6}$
	7.0	3.5	22	28	0.1		
	8.0	0.63	16	37	0.01	1000	$<10^{-6}$
WT[a]	8.0	3.8	15	0.4[b]	10	8.6	0.12

[a] Data for WT MurA from Marquardt (1993) and Marquardt et al. (1994). [b] Value measured using ^{32}P[PEP] based assay (D.H.K., unpublished data).

RESULTS

Site-Directed Mutagenesis of Cys115. C115A, C115S, C115D, C115E, and C115N mutants of MurA were overproduced to levels comparable to those observed for the overproduction of WT MurA (5−50 mg of pure protein/1 L of culture). Activity of C115A and C115S mutants of MurA was not detectable (activity <0.05% that of WT enzyme), in agreement with previous reports (Marquardt, 1993; Wanke & Amrhein, 1993). Catalytically active C115D and C115E mutants were characterized further. Surprisingly, we observed activity from the C115N mutant (ranging from 20 to 50% of the C115D mutant) that was fosfomycin-resistant and displayed the same pH dependence observed from the C115D and C115E mutants. However, tryptic digestion and mass spectrometry analysis[3] demonstrated the presence of significant amounts of deamidation of Asn115 to Asp115 (consistent with the level of activity observed), so the C115N mutant was not characterized further. Although there is some precedent for deamidation of asparagine and glutamine residues in proteins (Wright, 1991), the rate of deamidation observed in the case of the C115N mutant MurA ($t_{1/2}$ < 1 day) appears to be at least an order of magnitude greater than previously documented cases and is under further investigation.

Characterization of the C115D Mutant of MurA: Effect of pH, Kinetic Parameters, and Fosfomycin Resistance. The C115D mutant did retain enzymatic activity and was analyzed for the presence of copurifying PEP by the addition of 1 equiv of [^{14}C]UDP-GlcNAc and anion-exchange HPLC analysis of product formation. No enolpyruvyl-UDP-GlcNAc was formed from C115D MurA (data not shown), in contrast to a comparably purified WT control that was substantially the phospholactyl−enzyme adduct, as shown by the production of 0.5−1.0 equiv product as reported previously (Brown et al., 1994; Wanke & Amrhein, 1993).

As depicted in Figure 1, k_{cat}^{C115D} was strongly pH-dependent, titrating across the pH range examined (pH 5.5−9.0) with an apparent p$K_a \sim 6$, in contrast to the broad pH independence of k_{cat}^{WT}. Furthermore, k_{cat}^{C115D} was actually greater than k_{cat}^{WT} at pH <7. K_m (PEP)^{C115D} and K_m (UDP-GlcNAc)^{C115D} were determined at pH 6.0, 7.0, and 8.0 (Table 1), and neither showed significant variation with pH. K_m (UDP-GlcNAc)^{C115D} was observed to be equivalent to K_m (UDP-GlcNAc)^{WT}, but K_m (PEP)^{C115D} was ∼100-fold greater than K_m(PEP)^{WT}. $[k_{cat}/K_m(PEP)]^{C115D}$ also followed the pH dependence observed for k_{cat}^{C115D} and was decreased by >10-fold relative to $[k_{cat}/K_m(PEP)]^{WT}$.

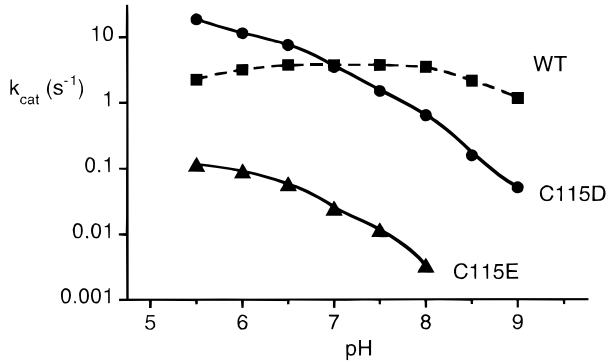


FIGURE 1: Contrasting pH dependences of k_{cat}^{WT} vs k_{cat}^{C115D} and k_{cat}^{C115E}.

Inhibition of the C115D mutant by fosfomycin was found to be competitive with respect to PEP, but the K_i was elevated ∼100-fold over the K_i for WT enzyme (Table 1) and was observed to increase from 1 mM at pH 8.0 to 2 mM at pH 6.0. In marked contrast to WT MurA, no time-dependent decrease in activity of the C115D mutant was observed even after prolonged (6 h) preincubation with UDP-GlcNAc and fosfomycin.

Characterization of the C115E Mutant of MurA. As shown in Figure 1, the C115E mutant was shown to follow a pH dependence similar to that of C115D enzyme. Activity was unaffected by preincubation with UDP-GlcNAc and 10 mM fosfomycin for 15 min [$t_{1/2}$ for inactivation of WT MurA by fosfomycin = 6 s (Marquardt et al., 1994)]. Both the pH dependence and fosfomycin resistance of C115E mutant activity rule out the possibility that the low level of activity observed (∼1% of C115D MurA) was due to chromosomally encoded WT MurA. Because of the low level of activity, this mutant was not characterized in further detail.

Characterization of the Reaction of C115D MurA with (Z)-FPEP. (*Z*)-FPEP was found to be a competitive inhibitor with respect to PEP with a K_i comparable to that determined previously for WT MurA [$K_i(FPEP)^{C115D} = 100$ μM (pH 6.0) and 80 μM (pH 8.0), compared to $K_i(FPEP)^{WT} = 40$ μM (pH 8.0)]. Figure 2 displays the time-dependent inhibition of C115D MurA by (*Z*)-FPEP in the presence of UDP-GlcNAc at pH 6.0, 7.0 and 8.0. The data in Figure 2 were fit to the equation $A(t) = (1 - A_\infty)\exp(-k_{obs}t) + A_\infty$, where $A(t)$ = normalized enzyme activity as a function of time and A_∞ = enzyme activity as $t \rightarrow \infty$. The values of k_{obs} and A_∞ are listed in Table 2. Inactivation proceeded to 30−50% ($A_\infty = 0.3-0.5$) of the initial enzyme activity, and the rate constant for enzyme inactivation, $k_{obs}(FPEP)^{C115D}$, followed a pH dependence similar to that observed for k_{cat}^{C115D}. The inset to Figure 2 shows the pH dependence of the rate constant for inactivation of WT enzyme by (*Z*)-FPEP, $k_{inact}(FPEP)^{WT}$, compared to the corresponding rate constant

[3] The 17-mer, FGQGQVSLPGG**X**TIGAR (residues 104−120), was isolated by tryptic peptide difference maps from the following MurA preparations, WT (X = Cys), C115D (X = Asp), and C115N (both X = Asn and X = Asp peptides in approximately equal amounts), and identified by microcapillary LC/ESI/MS on a Finnigan TSQ7000. X = Asp and X = Asn 17-mers were conclusively assigned by esterfication of the 17-mers that resulted in an additional 14 unit mass shift for Asp115 containing peptides relative to Asn115 and Cys115 peptides (T. A. Addona and W.S.L., unpublished data).

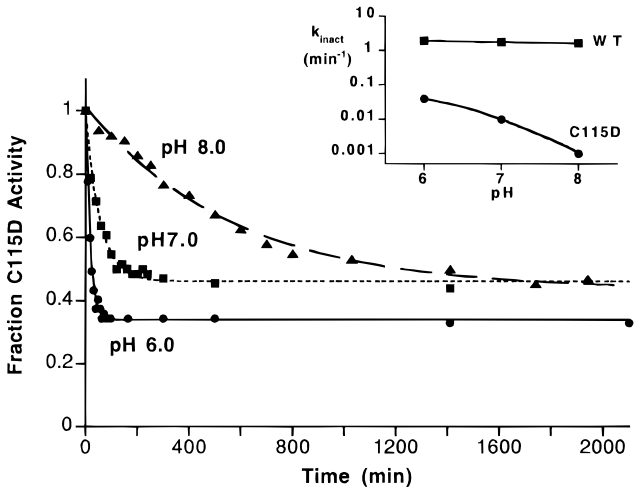


FIGURE 2: Time-dependent inhibition of C115D MurA at pH 6.0, 7.0, and 8.0. The data were fit to an exponential as described in the text. The inset shows the contrasting pH dependences of $k_{inact}(FPEP)^{WT}$ and the corresponding rate constant for fluoromethyl tetrahedral adduct (FT, defined in eq 1) formation, k_+^{C115D}.

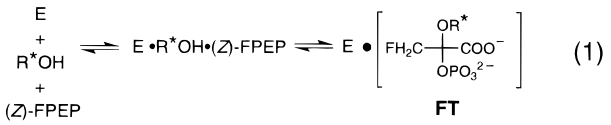
Table 2: Kinetic and Thermodynamic Parameters of (*Z*)-FPEP Inactivation of C115D MurA

pH	A_∞[a]	k_{obs} (min^{-1})[a]	K_{int}[b]	k_+ (min^{-1})[c]	k_- (min^{-1})[c]
6.0	0.34	0.061	2.0	0.041	0.020
7.0	0.47	0.019	1.1	0.010	0.0090
8.0	0.43	0.0017	1.3	0.00096	0.00074

[a] Parameters from fitting data in Figure 2 to $A(t) = (1 - A_\infty)\exp(-k_{obs}t) + A_\infty$, where $A(t)$ = normalized enzyme activity as a function of preincubation time. [b] $K_{int} = (1 - A_\infty)/A_\infty$. [c] Deduced from the following relationships: $k_+ + k_- = k_{obs}$ and $k_+/k_- = K_{int}$.

for C115D MurA inactivation (see Discussion). In contrast to the pH dependence of C115D MurA inactivation by FPEP, $k_{inact}(FPEP)^{WT}$ remained constant in the pH range 6.0−8.0.

The reaction of C115D MurA and (*Z*)-FPEP at pH 6.0 was also followed by anion-exchange HPLC with [^{14}C]UDP-GlcNAc. Enzyme concentration was present in excess of UDP-GlcNAc. Conversion of [^{14}C]UDP-GlcNAc to the fluoromethyl analog of the tetrahedral intermediate (FT, eq 1) proceeded to a final ratio of FT:UDP-GlcNAc of ∼2:1,



(1)

E, C115D MurA; R*OH, [^{14}C]UDP-GlcNAc;

with a rate constant of 0.05 min^{-1} (data not shown). The reverse reaction was examined by mixing [^{14}C]FT with excess enzyme and excess unlabeled UDP-GlcNAc and observing the washout of ^{14}C from FT into UDP-GlcNAc. The rate constant for washout was measured to be 0.02 min^{-1} (data not shown).

DISCUSSION

Site-Directed Mutagenesis of Cys115: Requirement of an Ionizable Side Chain for Residue 115. In this paper we have demonstrated that aspartate and, to a much lesser degree, glutamate can substitute for Cys115 at the active site of *E. coli* MurA, conferring complete resistance to time-dependent inactivation by fosfomycin. Steric considerations[4] probably account for the greatly decreased activity of the C115E mutant relative to the C115D enzyme. The inactivity of C115A and C115S mutants of MurA suggests that the carboxylate side chain of Asp115 substitutes for a specific catalytic function involving the ionization of the thiol group of Cys115. Consistent with the expected difference in pK_a between the −SH (8−11) and the −COOH (2−6) groups (Fersht, 1985), the activities of the WT and C115D (and C115E) enzymes display a dramatically different dependence on pH as shown in Figure 1. Because the enzymes differ at a single residue, Cys/Asp115, the broad pH dependence of k_{cat}^{C115D} suggests that the apparent pK_a in the pH profile of k_{cat}^{C115D} is due to the ionization of the Asp, for which a pK_a ∼ 6 is consistent. However, the interpretation of the pH rate profiles can be ambiguous (Knowles, 1976; Cleland, 1982). In the present case, for example, we cannot rule out the possibility that a change in rate-determining step in the C115D mutant has unmasked the pH dependence of a group other than residue 115 that is now expressed in the pH profile of k_{cat}^{C115D}, but not k_{cat}^{WT}. Alternatively, a change in rate-determining step may accompany the expression of the pK_a of Asp115 in the pH profile of k_{cat}^{C115D}.

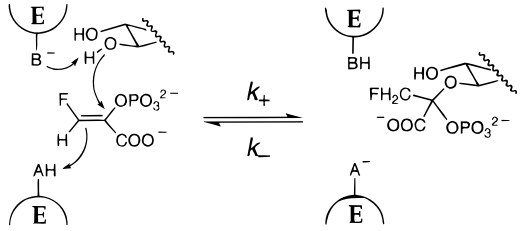
Mechanism of Inactivation of the C115D Mutant by (Z)-FPEP: Assignment of Cys/Asp115 as a General Acid. To further investigate the contrasting pH dependences of C115D and WT enzymes, we investigated the effect of pH on the reaction of C115D MurA with the pseudosubstrate, (*Z*)-FPEP. Previously, we have characterized the kinetics, thermodynamics, and stereochemistry of the reaction of WT MurA with the pseudosubstrate (*Z*)-FPEP (Kim et al., 1994, 1995a,b). In the case of WT MurA, we observed stoichiometric inactivation of enzyme activity due to the accumulation of two enzyme adducts, a covalent phosphofluorolactyl−enzyme adduct and FT, in slow equilibrium at the active site in a ratio of ∼1:1 (Kim et al., 1994, 1995a). Figure 2 shows the incomplete inactivation of C115D mutant activity by (*Z*)-FPEP in the presence of UDP-GlcNAc. As assessed by anion-exchange HPLC analysis using [^{14}C]UDP-GlcNAc, the kinetics and stoichiometry of accumulation of a single species, FT, fully accounted for the observed time-dependent inhibition. The incomplete nature of enzyme inactivation reflects the equilibration at the C115D enzyme active site between two nearly isoenergetic complexes—the inactive enzyme•FT complex and the enzyme•UDP-GlcNAc•(*Z*)-FPEP ternary complex (eq 1), which is active upon dilution into excess PEP. K_{int}, defined as the internal equilibrium ratio of enzyme•FT to enzyme•UDP-GlcNAc•(*Z*)-FPEP, and k_+ and k_-, the forward and reverse rate constants for interconversion between the two C115D enzyme complexes, were evaluated from the inactivation data in Figure 2 as described and summarized in Table 2.

The inset to Figure 2 compares the pH dependence of k_+^{C115D} to $k_{inact}(FPEP)^{WT}$, which is equivalent to k_+^{WT} because inactivation of WT MurA proceeds to completion. Mimicking the observed pH dependence of k_{cat}, k_+^{C115D} titrates with a pK_a = 6, while k_+^{WT} remains independent of pH over the range examined (pH 6.0−8.0). In contrast to the situation encountered in the interpretation of the pH dependence of

[4] Van der Waals volumes (in Å^3) of the amino acids: Cys, 86; Asp, 91; Glu, 109 (Creighton, 1993). In view of the 100-fold difference in activity between C115D and C115E enzymes, we focus our discussion regarding mechanism on the C115D mutant.

[5] On the other hand, the pH dependence of k_- is largely a function of the protonation state of BH of Scheme 2, which acts as the general acid in the decomposition of FT in the reverse direction.

Scheme 2: General Acid−Base Catalysis in MurA Autoinactivation by (*Z*)-FPEP



k_{cat}, the pH dependence of k_+ reflects the effect of pH on a single defined, cleanly isolated elementary step of catalysis—formation of the tetrahedral adduct arising from the protonation of C-3 of FPEP and attack of the 3′-oxygen of UDP-GlcNAc at C-2 of PEP (Scheme 2). The pH dependence of k_+^{C115D} reveals the dependence on the active site acid, AH (Scheme 2).[5] The contrasting, pH-independent behavior of k_+^{WT} strongly implicates Asp115 in the role of AH in the C115D enzyme and is consistent with the corresponding participation of Cys115 with pK_a > 9 as the general acid in WT MurA.

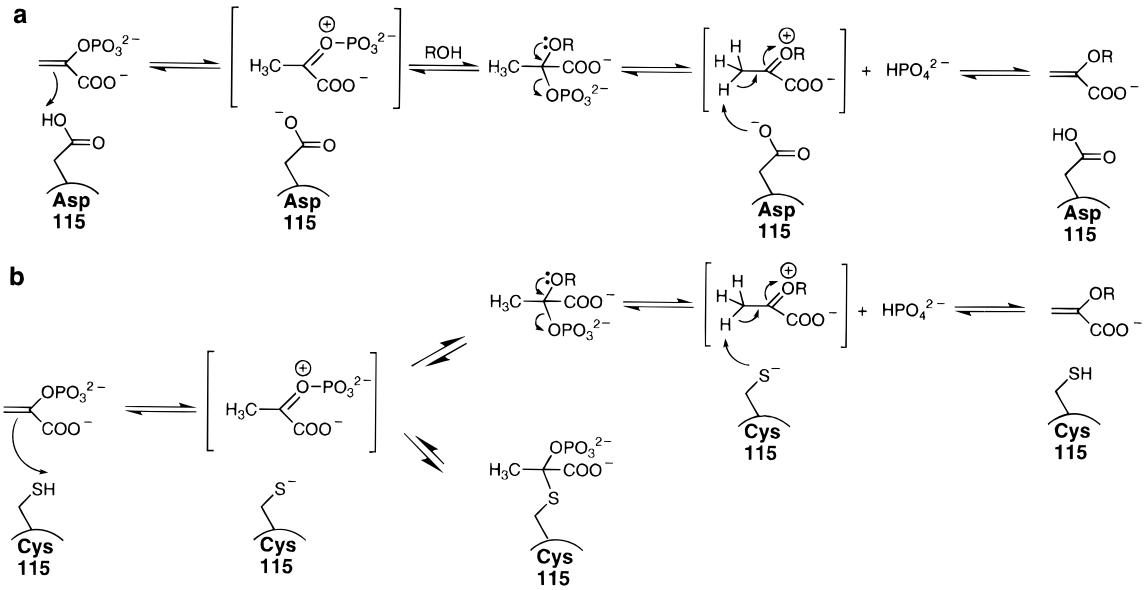
Extending these results to normal catalysis, we propose that Cys115 functions as an active site acid, involved in the initial protonation of the double bond of PEP and concomitant stabilization of the positive charge developing at C-2 of PEP in the enzyme-catalyzed addition of 3′-OH of UDP-GlcNAc to C-2 of PEP. In addition, we speculate that this residue may also function as the active site base in the elimination step, deprotonating the methyl group of the tetrahedral intermediate in the forward breakdown to enol ether product and providing electrostatic stabilization of the second oxocarbenium ion intermediate. The proposed mechanism of C115D and WT MurA involving oxocarbenium ion intermediates (Kim et al., 1995a), modified to include the participation of Asp/Cys115 in general acid−base catalysis, is illustrated in Scheme 3.

The Role of Cys115 in the Mechanism of MurA. The participation of cysteine in enzymic reactions has been most commonly observed in cases where the thiolate side chain plays the role of active site nucleophile in the formation of a covalent enzyme intermediate, for which there are a number of diverse examples (Walsh, 1979), including the cysteine proteases, thiolase, and tyrosine phosphatases (Cho et al., 1992). Precedent also exists for the participation of cysteine as an active site base, although fewer cases have been studied: in proline racemase (Rudnick & Abeles, 1975; Belasco et al., 1986) and glutamate racemase (Tanner et al., 1993), where two cysteine residues are proposed to function in each racemase in general acid−base catalysis as part of a two-base mechanism (Cardinale & Abeles, 1968), and thiolase, in which a cysteine residue distinct from the cysteine involved in covalent catalysis has been implicated as an active site base (Palmer et al., 1991).

Previous studies implicating Cys115 in the formation of a covalent phospholactyl−enzyme adduct (Cassidy & Kahan, 1975; Wanke & Amrhein, 1993; Brown et al., 1994), coupled with the present work indicates that in the MurA mechanism, Cys115 participates as *both* a general acid and an enzyme nucleophile (Scheme 3b). That this latter role is dispensable for enzymic activity is strongly suggested by the ability of the poorly nucleophilic β-carboxylate group of Asp115 to support high-level catalytic turnover (Scheme 3a) in place of the strongly nucleophilic thiolate of Cys115. We note, however, that the carboxylate group of Asp115 is well-suited to substitute for the thiolate side chain of Cys115 in the electrostatic stabilization of oxocarbenium ion intermediates, with ample precedent for such a role for active site aspartate and glutamate residues in the stabilization of carbocationic intermediates in enzymatic glycosyl transfer (Sinnott, 1990).

Although highly active, the catalytic efficiency of the C115D mutant relative to WT MurA is reduced by at least a factor of 10, as assessed by k_{cat}/K_m(PEP), in large part due to the 100-fold elevated K_m(PEP) of the C115D mutant. Because the K_i(FPEP) for both WT and C115D enzymes are comparable, the difference in K_m(PEP) is most likely not due to significantly decreased affinity for PEP at the active site of the C115D mutant, but rather, probably reflects other energetic changes along the reaction coordinate (e.g., a change in rate-determining step). The detailed kinetic and thermodynamic characterization of the C115D MurA reaction pathway and quantitative comparison with the reaction pathway of WT MurA should provide added insight into the respective roles played by Cys115 in WT MurA and Asp115 in the C115D mutant enzymes.

Scheme 3: Proposed Mechanism of the (a) C115D Mutant and (b) WT Enzyme



Implications of Fosfomycin Resistance of E. coli C115D MurA. Perhaps the most striking manifestation of the decreased nucleophilicity of Asp115 relative to Cys115 was the complete lack of time-dependent inactivation of C115D MurA by fosfomycin. Binding of fosfomycin to the C115D MurA active site still occurred, as determined by the reversible competitive inhibition with respect to PEP, but the 100-fold increased K_i relative to the WT MurA value (Table 1) suggests that decreased affinity and/or misalignment of the active site for fosfomycin, in addition to the replacement of the thiolate nucleophile, may also contribute to the fosfomycin resistance of the C115D mutant.

The principal mechanism of resistance to fosfomycin has been observed to be decreased uptake of the drug into the cytoplasm (Kahan et al., 1974), although plasmid-mediated fosfomycin resistance in clinical isolates involving glutathione-mediated deactivation of the drug has also been reported (Suárez & Mendoza, 1991). A temperature-sensitive mutation conferring fosfomycin resistance in an *E. coli* laboratory strain that appeared to produce a UDP-GlcNAc enolpyruvyl transferase with decreased sensitivity to fosfomycin and an elevated K_m(PEP) was reported (Venkateswaran & Wu 1972), but that mutational locus has not been characterized.

The fosfomycin resistance conferred on *E. coli* MurA by the Cys115-to-Asp115 substitution and the presence of aspartate at this position in the putative UDP-GlcNAc enolpyruvyl transferase of *M. tuberculosis* suggests a molecular mechanism contributing to the high-level fosfomycin resistance observed in *Mycobacteria* (G. A. C. Chung and K.D., unpublished data) as well as its *Actinomycetales* family members, *Actinomycetes*, *Nocardia* (Farina et al., 1995), and *Streptomyces*. Notably, insensitivity to fosfomycin in both fosfomycin-producing and non-fosfomycin producing strains of *Streptomyces* has been interpreted in terms of a likely endogenous mechanism for resistance (Suárez et al., 1989). We speculate that a resistant target enzyme may mediate producer immunity in *Streptomyces*. Characterization of the UDP-GlcNAc enolpyruvyl transferases initiating peptidoglycan synthesis in these organisms will allow the evaluation of these predictions.

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