Asparagine 23 and Aspartate 305 Are Essential Residues in the Active Site of UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase from *Enterobacter cloacae*†

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ABSTRACT: UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) catalyzes the transfer of the intact enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine (UDPNAG). This reaction constitutes the first committed step in the biosynthesis of the bacterial cell wall component peptidoglycan (murein). The transfer reaction involves the nucleophilic attack of the 3′-hydroxyl group of UDPNAG at the C-2 of PEP. The three-dimensional structure of MurA complexed with UDPNAG revealed an aspartate residue (D305 in the *En. cloacae* sequence) close to the 3′-hydroxyl group of UDPNAG, suggesting that it may act as an acid−base catalyst in the active center of the enzyme. In addition to aspartate 305, asparagine 23 also interacts with the 3′-hydroxyl group; however, its role in catalysis or binding of the UDPNAG substrate is unclear. To gain information on the role of these two amino acids in the MurA-catalyzed reaction we have exchanged D305 for alanine, cysteine, histidine, and glutamate, and N23 for alanine and serine using site-directed mutagenesis. While the D305 alanine, cysteine, and histidine mutant proteins do not have detectable enzymatic activity, the D305E mutant protein exhibits a low residual activity (ca. 0.1% of the wild-type enzyme). Unlike with wild-type MurA, no exothermic signal was obtained when the D305A and -E mutant proteins were titrated with UDPNAG, demonstrating that the affinity of the sugar nucleotide substrate is reduced as a result of the amino acid exchange. The reduced affinity to UDPNAG leads to a lower propensity of C115 to form either the *O*-phosphothioketal with PEP or the thioether with the antibiotic fosfomycin. These findings emphasize the dual role of D305 as a general base and an essential binding partner to UDPNAG in the active site of MurA. Similarly, the two N23 mutant proteins showed a much lower catalytic activity although binding of UDPNAG was not as much affected as in the case of the D305 mutant proteins. This result indicates that this amino acid residue is mainly involved in stabilization of transition states.

The biosynthesis of the sugar building block muramic acid, a structural component of the bacterial cell wall (peptidoglycan), starts with the transfer of the enolpyruvyl moiety of phosphoenolpyruvate to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine catalyzed by UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA)[1] as shown in Scheme 1. A similar reaction is found in the shikimate pathway which involves transfer of the enolpyruvyl moiety of phosphoenolpyruvate to the 5-hydroxyl group of shikimate 3-phosphate to form 5-enolpyruvylshikimate 3-phosphate (EPSP) catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase) (*1*). These two enzymes share a similar and unique 3D structure consisting of two "inside-out α/β-barrel" domains connected by a hinge region. For MurA, the X-ray structures of three forms of the protein have been reported: (i) the free unliganded protein (*2*, *3*); (ii) a complex of MurA, UDPNAG, and the covalently linked antibiotic fosfomycin (formation of this adduct is shown in Scheme 3) (*4*); and (iii) a complex of the C115A mutant protein with the (*Z*)-3-fluorophosphoenolpyruvate tetrahedral intermediate (*5*). The structure of the free protein represents an "open" and the latter two "closed" conformations of MurA. The closed forms reveal details with regard to the interactions of the sugar nucleotide substrate with amino acid residues in the active site. The pyranose ring of the substrate engages through its 3′- and 4′-hydroxyl groups in hydrogen bond interactions with both the carboxyl group of D305 and the amide group of N23 (Scheme 2, panel A). Both amino acid residues are strictly conserved in all known MurA sequences. They are in close structural proximity, with N23 interacting with K22 and the 3′-oxygen of the pyranose ring as shown in Figure 1 (panels B and C) and Scheme 2 (panel B). While in the UDPNAG/fosfomycin complex the carboxylate of D305 forms two hydrogen bonds to the 3′- and 4′-oxygen atoms, respectively (Figure 1, panel B), only one hydrogen bond to the 4′-oxygen in the structure of the tetrahedral intermediate obtained with UDPNAG and (*Z*)-3-fluorophos-

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[1] Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase; ITC, isothermal titration calorimetry; EP-UDPNAG, UDP-*N*-acetylenolpyruvylglucosamine; fosfomycin, (1*R*,2*S*)-1,2-epoxypropylphosphonic acid; MurA, UDP-*N*-acetylglucosamine enolpyruvyl transferase; MurB, UDP-*N*-acetylenolpyruvylglucosamine reductase; PEP, phosphoenolpyruvate; S3P, shikimate 3-phosphate; UDPNAG, UDP-*N*-acetylglucosamine.

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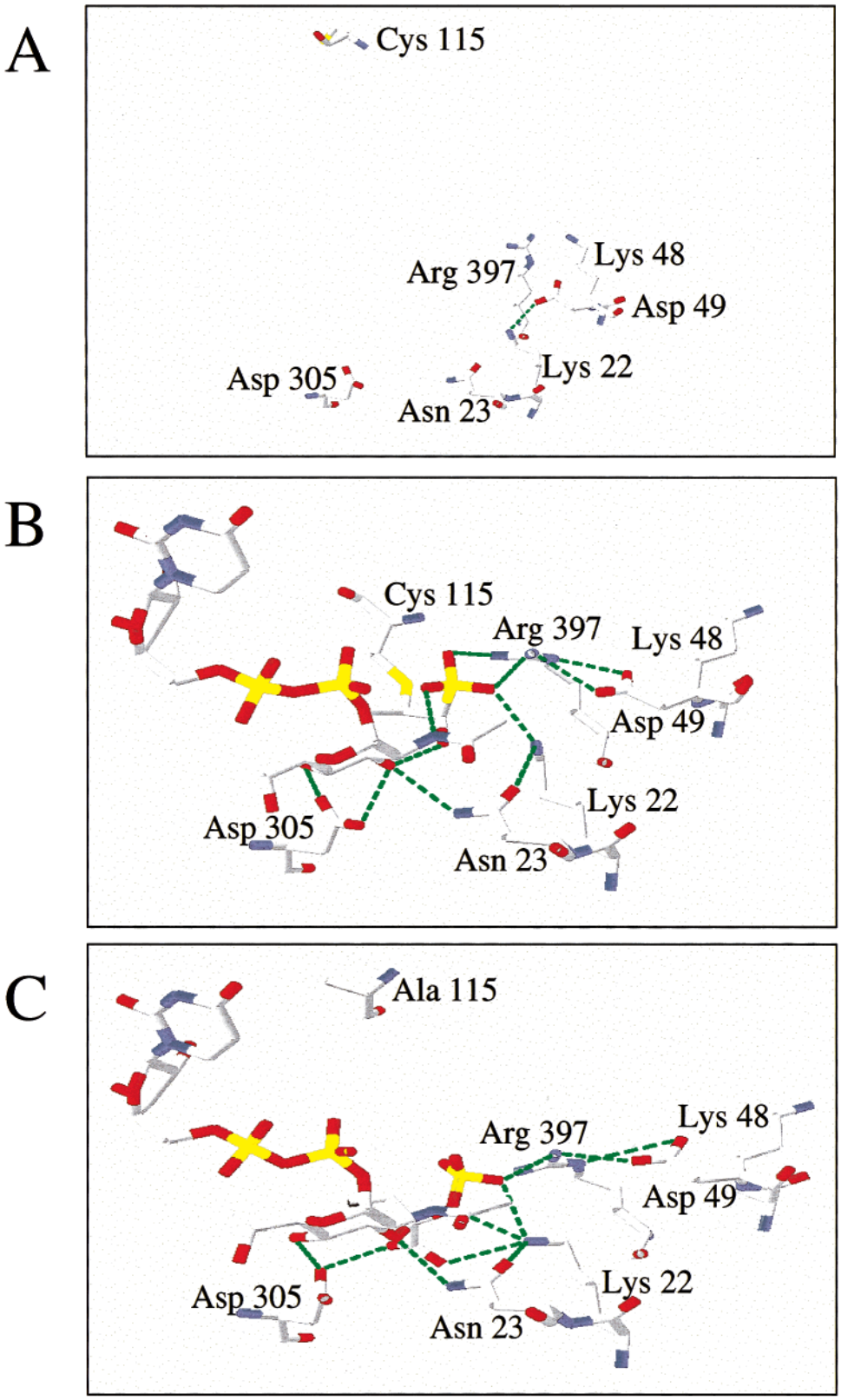
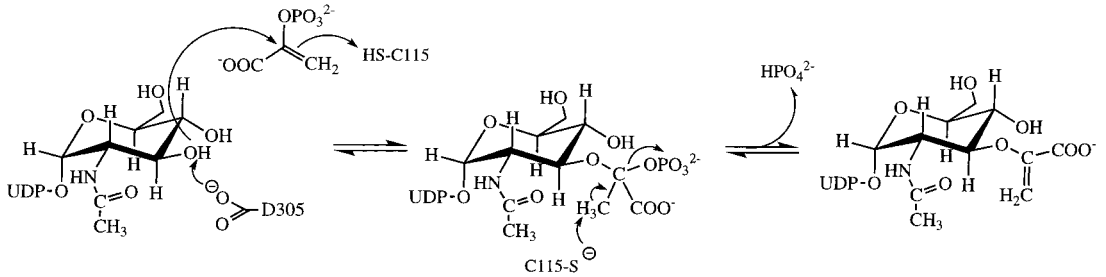


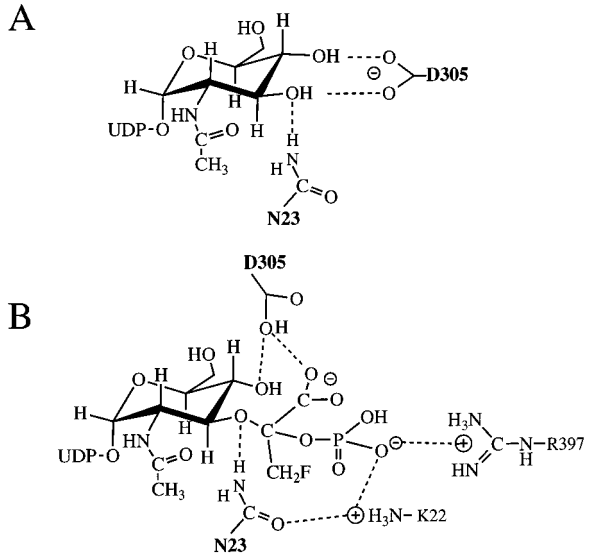
FIGURE 1: Interactions of N23 and D305 with UDPNAG in the active site of MurA (selected residues are shown for clarity). Panel A shows the structure of unliganded MurA (*2*); panel B, the structure of MurA with UDPNAG and the covalently linked antibiotic fosfomycin bound in the active center (*4*); and panel C, the structure of the C115A mutant MurA with the fluorinated tetrahedral reaction intermediate bound in the active center (*5*).

phoenolpyruvate was found (Figure 1, panel C, and Scheme 2, panel B). This finding is consistent with the proposed role of the carboxylate group as the base abstracting the proton from the 3′-hydroxyl group in order to facilitate its nucleophilic attack on C-2 of phosphoenolpyruvate. As this structure represents a reaction intermediate formed after the proton abstraction step, the (protonated) carboxylate group appears to swing away from the 3′-oxygen. These two available closed structures provoke several questions concerning the roles of N23 and D305. As for the asparagine, it

Scheme 1



Scheme 2



is conceivable that the amide side chain is involved in binding of the substrate and/or a reaction intermediate (Scheme 2, panel B). The asparagine also forms a hydrogen bond to the ϵ-amino group of K22 in both closed forms (see Figure 1, panels B and C, and Scheme 2, panel B). In the open form of MurA (Figure 1, panel A), however, the ϵ-amino group of K22 interacts ionically with the carboxyl group of D49 instead. This salt bridge is replaced during the formation of the closed conformation in favor of multiple interactions of the ϵ-amino group of K22 to the enolpyruvyl moiety of the tetrahedral intermediate and a hydrogen bond to N23. Therefore, N23 may also be involved in bringing about the conformational switch of the K22 side chain, resulting in a network of hydrogen bond interactions with the substrate(s) and the ensuing formation of the tetrahedral reaction intermediate. Interestingly enough, in the closely related EPSP-synthases, the asparagine is replaced by a serine which prompted us to exchange N23 in *En. cloacae* MurA for alanine and serine, respectively.

On the other hand, the carboxylate group of D305 is a good candidate for the proton-abstracting base as well as a putative binding partner to the sugar ring moiety (see Schemes 1 and 2). To determine the roles of these two amino acids experimentally, we have replaced D305 with alanine, cysteine, histidine, and glutamate, respectively, and N23 with alanine and serine, respectively, using site-directed mutagenesis. All six single mutant proteins were expressed in *E. coli* and isolated from crude extracts, and the purified proteins were analyzed with regard to their catalytic and binding properties.

MATERIALS AND METHODS

Chemicals. Fosfomycin and UDP-*N*-acetylglucosamine (sodium salt) were from Sigma, Buchs, Switzerland. Adenosine 5′-diphosphate (ADP, sodium salt), 1,4-dithio-DL-threitol, ethylenediaminetetraacetic acid (EDTA), isopropyl-β-D-1-thiogalactopyranoside, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and phosphoenolpyruvate (potassium salt) were from Fluka, Buchs, Switzerland. Tris was from BDH Laboratory Supplies, Poole, England. EP-UDPNAG was enzymatically synthesized and purified as described (*6*).

Enzymes. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, lactate dehydrogenase (EC 1.1.1.27) from bovine heart, pyruvate kinase (EC 2.7.1.40) from rabbit muscle, and trypsin (EC 3.4.21.4) from bovine pancreas were from Fluka. UDP-*N*-acetylenolpyruvylglucosamine reductase (EC 1.1.1.48, MurB) from *E. coli* was expressed as a glutathione-*S*-transferase (GST)-fusion protein and purified via a glutathione Sepharose column as described by Krekel (*7*).

Site-Directed Mutagenesis. Mutagenesis was carried out using the Quik-change site-directed mutagenesis kit from Stratagene. The following oligonucleotides were used to change the desired codon (codon change underlined):

N23A (AAC to GCC):

CTGGCGCGAAAGCCGCCGCGCTGCCGATCC (5′-3′)

GGATCGGCAGCGCGGCGGCTTTCGCGCCAG (3′-5′)

N23S (AAC to AGC):

CTGGCGCGAAAAGCGCCGCGCTGCCGATCC (5′-3′)

GGATCGGCAGCGCGGCGCTTTCGCGCCAG (3′-5′)

D305A (GAT to GCT):

CGGCATTCCCGACCGCTATGCAGGCTCAG(5′-3′)

CTGAGCCTGCATAGCGGTCGGGAATGCCG(3′-5′)

D305C (GAT to TGT):

CGGCATTCCCGACCTGTATGCAGGCTCAG(5′-3′)

CTGAGCCTGCATACAGGTCGGGAATGCCG(3′-5′)

D305E (GAT to GAG):

CGGCATTCCCGACCGAGATGCAGGCTCAG(5′-3′)

CTGAGCCTGCATCTCGGTCGGGAATGCCG (3′-5′)

D305H (GAT to CAT):

CGGCATTCCCGACCCATATGCAGGCTCAG(5′-3′)

CTGAGCCTGCATATGGGTCGGGAATGCCG(3′-5′)

The oligonucleotides were purified over Sep-Pak (C18) columns from Waters prior to PCR. In the PCR, the entire pKK233-2 plasmid containing the open reading frame coding for wild-type MurA from *En. cloacae* was amplified.

Mutations were confirmed by sequencing with an ABI 373 DNA sequencer (Applied Biosystems, CA) using dye terminator sequencing chemistry.

Expression and Purification of the Mutant Proteins. Wild-type MurA and mutant proteins were expressed and purified as described previously (*8*). Protein concentration was determined using an extinction coefficient of 24 020 M^{-1} cm^{-1} at 280 nm (*9*) or with the Bradford reagent from Pierce using bovine serum albumin (BSA) for calibration.

Enzyme Assays. Enzyme activity in the forward reaction (see Scheme 1, left to right) was determined using the phosphate assay developed by Lanzetta (*10*) and an enzyme-coupled assay described recently (*11*).

Enzyme activity in the reverse reaction (see Scheme 1, right to left) was also determined with an enzyme-coupled assay. MurA (10 μM) was incubated with 1 mM EP-UDPNAG and 10 mM sodium phosphate in 50 mM Tris/HCl, pH 7.4, containing 1 mM DTT at 25 °C. After 1 h incubation, aliquots (170 μL) of this reaction were transferred to a cuvette containing 830 μL of 50 mM Tris/HCl, pH 7.4, containing 1 mM DTT, 50 mM KCl, 50 mM $MgCl_2$, 1 mM ADP, 0.3 mM NADH, 10 units of pyruvate kinase, and 5 units of lactate dehydrogenase. The amount of PEP formed during incubation with the D305 and N23 mutant proteins was determined from the NADH consumed in the coupling reaction.

Fluorescence Measurements. The quench of the tryptophan fluorescence emission as a function of PEP concentration was used to determine the dissociation constant for PEP with the D305 and N23 mutant proteins as reported recently (*11*).

UDPNAG Binding Assays. To estimate dissociation constants of UDPNAG to wild-type MurA and the mutant proteins, UDPNAG (200 μM) was incubated with protein (84 μM) and then briefly centrifuged in 2 mL microconcentrators (Vivascience, CTA-membrane, 10 kDa cutoff) to recover ca. 20% of the total volume of the incubation mixture. The concentration of free UDPNAG in the filtrate and the retardate was determined by measuring the phosphate released upon addition of PEP and trace amounts of wild-type MurA using the Lanzetta assay (*10*).

Microcalorimetry. All microcalorimetry experiments were carried out in 50 mM Hepes/NaOH, pH 7.4, containing 2 mM DTT and 0.5 mM EDTA (standard buffer), filtered, and degassed by stirring under vacuum. Purified enzymes were passed through a PD10 column (Pharmacia) equilibrated with standard buffer prior to the experiment. The ligand used for titration was dissolved in exactly the same buffer as the enzyme, and both solutions were degassed directly before the measurement.

Ligand binding to MurA and the mutant proteins was analyzed with a Microcal Omega titration calorimeter equilibrated to 25 °C. In a typical experiment, a total of 25−30 aliquots (8 μL) of the ligand solution were injected into 1.4 mL of the protein solution under constant stirring at 200 rpm. The ligand was injected over a period of 7 s with 10 min between injections. The heats of binding (and/or reaction) were determined by integration of the observed peaks. To correct for the heat of dilution of the ligand, the heats evolving at the end of the titration, i.e., when all ligand binding sites were saturated, were subtracted from the heat of each injection. The corrected heats were then plotted against the ratio of ligand to enzyme concentration to generate the binding isotherm. Dissociation constants, heats of binding, and stoichiometries were obtained by fitting the binding isotherm according to the binding equations incorporated in the Omega instrument software.

Protein Chromatography. Separation of free MurA and the covalent adducts with either PEP or fosfomycin was performed by anion exchange chromatography (MonoQ) with an Äkta Explorer chromatography work station (Pharmacia) as described recently (*11*). The covalent MurA adduct was produced by incubation of 20 μM enzyme in the presence of UDPNAG (concentrations as given in the figure legends) and 200 μM PEP or fosfomycin for 15 min at 25 °C. An aliquot (500 μL) of the incubation mixture was then analyzed by chromatography as described above.

MALDI-TOF-MS Analysis of Fosfomycin Binding to D305 Mutant Proteins. MurA (100 μM) was incubated with 10 mM UDPNAG and 1 mM fosfomycin in 50 mM Tris/HCl, pH 7.4, for 30 min at 25 °C. The incubation mixture was then treated with 0.5 mg/mL trypsin at 25 °C in 50 mM Tris/HCl overnight. The tryptic digest was desalted using SepPak C18 cartridges using 50−100% acetonitrile as eluent. The eluate was dried in a speed-vac apparatus and redissolved in 20 μL of 0.1% trifluoroacetic acid. One microliter of this sample was deposited on a MALDI sample plate and mixed with 1 μL of a saturated dihydroxybenzoate solution in 0.1% trifluoroacetic acid/acetone (2:1, v/v). MALDI-TOF-MS spectra of the peptide fragments were recorded with a Voyager Elite mass spectrometer using the reflectron mode for increased mass accuracy. Interpretation of the peptide masses was based on the analysis reported recently (*12*).

RESULTS

Expression and Purification of the D305 and N23 Mutant Proteins. With the exception of the D305H mutation, the amino acid replacements in positions N23 and D305 had no adverse effect on the expression of soluble UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) as shown in Figure 2. Although expression of the D305H mutant protein was comparable with that of wild-type and the other mutant proteins studied, the majority of the protein was found in the particulate fraction after cell breakage, indicating that

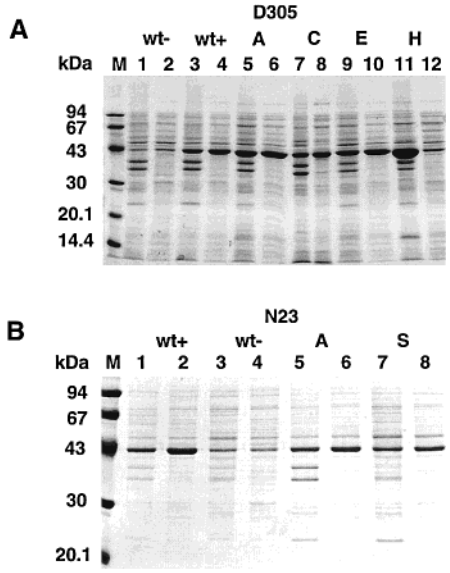


FIGURE 2: Expression of wild-type and D305 and N23 mutant proteins in *E. coli* strain JM105 as analyzed by SDS−PAGE. Odd numbers refer to the insoluble and even numbers to the soluble fraction of total protein after cell lysis by sonification. Abbreviations for expression experiments are as follows: wt−, wild-type MurA without induction; wt+, wild-type MurA induced with IPTG (0.1 mM). Panel A shows the expression of D305 mutant proteins: A, D305A; C, D305C; E, D305E; and H, D305H. Panel B shows the expression of N23 mutant proteins: A, N23A; and S, N23S. Molecular masses of the markers are indicated on the left side of the gel.

the mutant protein was less soluble (see Figure 2A, lanes 11 and 12). The ratio of soluble to insoluble protein was not affected by the growth temperature (25−37 °C). Therefore, this mutant protein was obtained in lower yields (11 mg/10 g wet cell paste) compared to wild-type protein [210 mg/10 g wet cell paste (*11*)]. Although the D305C mutant protein was expressed and soluble to similar levels as wild-type MurA, the final yield of protein was lower (16 mg/10 g wet cell paste) due to losses during purification, indicating lower stability of this mutant protein compared to wild-type MurA.

Enzymatic Activities of D305 and N23 Mutant Proteins. Replacement of D305 by either alanine, cysteine, or histidine was found to reduce enzymatic activity to levels near the estimated background activity of ca. 0.005% (see Table 1). This was found with both an assay based on the release of phosphate from PEP and an enzyme-coupled assay which relies on the reduction of UDP-*N*-acetylenolpyruvylglucosamine (EP-UDPNAG) by MurB at the expense of NADPH (see Materials and Methods). Only the D305E mutant protein exhibited significant activity in both enzyme assays employed in the forward reaction, i.e., on the order of 0.1−0.16% of that found for the wild-type enzyme (left to right in Scheme 1). However, none of the four D305 mutant proteins investigated showed activity above background levels in the reverse reaction (right to left in Scheme 1).

The N23A and N23S mutant proteins showed 0.35% and 0.12% of the wild-type activity in the phosphate assay and 0.1% in the enzyme-coupled assay in the forward reaction (see Table 1). In the reverse reaction, the activities were 4% and 2% for the N23A and N23S mutant proteins, respectively, in comparison with wild-type enzyme.

Table 1: Enzymatic Activities of the N23 and D305 Mutant Proteins As Compared to Wild-Type MurA

	% activity (wild-type = 100%)[a]		
	forward reaction		reverse reaction
mutant protein	phosphate assay[b]	coupled assay[c]	coupled assay[d]
N23A	0.35	0.1	4
N23S	0.12	0.1	2
D305A	nd[e]	nd	nd
D305C	nd	nd	nd
D305E	0.16	0.1	nd
D305H	nd	nd	nd

[a] Wild-type activity is 1.3 and 3.2 units mg^{-1} in the phosphate and coupled assay (forward reaction), respectively, and 1.7 units mg^{-1} in the reverse reaction. [b] Determination of phosphate was carried out as described in (*10*); background activity in this most sensitive assay was estimated to be ca. 0.005%. [c] The enzyme-coupled assay in the forward reaction was carried out as described in (*21*). [d] End-point determination of PEP generated in the reverse reaction using EP-UDPNAG and phosphate as substrates was performed as described under Materials and Methods. [e] nd = no activity above background level detectable.

The closely related enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) features a strictly conserved serine in the corresponding position of N23 in MurAs. Although this is certainly not the only replacement required to switch the substrate specificity from UDPNAG to S3P, the asparagine to serine exchange may contribute to a higher affinity of S3P binding. To test this possibility, we have checked the enzymatic activity of the N23S mutant protein with shikimate 3-phosphate (S3P) as the enolpyruvyl-accepting substrate. However, no activity was detected with either wild-type enzyme or the N23S mutant protein, indicating that this substitution alone does not alter the substrate specificity of MurA.

Binding of UDP-N-acetylglucosamine (UDPNAG) to the Mutant Proteins. Binding of UDP-*N*-acetylglucosamine (UDPNAG) to wild-type MurA can be conveniently measured by means of isothermal titration calorimetry (*11*), yielding a dissociation constant of 51 ± 11 μM. Neither the D305E nor the D305A mutant protein produced an exothermic signal as observed with wild-type enzyme (*11*). From the experimental setup used in the microcalorimetric experiments (ratio of protein to ligand and enzyme concentration), determination of a dissociation constant below or equal to $\approx$0.5 mM would have been feasible provided that deletion of a putative interaction between the ligand and the protein has no influence on the intensity of the exothermal signal. Hence, it can be concluded that the dissociation constant for UDPNAG is at least 10 times higher for the D305A and -E mutant proteins compared to that of wild-type enzyme. An estimation of the dissociation constant of UDPNAG to these mutant proteins was obtained by determining the concentration of free UDPNAG in equilibrium with UDPNAG bound to MurA as described under Materials and Methods. With this assay, the dissociation constant of UDPNAG to the D305E and D305A mutant proteins is 15- and 70-fold higher, respectively, than that for wild-type MurA.

Similarly, isothermal titration experiments with the N23 mutant proteins failed to generate a signal using UDPNAG as ligand. However, dissociation constants were estimated using the same approach as for the D305 mutant proteins. In contrast to these, the binding affinity is reduced only slightly for the N23S and N23A mutant proteins by a factor

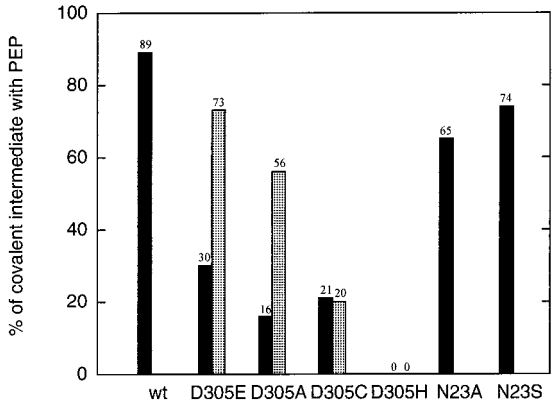


FIGURE 3: Formation of the *O*-phosphothioketal with the D305 and N23 mutant proteins as a function of UDPNAG concentration. Bar diagram of covalent adduct formed as the percentage of total protein with the N23 and D305 mutant proteins. Black bars represent the percentage of covalent adduct in the presence of stoichiometric amounts of UDPNAG and dotted bars the percentage of covalent adduct in the presence of a 100-fold excess of UDPNAG (2 mM) over the concentration of MurA (20 μM).

of ≈2 and ≈3, respectively. Apparently, replacement of N23 reduces the intensity of the exothermal signal significantly without having a major effect on the binding affinity of UDPNAG.

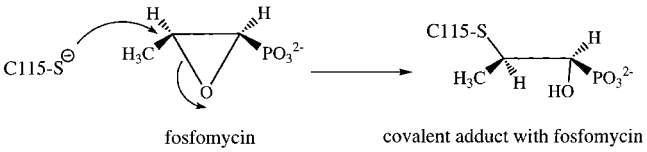
Binding of Phosphoenolpyruvate (PEP) to D305 and N23 Mutant Proteins in the Absence of UDPNAG. As binding of PEP to MurA is not associated with heat changes, isothermal titration microcalorimetry cannot be employed to determine dissociation constants (*11*). However, a fluorometric assay based on the quenching of the tryptophan fluorescence emission can be utilized to determine dissociation constants for the binding of PEP to MurA (*11*). In contrast to UDPNAG, binding of PEP to the D305 and N23 mutant proteins was unaffected by the amino acid exchanges in these two positions as judged by this method. The detailed results of several titrations performed with the D305 and N23 mutant proteins are summarized in Table 2.

Formation of the Covalent Adduct with PEP, the O-Phosphothioketal, in the Presence of UDPNAG. PEP has been demonstrated to react with the thiol group of cysteine 115 to form an *O*-phosphothioketal (*8*). This reaction occurs preferentially in the presence of UDPNAG. The amount of covalent adduct with PEP can be quantitated by means of anion-exchange chromatography (*11*). In fact, the presence of stoichiometric amounts of UDPNAG and an excess of PEP results in formation of 89% of this covalent adduct with wild-type MurA (*11*). As UDPNAG binding appears to be much weaker to the D305 mutant proteins, it was not unexpected to find reduced levels of *O*-phosphothioketal formation in the presence of stoichiometric concentrations of UDPNAG as shown in Figure 3 (black bars). In case of the D305A and D305E mutant proteins, formation of the *O*-phosphothioketal depends on the UDPNAG concentration, approaching the yield of the covalent adduct found with wild-type MurA at saturating concentrations (compare bar of wild-type to dotted bars of the D305A and D305E mutant proteins in Figure 3). On the other hand, formation of the covalent adduct with the D305C and D305H mutant proteins was not favorably affected by the UDPNAG concentration, indicating that UDPNAG binding is severely impaired in the latter two mutant proteins.

Table 2: Dissociation Constants for Binding of PEP to Wild-Type MurA and Mutant Proteins in the Absence of UDPNAG

protein	K_d (mM)
wild-type	0.24 ± 0.02
N23A	0.25 ± 0.01
N23S	0.23 ± 0.04
D305A	0.28 ± 0.04
D305C	nd
D305E	0.27 ± 0.01
D305H	0.37 ± 0.04

Scheme 3



fosfomycin covalent adduct with fosfomycin

The two N23 mutant proteins studied show slightly lower levels of the *O*-phosphothioketal when incubated with a stoichiometric concentration of UDPNAG as compared to wild-type enzyme, indicating a lower binding affinity of the sugar nucleotide (see Figure 3).

Formation of the Covalent Inhibitor Complex with the Antibiotic Fosfomycin in the Presence of UDPNAG. A similar but irreversible formation of a covalent adduct occurs with the antibiotic fosfomycin (Scheme 3) (*13*). This adduct is formed as a result of a nucleophilic attack of the thiol group of cysteine 115 on the epoxy ring of fosfomycin (*14*, *15*). Again, formation of this adduct is promoted in the presence of UDPNAG (*8, 16, 17*). Binding of fosfomycin in the presence of UDPNAG to wild-type MurA can be monitored by isothermal titration calorimetry, yielding an estimation of an apparent dissociation constant (*11*). ITC measurements with fosfomycin and the D305E mutant protein failed to produce a signal even at 100-fold excess of UDPNAG. This result is in keeping with the weaker binding of UDPNAG to these mutant proteins as demonstrated by ITC measurements with the sugar nucleotide substrate alone, estimation of dissociation constants, and the lack of covalent adduct formation with PEP (see above). Like the *O*-phosphothioketal, the enzymatically inactive fosfomycin adduct exhibits a distinct chromatographic mobility on anion exchange chromatography allowing the quantitation of adduct formation (*11*). Using this method, the amount of fosfomycin adduct formed was found to depend on the concentration of UDPNAG in the case of the D305E mutant protein (Figure 4). However, with the other three D305 mutant proteins, no fosfomycin adduct could be detected chromatographically. To obtain positive evidence for the absence of the fosfomycin adduct, the D305 mutant proteins were incubated with fosfomycin in the presence of a 100-fold excess of UDPNAG and digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry. As shown in Figure 5, reaction of the thiol group of C115 of wild-type MurA with fosfomycin and UDPNAG leads to the complete disappearance of the mass at 1618 Da (*12*) and concomitant appearance of a mass at 1756 Da (Figure 5, top panel). A similar result is obtained with the D305E mutant protein (Figure 5, second panel from bottom) but not with the D305A and D305H mutant proteins. The D305C mutant protein appears to give rise to some covalent attachment of fosfo-

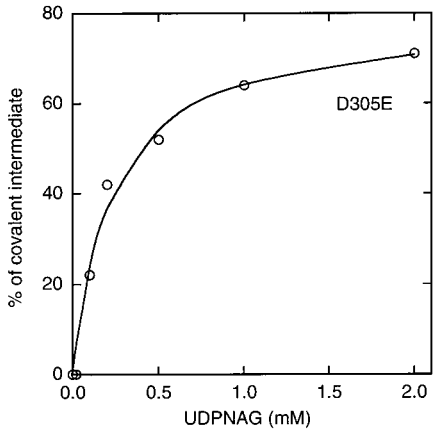


FIGURE 4: Formation of covalent adduct with the antibiotic fosfomycin. Binding of fosfomycin as a function of UDPNAG concentration. The concentration of MurA was 20 μM and that of fosfomycin 200 μM. The data points were fitted with a hyperbolic equation represented by the solid line.

mycin; however, as judged by the dominant mass peak at 1618 Da, the majority of the mutant protein remains unmodified. The mass peak at $MH^+ = 1660$ Da for wild-type MurA (Figure 5, top panel) corresponds to a fragment comprising amino acids 295−310 (calculated $MH^+ = 1658$ Da). This peptide fragment is produced by tryptic (R294) and chymotryptic (trace amount of chymotrypsin contained in the trypsin preparation used in the experiment) cleavage (F310) and, as it comprises amino acid 305, is subject to a mass shift corresponding to the introduced mutation (see labeled peaks in Figure 5). This mass spectrometric analysis clearly confirms that, with the exception of the D305E mutant protein (and, to a much smaller extent, the D305C mutant protein), fosfomycin is not covalently attached to the thiol group of C115 in the case of the D305A and D305H mutant proteins under the experimental conditions used.

Formation of the covalent fosfomycin adduct with the N23 mutant proteins in the presence of stoichiometric amounts of UDPNAG was first established by anion-exchange chromatography (data not shown). Under these conditions, the percentage of adduct formation was 72% and 78% with the N23S and N23A mutant protein, respectively, and hence slightly lower than with wild-type MurA. Hence, binding of fosfomycin to the N23A and N23S mutant proteins in the presence of UDPNAG was studied in more detail by microcalorimetry. ITC measurements are shown in Figure 6, and the thermodynamic parameters obtained from the titrations are summarized in Table 3. The apparent dissociation constant of fosfomycin is ca. 20- and 200-fold higher with the N23A and N23S mutant protein, respectively, demonstrating weaker binding as compared to wild-type MurA. With both N23 mutant proteins, binding of fosfomycin is associated with ca. 5-fold more negative binding entropy than found with wild-type enzyme. This entropic effect is partially compensated by a 2-fold more negative binding enthalpy. This result suggests that the initial binary complex of N23 mutant protein with UDPNAG is less ordered, and binding of UDPNAG is less favorable (ΔH is less exothermic) than is the case with wild-type enzyme.

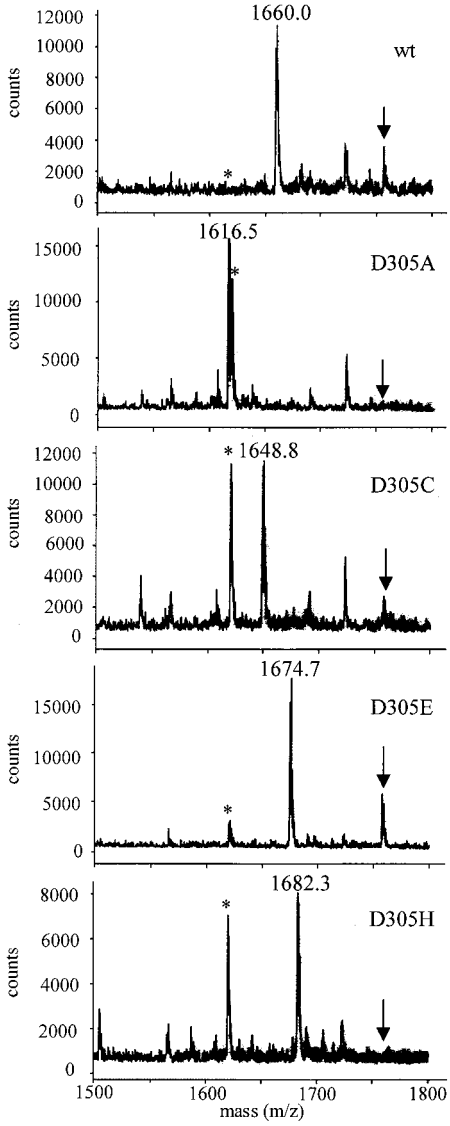


FIGURE 5: Mass spectral analysis of the D305 mutant proteins incubated with fosfomycin and a 100-fold excess of UDPNAG followed by tryptic digestion. The mass peak at 1618 (MH^+ peak marked with an asterisk) is due to the peptide fragment comprising amino acids 104−120 and disappears upon formation of a C115-bound covalent fosfomycin adduct, creating a new peak at 1759 (MH^+ peak marked with an arrow). The mass peak observed at 1660 for wild-type MurA is due to a peptide comprising amino acids 295−310 (calculated mass = 1658) and shifts with the D305 mutation introduced. The masses of these peptide are indicated in each panel.

DISCUSSION

The three-dimensional structure of MurA suggested a role for D305 as the base abstracting a proton from the 3′-hydroxyl group of UDPNAG, thereby facilitating the nucleophilic attack on C-2 of PEP (*4*). The low catalytic activity found with all four D305 mutant proteins clearly supports this role for the carboxylate side chain of D305 and emphasizes the importance of this step in the catalytic mechanism of the enolpyruvyl transfer reaction. In addition to its role in catalysis, D305 is obviously also important for binding of the sugar nucleotide substrate as demonstrated by the absence of an exothermal signal in ITC experiments and the higher dissociation constants. Binding of the sugar nucleotide substrate precedes the formation of the covalent adduct with either PEP [the *O*-phosphothioketal (*8*)] or the antibiotic fosfomycin (*14*). Therefore, it was not unexpected to find strong effects on the formation of these adducts in all D305 mutant proteins. In the case of the covalent PEP

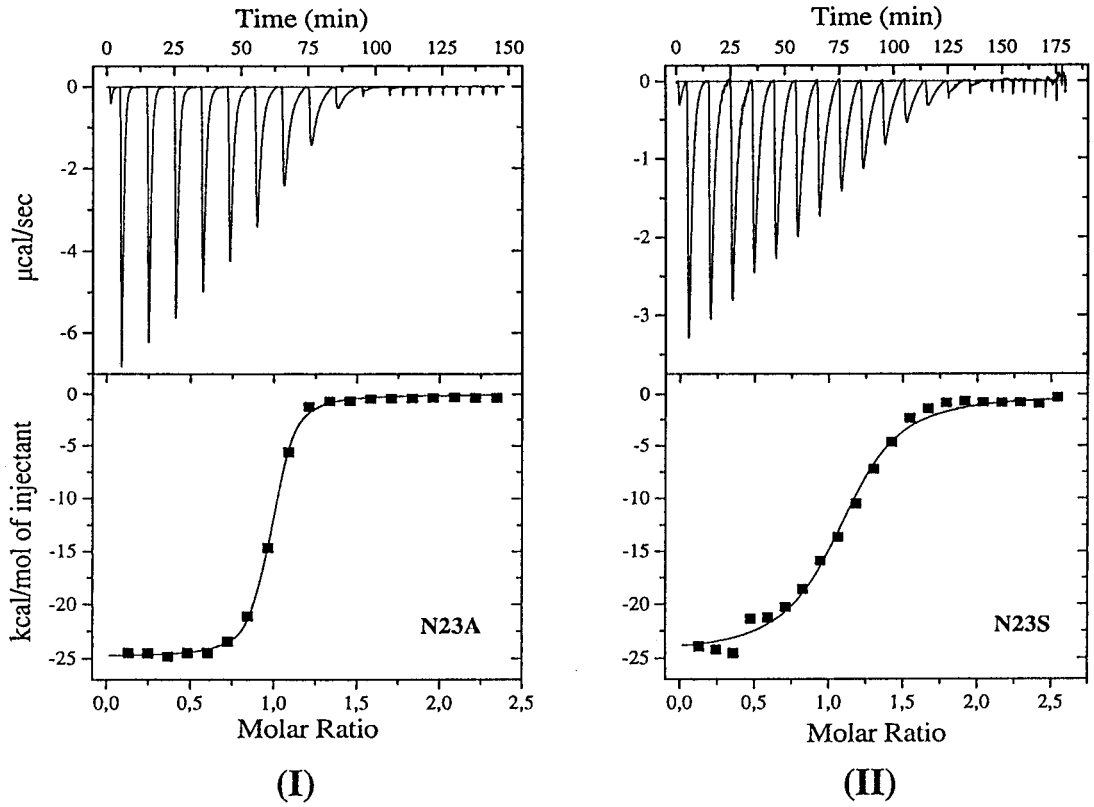


FIGURE 6: Isothermal titration calorimetry of N23 mutant proteins with the antibiotic fosfomycin in the presence of UDPNAG. Protein concentrations in the cell were 100−130 μM in the presence of 1 mM UDPNAG. The concentration of fosfomycin in the injection syringe was 2.5 mM. Top parts in panels I and II show the time-dependent release of heat (negative peaks) during the titration with fosfomycin. Bottom parts give the amount of heat measured per mole of fosfomycin injected as a function of the molar ratio of fosfomycin added per MurA. The solid curve represents the best fit using a single binding model (for values see Table 3).

Table 3: Thermodynamic Parameters of Fosfomycin Binding in the Presence of UDPNAG to Wild-Type MurA and the Two N23 Mutant Proteins As Determined by Isothermal Titration Calorimetry

protein	stoichiometry, n	K_d, nM	ΔG, kcal mol^{-1}	ΔH, kcal mol^{-1}	ΔS, cal mol^{-1} K^{-1}
wild-type[a]	0.86	<25	<−14.2	−10.9	>−11
N23A[b]	0.94	550 ± 100	−8.5 ± 0.1	−24.2 ± 0.6	−52.7 ± 2.1
N23S[c]	1.02	4800	−7.3	−24.3	−57.1

[a] Values taken from (*11*). [c] Values are the average of three measurements. [c] Values are the average of two measurements.

adduct, the low levels of *O*-phosphothioketal formed in the presence of stoichiometric amounts of UDPNAG could be increased 2.5- and 4-fold for the D305E and D305A mutant proteins, respectively, whereas with the D305C and D305H mutant proteins saturating UDPNAG concentrations had no effect on the low level of PEP adduct formation (20 and 0%, respectively). As judged from these experiments, the binding affinity of UDPNAG decreases in the order D305E >D305A > D305C > D305H. As deprotonation of the 3′-hydroxyl group is not required for the formation of the *O*-phosphothioketal, these results demonstrate that binding of UDPNAG to the D305 mutant proteins is diminished in comparison to wild-type protein. On the other hand, they also show that the carboxylate is not directly involved in stabilization of the *O*-phosphothioketal, as the D305A mutant protein lacking this group forms this covalent species to a similar level as the wild-type.

The ability to form a covalent adduct with fosfomycin was similarly affected by mutations at position 305. In fact, the D305E and, to a much lesser degree, the D305C mutant proteins were the only variants retaining the ability to covalently bind fosfomycin as demonstrated by anion-exchange chromatography and mass spectrometric analysis (Figures 4 and 5). Therefore, and in contrast to formation of the *O*-phosphothioketal, it appears that the carboxylate group is essential for the enzyme's ability to form the covalent fosfomycin adduct. The carboxylate group of D305 appears to orient the sugar nucleotide in such a way that binding interactions between the amino acid side chains in the active site, UDPNAG, and the antibiotic fosfomycin are in the correct spatial arrangement. Therefore, it is proposed that the covalent linkage of the C115 thiol group and fosfomycin takes place after binding of the latter to the active site of MurA in direct proximity to UDPNAG. This model is consistent with earlier studies of fosfomycin inhibition of MurA which have indicated formation of a fosfomycin−UDPNAG−MurA complex prior to covalent linkage of the antibiotic to the thiol group of C115 (*14*). In addition, reversible fosfomycin inhibition of the C115D mutant protein, competitive with respect to PEP, supports the existence of such a complex (*15*). However, the differences observed between the formation of the *O*-phosphothioketal and the fosfomycin complex, respectively, also indicate that the PEP binding site is not identical with the fosfomycin binding site. Unfortunately, a three-dimensional structure of the *O*-phosphothioketal required for a more precise comparative discussion of the factors involved in stabilizing the respective adducts has not been determined thus far.

Although N23 is strictly conserved in MurAs and despite the availability of two three-dimensional structures (see

Figure 1, panels B and C), its exact role in the active site is not as clearly defined as that of D305. As N23 engages in a hydrogen bond interaction with the 3′-hydroxyl group of UDPNAG, it was suggested to be involved in the binding of the latter (*4*). This involvement of N23 could be confirmed by our mutagenesis experiments although the effect on binding and concomitant formation of the *O*-phosphothioketal and fosfomycin adduct, respectively, are not as pronounced as with the D305 mutant proteins. On the other hand, the low activities measured for the N23A and N23S mutant proteins clearly indicate an involvement of this amino acid in catalysis, i.e., in stabilization of the transition state(s). The molecular basis of this stabilizing effect can be rationalized as follows: (i) the amide nitrogen forms a hydrogen bond to the oxyanion which is formed by deprotonation of the 3′-hydroxyl group by the carboxylate group of aspartate 305; and (ii) the carbonyl group participates in a network of hydrogen bond interactions which involve the side chains of R397 and K22 and the carboxylate and phosphate groups of the substrate (see Figure 1B,C), and hence N23 contributes to the stabilization of reaction intermediates occurring after the deprotonation event. The first role emphasizes that D305 and N23 work in concert to bring about the nucleophilic attack of the 3′-oxygen on the C-2 position of PEP. As a serine side chain could in principle fulfill this role as a hydrogen bond donor to the incipient 3′-oxyanion, it appears that this is not the major role of the asparagine. This conclusion is supported by the lower activity observed with the N23S mutant protein (see Table 1) and the 9-fold higher apparent dissociation constant with the antibiotic fosfomycin in comparison to the N23A mutant protein (see Table 3).

The thermodynamic parameters obtained from ITC measurements (Table 3) also demonstrate the importance of N23 in binding of reaction intermediate analogues such as the fosfomycin adduct. In the case of wild-type MurA, binding of UDPNAG is associated with a ΔH of -12.6 kcal mol^{-1}, and additional binding of fosfomycin to the preformed MurA−UDPNAG complex results in an additional ΔH of -10.9 kcal mol^{-1} (*11*). This amounts to an overall ΔH of -22.5 kcal mol^{-1}. In the case of the N23 mutant proteins, no exothermic signal was observed upon addition of UDPNAG alone, but titration with fosfomycin in the presence of UDPNAG resulted in an ΔH of -24 kcal mol^{-1}, i.e., the sum of the individual binding steps observed with wild-type MurA. This suggests that although binding of UDPNAG to the N23 mutant proteins is only slightly weaker as compared to wild-type enzyme, binding does not appear to result in the exact same binary complex as reflected by the lack of an exothermal signal in ITC experiments. However, addition of fosfomycin enforces the formation of a ternary complex with similar interactions as compared to wild-type MurA. The higher apparent dissociation constant of fosfomycin to the N23 mutant proteins results from a much more negative binding entropy, i.e., the entropic costs to have an alanine or a serine instead of an asparagine in this position. In the case of wild-type MurA, binding of UDPNAG is associated with a loss of entropy ($\Delta S = -23 \pm 5$ cal mol^{-1} K^{-1}) which was interpreted as being indicative of (partial) formation of a closed enzyme structure (*11*). It is conceivable that binding of UDPNAG to the N23 mutant proteins does not yield such a partially closed conformation and that only upon additional binding of fosfomycin is a closed conformation formed. This interpretation is in keeping with the large loss of entropy observed in ITC experiments with the N23 mutant proteins and fosfomycin.

Interestingly enough, a serine is found in all but 2 (out of 50 sequences aligned) 5-enolpyruvylshikimate-3-phosphate synthases (EPSPSs) in the corresponding position, indicating that the structure of the active site of EPSPS is designed such that the loss of stabilization, occurring in the N23 mutant proteins in MurA, is avoided or compensated. The current lack of a three-dimensional structure of EPSPS in its closed conformation, however, prevents a detailed discussion of this issue. Likewise, the nature and position of the proton-abstracting base in EPSPS remain an open question. Based on a sequence alignment performed by Marquardt (*18*), it was proposed that a conserved aspartic acid in position 313 (in the *E. coli* sequence) serves as a base in EPSPS (*4*). However, recent sequence information has shown that this aspartate is not conserved in all EPSPSs. Two other highly conserved aspartate residues emerge from a sequence alignment of these 50 EPSPS sequences (data not shown), i.e., aspartate 242 and 384 (*E. coli* sequence). A recent mutagenesis study has demonstrated that the D242A and D384A mutant proteins are catalytically inactive and deficient of S3P binding (*19*), similar to the effects observed in our study with the MurA D305 mutant proteins. Hence, both aspartic acid residues qualify for the role as an active site base in EPSPS. Comparison of the structures of *En. cloacae* MurA (*2*) and *E. coli* EPSPS (*20*) reveals that D305 in *En. cloacae* MurA and D242 in *E. coli* EPSPS are in similar locations in these two open forms of the enzymes and, therefore, it is proposed that D242 in EPSPSs is the active site base in *E. coli* EPSPS.

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