Lysine 22 in UDP-*N*-Acetylglucosamine Enolpyruvyl Transferase from *Enterobacter cloacae* Is Crucial for Enzymatic Activity and the Formation of Covalent Adducts with the Substrate Phosphoenolpyruvate and the Antibiotic Fosfomycin†

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ABSTRACT: UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) catalyzes the first committed step in the biosynthesis of the bacterial cell wall component peptidoglycan. The enzyme is the target of the antibiotic fosfomycin. A lysine residue (K22), strictly conserved in MurAs and the structurally and mechanistically related 5-enolpyruvylshikimate 3-phosphate synthases (EPSPS), is located near the active center of the enzyme. This residue is thought to be involved directly in the binding of the substrate phosphoenolpyruvate (PEP) and also to participate in the conformational change leading to the formation of the catalytically competent enzyme complex. Using site-directed mutagenesis, we have replaced this lysine with arginine (K22R), valine (K22V), and glutamate (K22E). These mutant proteins were expressed, purified, and characterized in comparison to wild-type MurA and a previously described inactive C115S mutant protein. It was found that all three K22 mutant proteins had less than 0.5% of the wild-type activity. Using isothermal titration calorimetry, it could be shown that the binding parameters for the UDP-sugar nucleotide substrate are not affected by the mutations, except for the K22E mutant protein. Similarly, binding of PEP was found to be unaffected in the K22 mutant proteins as demonstrated by tryptophan fluorescence quench titrations. On the other hand, the level of formation of a covalent adduct with either PEP or fosfomycin with the thiol group of cysteine 115 was diminished. The propensity to form an adduct with PEP decreased in the following order: wild type ≫ K22R > K22V > K22E. A comparable effect was found on the formation of the inhibitory covalent adduct of MurA and the antibiotic fosfomycin. These results are discussed in terms of an involvement of lysine 22 in a conformational change of MurA.

UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA)[1] catalyzes the first committed step in the biosynthesis of murein in eubacteria. The reaction involves the transfer of the enolpyruvyl moiety to the 3′-hydroxyl group of the sugar nucleotide substrate with the concomitant release of inorganic phosphate (Scheme 1). This reaction represents one of only four known examples of an enzymatic transfer of PEP. The other three reactions are catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, and 3-deoxy-D-*manno*-2-octulosonate-8-phosphate synthase. In contrast to the enol ether transfer reaction catalyzed by MurA and EPSPS, the reactions catalyzed by the latter two enzmyes involve a net aldol condensation leading to incorporation of the phosphoenolpyruvate moiety into a pyranose ring system (for a review, see ref *1*).

The mechanistic similarity of MurA and EPSPS among enolpyruvyl phosphate transferases is reflected by a close relationship of the three-dimensional topology of the enzymes (*2*−*4*). In fact, the three-dimensional structures of MurA and EPSPS are unique among protein structures and were originally described as an "inside-out α/β-barrel" (single domain) or "mushroom button" (both domains) (*2*). Moreover, MurAs and EPSPSs share a set of strictly conserved amino acids distributed over the entire length of the amino acid sequence. Among these amino acid residues is lysine 22 (position in *Enterobacter cloacae* MurA).

In the case of MurA, the structures of two very distinct conformations were reported. In the absence of substrates, an "open" conformation was observed by Schönbrunn et al. (*3*), while in the presence of UDPNAG and fosfomycin, a "closed" conformation was found by Skarzynski et al. (*4*). The major differences between these two structures can be summarized as follows (see Figure 1 for a graphical representation). (1) A loop region (amino acids 111−121 in the *En. cloacae* sequence), including a catalytically important cysteine residue, moves, during the transition from the open to the closed form, toward the UDPNAG binding site

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[1] Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; EP-UDPNAG, UDP-*N*-acetylenolpyruvylglucosamine; fosfomycin, (1*R*,2*S*)-1,2-epoxypropylphosphonic acid; ITC, isothermal titration calorimetry; 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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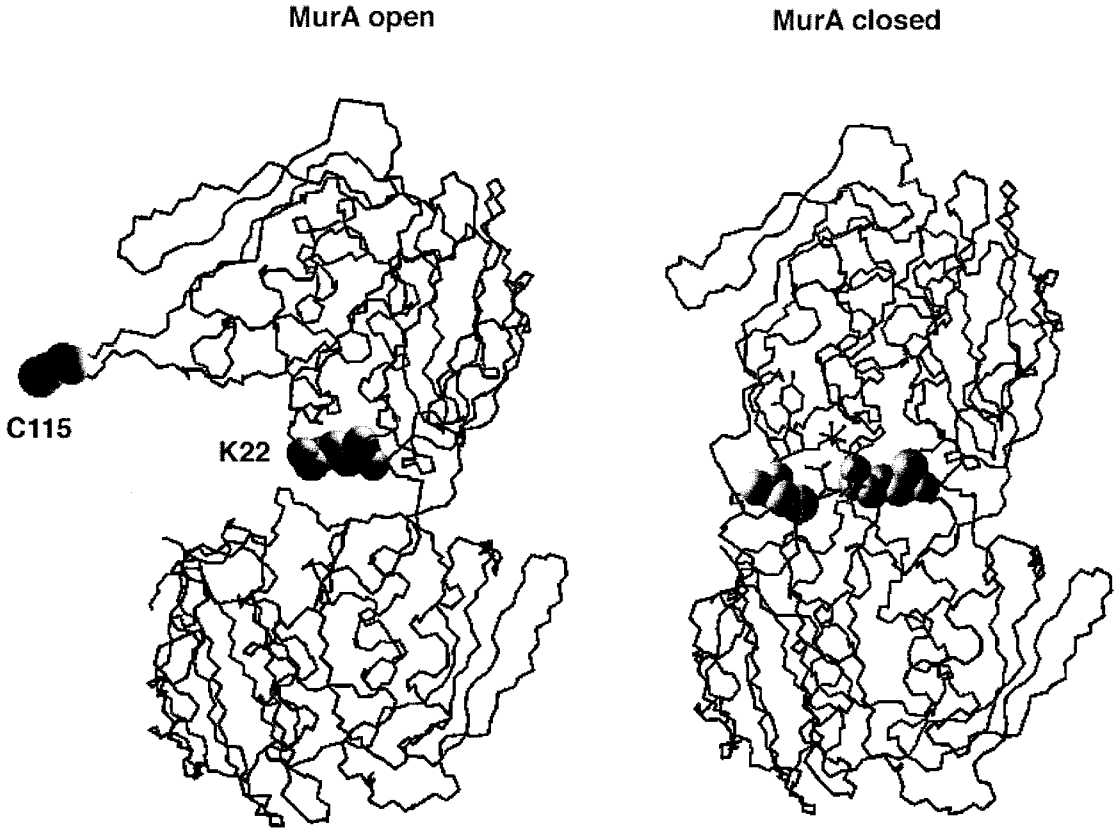
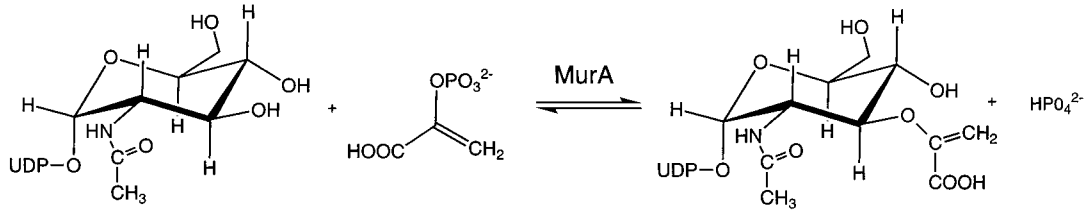


FIGURE 1: Three-dimensional structure of MurA in the open (left structure, PDB entry 1NAW) and closed (right structure with bound UDPNAG and fosfomycin, PDB entry 1UAE) conformation. The positions of cysteine 115 and lysine 22 are highlighted as space filling models.

Scheme 1



UDP-*N*-acetylglucosamine
UDPNAG

UDP-*N*-acetylenolpyruvylglucosamine
EP-UDPNAG

forming a lid on the active center, and (2) the interactions between two adjacent α-helices in the "hinge" region are reorganized. A salt bridge between the side chains of aspartate 49 and lysine 22 in the open form is abolished in the closed form in favor of an interaction of the lysine 22 side chain with asparagine 23 in the same helix and an oxygen of either the phosphonate group of the covalently bound inhibitor fosfomycin (*4*) or the phosphate group of the (*Z*)-3-fluorophosphoenolpyruvate-derived tetrahedral intermediate (*5*). Hence, it is conceivable that lysine 22 participates in the processes leading to the conformational change via reorganization of the interactions in the hinge region and in the binding of fosfomycin or PEP via interaction with the phosphonate or phosphate group, respectively. The importance of lysine 22 is also emphasized by its strict conservation among MurAs and EPSPSs. For the latter enzymes, the first evidence for the involvement of lysine 22 in catalysis and binding was obtained by chemical modification studies with pyridoxal 5′-phosphate (*6*). This was confirmed by site-directed mutagenesis experiments with the enzyme from *Petunia hybrida* (*7*). In this study, only the lysine to arginine mutant protein exhibited enzymatic activity (15% of that of wild-type EPSPS), while the K22A and K22E mutant proteins were catalytically inactive. A critical role of lysine 22 was also confirmed by recent investigations of the corresponding mutant protein from *Bacillus subtilis* (*8*) and *Escherichia coli* (*9*). Characterization of these mutant proteins indicated a role of this lysine in binding of the substrate S3P. In contrast, neither the open nor the closed structure of MurA suggests that lysine 22 is involved in binding of UDPNAG.

Here we describe the characterization of three proteins with mutations of the corresponding lysine 22 residue in MurA, i.e., K22R, K22E, and K22V. These mutant proteins were studied (i) to establish the function of the lysine in substrate binding, (ii) to establish its role in the conformational change occurring upon substrate binding, and (iii) to compare its role in MurA and EPSPS.

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 in ref *10*.

Enzymes. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, lactate dehydrogenase (EC 1.1.1.27) from bovine heart, and pyruvate kinase (EC 2.7.1.40) from rabbit muscle 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 (*11*).

Site-Directed Mutagenesis. Mutagenesis was carried out using the Quik-change site-directed mutagenesis kit from Stratagene. Oligonucleotides 29−33 bases in length were used to change the desired codon with respect to the codon usage of *En. cloacae*. The oligonucleotides were purified over Sep-Pak (C18) columns from Waters prior to PCR. In the PCR, the whole pKK233-2 plasmid containing the open reading frame encoding wild-type MurA from *En. cloacae* was amplified. Mutations were confirmed by sequencing with an ABI 373 DNA sequencer (Applied Biosystems, Foster City, 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 (*12*). The protein concentration was determined using an extinction coefficient of 24 020 M^{-1} cm^{-1} at 280 nm (*13*) or with the Bradford reagent from Pierce (Lausanne, Switzerland) using bovine serum albumin (BSA) for calibration.

Enzyme Assays. In the forward direction (left to right in Scheme 1), enzyme activities were determined on the basis of either the release of inorganic phosphate or an enzyme-coupled assay. In the first method, 10 μM enzyme was incubated in 50 mM Tris/HCl buffer (pH 7.4) containing 1 mM DTT at 25 °C with each substrate at 0.1, 1, and 10 mM. The amount of inorganic phosphate generated during the incubation was determined at different time points using the colorimetric assay described by Lanzetta et al. (*14*). The enzyme activity was then calculated via linear regression of the linear phase of the time course using the program SigmaPlot, version 4.1 (Jandel Scientific).

In the enzyme-coupled assay, MurA activity was coupled to the next step in murein biosynthesis, i.e., the activity of MurB which proceeds at the expense of NADPH. Consumption of NADPH (0.3 mM) was monitored at 340 nm in the presence of 1 μM wild-type MurA, or 10 μM K22 mutant proteins at substrate concentrations of 1 mM for UDPNAG and PEP. Data evaluation was carried out as described in the previous paragraph. All spectrophotometric measurements were carried out with a Uvikon 933 instrument (Kontron Instruments).

Enzyme activity in the reverse reaction (see Scheme 1) was also determined with an enzyme-coupled assay. MurA (2 μM) was incubated with 2 mM EP-UDPNAG and 10 mM sodium phosphate in 50 mM Tris/HCl (pH 7.4) containing 1 mM DTT at 25 °C. Aliquots (300 μL) of this reaction mixture were then transferred to a cuvette containing 700 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. In the case of the wild-type enzyme, the reaction was stopped by fosfomycin (2 mM final concentration). The amount of PEP formed during incubation with wild-type MurA or its K22 mutant proteins was determined from the amount of NADH consumed in the coupling reaction. The amount of PEP formed was plotted against the incubation time. The slope of this plot was taken as a measure of activity.

Fluorescence Measurements. The quench of the tryptophan fluorescence emission as a function of PEP concentration was measured with a fluorimeter from Kontron Instruments (model SFM25) using an excitation wavelength of 280 nm. All experiments were carried out at 25 °C in 50 mM Hepes/NaOH buffer (pH 7.4) containing 1 mM DTT. The protein concentration used in the titration experiments was 10 μM. The change of fluorescence at 338 nm was plotted against the concentration of PEP, and the data points were fitted with a hyperbolic function [$f(x) = -\Delta\text{fluorescence}_{max}[\text{PEP}]/(K_d + [\text{PEP}])$] using the program SigmaPlot, version 4.1 (Jandel Scientific).

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 (5 mM) was injected into 1.4 mL of the protein solution (200−300 μM) which was being stirred constantly at 200 rpm. The ligand was injected over a period of 10 s with 5 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 the stoichiometries were obtained by fitting the binding isotherm according to the binding equations incorporated in the Omega instrument software (Origin, version 2.9, MicroCal).

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 workstation (Pharmacia). The column (MonoQ HR 5/5) was equilibrated with 50 mM Bis-Tris-buffer/HCl (pH 7) (buffer A), and a step gradient with a final concentration of 0.4 M KCl was applied to elute the different forms of MurA (buffer B is buffer A containing

Table 1: Enzyme Activities of the K22 Mutant Proteins Compared to That of Wild-Type MurA

	% activity (wild-type = 100%)[a]		
mutant protein	forward reaction phosphate assay[b]	coupled assay[c]	reverse reaction coupled assay[d]
K22R	0.3	0.2	<0.5
K22V	0.03	0.1	~1
K22E	0.05	0.05	<0.5

[a] Wild-type activity is 1.3 and 3.2 units/mg in the phosphate and coupled assay (forward reaction), respectively, and 1.7 units/mg in the reverse reaction coupled assay. [b] Determination of phosphate was carried out as described in ref *14*. [c] The enzyme coupled assay in the forward reaction was carried out as described in ref *29*. [d] End point determination of PEP generated in the reverse reaction using EP-UDPNAG and phosphate as substrates was performed as described in Materials and Methods.

0.4 M KCl; 0 to 20% B in 5 columns volumes, 20 to 45% B in 25 column volumes, and, finally, 45 to 100% B in 1 column volume).

The covalent MurA adduct was produced by incubation of 20 μM enzyme in the presence of equimolar UDPNAG 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.

RESULTS

Expression and Purification of the K22 Mutant Proteins. As judged from SDS−PAGE analysis of crude bacterial extracts, all three K22 mutant proteins were expressed in quantities similar to those of wild-type MurA. Also, purification of the mutant proteins could be achieved following the procedure established for the wild-type enzyme (*12*). However, the mutant proteins exhibited reduced affinity for the reactive yellow resin used in the final purification step. In contrast to wild-type MurA, only approximately 40% of the mutant protein was bound to the reactive yellow material. The K22E mutant protein also eluted earlier from the column than did the K22R and K22V mutant proteins. Due to this altered chromatographic mobility, the overall yield of the homogeneous protein was only ca. 100 mg/10 g of wet cell paste for the K22 mutant proteins as compared to ca. 210 mg/10 g for wild-type MurA.

Determination of Enzyme Activities. Enzyme activities of wild-type MurA and the K22 mutant proteins are summarized in Table 1. In the forward reaction (left to right in Scheme 1), the activity decreases sharply in the following order: wild-type MurA ≫ K22R > K22V ≅ K22E. This was found independently with the two assay methods employed to assess enzyme activity. The background activity which is due to copurification of wild-type *E. coli* MurA is estimated to be on the order of 0.005%, i.e., 10−20-fold lower than that measured with the K22 mutant proteins. This estimation is based on activity measurements with the C115S (*11*) and D305A (A. Samland, N. Amrhein, and P. Macheroux, unpublished experiments) mutant proteins. In the reverse reaction (right to left in Scheme 1), only the K22V mutant protein exhibited detectable activity of ca. 1% as compared to that of the wild-type enzyme (see Table 1). This analysis clearly demonstrates the importance of lysine 22 for enzyme catalysis and suggests a distinct role for lysine 22 in the forward and backward reaction.

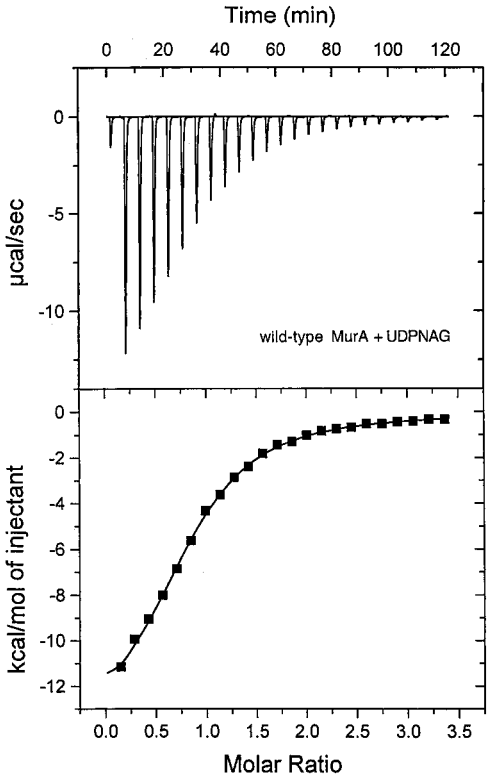


FIGURE 2: Isothermal titration calorimetry of wild-type MurA (220 μM) with UDPNAG (5 mM). The titration was carried out in 50 mM Hepes/NaOH (pH 7.4) containing 2 mM DTT and 0.5 mM EDTA at 25 °C and 200 rpm. The upper plot shows the time response of the exothermic signal as a function of the number of injected aliquots of UDPNAG (first injection was 1 μL, and all following injections were 8 μL). In the bottom graph, peak integrals are shown as a function of the molar ratio of UDPNAG and MurA.

Binding of UDP-N-Acetylglucosamine (UDPNAG) to Wild-Type and Mutant Proteins. In light of the recent finding that mutation of a lysine in an equivalent position in EPSPS resulted in the loss of S3P binding capacity (*8*, *9*), we set out to investigate characteristics of the binding of UDPNAG to the K22 mutant proteins. Dissociation constants for binding of UDPNAG to wild-type MurA and mutant proteins were determined by isothermal titration calorimetry (ITC). A representative titration is shown in Figure 2, and a summary of the collected data is given in Table 2. Titration of wild-type MurA with UDPNAG produces a series of negative peaks indicating exothermic binding of the sugar nucleotide (see Figure 2). A fit to these signals yields a K_d of 51 ± 11 μM which is in very good agreement with the value of 59 μM reported recently (*15*). The K22R and K22V mutant proteins exhibited dissociation constants similar to that of the wild-type enzyme, while the K22E mutant protein exhibited a higher K_d of 870 μM (see Table 2). This 17-fold increase of the dissociation constant is due to a more positive value for the binding enthalpy, while the binding entropy remained unchanged within the error of the experiment (Table 2). ITC measurements with the C115S mutant protein produced a slightly lower dissociation constant of 29 ± 1 μM. (*16*). Hence, it appears that the C115S replacement leads to a slight stabilization of the binary UDPNAG−MurA complex. This is remarkable since this amino acid is located in a loop in the upper domain of the protein (see Figure 1) which does not appear to participate in UDPNAG binding. However, UDPNAG binding is thought to induce a confor-

Table 2: Thermodynamic Parameters of UDPNAG Binding to Wild-Type MurA, the Three K22 Mutant, and the C115S Mutant Proteins As Determined by ITC[a]

protein	stoichiometry *n*	K_D (μM)	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
wild-type	0.84 ± 0.03	51 ± 11	−5.86 ± 0.12	−12.6 ± 1.5	−23 ± 5
K22R	0.93 ± 0.07	44 ± 8	−5.95 ± 0.12	−10.3 ± 0.5	−15 ± 1
K22V	0.96 ± 0.13	92 ± 4	−5.50 ± 0.02	−12.4 ± 1.7	−23 ± 6
K22E	0.98 ± 0.18	870 ± 260	−4.21 ± 0.19	−8.8 ± 1.0	−16 ± 4
C115S	0.96 ± 0.10	29 ± 1	−6.19 ± 0.02	−13.3 ± 1.2	−24 ± 4

[a] Values are the average of three measurements; protein concentrations were between 200 and 300 μM.

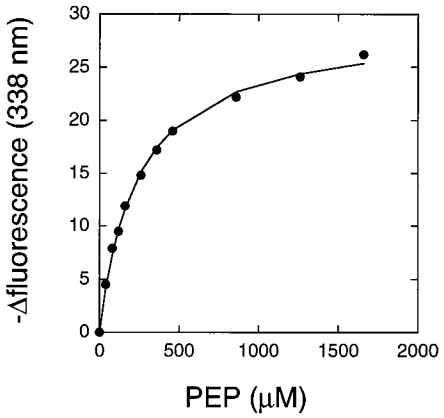


FIGURE 3: Titration of wild-type MurA with the substrate phosphoenolpyruvate (PEP) in the absence of UDPNAG. Tryptophan fluorescence emission quench was monitored as a function of phosphoenolpyruvate (PEP) concentration. Experimental data (●) were fitted with a hyperbolic function (solid line) as described in Materials and Methods. The fit results for this representative titration are 239 μM (K_d) and 29 ($-\Delta$fluorescence$_{max}$).

Table 3: Dissociation Constants for Binding of PEP to Wild-Type MurA and the K22 Mutant Proteins in the Absence of UDPNAG

protein	K_d (mM)[a]	protein	K_d (mM)[a]
wild-type	0.22	K22V	0.26
K22R	0.17	K22E	0.35

[a] Average of two titrations.

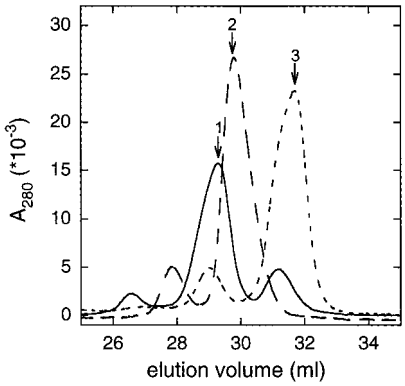


FIGURE 4: Chromatographic separation of wild-type MurA forms on a MonoQ column of free MurA (peak 1, solid line), a covalent adduct with fosfomycin (peak 2, dashed line), and a covalent adduct of MurA with PEP (peak 3, dotted line). The covalent adducts were prepared in the presence of UDPNAG as described in detail in Materials and Methods.

Table 4: Relative Formation of the *O*-Phosphothioketal with Wild-Type MurA and Mutant Proteins

	peak area in %		
protein	free MurA	covalent adduct	ratio of free/covalent adduct
wild-type	11	89	0.12
K22R	75	25	3
K22V	96	4	24
K22E	99	0.6	166

mational change which involves the movement of this loop toward the UDPNAG binding site ("closed" conformation as shown in Figure 1).

Binding of Phosphoenolpyruvate (PEP) to Wild-Type and Mutant Proteins in the Absence of UDPNAG. In contrast to UDPNAG, titration of MurA with PEP is not associated with detectable heat changes, and hence, dissociation constants could not be obtained by means of ITC. However, it was found that binding of PEP to MurA is associated with a partial quench of the tryptophan fluorescence emission (Figure 3). This property was exploited to obtain dissociation constants for PEP binding to wild-type MurA and the three K22 mutant proteins. A summary of the measured dissociation constants is compiled in Table 3. Since the dissociation constants determined for wild-type MurA and the K22 mutant proteins are comparable, it can be concluded that replacement of lysine 22 does not compromise the ability of MurA to bind PEP in the absence of UDPNAG.

Formation of the Covalent Adduct with PEP and with the Antibiotic Fosfomycin in the Presence of UDPNAG. It has been demonstrated that the thiol group of cysteine 115 is capable of reacting with PEP to yield a covalently bound *O*-phosphothioketal (*12*). This covalent adduct is generated preferentially in the presence of UDPNAG, indicating that formation of a "closed" conformation favors the reaction of the thiolate with PEP. Similarly, it was found that the presence of UDPNAG is a prerequisite for formation of the covalent linkage between fosfomycin and cysteine 115 (*17*). The covalent adduct with PEP can be separated from free MurA by anion exchange chromatography which allows the determination of the relative amounts of the two species (*12*). This altered chromatographic mobility was used to assess the formation of the covalent adduct with PEP for the K22 mutant proteins in comparison to wild-type MurA. Under the experimental conditions that were used (see Materials and Methods for details), 89% of the free MurA can be converted to the covalent adduct with PEP in the presence of UDPNAG (Table 4). The propensity to form a covalent adduct with PEP decreased drastically in the order wild type $\gg$ K22R > K22V > K22E as judged from the respective fractions of free MurA and covalent adduct (see Table 4).

As shown in Figure 4, the covalent adduct with fosfomycin also exhibits an altered chromatographic mobility compared to that of free MurA. This adduct was observed only with the wild type and the K22R mutant protein, while the other two K22 mutant proteins did not appear to form the covalent adduct (all experiments performed in the presence of UDPNAG as described for the experiments with PEP).

Table 5: Thermodynamic Parameters of Fosfomycin Binding to Wild-Type MurA and the Three K22 Mutant Proteins in the Presence of UDPNAG As Determined by ITC[a]

protein	stoichiometry n	K_D (nM)	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
wild-type	0.86	less than 25	less than −14.2	−10.9	greater than −11
K22R	0.85	460	−8.6	−14.1	−18
K22V	no binding observed				
K22E	no binding observed				

[a] Values are the average of two measurements. The concentration of the K22R mutant protein was 100 μM and that of the wild-type enzyme between 25 and 100 μM. A 10-fold excess of UDPNAG over protein was used in all titrations.

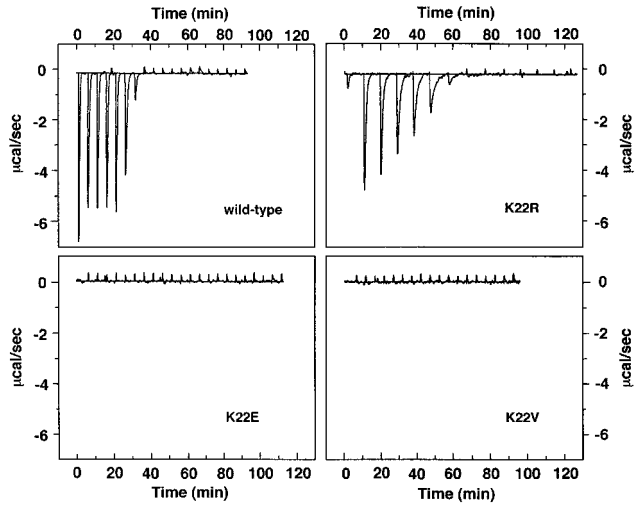


FIGURE 5: Isothermal titration calorimetry of wild-type MurA and the three K22 mutant proteins with fosfomycin in the presence of UDPNAG.

This was further substantiated by the determination of apparent dissociation constants for fosfomycin in the presence of UDPNAG using microcalorimetry. With wild-type MurA, binding was found to be very tight with an upper value for the K_d of approximately 25 nM (see Figure 5 and Table 5). The apparent dissociation constant for binding of fosfomycin to the K22R mutant protein in the presence of UDPNAG was at least 1 order of magnitude higher (K_d = 460 nM), indicating a much weaker binding compared to that of the wild-type protein. The other two mutant proteins exhibited no detectable binding of fosfomycin in the presence of UDPNAG, in agreement with the results obtained from the chromatographic experiments.

DISCUSSION

The characterization of three K22 MurA mutant proteins from *En. cloacae* has demonstrated the essential role of this lysine in catalysis. Enzymatic activity, in the forward direction, is decreased to ca. 0.3% in the K22R mutant protein, with a further decrease in the K22V and K22E mutant proteins. In the reverse reaction (right to left in Scheme 1), only the K22V mutant protein exhibited detectable activity. This result also suggests that lysine 22 plays a differential role in catalytic and/or binding events leading to substrate or product turnover.

Isothermal titration calorimetry revealed that binding of UDPNAG is basically unaffected in the K22R and K22V mutant proteins compared to that in the wild-type protein. In these proteins, binding of UDPNAG is enthalpically driven with a negative entropic contribution (Table 2). The only exception is the K22E mutant protein, which exhibits a dissociation constant that is 17-fold higher than that of wild-type MurA. This indicates that a negatively charged amino acid side chain produces a perturbation at or near the UDPNAG binding site, resulting in the increased dissociation constant.

The negative entropy that is observed indicates that UDPNAG binding is accompanied by a protein conformational change. This interpretation is in agreement with the structural rearrangements required for the "open" form to adopt the more tightly packed "closed" conformation of MurA (*3*, *4*). It appears that, with the exception of the K22E mutant protein, binding of UDPNAG and the mechanism of the accompanied conformational change are normal in the K22R and K22V mutant proteins.

As far as PEP binding in the absence of UDPNAG is concerned, only minor effects were found on the dissociation constant as judged from tryptophan fluorescence emission quench titrations. From this result, it can be concluded that the (initial) PEP binding site remains largely intact in all of the mutant proteins.

In contrast to UDPNAG and PEP binding, a massive effect was observed for the K22 mutant proteins on the formation of a covalent adduct with either PEP or fosfomycin in the presence of UDPNAG. In the case of wild-type MurA, the thiol group of cysteine 115 attacks C-2 of PEP to form a *O*-phosphothioketal or the C-2 of fosfomycin to yield a 2-[(*S*)-L-cysteinyl]-1-hydroxypropylphosphonic acid (*12*, *18*). These reactions are favored by the preformation of a binary UDPNAG−enzyme complex (*12*, *17*). Since the thiol group of cysteine 115 is readily accessible in the open form, acceleration of the nucleophilic reaction in the closed form of MurA appears to be a consequence of a lower pK_a value of the thiol group. Hence, it can be argued that generation of the covalent adduct reflects the ability of MurA to lower the pK_a value of the thiol group of cysteine 115 or, in other words, the ability to form the closed conformation. In fact, the decrease in activity found for the K22 mutant proteins in comparison to wild-type MurA is paralleled by their inability to form the covalent adduct with PEP (compare Tables 1 and 3). Hence, it could be argued that formation of the adduct is crucial for catalysis. However, studies with C115 mutant proteins have unequivocally demonstrated that a cysteine residue, and hence formation of a *O*-phosphothioketal adduct, is not required for enzyme activity (*16*). Moreover, kinetic studies in the same laboratory have shown that the *O*-phosphothioketal is in rapid equilibrium with a noncovalent tetrahedral intermediate formed during catalysis (*19*). In other words, the *O*-phosphothioketal is discussed as an "off-pathway" intermediate. In agreement with this interpretation of the kinetic results, the *O*-phosphothioketal adduct was shown to be catalytically competent (*12*) and could be detected as an early intermediate in the enzymatic

reaction as evidenced by time-resolved solid-state NMR spectroscopy (*20*). In view of this evidence, the decreased activity of the K22 mutant proteins cannot directly be ascribed to the inability to form the covalent *O*-phosphothioketal species. However, since the *O*-phosphothioketal adduct and the tetrahedral reaction intermediate are in equilibrium, it appears likely that alterations in the active site will affect the stability of both species. Examination of the three-dimensional structure of MurA in the presence of a transition state analogue reveals an interaction between the ϵ-amino group of lysine 22 and the phosphate group of 3-fluorophosphoenolpyruvate contributing to the stabilization of this species (*5*). However, if the number of contacts between amino acids and the transition state in the active site is considered, it appears unlikely that removal of a single interaction provides a full explanation for the loss of activity found for the K22 mutant proteins. Comparison of the three-dimensional structures of the open and closed conformations of MurA suggests that lysine 22 participates in the protein conformational change occurring upon substrate binding (*3*, *4*). This involvement of lysine 22 in the mechanism(s) governing a protein conformational change receives support from two observations. (i) The closed conformation with fosfomycin and UDPNAG bound cannot be formed with the K22V and K22E mutant proteins. (ii) The mutant proteins exhibited a progressive inability to form the covalent adduct with PEP. Therefore, we propose that the crucial role of lysine 22 as part of the conformational switch rationalizes the catalytic inactivity of the K22 mutant proteins. Since neither PEP nor UDPNAG binding is compromised by the lysine 22 replacements, it appears that this amino acid side chain is not directly involved in substrate recognition. This role is carried out by other, yet unidentified, amino acids which upon binding of PEP and/or UDPNAG relay their presence to lysine 22 which then acts as part of the mechanism leading to a closed protein conformation. The role and identity of amino acids involved in substrate recognition are currently subject to further investigations in our laboratory.

Comparison with Replacement of the Corresponding Lysine in EPSPS. Since lysine 22 is a strictly conserved amino acid in MurAs as well as EPSPSs, it can be hypothesized that this residue has a similar function in these enolpyruvyl transferases. Mutant proteins of EPSPS with amino acid exchanges in a corresponding position have been studied in the enzymes from *P. hybrida* (*7*), *B. subtilis* (*8*), and *E. coli* (*9*). In the first report (*7*), lysine 22 (in position 23 in the *P. hybrida* sequence) was replaced with either arginine, glutamate, or alanine. Of these three mutant proteins, only the lysine to arginine mutant protein retained some activity (15%), whereas the other two mutant proteins were inactive. Moreover, the lysine to arginine replacement did not affect the K_m values for S3P and PEP, in contrast to the lysine to alanine mutant protein which did not bind S3P. Studies with the EPSPS K19E mutant protein from *B. subtilis* (this position corresponds to position 22 in *En. cloacae*) (*8*) and the K22R mutant protein from *E. coli* (*9*) substantiated the loss of S3P binding. However, the lysine to arginine replacement in EPSPS from *P. hybrida* appears to exhibit a substantial residual activity of 15%, whereas the same mutation resulted in a complete loss of activity (below 0.01%) in the *E. coli* enzyme expressed in an *aro*$^-$ *E. coli* strain. Since the *P. hybrida* mutant proteins were expressed in an *aroA*$^+$ *E. coli* strain, it is unclear how much of the reported activities are due to a contamination of the protein preparation with *E. coli* wild-type EPSPS. Our results with the K22 MurA mutant proteins clearly indicate a crucial role for this residue in catalysis in keeping with the results obtained from the mutagenesis experiments with *E. coli* EPSPS. In contrast to EPSPS, lysine 22 in MurA does not participate in binding of the enolpyruvyl-accepting substrate UDPNAG, as judged by the dissociation constants found for UDPNAG with the wild-type enzyme and the K22R and K22V mutant proteins. This difference could be ascribed to the absence of a phosphomonoester group adjacent to the reacting hydroxyl group in UDPNAG and suggests that the phosphomonoester group of S3P may interact with the ϵ-amino group of lysine 22 in EPSPS. In fact, interactions between the phosphate group of S3P and the side chains of a lysine and an arginine residue of EPSPS have been inferred from REDOR-NMR measurements of the ternary complex (*21, 22*). The results from the mutagenesis experiments mentioned above support the conclusions drawn from the REDOR-NMR measurements. However, X-ray crystallographic data for the binary complex of EPSPS and S3P indicate a set of serine side chains as hydrogen bond donors for the phosphate group rather than lysine and arginine side chains (*23*). This would suggest that formation of the ternary complex is associated with the displacement of the serine residues by lysine and arginine residues.

As discussed above, the inability of the mutant proteins to form the covalent adduct with fosfomycin in the presence of the sugar nucleotide appears to reflect the inability to undergo a protein conformational change required for the formation of a catalytically competent complex with the substrates as shown in Figure 1 (right structure). A similar closure of the open form of EPSPS was inferred from various studies aiming to establish the existence of a closed conformation (*24*−*28*). Analogous to the substrate-induced conformational change in MurA, it can be envisaged that PEP and/or S3P binding induces a similar process in EPSPS. Interestingly enough, the herbicide glyphosate was shown to bind much tighter to S3P-complexed EPSPS than to uncomplexed EPSPS, quite similar to the binding preference of the antibiotic fosfomycin for the binary complex of UDPNAG and MurA.

However, in the case of the *E. coli* K22R EPSPS mutant protein, it was found that S3P does not bind to the mutant protein, and hence, the lack of glyphosate binding could be ascribed to the S3P binding deficiency (*9*). On the other hand, *P. hybrida* EPSPS, carrying the same amino acid replacement, exhibited a reduced affinity for glyphosate, albeit with retained S3P binding capacity, as judged by a similar K_m value (*7*). The latter observation suggests that glyphosate binding is not solely determined by S3P binding, but also relies on other factors, such as the ability to form a closed conformation, similar to the situation proposed for MurA.

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