Determination of the pK_a Value of C115 in MurA (UDP-*N*-Acetylglucosamine Enolpyruvyltransferase) from *Enterobacter cloacae*†

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ABSTRACT: The enzyme UDP-*N*-acetylglucosamine (UDP-NAG) enolpyruvyltransferase (MurA) catalyzes the formation of enolpyruvyl-UDP-NAG, a precursor in peptidoglycan biosynthesis. The residue at position 115 in MurA has been proposed to act as a general acid in the enzymatic reaction. This is also the primary site of action of the antibiotic fosfomycin. In this paper, the pK_a of Cys-115 has been determined to be 8.3, by titration of *Enterobacter cloacae* MurA with the alkylating agent iodoacetamide as a function of pH. Use of site-directed mutagenesis has established that only C115 is essential for catalysis, and the three other cysteine residues (C251, C354, and C381) are nonessential. Mass spectrometric analysis demonstrated that C115 is not alkylated at pH <7, but is alkylated significantly at pH >7. Measurement of the enzymatic inhibition by iodoacetamide as a function of pH showed maximum inhibition at pH >9, with a second-order rate constant of inhibition of 44 M^{-1} s^{-1} at pH 10. The presence of either one of the substrates did not influence the inactivation behavior, while the presence of both substrates resulted in a 5-fold reduction in the extent of alkylation. The covalent species that results from PEP bound to C115 of MurA exhibited 50−100-fold increased resistance against alkylation by iodoacetamide. These results imply that C115 is appreciably protonated at physiological pH and, therefore, is capable of acting as a proton donor in the enzyme-catalyzed reaction. However, it also implies that C115 is appreciably deprotonated at physiological pH also, whereupon the resultant thiolate nucleophile may play an important role in the formation of the covalent *O*-phosphothioketal species, whose role in catalysis is yet to be established.

MurA[1] catalyzes the first committed step in the formation of the bacterial cell wall heteropolymer peptidoglycan (murein). Murein is a unique structural component of the cell wall of eubacteria, and hence, its biosynthetic pathway is an important target for antibiotics currently in use or under development (*1*). The MurA reaction involves the unusual transfer of the intact enolpyruvyl moiety from phosphoenolpyruvate to the 3′-hydroxyl group of UDP-*N*-acetylglucosamine with concomitant release of inorganic phosphate (*2*, *3*). MurA is inhibited specifically by the antibiotic fosfomycin, 1(*R*),2(*S*)-1,2-epoxypropylphosphonic acid, which is in pharmaceutical use (*4*).

Another enolpyruvyltransferase, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), catalyzes the sixth step of the shikimate (prechorismate) pathway, which leads to the aromatic amino acids and their many derivatives (*5*). The shikimate pathway occurs in microorganisms, fungi, and plants and, more recently, has been discovered in apicomplexan organisms (*6*), emphasizing the importance of this pathway as an attractive target for the development of therapeutically useful drugs. Since EPSPS is inhibited by glyphosate (*7*), the active ingredient of Roundup, one of the world's most widely used broad-spectrum herbicides, EPSPS catalysis and inhibition by glyphosate have been characterized in great detail (*8*).

The overall sequences of MurA and EPSPS are about 20% identical (*9*, *10*). Structural analysis of EPSPS and MurA has revealed similar three-dimensional topologies, consisting of two globular domains (with an inside-out $\alpha-\beta$-barrel structure), connected by a hinge region (*9*, *11*, *12*). Recently, about 10 putative active site residues in EPSPS have been investigated by site-directed mutagenesis (*13*). Among the residues that were found to be essential for EPSPS catalysis (e.g., K22 and R100), there are highly conserved equivalent residues in MurA, involved in ligand binding, as shown by X-ray structural analysis (*12*, *14*).

Catalysis by both MurA and EPSPS can be envisaged as a series of proton transfer reactions: (i) C-3 of PEP is transiently protonated and thus sp^3-hybridized, (ii) the nucleophilic attack by the hydroxyl group of UDP-NAG or

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[1] Abbreviations: CABS, 4-(cyclohexylamino)-1-butanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase (EC 2.5.1.19); HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MES, 2-morpholinoethanesulfonic acid; MurA, UDP-*N*-acetylglucosamine enolpyruvyltransferase (EC 2.5.1.7); MurB, UDP-*N*-acetyl enolpyruvyl-glucosamine reductase (EC 1.1.1.148); PEP, phosphoenolpyruvate; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); PCR, polymerase chain reaction; TFA, trifluoroacetic acid; UDP-NAG, UDP-*N*-acetylglucosamine.

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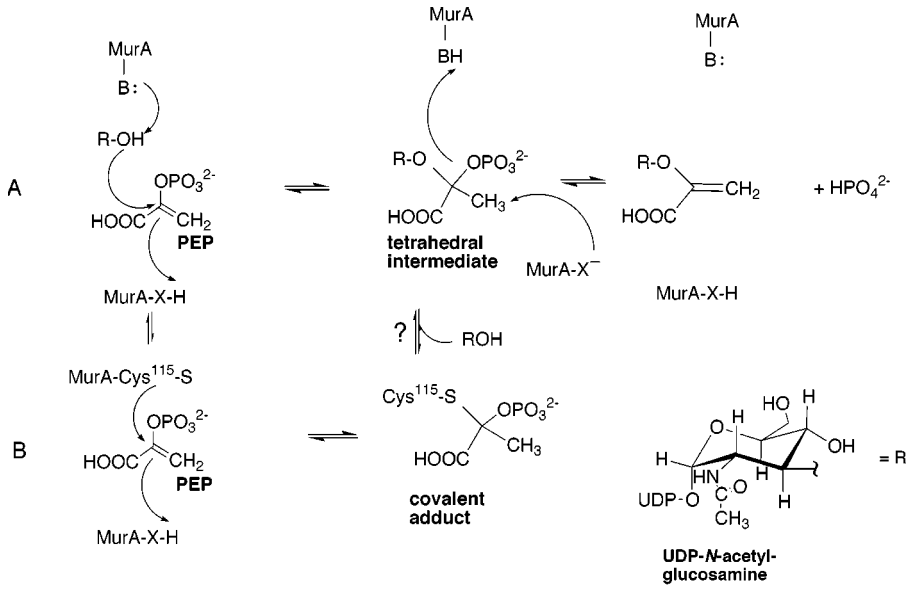
Scheme 1[a]



[a] The mechanism of the MurA reaction and the role of postulated active site general acids and bases. Reaction path A depicts the series of events without involvement of a protein nucleophile binding PEP covalently. The active site acid "X" is probably either C115 or D115. Path B depicts its behavior as a nucleophile that leads to the formation of the covalent species. The equilibrium labeled with a question mark is hypothetical.

EPSP is facilitated by deprotonation, and (iii) the released orthophosphate receives a proton (Scheme 1). Despite extensive studies on EPSPS, none of the residues directly involved in those proton transfer reactions have been identified unequivocally to date.

In MurA, a potential proton donor at position 115 is necessary for enzymatic activity. The C115S or C115A MurA mutants are inactive, while C115D or C115E mutants exhibit considerable residual activity (*15*). The pH optimum of the C115D mutant MurA is shifted toward a lower pH value, compared with that of the wild-type enzyme. This mutant was also found to behave differently toward the inhibitor (*Z*)-F-PEP, and on the basis of those findings, a pK_a value of >9 was tentatively assigned to C115 (*15*). The C115D mutant MurA from *Escherichia coli* exhibited significant resistance against fosfomycin inhibition (*15*). Among the MurAs sequenced to date, 22 possess a Cys residue at the position corresponding to position 115 in the *E. coli* enzyme, while seven sequences were found with an aspartate residue at this position. In the latter group are the MurAs from *Mycobacteria*, which have been found to be fosfomycin-resistant (*16*). The sequence stretch, in which residue 115 occurs, is one of the most highly conserved regions in MurA (*10*), and structurally, it comprises a flexible loop, held between two proline residues. This loop undergoes pronounced conformational changes upon binding of ligands (*12*, *14*, *17*).

Cysteine 115 can also act as a nucleophile. The site of action of fosfomycin on MurA has been determined to be Cys115 among the four Cys residues in the MurAs from both *Enterobacter cloacae* and from *E. coli* (since both are *Enterobacteria*, the sequences of MurAs from those two organisms are >95% identical) (*18*, *19*). It has been shown that MurA is able to bind PEP covalently via Cys115 as the *O*-phosphothioketal of pyruvic acid (*18*, *19*). In the presence of UDP-NAG, this species is able to turn over to products. Both observations imply that the role of C115 in these reactions is that of a nucleophile, attacking C-2 of fosfomycin or PEP. These findings gave rise to the hypothesis that in MurA catalysis, C115 first protonates C-3 of PEP and then nucleophilic attack by the Cys115 thiolate at C-2 of PEP occurs, followed by nucleophilic attack by the 3′-hydroxy group of UDP-NAG at C-2 of PEP (Scheme 1) (*18*, *20*). The covalent adduct and noncovalent tetrahedral intermediate have been found to rapidly equilibrate (*21*−*23*). On the basis of the kinetic properties of the C115D mutant MurA from *E. coli*, it was suggested that the function of C115 as a nucleophile is not mechanistically required in the catalytic cycle (*15*).

To define better the role of the thiol group of C115 in MurA catalysis, we have determined its pK_a value in the absence and presence of substrates. For this purpose, we used iodoacetamide as a probe for the deprotonation of the C115 thiol group. As C115 is crucial for MurA activity, alkylation can be monitored by following the decrease of activity upon incubation with iodoacetamide. Alkylation of C115 was also detected by means of mass spectrometry. Parallel experiments using site-directed mutagenesis of the three other cysteine residues in MurA were carried out to investigate the role of these cysteine residues, if any, in catalysis.

MATERIALS AND METHODS

Chemicals. PEP, K^+ salt, iodoacetamide, HEPES, MES, sodium citrate, and ammonium bicarbonate of highest available purity were from Fluka (Buchs, Switzerland, or Milwaukee, WI). DTT was from Boehringer Mannheim (Indianapolis, IN). Trizma base was from Fisher Scientific (Fair Lawn, NJ). Dihydroxybenzoic acid was from Aldrich (Buchs, Switzerland). EDTA was from J. T. Baker (Pillipsburg, NJ). All other reagents were from Sigma (St. Louis, MO).

Enzymes. Trypsin (EC 3.4.21.4) from bovine pancreas (12 081 units/mg, chymotrypsin activity of ≤0.2%) and glucose oxidase (EC 1.1.3.4, 137 units/mg) were from Fluka.

MurA from *Ent. cloacae* was expressed in *E. coli* and purified as described previously (*18*). Protein concentrations

were determined using the Bradford method (*24*) with bovine serum albumin as the protein standard or using an extinction coefficient of 24 020 M^{-1} cm^{-1} at 280 nm (*25*).

MurB from *E. coli* was expressed as a glutathione *S*-transferase (GST) fusion protein and purified as described in ref *10*.

The covalent adduct of MurA and PEP was obtained by purification as described previously (*18*), omitting the incubation step of MonoQ material with UDP-NAG before it was subjected to chromatography on reactive yellow. About 70% of this material was found to occur as the covalent species, as described in ref *18*.

Site-Directed Mutagenesis of Cys354 to Ser. A polymerase chain reaction (PCR) cassette method was used for site-directed mutagenesis to generate the mutant C354S MurA (*26*). In the expression vector pKK233-2 with the MurA gene in a *Nco*I insert (*27*), a *Bst*EII−*Ssp*I fragment was replaced with a PCR product, carrying the desired mutation. The mutagenic primers had the following sequences: 5′ c acg gtg att tct cac ggt gtt gag and 5′ cc gtg aga aat cac cgt gtt ac (reverse). The underlined sequences encode the Cys−Ser exchange. The DNA sequence of the mutant gene was confirmed by sequencing on an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) using dye-terminator chemistry.

Alkylation of MurA with Iodoacetamide and Activity Assays. For the alkylation and activity-monitoring experiments, ca. 1 mg (∼20 nmol) of pure protein was transferred into the appropriate inactivation buffer by passage through a PD10 desalting column (Amersham Pharmacia Biotech, Piscataway, NJ). The final protein concentration was determined by measuring absorption at 280 nm. Iodoacetamide was made fresh for each set of inhibition experiments in the inactivation buffer and kept in the dark. Alkylation was carried out at 22 °C in the dark. The following buffers (50 mM) were used: CABS at pH 9.5−10.5, CHES at pH 9−10, Tris-HCl at pH 8−9, HEPES at pH 6.5−8, PIPES at pH 6−7, and MES at pH 5.8−6.5. The buffers contained 2 mM EDTA (Na^+ salt) and were equilibrated with nitrogen gas overnight. Except for the maximal and the minimal pH values, rate constants were determined in two different buffer systems independently for a given pH.

At given time points, aliquots from the inactivation assay mixtures were diluted 50-fold for the coupled activity assay described by Schönbrunn et al. (*17*). The activity assay mixture contained 1 mM DTT, which is a ca. 50-fold molar excess over iodoacetamide and hence able to quench the alkylation reaction completely, as deduced from linear reaction rates obtained in a 3 min period. Inhibition assays in the presence of substrates were preincubated for 15 min before iodoacetamide was added.

Detection of Iodoacetamide-Labeled Fragments by MALDI-TOF Spectrometry. MurA (100 μM) was incubated with a 10-fold molar excess of iodoacetamide in the appropriate buffer (200 mM Tris-HCl at pH 7−9, 200 mM MES at pH 6−7, and 200 mM sodium citrate/phosphate at pH 5−6) in a total volume of 100 μL for 15 min at 22 °C. Unreacted iodoacetamide was removed by passage through a PD10 column (Amersham Pharmacia Biotech), with a change of the buffer to 0.1 M ammonium bicarbonate (pH 8.7). The protein fractions were pooled and concentrated by lyophilization (Savant Speedvac) to a volume of 100 μL. Trypsin was added to a final concentration of 0.5 mg/mL, and the samples were incubated for 3 h at 37 °C. Ten microliters of the digest was diluted with 40 μL of 10% TFA. 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% TFA/acetone (2:1, v/v). MALDI-TOF spectra of the peptide fragments were recorded with a Voyager Elite mass spectrometer using the reflectron mode for increased mass accuracy.

Determination of MurA Activity as a Function of pH. Enzyme activity was determined on the basis of the release of inorganic phosphate (*30*). MurA (0.4 μM) was incubated in the following buffers (50 mM), all containing 1 mM DTT and 2 mM EDTA: CABS at pH 9.5−11, Tris at pH 7.2−9.5, and PIPES at pH 5.8−7.2. Measurements were carried out at 22 °C.

Analysis of Data. Data were analyzed and curves fitted using Sigmaplot (version 4.1, Jandel Scientific). As described in ref *28* for thioredoxin, the reaction of stoichiometric concentrations of MurA and iodoacetamide follows second-order reaction kinetics. The concentrations of alkylated MurA ($c_{MurAalkyl}$) were derived from MurA activity at time zero ($=c_{MurA0}$) and the activity at a given time point. The experimental data were fitted to the following equation:

$$k_{app} = \frac{c_{MurAalkyl}}{t c_{MurA0}(c_{MurA0} - c_{MurAalkyl})} \quad (1)$$

The obtained rate constants were fitted to the Henderson−Hasselbalch equation to derive the pK_a value:

$$k_{app} = k_{min} + \frac{k_{max} - k_{min}}{1 + 10^{pK_a - pH}} \quad (2)$$

where k_{max} is the highest and k_{min} the lowest measured k_{app} value, respectively. The pH-dependent turnover rates (v) were fitted to

$$\log v = \frac{C}{1 + ([H]/K_a) + (K_b/[H])} \quad (3)$$

where C represents the pH-independent value of the parameter, K_a and K_b are the acid dissociation constants, and [H] is the proton concentration.

RESULTS

Mutational Analysis of the Four Cysteine Residues in MurA from Ent. cloacae. Data on the C115S MurA mutant from *Ent. cloacae* and the C115A mutant from *E. coli* have been published (*18*, *19*). We have extended the mutational analysis to the three other cysteine residues, i.e., C251, C354, and C381 in MurA from *Ent. cloacae*, by changing each individually to serine. Preliminary results on the C251S and C381S mutants have been published (*18*, *19*). The C354S mutation was obtained using a PCR-based protocol. Kinetic evaluation of the mutant proteins was performed using the coupled assay in which EP-UDP-NAG is reduced to lactyl-UDP-NAG by MurB, the second enzyme in murein biosynthesis. None of the single-point mutant proteins were found to have significantly impaired catalytic activity (Table 1). From this, we conclude that Cys115 is the only essential

Table 1: Kinetic Parameters of Wild-Type MurA and Single-Point-Mutated MurAs at 37 °C[a]

	K_M(PEP) (μM)	K_M(UDP-NAG) (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\times 10^6$ M^{-1} s^{-1}) PEP	UDP-NAG
wild-type	8.3 ± 2	80 ± 7	3 ± 0.4	0.36	0.048
C115S	nd[b]	nd	nd	nd	nd
C251S	9.3 ± 2	90 ± 10	2.8 ± 0.2	0.28	0.031
C354S	11 ± 2	85 ± 7	3.1 ± 0.3	0.28	0.036
C381S	9.8 ± 2.5	93 ± 11	2.7 ± 0.2	0.26	0.045

[a] The kinetic measurements were performed with the purified enzyme using a coupled assay with EP-UDP-NAG-reductase (MurB), monitoring the oxidation of NADPH. The concentration of the respective other substrate was 1 mM. The values are the averages of three independent measurements. [b] nd, not detectable.

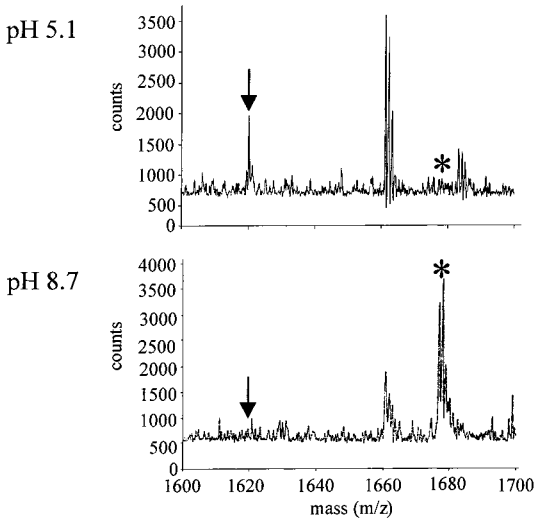


FIGURE 1: Analysis of tryptic fragments of MurA treated with iodoacetamide at pH 5.1 and 8.7 by MALDI-TOF mass spectrometry. MurA (100 μM) was incubated with 1 mM iodoacetamide in the appropriate buffer for 15 min at 22 °C. The enzyme was then digested with 0.5 mg/mL trypsin. The mass peak at *m*/*z* 1620 (indicated with an arrow) represents the peptide fragment comprising amino acid residues 104−120, whereas the mass peak at *m*/*z* 1677 (indicated with an asterisk) represents the same peptide fragment with the covalent modification (IAA linked to Cys115). The mass difference of *m*/*z* 57 accounts for the mass of the added acetamide moiety. The peak at *m*/*z* 1660 comprises amino acid residues 295−310 and is the result of residual chymotrypsin activity within the trypsin preparation.

cysteine residue required for enzymatic activity of MurA from *Ent. cloacae*.

MALDI-TOF Spectrometric Analysis of Tryptic Fragments of Free MurA after Incubation with Iodoacetamide at Different pH Values. MALDI-TOF spectrometric analysis has previously allowed identification of 82% of the tryptic fragments of MurA (*29*). The alkylation of Cys115 results in a stably modified peptide species so that a defined mass shift of 57 is expected for the alkylated fragment. The loop segment containing C115 and comprising residues 104−131 appears as a single MH^+ ion with a mass (*m*/*z*) of 1618 Da. Reaction of MurA with a 10-fold molar excess of iodoacetamide prior to tryptic digestion resulted in the disappearance of this mass peak, and the concomitant appearance of a mass peak at 1677 Da (Figure 1). The two peptides with masses of 2025 and 1933 Da containing cysteines 354 and 381, respectively, were not affected by treatment with iodoacetamide. The covalent adduct of C115 MurA with PEP is not observable by means of mass spectrometry (*18*). When the thiol proup of C115 was masked by formation of the *O*-phosphothioketal adduct with PEP, no alkylated loop fragment could be detected (not shown). As MALDI-TOF spectrometric analysis is only semiquantitative, any deduction relating peak height or area to the susceptibility to alkylation as a function of pH is not possible. However, it should be noted that complete alkylation of the C115-containing fragment was observed at pH >7.

Kinetic Analysis of Site Specific Inactivation of MurA with Iodoacetamide. To determine the pK_a value of C115, MurA was incubated with equimolar concentrations of iodoacetamide at various pH values. DTT, present in the storage buffer, would interfere, and was removed by gel filtration prior to the experiments. The buffers were equilibrated with nitrogen gas, and contained EDTA. The chosen conditions resulted in second-order rate kinetics of inactivation, depending on the pH value of the solution (Figure 2). The highest k_{app}, measured at pH 10, was 44 M^{-1} s^{-1}, and the lowest value was determined to be 0.1 M^{-1} s^{-1} at pH 5.8. The presence of either one of the substrates only marginally influenced the titration behavior (Figure 3). With 1 mM UDP-NAG present, the rate constants were slightly lower, although the pK_a value itself was unaffected. The presence of 1 mM PEP did not change the behavior at all from that found with the free enzyme. Incubation with both substrates resulted in a pronounced protection against alkylation, and a 5-fold lower k_{app} was obtained at pH 9 (5 M^{-1} s^{-1}). The covalent adduct (the *O*-phosphothioketal) exhibited significantly increased stability. When alkylated with iodoacetamide, a 50−100-fold lower k_{app}, as compared to that observed with free enzyme, was determined (0.8 M^{-1} s^{-1} at pH 10 and 0.2 M^{-1} s^{-1} at pH 9).

pH Dependence of Activity. As the pK_a value of C115 turned out to be indeed higher than 8, we determined the pH profile of MurA activity for pH values between 6 and 11. Activity was measured by determining the rate of release of inorganic phosphate (*30*), thus ruling out interference from the pH−activity profile of the coupled enzyme, MurB. The pH profile of MurA exhibited a typical bell-shaped curve (see Figure 4), with two pK_a values of 6.5 and 9.3.

DISCUSSION

Cys115 plays an important role in the formation of the covalent adduct of MurA (*18*, *20*) and the adduct with the inhibitor fosfomycin (*12*, *18*, *19*). As outlined by Kim et al. (*15*), the kinetic analysis of the MurA mutant C115D from *E. coli* strongly emphasized the importance of an acid function at position 115. The dependence of the pH optimum of an enzymatic reaction reflects the pK_a of a single ionizable group only if the step involved is rate-limiting for the overall reaction (*31*). In the case of an enzymatic reaction like that catalyzed by MurA, any extrapolation from the reaction velocity as a function of pH to specific acidic or basic amino acid residues is further complicated by the fact that other acidic or basic groups are involved in catalysis. Kim et al. determined a pK_a value of >9 from the inactivation behavior of (*Z*)-F-PEP toward wild-type and mutant C115D MurA, although they did not elevate the pH beyond 8. To shed more light on the role of C115 in MurA catalysis, we have

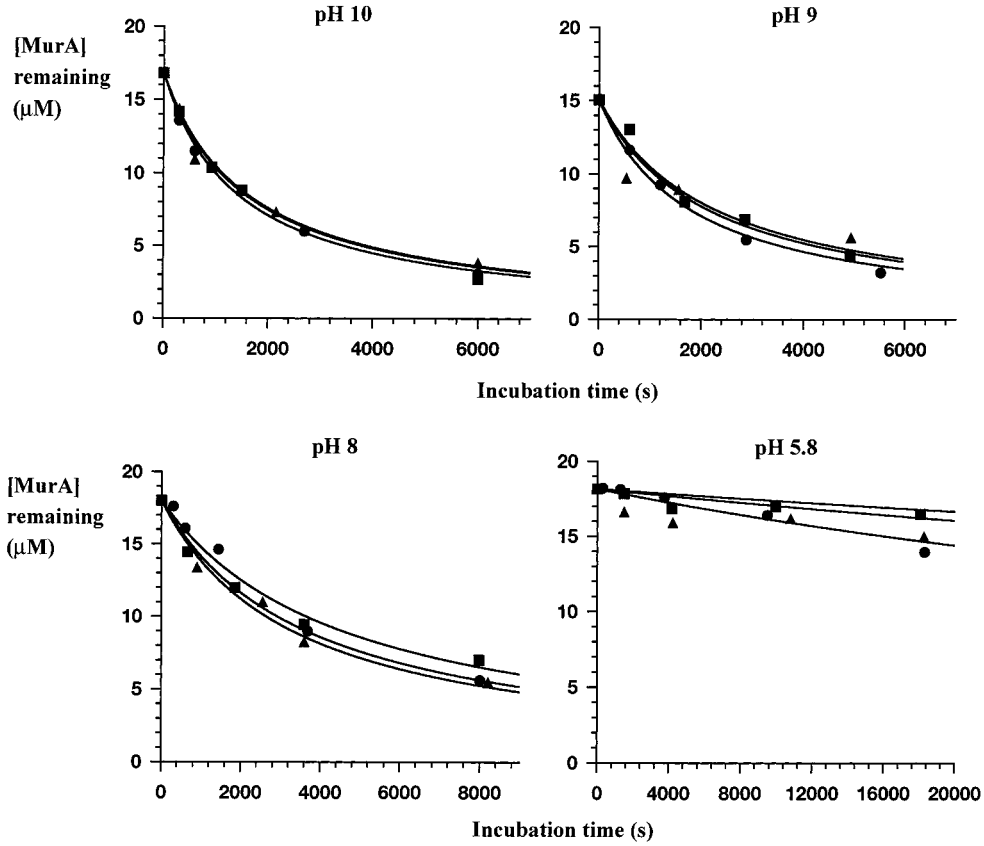


FIGURE 2: Inactivation kinetics of the reaction of equimolar amounts of iodoacetamide and MurA. For each set of experiments, 10 nmol of MurA was transferred into the appropriate buffer and cleansed from DTT by passage through a PD10 column. Iodoacetamide was made up fresh in the same incubation buffer. At the indicated time points, the assay mixture was diluted 50-fold into assay buffer containing 1 mM DTT to ensure complete quenching of protein alkylation. MurA activity was monitored in a coupled reaction with MurB. The concentration of remaining MurA was derived from residual activity at given time points. The experimental data [(●) no ligand, (■) 1 mM UDP-NAG, and (▲) 1 mM PEP] and the respective fits to eq 1 (see Materials and Methods) are shown.

investigated the sensitivity of this residue toward alkylation with the cysteine specific probe iodoacetamide, in an attempt to determine the pK_a value for this functional group.

Using site-directed mutagenesis, we have demonstrated that C115 is the only catalytically essential cysteine residue in MurA from *Ent. cloacae* required for enzymatic activity. These observations are in keeping with X-ray crystallographic data, which indicate that all other cysteine residues, i.e., C251, C354, and C381, reside in a hydrophobic core of domain I of MurA, a region not in the vicinity of the active site of the enzyme (*9*). The ligand-bound structures of MurA also showed none of them to participate in the active site (*12*, *14*).

The mass spectrometric analysis of tryptic fragments after alkylation provided evidence that C115 is the only thiol group modified by iodoacetamide and that the appearance of the alkylated mass peak corresponding to the C115-containing fragment is dependent on the pH. Other tryptic fragments containing cysteine residues were not found to be alkylated. Furthermore, the covalent adduct of MurA with PEP is significantly protected against the alkylation reaction. Hence, not only are the other three Cys residues not involved in the function of MurA, they are obviously also not accessible to iodoacetamide, or their pK_a values are, due to their hydrophobic environment, shifted toward pH values higher than those we chose in our experiments.

As C115 in MurA can be involved in covalent bond formation, e.g., with fosfomycin or (*Z*)-F-PEP, and as this results in inactive enzyme (*18*, *23*), we decided to perform a quantitative analysis of the reactivity of C115 by observing the decrease in MurA activity as a function of incubation time with iodoacetamide and pH. We performed our experiments following the protocols outlined by Kallis and Holmgren for thioredioxin (*28*). The pK_a value of 8.3 for the thiol group of C115, determined for the free enzyme, is close to values reported for free cysteine, which range between 8.3 and 9.1 (*31*, *32*). Compared to that of thioredoxin, the apparent second-order rate constants of 0.1−44 M^{-1} s^{-1} for the reaction of C115 of MurA and iodoacetamide are significantly lower. For thioredoxin, rate constants between 50 M^{-1} s^{-1} at the lowest pH and 200 M^{-1} s^{-1} at the highest pH were found in a bisigmoidal titration curve, apparently reflecting the deprotonation behavior of two cysteines, C32 and C35 (*28*). Second-order rate constants similar to and even lower (0.1−10 M^{-1} s^{-1}) than that for C115 in MurA were reported by Marchal and Branlant (*33*), for C308 in the apo form of nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase. The pK_a value of this residue was found to decrease by 2 pH units, and the second-order rate constants were found to increase 20-fold upon the NADPH-dependent transition of the enzyme from the apo to the holo form.

For C115, we found no shift in the pK_a value and only a slight reduction of the second-order rate constants in the presence of 1 mM UDP-NAG. Catalysis of MurA has been shown to follow an induced fit mechanism. While the K_d

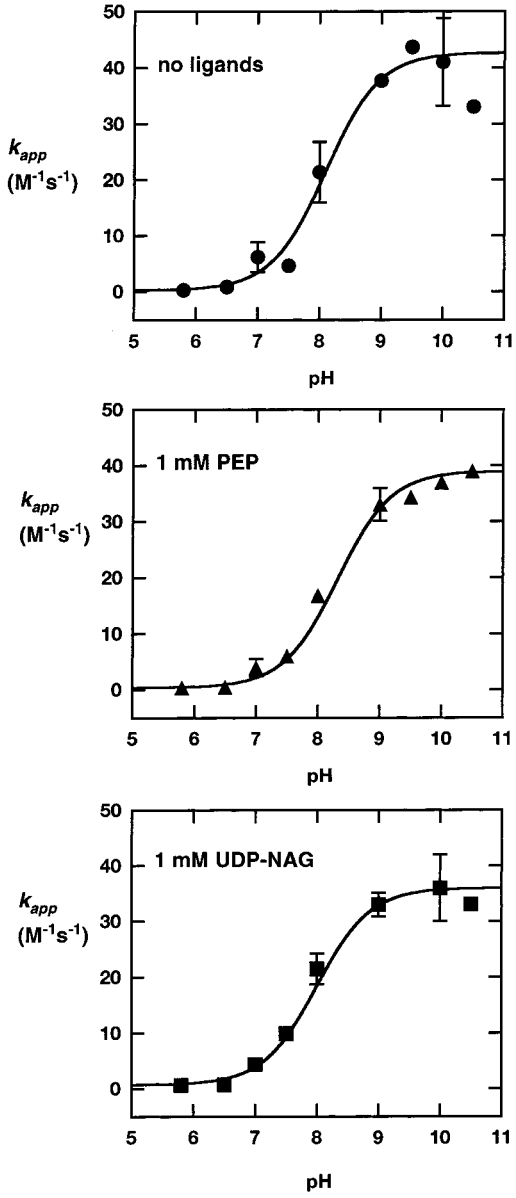


FIGURE 3: Apparent second-order rate constants of the reaction of iodoacetamide with MurA as a function of pH. The apparent second-order rate constants were derived from the inhibition kinetics as shown in Figure 2 and displayed as a function of pH. The curves represent the fits to eq 2 (see Materials and Methods).

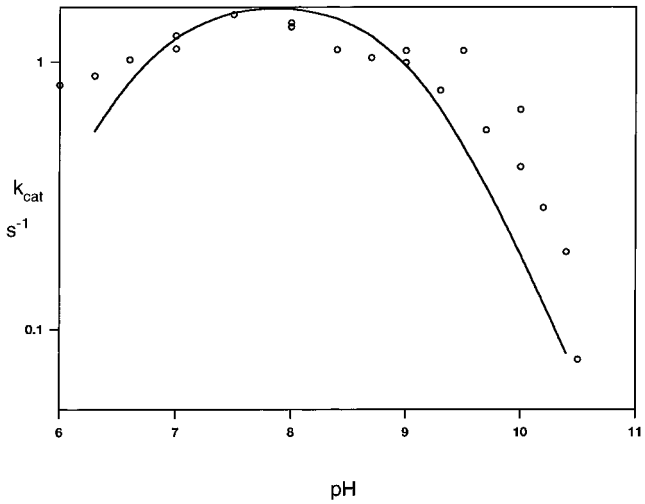


FIGURE 4: Maximal velocity of the enolpyruvyl transfer reaction catalyzed by wild-type MurA as a function of pH. The activity was determined by assessing the release of inorganic phosphate under substrate saturating conditions (1 mM each) at 22 °C. The symbols represent the experimentally determined values, while the curve is a fit to eq 3 (see Materials and Methods).

value of free enzyme for PEP was reported to be 220−240 μM (*34*, *35*), a K_M value of 8.3 μM for MurA toward PEP under UDP-NAG-saturating conditions was determined (Table 1). It has been shown that binding of UDP-NAG induces conformational changes in MurA, presumably resulting in an overall more closed structure, and a local movement of the C115-containing loop toward the catalytic cleft (*29*, *34*), resulting in an increased affinity toward PEP. This process is thought to lead to an activation of C115 by the change of its environment compared to the unliganded state (*17*). On the basis of our titration data, this activation results neither in a shift of the pK_a value nor in enhanced reactivity of C115 with iodoacetamide. An unchanged pK_a value of C115 upon binding of UDP-NAG corroborates the function of this residue as a proton donor, and deprotonation would only be triggered by binding of PEP. The slightly decreased rate constants for the reaction with iodoacetamide might reflect a reduced accessibility of this residue.

The presence of both substrates at saturating concentrations (1 mM) strongly protected MurA against alkylation. It was previously shown that resistance of MurA toward proteolysis is significant when both substrates are present (*29*). It was also demonstrated that structural elements which contain residues participating in catalysis are the most protected (*29*). While the higher resistance against trypsin in the presence of both substrates can be explained by a more closed structure in the presence of substrates and products, the protective effect against iodoacetamide probably derives also from the appearance of covalent adduct, which was found to be the most protected species (50−100-fold lower rate constants).

The pK_a value of the functional group whose protonation is critical for MurA activity is about 9.3, as derived from the pH−activity profile (Figure 4). Thus, while at pH 9.3 only 5% of the cysteine residues of MurA are protonated, as deduced from the inactivation data (Figure 3), still half of the enzymatic activity is retained. This implies that deprotonation of C115 is not a rate-limiting step in MurA catalysis.

Walsh and co-workers have suggested that a rapid equilibrium exists between the covalent species and the tetrahedral intermediate (*22*, *23*, *36*). Given a pK_a value of 8.3 for C115, a considerable fraction of the cysteine residues would occur in the highly nucleophilic thiolate form at pH 8. Hence, a study of the appearance of the covalent species as a function of pH will be important to understanding its role in MurA catalysis, and the corresponding experiments are underway in this laboratory. The function of C115 as a nucleophile may not be required for catalysis, but the catalytic efficiency of the MurA C115D mutant of the *E. coli* enzyme was found to be greatly reduced (*21*). It will be interesting to compare the catalytic efficiencies of MurAs naturally carrying an aspartate residue at position 115. The precise role played by the covalent species in the catalysis or maintenance of activity

of MurAs possessing a cysteine residue in position 115 remains to be established.

REFERENCES

1. Bugg, T. D., and Walsh, C. T. (1992) *Nat. Prod. Rep. 9*, 199−215.
2. Li, Y., and Evans, J. N. S. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4612−4616.
3. Walsh, C. T., Benson, T. E., Kim, D. H., and Lees, W. J. (1996) *Curr. Biol. 3*, 83−91.
4. Reeves, D. S. (1994) *J. Antimicrob. Chemother. 34*, 853−858.
5. Schmid, J., and Amrhein, N. (1999) in *Plant Amino Acids: Biochemistry and Biotechnology* (Singh, B. K., Ed.) Marcel Dekker, New York.
6. Roberts, F., Roberts, C. W., Johnson, J. J., Kyle, D. E., Krell, T., Coggins, J. R., Coombs, G. H., Milhous, W. K., Tzipori, S., Ferguson, D. J., Chakrabarti, D., and McLeod, R. (1998) *Nature 393*, 801−805.
7. Steinrücken, H. C., and Amrhein, N. (1984) *Eur. J. Biochem. 143*, 351−357.
8. Franz, J. E., Mao, K. M., and Sikorski, J. A. (1997) *Glyphosate: A unique global herbicide*, American Chemical Society, Washington, DC.
9. Schönbrunn, E., Sack, S., Eschenburg, S., Perrakis, A., Krekel, F., Amrhein, N., and Mandelkow, E. (1996) *Structure 4*, 1065−1075.
10. Krekel, F. (1999) Ph.D. Thesis, Swiss Federal Institute of Technology, Zürich, Switzerland.
11. Stallings, W. C., Abdelmeguid, S. S., Lim, L. W., Shieh, H. S., Dayringer, H. E., Leimgruber, N. K., Stegeman, R. A., Anderson, K. S., Sikorski, J. A., Padgette, S. R., and Kishore, G. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5046−5050.
12. Skarzynski, T., Mistry, A., Wonacott, A., Hutchinson, S. E., Kelly, V. A., and Duncan, K. (1996) *Structure 4*, 1465−1474.
13. Shuttleworth, W. A., Pohl, M. E., Helms, G. L., Jakeman, D. L., and Evans, J. N. S. (1999) *Biochemistry 38*, 296−302.
14. Skarzynski, T., Kim, D. H., Lees, W. J., Walsh, C. T., and Duncan, K. (1998) *Biochemistry 37*, 2572−2577.
15. Kim, D. H., Lees, W. J., Kempsell, K. E., Lane, W. S., Duncan, K., and Walsh, C. T. (1996) *Biochemistry 35*, 4923−4928.
16. De Smet, K. A., Kempsell, K. E., Gallagher, A., Duncan, K., and Young, D. B. (1999) *Microbiology 145*, 3177−3184.
17. Schönbrunn, E., Eschenburg, S., Krekel, F., Luger, K., and Amrhein, N. (2000) *Biochemistry 39*, 2164−2173.
18. Wanke, C., and Amrhein, N. (1993) *Eur. J. Biochem. 218*, 861−870.
19. Marquardt, J. L., Brown, E. D., Lane, W. S., Haley, T. M., Ichikawa, Y., Wong, C. H., and Walsh, C. T. (1994) *Biochemistry 33*, 10646−10651.
20. Ramilo, C., Appleyard, R. J., Wanke, C., Krekel, F., Amrhein, N., and Evans, J. N. S. (1994) *Biochemistry 33*, 15071−15079.
21. Kim, D. H., Lees, W. J., and Walsh, C. T. (1995) *J. Am. Chem. Soc. 117*, 6380−6381.
22. Lees, W. J., and Walsh, C. T. (1995) *J. Am. Chem. Soc. 117*, 7329−7337.
23. Kim, D. H., Tucker-Kellogg, G. W., Lees, W. J., and Walsh, C. T. (1996) *Biochemistry 35*, 5435−5440.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−245.
25. Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) *Anal. Biochem. 200*, 74−80.
26. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., and Struhl, K. (1994) *Current Protocols in Molecular Biology*, Greene Associates and Wiley-Interscience, New York.
27. Wanke, C., Falchetto, R., and Amrhein, N. (1992) *FEBS Lett. 301*, 271−276.
28. Kallis, G. B., and Holmgren, A. (1980) *J. Biol. Chem. 255*, 10261−10265.
29. Krekel, F., Oecking, C., Amrhein, N., and Macheroux, P. (1999) *Biochemistry 38*, 8864−8878.
30. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem. 100*, 95−97.
31. Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Co., New York.
32. Lindley, H. (1960) *Biochem. J. 74*, 577−584.
33. Marchal, S., and Branlant, G. (1999) *Biochemistry 38*, 12950−12958.
34. Schönbrunn, E., Svergun, D. I., Amrhein, N., and Koch, M. H. (1998) *Eur. J. Biochem. 253*, 406−412.
35. Samland, A. K., Amrhein, N., and Macheroux, P. (1999) *Biochemistry 38*, 13162−13169.
36. Lees, W. J., Benson, T. E., Hogle, J. M., and Walsh, C. T. (1996) *Biochemistry 35*, 1342−1351.

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