Site-directed mutagenesis and chemical modification of the two cysteine residues of the UDP-*N*-acetylmuramoyl:L-alanine ligase of *Escherichia coli*

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Abstract Site-directed mutagenesis and chemical modification of the two cysteine residues of the MurC L-alanine-adding enzyme from *Escherichia coli* were undertaken to study their possible role in activity and stability. Their replacement by alanine was not critical for activity. However, C230 played a role in enzyme stability and substrate binding. *N*-Ethylmaleimide alkylation led to monoalkylated and dialkylated proteins. The monoalkylated protein had mostly unmodified C230 residues. The extent of alkylation of C230 paralleled the loss of activity, whereas that of C426 did not. Protection against inactivation by β,γ-imidoadenosine 5′-triphosphate implied the involvement of C230 in the ATP binding site.

Key words: MurC synthetase; Cysteine residue; Site-directed mutagenesis; *N*-Ethylmaleimide

1. Introduction

Peptidoglycan is an essential polymer of the cell walls of eubacteria. Its cytoplasmic precursor, UDP-MurNAc-pentapeptide, is assembled in the bacterial cytoplasm by the stepwise addition of L-alanine, D-glutamate, diaminopimelate or L-lysine, and a dipeptide (generally D-alanyl-D-alanine) to UDP-MurNAc. Each step is catalyzed by a specific enzyme belonging to the well-defined family of the closely functionally related Mur synthetases [1,2]. In the first step the UDP-MurNAc:L-alanine ligase is responsible for the formation of UDP-MurNAc-L-Ala. This enzyme, and the Mur synthetases in general, catalyze the formation of an amide or peptide bond concomitantly with the hydrolysis of ATP into ADP and P_i. The mechanism implies the carboxyl activation of the nucleotide substrate UDP-MurNAc by ATP, followed by the nucleophilic attack by the amino group of L-alanine. An acylphosphate intermediate involving the carboxyl group of UDP-MurNAc and the γ-phosphoric group of ATP was demonstrated [3–5]. The *murC* gene encoding the L-alanine ligase is located at 2 min on the *Escherichia coli* chromosome in the *mra* cluster and contains 1473 nucleotides translated into a 491-amino acid protein with a molecular mass of 53 625 Da [6,7]. The enzyme was overproduced in *E. coli*, purified [3,8,9] and shown to exist in an equilibrium between monomeric and dimeric active forms [10].

The L-alanine-adding activity from various organisms was found to be markedly increased by 2-mercaptoethanol [11–13]. In this respect, it appeared to be unique among the Mur synthetases involved in the formation of UDP-MurNAc-pentapeptide. In *E. coli* the MurC activity was initially detected at a very low level in comparison with synthetases MurD, MurE, and MurF [14]. The presence of 2-mercaptoethanol during the preparation of crude extracts led to an increased MurC activity up to the level of the other synthetases [12]. On the other hand, the presence of thiol-modifying reagents led to the loss of activity which was partially avoided by addition of ATP or ATP+UDP-MurNAc [8]. These results suggested that a cysteine residue might play a role in the stability of the enzyme and in its catalytic process. There are only two cysteine residues in the MurC sequence (C230 and C426). The study of the effects of their replacement by alanine by site-directed mutagenesis, and that of their specific chemical modification by *N*-ethylmaleimide alkylation, was undertaken.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

E. coli JM83 and PC 2453 (*murC* [Ts], Phabagen Collection, Department of Molecular Cell Biology, State University of Utrecht, Utrecht, The Netherlands) were used as hosts for plasmids as well as for the overproduction of wild-type and mutant proteins. *E. coli* strain BMH71-18 *mutS*, defective in mismatch repair, was used in site-directed mutagenesis experiments [15]. Plasmid pAM1005 carrying the wild-type *murC* gene in the p*Trc*99A vector has been previously described [8]. 2YT medium [16] was used supplemented with ampicillin (100 μg/ml) for growing cells and growth was measured by turbidity at 600 nm.

2.2. DNA techniques and E. coli cell transformation

Small- and large-scale plasmid preparations were carried out by the alkaline method [17]. DNA sequencing was performed according to Sanger et al. [18], using the T7 sequencing kit from Pharmacia. Plasmid DNA was introduced into *E. coli* by transformation of $CaCl_2$-treated cells or by electroporation.

2.3. Site-directed mutagenesis

Site-directed mutagenesis of the *E. coli* MurC enzyme was performed using the Transformer site-directed mutagenesis kit purchased from Clontech (Palo Alto, CA) based on the method of Deng and Nickoloff [15]. This method works by simultaneous annealing of two oligonucleotide primers to one strand of a denatured plasmid, one

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Abbreviations: ADPNP, β,γ-imidoadenosine 5′-triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylene diaminetetraacetic acid; ESI, electrospray ionization; MS, mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; MurC, UDP-MurNAc:L-alanine ligase, L-alanine-adding enzyme; MurNAc, *N*-acetylmuramic acid; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; SDS, sodium dodecylsulfate; WT, wild-type

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primer introducing the desired mutation into the gene and the other primer mutating a unique site in the plasmid vector sequence for the purpose of selection. The latter primer, which is common to the two mutagenesis experiments (5′-TTGGTGCGGACATCTCGGTAG-3′), was defined for suppression of the unique *Eco*RV site within the *lacI*^{q} gene of target plasmid pAM1005 (with no change in the amino acid sequence of LacI) [1]. The sequences of the two other oligonucleotides used for replacement of cysteines 230 and 426 by alanine are respectively: 5′-GCGGTGATGGCTGTTGATGATCCG-3′ and 5′-CGTTCGCTGGCTCGGCACAATTCGT-3′. The strains carrying the *murC* mutated plasmids are JM83(pAM1005-C230A) and JM83(pAM1005-C426A).

2.4. Purification of wild-type and mutated UDP-MurNac: L-alanine ligases

Cultures of JM83(pAM1005), JM83(pAM1005-C230A), and JM83(pAM1005-C426A) were carried out at 30°C in 2YT medium supplemented with ampicillin (100 μg/ml). IPTG (1 mM) was added at $OD_{600} = 0.2$, and cells were harvested after 3 h of induction. Purification of wild-type and mutated MurC ligases was performed as described by Liger et al. [8]. Finally, for long storage times proteins were lyophilized in 20 mM Tris-HCl, pH 7.5, containing mannitol (50 mg/ml), 5 mM DTT, 5 mM EDTA and 0.02% SDS. The wild-type protein was stored at −20°C at 9.8 mg/ml in 240 mM KH_2PO_4, pH 7.2, containing 2.5 mM DTT and 2.5 mM EDTA.

2.5. Standard assay for UDP-MurNac: L-alanine ligase activity

The L-alanine-adding activity was routinely assayed by following the addition of either radiolabeled or unlabeled L-alanine to UDP-MurNAc. In the first case, the assay was carried out according to Liger et al. [8] in a mixture containing, in a 50 μl final volume, 0.1 M Tris-HCl, pH 8.6, 20 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM (*R*)-(−)-1-aminoethylphosphonic acid, 5 mM ATP, 0.5 mM UDP-MurNAc, 0.5 mM L-[^{14}C]alanine (2 kBq), enzyme (25 μl), and BSA (1 mg/ml). Appropriate dilutions of the enzyme were performed in 20 mM KH_2PO_4, pH 7.2, 2.5 mM EDTA, and 2.5 mM DTT. After 30 min at 37°C reactions were stopped with acetic acid. Substrates and products were separated on a Nucleosil $5C_{18}$ column (4.6×150 mm; Altech France, Templemars, France) by isocratic elution with 50 mM ammonium formate, pH 4.3, at a flow rate of 0.6 ml/min and detection with a radioactive flow detector model LB506-C1 (Berthold, Bad Wildbad, Germany) using the Quicksafe Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.6 ml/min. Quantification was carried out with a computer connected to the detector and equipped with the Berthold HPLC software. In the second case the activity was determined by quantifying UDP-MurNAc-L-alanine generated in a mixture (final volume 20 μl) containing 50 mM Tris-HCl, pH 8.6, 0.5 mM UDP-MurNAc, 5 mM ATP, 20 mM $MgCl_2$, 1 mM L-alanine, 25 mM $(NH_4)_2SO_4$, 1 mM (*R*)-(−)-1-aminoethylphosphonic acid and enzyme (10 μl). Appropriate dilutions of the enzyme were performed in 20 mM K_2HPO_4, pH 7.2, containing 1 mg/ml BSA, 2.5 mM EDTA and 2.5 mM DTT. After 30 min at 37°C reactions were stopped by addition of acetic acid. Substrates and products were separated on a reverse-phase Nucleosil $5C_{18}$ column (4.6×250 mm) by isocratic elution with 50 mM ammonium dihydrogenophosphate, pH 3.6, at a flow rate of 1 ml/min. Detection was performed by following absorbance at 260 nm.

2.6. Chemical modification by N-ethylmaleimide

Before alkylation by NEM, the wild-type and mutant MurC proteins were freshly dialyzed against 50 mM sodium phosphate buffer, pH 7.5, to eliminate DTT. Proteins (1 mg/ml) were then incubated for 60 min at 37°C in presence of NEM in 50 mM sodium phosphate buffer, pH 6.5. Alkylation was stopped by dilution with an equal volume of the same buffer containing 100 mM DTT. The chemically modified protein was used for determining its enzymatic activity, its mass spectrum, and its peptide map after trypsin digestion. Protection experiments were performed by incubating the protein with 5 mM β,γ-imidoadenosine 5′-triphosphate (ADPNP) in 50 mM sodium phosphate buffer, pH 6.5, and 5 mM $MgCl_2$, for 30 min prior to the addition of NEM.

2.7. Mass determination and sequence analysis

Prior to MS analysis, proteins (50–100 μg) were desalted on a reverse-phase Vydac $5C_4$ column (4.6×150 mm, 214TP5415) eluted with a linear gradient from 30 to 65% solvent B in 35 min, at a flow rate of 1 ml/min, where solvent A contained water:TFA (100:0.1 v/v) and solvent B acetonitrile:TFA (100:0.1 v/v). The triple-stage quadrupole mass analyzer Finnigan MAT TSQ 7000, equipped with an electrospray ionization (ESI) source, scanned from *m/z* 800 to 2500 in the single quadrupole mode. Data were acquired in the profile mode using positive ion detection. Mass spectrometry data were processed with Finnigan MAT ICIS software.

2.8. Tryptic digestion of UDP-MurNAc: L-alanine ligase and mapping

Unmodified and modified ligases (100 μg) were digested by trypsin (trypsin/ligase ratio 1/100 w/w) in 50 mM sodium phosphate, pH 7.5, and 1 mM DTT, at 37°C for 60 min. Digestion was stopped by addition of TFA. Peptide samples were separated by reverse-phase HPLC, using a 600E pump (Waters) and an automatic 712 WISP injector (Waters), on a C_{18} Vydac column (4.6×250 mm, 218TP54) at 1 ml/min with a gradient using water with 0.1% TFA (v/v) as solvent A and acetonitrile with 0.1% TFA (v/v) as solvent B. Peptides were detected with UV detector 785A (Applied Biosystems) at 220 nm. Fluorescence, detected with Waters 740 fluorescence detector ($\lambda_{excitation}$ 280 nm and $\lambda_{emission}$ 348 nm), and UV spectra, measured with Waters 991PDA photodiode array detector, were used to assist the identification of the tryptic peptides. LC-MS was used to determine the mass of the peptides. For each peptide, a sequence was obtained using the PEPM program (Finnigan). When possible the identification was verified by LC-MS-MS. Some ambiguities were resolved by automated Edman sequencing after collection of the peptides. Peptide sequencing was performed on model 470 A sequencer (Applied Biosystems) connected on-line to a 120PTH analyzer (Perkin-Elmer).

3. Results

3.1. Site-directed mutagenesis of the two cysteines

Two plasmids harboring the desired mutations were derived from the MurC-overproducing plasmid pAM1005 [8]. Their DNA sequence encoded an alanine instead of the 230 or 426 cysteine residue. After transformation in an *E. coli murC ts* host, only the C426A mutated plasmid gave thermoresistant transformants at 42°C on plates, whereas the C230A mutated plasmid could not effectively restore growth under the same conditions. However, in liquid medium both strains grew well at 30°C and 42°C. Crude extracts were prepared from each mutant grown at 30°C, and SDS-PAGE showed that the mutated MurC proteins were overproduced at the same very high level as the wild-type overproducing strain (data not shown).

3.2. Enzymatic properties of the C230A and C426A MurC proteins

The L-alanine-adding activity of the crude extracts from JM83(pAM1005), JM83(pAM1005-C230A), and

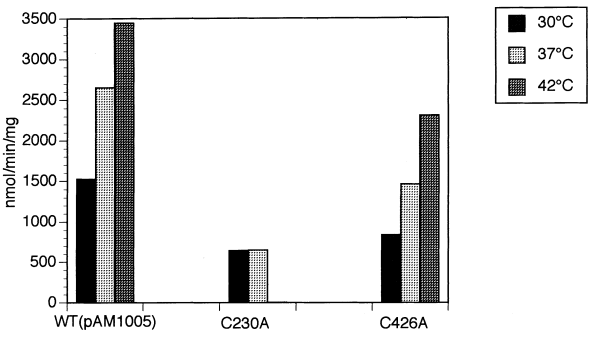

Fig. 1. Enzymatic properties of the MurC mutants. The L-alanine ligase activity was assayed at different temperatures on crude extracts from wild-type and mutant *murC* strains grown at 30°C under the standard conditions described in Section 2.

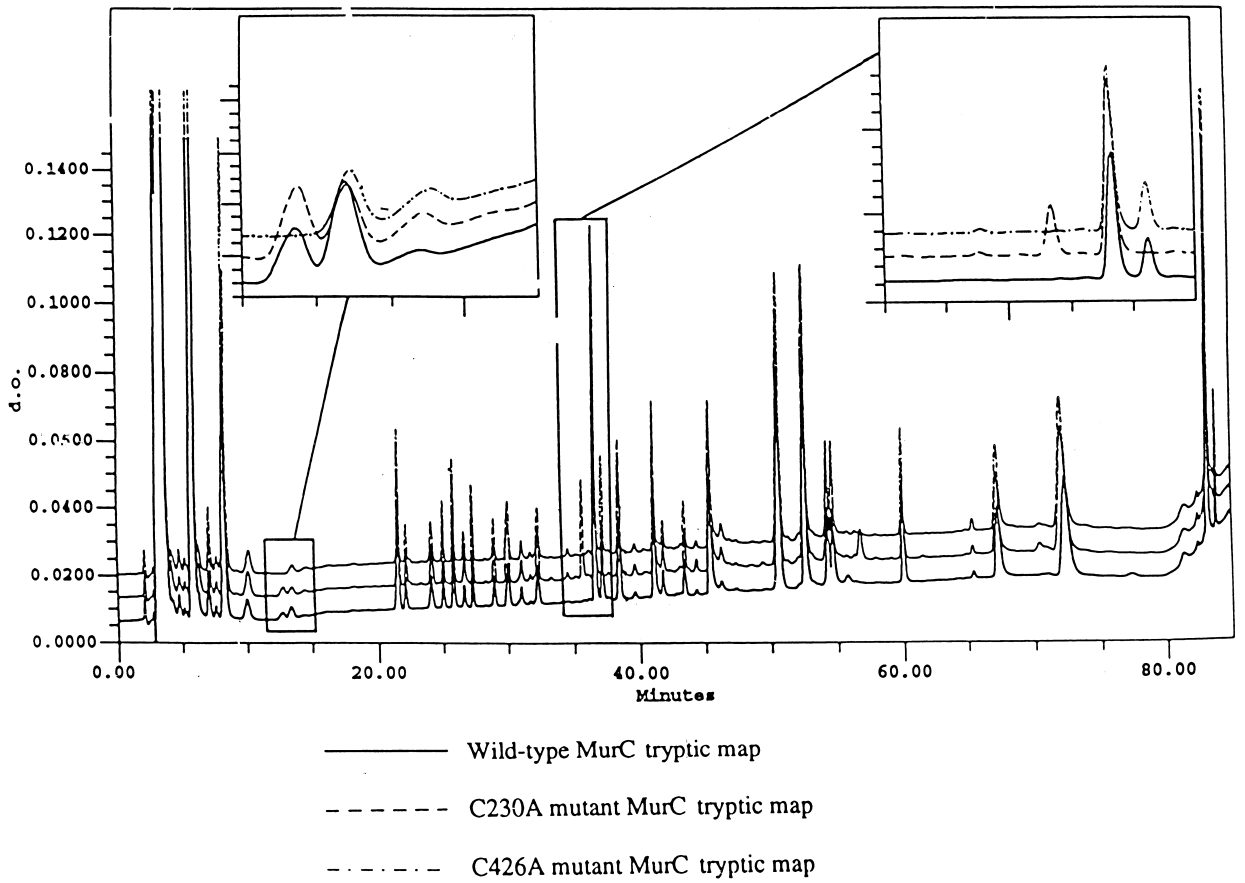

Fig. 2. Identification of the cysteine-containing peptides. Tryptic peptide maps by RP-HPLC of the wild-type and mutant MurC proteins (see Section 2 for details). The first frame contains peptide 424–427 (retention time 12.7 min), which is absent in the mutant C426A MurC protein. The second frame contains peptide 227–237 (retention time 37.3 min). The homologous peptide of the mutant C230A MurC protein was eluted at 35.7 min.

JM83(pAM1005-C426A) grown at 30°C was assayed in the presence of DTT at 30°C, 37°C, and 42°C (Fig. 1). At 30°C and 37°C very high levels of activity were observed with all three extracts. The two cysteine residues were thus not essential for the catalytic activity of MurC. At 42°C the MurC activity of the C230A mutant was completely lost. This extreme thermosensitivity explained the results of the complementation experiments carried out on plates with C230A transformants. On the other hand, the activity of both wild-type and C426A MurC increased at 42°C. Similar results were obtained when the L-alanine-adding activity of the purified wild-type and mutant MurC proteins was assayed at 30 and 37°C under the same conditions (data not shown).

The apparent K_m values of the purified mutant proteins for the ATP, L-alanine, and UDP-MurNAc substrates were determined. The most important changes were observed with the C230A protein. The K_m value for ATP was 1.4 mM instead of 0.45 mM with the wild-type protein. The apparent affinities for the two other substrates also decreased. The K_m values for UDP-MurNAc and L-alanine were respectively 1.1 mM and 0.3 mM instead of 0.1 mM and 0.04 mM with wild-type MurC. On the other hand, the K_m values of the C426A protein increased only to a minor extent.

3.3. Stability of the purified enzymes in absence of reducing reagents

The UDP-MurNAc:L-alanine ligase activity was also determined for the wild-type and mutant MurC purified proteins after dialysis against 50 mM phosphate buffer, pH 7.5, to remove DTT which is undesirable in the NEM alkylation experiments. Removal of DTT led to a further increase of the thermosensitivity of C230A MurC at 37°C, but also to a slight thermosensitivity of wild-type MurC and C426A MurC. After 60 min at 37°C, wild-type MurC lost 28% of its activity, C426A MurC 36% and C230A MurC 68%.

Table 1
Effect of NEM alkylation on the UDP-MurNAc:L-alanine ligase activity

NEM		MurC wild-type		MurC C426A		MurC C230A	
		ADPNP		ADPNP		ADPNP	
		−	+	−	+	−	+
30°C	−	7.0	7.5	9.9	11.4	5.0	5.1
	+	4.2	5.0	7.9	9.6	1.3	2.4
37°C	−	10.3	11.3	12.4	12.6	0.8	0.7
	+	5.0	8.2	6.7	10.4	0.5	0.6

Experiments were carried out in the presence or absence of NEM (5 mM) and in the presence or absence of ADPNP (5 mM) as described in Section 2. The incubation medium contained 50 mM sodium phosphate, pH 6.5, enzyme (1 mg/ml) and $MgCl_2$ (5 mM). Mixtures were diluted 4000-fold before the enzymatic assay. Activities are expressed as nmol/min/μg protein.

3.4. Ligase activity of wild-type and mutant MurC protein after NEM alkylation

Wild-type and mutant MurC proteins were treated with NEM, which is a well-established alkylating reagent of protein cysteine residues [19]. It was previously shown that the wild-type L-alanine ligase activity decreased after NEM treatment and that ATP or ATP+UDP-MurNAc had a partial protective effect against this inactivation [8]. Wild-type and the two mutated MurC proteins were treated with NEM in the absence or presence of ADPNP, a non-hydrolyzable ATP analogue. Considering the high thermosensitivity of the C230A protein, experiments were carried out at both 30 and 37°C (Table 1). As expected experiments at 37°C with C230A MurC led to too low significant values. The other results were similar at both temperatures. The loss of activity upon alkylation was greatest with C230A MurC and the protective effect of ADPNP was greatest with C426A MurC.

3.5. Extent of NEM alkylation of wild-type MurC

The stoichiometry of the labeling of wild-type MurC by NEM was determined by mass spectrometry. The untreated protein showed a mass of 53 638 ± 16 Da, the theoretical mass being 53 625 Da. TFA adducts (53 754, 53 881, and 53 991 Da) were also detected. When wild-type MurC was treated with 5 mM NEM its mass spectrum showed four species reduced to only two after TFA correction; 72% (experimental mass: 53 765 ± 16 Da; theoretical mass: 53 750) corresponded to the alkylation of one residue, and 28% (experimental mass: 53 889 ± 16 Da; theoretical mass: 53 875) corresponded to two alkylated residues. There was no unalkylated protein. In the presence of 5 mM ADPNP a unique species (53 770 ± 16 Da) corresponding to one alkylated residue was detected.

3.6. Identification of the NEM-alkylated cysteine residues by peptide mapping

In order to correlate variations of activity with chemical modification by NEM it was essential to ascertain the extent and specificity of alkylation of each cysteine residue in species 53765 and 53889. For this purpose the trypsin map of wild-type MurC was established. By MS-MS sequencing, 21 peptides were identified. They represented 53% of the total amino acid sequence. Because of sodium and potassium adducts 10 other peptides could not be fragmented by MS-MS. They were collected and identified by Edman sequencing. By these two methods 81% of the protein sequence was identified. The missing peptides not identifiable by LC-MS seemed to be either large peptides or di- and tripeptides. Similar peptide mapping experiments were carried out with C230A and C426A MurC proteins. In all cases the cysteine-containing peptides (227–237 and 424–427) were clearly identified (Fig. 2).

The effect of alkylation with 5 mM NEM on the peptide maps of wild-type, C230A and C426A MurC proteins was investigated at 37°C. In wild-type and C230A MurC proteins only peptide 424–427 containing cysteine 426 disappeared completely from the map. In wild-type and C426A MurC proteins the area of peptide 227–237 containing cysteine 230 decreased by 37 and 45%, respectively. In both cases, the accompanying decrease of enzymatic activity (Table 1) seemed to be directly correlated with the area decrease and therefore with the extent of alkylation of cysteine 230. In wild-type and C426A MurC proteins the alkylation of C230 in peptide 227–237 was greatly hindered by the presence of 5 mM ADPNP.

4. Discussion

The purpose of this study was to examine by site-directed mutagenesis and chemical modification the role of the two cysteine residues of the MurC synthetase of *E. coli* on its enzymatic activity and stability. Residues C230 and C426 are not directly involved in catalysis since their replacement by alanine was not critical for the activity at least at 30°C and 37°C in the presence of DTT. In the alignments of the Mur synthetases from various organisms the two cysteine residues of MurC of *E. coli* appear poorly conserved [1]. It was also noteworthy that they are not even present in all MurC synthetases. They are found in the same position in the MurC sequence of *Haemophilus influenzae* but only C230 is found in *Bacillus subtilis, Mycoplasma tuberculosis*, and *Synechocystis* spp. They are both absent from the *Borrelia burgdorferi, Helicobacter pylori* and *P. gingivalis* MurC enzymes. Nevertheless, C230 seems to play a specific role in the thermostability of the enzyme from *E. coli* whereas C426 does not.

NEM alkylation of wild-type MurC led mainly to two molecular species: 72% monoalkylated and 28% dialkylated proteins. Since peptide mapping experiments showed that C426 was completely modified by NEM treatment, the monoalkylated species had mostly unmodified C230 residues. This was substantiated by the agreement between the 37% disappearance of the C230-containing peptide and the 28% of the dialkylated species. Therefore, C426 is more readily accessible to NEM than C230 is. At 37°C the extent of alkylation of C230 in C426A MurC closely paralleled the lost of activity (Table 1), thereby indicating that its blocking by NEM leads to total inactivation of the enzyme, whereas the alkylation of C426 in C230A MurC does not (26% residual activity). It is only the poor accessibility of 5 mM NEM to C230 which limits enzyme inactivation and explains why the loss of activity was greatest with C230A MurC. In the presence of ADPNP only C426 was alkylated, since the C230-containing peptide 227–237 was not modified in wild-type and C426A MurC proteins and their activity was preserved. These results strongly suggested the involvement of C230 in the ATP binding site which is also substantiated by the increased K_m value of C230A MurC for ATP. The increased K_m values for the two other substrates could be a consequence of the ordered binding of the substrates, ATP being bound first followed by UDP-MurNAc and L-alanine [1,5]. Therefore, both the site-directed mutagenesis and NEM alkylation experiments point to the importance of C230 for the stability and activity of MurC. Presumably, the low MurC ligase activity of crude extracts from *E. coli* prepared in the absence of any reducing reagent [14] could be due to some kind of oxidative alteration of this residue. However, the chemical nature of this modification as well as knowing whether we are dealing with an intra- or intermolecular process remain open questions.

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