Inhibition of membrane-bound lytic transglycosylase B by NAG-thiazoline

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Abstract The lytic transglycosylases cleave the bacterial cell wall heteropolymer peptidoglycan with the same specificity as the muramidases (lysozymes), between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues, with the concomitant formation of a 1,6-anhydromuramoyl residue. The putative catalytic residue in the family 3 lytic transglycosylase from *Pseudomonas aeruginosa*, Glu162 as identified by sequence alignment to the homologous enzyme from *Escherichia coli*, was replaced with both Ala and Asp by site-directed mutagenesis. Neither mutant enzyme differed structurally from the wild-type enzyme, as judged by CD spectroscopy, but both were enzymatically inactive confirming the essential role of Glu162 in the mechanism of action of this lytic transglycosylase. The β-hexosaminidase inhibitor NAG-thiazoline was shown to inhibit the activity of lytic transglycosylase activity, thus providing the first direct evidence that the formation of the 1,6-anhydromuramoyl residue may proceed through an oxazolinium ion intermediate involving anchimeric assistance. Using surface plasmon resonance and difference absorbance spectroscopy, K_d values of 1.8 and 1.4 mM, respectively, were determined for NAG thiazoline, while its parent compound *N*-acetylglucosamine neither inhibited nor appeared to bind the lytic transglycosylase with any significant affinity.

Keywords: Lytic transglycosylase; Peptidoglycan; Inhibition; Catalytic residue; Mechanism; *Pseudomonas aeruginosa*

1. Introduction

Lytic transglycosylases (LTs), a class of bacterial autolysins, function to cleave peptidoglycan at the same site as lysozymes (EC 3.2.1.17; peptidoglycan *N*-acetylmuramoyl hydrolase), specifically the β-(1→4)-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues. However, LTs are catalytically distinct from the hydrolytic lysozymes because they cleave peptidoglycan with the concomitant formation of 1,6-anhydro-MurNAc (AnhMurNAc) residues [1]. These enzymes contribute to the metabolism of peptidoglycan but their exact role has not been determined. They have been implicated as space makers for the insertion of new peptidoglycan into the cell wall during peptidoglycan remodeling and cell growth and as cell wall 'zippers' during cell division [2,3]. They may also function in the recycling of old wall material and macromolecular transport systems of Gram-negative bacteria [recently reviewed in 4].

LTs appear to be ubiquitous in the eubacteria that produce peptidoglycan (viz., all but the cell wall-less mycoplasmas) and even in some lytic bacteriophages [5]. The Gram-negative, human opportunistic pathogen *Pseudomonas aeruginosa* produces four family 3 LTs [5]. The genes encoding all four have been cloned and expressed in *Escherichia coli* [6,7]. One of these, MltB, shares 66% amino acid identity with the *E. coli* enzyme and both have been demonstrated to be lipoproteins [6,8,9]. All four family 3 enzymes from *P. aeruginosa* contain an invariant glutamyl residue which comprises one of the family's consensus sequences [5] and has been identified as the putative catalytic acid/base in *E. coli* MltB [11].

Determination of the crystal structure of a naturally produced, soluble derivative of the family 3 *E. coli* membrane-bound LT B (MltB; Slt35) complexed with GlcNAc [10], GlcNAc-MurNAc-L-Ala-D-Glu, and the inhibitor bulgecin [11] by Dijkstra and his colleagues led to the identification of four sugar-binding subsites within the enzyme with the invariant Glu162 poised between the −1 and +1 binding site. No other potential catalytic residue was observed in close proximity to this catalytic center. A similar situation was also found with the complex formed between Slt70, a family 1 LT from *E. coli*, and either a 1,6-anhydromuropeptide [12] or bulgecin [13]. The presence of a single catalytic residue at the active center of LTs indicates that these enzymes appear to use a different mechanism of action compared to most glycosidases, including lysozyme, which employ a catalytic diad of acidic residues [14]. In the double-displacement reaction mechanism of hen egg-white lysozyme, Glu35 remains protonated at physiological pH to act as a catalytic acid, while deprotonated Asp52 serves as a nucleophile to stabilize the putative oxocarbenium transition state formed during hydrolysis resulting in a covalent intermediate [15]. With the LTs, the

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Abbreviations: AnhMurNAc, 1,6-anhydro-*N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; HPAEC, high pH anion-exchange chromatography; IPTG, isopropyl-β-D-thiogalactoside; LT, lytic transglycosylase; Mlt, membrane-bound lytic transglycosylase; MurNAc, *N*-acetylmuramic acid; NAG-thiazoline, *N*-acetylglucosamine thiazoline; sMlt, soluble derivative of membrane-bound lytic transglycosylase

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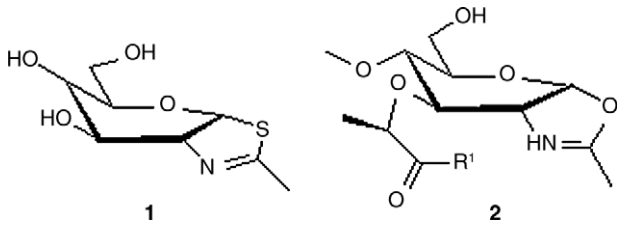

Fig. 1. Structures of NAG-thiazoline (1) and the proposed reaction intermediate of LTs, muramoyl oxazoline (2).

apparent presence of only a single catalytic acid led us and others to investigate whether these enzymes behave like the glycosyl hydrolase family (CAZy) 18 chitinases and chitobiases, family 20 *N*-acetyl-β-hexosaminidases, and the family 23 goose-type lysozymes. Each of these glycosidases was found to lack a carboxylate suitably positioned to stabilize the oxocarbenium ion transition state [16–20] and based on kinetic and X-ray crystallographic analyses of enzyme–inhibitor interactions together with theoretical considerations, hydrolysis has been proposed to involve a substrate-assisted mechanism and an oxazoline intermediate [18,21–29].

Recently, we have shown that the β-hexosaminidase inhibitor *N*-acetylglucosamine thiazoline (NAG-thiazoline) [23,30] (Fig. 1) causes morphological changes to *E. coli* when added alone to growing cultures, suggesting that the peptidoglycan biosynthetic complexes of LTs and penicillin-binding proteins are affected [31]. Furthermore, NAG-thiazoline protects the cells from penicillin-induced lysis, a process requiring autolysins including the LTs. In this study, we confirm the identification of Glu162 as an essential catalytic residue and we show that NAG-thiazoline inhibits the enzymatic activity of an engineered soluble derivative of *P. aeruginosa* MltB (sMltB).

2. Materials and methods

2.1. Chemical reagents and enzymes

Deoxynucleoside triphosphate set, PCR grade, Expand™ long template PCR system, FastStart *Taq* DNA polymerase, glycine, isopropyl-β-D-thiogalactoside (IPTG), and *Pwo* polymerase were from Roche Molecular Biochemicals (Laval, PQ), while BCA protein assay kit was from Pierce (Rockford, IL). All other chemicals were supplied by either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Nepean, Ont.) and were of reagent grade or HPLC-grade where appropriate.

NAG-thiazoline (Fig. 1) was prepared from peracetylated glucosamine using Lawesson's reagent as previously described [31]. For inhibition and binding studies, NAG-thiazoline concentrations were corrected to reflect the purity of the sample as determined by NMR using a Bruker Avance 400 and CD_3OD (referenced at 4.87 ppm and 3.30 ppm for ^1H and 49.15 ppm for ^{13}C) as solvent.

2.2. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used and created in this study are summarized in Table 1. Cultures were routinely grown in Luria–Bertani (LB; 1% tryptone peptone, 1% NaCl, and 0.5% yeast extract) broth or LB-agar plates at 37 °C. For protein expression experiments, cells were grown in Super Broth (3.2% tryptone peptone, 0.5% NaCl, and 2% yeast extract) at both 18 and 37 °C as appropriate. When necessary, growth media were supplemented with antibiotics at the following concentrations: ampicillin (Ap; 100 μg/ml), kanamycin (Km; 30 μg/ml), or chloramphenicol (Cam; 34 μg/ml).

2.3. Isolation and purification of sMltB and its derivatives

The production, isolation, and purification to homogeneity of a soluble derivative of *P. aeruginosa* MltB (sMltB) from freshly transformed *E. coli* with pNBAC 54-1 (encoding His_6-tagged sMltB) were conducted as previously described [6].

2.4. Site-directed mutagenesis

The QuikChange™ site-directed mutagenesis system (Stratagene, LaJolla, CA) was used to create single amino acid replacements within sMltB. Conditions for the mutagenesis PCR were those suggested by the manufacturer using *Pfu*Turbo DNA polymerase. Plasmid pNBAC54-1 was used as template in a PCR using the following primers:

Glu162 → Ala, 5′-GGCATCATCGGCGTGG**CC**ACCCGCTGGGGGCGTG-3′ and 5′-CACGCCCCCAGCGGGT**GG**CCACGCCGATGATGCC-3′; Glu162 → Asp, 5′-GGCATCATCGGCGTGGA**T**ACCCGCTGGGGGCGTG-3′ and 5′-CACGCCCCCAGCGGGT**A**TCCACGCCGATGATGCC-3′, with the changed nucleotides denoted by bold and underlined type. After PCR, the wild-type plasmid was digested with *Dpn*I (specific for methylated DNA) leaving only mutated plasmid DNA. The *Dpn*I-digested material was transformed into competent *E. coli* DH5β cells and plated on LB agar containing 30 μg/ml Km. Plasmids pNBAC-ME162A and pNBAC-ME162D encoding the Glu162 → Ala and Glu162 → Asp mutants of sMltB, respectively, were isolated from several resulting colonies and sequenced to ensure that only the desired mutation had been made.

2.5. Expression and purification of sMltB mutants

Escherichia coli BL21-λDE3 was freshly transformed with pNBAC-ME162A and pNBAC-ME162D and used to inoculate cultures of Super Broth supplemented with appropriate antibiotics. The inoculated cultures were incubated at 37 °C until early exponential phase (OD_{600} approximately 0.5). The cultures were then cooled to 18 °C for 15 min prior to the addition of IPTG (freshly prepared in water and filter sterilized) to a final concentration of between 0.05 and 0.1 mM. Induced cultures were incubated for up to 18 h at temperatures ranging from 18 to 37 °C. After incubation, the cells were collected by cen-

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Reference or source
E. coli strains		
DH5α	K-12 ϕ 80d *lacZ*_M15 *endA1 hsdR17* ($r_K^-m_K^-$) *supE44* thi-1 *GyrA96 relA1* ρ(lacZYA-argF) U169 F^-	Clonetech
BL21[λDE3] pLysS	F^- *ompT hsdS*$_B$($r_B^-m_B^-$) *gla dcm met* (DE3) pLysS (Cm^R)	Novagen
Plasmids		
pET30a(+)	IPTG inducible expression vector; Km^R	Novagen
pNBAC54-1	pET30a(+) derivative containing *mlt*B on a *Nde*I/*Xho*I fragment encoding an N-terminal truncation of Arg-2 to Cys-17; Kan^R	[13]
pNBAC-ME162A	PNBAC54-1 derivative containing the site-specific change encoding Glu162 → Ala	This work
pNBAC-ME162D	PNBAC54-1 derivative containing the site-specific change encoding Glu162 → Asp	This work

trifugation (5000 × *g*, 15 min, 4 °C), washed, and then disrupted by sonication as previously described [6].

2.6. Assay for LT activity and inhibition

The peptidoglycan substrate was isolated and purified from cultures of *P. aeruginosa* PAO1 grown for 8 h at 37 °C in LB broth as previously described [32]. The isolated peptidoglycan was treated with DNase, RNase, and Pronase and re-isolated by centrifugation as described by Glauner [33]. Assays of cleavage reactions based on the release of soluble muropeptides from insoluble peptidoglycan suspended in 50 mM ammonium acetate buffer, pH 5.8, were conducted as previously described by Blackburn and Clarke [34]. For inhibition studies, the data were plotted as percent residual activity based on the control reaction conducted in the absence of NAG-thiazoline.

The zymography protocol employed in this study was similar to that described by Watt and Clarke [35] and Bernadsky et al. [36] using purified peptidoglycan incorporated into 0.75 mm thick 12.5% polyacrylamide gels to a final concentration of 0.1%.

2.7. Determination of K_d for NAG-thiazoline

The binding of ligands to sMltB was determined by both surface plasmon resonance (SPR) and difference absorption spectroscopy. SPR experiments was performed using a BIAcore 2000 instrument (BIAcore, Inc., NJ) and the buffer conditions described by Vollmer et al. [37]. Purified sMltB in 10 mM sodium acetate buffer, pH 5.5, was immobilized onto the surface of a BIAcore CM5 sensor chip via free amino groups, and remaining binding sites were blocked by the injection of 1 M ethanolamine. Control lanes on the same sensor chip were similarly treated but for the omission of protein. Varying concentrations of analytes (GlcNAc or NAG-thiazoline) were passed over the surface of the control and sMltB-His_6 -coupled lanes at a flow rate of 30 μl/min. The dissociation constants (K_d) were calculated from sensograms with the aid of BIAevaluation, version 3.1.

Absorbance spectra of purified sMltB (2.16 μM) in 10 mM ammonium acetate buffer, pH 6.5, containing 100 mM NaCl were collected using a Beckman DU-70 spectrophotometer (Beckman Coulter Canada Inc., Mississauga, Ont.). Quantitative binding experiments were performed by collecting scans of sMltB after the sequential addition of NAG-thiazoline and incubation for 90 s at room temperature. Each scan was corrected for dilution by multiplying with a correction factor $[(\sum V_t + V_i)/V_i$, where V_t is the titration volume and V_i is the initial volume]. Scans were normalized by subtraction of the scans of both ligands in the absence of enzyme at zero ligand concentration [38]. The troughs to peak heights at ΔA_{277}–ΔA_{244} nm were plotted versus the total ligand concentration, and dissociation constant K_d was derived by plotting ΔA versus ΔA/[NAG-thiazoline].

Other analytical methods. Sodium dodecyl sulfate (SDS)–polyacrylamide gels were prepared using the discontinuous buffer system of Laemmli [39] and sample buffer containing SDS and 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue R-250 as described by Bollag et al. [40]. Protein concentrations were measured using the Pierce BCA protein assay kit with BSA serving as the standard. Far-UV CD spectra were measured using a JASCO 600 Spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) and estimation of secondary structure content from CD profiles was made using the JASCO 600 software. Amino acid and amino sugar analysis were performed on a Beckman System Gold amino acid analyzer as previously described [6,32,34].

3. Results and discussion

3.1. Replacement of the catalytic acid of sMltB

Studies were initiated with sMltB, a family 3 LT [5], to evaluate the effect on activity of changing the invariant and putative catalytic residue, Glu162, equivalent to Glu162 in *E. coli*. The QuikChange mutagenesis system was used to introduce changes in cloned *mltB* [6] and the desired mutations were confirmed by the complete nucleic acid sequencing of the mutated genes. After testing a variety of induction temperatures and IPTG concentrations to minimize the formation of inclusion bodies, optimal expression of the sMltB derivatives in freshly transformed *E. coli* BL21-λ DE3 was achieved with 0.1 mM IPTG at 18 °C. Cells were harvested after 3 h incubation and proteins were released by sonic disruption. The isolation of the sMltB mutants was facilitated by the presence of C-terminal His-tags. Following the affinity chromatography on Ni^{2+}–NTA agarose, the isolated proteins were purified to apparent homogeneity by a combination of cation-exchange chromatography on Mono S and gel permeation chromatography on Superdex-75. This protocol routinely resulted in the preparation of 0.3–0.5 mg purified protein per liter of cell culture.

Far-UV circular dichroism spectroscopy was used to evaluate and compare the secondary structures of sMltB, and the generated mutants. Representative CD spectra of the wild-type and Glu162 → Ala mutant of sMltB are presented in Fig. 2 The shapes of the CD spectra were identical for all proteins tested and showed strong signals around 197, 209 and 222 nm, typical of predominantly β-helical proteins. Estimation of secondary structure content from the CD profiles was made using the JASCO 600 software and it is summarized in Table 2. The CD estimates of secondary structure for sMltB are similar to values for *E. coli* Slt35 as determined directly by X-ray crystallography [10,11]. Thus, in addition to sharing 72% sequence identity, it appears likely that these two family 3 lytic transglycosylases have very similar three-dimensional structures. Additionally, these data indicate that site-specific replacement

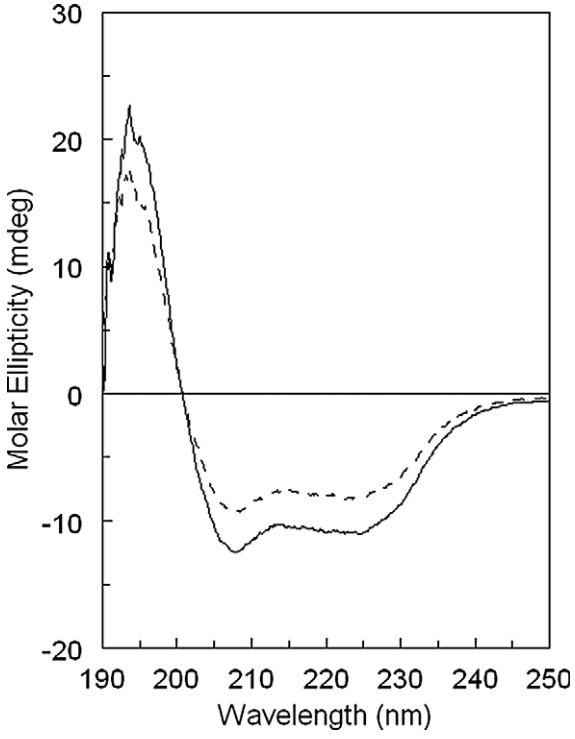

Fig. 2. CD spectra of sMltB derivatives. Far-UV CD measurements were made with purified wild-type (solid line) and Glu162 → Ala (dashed line) sMltB at room temperature in a nitrogen gas atmosphere. Enzymes were diluted to approximately 200 μg/ml in 50 mM acetate buffer, pH 5.8, containing 0.1% Triton X-100 and scanned four times from 190 to 250 nm. The CD measurements of the buffer alone have been subtracted from the data presented.

Table 2
Secondary structure estimation of *P. aeruginosa* sMltB by CD spectroscopy and comparison to *E. coli* Slt35

Enzyme	α-Helical	β-Sheet	β-Turn	Random coil
SMltB w.t.	53.8	6.7	19.1	20.5
E162A	55.2	7.7	16.4	20.6
E162D	53.0	8.8	16.4	21.8
Slt35[a]	48.1	8.4	12.7	30.7

[a] Secondary structure content determined from X-ray crystallographic structures [14,15].

of the putative catalytic Glu residue did not lead to significant changes in the secondary structures of the sMltB derivatives.

3.2. Activity of sMltB and mutant proteins

The activity of sMltB and its mutant derivatives was evaluated by three methods, zymography, examining the profiles of produced soluble muropeptides by high pH anion-exchange chromatography (HPAEC)-PAD, and using the previously established kinetic assay for LTs [6]. Representative results for both of the former two analyses are presented in Fig. 3. The wild-type enzyme was shown to be catalytically active by zymography as demonstrated by the zone of clearing in the stained peptidoglycan-containing zymogram gel (Fig. 3, inset). Incubation of the enzyme with insoluble peptidoglycan substrate and analysis of the soluble reaction products by HPAEC yielded the same profile of soluble muropeptide components as previously observed [6]. Thus, three major muropeptides were detected, which can be identified as anhydrodisaccharide-tetrapeptide, anhydrodisaccharide-tripeptide, and cross-linked anhydrodisaccharide-tetrapeptide-anhydrodisaccharide-tripeptide based on their retention times of 51, 55, and 62 min, respectively, on the Dionex CarboPac PA1 anion-exchange column and previously assigned molecular masses [6]. Using the kinetic assay, the specific activity and k_{cat} of the wild-type sMltB were determined to be 535 nmol min^{-1} mg^{-1} and 0.45 s^{-1}, respectively. In contrast, the mutant Glu162 → Ala sMltB was completely devoid of lytic activity against peptidoglycan as judged using each of the assays under the experimental techniques employed. This lack of apparent activity provides strong evidence to confirm the identification of Glu162 as an essential amino acid residue, which presumably serves as the catalytic acid/base. These data are consistent with previous findings involving *E. coli* Slt70 and Slt35 in which the respective single putative catalytic glutamyl residues were replaced [10,41]. Interestingly, shortening the length of the glutamyl side chain by a methyl group in *P. aeruginosa* sMltB had the same effect as removing the functional carboxylic acid altogether as the Glu162 → Asp sMltB was also completely inactive (Fig. 3).

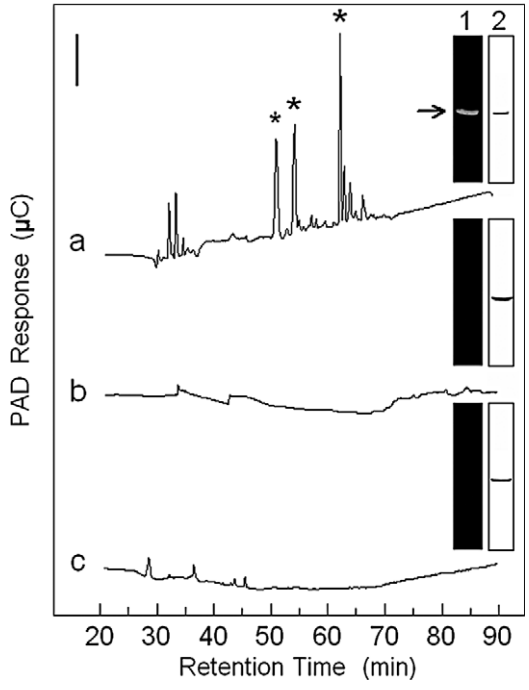

Fig. 3. Effect on activity of a site-directed replacement of Glu162 to Ala or Asp in sMltB. The soluble products released from insoluble peptidoglycan by (a) wild-type, (b) Glu162 → Ala, and (c) Glu162 → Asp sMltB were analyzed by HPAEC and zymography. The solid bar denotes a PAD response of 0.25 μC, and the asterisks identify the three major muropeptides released with retention times of 51, 55, and 62 min. Insets: Zymogram analysis. Lane 1, zymogram; lane 2, corresponding protein sample stained with Coomassie Brilliant Blue. The arrow denotes the zone of clearing in the zymogram of wild-type sMltB.

3.3. Inhibition of sMltB by NAG-thiazoline

The apparent absence of a second potential catalytic residue in LTs proximal to the glycosidic oxygen of the scissile bond in the peptidoglycan substrate has led to the suggestion [6,11,13] that the mechanism of action of these enzymes may follow that of some *N*-acetylglucosaminidases and chitinases [18,21–29]. The latter enzymes are thought to hydrolyze *N*-acetylglucosaminyl polymers by invoking substrate-assisted catalysis involving the *N*-acetyl group of the aminosugar substrate at subsite −1. Evidence in support of this hypothesis has been provided, in part, by using NAG-thiazoline in both inhibition and crystallographic studies of a bacterial hexosaminidase [23,27,30]. Hence, NAG-thiazoline was synthesized in 46% overall yield from GlcNAc by treatment with Lawesson's reagent and evaluated as an inhibitor of sMltB. A 61% decrease in apparent activity was observed for sMltB when assayed in

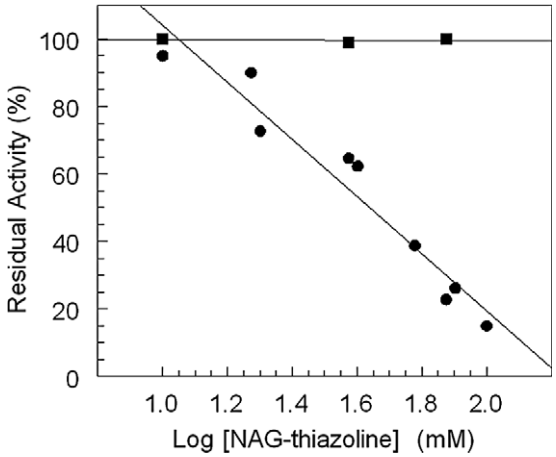

Fig. 4. Inhibition of sMltB by NAG-thiazoline. sMltB in 50 mM sodium acetate, pH 5.8, was treated with 0–80 mM (●) NAG-thiazoline or (■) GlcNAc and then assayed for residual activity by monitoring the release of soluble fragments from an insoluble peptidoglycan substrate. Representative data are presented as a percentage of residual activity in the absence of ligand.

the presence of 60 mM NAG thiazoline (Fig. 4). As expected, this inhibition was found to be reversible as enzymatic activity was recovered upon dilution of the compound from the enzyme. In contrast, GlcNAc, the parent compound of NAG-thiazoline, at concentrations up to 75 mM under similar conditions had no apparent effect on enzyme activity. Given the inherent heterogeneity of the insoluble peptidoglycan substrate, the complexity of the assay for sMltB, and the possibility that the small ligand interacts with the insoluble heteropolymeric substrate (which may account for the relatively poor inhibition observed), the determination of a K_i for the inhibition was not attempted. Instead, the binding constants for the interaction of sMltB with NAG-thiazoline were determined as described below.

3.4. Determination of K_d for NAG-thiazoline

Using a BIAcore CM5 sensor chip bound with sMltB, a dose-dependent interaction with NAG-thiazoline was observed (Fig. 5). In contrast, injection of up to 200 μM GlcNAc to the same sensor chip did not generate a similar dose-dependent response (Fig. 5 inset). Analysis of the sensograms provided a K_d value of 1.77 ± 0.13 mM for NAG-thiazoline. This value is in close agreement with the K_d value obtained by difference UV absorbance spectroscopy. Difference spectra showed a negative trough at 277 nm with a red shift upon binding of NAG-thiazoline to the protein (Fig. 6). This red shift indicates a conformational change of an aromatic residue to a more non-polar environment. Saturation curves of sMltB titrated with NAG-thiazoline and transformation of the data (Fig. 6, inset) gave a K_d of 1.36 ± 0.077 mM. Again, in contrast, addition of GlcNAc showed a small positive peak at 277 nm which can be attributed to either non-specific binding to the protein or binding to another subsite of the enzyme's substrate binding cleft. These data thus provide further evidence for the specificity of binding that the thiazoline moiety confers.

3.5. Concluding remarks

Dijkstra and co-workers [11] have proposed that the putative oxocarbenium ion transition state formed during the cleavage of peptidoglycan by LTs is stabilized by the *N*-acetyl oxygen of MurNAc at subsite −1 either by a charge–charge

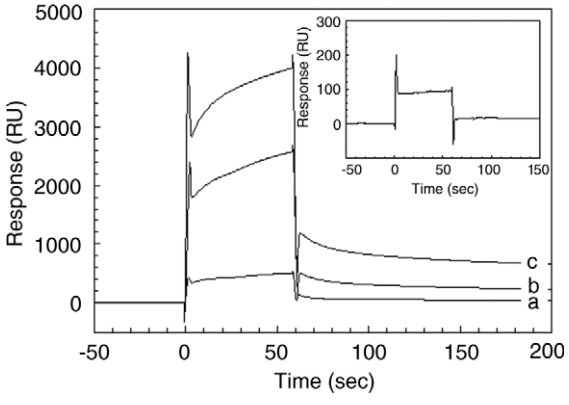

Fig. 5. SPR sensogram of molecular interactions between sMltB and NAG-thiazoline. The sensograms show the interaction for 60 s of three concentrations of NAG-thiazoline (a, 20; b, 100; and c, 200 μM) with sMltB immobilized onto a CM5 sensor chip using a BIAcore 2000. Inset: SPR sensogram obtained by a single injection of 200 μM GlcNAc to the same sensor chip.

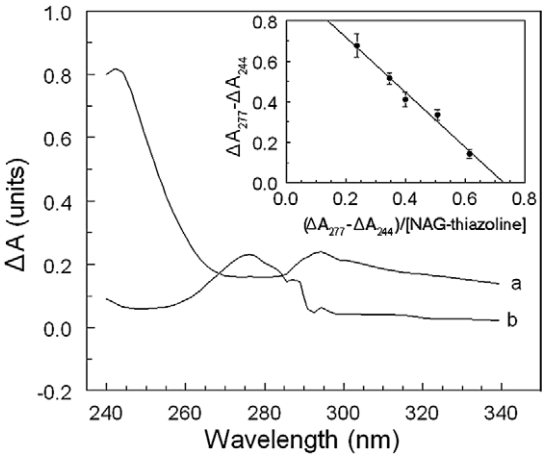

Fig. 6. UV difference absorbance spectra of sMltB complexed with ligands NAG-thiazoline or *N*-acetyl glucosamine. Representative absorbance difference spectra were obtained after incubating sMltB (2.16 μM) in 10 mM ammonium acetate buffer, pH 6.5 containing 100 mM NaCl for 90 s with (a) 2.19 mM NAG-thiazoline, and (b) 2.18 mM GlcNAc. The scans were normalized by subtraction of the scan at zero ligand concentration. Inset: Determination of the K_d value of sMltB for NAG-thiazoline ($n = 3$).

interaction or by the formation of a covalent oxazolinium intermediate. Given our current observations with NAG-thiazoline, it is tempting to suggest that we have provided the first evidence for the latter mechanism involving an oxazolinium ion intermediate. Thus, the catalytic glutamyl residue (Glu162) is proposed to serve as an acid catalyst to donate a proton to the glycosidic oxygen of the linkage to be cleaved leading to the formation of an intermediate with oxacarbenium ion character (Fig. 7). In the absence of an anion/nucleophile in close proximity to stabilize this oxacarbenium intermediate, the LTs would employ anchimeric assistance of the MurNAc 2-acetamido group resulting in the formation of an oxazolinium ion intermediate. With the shielding of the intermediate from solvent water [11], this would then be followed by abstraction of the C-6 hydroxyl proton of the oxazolinium species involving the same catalytic glutamyl residue which now serves as the base catalyst leading to the formation of 1,6-anhydromuramic acid product.

Mechanism-based inhibitors are expected to bind active sites with high affinity. This was indeed the case for NAG-thiazoline when serving as a reaction intermediate analogue of *N*-acetylglucosaminidases [23]. NAG-thiazoline is derived directly from GlcNAc, which is the repeating subunit of *N*-acetylglucosaminidase's homopolymeric substrate chitin, and the inhibition constant of this inhibitor for the enzyme is four orders of magnitude lower than that of its parent (compare 0.28 μM to 6 mM, respectively) [23]. The K_d value obtained for NAG-thiazoline with sMltB was comparatively much higher, but this needs to be considered in the context that the parent compound GlcNAc does not bind the enzyme with any measurable affinity. Moreover, the situation of employing monomeric inhibitors to probe enzymes that act on branched- and/or hetero-polymeric substrates appears to be more complex. The example of the monosaccharide and transition-state inhibitor gluconolactone serves to illustrate this point well. Notwithstanding its use and value over the past 40 years to probe the mechanism of action of a variety of the hydrolytic

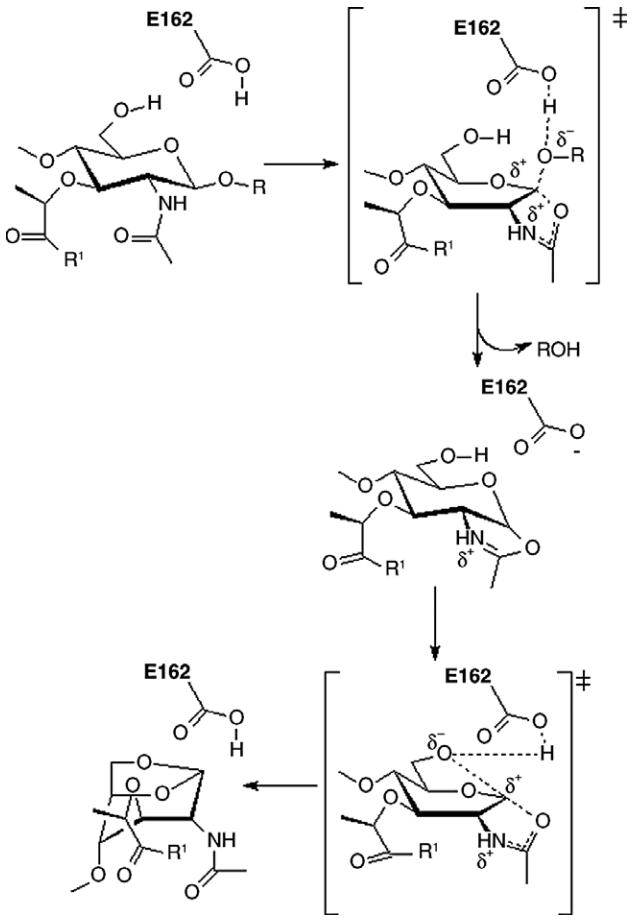

Fig. 7. Proposed reaction mechanism of MltB. Glu162 serves initially as an acid to protonate the glycosidic linkage to be cleaved leading to the formation of the muramoyl oxazolinium-ion intermediate, and then as a base to abstract the C-6 hydroxyl proton promoting collapse of the oxazolinium ion and concomitant formation of the 1,6-anhydromuramoyl product.

carbohydrases including lysozyme, gluconolactone typically binds with K_d values in the mM range. As with the LTs, the reaction center of lysozyme involves a muramoyl residue and so like NAG-thiazoline, gluconolactone would represent a very simple inhibitor. In fact, gluconolactone and other monosaccharide-derived lactones, including that of GlcNAc, proved to be too simple in structure for lysozyme as numerous attempts by Phillips and co-workers to bind them into subsite D at the catalytic center of lysozyme failed [42]. The lactone was subsequently shown to be a transition state analogue of the enzyme when it comprised a tetrasaccharide (viz. tetra-GlcNAc), thereby permitting more extensive contacts with the enzyme's binding subsites [43].

In the light of these and other findings regarding carbohydrases and simple transition-state analogues of their complex substrates, it is not too surprising that NAG-thiazoline was found to bind sMltB with relatively weak affinity. This view is consistent with our recent findings that the key features of ligands associated with binding to sMltB include the D-lactyl side chain and associated peptide at C-3 of muramoyl residues [44]. Indeed, an extensive network of contacts is seen in the crystal structure of *E. coli* Slt35-ligand complexes between the enzyme and both the D-lactyl moiety and the stem peptide of the ligands [11]. At subsite −1, which is adjacent to the site of cleavage, these include a hydrogen bond between the invariant Arg188 residue and the carbonyl of the D-lactyl group, and a salt bridge between the highly conserved Arg187 residue [5] and the free β-carboxyl group of the γ-glutamyl residue of the stem peptide. Recognizing that NAG-thiazoline lacks both lactyl and peptidyl side chains in addition to being only monomeric, thereby precluding important binding contacts in subsites beyond the catalytic center of the enzyme, efforts have been directed towards the synthesis of thiazoline derivatives that more closely mimic the muramyl substrate with associated stem peptide. Unfortunately, success has not been achieved to date due to the instability of the thiazoline moiety associated with these derivatives during de-protection reactions. Nonetheless, we are encouraged to continue this research in the light of our recent observations that NAG-thiazoline causes changes to both morphology and cell-surface hydrophobicity of growing *E. coli* [31].

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