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Structure-Activity Relationship Studies of the Two-Component Lantibiotic Haloduracin

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SUMMARY

The lantibiotic haloduracin consists of two post-translationally processed peptides, Halα and Halβ, which act in synergy to provide bactericidal activity. An in vitro haloduracin production system was used to examine the biological impact of disrupting individual thioether rings in each peptide. Surprisingly, the Halα B ring, which contains a highly conserved CTLTXEC motif, was expendable. This motif has been proposed to interact with haloduracin's predicted target, lipid II. Exchange of the glutamate residue in this motif for alanine or glutamine completely abolished antibacterial activity. This study also established that Halα-Ser26 and Halβ-Ser22 escape dehydration, requiring revision of the Halβ structure previously proposed. Extracellular proteases secreted by the producer strain can remove the leader peptide, and the Halα cystine that is dispensable for bioactivity protects Halα from further proteolytic degradation.

INTRODUCTION

Lantibiotics are a class of cyclic peptides produced by Gram-positive bacteria. They are ribosomally synthesized as inactive precursor peptides that undergo posttranslational modification to generate their mature, biologically active forms. More than 50 lantibiotics are known to date, most of which function as antimicrobial peptides (Chatterjee et al., 2005b; Cotter et al., 2005; Willey and van der Donk, 2007). Notably, the most extensively studied member, nisin, has been used worldwide for decades in the food industry without the development of widespread antibiotic resistance (Delves-Broughton et al., 1996). Moreover, other lantibiotics, such as mersacidin, lacticin 3147, and microbisporicin, are effective against methicillin-resistant *Staphylococcus aureus* and other multidrug-resistant pathogens (Castiglione et al., 2008; Galvin et al., 1999; Kruszewska et al., 2004).

All lantibiotics contain the characteristic lanthionine (Lan) and/or methyllanthionine residues (MeLan), and also typically the unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) that are introduced by posttranslational modification. Lantibiotic precursor peptides (LanA) consist of a structural region that will be transformed into the antimicrobial peptide and an N-terminal leader sequence that does not undergo modification. Specific Ser and Thr residues in the structural region are enzymatically dehydrated to yield Dha and Dhb, respectively (Figure 1). Through a Michael-type addition, Cys residues react with these dehydro amino acids to form the thioether rings, Lan from Ser and MeLan from Thr. The precise roles of the leader peptide are still under debate (Furgerson Ihnken et al., 2008; Levengood et al., 2007; Patton et al., 2008), but following modification it must be removed by proteolysis to generate the active lantibiotic (Chen et al., 2001; Corvey et al., 2003; Li et al., 2006; McClerren et al., 2006; van der Meer et al., 1994; Xie et al., 2004).

A subclass of lantibiotics consists of the two-peptide systems. These compounds are formed from two precursor peptides posttranslationally modified to create two distinct products that act in synergy to provide bactericidal activity (Garneau et al., 2002). Currently, seven two-component lantibiotics are known, including the best-studied example, lacticin 3147 (Ryan et al., 1999), and the most recently identified member, haloduracin (Lawton et al., 2007; McClerren et al., 2006). For haloduracin, produced by the Gram-positive alkaliphilic bacterium *Bacillus halodurans* C-125, the unmodified peptides HalA1 and HalA2 are each dehydrated and cyclized by two dedicated bifunctional enzymes, HalM1 and HalM2, respectively. During this process, one of the four Ser/Thr residues in the structural region of HalA1 and one of the eight Ser/Thr residues in the structural peptide of HalA2 escape dehydration by their respective modification enzymes. Proteolytic removal of the leader peptide results in the mature, active components, Halα and Halβ. The HalA1 peptide is predicted to have 32% and 44% amino acid sequence identity with the precursor peptides from mersacidin (P43683) and lacticin 3147 A1 (O87236), respectively. Both mersacidin and the mature A1 component of lacticin 3147 bind to lipid II, an essential precursor in peptidoglycan biosynthesis (Brötz et al., 1998; Wiedemann et al., 2006). A sequential model has been proposed for the bactericidal action of two-peptide lantibiotics in which the α (A1) peptide first binds to lipid II and the α:lipid II complex serves as a docking site for the β (A2) peptide. Once recruited, the extended conformation of the β component is believed to facilitate pore formation in the bacterial membrane (Morgan et al., 2005; Wiedemann et al., 2006).

Herein, the structural features essential for biological activity of haloduracin were investigated using a recently developed in vitro biosynthetic system (McClerren et al., 2006). A number of haloduracin analogs were constructed and the impact of the

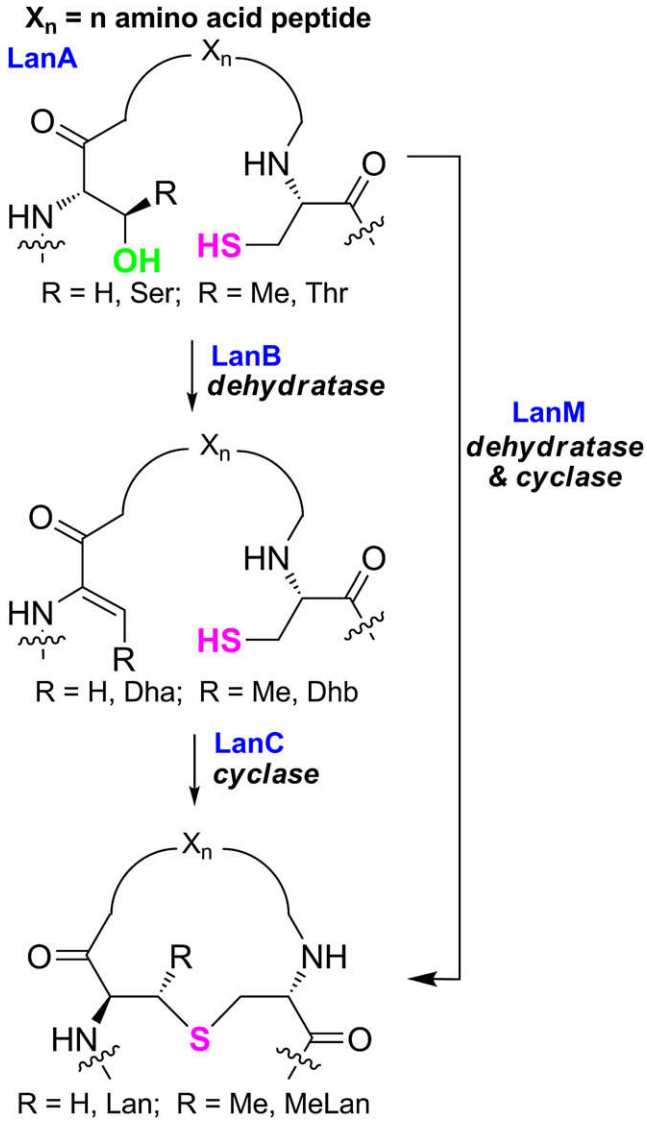

Figure 1. General Overview of the Common Steps in Lantibiotic Biosynthesis

LanA, lantibiotic precursor peptide.

alterations on antibiotic activity was assessed. In addition, this study suggests that some or all of the proteolytic process that removes the leader peptides may be performed by extracellular proteases secreted by the *B. halodurans* producing strain.

RESULTS

Structures of Halα and Halβ

Based on the mass of haloduracin prepared enzymatically in vitro as well as the products isolated from *B. halodurans* C-125, one Ser/Thr residue escapes dehydration in both Halα and Halβ (McClerren et al., 2006). To identify the locations of these residues and to examine their biological significance, specific Ser/Thr residues were mutated in the substrate peptides to Ala. The mutant substrates were subjected to HalM1 or HalM2 and analyzed by MALDI-TOF MS. For HalA1, three of four residues undergo dehydration. Comparison of its sequence to that of other lantibiotics suggested that Ser26 escaped dehydration (McClerren et al., 2006; Figure 2A). To test this hypothesis the S26A mutant of HalA1-Xa was constructed by site-directed mutagenesis and processed in vitro with HalM1. As discussed previously (McClerren et al., 2006), this substrate contains a Factor Xa recognition site for leader peptide removal. Upon subjection to HalM1 under standard assay conditions (McClerren et al., 2006), three dehydrations were still observed by MS (see Figure S1A available online), thus indicating that Ser26 is indeed the residue that is not acted upon by HalM1. For Halβ, it was established that seven of eight Ser/Thr residues undergo dehydration in the structural region of HalA2 (McClerren et al., 2006). Based on homology to other lantibiotics it was proposed that Thr18 was not dehydrated. However, when the T18A mutant of HalA2-Xa was treated with HalM2, a product with 6-fold dehydration was observed (Figure S1B). This finding strongly suggests that Thr18 is normally dehydrated by HalM2. FTMS/MS data obtained previously narrowed down the other possible candidates for the residue that is not dehydrated to Thr17, Thr21, or Ser22 (McClerren et al., 2006). Thr17 and Thr21 are believed to be involved in methyllanthionine rings that are highly conserved in two-component lantibiotics (McClerren et al., 2006). Thus, HalA2-Xa S22A was constructed and subjected to HalM2 catalysis. MS analysis showed that this mutant was dehydrated 7-fold (Figure 3), demonstrating that Ser22 escapes dehydration by HalM2 in wild-type HalA2. The structure for Halβ has been revised to reflect these data (Figure 2B).

The cyclization reaction is more difficult to examine because it does not involve a change in mass. In previous studies, techniques were developed that report on noncyclized cysteines through derivatization of their thiol groups (Li and van der Donk, 2007; Li et al., 2006; McClerren et al., 2006; Paul et al., 2007). Control reactions with unmodified HalA1 and HalA2 substrates indicated that iodoacetamide (IAA) was more effective for HalA1, whereas *p*-hydroxymercuribenzoic acid (PHMB) (Li and van der Donk, 2007; Paul et al., 2007; Pitts and Summers, 2002) showed better derivatization efficiency for HalA2. Hence, throughout this study IAA was used to test cyclization of HalA1 mutants, and PHMB was used for HalA2. The reasons behind the different derivatization efficiency of these peptides were not investigated but are likely related to their different solubilities. The products of the enzyme assays with HalA1-Xa S26A, HalA2-Xa T18A, and HalA2-Xa S22A were treated with reductant followed by thiol derivatizing agent and monitored by MALDI-TOF MS, showing that none of the assay products displayed adducts (beyond the two expected for the cysteine residues of the disulfide in Halα) and demonstrating that these mutants were cyclized (Figure S1C–E). Removal of the leader peptide for evaluation of antimicrobial activity of the haloduracin analogs produced was accomplished using the previously described Factor Xa engineered system (McClerren et al., 2006). Briefly, in substrates denoted HalA1/2-Xa, the four amino acids in the leader peptides immediately prior to the structural region of HalA1 (Val-Asn-Gly-Ala) and HalA2 (Val-His-Ala-Gln) were replaced with the Factor Xa recognition site (Ile-Glu-Gly-Arg; Figure 2). After treatment of the HalM1 and HalM2 enzymatic products with Factor Xa and confirmation of proteolytic processing by MALDI-TOF MS, agar diffusion assays against a haloduracin-sensitive strain,

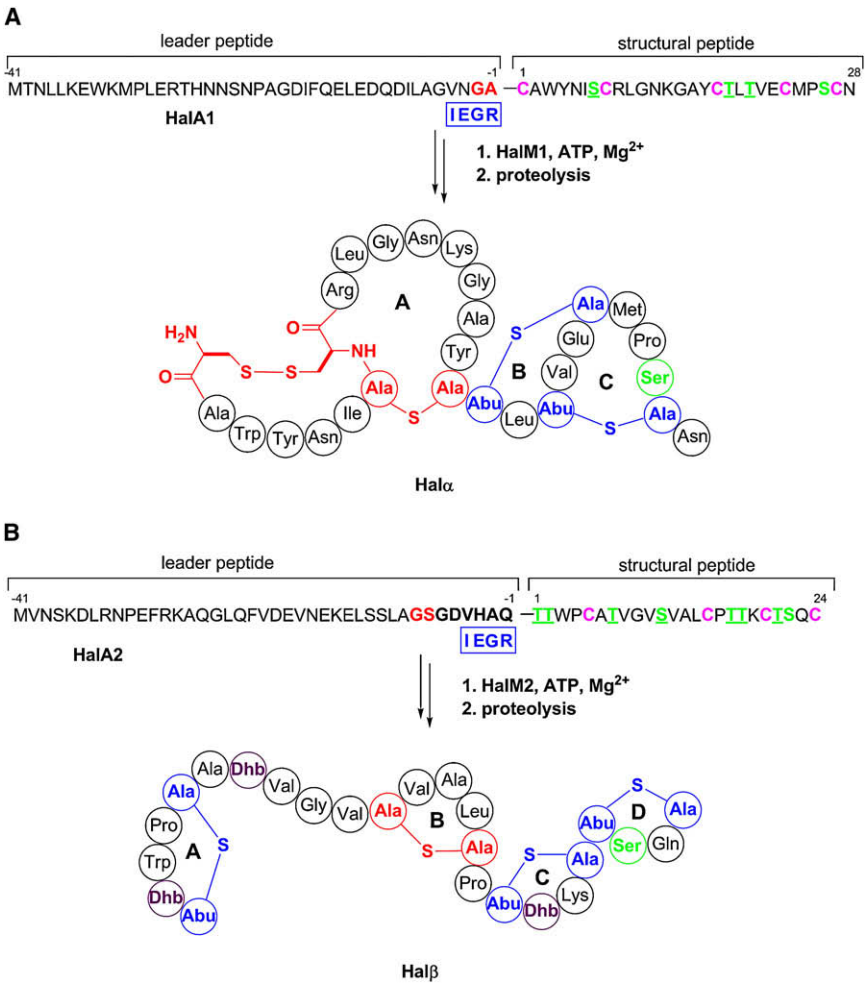

Figure 2. Biosynthesis of Halα and Halβ

(A and B) In substrates designated HalA1-Xa or HalA2-Xa, the four amino acids at positions −1 to −4 were replaced by IEGR (boxed in blue). In (B), the revised structure of Halβ is shown based on the results in this study.

Lactococcus lactis CNRZ 117, were performed. The data showed that the two residues that escape dehydration (Halα Ser26 and Halβ Ser22) are not necessary for antibacterial activity as replacement with Ala still resulted in zones of growth inhibition. Furthermore, when an Ala residue was installed in place of Dhb18 of Halβ, bioactivity was retained (Figure 4, regions 14–16).

Thioether Ring Disruption in Halα and Halβ

To evaluate the significance of each sulfide ring in Halα and Halβ, they were disrupted individually by converting Cys residues to Ala in the substrate peptides. Previously it was shown that the cystine linkage in Halα was not required for bacterial growth inhibition (McClerren et al., 2006). Even when the Cys thiols were alkylated by treatment with iodoacetamide (IAA), a zone of inhibition was observed in agar diffusion assays. The nonessential nature of the cystine was confirmed in this study by generation of the HalA1-Xa C8A peptide and incubation with HalM1, resulting in a 3-fold dehydrated product (Figure 5A). Subsequent analysis with IAA for cyclization as discussed previously showed that the mutant contained a single reactive Cys (Cys1), indicating that three Cys residues were unavailable for alkylation (Figure 5B), a result consistent with formation of three thioether rings. The HalM1 product was treated with Factor Xa protease to remove the leader sequence, and tested against the bacterial indicator strain, resulting in a zone of inhibition (Figure 4, region 3) and confirming that the disulfide is dispensable.

Using a similar strategy, the A, B, and C rings were disrupted by mutating Cys17, Cys23, and Cys27 of HalA1-Xa to Ala, respectively. MALDI-TOF MS analysis of the assay products prepared by incubation of the HalA1-Xa Cys-to-Ala mutants with HalM1 showed that 3-fold dehydration occurred for all (Figure S2), and IAA analysis revealed that all of these HalM1-processed mutants contained two reactive Cys residues corresponding to Cys1 and Cys8. The IAA derivatization studies suggest that all other Cys residues underwent the anticipated cyclization in these mutants. Subsequent removal of the leader peptide and qualitative antimicrobial assays showed that the C ring was essential for bioactivity because the C27A mutant did not produce a zone of inhibition (Figure 4, region 6). Surprisingly, the B ring proved to be expendable as Halα-C23A gave a clear inhibition zone when antibiotic activity was assessed in three independent experiments (Figure 4, region 5). A small but reproducible zone was observed for the C17A mutant, suggesting the A ring is important but not absolutely essential (region 4).

Evaluation of the importance of the Lan and MeLan rings in Halβ was similarly completed. The A, B, C, and D rings were individually disrupted by conversion of Cys5, Cys15, Cys20, and Cys24 of HalA2-Xa to Ala, respectively. Incubation with HalM2 and analysis by MALDI-TOF MS revealed seven dehydrations for all four mutants as shown for the C5A mutant in Figure 5C (for all other mutants, see Figure S3). Subsequent analysis with PHMB for cyclization showed that the mutants C5A, C20A, and C24A did not contain any free thiols, suggesting they underwent complete cyclization as shown for C5A in Figure 5D (for other mutants, see Figure S3). However, C15A did display multiple adducts derived from PHMB (Figure S3C), indicating that disruption of the B ring also affected ring formation of one or more of the other rings. The consequences of removal of one or more rings on antimicrobial activity were evaluated by agar diffusion assays after leader peptide removal. The mutant that lacked the A ring (C5A) retained antibiotic activity (Figure 4, region 8). No zone of inhibition was observed for the C15A mutant of Halβ, and only weak zones of growth inhibition

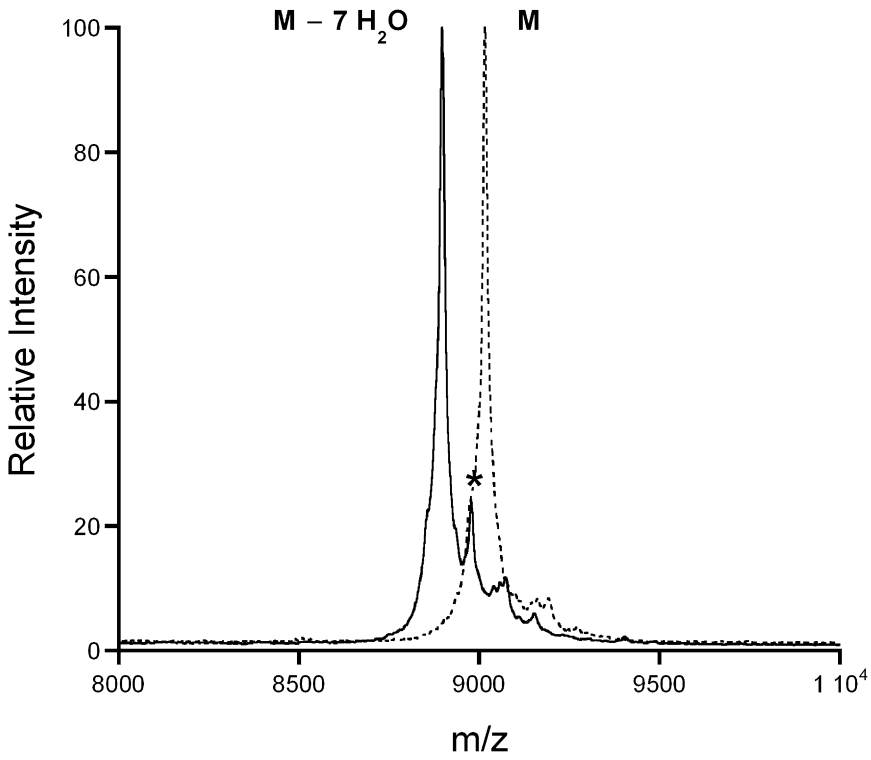

Figure 3. MALDI-TOF MS of HalA2-Xa S22A
HalA2-Xa S22A before (dashed line) and after (solid line) incubation with HalM2. The asterisks (*) indicate a phosphorylated peptide (Chatterjee et al., 2005a).

were observed for the C20A and C24A mutants (Figure 4, regions 9–11).

A Highly Conserved Glutamate Residue in Halα Is Essential for Bioactivity

It was recently shown that a highly conserved glutamate in mersacidin (Glu17) was essential for binding to its target lipid II (Brötz et al., 1998; Hsu et al., 2003; Szekat et al., 2003). Because of the high degree of homology between the C ring of mersacidin and the B ring of Halα (Figure 6A), the corresponding Glu22 could play a similar role in the mode of action of haloduracin. Therefore, Glu22 in HalA1-Xa was mutated to Ala and Gln. Upon treatment with HalM1, these mutants underwent three dehydrations as expected (Figure 6B). Cyclization was confirmed using IAA treatment under reducing conditions followed by MS analysis. The two IAA adducts observed were assigned to the two Cys residues that form the disulfide ring of HalA1-Xa (Figure 6C). Bioactivity analysis after removal of the leader peptide with Factor Xa showed that antibacterial activity was completely abolished under the conditions used (Figure 4, regions 12 and 13).

Proteolytic Processing

Leader peptide removal for HalA1 and HalA2 is proposed to be achieved in vivo by a single enzyme termed HalT (Lawton et al., 2007; McClerren et al., 2006). The predicted cleavage site for LanT protease domains is after a double-Gly recognition motif (GG, GA, or GS sequences; Håvarstein et al., 1995; Nes and Tagg, 1996). For HalA1, proteolysis behind the predicted cleavage site Gly-2Ala-1 (Figure 2A) was confirmed by mass spectrometry on haloduracin α produced by *B. halodurans* C-125 (McClerren et al., 2006). For HalA2, the cleavage was predicted to take place after Gly-8Ser-7 (Figure 2B), yet the mass spectrometric data on Halβ produced by *B. halodurans* C-125 showed that residues Gly-6 through Gln-1 were also removed (Figure 2B). Therefore, if HalT cleaves after the GlySer sequence, then an additional N-terminal proteolytic step must occur. The biological significance of the removal of the residues GDVHAQ was examined by the generation of a mutant of HalA2, designated HalA2-XaGS, with the Factor Xa cleavage signal immediately following the predicted HalT cleavage signal at Gly-8Ser-7 (Figure S4A). This construct was incubated with HalM2 to yield the expected 7-fold dehydrated product (Figure S4B) and was further processed by treatment with Factor Xa. When the resulting product designated GDVHAQ-Halβ was tested for antibiotic activity in the presence of Halα, inhibition zones comparable to in vivo and in vitro prepared haloduracin were observed (Figure S4D). This data indicated that the additional six amino acids on the N terminus of this mutant product do not interfere with the bioactivity of haloduracin.

We investigated the possibility that N-terminal trimming of the GDVHAQ-Halβ product occurred on the agar plate by extracellular proteases from the reporter strain during the bioassay, and that the trimmed Halβ product was actually responsible for the observed zone of inhibition. Furthermore, we investigated whether the producer strain *B. halodurans* C-125 secretes a protease that could perform N-terminal trimming of longer Halβ products. For this purpose, the HalA2-XaGS Q23A mutant was generated. The Q23A mutation was introduced so that Halβ generated by processing of GDVHAQ-Halβ could be distinguished from Halβ produced endogenously by *B. halodurans* C-125. This mutation was chosen because an Ala is present at this position in lacticin 3147 A2. The peptide was expressed in *E. coli*, purified, and subjected to HalM2 modification, resulting in seven dehydrations and complete cyclization (Figures S5A and S5B). Subsequent Factor Xa cleavage yielded GDVHAQ-Halβ Q23A as observed by MALDI-TOF MS. Incubation with the *L. lactis* CNRZ 117 indicator strain did not result in processing of GDVHAQ-Halβ Q23A (Figure 7A). However, the culture supernatant from the *B. halodurans* C-125 producer strain did contain one or more proteases that cleanly removed the GDVHAQ sequence, resulting in Halβ Q23A (Figure 7A). In a second experiment, full-length HalA2-XaGS Q23A processed by HalM2 was incubated with the culture supernatant. Once more, proteolytic processing occurred, removing the entire leader sequence and producing Halβ Q23A (Figure 7B). To examine the fate of Halα

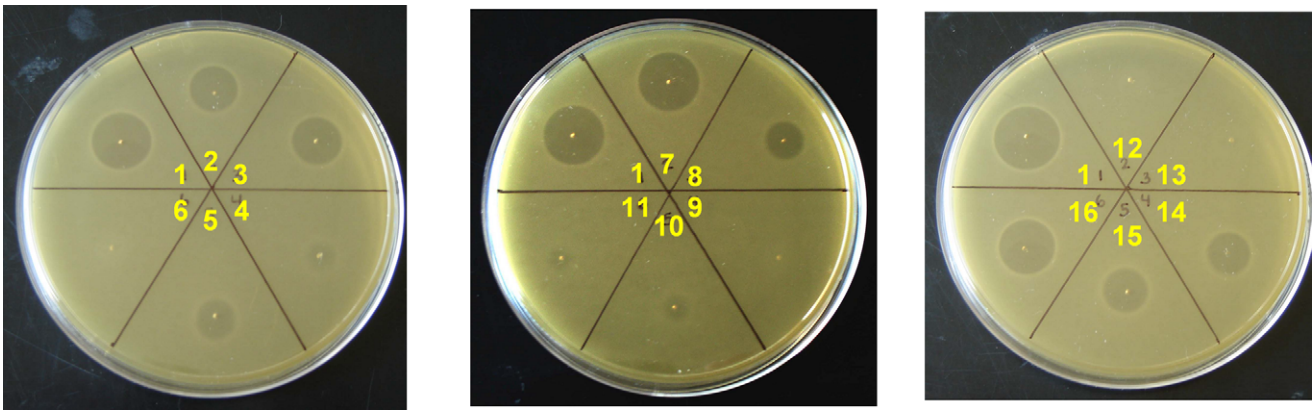

Figure 4. Antimicrobial Activity Assays for Haloduracin Mutants against the Indicator Strain *L. lactis* CNRZ 117

In vitro refers to compound produced with purified HalM enzymes and subsequent proteolysis with Factor Xa. In vivo refers to Halα or Halβ isolated and purified from *B. halodurans* C-125. Region 1, Halα + Halβ (both in vivo); region 2, Halα (in vitro) and Halβ (in vivo); region 3, Halα C8A (in vitro) + Halβ (in vivo); region 4, Halα C17A (in vitro) + Halβ (in vivo); region 5, Halα C23A (in vitro) + Halβ (in vivo); region 6, Halα C27A (in vitro) + Halβ (in vivo); region 7, Halα (in vivo) + Halβ (in vitro); region 8, Halα (in vivo) + Halβ C5A (in vitro); region 9, Halα (in vivo) + Halβ C15A (in vitro); region 10, Halα (in vivo) + Halβ C20A (in vitro); region 11, Halα (in vivo) + Halβ C24A (in vitro); region 12, Halα E22A (in vitro) + Halβ (in vivo); region 13, Halα E22Q (in vitro) + Halβ (in vivo); region 14, Halα S26A (in vitro) + Halβ (in vivo); region 15, Halα (in vivo) + Halβ Dhb18Ala (in vitro); region 16, Halα (in vivo) + Halβ S22A (in vitro).

under these conditions, purified Halα was incubated with the culture supernatant of *B. halodurans* C-125 with and without pretreatment with the reductant tris(2-carboxyethyl)phosphine (TCEP) to reduce the N-terminal disulfide and subsequent alkylation by IAA. Interestingly, the Halα that was not treated with TCEP/IAA did not undergo any proteolysis as judged by MALDI-TOF MS (Figure S5D), but the sample preincubated with TCEP/IAA was proteolytically processed to peptides corresponding to loss of the N-terminal three and five amino acids (Figure 7C).

DISCUSSION

The ribosomal origin of the lantibiotics has provided a convenient means to conduct structure-activity relationship studies via the generation of analogs by mutagenesis of the prepeptides. Such mutagenesis studies have been carried out both in vivo in the producer strains or a suitable heterologous host, and in reconstituted in vitro systems (Chatterjee et al., 2005b; Cotter et al., 2005; Lubelski et al., 2008). In this study, haloduracin mutants were produced in vitro and analyzed for antibacterial activity with a focus on the posttranslational modifications. The outcome with the S26A mutant of HalA1 confirmed the previous hypothesis based on sequence homology with other lantibiotics that this residue is not dehydrated in Halα. However, the findings with both HalA2 T18A and S22A mutants are inconsistent with the previously proposed structure of Halβ based on sequence homology (McClerren et al., 2006). Both mutants strongly suggest that Ser22 in Halβ is not dehydrated, a result that was confirmed for wild-type HalA2 by postsource decay MS in a study on the directionality and processivity of HalM2 (unpublished data). The observation that the residues that escape dehydration in both Halα and Halβ are serines is in line with a previous study that compared all known lantibiotics and concluded that Ser residues escape dehydration with a much higher frequency than Thr residues (Rink et al., 2005). The underlying reason for this trend is not known. In the case of haloduracin, the flanking residues of Ser26 in Halα (Pro and Cys) and Ser22 in Halβ (Thr/Dhb and Gln) are not obvious deactivating residues. For instance, a Pro flanking Thr17 and Thr/Dhb flanking Thr2 and Thr17 in HalA2 did not negatively affect dehydration by HalM2. Although the molecular logic of why lantibiotic synthetases sometimes skip a Ser/Thr is not known, a potential evolutionary reason could be that the unmodified Ser/Thr in question is important for the biological activity of the final compound. It appears, however, that this is not the case for Ser26 in Halα and Ser22 in Halβ, as shown by antimicrobial assays of analogs in which these residues were mutated. Similarly, the Thr17, Thr19, and Thr23 residues that remain unmodified in lacticin 3147 A2 (but have no homologous residues in haloduracin) were not critical for its biological activity (Cotter et al., 2006).

The corollary of Ser22 escaping dehydration in Halβ is that Thr18 must be dehydrated, in contrast to the homologous residue in lacticin 3147 A2 (Thr23), which is not dehydrated according to NMR studies (Martin et al., 2004). The differences between these two lantibiotics illustrate that the use of sequence homology to predict the structure of lantibiotics is perilous. This finding is relevant for the currently structurally uncharacterized two-component lantibiotics Smb and BHT for which the genes were recently reported. These predict 7 (BHT) or 8 (Smb) Ser/Thr in the A-peptide and 7 Ser/Thr in the B peptide (Hyink et al., 2005; Yonezawa and Kuramitsu, 2005) (Figure S6), but the masses observed for the mature peptides suggest only five of these residues are dehydrated in each peptide for Smb (Petersen et al., 2006). The BHT/Smb peptides have sequence homology with both haloduracin and lacticin 3147 (Figure S6), but based on the structures of these two lantibiotics it cannot be concluded which residues escape dehydration in Smb/BHT.

The thioether rings in lantibiotics are believed to be the basis for their biological activity, and in general, disruption of a ring results in abolishment of antimicrobial activity (Bierbaum et al., 1996; Chatterjee et al., 2005b, 2006; Chen et al., 1998; Kuipers et al.,

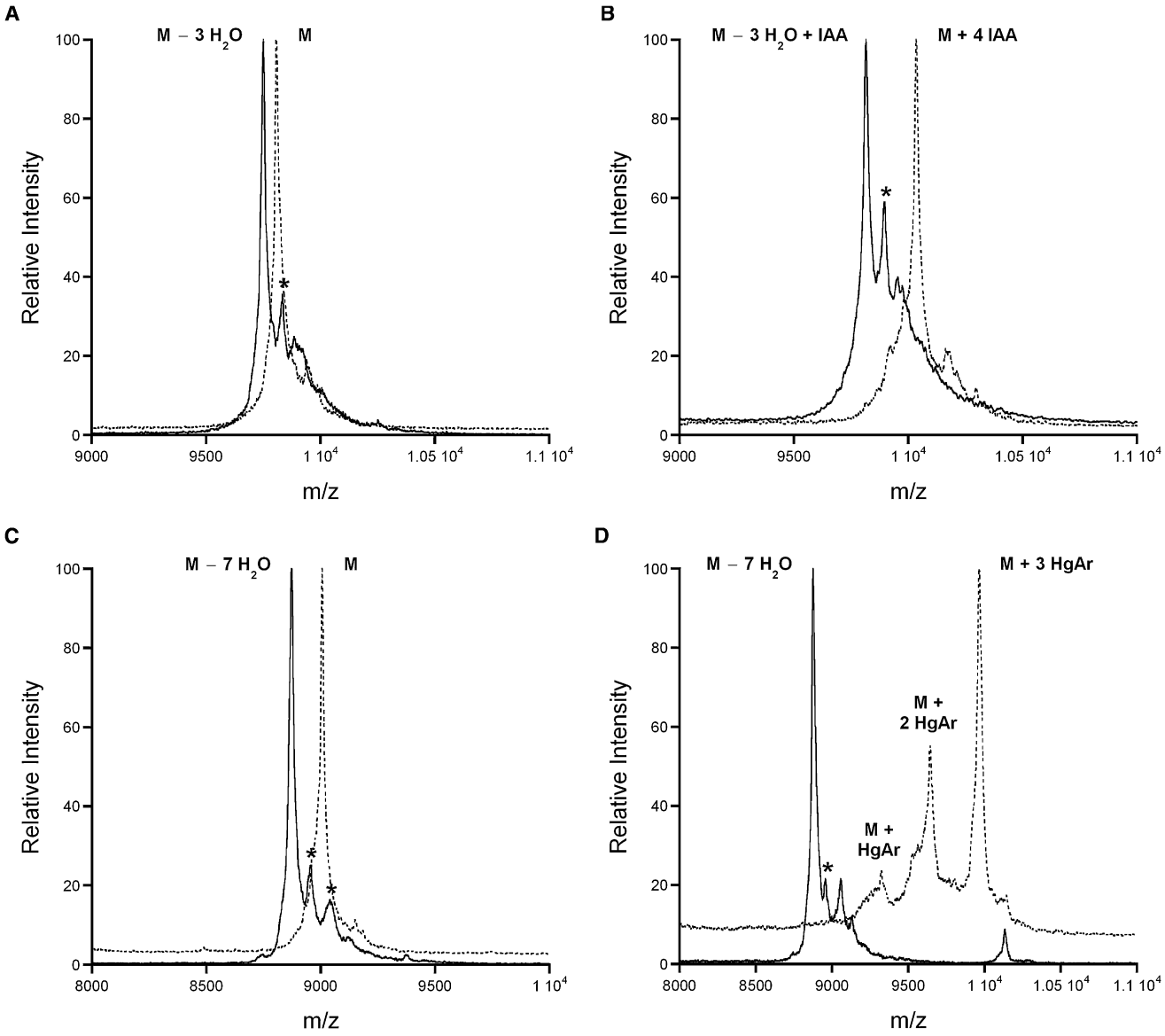

Figure 5. Representative MALDI-TOF MS Data of the HalA1-Xa or HalA2-Xa Cys-to-Ala Mutants Treated with HalM1 or HalM2

The asterisks (*) indicate phosphorylated products (Chatterjee et al., 2005a).

(A) HalA1-Xa C8A before (dashed line) and after incubation with HalM1 (solid line).

(B) HalA1-Xa C8A treated with TCEP/IAA before (dashed line) and after HalM1 (solid line).

(C) HalA2-Xa C5A before (dashed line) and after incubation with HalM2 (solid line).

(D) HalA2-Xa C5A treated with TCEP/PHMB before (dashed line) and after HalM2 treatment (solid line).

1996; Ottenwälder et al., 1995; van Kraaij et al., 2000). At present only limited information is available regarding the importance of the Lan and MeLan rings in each peptide of two-component lantibiotics. Ala scanning and random mutagenesis have been applied to the genes encoding the precursor peptides of lacticin 3147, resulting in a large amount of valuable data (Cotter et al., 2006; Field et al., 2007). However, the absence of bioactivity of several of the mutants involving the thioether rings resulted from abolished production and hence provided no direct information regarding their importance for activity. In the current study, a comprehensive examination of the importance of each (methyl)lanthionine in haloduracin for biological activity was performed, taking advantage of its in vitro reconstituted biosynthesis. These experiments show that mutation of Cys17, Cys23, and Cys27 resulted in Halα analogs in which the A, B, and C rings were disrupted, respectively, without any evidence that the remaining rings were not formed. The qualitative antimicrobial activity of these mutants shows that the C ring of Halα is essential for activity. Its A ring is important but not essential, with a weak zone of inhibition observed, and the B ring is not required. The retention of bioactivity upon disrupting the B ring of Halα was surprising given its high level of conservation in comparison with mersacidin

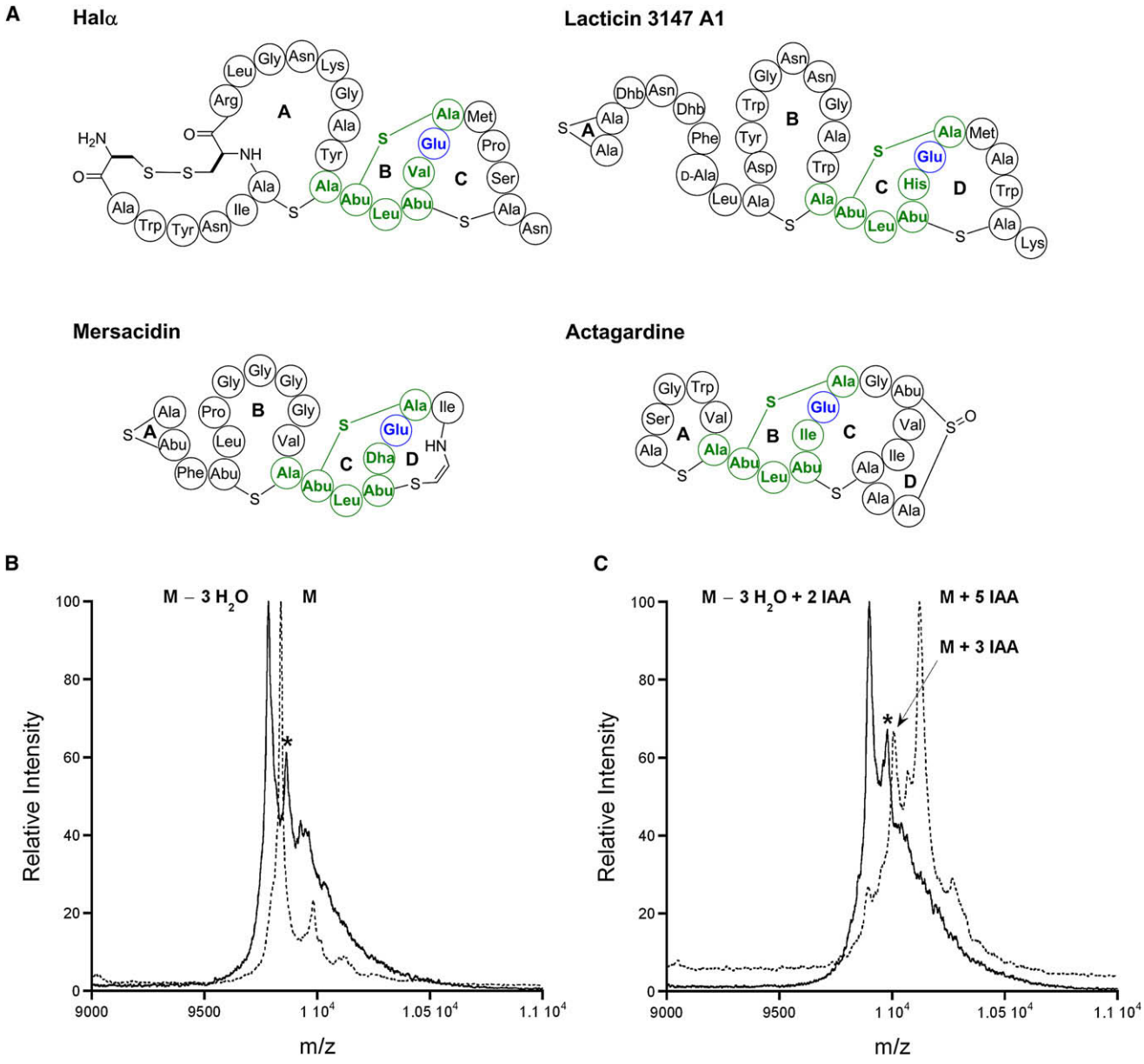

Figure 6. Importance of Glu22 for Bioactivity

(A) Structures of Halα, lacticin 3147 A1, mersacidin, and actagardine. Residues colored green represent the proposed lipid II binding motif (CTLTXEC); the Glu residue is blue.

(B) HalA1-Xa E22Q before (dashed line) and after incubation with HalM1 (solid line). The asterisks (*) indicate phosphorylated peptides (Chatterjee et al., 2005a).

(C) HalA1-Xa E22Q treated with TCEP/IAA before (dashed line) and after HalM1 (solid line).

(C ring; Chatterjee et al., 1992), actagardine (B ring; Zimmermann et al., 1995), and lacticin 3147 A1 (C ring; Ryan et al., 1999; Figure 6). These three lantibiotics bind to the peptidoglycan precursor lipid II (Brötz et al., 1998; Wiedemann et al., 2006; Zimmermann and Jung, 1997), and the CTLTXEC motif encompassing the conserved rings is believed to be important for this activity. For instance, alanine scanning mutagenesis carried out for lacticin 3147 A1 in vivo (Cotter et al., 2006) showed this region was highly sensitive to alteration with respect to bioactivity. In addition, when the C ring in lacticin 3147 A1 was opened, bioactivity was abolished (Cotter et al., 2006; Field et al., 2007). At present it is not known why disruption of the B ring of Halα is not detrimental to bioactivity, especially as the conserved Glu residue in the CTLTXEC motif is critical. The abolishment of antimicrobial activity for Halα E22A and E22Q is in agreement with previous studies on mersacidin in which the Glu was substituted with Ala and antibiotic activity was lost (Szekat et al., 2003).

The qualitative SAR studies on Halβ resulted in removal of its A–D rings. For the mutants disrupting the A, C, and D rings, the rings not targeted by mutagenesis were still formed by HalM2 according to the PHMB data, but mutation of Cys15 resulted in disruption of cyclization for other rings in addition to the B ring. These

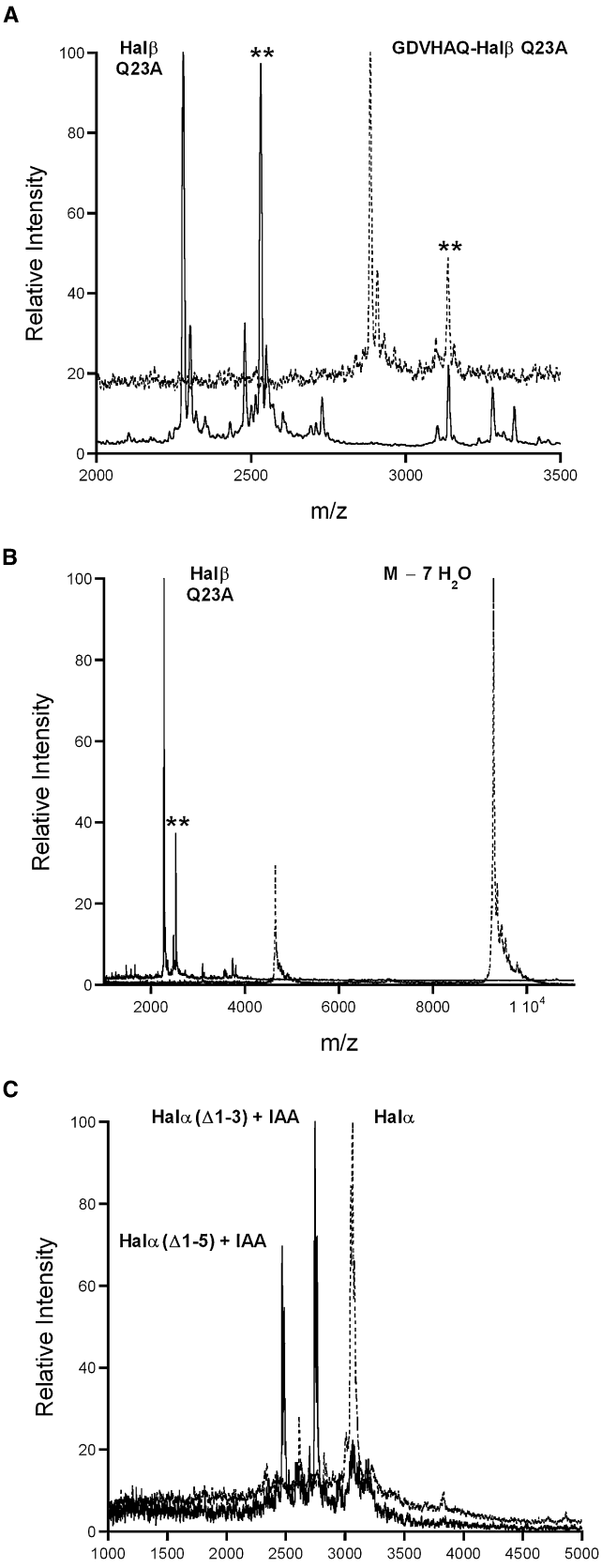

Figure 7. Proteolytic Processing of Haloduracin

(A) HalA2-XaGS Q23A treated with HalM2 and Factor Xa resulted in GDVHAQ-Halβ Q23A. This peptide was used to monitor proteolytic processing. GDVHAQ-Halβ treated with supernatant of *L. lactis* CNRZ 117 (dashed line) or *B. halodurans* C-125 (solid line). GDVHAQ-Halβ Q23A calculated (calcd) 2883 Da; observed (obsd) 2886 Da. A TCEP adduct was observed at 3136 Da (Δm = 250 Da, calcd 3133 Da) and is labeled with a double asterisk (**). Halβ Q23A calcd 2276 Da; obsd 2281 Da. A TCEP adduct was observed at 2532 Da (Δm = 250 Da, calcd 2526 Da) and is labeled with a double asterisk (**).

(B) HalA2-XaGS Q23A treated with HalM2 and then supernatant of *L. lactis* CNRZ 117 (dashed line), calcd 9290 Da (M + H – 7 H_2O); obsd 9290 Da. HalA2-XaGS Q23A treated with HalM2 and then supernatant of *B. halodurans* C-125 (solid line), calcd 2276 Da (Halβ Q23A); obsd 2279 Da.

(C) Halα isolated from *B. halodurans* C-125 was treated with TCEP/IAA. The resulting bisalkylated peptide was then incubated with supernatant of *L. lactis* CNRZ 117 (dashed line) or *B. halodurans* C-125 (solid line). Halα + 2 IAA calcd 3163 Da; obsd 3165 Da. Halα(Δ1–3) + IAA calcd 2745 Da; obsd 2748. Halα(Δ1–5) + IAA calcd 2468 Da; obsd 2469.

findings illustrate the danger of reaching conclusions regarding the importance of certain residues for bioactivity on the basis of mutagenesis studies without confirming that the mutation did not interfere with ring formation. Based on the experimental data, it can be concluded that the A ring in Halβ is dispensable and that the C and D rings are important but not essential. No conclusions can be made regarding the importance of the B ring because the observed lack of bioactivity of the Halβ C15A mutant could be due to disruption of multiple rings.

This study also provides some additional insights into proteolytic processing of haloduracin. Similar to the two-component lantibiotics cytolysin and plantaricin W (Cox et al., 2005; Holo et al., 2001), the mass of the mature Halβ peptide is smaller than that predicted by the cleavage site for the N-terminal protease domain of HalT. Additional proteolysis for cytolysin is carried out by a designated protease, termed CylA, which was shown to be necessary for antibiotic activity (Cox et al., 2005). No CylA homologs have been identified in the *B. halodurans* C-125 genome, yet six amino acids must be removed from the N terminus of the product that is expected to be secreted by HalT. The observed removal of these amino acids upon exposure to the supernatant of *B. halodurans* cell culture strongly suggests that one or more secreted proteases are responsible for this process. When Halα was first reduced, opening the disulfide ring at its N terminus, followed by alkylation of Cys1 and Cys8 with IAA, it too became susceptible to additional N-terminal proteolysis. Because the disulfide is shown not to be important for antimicrobial activity in haloduracin or in plantaricin W (Holo et al., 2001; McClerren et al., 2006), it may be conserved to protect the peptide from such degradation in the extracellular environment. Similarly, the N-terminal lanthionine in lacticin 3147 A1, which is also not required for antimicrobial activity (Cotter et al., 2006), may protect this compound from proteolysis. Interestingly, the great majority of the lantibiotics in the two-component class have cyclic structures at or near their N termini (Figure S6).

SIGNIFICANCE

This study is the first systematic analysis of the SAR of the individual thioether rings in a two-component lantibiotic.

Using an in vitro biosynthetic system, the C ring and Glu22 of Halα of haloduracin are shown to be essential for antimicrobial activity with wild-type Halβ. The A ring of Halα is important but not essential, and surprisingly, given its high conservation in lantibiotics, the B ring is not required. The C and D rings of Halβ were important but not absolutely required for its synergistic activity with wild-type Halα. The disulfide ring present at the N terminus of Halα is not important for its antimicrobial activity but protects the compound from proteolytic degradation by proteases secreted by the producer strain. Collectively, these findings provide new insights into the roles and importance of the crosslinks found in two-component lantibiotics. This study also highlights the value of using an in vitro biosynthetic system for SAR studies.

EXPERIMENTAL PROCEDURES

Construction of all overexpression plasmids and procedures for peptide purification are described in the Supplemental Data along with tables of all masses of the ions observed in the mass spectra shown in this work.

Enzymatic Assays for Dehydration Activity

Purified HalA peptides were each dissolved in HalM assay buffer (50 mM Tris [pH 8.3] for HalA1 peptides and 50 mM MOPS [pH 7.2] for HalA2 peptides) to a final concentration of 0.3 mg/ml, and $MgCl_2$, ATP, and TCEP were added to final concentrations of 10 mM, 2.5 mM, and 1 mM, respectively (final volume 20 μl). Wild-type and mutant HalA1-Xa were incubated in separate reactions with HalM1; wild-type and mutant HalA2-Xa were incubated with HalM2 with a final enzyme concentration of ~0.2 mg/ml enzyme. Activity assays were incubated at 25°C for 3 hr and then quenched by addition of 5% TFA to a final concentration of 0.5% TFA and analyzed by MALDI-TOF MS. Samples were desalted prior to MS using 10 μl C-18 ZipTip (Millipore; Billerica, MA) and eluted with 4 μl of α-hydroxyl cinnamic acid. From this solution, 3 μl was applied to the MALDI-TOF MS target and analyzed.

Evaluation of Cyclization Activity

For HalM1 assays, 1 μl of 100 mM iodoacetamide (IAA) was added directly to 20 μl of HalM1 assay (~5 mM final concentration) and incubated at 25°C for 90 min in the dark. For HalA1 starting material samples, 0.3 mg/ml peptide was incubated in 50 mM Tris [pH 8.3] supplemented with 1 mM TCEP for 20 min prior to addition of IAA, and the reaction was carried out as described. Samples were desalted by C-18 ZipTip (Millipore) and analyzed by MALDI-TOF MS. For HalM2 assays, 20 μl aliquots were dried via centrivap and resuspended in 6 μl of 10 mM TCEP and 4 M guanidine hydrochloride [pH 8]. Samples were incubated at 25°C for 20 min, 3 μl of saturated *p*-hydroxymercuribenzoic acid (PHMB) solution was added, and the reaction mixture was incubated at 25°C for 2 hr in the dark. For HalA2 starting material samples, 0.3 mg/ml peptide was dried via centrivap and PHMB assays were carried out as described. Samples were checked for thiol modification by ZipTip (Millipore) desalting and eluting in 4 μl of α-hydroxyl cinnamic acid to run MALDI-TOF MS.

Antibacterial Activity Assays

Larger-scale enzymatic assays (100 μl) were carried out and then dried via centrivap. The products were resuspended in 100 μl of Factor Xa cleavage buffer (20 mM HEPES [pH 8.0], 100 mM NaCl, 2 mM $CaCl_2$), and 3 μl of 1 mg/ml Factor Xa (New England Biolabs; Ipswich, MA) was added. Cleavage reactions were incubated at 25°C for 4 hr and analyzed by MALDI-TOF MS. Samples were dried by centrivap and resuspended in 5 μl of sterile water. Any insoluble material was removed by centrifugation at 14 krpm for 5 min in a benchtop microcentrifuge. Solid agar diffusion assays were used to assess bactericidal activity. An overnight culture of a haloduracin-sensitive strain, *Lactococcus lactis* CNRZ 117 (Centre National de Recherches Zootechniques; Jouy-enJosas, France), was grown in M17 medium (Difco) supplemented with 0.5% glucose at 30°C under static conditions for 15–20 hr. Plates were prepared by addition of 500 μl of 20% glucose (0.5% final concentration) and 150 μl of dense culture to 20 ml of molten M17 agar (50°C) that was then allowed to solidify in a Petri dish at 25°C. Authentic haloduracin was isolated from *Bacillus halodurans* C-125 (American Type Culture Collection) as described previously (McClerren et al., 2006) and then further purified by RP-HPLC to separate Halα and Halβ (T. Oman and W.v.d.D., unpublished data). For positive control samples, purified Halα and Halβ peptides were combined in a 1:1 ratio (10 μl each of 10 μM solutions). For evaluation of the consequences of mutations, purified Halα or Halβ peptides (5 μl of 10 μM solution) were combined with in vitro prepared mutants (5 μl concentrated HalM products from 100 μl-scale assays). Samples were applied to small wells that were manually formed in the agar plate. The seeded strain was allowed to grow at 25°C for 12 hr, and antibacterial activity was qualitatively determined by the presence or absence of a growth inhibition zone.

Proteolytic Processing

HalA2-XaGS and HalA2-XaGS Q23A were incubated with HalM2 and treated with Factor Xa (50 μl scale). The resulting products, GDVHAQ-Halβ and GDVHAQ-Halβ Q23A, were analyzed for antimicrobial activity using solid agar diffusion assays as described. To test if the six N-terminal amino acids are removed by an external protease, these products were incubated with the cell-free supernatants from *B. halodurans* C-125 and *L. lactis* CNRZ 117 cultures. *B. halodurans* C-125 was grown in brain heart infusion (BHI) medium (Bacto or BBL) for 96 hr at 30°C with vigorous agitation. Cultures were centrifuged (5000 × g, 10 min), and cell-free supernatant was used for assays with GDVHAQ-Halβ Q23A. In addition, the cells were washed with 1 M NaCl. After centrifugation, the cell-free 1 M NaCl wash was also used for assay with GDVHAQ-Halβ Q23A. Production of haloduracin from GDVHAQ-Halβ Q23A was analyzed by MALDI-TOF MS. Both the cell-free supernatant and the 1 M NaCl wash contained proteolytic activity as discussed in Results. *L. lactis* CNRZ 117 was grown as described and its culture supernatant was used for analogous experiments as described for *B. halodurans.* Identical procedures were also used to monitor proteolytic processing of Halα and HalM2-processed HalA2.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and six figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/10/1035/DC1/.

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