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Dissecting the catalytic and substrate binding activity of a class II lanthipeptide synthetase BovM

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ABSTRACT

LanM proteins are the synthetases of the class II lanthipeptides, which are responsible for lanthionine or methyllanthionine formation in lanthipeptides. LanMs are bifunctional enzymes with N-terminal dehydratase and C-terminal cyclase domains. However, the catalytic and especially the substrate binding function of LanM are not fully investigated. In this study, we analyzed the function of conserved residues of BovM, which is the synthetase of lanthipeptide bovicin HJ50, with alanine substitution method. Mass spectrometry (MS) and surface plasmon resonance (SPR) analyses showed six hydrophilic residues (e.g. Asp247) were involved in the dehydration activity of BovM and four hydrophobic residues (e.g. Ile254) were responsible for the substrate binding of BovM. In addition, a conserved Asp155 was proposed to be general base in the elimination of phosphates during the dehydration reactions. This research of BovM shed a light on the catalytic and substrate binding mechanism of LanM proteins.

1. Introduction

Lanthipeptides are ribosomal-synthesized, post-translational modified peptides containing special cyclic amino acids lanthionine (Lan) and 3-methyllanthionine (MeLan). Lanthipeptides possessing antimicrobial activity are called lantibiotics [1,2]. The special residues endue lanthipeptides with biological activity along with thermal stability and proteolytic resistance [3,4]. The Lan and MeLan are generated by dehydration of Ser and Thr residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively and subsequent cyclization with Cys residues [2]. These modifications are executed by dedicated dehydratase LanB and cyclase LanC in class I lanthipeptides while in class II, III and IV lanthipeptides are executed by multifunctional enzymes LanM, LanKC and LanL, respectively [2]. The modified lanthipeptide precursor LanA is secreted and processed to remove the N-terminal leader peptide, thereby producing the active lanthipeptide [1]. Due to the potential in clinical application and the convenient genetic manipulation, the lanthipeptide engineering have drawn more and more attention in recent years [2,5].

LanM proteins are class II lanthipeptide synthetase, which possess broad substrate specificity which can introduce thioether linkages into unnatural substrates [6,7]. Thus, they are attractive options to introduce constrained structure to biological peptides, rendering them with high stability [8]. LanMs are bifunctional enzymes containing an N-terminal dehydratase domain and a C-terminal cyclase domain [9,10]. LanMs share about 30% sequence identity, and their dehydratase domains show no homology to any other protein families while their cyclase domains are homologous to cyclase LanC. The triad zinc ligands in cyclase domain have been confirmed in LctM (Cys781, Cys836 and His837), the synthetase of lacticin 481. Thus, the cyclization function of LanM is proposed to be accomplished by Michael-addition assisting by the chelating zinc [11]. However, the dehydration mechanism of LanM is elusive. On the other hand, no substrate binding site has been identified in LanM, which is important to the catalytic function of LanM. LanMs share approximate 30 highly conserved residues [12]. This provided clues for investigating the function of LanMs. Mutagenesis of some conserved residues of dehydratase domain of LctM released evidence that the dehydration function is executed by phosphorylation of Ser/Thr and subsequent elimination of the phosphates [13,14]. The conserved Arg399 and Thr406 in dehydratase domain of LctM are responsible for the elimination reaction, which was also observed in a newly identified ProcM (Arg510 and Thr516) [14,15]. However, these two residues are not sufficient to fulfill the elimination function, indicating some other residues remain to be discovered. On the other hand,

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although a substrate binding pocket is speculated to be constituted by several residues scattered in LanM [12,16], no such residue has been identified.

Bovicin HJ50 is a class II lantibiotic produced by *Streptococcus bovis* HJ50 isolated from milk [17]. It is reminiscent of lacticin 481 in ring topology but differed by containing an uncommon disulfide bond (Fig. 1A) [18]. The biosynthetic gene cluster of bovicin HJ50 has been sequenced containing the precursor gene *bovA* and modification gene *bovM* [19]. The modification function of bovicin HJ50 synthetase BovM has been reconstituted in *Escherichia coli* by co-expression of precursor BovA and BovM, achieving native modification to His_6-BovA [20]. In this study, we successfully reconstituted the catalytic activity of BovM *in vitro*, and further investigated the functions of the conserved residues by site-directed mutagenesis, mass spectrometry (MS) and surface plasmon resonance (SPR) analyses. Our results identified a novel Asp residue that participated in the elimination of phosphates during the dehydration reactions, and determined the conserved residues that were responsible for substrate binding activities.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli DH5α and BL21 (DE3) were cultured in LB medium at 37 °C and used for plasmids cloning and protein expression, respectively. *S. bovis* HJ50 was cultured in M17 medium supplemented with 0.3 M sucrose at 37 °C. *Micrococcus flavus* NCIB 8166 was cultured in S1 medium at 30 °C.

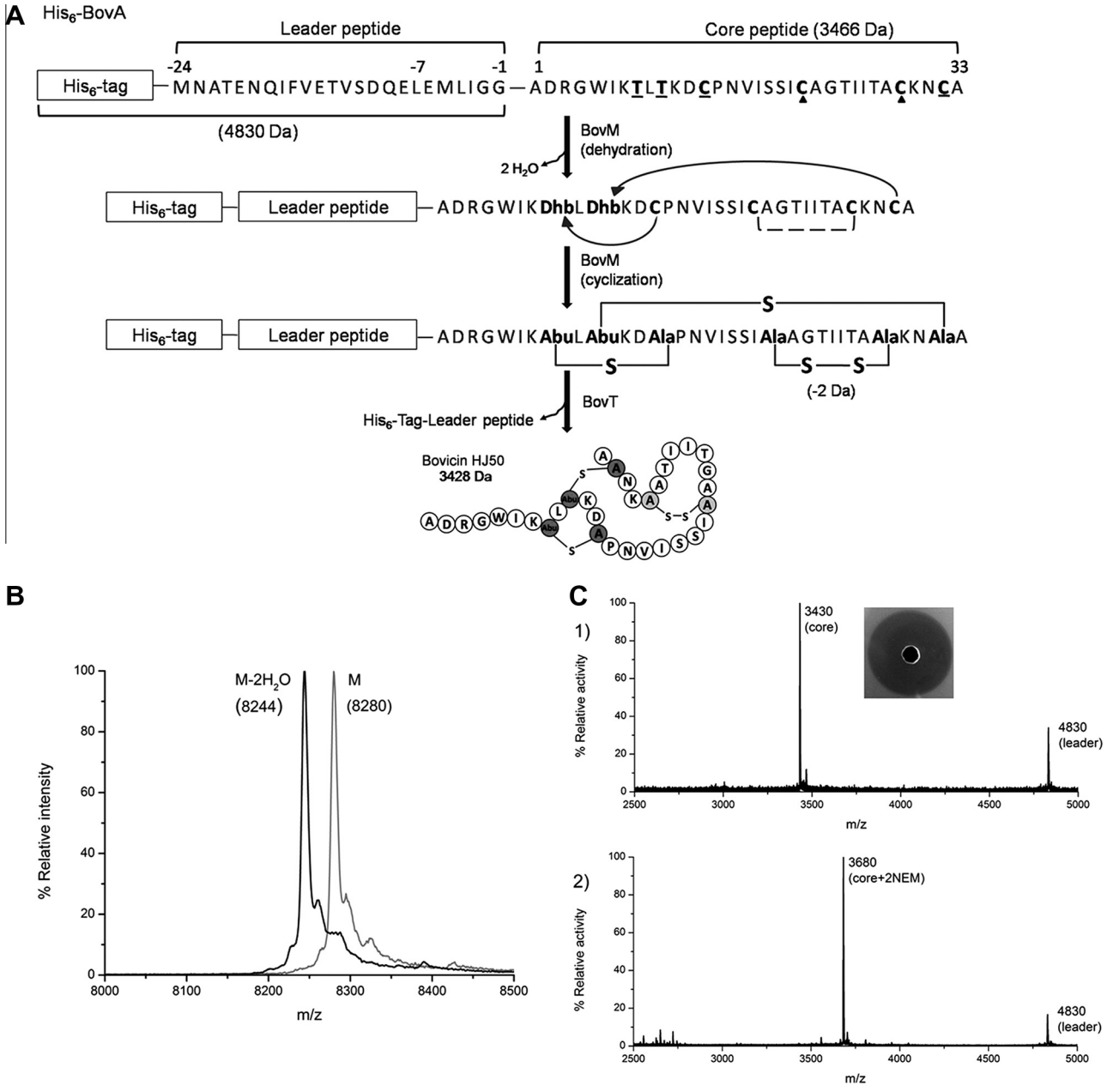

Fig. 1. (A) *in vitro* modification of His_6-BovA by BovM. (B) Mass spectra of 6 μM His_6-BovA treated with 0.08 μM BovM for 0 min (Gray) and 15 min (Black). (C) Mass spectra of (1) His_6-BovA incubated with BovM for 15 min followed by treated with BovT15 and inhibition zones of the digested products against *M. flavus* NCIB8166 and (2) subsequently treated with NEM for 30 min at 4 °C.

2.2. Molecular biology protocols

General molecular biology manipulations were carried out according to the established protocols [21]. PCR was performed with Q5 High-Fidelity DNA polymerase (NEB). The PCR products were purified with the AxyPrep PCR cleanup kit (Axygen). Restriction digestions were performed by Fast-Digest restriction enzyme (Thermo scientific). DNA ligation was executed by T4 DNA ligase (Thermo scientific). Plasmids from *E. coli* were isolated by AxyPrep plasmid miniprep kit (Axygen).

2.3. Construction of plasmids

The construction of plasmid pET-bovA has been reported previously [20]. The *bovM* gene was amplified from the genome of *S. bovis* HJ50, digested by *Bam*HI and *Sal*I restriction enzymes and ligated into pET-28a generating pET-bovM. Expression vectors of site-directed mutagenesis mutants of BovA and BovM were constructed utilizing site-directed mutagenesis protocols described by Chiu [22]. These mutations were identified by sequencing, and plasmids containing correct mutations were transformed into *E. coli* BL21 (DE3) for protein expression.

2.4. Over-expression and purification of proteins

Purification of His_6-BovA and BovT150 was performed as previous reports [20]. *E. coli* BL21 (DE3) harboring pET-bovM or its mutants were cultured in l L LB with 50 μg/ml kanamycin at 37 °C till the OD_{600nm} reaching 0.6–0.8, and induced by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 18 °C for 20 h. After that, the cells were harvested and resuspended in 40 ml start buffer (50 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) and lysed by sonication. The cellular debris were centrifuged and the supernatants were filtrated by 0.45 μm filter followed by loaded onto 1 ml Histrap FF packing (GE healthcare) that was pre-equilibrated by start buffer. After washed by 20 ml wash buffer (start buffer plus 40 mM imidazole) to remove the unspecific binding proteins, the protein was eluted by 5 ml elution buffer (start buffer plus 480 mM imidazole). The eluted His_6-BovM solutions were concentrated by ultrafiltration (30 KD cut-off) and loaded onto Superdex™ 200 10/300 GL size exclusion column (GE healthcare) which was pre-equilibrated by assay buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM $MgCl_2$, pH 7.4) or HBS-T buffer (10 mM HEPES-NaOH, 150 mM NaCl, 0.005% Tween-20, pH 7.4). Collected protein solutions were concentrated by ultrafiltration (30 KD cut-off). The purity of acquired protein was judged by SDS–PAGE as standard protocols [21]. The protein concentrations were determined by BCA protein-assay kit (Axygen).

2.5. Activity assays of BovM mutants to BovA by MALDI-TOF MS

The lyophilized His_6-BovA was redissolved in assay buffer. His_6-BovA and BovM or its mutants were added into the reaction mixture to a final concentration of 6 μM and 0.08 μM, respectively, along with 2.5 mM ATP and 1 mM DL-Dithiothreitol (DTT). The reactions were proceeded at 25 °C, and terminated by 0.5% trifluoroacetic Acid (TFA) at different time points from 15 min to 4 h. All of the reaction mixtures were then desalted by zip-tip (MILLIPORE), and the molecular weight (MW) of products were tested by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Digestion of BovT150 and modification of N-ethylmaleimide (NEM) were performed as previous report [20], and the products were also identified by MALDI-TOF MS. The antimicrobial activities of products were tested by agar well diffusion assay against *M. flavus* NCIB 8166.

2.6. Affinity measurement of BovM derivatives and BovA by surface plasmon resonance (SPR)

SPR measurements were executed by Biocore3000 (GE Healthcare). Lyophilized His_6-BovA was dissolved in 10 mM acetate (pH 5.5) to a final concentration of 25 μg/ml, and immobilized on a CM5 chip by amide coupling to 1000 RU. His_6-BovM and its mutants dissolving in HBS-T buffer were injected through this chip as analytes at a flow rate of 30 μl/min for 180 s followed by eluted with HBS-T buffer for 360 s. The concentrations of these analytes were in 2-fold gradient dilution from 1.25 μM to 0.039 μM. After every detection, the chip was regenerated by injection of 50 mM NaOH at a flow rate of 10 μl/min for 100 s. All tests were performed at 25 °C. Collected data were analyzed by BIAevaluation 4.1 software (GE Healthcare). The SPR curves were fitted by a 1:1 (Langmuir) binding model and local fitted R_{max} to get the equilibrium constants K_D, which was regarding to the protein–protein affinity.

3. Results

3.1. in vitro modification of BovA by BovM

To investigate the function of BovM, we reconstituted the catalytic activity of BovM *in vitro*. BovM and its substrate BovA with N-terminal 6 × His (His_6-BovM and His_6-BovA) were expressed in *E. coli*, and purified to homogeneity (>95%) by size exclusion chromatography (SEC) and reversed-phase high performance liquid chromatography (RP-HPLC), respectively. His_6-BovM was incubated with His_6-BovA in the reaction buffer containing ATP, Mg^{2+} and DTT [10], and the product was identified by MALDI-TOF MS analysis which exhibited a mass peak of 8244.2 Da. The 36 Da decrease in comparison with theoretical molecular weight of His_6-BovA (m/z 8280.3) was in good accordance with His_6-BovA in 2-fold dehydration (Fig. 1B). This result demonstrated that the BovA could be dehydrated by BovM *in vitro*. To further confirm whether the modification was correct, the product was digested by BovT150, the C39 peptidase domain of BovT, to remove the leader peptide. Subsequent MALDI-TOF MS analysis showed a mass peak of 3430.3 Da, which was identical to the mass of wild-type bovicin HJ50 (3428 Da) without disulfide linkage. Chemical modification with NEM (125 Da), a thio-specific reagent which could covalently bond to free thiols, generated a product with two NEM additions (ΔM = 250 Da), which confirmed the opening of the disulfide bridge. This also implied the formation of two thioether bridges, which was further reinforced by that the modified and digested product displayed antimicrobial activity against indicator strain *M. flavus* NCIB8166 as authentic bovicin HJ50 (Fig. 2C). Collectively, these results fully supported that His_6-BovA could be modified by His_6-BovM *in vitro*, and this *in vitro* reaction system could be applied for studying BovM function.

3.2. Generation of conserved residue substitution mutants of BovM and their dehydration activity assays

BovM (837 residues) consists of an N-terminal dehydratase domain (1–460 residues) and a C-terminal cyclase domain (536–837 residues) (Fig. 2A). Sequence alignment of BovM with selected LanM proteins revealed 25 conserved residues containing negative charge residues (Asp155, Asp247, Asp264, Glu266, Glu446), positive charge residues (His157, Lys175, His249, Arg393, His672), polar residues (Tyr230, Asn252, Thr399, Tyr402, Tyr556, Cys715, Cys761) and hydrophobic residues (Ile254, Leu290, Phe365, Phe458, Leu597, Trp714, Leu760, Leu771) (Fig. 2B). Except for the reported zinc ligands His672, Cys715 and Cys761 [11], the functions of others were studied in this work.

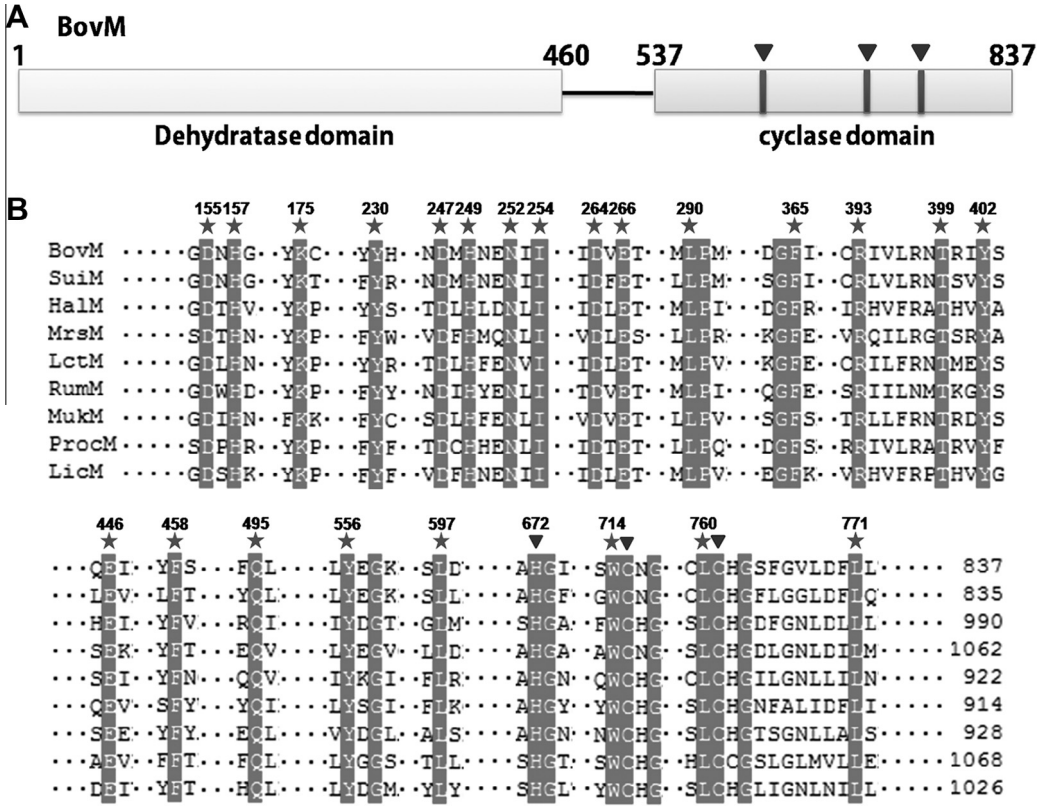

Fig. 2. Sequence analyses of BovM (A) Schematic diagram of two functional domains of BovM. (B) Sequence alignment of selected LanMs. The conserved residues were shown by dark color. The mutated residues were shown by stars. The cyclase active sites were shown by black triangle.

To investigate the contributions of these conserved residues to function of BovM, the Ala substitution mutants of these residues were constructed and purified. The relative activities of BovM mutants were monitored by the substrate completely consumption time. His_6-BovA was catalyzed by them in the same conditions as above-mentioned *in vitro* reaction system. As determined, wild-type BovM (WT) fully dehydrated His_6-BovA within 15 min. Thus, the assay time of BovM mutants were ranging from 15 min to 4 h, and the modified products were identified by MS analysis (Fig. 3). The results showed that, except mutants Y556A and L771A possessed similar dehydration time to WT (Fig. 3A), the other mutants possessed decreased dehydration activity (described in followed sections), indicating these residues might participate in the function of BovM. In addition, soluble expression of Y402A, F458A, W714A and L760A was unsuccessful, implying that mutations of these residues might influence the stability of BovM.

3.3. Key residues of BovM responsible for the elimination of phosphates

The dehydration reaction catalyzed by LanM consists of phosphorylation and elimination of phosphates, and disruption of the phosphates elimination activity would result in generation of phosphorylated products. The MS results showed that mutants D155A, H157A, R393A, T399A of BovM predominantly converted His_6-BovA into single phosphorylated products (ΔM = +80 Da) in 4 h. After incubation with D155A for 1 h, His_6-BovA were completely converted to single-phosphorylated products, while no dehydrated product was detected after 4 h (Fig. 3B). This was also observed in mutants of R393A, T399A and H157A. However, H157A produced trace amounts of 2-fold dehydrated products after 4 h (Fig. 3C). MALDI-TOF MS analysis about the products that were digested by BovT150 showed single phosphorylated core peptide, demonstrating that the phosphate group situated in the core peptide (data not shown). These results suggested that these four residues participated in the elimination of phosphates group during the dehydration reaction.

3.4. Key residues of BovM involved in dehydration function

The N-terminal conserved residues of BovM might be responsible for its dehydration activity. The MS analyses revealed nine mutants of polar residues of BovM with low dehydration activity. K175A did not produce modified product in 1 h, and a mixture of 2-fold dehydrated substrates, single phosphorylated substrates and most of the unmodified His_6-BovA were detected after 4 h (Fig. 3D). Y230A produced 2-fold dehydrated His_6-BovA along with considerable amounts of unmodified and trace amount of single phosphorylated products after incubation with His_6-BovA for 4 h (Fig. 3E). D264A and E446A required 4 h to convert the substrates completely to 2-fold dehydrated products (Fig. 3F) whereas Q495A required about 2 h (Fig. 3G). The other four mutants (D247A, N252A, H249A and E266A) showed drastically decreased dehydration activity. A small amount of 2-fold dehydrated products were detected after incubation of His_6-BovA with these mutants for 4 h, leaving most of the unmodified substrates (Fig. 3H). Collectively, the results suggested that these polar residues were responsible for the dehydration activity of BovM.

To investigate the roles in the substrate binding of these residues, the substrate affinities of these mutants were tested by SPR. His_6-BovA was conjugated onto a CM5 chip as the ligand, the analytes His_6-BovM and its mutants were injected to flow past the chip. The SPR data were collected and fitted by a 1:1

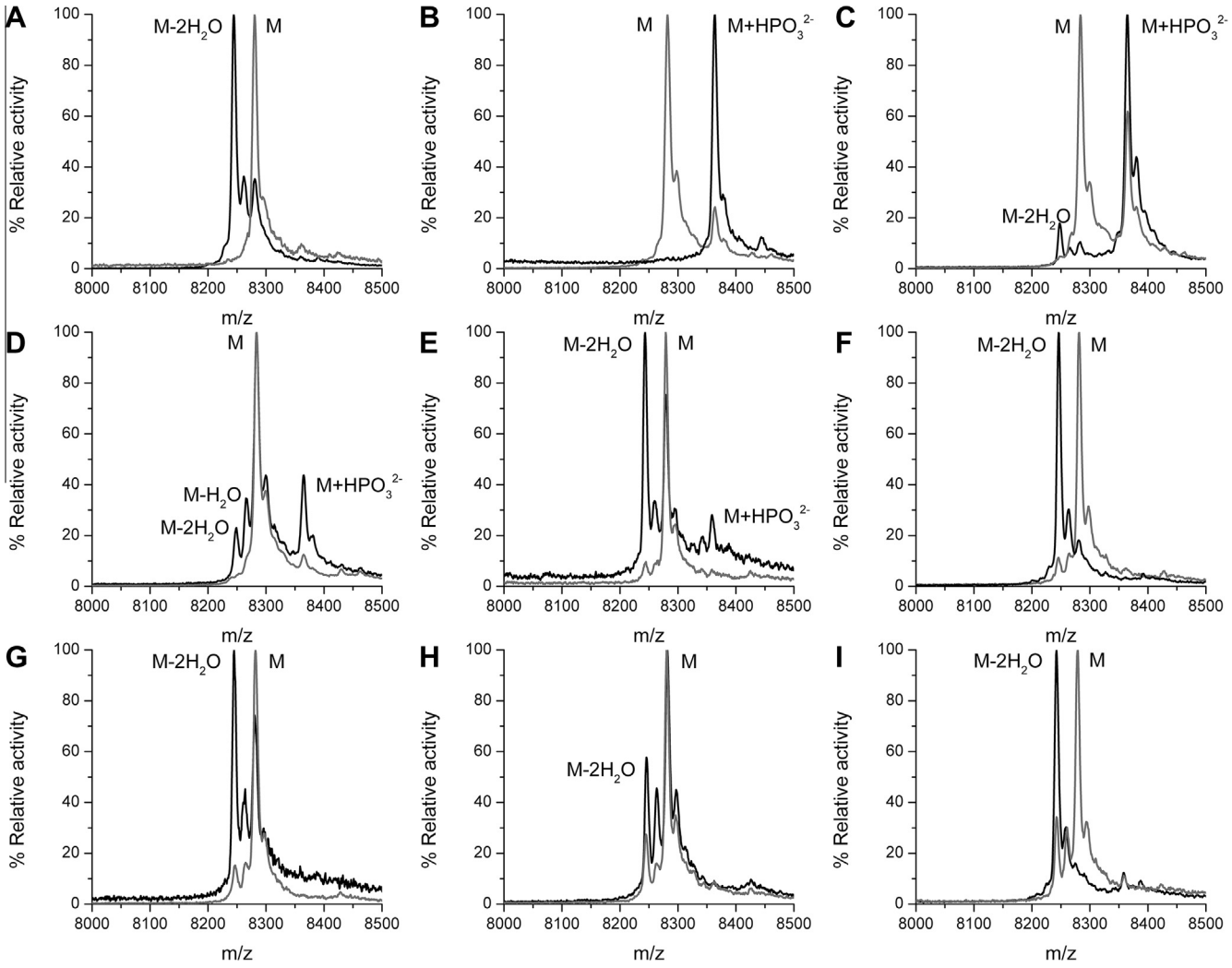

Fig. 3. Mass spectra of His_6-BovA (6 μM) after treated by partial BovM mutants (0.08 μM) for short (gray) and long (black) time at 25 °C. (A) Y556A 0 and 15 min, (B) D155A 30 min and 2 h, (C) H157A 30 min and 4 h, (D) Y230A 1 h and 4 h, (E) D247A 30 min and 4 h, (F) K175A 30 min and 4 h, (G) D264A 30 min and 4 h, (H) Q495A 15 min and 2 h and (I) L290A 15 min and 1 h.

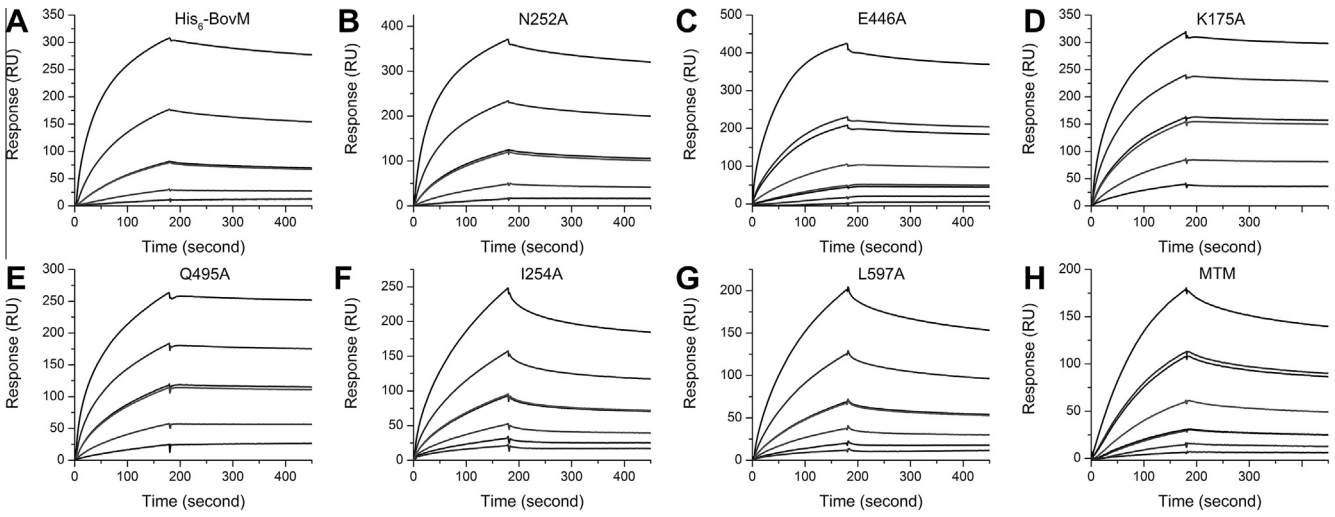

Fig. 4. SPR analyses of interaction between BovM or its representative mutants and His_6-BovA. His_6-BovA was binding on the chip as ligand and the analytes (A) BovM, (B) N252A, (C) E446A, (D) K175A, (E) Q495A, (F) I254A, (G) L597A, (H) MTM were injected in the concentration of 1.25, 0.625, 0.3125, 0.156, 0.078 and 0.039 μM.

Table 1
Kinetic constants for interaction between BovM or its mutants and His_6-BovA.

Enzyme	k_a (M^{-1} S^{-1})	k_d (S^{-1})	K_D (nM)
WT	1.83×10^4	3.01×10^{-4}	16.4
Y230A	1.82×10^4	2.58×10^{-4}	14.2
D247A	1.07×10^4	1.21×10^{-4}	11.3
H249A	1.36×10^4	2.14×10^{-4}	15.4
N252A	2.03×10^4	3.60×10^{-4}	17.7
E266A	2.80×10^4	5.21×10^{-4}	18.6
E446A	1.69×10^4	2.93×10^{-4}	17.3
K175A	1.86×10^4	9.46×10^{-5}	5.1
D264A	1.46×10^4	6.52×10^{-5}	4.5
Q495A	1.73×10^4	1.75×10^{-5}	1.0
I254A	7.58×10^3	6.28×10^{-4}	82.9
L290A	5.45×10^3	6.09×10^{-4}	112
F365A	7.48×10^3	4.71×10^{-4}	63.1
L597A	8.19×10^3	6.97×10^{-4}	85.1
MTM	6.53×10^3	7.97×10^{-4}	122

(Langmuir) binding model and local fitted R_{max}. The SPR curves of representative mutants were shown in Fig. 4, and the calculated affinity constants (K_D) of these mutants and WT were listed in Table 1. The k_a referred to the association rate and the k_d referred to the dissociation rate, $K_D = k_a/k_d$. The K_D of WT was 16.4 nM (Fig. 4A), similar to another LanM protein NukM ($K_D = 12$ nM) [16]. The K_D of Y230A, D247A, H249A, N252A, E266A and E446A were14.2 nM, 11.3 nM, 15.4 nM, 17.7 nM, 18.6 nM and 17.3 nM, respectively (Fig. 4B and C), showing no significant difference to that of WT. In contrast, the K_D of K175A, D264A and Q495A were 5.4 nM, 4.5 nM and 1 nM, respectively, showing higher affinity to BovA than that of WT (Fig. 4D and E). These results suggested that the Tyr230, Asp247, His249, Asn252, Glu266 and Glu446 were irrelevant with binding activity of BovM, while the Lys175, Asp264 and Gln495 might be responsible for the substrate release of BovM.

3.5. Conserved residues of BovM contributing to substrate binding

In contrast to the residues mentioned above, the MS results showed that the hydrophobic residues mutants, I254A, L290A, F365A and L597A, achieved 2-fold dehydration of His_6-BovA ranging from 30 min to 45 min, exhibiting slightly lower activity than WT (Fig. 3I). No phosphorylated intermediate or unmodified product was detected after catalyzing for 1 h. These results indicated that these hydrophobic residues may not directly participate in the catalytic activity of BovM. Furthermore, SPR assay was employed to test the substrate affinity of these mutants. The K_D of I254A, L290A, F365A and L597A were 82.9 nM, 112 nM, 63.1 nM, and 85.1 nM, respectively, indicating that the affinities of these four mutants towards His_6-BovA were all decreased compared to that of WT. After that, the four hydrophobic residues in BovM were then all mutated to Ala, acquiring a multiple mutant (MTM), and the catalytic and binding activities were tested as described above. Trace amount of 2-fold dehydrated products were detected along with most of the unmodified substrate after 4 h, showing that the catalytic activity of MTM was drastically decreased. The K_D of the multiple mutant was 122 nM, exhibiting lower affinity to BovA than that of single mutants (Fig. 4H). Collectively, these results suggested that the hydrophobic residues Ile254, Leu290, Phe365 and Leu597 participated in the substrate binding of BovM.

4. Discussion

LanM proteins are posttranslational modification enzymes of class II lanthipeptides with broad substrate specificity. Engineering of LanMs as tool enzymes has drawn much attentions in recent years [8,15]. Despite identification of some critical residues of the catalytic function of LanMs [11,14], the dehydration and substrate binding mechanism of LanM stays to be an open question. In this study, we studied BovM, the synthetase of bovicin HJ50, by MS and SPR and found two veiled residues Asp155 and His157 participating the elimination of phosphates in the dehydration reaction; in addition, four hydrophobic residues were revealed to be responsible for the substrate binding of BovM. This work provided new clues on the dehydration and substrate binding mechanism of LanMs.

The elimination of phosphates is essential process in the dehydration reaction catalyzed by LanM [13]. In this work, we found that the mutants R393A and T399A lost the elimination activity, which resembled to the previous reports about LctM (Arg399 and Thr406) [14]. Furthermore, we found two new mutants, the mutant D155A showed phosphorylation activity but lost the elimination activity while H157A showed low elimination activity with predominant phosphorylation activity. All these four residues contain an active side chains with different charge and most likely coworked in the general acid-base mechanism [14]. Concretely, we speculated that the Arg393 ($pK_a = 12.48$) acted as a general acid, the Asp155 ($pK_a = 3.86$) acted as a general base and the His157 and (or) the Thr399 acted as activator(s). In addition, these four residue situated in different part of BovM, blanked by the other key residues, indicating that there was no independent phosphates elimination domain in BovM. This is different from LanKC and LanL, both of which possess separate protein kinase and pSer/Thr lyase domains [23,24].

The hydrophobic Ile254, Leu290, Phe365 and Leu597 were revealed to be responsible for the substrate binding of BovM. Mutants of these residues showed higher KD than that of WT ($\times 5$–$\times 10$), meaning that these mutants possessed lower substrate affinity. Additionally, multiple mutants showed similar affinity to single mutants but decreased dehydration activity. We speculated that these residues situated in a substrate binding cassette that interacts with BovA. In addition, some non-conserved residues that also participate in the substrate recognition of BovM remain to be revealed. Previous reports have shown that the hydrophobic residues in leader peptide of lanthipeptides are important for the modification of synthetases in class I and class III lanthipeptides [25–28]. Likewise, a conserved hydrophobic residue Leu-7 in the leader peptide of class II lanthipeptides is found important for the catalysis of LanM [29]. Mutation of Leu-7 of BovA to Ala drastically slowed down whereas to Asp totally hindered the modification of BovM (data not shown), indicating the importance of hydrophobic interaction for substrate binding of BovM. Moreover, the amphipathic helix containing hydrophobic residues in the C-terminal of the leader peptide is also important for the substrate binding of LanM [30]. Thus, we interpreted that the conserved hydrophobic residues interacted with the hydrophobic residues in the leader peptide of BovA.

In summary, this study further studied the dehydration function of LanMs and provided clues on the substrate binding mechanism of LanMs. We identified 22 conserved residues that were responsible for the catalytic function of BovM, among which Asp155 and His157 were responsible for the phosphate elimination of BovM that were not reported in other LanMs. In addition, four conserved hydrophobic residues were unveiled to be responsible for the substrate binding of BovM. More precise roles of these residues will require the structure information of these enzymes, which so far is not available. Future work will focus on the acquirement of X-ray structure of BovM, which would further elucidate the function mechanism of LanMs and facilitate engineering of LanMs as tool enzymes.

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