RESEARCH LETTER

Identification of residues essential for the catalytic activity of Sec11b, one of the two type I signal peptidases of *Haloferax volcanii*

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Abstract

Sec11b is one of two signal peptidases (SPases) in the haloarchaeon *Haloferax volcanii*. Site-directed mutagenesis revealed Ser-72, His-137 and Asp-187 as essential for signal peptide cleavage. Thus, like the SPase of the methanoarchaeon *Methanococcus voltae*, *H. volcanii* Sec11b uses a catalytic mechanism reminiscent of its eukaryal rather than its bacterial counterpart. The availability of an additional model system to study the archaeal SPase, now in the form of the purified protein, promises additional insight into the behavior of this enzyme.

Introduction

Type I signal peptidases (SPases) are integral membrane proteins responsible for the removal of signal peptides (SPs) from preproteins following their translocation across biological membranes (Dalbey *et al.*, 1997; Paetzel *et al.*, 2000, 2002; van Roosmalen *et al.*, 2004). While fulfilling similar roles, the bacterial and eukaryal versions of the enzyme differ in terms of their catalytic mechanisms, their sensitivities to inhibitors and their oligomeric status. In bacteria like *Escherichia coli*, the conserved nucleophilic Ser-90 (*E. coli* numbering) and the proposed general base Lys-145 are believed to form the catalytic dyad responsible for the proteolytic action of the enzyme (Black, 1993; Tschantz *et al.*, 1995; van Dijl *et al.*, 1995; Paetzel *et al.*, 1997). In eukaryal type I SPases, responsible for the cleavage of SPs following preprotein translocation into the endoplasmic reticulum (ER), the lysine found in the bacterial catalytic dyad is replaced with an essential histidine residue (Dalbey & von Heijne, 1992; van Valkenburgh *et al.*, 1999). A similar histidine–lysine exchange is also detected in a limited number of Gram-positive bacterial SPases, exemplified by *Bacillus subtilis* SipW (Tjalsma *et al.*, 2000; van Roosmalen *et al.*, 2004). In SipW, however, the histidine residue can be replaced by a lysine with no effect on enzymatic function (Tjalsma *et al.*, 2000). Site-directed mutagenesis studies have also revealed the essential nature of two aspartic acid residues (the equivalents of *E. coli* Asp-273 and Asp-280) in the yeast enzyme (van Valkenburgh *et al.*, 1999). In the *E. coli* enzyme, by contrast, neither Asp residue is essential (Klenotic *et al.*, 2000). Thus, while its catalytic mechanism remains to be elucidated, the eukaryal SPase may rely on either a Ser–His dyad or a Ser–His–Asp triad for its catalytic activity, rather than the Ser–Lys dyad used by the bacterial enzyme (van Valkenburgh *et al.*, 1999). Indeed, it remains to be determined whether the catalytic subunit of the eukaryal enzyme assumes the same protein fold as its bacterial counterpart. Finally, differences in catalytic mechanism suggested by amino acid profiles are further reflected in the availability of inhibitors specific for either the bacterial or the eukaryal enzyme (Dalbey *et al.*, 1997; Paetzel *et al.*, 2000, 2002; van Roosmalen *et al.*, 2004).

If only a basic understanding of the catalytic mechanism of SPases in bacteria and eukarya is available (Klenotic *et al.*, 2000; Paetzel *et al.*, 2002; van Roosmalen *et al.*, 2004), then even less is known of SP cleavage in Archaea (Eichler, 2002; Ng *et al.*, 2007). Archaeal SPases lack the conserved lysine of the bacterial Ser–Lys catalytic dyad and, like their eukaryal counterparts, have replaced the bacterially conserved lysine with a histidine residue (Eichler, 2002; Ng *et al.*, 2007).

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Site-directed mutagenesis studies of SPase from the methanoarchaeon *Methanococcus voltae* expressed in *E. coli* (Ng & Jarrell, 2003; Bardy *et al*., 2005) have confirmed the essential nature of the Ser-90 and His-145 equivalents, as in the eukaryal enzyme (van Valkenburgh *et al*., 1999). In particular, His-122 cannot be replaced by Lys. Thus, in terms of catalysis, archaeal SPases may be more reminiscent of their eukaryal rather than their bacterial counterparts. Differences between the modes of action of the eukaryal and archaeal enzymes do, however, exist. As mentioned, whereas the equivalents of the well-conserved Asp-273 and Asp-280 residues are essential for the action of the yeast enzyme (van Valkenburgh *et al*., 1999), only the latter is essential for *M. voltae* SPase activity (Bardy *et al*., 2005). It is also conceivable that the archaeal enzyme functions in an SipW-like manner. Clearly, numerous questions concerning the mechanism of the archaeal SPase remain.

Recently, two type I signal peptidases from the halophilic archaeon *Haloferax volcanii*, termed Sec11a (GenBank accession no. AY940668) and Sec11b (GenBank accession no. AY940669), were cloned, expressed, purified and partially characterized (Fine *et al*., 2006). Of the two, only Sec11b is apparently essential, given the inability to generate a chromosomal deletion strain lacking the encoding gene. Now, to determine whether the equivalents of Ser-90, His-145 and Asp-280, shown to be essential for the function of the *M. voltae* enzyme, are also crucial for the catalytic activity of *H. volcanii* Sec11b, site-directed mutagenesis has been used, together with a previously developed *in vitro* assay for this haloarchaeal enzyme (Fine *et al*., 2006).

Materials and methods

SPase activity assay

In the SPase activity assay described previously (Fine *et al*., 2006), a polyhistidine-tagged version of the chimeric SPase substrate, formed between the 34 amino acid residue SP of the *H. volcanii* S-layer glycoprotein and the *Clostridium thermocellum* cellulose binding-domain (CBD), is combined with a cellulose-bound chimera of *C. thermocellum* CBD fused to *H. volcanii* Sec11b. Both chimera were created at the gene level.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Quickchange (Stratagene) protocol according to the manufacturer's instructions, together with plasmid pCBD-Sec11b (Fine *et al*., 2006) as a template. The oligonucleotide primers used to introduce the various mutations are listed in Table 1. The introduction of mutations was confirmed by sequencing.

Results and discussion

As a first step in identifying Sec11b residues important for the catalytic activity of the enzyme, cellulose-purified CBD-Sec11b was combined with the SP-CBD substrate. Such incubation led to the rapid release of the SP, reflected in the conversion of SP-CBD to the mature CBD (Fig. 1a, left panel). Control experiments confirmed that the mature CBD domain did not originate from the enzyme-based chimera (middle panel) or from spontaneous breakdown of

Table 1. Primers used in this study

Primer	Sequence
S72A-F	GGTCGCCGTCGAAAGCGG**GGC**CATGGAACCGCACATGCAC
S72A-R	GTGCATGTGCGGTTCCATG**GCC**CCGCTTTCGACGGCGACC
H137K-F	CAGGTCCCCCGATTAT**CAA**GCGGGCGATGTTCTGGGTCGAC
H137K-R	GTCGACCCAGAACATCGCCCGC**TTG**ATAATCGGGGGACCTG
D181A-F	GCTTCATCACGAAAGG**CGC**CAACAACCCCCGGTACGACCAAG
D181A-R	CTTGGTCGTACCGGGGGTTGTTG**GCG**CCTTTCGTGATGAAGC
D181E-F	GCTTCATCACGAAAGG**CGA**GAACAACCCCCGGTACGACCAAG
D181E-R	CTTGGTCGTACCGGGGGTTGTTC**TCG**CCTTTCGTGATGAAGC
D187A-F	ACAACAACCCCCGGTA**CGC**CCAAGTAAGCGGCATCGCCGA
D187A-R	TCGGCGATGCCGCTTACTTGG**GCG**TACCGGGGGTTGTTGT
D187N-F	ACAACAACCCCCGGTA**CAA**CCAAGTAAGCGGCATCGCCGA
D187N-R	TCGGCGATGCCGCTTACTTGG**TTG**TACCGGGGGTTGTTGT
K79A-F	GGAACCGCACATGCA**CGC**CGGCGACCTCGTGTTCATCAC
K79A-R	GTGATGAACACGAGGTCGCCG**GCG**TGCATGTGCGGTTCC
K115A-F	CGGAGGTCGACTACCG**GGC**GTTCGGCGGCCCCGGGAGCGTC
K115A-R	GACGCTCCCGGGGCCGCCGAACGC**CCG**GTAGTCGACCTCCG

The codon introducing the mutation is shown in bold. F, forward primer; R, reverse primer.

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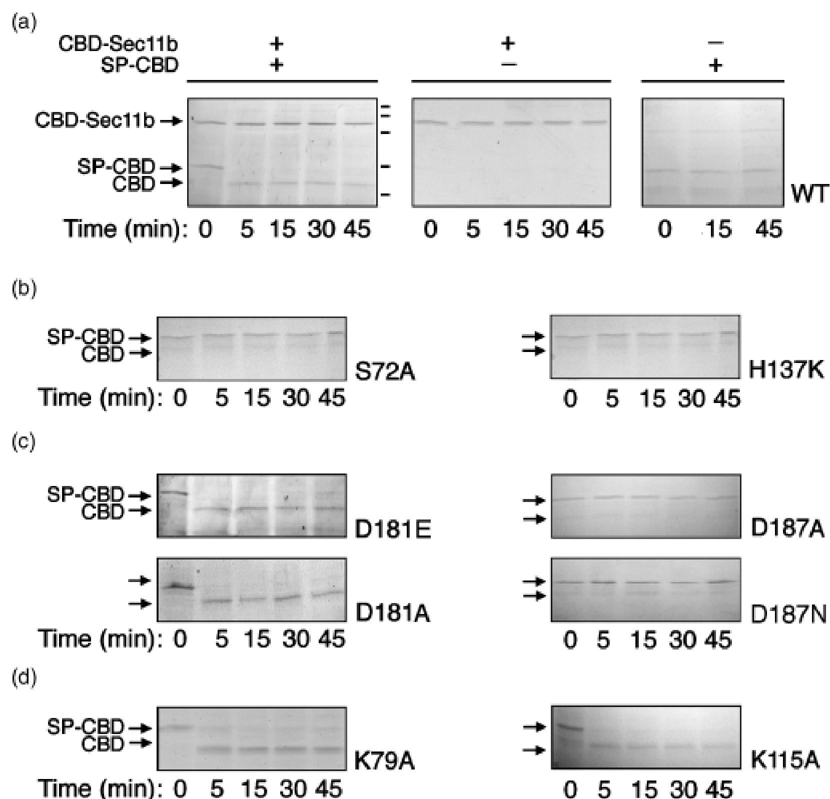

Fig. 1. Site-directed mutagenesis reveals the involvement of Ser-72, His-137 and Asp-187 in the catalytic mechanism of *Haloferax volcanii* Sec11b. (a) Sec11b activity was assayed as described previously (Fine *et al*., 2006). Purified CBD-Sec11b (50 μg) and SP-CBD (15 μg) were combined in buffer (0.7% Triton X-100, 3 M NaCl, 50 mM Tris-HCl, pH 7.2) at 40 °C for up to 45 min. At various times, aliquots were removed and analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining. Left panel, CBD-Sec11b and SP-CBD; middle panel, CBD-Sec11; right panel, SP-CBD. The positions of CBD-Sec11b, SP-CBD and CBD are depicted by arrows on the left. The effects of (b) S72A (left panel) and H137K (right panel), (c) D181E and D181A (upper and lower left panels, respectively) and D187A and D187N (upper and lower right panels, respectively), and (d) K79 (left panel) and K115 (right panel) mutations on Sec11b activity were studied by the *H. volcanii* SPase *in vitro* assay.

the SP-CBD substrate (right panel). The ability of cellulose-bound Sec11b to cleave the SP from the SP-CBD chimera effectively also points to *H. volcanii* SPase functioning as a single subunit, given that no other proteins are required for the catalytic activity.

Next, efforts were directed at addressing the essential nature of the conserved Ser and His residues in Sec11b, i.e. S72 and H137. In numbering *H. volcanii* Sec11b residues, the Met lying 40 residues upstream of the predicted transmembrane domain of the protein and the site of CBD linkage was arbitrarily considered as the start of the protein, because the N-terminus of *H. volcanii* Sec11b has yet to be experimentally confirmed (Fine *et al*., 2006). As shown in Fig. 1b, neither the S72A nor the H137K mutants retained any catalytic activity, pointing to the essential nature of these residues [as in the eukaryal SPase (Dalbey & von Heijne, 1992; van Valkenburgh *et al*., 1999)] and confirming that the conserved His cannot be replaced by Lys, as for the *M. voltae* enzyme (Bardy *et al*., 2005) but in contrast to SipW-like bacterial SPases (Tjalsma *et al*., 2000). Subsequent efforts addressed the crucial nature of D181 and D187, i.e. equivalents of those Asp residues considered in earlier studies of the yeast and *M. voltae* enzymes (van Valkenburgh *et al*., 1999; Bardy *et al*., 2005). In the case of *H. volcanii* Sec11b, D181E and D181A mutants presented activity similar to the wild-type enzyme, suggesting that Asp-181 is not crucial for Sec11b function (Fig. 1c, left panels). By contrast, replacing D187 with an Ala or Asn resulted in abolishment of activity (Fig. 1c, right panels). Thus, like S72 and H137, D187 is essential for *H. volcanii* Sec11b activity. Finally, despite the inability of Lys to replace His137, two of the three Lys residues of *H. volcanii* Sec11b, i.e. K79 and K115, were replaced with Ala residues to confirm whether this amino acid, nonetheless, participates in the catalytic mechanism of the enzyme. As shown in Fig. 1d, both K79A and K115A mutants were as active as the native enzyme, implying that Lys is not involved in Sec11b activity.

The results of these studies point to *H. volcanii* Sec11b as behaving like its *M. voltae* counterpart, likely relying on a Ser–His–Asp catalytic triad. As such, the archaeal SPase clearly differs from *E. coli*- or SipW-like bacterial SPases (Black, 1993; Tschantz *et al*., 1995; van Dijl *et al*., 1995; Paetzel *et al*., 1997; Tjalsma *et al*., 2000). Moreover, the requirement for only the equivalent of *E. coli* Asp-280 apparently distinguishes the archaeal enzyme from the eukaryal SPase, exemplified by yeast Sec11, where both Asp-273 and Asp-280 equivalents are necessary for activity (Dalbey & von Heijne, 1992; van Valkenburgh *et al*., 1999). The shared requirement for the equivalents of Ser-90, His-145 and Asp-280 by archaeal and eukaryal SPase implies, nonetheless, that a deeper understanding of the catalytic mechanism of the archaeal enzyme could help elucidate the

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mode of action of its eukaryal counterpart. Such insight could aid in the development of novel antibiotic agents aimed at the bacterial enzyme, a potential target for novel antimicrobials (Paetzel *et al.*, 2002; Bruton *et al.*, 2003).

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