Different Mechanisms for Thermal Inactivation of *Bacillus subtilis* Signal Peptidase Mutants*

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The type I signal peptidase SipS of *Bacillus subtilis* is of major importance for the processing of secretory precursor proteins. In the present studies, we have investigated possible mechanisms of thermal inactivation of five temperature-sensitive SipS mutants. The results demonstrate that two of these mutants, L74A and Y81A, are structurally stable but strongly impaired in catalytic activity at 48 °C, showing the (unprecedented) involvement of the conserved leucine 74 and tyrosine 81 residues in the catalytic reaction of type I signal peptidases. This conclusion is supported by the crystal structure of the homologous signal peptidase of *Escherichia coli* (Paetzel, M., Dalbey, R. E., and Strynadka, N. C. J. (1998) Nature 396, 186–190). In contrast, the SipS mutant proteins R84A, R84H, and D146A were inactivated by proteolytic degradation, indicating that the conserved arginine 84 and aspartic acid 146 residues are required to obtain a protease-resistant conformation. The cell wall-bound protease WprA was shown to be involved in the degradation of SipS D146A, which is in accord with the fact that SipS has a large extracytoplasmic domain. As WprA was not involved in the degradation of the SipS mutant proteins R84A and R84H, we conclude that multiple proteases are responsible for the thermal inactivation of temperature-sensitive SipS mutants.

Bacterial proteins that are exported from the cytoplasm via the general secretion pathway are synthesized with an amino-terminal signal peptide. The signal peptide is required for the targeting of pre-proteins to the membrane and the initiation of protein translocation across this membrane (for review, see Ref. 1). During, or shortly after, the translocation of secretory pre-proteins, signal peptides are removed by type I signal peptidases (SPases),

which is a prerequisite for the release of the mature protein from the trans side of the membrane (for review, see Ref. 2).

Type I SPases belong to a special class of serine peptidases (peptidase classification: clan SF, family S26; Ref. 3) with conserved serine and lysine residues, which are essential for catalysis, most likely by forming a serine-lysine catalytic dyad (see Refs. 2, 4, and 5). As demonstrated by the crystallographic analysis of the type I SPase of *Escherichia coli* (also known as leader peptidase), the active site serine residue acts as a nucleophile attacking the carbonyl carbon of the scissile peptide bond at the SPase recognition site, whereas the unprotonated form of the lysine ε-amino group probably serves to activate the hydroxyl group of the serine residue. A similar catalytic mechanism has been proposed for the structurally related LexA-like proteases (4–8), which employ a serine-lysine catalytic dyad for self-cleavage (7, 9, 10).

In the Gram-positive bacterium *Bacillus subtilis* five chromosomally encoded type I SPases have been identified, which are denoted SipS, SipT, SipU, SipV, and SipW (11–13). SipS and SipT are of major importance for protein secretion, whereas SipU, SipV, and SipW contribute only to a minor extent to the processing of secretory pre-proteins (12–14). Cells depleted of functional SipS and SipT stop growing and lyse, showing that the presence of either SipS or SipT is essential for growth and viability. This was demonstrated with a *B. subtilis* strain in which the chromosomal copies of the sipS and sipT genes were disrupted and functionally replaced by one of five different plasmid-borne genes for temperature-sensitive SipS mutant proteins (i.e., L74A, Y81A, R84A, R84H, and D146A; Ref. 13). In the present study, we have investigated the mechanism of thermal inactivation of these five SipS mutant proteins. The results show that SipS L74A and Y81A are structurally stable at high temperature, indicating that the substituted residues are, in addition to the putative active site serine (Ser-43) and lysine residues (Lys-83), involved in catalysis. By contrast, SipS R84A, R84H, and D146A are prone to proteolytic degradation, in particular at high temperature, showing that the residues at these positions are required to maintain resistance to proteases. Interestingly, a cell wall-bound protease, WprA, was shown to be involved in the degradation of SipS D146A, but not in the degradation of SipS R84A and R84H.

**EXPERIMENTAL PROCEDURES**

Plasmids, Bacterial Strains, and Media—Table I lists the plasmids and bacterial strains used. TY medium was prepared as described in Ref. 15. GCHE medium was prepared as described in Ref. 16. Antibiotics were used in the following concentrations: chloramphenicol, 5 μg/ml; erythromycin, 1 μg/ml; kanamycin, 10 μg/ml.

Transformation of *B. subtilis*—*B. subtilis* was transformed by heat shock. *B. subtilis* was transformed into GCHE medium until an optical density at 600 nm of 0.8, subsequent addition of DNA to the culture, and continued growth for 4 h. Western Blot Analysis—Western blotting was performed using a semi-dry system as described by Kyhse-Andersen (17). After separation by SDS–polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Roche Molecular Biochemi-
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**TABLE I**  
**Plasmids and bacterial strains**

| Plasmids/strains | Relevant properties | Ref. |
|------------------|--------------------|------|
| pGDL41           | Encodes pre(A13i)-β-lactamase and SipS of *B. subtilis*; Ap<sup>R</sup>, Km<sup>R</sup> | 11   |
| pS-L74A          | Encodes SipS L74A; otherwise identical to pGDL41 | 6    |
| pS-Y81A          | Encodes SipS Y81A; otherwise identical to pGDL41 | 6    |
| pS-R84A          | Encodes SipS R84A; otherwise identical to pGDL41 | 6    |
| pS-R84H          | Encodes SipS R84H; otherwise identical to pGDL41 | 6    |
| pS-D146A         | Encodes SipS D146A; otherwise identical to pGDL41 | 6    |
| *B. subtilis* strains |                        |      |
| 8G5 sipS (∆S)    | trpC2, tyr, nic, ura, met, ade, sipS | 14   |
| ∆ST (pGDL41)     | trpC2, tyr, nic, ura, met, ade, sipS, sipT; Cm<sup>R</sup>; contains pGDL41 | 13   |
| ∆ST (pS-x)       | trpC2, tyr, nic, ura, met, ade, sipS, sipT; Cm<sup>R</sup>; contains plasmid encoding mutant SipS (x indicates the position and type of amino acid substitution in the corresponding mutant proteins) | 13   |
| KS408            | KS408 derivative; *wprA*-pMutin2; IPTG-dependent transcription of *wprA*; Cm<sup>R</sup>, Em<sup>R</sup> | 20   |
| *wprA*-pMutin2   | like 8G5 sipS; *wprA*-pMutin2; IPTG-dependent transcription of *wprA*; Em<sup>R</sup> | This study |
| W∆S              | Like 8G5 sipS; *wprA*-pMutin2; IPTG-dependent transcription of *wprA*; Em<sup>R</sup> | This study |

*Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Em, erythromycin.

RESULTS

*Stability of SipS Mutant Proteins at 48 °C*—We have shown previously that the SipS mutant proteins R84A, R84H, and D146A are prone to proteolytic degradation at 37 °C, showing very low levels of SPase activity (6). Nevertheless, at this temperature sufficient active molecules of SipS R84A, R84H, or D146A are produced in *B. subtilis* to replace the wild-type forms of SipS and SipT (13). To investigate whether the inactivity of these three mutant proteins at 48 °C (13) was due to increased proteolytic degradation, Western blotting experiments were carried out with *B. subtilis* 8G5 sipS (∆S; lacking the chromosomal sipS gene) transformed with plasmid pS-L74A, pS-R84A, or pS-D146A (Table I). As shown in Fig. 1 (A and B), SipS R84A, R84H, or D146A were detectable at 37 °C, but not at 48 °C. Furthermore, at 37 °C all three mutant proteins were present in reduced amounts compared with wild-type SipS, the D146A mutant protein being most unstable. Examination of the accumulation of the hybrid precursor pre(A13i)-β-lactamase (11) in *B. subtilis* ∆ST (lacking the chromosomal sipS and sipT genes) transformed with plasmid pS-R84A, pS-R84H, or pS-D146A (Table I). As shown in Fig. 1 (A and B), SipS R84A, R84H, or D146A were detectable at 37 °C, but not at 48 °C. Furthermore, at 37 °C all three mutant proteins were present in reduced amounts compared with wild-type SipS, the D146A mutant protein being most unstable. Examination of the accumulation of the hybrid precursor pre(A13i)-β-lactamase (11) in *B. subtilis* ∆ST (lacking the chromosomal sipS and sipT genes) transformed with plasmid pS-R84A, pS-R84H, or pS-D146A (Table I). As shown in Fig. 1 (A and B), SipS R84A, R84H, or D146A were detectable at 37 °C, but not at 48 °C. Furthermore, at 37 °C all three mutant proteins were present in reduced amounts compared with wild-type SipS, the D146A mutant protein being most unstable.

Similarly, the stability of the SipS mutant proteins L74A and Y81A, which compared with SipS R84A, R84H, and D146A, showed a relatively high activity at 37 °C (6) and which were unable to replace SipS and SipT at 48 °C (13), was investigated by Western blotting, using *B. subtilis* strains lacking the chromosomal sipS genes) transformed with plasmids pS-L74A or pS-Y81A. Unexpectedly, both at 37 and 48 °C, the levels of SipS L74A and Y81A were comparable with those of wild-type SipS (Fig. 1, A and B). The impaired SPase activity of SipS L74A and Y81A at 48 °C was reflected by the increased accumulation of pre(A13i)-β-lactamase in *B. subtilis* ∆ST transformed with plasmids pS-L74A or pS-Y81A, as compared with *B. subtilis* ∆ST producing wild-type SipS (Fig. 1C). These results show that leucine 74 and tyrosine 81 are very important for catalysis at 48 °C, but not for protease resistance of SipS.

*SipS D146A Is a Substrate for the Cell Wall-bound Protease WprA*—As SipS is a type II membrane protein, the largest (carboxyl-terminal) part of which is exposed to the extracytoplasmic side of the membrane (11), it seems likely that proteases residing in the membrane or cell wall are responsible for the degradation of the SipS R84A, R84H, and D146A mutant proteins. As a first approach to identify the proteases responsible for their degradation, we tested the stability of SipS D146A, the most unstable mutant, in *B. subtilis* strains lacking (putative) membrane-bound proteases such as FtsH, HtrA, protease IV or Tsp (for a review on the *E. coli* homologues of these proteases, see Ref. 18), or the cell wall-bound protease WprA (19, 20). Strikingly, none of the membrane-bound proteases appeared to be involved in the degradation of SipS D146A (data not shown). In contrast, at 37 °C, *B. subtilis* W∆S (depleted of WprA, lacking wild-type SipS) accumulated approximately 10-fold higher levels of SipS D146A than *B. subtilis* ∆S (Fig. 2), showing that WprA is involved in the degradation of SipS D146A. Interestingly, the levels of SipS R84A and R84H were...
DISCUSSION

In the present studies, we show that temperature-sensitive mutants of SipS are inactivated via different mechanisms. Two major classes of temperature-sensitive SipS mutants were identified. The first class of mutant proteins, consisting of SipS L74A and Y81A, is structurally stable but has impaired catalytic activity at 48 °C, while the second class of mutant proteins, consisting of SipS R84A, R84H, and D146A, is sensitive to proteolysis, in particular at 48 °C.

The observation that SipS L74A and Y81A are structurally stable, showing a particularly reduced enzymatic activity at 48 °C, indicates that leucine 74 and tyrosine 81 are involved in catalysis. This is a novel conclusion, which was not evident from previous site-directed mutagenesis experiments with SipS or other type I SPases (for review, see Ref. 2), but which is supported by the recently published crystal structure of the E. coli SPase I (5). As evidenced by the latter structure, the side chains of phenylalanine 133, tyrosine 143, and methionine 270, and the main chain atoms of methionine 270, methionine 271, not increased in the absence of WprA, indicating that these SipS mutants are not substrates for WprA. Despite the improved stability at 37 °C, SipS D146A was not detected immunologically in cells of B. subtilis WΔS (pS-D146A) grown at 48 °C (data not shown). Similarly, at 48 °C, SipS R84A and R84H were not detected in cells of B. subtilis WΔS transformed with pS-R84A or pS-R84H (data not shown).

Suppression of the Temperature Sensitivity of B. subtilis ΔST (pS-D146A) by WprA Depletion—We have shown previously that the production of SipS D146A, to levels that are below the detection limit for Western blotting, can be sufficient for growth of strains lacking SipS and Stp, provided that the temperature is not raised above 42 °C (13). To determine whether the depletion of WprA in B. subtilis ΔST (pS-D146A) could suppress the temperature sensitivity of this strain, even though the depletion of WprA did not result in the accumulation of detectable amounts of SipS D146A at 48 °C (see above), a wprA mutation was introduced into this strain. As shown in Fig. 3, the depletion of WprA resulted in the suppression of the temperature sensitivity of B. subtilis ΔST (pS-D146A); while B. subtilis ΔST (pS-D146A) stopped growing and started to lyse upon a temperature shift from 37 to 48 °C, the corresponding WprA-depleted strain continued to grow normally at 48 °C. These observations show that depletion of WprA resulted in increased levels of active SipS D146A even at 48 °C.

2 R. E. Dalbey, personal communication.
teins, either before, during, or after membrane insertion. The latter possibility is particularly intriguing since it would require retrograde transport to the cytoplasm, as documented recently for the degradation of ER luminal proteins (22–25).

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