Mutational Evidence of Transition State Stabilization by Serine 88 in *Escherichia coli* Type I Signal Peptidase[†,‡]

Joseph L. Carlos,[§] Philip A. Klenotic,[§] Mark Paetzel,[∥] Natalie C. J. Strynadka,[∥] and Ross E. Dalbey*[,§]

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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ABSTRACT: Type I signal peptidase (SPase I) catalyzes the hydrolytic cleavage of the N-terminal signal peptide from translocated preproteins. SPase I belongs to a novel class of Ser proteases that utilize a Ser/Lys dyad catalytic mechanism instead of the classical Ser/His/Asp triad found in most Ser proteases. Recent X-ray crystallographic studies indicate that the backbone amide nitrogen of the catalytic Ser 90 and the hydroxyl side chain of Ser 88 might participate as H-bond donors in the transition-state oxyanion hole. In this work, contribution of the side-chain Ser 88 in *Escherichia coli* SPase I to the stabilization of the transition state was investigated through in vivo and in vitro characterizations of Ala-, Cys-, and Thr-substituted mutants. The S88T mutant maintains near-wild-type activity with the substrate pro-OmpA nuclease A. In contrast, substitution with Cys at position 88 results in more than a 740-fold reduction in activity (k_{cat}) whereas S88A retains much less activity (>2440-fold decrease). Measurements of the kinetic constants of the individual mutant enzymes indicate that these decreases in activity are attributed mainly to decreases in k_{cat} while effects on K_m are minimal. Thermal inactivation and CD spectroscopic analyses indicate no global conformational perturbations of the Ser 88 mutants relative to the wild-type *E. coli* SPase I enzyme. These results provide strong evidence for the stabilization by Ser 88 of the oxyanion intermediate during catalysis by *E. coli* SPase I.

Proteins that are exported across the bacterial cell membrane generally contain amino-terminal signal peptides that are cleaved during translocation of the exported protein across the lipid bilayer. Cleavage of these signal peptides is accomplished by type I signal peptidases (SPase I).[1] Since these enzymes are essential for the viability of most bacteria (*1*−*3*), they are currently of interest as possible targets for the design of novel antibiotics (*4*).

Type I SPases identified in eubacteria and the mitochondria and chloroplasts of higher eukaryotes appear to be unconventional Ser proteases. They are classified into a novel class distinguished by a conserved Ser/Lys dyad catalytic mechanism (*5*). Included in this classification (Clan SF) are the LexA repressor protein, *Bacillus subtilis* SPase I (SipS), Tsp protease, and tricorn protease (*6*). The catalytic system employed by these endopeptidases is hypothesized to occur via the use of a Lys side chain as a general base in the deprotonation of a nucleophilic Ser residue. In contrast, the Lys general base is replaced by a His in the proposed catalytic mechanism of SPase I enzymes found in archea and the endoplasmic reticulum. No conserved Lys residue has been found to be necessary for catalysis in these enzymes (*7*).

The most direct evidence to date of the catalytic mechanism of type I signal peptidases has been provided by the crystal structure of the inhibitor-bound soluble fragment of *Escherichia coli* SPase I (*8*). The structure reveals the â-lactam type inhibitor [allyl (5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate] carbonyl (C7) covalently bonded to the Ser 90 Oç. The structure also shows that the only titratable residue in the vicinity of the nucleophilic Ser 90 side chain is Lys 145. The Lys 145 Nœ, at 2.9 Å away from the Ser 90 Oç, is properly positioned to function as the general base during catalysis. An additional feature of the catalytic mechanism of *E. coli* SPase I revealed by the crystal structure is the likely formation of a transition-state oxyanion

catalytic activity are shown to be mainly due to decreases in k_{cat}, while effects on K_m were found to be negligible. All three mutants displayed similar thermal stability and CD spectra compared to wild-type enzyme. Finally, the calculated differential free energy of transition-state stabilization provided by Ser 88 was calculated to be 5.2 kcal/mol. This is consistent with the loss of an oxyanion−hydroxyl interaction (*9*).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The *E. coli* strains BLR(DE3) and BL21(DE3) were purchased from Novagen. The *E. coli* IT41 temperature-sensitive SPase I strain was obtained from Dr. Yoshikazu Nakamura (*10*). The pETlep vector, expressing wild-type 6-His-tagged *E. coli* SPase I enzyme under IPTG-inducible T7 control was constructed as described in Klenotic et al. (*11*). The modified pET-23a vector expressing the pro-OmpA nuclease A fusion protein was a gift from Dr. Mark O. Lively.

Site-Directed Mutagenesis and DNA Manipulations. Oligonucleotide PCR-directed mutagenesis was performed on the pETlep vector using the Quikchange system (Stratagene). Plasmid isolations were carried out with the Qiagen midiprep kit and plasmid DNA sequencing was accomplished by the dideoxy chain-terminator method (*12*) with the Sequenase 2.0 derivative of T7 DNA polymerase (U.S. Biochemical Corp.) or by the fluorescent dye termination method (ABI Prism AmpliTaq FS dye terminator cycle sequencing kit) on a Perkin-Elmer/Applied Biosystems model 373A automated DNA sequencer at The Ohio State University Biopolymer Facility. Sequencing primers were purchased from Integrated DNA Technologies. *E. coli* host strain BLR(DE3) was transformed with the pETlep vector, bearing the wild-type or mutant SPase I gene, by the calcium chloride method (*13*) and selected in 100 íg/mL ampicillin and 12.5 íg/mL tetracycline.

Purification of 6-His-Tagged SPase I Ser 88 Mutants and Pro-OmpA Nuclease A Substrate. Wild type and S88A, S88C, and S88T SPase I mutants containing a 6-His tag were purified on an anion-exchange nickel affinity chromatography system as described by Klenotic et al. (*11*

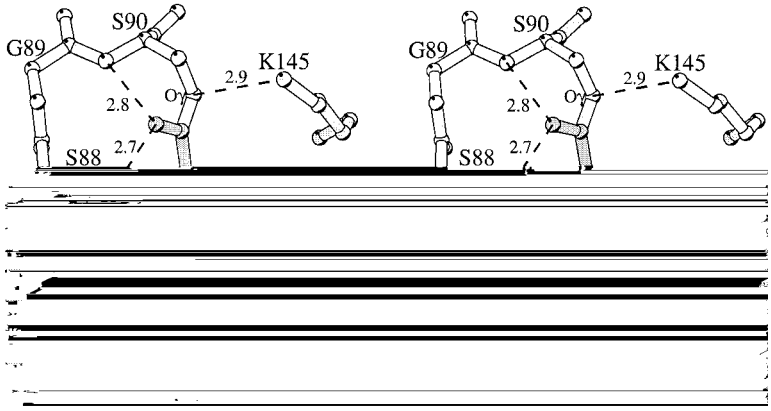

FIGURE 1: Oxyanion hole of *E. coli* signal peptidase. A signal peptidase−signal peptide acyl-enzyme complex is modeled on the basis of the crystal structure of the *E. coli* signal peptidase−inhibitor complex (*8*). The main-chain amide of Ser 90 and the side-chain hydroxyl of Ser 88 are the only potential hydrogen-bond donors in the environment of the substrate's scissile carbonyl oxygen. The side chain of Ser 88 is shown in black. The dashed lines indicate H-bond distances (in angstroms). The P1 through P3 residues of the substrate are shown in gray. This stereo figure was prepared with the program MOLSCRIPT (*32*).

CD Spectroscopy. The far-ultraviolet circular dichroism spectra for wild-type and Ser 88 mutant SPase I enzymes were obtained on a Jasco spectrapolarimeter J-500C instrument at a constant cuvette temperature of 4 °C. As determined by use of a molar extinction coefficient of 44 000 cm^{-1} M^{-1} at 280 nm (*16*), all protein samples were at a concentration of 10 μM in 5 mM potassium phosphate, pH 8.0, and 1% *n*-octyl β-glucopyranoside detergent.

Thermostability. Wild-type and mutant enzymes in 20 mM potassium phosphate, pH 8.0, and 1% Triton X-100 were preincubated at the indicated temperatures for 1 h. Residual activities were then measured by the addition of 1 μl of heat-treated enzyme to 10 μL of substrate pro-OmpA nuclease A at a final concentration of 4 μM. Each reaction was then incubated at 37 °C for 30 min, stopped by the addition of 2.5 μL 5× SDS sample buffer, and quenched in a dry ice−ethanol bath. After resolution of substrate and cleavage product by SDS−17.2% PAGE and staining, percent processing was quantitated as in the kinetic assays.

Molecular Modeling. The program O (*17*) was used to perform the molecular modeling studies.

RESULTS

The inhibitor-bound crystal structure of the soluble catalytic fragment of *E. coli* SPase I (*8*) showed that the Ser 90 backbone amide and the rotated Ser 88 hydroxyl side chain could participate as H-bond donors in the stabilization of a tetrahedral oxyanion intermediate during the course of catalysis. A model of these interactions with a peptide substrate is shown in Figure 1. It is clear from this model that Ser 88 may play an important catalytic role.

To confirm the role of Ser 88 in the proposed interactions, site-directed mutagenesis of full-length wild-type *E. coli* SPase I (pETlep vector) was performed to introduce Ala, Cys, and Thr at the Ser 88 position. Each mutant protein was isolated to homogeneity as previously described (*11*) and the expression vector DNA was sequenced to confirm the presence of each mutation.

S88T Maintains Near-Wild-Type Activity but S88A and S88C Exhibit Substantially Reduced Activity in Vivo and in Vitro. Purified pro-OmpA−nuclease A fusion protein (*14*) was used as an in vitro substrate to study the *E. coli* SPase I Ser 88 mutants. With the wild type as a control, the in vitro effects of enzyme serial dilutions on pro-OmpA−nuclease A processing were assessed for the S88A, S88C, and S88T mutants. As shown in Figure 2A, the S88A and S88C mutants displayed slight processing of the substrate at the stock 0.1 mg/mL ("1" dilution) enzyme concentration and no visible processing at 0.01 mg/mL ("10^1" dilution). S88C maintains slightly greater enzymatic processing of the pro-OmpA−nuclease A substrate than the S88A mutant. Contrary to S88A and S88C, the S88T mutant exhibited processing down to a 10^3 stock enzyme dilution. The S88T in vitro activity was comparable to that of the wild-type enzyme.

In the temperature-sensitive SPase I-deficient IT41 strain (*10*), the processing of radiolabeled pro-OmpA by plasmid-encoded *E. coli* SPase I constructs can be examined in vivo. In this system, the enzymatic activities of wild-type, S88A, S88C, and S88T proteins in their native intracellular membrane environments were compared. Figure 2B shows that wild type is slightly more active than the S88T enzyme. Approximately 50% cleavage of the pro-OmpA substrate has occurred after 10 s with the S88T enzyme, whereas at the same time point greater than 50% pro-OmpA processing has already occurred with the wild-type enzyme. Also, similar to the in vitro assay results, the S88A and S88C mutants displayed very little activity in vivo. The S88A mutant showed 50% processing occurring at slightly over 40 s chase whereas the S88C mutant displayed 50% processing at slightly under 40 s (Figure 2B). The S88A and S88C SPase 1 activities were only slightly greater than that of the negative control cell line, which carried no plasmid encoded SPase I (Figure 2B, "no plasmid").

Decrease in Activity of Ser 88 SPase I Mutants Is Mainly Due to a Reduction in k_{cat}. To determine more precisely the effects on catalysis of the Ser 88 mutations, the kinetic constants for each mutant were measured by the pro-OmpA−nuclease A in vitro assay. At constant enzyme with various substrate concentrations, initial velocities were determined from plots of pro-OmpA nuclease A processed versus time. The k_{cat} and K_m constants were then determined from initial velocity versus substrate concentration plots. The data are summarized in Table 1. Substitution of the hydroxyl side

chain by a sulfhydryl in the S88C mutant resulted in a drastic 740-fold decrease in k_{cat}

modeled side-chain hydroxyl group of the S88T mutant superimposes on the modeled Ser 88 hydroxyl. The side-chain methyl group on the S88T mutant points into the solvent and lies adjacent to, but does not clash with, the modeled substrate P2 side chain. In the S88C mutant, the longer Cβ−Sγ bond distance and the larger van der Waals radius of the Sγ results in a potential clash with the signal peptide main chain. Main-chain or side-chain adjustments may allow for the Cys 88 thiol to contribute some oxyanion stabilization and thus explain its higher activity than the S88A mutant. It should be noted that sulfur atoms are capable of forming only weak hydrogen bonds (*19*) and that we cannot be sure of the ionization state of the cysteine thiol group. Theoretically, at the assay reaction conditions (pH = 8.0) the thiol would be approximately 50% ionized. Modeling of the S88A mutant reveals that there would not be enough room to introduce a mediating water as a hydrogen-bond donor.

An updated model of the proposed catalytic mechanism of *E. coli* SPase I (*20*), which includes the transient H-bonding interactions of the transition state, is shown in Figure 4. In this scheme the amide backbone of the catalytic Ser 90 and the hydroxyl side chain of Ser 88 provide electrophilic assistance to the transition-state oxyanion. Also, the K145Nα−scissile carbonyl oxygen distance in Figure 1 is 4.5 Å. Thus the protonated K145 residue possibly also

Quantitation of the kinetic constants of the *E. coli* SPase I Ser 88 mutants compared to those of wild-type enzyme

(*25*) and of a distal Thr-stabilized interaction in subtilisin BPN′ (*31*) that could be replaced with a Ser residue while