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Backbone NMR assignments of DFP-inhibited mature subtilisin E

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Abstract

Here we report the backbone chemical shifts of the DFP-inhibited mature subtilisin E, which was uniformly labeled by ¹³C, ¹⁵N with a supplement of excess calcium.

Keywords

Subtilisin; Intramolecular chaperone; Serine protease; Propeptide

Biological context

Subtilisin E (SBE), a member of the subtilase superfamily, is an alkaline serine protease from *Bacillus subtilis* 1168. Subtilisin and subtilisin-like proteases, such as subtilisin BPN' from *Bacillus amyloliquefaciens* and Esperase[®] from *Bacillus lentus* have been intensively studied for their biochemical and biophysical properties. For decades, subtilisins have also been a great model protein for studying protein engineering and intramolecular chaperone-mediated protein folding (Inouye, 1991). The enzymatically active domain of SBE consists of 275 amino acids; however, it is produced as a pre-pro-subtilisin precursor containing a 29-residue pre-sequence for signaling protein secretion and a 77-residue propeptide as an intramolecular chaperone essential for correct folding of SBE.

Previous studies have shown that the maturation process is important for SBE to become functionally active *in vivo* (Ikemura et al., 1987) and *in vitro* (Ikemura and Inouye, 1988). Formation of the native structure is the first step of maturation, which depends on the propeptide and requires calcium ion for stability (Yabuta et al., 2002), and is followed by the removal of the propeptide. Recently, an engineered Sbt70 derived from subtilisin BPN', sharing 86% sequence identity to SBE, was shown to fold through an altered pathway, which was propeptide and calcium independent (Bryan et al., 1995). Here, we report the backbone resonance assignments of the mature subtilisin E folded through a native maturation process and inhibited by diisopropyl fluorophosphate (DFP), an irreversible serine protease inhibitor. The assignments provide important information for further study on the propeptide-mediated subtilisin folding through a native pathway.

Methods and experiments

The SBE precursor containing propeptide was expressed in *E. coli* using pET11a vector with M9 minimal medium supplemented with $^{15}\text{NH}_4\text{Cl}$ (1 g/L) and ^{13}C -glucose-C6 (1 g/L). Induced by isopropyl β -D-1 thiogalactopyranoside (IPTG), the prosubtilisin was produced into inclusion bodies, so that its expression is not toxic to the cells. SBE used in this study has two amino acid substitutions, V4F and S85A, which have no effect on either enzymatic activity or autoprocessing. Purification of the SBE precursor was carried out using a cation exchange column (CM SepharoseTM CL-6B) with a sodium chloride gradient under denaturing conditions in 5 M urea at pH 5. Mature and active SBE was obtained by rapid dilution of the precursor into refolding buffer (50 mM Tris-HCl 0.5 M $(\text{NH}_4)_2\text{SO}_4$ 10 mM CaCl_2 pH 7) at room temperature allowing the maturation process, including autoprocessing of the propeptide, to be fully completed. Active SBE was concentrated down to 10 ml using a Millipore stirred cell followed by addition of 5 mM DFP to prevent autoproteolysis. The DFP-modified mature SBE was further purified by gel filtration column (HiLoadTM SuperdexTM 75) and transferred into sample buffer (25 mM MES-NaOH 100 mM CaCl_2 pH 6.5) followed by addition of 10% D_2O and 0.05% NaN_3 for NMR data acquisition. Note that DFP-modified SBE was highly stable as no apparent autoproteolysis was observed even after 4 weeks incubation at 30°C.

All NMR spectra were acquired at 30°C on a Varian 600MHz spectrometer with a cold-probe. Data were processed using NMRPipe (Delaglio et al., 1995) and analyzed by Sparky (Goddard and Kneller) and CARA (Keller, 2004; <http://www.nmr.ch/>). Assignments were based on the following triple resonance experiments: HNCA, HN(CO)CA, HNCO, HN(CA)CO, HNCACB, CBCA(CO)NH, HC(CO)NH (Ferentz and Wagner, 2000) and ^{15}N -edited NOESY (Sattler et al., 1999). The analysis of triple resonance connectivity was done with assistance of PINE (supported by the National Magnetic Resonance Facility at Madison; <http://miranda.nmr.fam.wisc.edu/PINE/>) and AutoLink (supported by the University of Zurich, Switzerland; <http://www.nmr.ch/>).

Assignments and data deposition

As shown in the ^{15}N , ^1H -HSQC spectrum (Fig. 1), 252 crosspeaks were assigned to specific residues (97%) with Gln 2, Ser 33, Ser 78, Gln 103, Tyr 104, Ser 125, Thr 133, Ala 134 and Ser 161 missing due to indistinguishable overlap. The backbone resonance assignments were obtained and the chemical shifts of assigned ^1H , ^{13}C , ^{15}N were deposited in the BioMagResBank (<http://www.bmrb.wisc.edu/>) with the accession number 15706. Based on the assigned chemical shifts, secondary structure propensities could be predicted using the Secondary Structure Propensity (SSP) algorithm developed by Forman-Kay and colleagues (Marsh et al., 2006). Comparison of DFP inhibited SBE with the Sbt70 derived from subtilisin BPN' shows that the secondary structure propensities of the two proteins are very similar despite eight mutations at positions K43N, M50F, A73L, Q206V, Y217K, N218S, S221A, Q271E, and a nine residue deletion at positions L75 to G83 containing the calcium binding site.

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Fig. 1. ^1H , ^{15}N -HSQC spectrum of DFP-inhibited SBE. Resonance assignments are indicated in one letter amino acid code and residue number. The insets show the resonance of G258 and an expanded view of the central part of the spectrum respectively and are shown at the optimized contour level.

Fig. 2. Secondary structure propensity scores. ^{13}C α - and β -carbon chemical shifts are used in the calculation. (a) DFP inhibited SBE; (b) Sbt70 (BMRB accession number: 15250)