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Crystallization and preliminary crystallographic analysis of *Achromobacter* protease I mutants

Len Ito,[a,b]* Kentaro Shiraki,[c] Tatsuya Uchida,[b] Masaki Okumura[b] and Hiroshi Yamaguchi[b]

[a]Japan Synchrotron Radiation Research Institute (SPring-8), 1-1-1 Kouto, Sayo, Hyogo 679-5198, Japan, [b]School of Science and Technology, Kwansei Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan, and [c]Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, Japan

Correspondence e-mail: l-ito@spring8.or.jp

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Achromobacter protease I (API), a serine protease, shows an order of magnitude higher activity than bovine trypsin. The optimum pH of mutant enzymes with His210 replaced by Ser (H210S) and Trp169 replaced by Phe (W169F) has been shown to shift from approximately pH 9 (wild-type enzyme) to approximately pH 7 while retaining high activity. The mutants were crystallized by the hanging-drop vapour-diffusion technique with 2 *M* ammonium sulfate as the precipitant. The space group of the W169F mutant crystal was $P1$, with unit-cell parameters $a = 42.6$, $b = 34.7$, $c = 69.5$ Å, $\alpha = 91.8$, $\beta = 97.5$, $\gamma = 89.9^\circ$, while the space group of the H210S mutant crystal was $P2_1$, with unit-cell parameters $a = 42.4$, $b = 34.2$, $c = 67.7$ Å, $\beta = 97.6^\circ$. Diffraction data were collected from W169F and H210S crystals to resolutions of 2.0 and 2.3 Å, respectively.

1. Introduction

Achromobacter protease I (API) consists of 268 amino-acid residues with a low sequence similarity to other serine proteases (approximately 10%). API has several unique properties that are distinct from those of other trypsin-type serine proteases, such as a relatively higher protease activity than bovine trypsin, a wide pH optimum ranging from 8.5 to 10.7, restricted Lys specificity, including the Pro–Lys bond, and structural stability against denaturation with 0.1% SDS and 4 *M* urea (Masaki, Fujihashi *et al.*, 1981; Masaki, Tanabe *et al.*, 1981). Because of these unique properties, API has been an effective tool for peptide fragmentation in protein-sequence analysis (Morihara *et al.*, 1986; Sakiyama & Masaki, 1994). However, its property of having its optimum pH shift in the alkaline region is a disadvantage, because a neutral pH (typically pH 6–7) is preferred when a protease cleaves a substrate protein to peptide fragments (Tsunasawa *et al.*, 1989).

X-ray crystallographic analysis of API (PDB code 1arb; Tsunasawa *et al.*, 1989) revealed that the apparent tertiary structure of the protein is similar to that of bovine trypsin. However, a careful structural comparison between API and trypsin-type serine proteases showed that API has a critically different arrangement of aromatic amino acids around the active site (PDB code 1arb). We previously reported the contribution of the electrostatic interaction Asp113–His210, which is supported by Trp169, and the optimum pH shift of the His210 mutant from about pH 9 (in the wild-type enzyme) to approximately pH 7 while retaining high activity. In particular, we previously reported that the k_{cat}/K_m of H210S is two times higher than that of wild-type API (Shiraki & Sakiyama, 2002). In contrast, the k_{cat}/K_m of W169F is similar to that of wild-type API (Shiraki, Norioka, Li & Sakiyama, 2002). The molecular mechanism has not been elucidated, but we have hypothesized that the structure of the aromatic stacking between His210 (in the substrate-binding S1 site) and Trp69 (in close proximity to subsite S1) plays an important role in the high catalytic potency of API. In this article, we present preliminary X-ray studies of the API mutants. The H210S mutant crystallized in space group $P2_1$, whereas the W169F mutant crystallized in space group $P1$.

Table 1
Data-collection and diffraction data statistics.

Values in parentheses are for the highest resolution shell.

	H210S	W169F
Detector	R-AXIS IV	R-AXIS IIc
Wavelength (Å)	1.5418	1.5418
Crystal-to-detector distance (mm)	120	100
Oscillation angle (°)	1.8	1.5
Total oscillation range (°)	183.6	180
Space group	$P2_1$	$P1$
Unit-cell parameters		
a (Å)	42.4	42.6
b (Å)	34.2	34.7
c (Å)	67.7	69.5
α (°)	90.0	91.8
β (°)	97.6	97.5
γ (°)	90.0	89.9
Resolution (Å)	41.88–2.30 (2.42–2.30)	68.89–2.00 (2.07–2.00)
Multiplicity	3.6 (3.6)	1.9 (1.8)
Completeness (%)	99.8 (99.3)	86.5 (71.0)
$R_{\rm merge}$†	0.089 (0.165)	0.085 (0.271)
$\langle I/\sigma(I)\rangle$	14.4 (8.2)	10.8 (2.6)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation and $\langle I(hkl)\rangle$ is the mean intensity of the reflection.

2. Methods and results

2.1. Protein expression and purification

Single-stranded DNA for mutagenesis was obtained from plasmid pKYN200 (Ohara *et al.*, 1989). Mutagenesis was performed according to the uracil-DNA-mediated method (Kunkel, 1985). The mutagenic primers were 5′-CGGCGCCGCCGCCGAAGGCCACGAACG-3′ for the W169F mutant and 5′-CGACGGGCCCCCGGACAGCTG-GCCGAGCAC-3′ for the H210S mutant. *Escherichia coli* strain JM109 was used for preparation of the plasmid and phage DNA. The mutant genes were identified by DNA sequencing and then subcloned into the expression vector pKYN200. Transformants of *E. coli* strain JA221 cells were grown on Luria–Bertani medium supplemented with 50–100 µg ml^{-1} ampicillin. The expressed mutated APIs were purified by affinity chromatography on hen egg-white ovomucoid. A periplasmic fraction prepared from 1 l culture medium was passed through a DEAE-cellulose column (3.6 × 50 cm) equilibrated with 10 m*M* Tris–HC1 pH 9.0. After the pH of the unadsorbed fraction had been adjusted to 8.0 by the addition of glacial acetic acid, the fraction was applied onto an ovomucoid Sepharose 4B column (chicken ovomucoid content 0.3 mmol per millilitre of wet gel; 1.6 × 25 cm) equilibrated with 50 m*M* Tris–HCl pH 8.0. After the column had been successively washed with 50 m*M* Tris–HCl pH 8.0 and distilled water, the adsorbed mutated API was eluted with 10 m*M* ammonium acetate pH 3.0. The eluted solution was immediately neutralized carefully by adding diluted ammonium hydroxide since API is unstable below pH 4.0 (Norioka *et al.*, 1994). The amount of mutant protein obtained from 1 l culture medium was 0.2–0.4 mg.

2.2. Crystallization

The API mutants H210S and W169F were prepared as described previously (Shiraki & Sakiyama, 2002; Shiraki, Norioka, Li & Sakiyama, 2002; Shiraki, Norioka, Li, Yokota *et al.*, 2002). The API mutants were crystallized using the hanging-drop vapour-diffusion method. Hanging drops were obtained by mixing 5.0 µl protein solution (10–20 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0) and 5.0 µl reservoir solution and were equilibrated against 1 ml reservoir solution consisting of 2 *M* ammonium sulfate and 5% 2-propanol in 10 m*M* Tris–HCl pH 8.0 at room temperature; the crystals reached dimensions of above 0.2 × 0.2 × 0.1 mm after one week.

2.3. X-ray diffraction study

Crystals were mounted and sealed in a glass capillary with a small amount of mother liquor. X-ray diffraction data for the W169F and H210S mutant crystals were collected on R-AXIS IIc and R-AXIS IV detectors, respectively, using a rotating-anode generator at room temperature. The W169F crystal, which belonged to space group $P2_1$ with unit-cell parameters a = 42.6, b = 34.7, c = 69.5 Å, α = 91.8, β = 97.5, γ = 89.9°, diffracted to 2.0 Å resolution. The H210S crystal, which belonged to space group $P2_1$ with unit-cell parameters a = 42.4, b = 34.2, c = 67.7 Å, $\alpha = \gamma$ = 90.0, β = 97.6°, diffracted to 2.3 Å resolution. Data were processed using the *DENZO* and *SCALEPACK* programs (Otwinowski, 1992) for the W169F mutant crystal and the *MOSFLM* (Leslie, 1992) and *SCALA* programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) for the H210S mutant crystal. The data-processing statistics are shown in Table 1.

Assuming the presence of one molecule of the H210S mutant in the asymmetric unit, the $V_{\rm M}$ value (Matthews, 1968) was calculated to be 1.7 Å^3 Da^{-1}, corresponding to a solvent content of 26.2%. The $V_{\rm M}$ value calculation for the W169F mutant ($V_{\rm M}$ = 1.8 Å^3 Da^{-1}, corresponding to a solvent content of 29.4%) indicated the probable presence of two molecules in the asymmetric unit, designated chains *A* and *B*. The crystal structure was determined using the molecular-replacement (MR) method. The MR calculation was performed using *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 package. The structure of API (PDB code 1arb) was used as the search model. The *R* factor and correlation coefficient for the H210S mutant obtained from the MR calculations were 33.8% and 69.2%, respectively. The *R* factor and correlation coefficient for the W169F mutant obtained from the MR calculations were 34.8% and 69.5%, respectively. Structure determination and refinement are currently under way.

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