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Role of Disulphide Bonds in a Thermophilic Serine Protease Aqualysin I from *Thermus aquaticus* YT-1

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A thermophilic serine protease, Aqualysin I, from *Thermus aquaticus* YT-1 has two disulphide bonds, which are also found in a psychrophilic serine protease from *Vibrio* sp. PA-44 and a proteinase K-like enzyme from *Serratia* sp. at corresponding positions. To understand the significance of these disulphide bonds in aqualysin I, we prepared mutants C99S, C194S and C99S/C194S (WSS), in which Cys69-Cys99, Cys163-Cys194 and both of these disulphide bonds, respectively, were disrupted by replacing Cys residues with Ser residues. All mutants were expressed stably in *Escherichia coli*. The C99S mutant was 68% as active as the wild-type enzyme at 40°C in terms of k_{cat} value, while C194S and WSS were only 6 and 3%, respectively, as active, indicating that disulphide bond Cys163-Cys194 is critically important for maintaining proper catalytic site conformation. Mutants C194S and WSS were less thermostable than wild-type enzyme, with a half-life at 90°C of 10 min as compared to 45 min of the latter and with transition temperatures on differential scanning calorimetry of 86.7°C and 86.9°C, respectively. Mutant C99S was almost as stable as the wild-type aqualysin I. These results indicate that the disulphide bond Cys163-Cys194 is more important for catalytic activity and conformational stability of aqualysin I than Cys67-Cys99.

Key words: disulphide bond, serine protease, subtilase, thermostability, activity.

Abbreviations: DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; MES, 2-(*N*-morpholino) ethane sulphonic acid; *N*-sucAAPFpNA, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; ODA, oligonucleotide-directed dual amber; PMSF, phenylmethanesulphonyl fluoride; SPRK, proteinase K-like enzyme from *Serratia* sp.; TCA, trichloroacetic acid; VPR, proteinase from *Vibrio* PA-44.

In recent years, there has been an increasing interest in hyperthermophilic and psychrotrophic micro-organisms because they are promising as a source of useful enzymes intended for biotechnological applications in various uses. The molecular basis of hyperthermophilicity and psychrotrophy of their protein components is particularly interesting from both basic and practical standpoints, for it would enable us to construct genetically engineered proteins that could work under a variety of conditions. Various intra-molecular interactions including ionic interaction, hydrogen bonding, hydrophobic interaction and disulphide bonds are assumed to be involved in stabilizing and maintaining the enzyme structure that is necessary to carry out its catalytic function (*1*), but their contribution has not been fully defined in individual cases. In our recent study, we demonstrated that Pro residues in surface loops of aqualysin I were contributing significantly to its hyperthermophilicity (*2*). To further extend the understanding on the molecular bases of stabilization of this enzyme against heat we attempted to examine the role of disulphide bonds by site-directed mutagenesis.

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Subtilisin-like protease superfamily members are a group of closely related proteases that are classified in S8 family in serine protease superfamily, according to the MEROPS peptidase database (http://merops.sanger.ac.uk/). They show a remarkable conservation of the arrangement of amino acids in the active site as well as of overall structures consisting of α/β protein scaffold, but their temperature-stability profiles differ widely, being psychrophilic, mesophilic and hyperthermophilic according to the characteristics of organisms from which they are derived. Because of these characteristics they seem suitable for comparative studies on the basis of thermostability.

Aqualysin I is an alkaline serine-protease produced by a gram-negative thermophilic bacterium *Thermus aquaticus* YT-1 (*3*, *4*). The aqualysin I gene was cloned and expressed in *Escherichia coli* (*5–9*). Based on analysis of sequence homology with more than 100 subtilisin-like serine proteases, aqualysin I is classified into the proteinase K family, which consists of a group of Gram-negative bacteria-derived proteinases within the subtilase superfamily (*1*). The mature protein is processed from a precursor peptide through removal of the N- and C-terminal peptide fragments, and these N- and C-terminal pro-sequences fulfil vital roles in protein folding, maturation and secretion (*10–18*).

Aqualysin I has four Cys residues, Cys67, Cys99, Cys163 and Cys194, that participate in forming two disulphide bonds, Cys67-Cys99 and Cys163-Cys194, and is active up to about 80°C (*5*, *19*). These disulphide bonds are conserved in a subtilisin-like serine protease from psychrotrophic *Vibrio* sp. PA44 (VPR) (*20–22*) and in a proteinase K-like enzyme from psychrotrophic *Serratia* sp. (SPRK) (*23*, *24*), which has 60% and 61% sequence identity to aqualysin I, respectively, at corresponding positions. Even though VPR has an additional disulphide bond Cys277–Cys281, it shows cold-adapted properties, showing higher catalytic activity at lower temperature in the range <55°C and lower thermal stability than aqualysin I and SPRK, the latter being active at 65–70°C. On the other hand, proteinase K, a related mesophilic enzyme from *Tritirachium album* Limber, has two disulphide bonds, though at different positions from aqualysin I, VPR and SPRK, and is active at 40–70°C. Moreover, pyrolysin from hyperthermophilic archaea *Pyrococcus furiosus* and stetterlysin from extremely thermophilic archaea *Thermococcus stetteri* have no cysteine residues (*25*). In view of these accumulated lines of evidence disulphide bonds in subtilases may not seem to be directly related to the thermostability of these enzymes (*20–25*).

However, other studies in the literature indicated that introduction of disulphide bonds into subtilisin and other related serine proteases at positions where they are found in aqualysin I improved the thermostability of those enzymes to some extent (*26–29*). It was also noted that a serine protease from the extremely thermostable eubacterium *Aquifex pyrophilus* has eight cysteine residues, as compared to four in aqualysin I, and most of these cysteine residues form disulphide bonds in the putative mature form (*30*). This may imply that disulphide bonds at appropriate locations play an indismissible role in eliciting thermostability of serine proteases. In order to help in clarifying this controversial issue, we attempted to construct and stably express disulphide bond-deficient aqualysin I mutants, and examined their catalytic activity and thermostability to elucidate the exact role of two disulphide bonds in aqualysin I.

MATERIALS AND METHODS

Strain and Growth Medium—*Escherichia coli* TG1{*supE thi-1* Δ(*lac-proAB*)Δ(*mcrB-hsdSM*)5(rk$^-$mk$^-$) [F', *traD36, proAB, lacI^{q}ZΔM15*]} and MV1184 {*ara*, Δ(*lac-proAB*), *rpsL*, *thi*(Φ80*lacZ*ΔM15), Δ(*srl-recA*) 306::Tn*10* (*tet^r*)/F' [*traD36, proAB$^+$, lacI^q, lacZ*ΔM15]} were used as an expression host and a gene-engineering host, respectively.

LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl, pH 7.0) was used for pre-culture and growth of bacteria. Solid medium contained bacto-agar (1.5%). Ampicillin (final conc. 50–100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (50 μg/ml) were added to culture media when needed.

Construction of Expression Plasmid and Mutant Plasmids—pAQNΔC105, with an insert coding for aqualysin I with a signal peptide and an N-terminal pro-sequence, but without a C-terminal pro-sequence, was a kind gift from Prof. Matsuzawa at the Department of Bioscience and Biotechnology, Aomori University. An expression plasmid pMAQΔc2, which was designed to produce AQN as a fusion protein with maltose-binding protein (MBP) on expression, was constructed based on pAQNΔC105 and pMAL plasmids (New England Biolabs Inc.) as described previously (*2*).

To construct plasmids with a mutated aqualysin I gene, site-directed mutagenesis was carried out with ODA–PCR method (Mutan®-Super express Km; TaKaRa) using pMAQΔc2 as a template. Oligonucleotide primers used for site-directed mutagenesis are shown below with mismatched bases underlined and triplets coding for Ser indicated by dots; 5′-GAACCGTTG$\dot{C}\underline{T}$GTCCAGGACG-3′ (AQ-C99S) and 5′-GAAGAGATCTACG$\dot{C}\underline{T}$ACTACC GTAG TTGG-3′ (AQ-C194S). The fragments containing either of these mutation were inserted into an expression vector, pMAQΔc2, and the plasmids with mutated aqualysin I gene, thus produced, were designated pMAQ-C99S and pMAQ-C194S, respectively. A plasmid with both C99S and C194S mutations, pMAQ-WSS, was then constructed by recombination between pMAQ-C99S and pMAQ-C194S. The nucleotide sequences around the mutation sites as well as other parts of the gene were confirmed by DNA sequencing.

Gene Engineering and Chemical Reagents—Gene engineering experiments were carried out essentially according to Sambrook and Russell (*31*). Enzymes for gene engineering were purchased from TaKaRa, and used according to the manufacturer's instructions. Chemical reagents used were of the highest quality available from Wako Pure Chemicals (Tokyo), Kanto Kagaku Co. (Tokyo) and Sigma Chemicals (St. Louis).

Purification and Activity Measurement of Wild-type and Mutant Aqualysin I—After induction by IPTG (isopropyl β-D-thiogalactopyranoside) at $OD_{660} = 0.8$, the transformants were further cultivated overnight in LB medium. The cells were harvested by centrifugation and sonicated, and the crude extract was subjected to heat treatment (70°C, 1 h), hydrophobic chromatography (Butyl Sepharose; Amersham Biotech.) and cation exchange chromatography (Resource S; Amersham Biotech.) as described previously (*2*). The enzyme was purified to homogeneity through these steps to give a single band on SDS–PAGE on staining with CBB R-250 (*32*). Enzyme activity was measured at 40°C with *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (*N*-sucAAPFp NA) as a substrate in 50 mM HEPES-NaOH (pH 7.5) containing 1 mM $CaCl_2$. The change in absorbance at 410 nm was continuously monitored, and the activity was estimated with $\varepsilon_{410} = 8{,}680\ M^{-1}cm^{-1}$ as a molar absorption coefficient of *p*-nitoroaniline. One unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of *p*-nitroaniline from substrate in 1 min. In cases of using casein as a substrate, 50 μl of enzyme solution at an appropriate concentration was added to 2,950 μl of 1% casein (MERCK) and incubated for an appropriate period of time, and the reaction was stopped by pouring 500 μl aliquot of the sample mixture into 750 μl of 5% trichloroacetic acid (TCA) solution. The mixture was further incubated for 30 min at room temperature,

then centrifuged at 3,000 × *g* for 15 min, and the proteolytic activity was estimated by measuring the absorbance of the supernatant at 280 nm. One unit of enzyme, using casein as a substrate, was defined as the amount of enzyme that causes an increase of 0.002 absorbance unit at 280 nm in 1 min. Protein concentration was measured with the micro-assay method (Bio-Rad), which is based on the Bradford method (*33*), using bovine serum albumin (BSA) as a standard.

Determination of Temperature Dependence of Proteolytic Activity and Heat Stability of Aqualysin I—To examine the temperature dependence of the enzyme activity, 2,450 μl of 1% casein solution (50 mM Tris–Cl, pH 8.5) was pre-incubated at an appropriate temperature for 5 min, and then 50 μl of enzyme solution was added. The enzyme reaction was stopped at appropriate times (0.5–5 min) by transferring a 500 μl aliquot of the reaction mixture into a tube containing 750 μl of 5% TCA solution as a stop solution. After 30 min incubation at room temperature, the mixture was centrifuged and the activity was estimated from A_{280} of the supernatant as described earlier.

To examine the heat stability, the enzyme was diluted with 20 mM MES–NaOH buffer (pH 6.0, 1 mM $CaCl_2$) to give a 50 μg/ml solution. The enzyme solution was incubated for an appropriate time at various temperatures (70–100°C), and then was cooled quickly. The remaining activity was measured with 3 mM *N*-sucAAPFpNA as a substrate at 40°C as described earlier.

Differential Scanning Calorimetry (DSC) Analysis—For DSC analysis, purified wild-type aqualysin I and mutants were treated with 25 mM phenylmethane sulphonyl fluoride (PMSF) dissolved in methanol for 30 min to prevent autolytic degradation during the calorimetric measurements. After complete inactivation of the protease activity was confirmed, the samples were dialysed overnight against 20 mM phosphate buffer (pH 7.4) and filtered through a nitrocellulose filter (0.45 μm pore size). DSC measurements were kindly performed at Nihon SiberHegner K.K. using VP-Capillary DSC Platform system (MicroCal) at 0.5–0.8 mg/ml protein concentration under the condition as follows: Scan range, 25–110°C; scan rate, 250°C/h; feedback/gain, High; filter period, 2 s; pre-scan thermostat, 15 min.

RESULTS

Purification of Wild-type Aqualysin I and Its Mutants—Mutant aqualysin I constructs, pMAQ-C99S, pMAQ-C194S and pMAQ-WSS, which were derived from pMAQΔc2, were expressed and the protein products were purified to homogeneity in essentially the same way as wild-type aqualysin I, except that C194S and WSS required longer heat treatment than wild-type enzyme (Fig. 1).

Temperature Dependence of the Activity of Wild-type Aqualysin I and Its Mutants on Casein—Figure 2 compares the temperature dependence of the caseinolytic activity of wild-type aqualysin I and its mutants. The C99S mutant gave similar temperature-activity profile with wild-type enzyme in a range of 50–90°C showing full activity at around 90°C. In contrast, the activities of the C194S and WSS mutants were definitely lower than wild-type enzyme at temperatures above 60°C. The activity of these mutants only slightly increased with increasing temperature so that at higher temperatures, at which wild-type enzyme gave its full activity, these mutant enzymes showed only marginal caseinolytic activity.

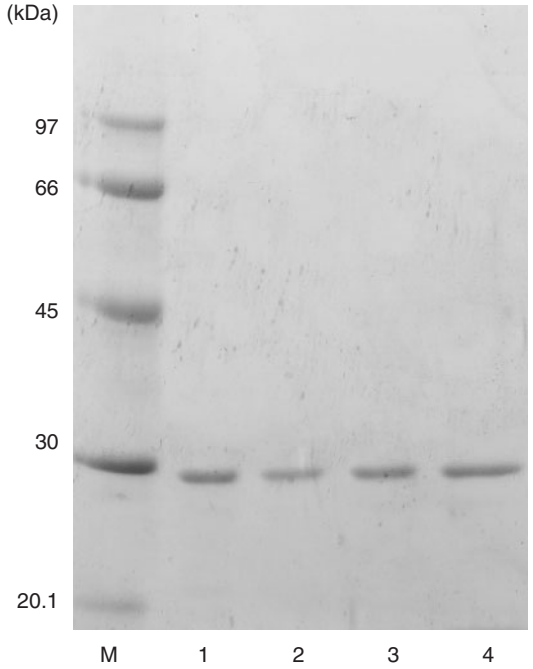

Fig. 1. **SDS–PAGE of wild-type aqualysin I and its mutants.** Wild-type and mutant enzymes purified as described in the text were inactivated with 25 mM PMSF (final concentration) and analysed by SDS–PAGE according to Laemmli (*32*) using a 10% polyacrylamide gel. Lane 1, wild-type aqualysin I; lane 2, C99S; lane 3, C194S; lane 4, WSS; lane M, Low Molecular Weight Marker (molecular mass; 20.1, 30.0, 45.0, 66.0, 97.0 kDa, GE Healthcare).

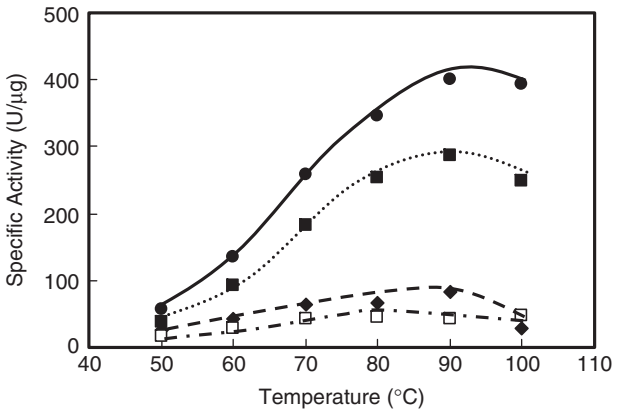

Fig. 2. **Temperature dependence of activity of wild-type aqualysin I and its mutants.** Temperature dependence of the activity was measured as described in MATERIALS AND METHODS section with casein as substrate. Filled circle, filled square, filled triangle and open square represent the activity of wild-type aqualysin I, C99S, C194S and WSS, respectively.

Activity of Wild-type Enzyme and Its Mutants on a Synthetic Substrate—The kinetic parameters of wild-type and mutant enzymes towards a synthetic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, are shown in Table 1. The table indicates that K_m values of mutants were increased 1.7- to 3.9-fold the value of wild-type enzyme. The k_{cat} value of C99S was 2/3 that of wild-type enzyme, while the k_{cat} values of C194S and WSS was decreased to 1/15 and 1/30, respectively, as compared to the value of wild-type enzyme.

Table 1. **Kinetic parameters of wild-type aqualysin I and its mutants.**[a]

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild-type	91.6 ± 2.75	0.79 ± 0.04	115.9 ± 5.34
C99S	62.0 ± 2.86	1.37 ± 0.02	45.9 ± 2.69
C194S	6.4 ± 0.13	2.17 ± 0.03	3.0 ± 0.42
WSS	3.2 ± 0.57	3.06 ± 0.05	1.1 ± 0.21

[a]Parameters were determined at 40°C with *N*-sucAAPFpNA as substrate.

Thermostability of Wild-type Enzyme and Mutants—The activity of wild-type aqualysin I and mutants remaining after heat treatment at temperatures ranging from 70°C to 100°C was determined at 40°C with *N*-suc AAPFpNA as a substrate and the results are illustrated in Fig. 3. The residual activity of wild-type enzyme and C99S followed rather similar time course, and that of C194S and WSS also did. Thus at 90°C, half-lives of wild-type enzyme and C99S mutant were about 40 and 30 min, respectively, whereas those of C194S and WSS mutants were only about 10 min. The latter mutants

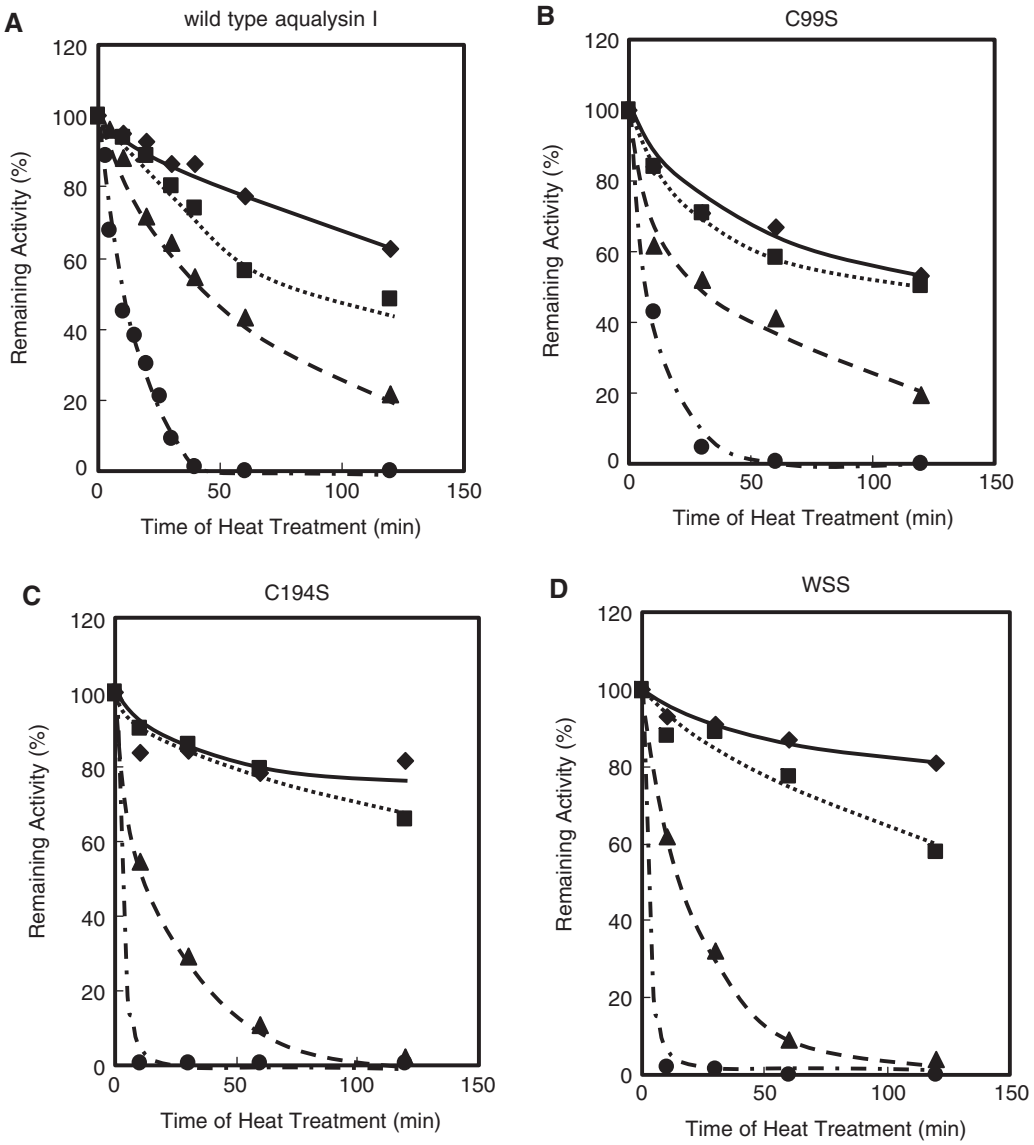

Fig. 3. **Heat stability of wild-type aqualysin I and its mutants.** Heat stability was determined as described in MATERIALS AND METHODS section. Remaining activity after heat treatment at 70°C (filled diamond), 80°C (filled square), 90°C (filled triangle) and 100°C (filled circle) for the indicated time periods relative to that before heat treatment of wild-type aqualysin I (A), C99S mutant (B), C194S mutant (C) and WSS mutant (D) is plotted.

Table 2. **The effect of dithiothreitol (DTT) on thermostability of wild-type aqualysin I and C99S mutant.**

	Remaining activity (%)	
	Wild type	C99S
No treatment	100	100
Treatment at 90°C for 30min	76	63
Treatment at 90°C for 30 min + 100 mM DTT	34	41

The remaining activity was determined at 40°C with *N*-suc AAPFpNA as substrate after heat treatment at 90°C for 30 min.

were completely inactivated in 10 min at 100°C, while wild-type enzyme and C99S mutant retained about 40% of the original activity under the same condition. This indicates that disruption of disulphide Cys163–Cys194 more significantly affected the thermostability of aqualysin I than that of Cys67–Cys99. Under reducing conditions, the stability of wild-type enzyme and C99S mutant was decreased considerably to a level very similar to that of C194S and WSS mutants. Thus, the remaining activities after treatment of wild-type enzyme and C99S mutant at 90°C for 30 min in the presence of DTT were 34 and 41%, respectively, of the activity before treatment (Table 2), which were comparable with the activity of C194S and WSS mutants remaining after 30 min at 90°C (Fig. 3). The half-lives of wild-type enzyme and C99S mutant at 80°C were about 100 min and 110 min, respectively, while under the same conditions, those of C194S and WSS mutants were longer than 180 min. The inactivation of wild-type enzyme and C99S mutant may be due to autolysis, and the differential stability observed here likely reflects differential activity of these wild-type and mutant aqualysin I's.

DSC was carried out on wild-type aqualysin I and mutants after treatment with PMSF to prevent their autolysis. The transition temperatures for C194S (86.7°C) and WSS(86.9°C) were lower than that for the wild-type enzyme(94.0°C) and C99S (89.8°C) mutant in accordance with the results with heat-inactivation experiments, although the decrease in the transition temperature was not as pronounced as with P5N and P7I mutants of aqualysin I reported previously (*2*). These results indicate that disulphide bond Cys163–Cys194 contributes more than the other disulphide bond (Cys67–Cys99) to the thermostability of aqualysin I, but the contribution may not be very profound.

DISCUSSION

Aqualysin I is active and stable up to about 80°C (*5*, *19*) and has two disulphide bonds. It was reported that the introduction of disulphide bonds into subtilisin and other related proteases at positions where they are found in aqualysin I improved the thermostability of those proteases (*26–29*). Based on these findings it has been assumed that disulphide bonds are at least partly responsible for the thermophilicity of aqualysin I, but the mechanism by which they contribute to thermostability of the enzyme and whether these disulphide bonds equally contribute to fulfil stabilizing roles has remained unknown, for the aqualysin I mutants targeted at Cys residues were difficult to be expressed and processed correctly in *E. coli* (*5–9*).

Aqualysin I precursor consists of four regions, namely, a signal peptide, an N-terminal pro-sequence, the main-body region and a C-terminal pro-sequence. In *E. coli*, the expression of mature aqualysin I requires at least the N-terminal pro-sequence, which works as intra-molecular chaperon (*10*, *11*), to precede the main-body, and the precursor is cleaved autolytically in cell extracts through heat treatment for 1 h at 70°C to yield correctly folded mature aqualysin I protein. This system allowed the expression of wild-type aqualysin I, but turned out not to be applicable to the expression of Cys-mutants. More recently, we constructed a MBP-aqualysin I fusion protein expression system, in which MBP protein was fused to the N-terminal pro-sequence of aqualysin I (*2*). This fusion system allowed us to obtain much more efficient and stable expression in *E. coli* of aqualysin I and its mutants, including those targeted at Cys residues.

Aqualysin I Cys-mutants thus expressed were purified to homogeneity in essentially the same way as wild-type aqualysin I, except that C194S and WSS required longer heat treatment than the wild-type enzyme (Fig. 1). The latter result is consistent with the fact that C194S and WSS had lower activity than wild-type aqualysin I and C99S, since the release of N-terminal pro-sequence to yield the mature enzymes requires autocatalytic cleavage of the N-terminal pro-sequence during the heat treatment as mentioned above.

Wild-type aqualysin I and the three Cys-mutants had very similar secondary structures as judged through circular dichroism measurement (data not shown). Nevertheless, catalytic activity as well as thermostability of these mutants was quite distinct, indicating that two disulphide bonds are differently contributing to the activity and stability of the enzyme. The loss of N-terminus-proximal disulphide bond (Cys67–Cys99) due to C99S mutation did not much affect the enzyme activity as measured by both casein and synthetic *N*-sucAAPFpNA as substrates. The temperature dependence of the caseinolytic activity of C99S mutant was almost indistinguishable from that of the wild-type aqualysin I. In contrast, the loss of the other one (Cys163–Cys194) due to C194S mutation led to a marked decrease in the activity of aqualysin I with both natural and synthetic substrates. Quantitatively, the k_{cat}/K_m value of the C194S mutant was only <3% the value for the wild-type enzyme, while the C99S mutant retained 40% the k_{cat}/K_m value of the wild-type enzyme. This indicates that Cys163–Cys194 is much more important than Cys67–Cys99 for the proteolytic activity of aqualysin I. The differential effect of mutation on the catalytic efficiency is mainly due to the change in k_{cat} values, suggesting that the loss of disulphide bonds did not profoundly affect the enzyme–substrate interactions (Table 1). This is different from the situation with thrombin where destruction of a disulphide bond induced relocation of a side chain as well as back bone groups in primary specificity pocket, leading to an increase in the K_m value (*34*). The catalytic properties of C99S/C194S double mutant as revealed by kinetic analysis with

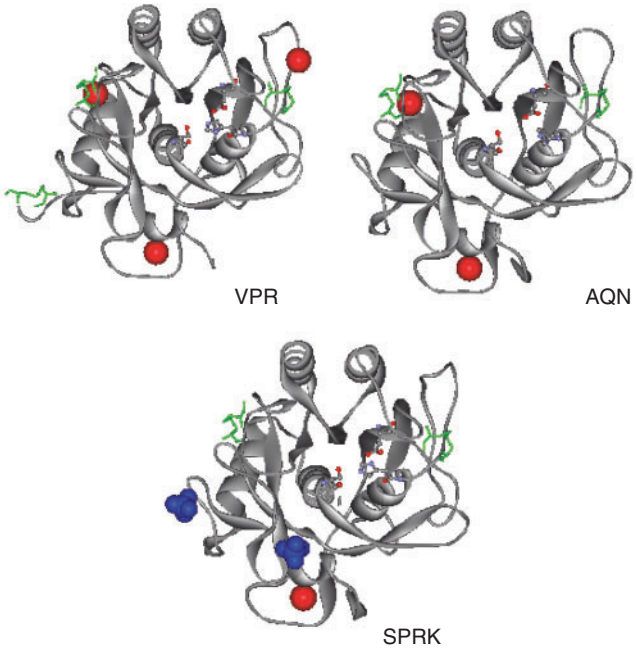

Fig. 4. **Structure of VPR (1SH7), SPRK (2B6N) and AQN.** The position of disulphide bonds and amino acid residues constituting the active site (Asp, His and Ser) are shown with stick model in green, and with ball-and-stick model in elemental colours, respectively. The calcium ions (red) and sulphate ions (blue) are represented with CPK model.

N-sucAAPFpNA as a substrate were consistent with independent contribution of the two disulphide bonds in aqualysin I to the catalytic properties of the enzyme, the contribution of Cys163–Cys194 being much greater.

Crystal structure analysis of VPR has been published (*22*, Fig. 4), and is very similar to that of aqualysin I (Ohta, T., personal communication). The C-terminus-proximal disulphide bond (Cys163–Cys194) is found in a region close to the calcium-binding site and substrate-binding pocket S1 in VPR. On the other hand, the N-terminus-proximal disulphide bond (Cys67–Cys99) is located closer to the active site composed of Asp39, His70 and Ser222. It is not clear at present how the destruction of the former disulphide bond not quite close to the active site profoundly affected the conformation of the catalytic site leading to an extensive loss of catalytic activity. It could be that the disruption of disulphide bond in the neighbour of Asn157, which mediated stabilization of oxyanion hole in the transition-state in subtilases (*35–37*), might somehow distort the spatial arrangement of amino acid residues involved in transition-state stabilization, or that hydroxy group of serine residue replaced for Cys194 might interact with other residue(s) to adversely affect the catalytic activity. It may be pertinent to note here that wild-type enzyme and C67S mutant were inactivated at 90°C under reducing condition in a time course similar to that of C194S mutant (Table 2), but were not immediately inactivated drastically as would be expected if the presence of disulphide itself is indispensable for catalytic activity. This may seem to be in favour of the possibility that the substitution of Ser194 for Cys194 may be the major cause for the inactivation due to C194S mutation. Significance of Cys194 in the form of disulphide in the maintenance of the active structure of aqualysin I remains to be elucidated. Differential catalytic activity among wild-type and mutant enzymes was not due to the differential affinity for calcium ions. This was confirmed by the fact that calcium ion-dependence of activity on *N*-sucAAPFpNA measured in a range of 0–10 mM after removal of tightly bound calcium ion by dialysis against 20 mM MES-NaOH containing 10 mM EDTA (ethylene diamine tetraacetic acid) at pH 6.0, was indistinguishable among the wild-type enzyme and three mutants (data not shown).

The two disulphide bonds Cys67–Cys99 and Cys163–Cys194 differently contribute also to the thermostability of aqualysin I. Thus at higher temperature, at 90°C and 100°C, the residual activity of C194S and WSS mutants decreased much rapidly than the wild-type enzyme and C99S mutant, indicating that the active conformation of C194S and WSS mutants is more unstable against heat treatment than the wild-type enzyme. The heat stability of C99S mutant was similar to that of the wild-type enzyme at 100°C, although the former was inactivated a little faster than the latter at 90°C. These results indicate that disulphide bond Cys163–Cys194 is more important than Cys67–Cys99 in maintaining enzymatically active conformation of aqualysin I at elevated temperatures. This is consistent with the results that the transition temperature for C194S mutant in DSC measurement (86.7°C) was definitely lower than that for C99S mutant (89.8°C). The transition temperature for C99S mutant was lower than that for the wild-type enzyme by 4.2°C. This is also consistent with the report by Takagi *et al*. (*26*) showing that introduction of Cys61–Cys98 disulphide bond, which is homologous to Cys67–Cys99 of aqualysin I, into subtilisin E led to a 4.5°C increase in the transition temperature. The residual activity of both wild-type aqualysin I and C99S decreased significantly more rapidly than C194S and WSS mutants at 70°C and 80°C. This may be a result of faster autolysis of these enzymes due to their markedly higher catalytic activity as compared to the other two mutants.

Although Cys163–Cys194 definitely contributes to the thermostability of aqualysin I in accordance with previous observations on several related enzymes, the stabilizing effect exerted by this disulphide bond was not as large as that contributed by Pro7 or even Pro5 that was analysed in detail in our previous report (*2*). The time course of heat inactivation of Cys163–Cys194 disulphide bond-deficient mutants at 90°C and 100°C were very similar to that of P268T, which was only a little less heat-stable than wild-type aqualysin I and had transition temperature of 86.5°C in DSC analysis that is only a little lower as compared to 94.0°C of the wild-type enzyme (*2*). These results are compatible with the present results of DSC analysis in which C194S and WSS exhibited transition temperatures, 86.7°C and 86.9°C, respectively, that is almost the same with that of P268T. Heat stability of proteins are determined by cumulative stabilizing as well as destabilizing effects resulting from various interactions and structural factors including weak bonds between amino acid residues,

solvent accessibility and occurrence of residues that tend to make protein conformation thermolabile (*1*, *2*, *21*, *23*, *38–41*). Disulphide bonds are among such stabilizing interactions (*26–29*), but their number and localization among related proteases in subtilase family do not seem explicitly correlated with their thermostability (*22*, *24*, *41*). This may imply that disulphide bonds do not play a major role in conferring thermostability of these enzymes. Our present results indicating that only Cys163–Cys194 fulfilled rather minor stabilizing function in aqualysin I and that the other disulphide bond did not contribute the thermostability are consistent with this notion. In the case of aqualysin I, proline residues in the N-terminal loop structure contributed much more greatly to the thermostability of the enzyme, as we showed in our previous work.

In conclusion, the disulphide bond Cys163–Cys194 somehow fulfils an important role in the construction of functional catalytic site of aqualysin I, but only moderately contributes to the conformational stability of the enzyme at elevated temperatures. The other disulphide bond Cys67–Cys99 neither did contribute much to the construction of active conformation nor to its maintenance at higher temperatures.

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