Note

Application of a Metal Switch to AQUALYSIN I, a Subtilisin-type Bacterial Serine Protease, to the S3 Site Residues, Ser102 and Gly131

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We applied ‘metal switch’ experiments to the S3 site residues, Ser102 and Gly131, of aqualysin I, a subtilisin-type serine protease. We showed that two histidines introduced at these positions did take part in histidine-metal-histidine bridge formation, and metal ions inhibited the protease activities. These results indicate that two histidines are near each other, and both side chains are metal-accessible. This is the first report on application of the metal-switch technique to a subtilisin-related enzyme.

Key words: aqualysin I; subtilisin; Thermus aquaticus YT-1; metal-switch; S3 site

Since the appearance of site-directed mutagenesis techniques, many attempts have been made to alter the profiles of enzymes using a microbial protease, subtilisin BPN’, a well-known group of enzymes which have been often used as model enzymes. Aqualysin I, one of the subtilisin-related bacterial proteases, originated from a thermophilic bacterium, Thermus aquaticus YT-1, is a cysteine-containing alkaline serine protease, like fungal proteinase K.1) The kinetical properties as well as maturation mechanism of aqualysin I are well characterized.2-13) Our results and recent reports indicated that the tertiary structures of these subtilisin enzymes resemble each other.14) These proteins contain subsites, among them the S1, S2, S3, and S4 sites, that are important in substrate recognition. Studies on subtilisins showed that the S1, S2, and S4 sites form pockets on the substrate binding site.15-22) On the other hand, crystallographic analyses showed that the S3 site is on the edge of the substrate binding site, not forming a pocket.23-25) We applied the designing strategy to the S3 site of the enzyme, which does not form any pocket (see illustrations in Fig. 1). The substrate-accepting cleft is mainly formed by two parallel strands, in anti-parallel orientation to the substrate strand. No bulky side chains are on either substrate accepting strand (Gly101-Gly103 and Ser128-Gly131), especially on the enzyme surface side. From the computational simulation and the experimental results, we

Fig. 1. Schematic Representation of a Metal Switch.

The substrate binding strands Gly101-Gly103 and Ser128-Gly131 are indicated with the P3 side chain of the substrate (above). Scissors represent for the active site serine residue. The introduced two histidines at the position of Ser102 and Gly131 are illustrated with metal ion (below). If the introduced two histidine residues can act as a metal switch acceptor, the metal ion should bind to the place between the histidines, closing the entrance of the substrate binding site and inhibiting the enzymatic activity.

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Abbreviations: HEPES, N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Suc-, Succinyl- (3-Carboxypropyl-); -pNA, p-nitroanilide
found that the S3 site is formed by two residues, Ser102 and Gly131. The computational simulation also suggested that these residues on the S3 site should be on the edge of the substrate accepting strands, and their side chains should be exposed toward the solvent, being near each other.

In this paper, we applied a 'metal-switch' to the S3 site residues of aqualysin I, replacing Ser102 and Gly131 by histidine residues (see Fig. 1). If both introduced side chains of these residues are near as we had expected, the activity of the mutant enzyme should be switched by the addition of a metal ion by making a histidine-metal-histidine bridge as in the cases of trypsin and chymotrypsin.

The wild-type enzyme was prepared from the culture medium of *Thermus aquaticus* YT-1 as described previously. The mutant enzyme (Ser102/ Gly131) was prepared from *E. coli* according to the method previously described (see the legend of Fig. 2).

![Fig. 2. Metal Inhibition Assays of Enzyme Activities.](image)

(A) Residual activities of wild-type enzyme (open bars) and the mutant enzyme (closed bars). The enzyme solution (40 μL; containing about 50 nm enzyme) was mixed with the metal or EDTA solution (40 μL), and the mixture was incubated at room temperature for 15 minutes. The hydrolysis of the substrate was chased at 410 nm, 40°C after the addition of the substrate solution (200 μL; about 2 mm). Chromogenic tetrapeptide suc-Ala-Ala-Pro-Phe-pNA was used as substrate. 'Control' represents the enzyme activities without the metal ion nor EDTA solution, which is defined as 100% (corresponding to about 0.3 and 0.2 μM/min for wild-type and the mutant enzymes, respectively). The final concentration of each metal ion was as follows: Cu^{2+}, 0.71 mM; Zn^{2+}, 0.71 mM; Ni^{2+}, 0.71 mM; Ca^{2+}, 7.1 mM; and Mg^{2+}, 7.1 mM. The final concentration of EDTA was 7.1 mM. Experimental errors were less than 20%.

(B) Restoration experiments of inhibited activity of the mutant enzyme by metal ion. Open bars represent the inhibited activities by the metal ion, and hatched bars represent the restored activities of the mutant enzyme by the addition of EDTA solution. Divalent cation solution (5 mM; 100 μL) and the enzyme solution (50 mM; 100 μL) were mixed and were incubated for 15 minutes at room temperature. After incubation, 30 μL of the mixed solution was fractionated and was mixed with 20 μL of EDTA (100 mM) or Milli-Q water, then another incubation was done for 15 min at room temperature. Then, 200 μL of substrate solution was added to the mixture, and the proteolytic activity was monitored at 410 nm. Reactions were done at 40°C. 'Control' represents the enzyme activities without the metal ion. The final concentration of each metal ion is 0.67 mM. Experimental errors were less than 20%.

As is often reported in the cases of metal-switch experiments of trypsin and chymotrypsin, there is some metal dependency: the histidine-metal-histidine bridge formation requires such metal ions as copper, zinc, or nickel. Moreover, if two introduced histidines are near enough to form such a bridge, the enzyme activity of the mutant enzyme should be regulated by addition of the metal ions. We did three kinds of metal-switching assays. The tetrapeptide chromogenic substrate suc-Ala-Ala-Pro-Phe-pNA was used as the substrate for the reason that this substrate has been used as a standard substrate for aqualysin I and is efficiently hydrolyzed by this enzyme.

At first, we examined whether the mutant enzyme could be inhibited by the metal ions or not. The results are summarized in Fig. 2A. Assays were done under the standard conditions for aqualysin I. The results show the inhibition of the mutant enzyme by three divalent ions, copper, zinc, and nickel: especial-
Fig. 3. Metal-Switch Experiment by Manipulating the Zinc Ion Concentration.

The chromogenic tetrapeptide Suc-Ala-Ala-Pro-Phe-pNA was used as substrate. Assays were done at 40°C, pH 7.5 (50 mM HEPES, 0.5 mM CaCl₂). Reactions were started by the addition of 20 μl of enzyme solution (30 nM) to 200 μl of substrate solution (1 mM), and the proteolytic activity was chased at 410 nm. After 110 s, the concentration of zinc ion was adjusted to 0.42 mM by adding 20 μl of 5 mM ZnCl₂ in HEPES buffer. The assay was then continued. After 220 s, the activity was restored by adding 20 μl of 10 mM EDTA to the mixture. The activity of mutant aqualysin I was again inhibited by the addition of 60 μl of zinc ion, and after the activity was monitored for a short period of time, the inhibition was again relieved by chelation with 60 μl of 10 mM EDTA.

ly strong inhibition by copper and zinc ions. The activity of the wild-type enzyme was slightly affected by copper ion; maybe for the reason that this metal can also bind to the calcium binding site or other sites of aqualysin I as to slightly reduce the enzyme activity.³,⁴ Calcium and magnesium ions, and EDTA, had little or no effect to the activity of both wild-type and mutant enzymes. These results strongly suggest that the introduced histidines can form the histidine-metal-histidine bridge.

For the next step, we examined the ability of restoration, by the addition of chelator EDTA, of the inhibited activity of the mutant enzyme by the metal ions. We tested three metal ions, copper, zinc, and nickel, which inhibited the mutant enzyme. The results are summarized in Fig. 2B. The proteolytic activity of the mutant enzyme was also reduced by these metal ions. The tendency of inhibition by copper, zinc, and nickel ions shown in Fig. 2A and Fig. 2B was almost the same. The slight differences maybe come from the assay conditions: differences of the final concentration of metal ions, differences of the incubation time. As a whole the results show that the activity of the mutant enzyme was inhibited by the addition of metal ions, and the inhibited activities can be easily restored by the addition of EDTA.

Additionally, we did another metal-switch experiment as described by Higaki et al.²⁰ The enzyme activity was manipulated by the zinc ion concentration (see Fig. 3). The enzyme activity was switched by the addition of metal and chelator. From these experimental data, we tried to evaluate the inhibition constants of the metal ions toward the mutant enzyme: the values were around 0.1 mM for copper and zinc ions. The details need further investigation.

Our data show that by introducing two histidine residues, both at Ser102 and Gly131, the catalytic activities of aqualysin I was inhibited by metal ions. These data indicate that the activity of aqualysin I was regulated by application of the ‘metal switch’ technique. The results also suggested that no histidine residue at Ser102 and Gly131 are near, and the side chains of which are at the position close enough to form the metal bridge as shown in this paper. As far as it is concerned with designing the substrate specificity of aqualysin I, the introduction of this metal-switch is not so important, because the introduced metal switch could not achieve complete inhibition of enzyme activity: the inhibition constants of the metal ions are around 0.1 mM, not as strong inhibition as report by Higaki et al.²⁰ The important feature of this study is that we could have applied the metal switch technique to the substrate binding site of the enzyme.

Since the appearance of the metal chelation technique by histidine residues, many attempts have been done to apply this technique to the enzymes. The most famous examples are found with trypsin-type serine proteases.²⁶-²⁹ They applied the histidine-metal-histidine bridge formation to the active site of the enzyme, including the histidine within the catalytic triad. Their ideas are good, of course, but lack the possibilities of applications to other enzymes, because not very many enzymes include histidine residues in the active site. Aqualysin I belongs to the subtilisin-type serine proteases, a different type of proteases. Although this enzyme has a histidine residue in the catalytic triad, the computer simulation also showed that it is impossible to apply the same strategy as to a trypsin-type enzyme, mostly because the tertiary structure of subtilisin enzymes differ from those of trypsin enzymes. So, this is the first report on application of the metal-switch technique not to the active site but to the substrate binding site, and moreover the first application to subtilisin-related enzymes.

References

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Application of Metal Switch to Aqualysin I


