A Bound Water Molecule Is Crucial in Initiating Autocatalytic Precursor Activation in an N-terminal Hydrolase*

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Cephalosporin acylase (CA) is a member of the N-terminal hydrolase family, which is activated from an inactive precursor by autoproteolytic processing to generate a new N-terminal nucleophile Ser or Thr. The gene structure of the precursor cephalosporin acylases generally consists of a signal peptide that is followed by an α-subunit, a spacer sequence, and a β-subunit. The cephalosporin acylase precursor is post-translationally modified into an active heterodimeric enzyme with α- and β-subunits, first by intramolecular cleavage and, second, by intermolecular cleavage. Intramolecular autocatalytic proteolysis is initiated by nucleophilic attack of the residue Ser-1β onto the adjacent scissile carbonyl carbon. This study determined the precursor structure after disabling the intramolecular cleavage. This study also provides experimental evidence showing that a conserved water molecule plays an important role in assisting the polarization of the OG atom of Ser-1β to generate a strong nucleophile and to direct the OG atom of the Ser-1β to a target carbonyl carbon. Intramolecular proteolysis is disabled as a result of a mutation of the residue Ser-1β. The gene structure of the open reading frame of CAs generally consists of a signal peptide that is followed by an α-subunit, a spacer sequence, and a β-subunit, all of which are translated into a single polypeptide chain, the CA precursor. The precursor is post-translationally modified into an active heterodimeric enzyme with α and β subunits, first by intramolecular cleavage process and, second, by intermolecular cleavage process.

Ntn hydrolases are generally translated into a precursor enzyme. Several Ntn hydrolases are activated through autoproteolytic processing by the Ser, Thr, or Cys residues. Autoproteolytic processing occurs in an intramolecular manner in several Ntn hydrolases. These include glutamine 5-phospho-ribose-1-pyrophosphate amidotransferase, penicillin G acylase, proteasome β subunit (PS), glycosyl asparaginase (GA), and cephalosporin acylase (2–4).

The gene structure of the open reading frame of CAs generally consists of a signal peptide that is followed by an α-subunit, a spacer sequence, and a β-subunit, all of which are translated into a single polypeptide chain, the CA precursor. The precursor is post-translationally modified into an active heterodimeric enzyme with α and β subunits, first by intramolecular cleavage process and, second, by intermolecular cleavage process.

The heterodimeric enzyme is envisaged from the structural studies of the active CA and precursor CA, in which the nascent polypeptide (precursor) of cephalosporin acylase is autoproteolytically activated through a two-step autocatalytic process upon folding (3, 5). The first step is an intramolecular cleavage process by the N-terminal Ser at the beginning of the β-subunit. This results in an α-subunit, a spacer peptide that is attached to the C terminus of the α-subunit, and a β-subunit. The second step is an intermolecular event, which is mediated by the newly generated N-terminal Ser or Thr of the β-subunit by means of the intramolecular cleavage. The second event results in further cleavage at the second scissile bond and finally releases the spacer peptide (5, 6).

The study for the autoproteolytic mechanism of intermolecular cleavage was carried out in several precursor structures of N-terminal hydrolase. The structures of the GA and PS precursors were determined, and the autoproteolytic mechanisms for intramolecular cleavage by the Thr residue were proposed (4, 7). Interestingly, two Ntn hydrolases (GA and PS) proceed to autoproteolysis along different paths even if the same amino acid (Thr) was used as a key nucleophile for autoproteolysis. This means that a water molecule enhances the nucleophilicity of the Thr Oy at the +1 position in PS, although the Asp-151 carboxylate at the −1 position promotes the nucleophilicity of the Thr-152 at the +1 position in GA. The intramolecular proteolysis proceeds to a N → O or N → S acyl shift (8–10) and leads to an ester or thioester that is subsequently hydrolyzed to carbonyl and amino groups. The resulting N-terminal Ser, Thr, or Cys residue becomes exposed to the solvent to participate in the nucleophilic catalytic center for the Ntn hydrolases (2, 7, 11).
The structures of CA from *Pseudomonas diminuta* KAC-1 (CAD) and a precursor CAD were determined (12, 13). Also, the mechanism of the intramolecular cleavage of the scissile peptide bond at the N-terminal side of the catalytic Ser-1β was proposed. Nucleophilic attack on the carbonyl carbon at the N-terminal side by the OG atom of Ser-1β results in ester formation between the carbonyl carbon of Gly-169s (amino acid immediately before the nucleophilic Ser-1β) and the OG atom of Ser-1β. Initially, the hydroxyl of Ser-1β is assisted by the conserved water, which is stabilized by four hydrogen bonds in a pseudo-tetrahedral geometry, and may accept a proton from the hydroxyl group. As a result nucleophilic attack takes place on the main chain carbonyl carbon of the Gly-169s. The detailed pattern of autoproteolysis in the precursor CA is somewhat different from the previously determined precursor structures of the Ntn hydrolase families such as GA (7) and PS (4) in terms of both the type of the nucleophilic residue and the reaction pattern.

A previous structural study of the precursor, CAD, revealed that conserved water (present in both the precursor CAD and the mature CAD) plays an important role in assisting the OG atom of Ser-1β so that the nucleophilic OG atom can carry out an attack on the scissile carbonyl carbon of Gly-169s (13). The study proposed that the bound water assists the autocatalytic proteolysis of intramolecular cleavage. However, the role of bound water has not been fully examined due to insufficient experimental data.

This study examined the role of bound water. A mutant CA that had lost the capability of autocatalytic proteolysis and remained a precursor enzyme was obtained. The structure of the mutant CA was determined at a 2.5-Å resolution. The precursor structure that was disabled in the autocatalytic proteolysis lost the bound water from the active site. A comparison of the active sites between the two different precursors with and without the autocatalytic proteolysis provided data that showed that the absence of bound water abolished the capability of carrying out the autoproteolytic intramolecular cleavage from the precursor CA. Also, the disability of the intramolecular proteolysis was the result of a mutation of the residues, causing a conformational distortion to the active site. This study provides the first evidence that a bound water molecule can play a critical role in initiating the beginning of intramolecular cleavage in the post-translational modification of the precursor enzyme.

**EXPERIMENTAL PROCEDURES**

**Crystallization**—The mutant CAD of the F177β residue to Pro (F177βP), with no capability of autocatalytic proteolysis (14) from *P. diminuta* KAC-1 (GenBank accession number AF251710; Ref. 16), was prepared by site-directed mutagenesis and subcloned into Escherichia coli BL21(DE3) using the overexpression vector pET24d(+). The protein was then purified using His-tag affinity chromatography. The precursor CAD concentration was 10 mg ml\(^{-1}\). The protein was then purified using His-tag affinity chromatography. The precursor CAD concentration was 10 mg ml\(^{-1}\) in a storage buffer (50 mM sodium phosphate, pH 7.0, and 150 mM NaCl). The F177βP precursor CAD crystals grew overnight at 21°C from hanging drops containing 3 µl of a protein solution (10 mg ml\(^{-1}\) precursor CAD, 50 mM sodium phosphate, pH 7.0, and 150 mM NaCl) and 3 µl of a reservoir solution (16% (w/v) polyethylene glycol 8000, 10 mM dithiothreitol, 200 mM magnesium acetate, and 100 mM sodium cacodylate, pH 7.0) for 3 days before being flash-cooled in a 100 K gaseous nitrogen stream.

**Data Collection**—The data set for the F177βP precursor CAD crystal was collected to a resolution of 2.5 Å at the Pohang Accelerator Laboratory 6MX beamline from frozen crystals using a wavelength of 0.91005 Å. The data were indexed and integrated using DENZO and scaled by SCALEPACK (17).

**Refinement**—The structure of the F177βP precursor CAD yielded an excellent initial crystallographic model that is based on the structure of the S1βA precursor CAD (15). Therefore, only minor adjustments were needed during the course of refinement. *F*\(_c\) – *F*\(_d\) difference Fourier maps provided an excellent guide for locating the residues (Fig. 1). The modeled residues were refined using the program O (18) based on the structure of the S1βA precursor CAD. All of the crystallographic refinements were carried out using the CNS program (19) with a maximum-likelihood refinement. The model geometry was confirmed by PROCHECK (20). The structure of the F177βP precursor CAD is quite similar to that of the S1βA precursor CAD (13) except for the mutation sites, the deviations in the Cα of the main chain backbone, and the residue conformation of the active site. Table I shows the data and refinement statistics. The figures were generated by MOLSCRIPT (21), BOBSCRIPT (22), and RASTER3D (23).

**RESULTS AND DISCUSSION**

**Site-directed Mutagenesis of CAD Yields a Different Status of Intramolecular and Intermolecular Cleavage**—The CAD precursor of the wild-type spontaneously conducts intramolecular cleavage by autocatalytic proteolysis at the scissile peptide bond between the Gly-169s and Ser-1β residues upon folding after protein synthesis. Subsequently, the processed form of CAD performs intermolecular cleavage at the peptide bond between the Gly-158s and Glu-159s residues (3, 13, 14). Many mutants of the active site residues were examined to determine whether they are properly post-translated to an active heterodimeric form of CAD. Some of the active site mutants did not undergo proper intramolecular or intermolecular cleavage (Fig. 2, a and b).

In particular the mutation of Ser-1β to Ala (S1βA) completely lost its intramolecular autoproteolytic activity (24) because the OG atom from the side chain of Ser-1β plays an important role in nucleophilic attack at the scissile peptide bond. The 77-kDa size of the S1βA protein, which contains the α-subunit, spacer, and β-subunit, appears as a single band that corresponds to the non-processed CAD (Fig. 2a). The S1βA precursor CAD could represent a wild-type precursor CAD with

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(1) The notation of the amino acid sequence in precursor CAD is identical to the CAD up to residue 158s (12). The α and β subunits are indicated by attaching α and β, respectively, to the end of the residue number. In addition, the spacer residue is represented by an “s” at the end of a residue number, such as 158s. The spacer sequence of the precursor CAD is from residue 159s to 169s in this structure (13, 14). The mutation of Phe-177 to Pro is labeled as F177βP. The same notations are used for the other mutants accordingly.
autocatalytic intramolecular cleavage (13) once the mutated Ala residue at the β1 position is modeled to Ser. This is because the overall conformation of the precursor S1βA CAD structure is similar to the mature CAD structure. In fact, a r.m.s. deviation of 0.18 Å is obtained after superimposing the 672 common Ca atoms to a wild-type mature CAD (13). Furthermore, the Cos of the nine active site residues of the mature CAD, which directly interacted with the substrate, glutaryl-7-aminocephalosporanic acid, are superimposed onto the corresponding Ca atoms of the S1βA precursor CAD with a r.m.s. deviation of 0.22 Å (13).

One of the most interesting mutations is F177P to Pro (F177βP). The intramolecular cleavage is interrupted even though it possesses a catalytic nucleophile for intramolecular cleavage, Ser-1β (13). The F177βP mutant is believed to be a conformational mutant; therefore, it cannot undergo nucleophilic attack at the scissile peptide for intramolecular cleavage.

Other mutants show various stages of intramolecular and intermolecular cleavage (Fig. 2, a and b). The Ser-1β to Cys-1β (S1βC) mutation undergoes intramolecular cleavage, but its intermolecular cleavage is interrupted (Fig. 2a). The Y33βF processes are normal to the mature heterodimeric CAD with the right-sized α and β subunits. Y33βS completes the intramolecular cleavage, but the intermolecular cleavage is interrupted. Y33βI accomplishes the intramolecular cleavage in part so that it produces mixed bands of sα and a (Fig. 2a). Four of the five Ser-152α mutants can normally undergo intramolecular and intermolecular cleavage to produce mature CADs, but only the intermolecular cleavage of the S152αP mutant is interrupted (Fig. 2b). Only the L24βP mutant of the three L24β mutations proceeds to a mature CAD, whereas, L24βR can undergo partial intermolecular cleavage, and the L24βI mutant cannot undergo intermolecular cleavage (Fig. 2b). It appears that the S152αP, L24βF, and L24βR mutants contain a trace amount of the precursor forms of the 77-kDa size, as shown in the SDS-PAGE (Fig. 2b). They all contain bulky side chains, but further investigation may be required to determine the relationship with the intermolecular cleavage.

The F177βP mutant remains a non-processed precursor (the F177βP precursor CAD); therefore, its structure is very interesting for understanding the intramolecular cleavage from a mechanistic point of view. The conformational or functional moiety in the F177βP mutant may be damaged due to its mutation so that it cannot accomplish the catalytic action of intramolecular cleavage. For that reason, the structure of the precursor F177βP CAD was determined at a 2.5-Å resolution, and the structure was compared with that of the structure of the S1βA precursor CAD.

Structure Determination of F177βP Precursor CAD—The structure of the F177βP precursor CAD was determined at a 2.5-Å resolution (Table I). In the course of the structure refinement, the $F_{o} - F_{c}$ difference Fourier map, with neighboring residues around P177β and Ser-1β that were omitted from the phase calculation (Fig. 1, see also Fig. 5, a and b), showed a clear positive density for these sequence regions before adding any information of backbone residues in the unbiased map. The residues were easily adjusted into the $F_{o} - F_{c}$ difference maps using the O program (18). They were then refined using CNS (19) to an $R_{cryst}$ of 22.3% and an $R_{free}$ of 26.7% (Table I). The
S1βA precursor CAD was determined previously at a 2.5-Å resolution. It was refined to good geometry (R_{cryst} of 20.3%, R_{free} of 23.7%) (13). The space group of the F177BP crystal was P4_12_2, which is the same as the S1βA precursor CAD. Therefore, the structure determination was straightforward and guided by the S1βA precursor CAD structure. The overall conformation of the F177BP structure was refined using the CNS program (19). The neighboring regions of F177BP and Ser-1β were manually corrected based on the F_{obs} − F_{calc} difference maps using the O program (18).

The overall conformation of the F177BP precursor CAD was quite similar to the S1βA precursor CAD. However, the Cos of the F177BP precursor CAD was not superimposed onto those of the S1βA precursor CAD as much as the S1βA precursor CAD was superimposed onto those of the mature CAD (Fig. 3). The r.m.s. deviation for the 62 Cos of the active site pockets between the F177BP precursor CAD and the S1βA precursor CAD was 0.573 Å, whereas the r.m.s. deviation of the 51 Cos between the S1βA precursor CAD and the mature CAD was 0.125 Å (11 residues of the spacer peptide were not used for the superposition) (Fig. 3). The superposition among the three CADs (mature CAD, S1βA, and F177BP) showed that the S1βA precursor CAD was similar to the mature CAD. However, the F177BP precursor CAD was considerably deviated from both the mature CAD and the precursor S1βA structures.

This comparison suggests that the active site of the F177BP precursor CAD was significantly distorted from that of the wild-type CAD. It is clear that the mutation of F177β to Pro imposes much strain on the main chain backbone of F177BP. Therefore, it induces significant conformational changes in the active site. This conformational distortion is leveraged into a non-processed precursor CAD for intramolecular cleavage, resulting in the F177BP precursor CAD. It will be interesting to investigate how one amino acid substitution is linked to abolishing the intramolecular cleavage in the post-translational modification. Conceivably, if these conformational mutations occur in nature, then they will have an influence upon the selection procedure in the post-translational modification.

**Model of Autocatalytic Proteolysis in Intramolecular Cleavage**—The structures of the S1βA precursor CAD and the mature CAD led us to previously propose the model of the autocatalytic intramolecular cleavage (Fig. 4, a and b) (13). A nucleophilic attack on the carbonyl carbon of Gly-169s by the OG atom of Ser-1β resulted in an ester formation between the carbonyl carbon of Gly-169s and the OG atom of Ser-1β. Previously, the hydroxyl of Ser-1β was assisted by the conserved water (WAT1 in Figs. 4, a and b, and 5a) and by the four hydrogen bonds in pseudo-tetrahedral geometry. WAT1 may enable the hydroxyl of Ser-1β to be precisely positioned and assist the polarization of the hydroxyl for nucleophilic attack. A tetrahedral intermediate can form after the OG atom of Ser-1β carries out the nucleophilic attack on the main chain carbonyl carbon of Gly-169s. Subsequently, the resulting oxyanion may move toward WAT2 (Figs. 4b and 5a) to be stabilized by the hydrogen bonds from the plausible oxyanion hole, which is not formed in the nucleophilic attack stage, but it may form in the transition state, consisting of WAT2 (Fig. 5a) and the main chain NH of His-23β. The oxyanion intermediate may then collapse, resulting in a shift in the linkage of the amide bond to an ester bond (N → O acyl shift) (3, 25). Finally, the conserved water, WAT1, may carry out a second nucleophilic attack on the carbonyl carbon of the newly formed ester intermediate, which would result in a free N-terminal Ser-1β and a carboxylate of Gly-169s (13).

In the proposed model the conserved water, WAT1, played a key role in assisting the critical nucleophilic residue of Ser-1β (13). The hydroxyl of Ser-1β was indispensable in the autocatalytic intramolecular cleavage. The loss of the hydroxyl group of Ser-1β resulted in a disturbance in the intramolecular cleavage in the S1βA precursor CAD (13). The conserved waters, WAT1 and WAT2, were also presumed to play major roles in the autocatalytic proteolysis (Fig. 4, a and b, and Fig. 5a). However, the exact roles of the two conserved waters could not be determined, as suggested in the proposed model (13), due to the lack of experimental evidence.

**TABLE I** Summary of the crystallographic data

| Parameter                  | Value     |
|----------------------------|-----------|
| Space group                | P4_12_2   |
| Unit cell dimensions (Å)   | a = b = 73.7, c = 383.7 |
| Wavelength (Å)             | 0.91005   |
| Resolution range (Å)       | 20–2.5 (2.59–2.50) |
| Completeness (%)           | 95.7 (99.9) |
| Reflections, total         | 242735    |
| Reflections, unique        | 37824     |
| R_{sym} (%)                | 10.2 (47.6) |
| I/σ                        | 8.2 (5.8) |
| X-ray source               | PAL 6MX   |
| Refinement statistics      |           |
| Resolution (Å)             | 20–2.5    |
| R_{cryst} (%)              | 22.3      |
| R_{free} (%)               | 26.7      |
| No. of non-H atoms         | 5374      |
| Protein                    | 225       |
| Water                      | 225       |
| r.m.s.d. (Å)               | 0.0072    |
| Angles (°)                 | 1.35      |
| Average B-factors          | 37.6      |

a Values within the parentheses are for the last shell of data.

b R_{cryst} = \frac{2|I|−|I|}{|I|}

c R_{free} = \frac{2|I_{obs}|−|I_{calc}|}{|I_{obs}|}

d Bonds (Å)

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**Fig. 3.** Superposition of active site Cos among precursor CADs and wild-type CAD. The 62 Cos atoms that are located in close proximity to the active site clefts are used to superimpose the S1βA and F177BP precursor CADs. The 51 Cos atoms for the wild-type mature CAD are used (11 residues of the spacer peptide are omitted from the mature CAD). The side chains of the mutated residues, F177β and S1βA, are shown in grey. The Cu traces of the F177BP precursor, the S1βA precursor, and the wild-type CAD are colored in green, yellow, and red, respectively.

The two water molecules are bound to the active site and may enable the hydroxyl of Ser-1β to be precisely positioned and assist the polarization of the hydroxyl for nucleophilic attack. A tetrahedral intermediate can form after the OG atom of Ser-1β carries out the nucleophilic attack on the main chain carbonyl carbon of Gly-169s. Subsequently, the resulting oxyanion may move toward WAT2 (Figs. 4b and 5a) to be stabilized by the hydrogen bonds from the plausible oxyanion hole, which is not formed in the nucleophilic attack stage, but it may form in the transition state, consisting of WAT2 (Fig. 5a) and the main chain NH of His-23β. The oxyanion intermediate may then collapse, resulting in a shift in the linkage of the amide bond to an ester bond (N → O acyl shift) (3, 25). Finally, the conserved water, WAT1, may carry out a second nucleophilic attack on the carbonyl carbon of the newly formed ester intermediate, which would result in a free N-terminal Ser-1β and a carboxylate of Gly-169s (13).
Autocatalytic Proteolysis of Cephalosporin Acylase

345

Fig. 4. A proposed model of intramolecular cleavage in CAD (13). a, the conserved water, WAT1, of the S1βA precursor CAD. The OG atom of Ser-1β in the S1βA precursor CAD was modeled after the torsional geometry of the OG atom of the Ser-1β residue of the wild-type CAD structure (13). The modeled residue is represented as S1βA in the figure. In the model the conserved WAT1 is hydrogen-bonded to two proton acceptors and two donors in pseudo-tetrahedral geometry, which are also involved in the chemical catalysis of CAD (26). The residues of the S1βA precursor CAD are shown in yellow in the ball and stick model. The dotted lines represent the hydrogen bonds ranging from 2.8 to 2.9 Å, a, a proposed mechanism of intramolecular cleavage for CAD (13). The arrows represent nucleophilic attack. The boxed residues represent each amino acid. WAT1 and WAT2 represent the conserved waters. This 90° rotation of the hydroxyl to the correct place (Fig. 5, a and b). The numbers near the lines represent the distances between the two atoms. The dotted lines represent hydrogen bonding. Ser-1β is the key nucleophilic center for intramolecular cleavage. The first nucleophilic attack takes on the scissile carbonyl carbon of Gly-169β. The OG atom of Ser-1β is assisted by WAT1, which forms four hydrogen bonds in a pseudo-tetrahedral geometry. The WAT2 is 3.9 Å away from the carbonyl oxygen of Gly-169β before the nucleophilic attack occurs. The carbonyl oxygen of Gly-169β is hydrogen-bonded to the main chain NH of the His-23β residue. The WAT2 is also hydrogen-bonded to the side chain of the His-23β residue (13, 14, 26).

$F_o - F_c$ difference Fourier map was calculated for the F177βB precursor CAD (Fig. 5b) under exactly the same conditions that are shown in Fig. 5a. Surprisingly, the $F_o - F_c$ difference map of the F177βB precursor CAD had no electron density where the peak density of WAT1 was placed in the S1βA precursor CAD (Fig. 5b). Indeed, a mutation of F177βB is the key nucleophilic center for intramolecular cleavage. The hydroxyl position of the nucleophile, Ser-1β, was not visible in the S1βA precursor CAD (Fig. 5a) because Ser-1β had mutated into Ala (13). The hydroxyl was modeled into the Ala1β residue of the S1βA precursor CAD, guided by the conserved WAT1 water out of the position. The disappearance of the electron density from the water, WAT1, was not an artifact, because the difference electron density for the other conserved water, WAT2, recurred at 3.3 σ in the F177βB precursor structure (Fig. 5b). It turns out that the disappearance of WAT1 prevented the F177βB precursor CAD from carrying out autocatalytic intramolecular cleavage.

The hydroxyl position of the nucleophile, Ser-1β, was not visible in the S1βA precursor CAD (Fig. 5a) because Ser-1β had mutated into Ala (13). The hydroxyl was modeled into the Ala1β residue of the S1βA precursor CAD, guided by the conformation of the mature CAD (Fig. 4, a and b, and Fig. 5a) (13). In the F177βB precursor CAD, the hydroxyl of Ser-1β can be observed in the $F_o - F_c$ difference map, which enabled positioning of the hydroxyl to the correct place (Fig. 5b and Fig. 6). The position of the hydroxyl of Ser-1β in the F177βB precursor CAD was rotated 90° away from that of the S1βA precursor CAD (Fig. 5, a and b, and Fig. 6). The movement of the hydroxyl of Ser-1β in the F177βB precursor CAD occurred because of the disappearance of the conserved water, WAT1, that abolished the hydrogen bonding of the hydroxyl of Ser-1β to the WAT1 water. This 90° movement prevented the hydroxyl of the cata-
lytic Ser-1β from carrying out the nucleophilic attack on the scissile bond between Gly-169s and Ser-1β.

Conformation of the F177βP Precursor CAD Forces the Elimination of the Conserved Water—The 62 Cαs in the active site of the F177βP precursor CAD were deviated by a r.m.s. deviation of 0.57 Å from those of the S1βA precursor CAD (Fig. 3). The main chain backbones of the active site for the two structures (Fig. 6) were offset by the r.m.s. deviation of 0.683 Å from each other. Regardless of the offset for the main chain backbones for the two precursor structures, it appears that the overall conformations of the active site residues are similar.

A close examination of the two active sites suggests why the conserved water WAT1 moves out of the active site. In the F177βP precursor CAD, two parallel β-sheets are compressed inward. Therefore, the interstitial space, where the conserved water WAT1 should be bound, becomes narrower when compared with the S1βA precursor CAD (Fig. 6). The position of the conserved water, WAT1, is located in the compressed interstitial space between the main chain nitrogen N of Val-70β and the carbonyl carbon C of Gly-169s in the F177βP precursor CAD. The distances between the two atoms (i.e. the main chain nitrogen N of Val-70β and the carbonyl carbon C of the Gly-169s) of the S1βA and F177βP precursor CADs are 5.6 and 4.6 Å, respectively. In the case of the F177βP precursor CAD, the distance of 4.6 Å was too short so the conserved water, WAT1, could not withstand the van der Waals contacts from the carbonyl carbon C of Gly-169s and the adjacent residue (Gly-168s) in the F177βP precursor CAD. The directions of the hydroxyls for the F177βP precursor and S1βA precursor are 90° away from each other because the hydroxyl of the F177βP precursor is not hydrogen-bonded to the conserved WAT1.

Abundance of the conserved water WAT1 abolishes intramolecular cleavage. Co traces of the F177βP and the S1βA precursors are colored green and yellow, respectively. The green-colored letters represent residues of the F177βP precursor, and the yellow letters represent the S1βA precursor. Black letters are common for both structures. Distances (dotted lines) between the main chain nitrogen N of Val-70β and the carbonyl carbon C of Gly-169s are 4.6 and 5.6 Å for the F177βP and S1βA precursor CADs, respectively. The crucial water, WAT1, for the intramolecular cleavage is not present in the F177βP precursor CAD due to the reduced distance between the main chain nitrogen N of Val-70β and the carbonyl carbon C of Gly-169s when compared with the S1βA precursor CAD. The catalytic nucleophile Ser-1β is shown in yellow for the S1βA precursor and in green for the F177βP precursor. The directions of the hydroxyls for the F177βP precursor and S1βA precursor are 90° away from each other because the hydroxyl of the F177βP precursor is not hydrogen-bonded to the conserved WAT1.

Role of the Conserved Water WAT1 in Intramolecular Cleavage—The precursor structures of GA and PS, which are other subfamilies of Ntn hydrolase, were determined in order to understand the autoproteolytic mechanism for intramolecular cleavage (4, 7). These two Ntn hydrolases use Thr as a key catalytic nucleophile for intramolecular autoproteolysis. Bound water enhances the nucleophilicity of the Thr Oγ at the +1 position in the PS precursor (4), whereas the Asp-151 carboxylate at the −1 position promotes the nucleophilicity of the Thr-152 at the +1 position in the GA precursor (7). Consequently, the hydroxyl of the catalytic Thr attacks the adjacent scissile peptide bond to carry out intramolecular cleavage (4, 7–10).

On the other hand, two precursor structures of the CAD mutants (S1βA and F177βP) revealed the role of the bound water WAT1 that corresponds to the bound water of the catalytic Thr-152 in the PS precursor (4, 27) and the carboxylate of the Asp-151 in the GA precursor (7). Therefore, the conserved water WAT1 of the CAD precursor would assist the polarization of the Ser-1β hydroxyl by the four hydrogen bonds in pseudo-tetrahedral geometry, the same as the bound water of the GA precursor and the Asp-151 carboxylate of the PS precursor (4, 7, 13, 27).

In the GA precursor the second carboxylate oxygen of Asp-151 is positioned to act as a general base and deprotonate the hydroxyl oxygen of the catalytic Thr-152 to enhance its nucleophilicity (7). This was further supported by evidence that the equivalent Asp in human GA precursor (15) is likely to mainly be in its basic form (7, 15). By contrast, the hydroxyls of the catalytic Ser-1β and Thr-1 in the CAD (13) and PS (4) precursors are polarized by the conserved waters that are weakly electronegative when compared with the Asp-151 carboxylate of the GA precursor. The question arises, Are conserved waters capable of enhancing the nucleophilicity of the hydroxyls in themselves? Evidence positively supports the conclusion that structural conformation may also promote nucleophilic reactions of hydroxyls to scissile peptide bonds (4, 7, 13, 27). First, there are no amino acid bases that act as proton acceptors that are close to the Oγ of the hydroxyls in both the CAD and PS precursors, whereas conserved waters are fully occupied in an ideal position for hydrogen bonding that is close to the hydroxyl of the catalytic nucleophiles (4, 13, 27). Second, a positive charge of the neighboring Lys-32 in the PS precursor may shift the intrinsic pKₐ of the conserved water and the hydroxyl of the catalytic Thr-1, thereby enhancing their nucleophilicity (27). Third, the angles of the N-Cα-C’ (α angle) of Gin-168s and Gin-169s in the CAD precursor are 121.4° and 102.6°, which deviate from the ideal value (112.5° and 111.2°, respectively) by 3σ (13). The bonding geometries of the scissile amino acid (Gly-169s) and the adjacent residue (Gly-168s) are significantly distorted; therefore, these distortions may raise the energy for each distorted residue (7, 13). Possibly all three elements may work cooperatively for the nucleophilic reactions of the catalytic Ser-1β and Thr-1 in the CAD and PS precursors.

Alternative Role of the Conserved Water WAT1 in Intramolecular Cleavage—The conserved WAT1 in the CAD precursor coordinates the three parts of the polypeptide chain and the hydroxyl of the catalytic Ser-1β through hydrogen bonding. The hydrogen bonding of WAT1 to Ser-1β directs the hydroxyl side chain to a position where it can precisely carry out a nucleo-
philic attack on the carbonyl carbon of Gly-169s. Removal of WAT1 in the F177βP precursor CAD consequently leads to a structural rearrangement, which results in a rotation of the hydroxyl of Ser-1β to a catalytically unfavorable position that is presumably a preferred rotamer in that conformation. Apparently, WAT1 plays a role in maintaining the active conformation of the Ser-1β catalytic site in addition to enhancing the polarization of the Ser-1β hydroxyl.

In the ββ subunit of the PS precursor (9), which has Lys-33 replaced by Arg, the nucleophilic reaction by the catalytic Thr-1 did not occur because the Thr-1 hydroxyl was pushed away by the bulkier Arg-33. This defect for autoproteolysis was attributed to the structural lability of the catalytic Thr-1 site, which could be caused by mutations of the active site residues. In the GA precursor (7) the residues Trp-11, Asp-151, and Thr-203 of the active site are coordinated through hydrogen bonding so as to direct the hydroxyl of the catalytic Thr-1 to a catalytic position. Mutagenesis for these residues showed critical effects for autoproteolysis, resulting in breaking the side-chain geometry of the catalytic Thr-1 to the scissile peptide bond. In the F177βP precursor CAD, WAT1 primarily maintained the direction of the hydroxyl of the catalytic Ser-1β to a catalytic position. The removal of WAT1 carried away the position of the hydroxyl by 90°, which resulted in losing autoproteolysis. Presumably, any structural defect that interrupts the catalytic position of the hydroxyl to the scissile peptide bond may have a critical effect on autoproteolysis.

It is alternatively conceivable that the mere distortion of the polypeptide chain in the active site areas may interrupt the structural conformation for autoproteolysis in itself, since it could rearrange the position of the hydroxyl of the catalytic Ser or Thr regardless of the disappearance of the conserved WAT1. This conclusion is implicated in the F177βP precursor structure so that the mutation effect of F177β (even though not directly involved in the autocatalytic proteolysis reaction) is transferred to the Ser-1β catalytic site via a strain of the main chain backbone. This structural work of the F177βP precursor CAD may be the first detailed structure showing that a bound water molecule plays these important roles in determining the initiation of the intramolecular cleavage in post-translational modification.

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