The three-dimensional structure of nylon hydrolase and the mechanism of nylon-6 hydrolysis*

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Background: Biodegradation of polyamides is important from the industrial and environmental point of view.

Results: We identified the catalytic residue of nylon hydrolase as Thr267, and enhanced the protein thermostability by 36°C ($T_m=88°C$) by introducing mutations at the subunit interfaces of tetramer structure.

Conclusion: We revealed the mechanism of nylon-6 hydrolysis.

Significance: We established an approach to biodegrade polymeric nylon-6.

SUMMARY

We performed x-ray crystallographic analyses of the 6-aminohexanoate oligomer hydrolase (NylC) from Agromyces sp. at 2.0 Å-resolution. This enzyme is a member of the N-terminal nucleophile (N-tn) hydrolase superfamily that is responsible for the degradation of the nylon-6 industry byproduct. We observed four identical heterodimers (27kDa+9kDa), which resulted from the autoprocessing of the precursor protein (36kDa) and which constitute the doughnut-shaped quaternary structure. The catalytic residue of NylC was identified as the N-terminal Thr267 of the 9kDa-subunit. Furthermore, each heterodimer is folded into a single domain, generating a stacked αββα core structure. Amino acid mutations at subunit interfaces of the tetramer were observed to drastically alter the thermostability of the protein. In particular, four mutations (D122G/H130Y/D36A/E263Q) of wild-type NylC from Arthrobacter sp. (plasmid pOAD2-encoding enzyme), with a heat denaturation temperature of $T_m=52°C$, enhanced the protein thermostability by 36°C ($T_m=88°C$), whereas a single mutation (G111S or L137A) decreased the stability by approximately 10°C. We examined the enzymatic hydrolysis of nylon-6 by the thermostable NylC mutant. Argon-cluster secondary ion mass spectrometry analyses of the reaction products revealed that the major peak of nylon-6 (m/z 10,000-25,000) shifted to a smaller range, producing a new peak corresponding to m/z 1500-3000 after the enzyme treatment at 60°C. In addition, smaller fragments in the soluble fraction were successively hydrolyzed to dimers and monomers. Based on these data, we propose
that NylC should be designated as nylon hydrolase (or nylonase). Three potential uses of NylC for industrial and environmental applications are also discussed.

Nylons are synthetic polymers that contain recurring amide groups (R-CO-NH-R') as integral parts of their main polymer chains. The high strength, elasticity, abrasion resistance, chemical resistance and shape-holding characteristics of nylons over wide temperature ranges make these polymers suitable for the production of fibers and plastics. Currently, the worldwide production of nylons is estimated to be three to four million tons per year. Nylons tend to be partially crystalline, and the degree of crystallinity affects the properties of nylons, such as the melting points, strength and rigidity. Two forms of crystals (α and γ) have been reported (1). In the α-form, each polymer chain is stabilized by hydrogen bonds with adjacent chains aligned in an anti-parallel orientation, whereas the chains are parallel in the γ-form. The α-form of nylon-6 is generally more stable (1).

Nylon-6 is produced by the ring-cleavage polymerization of ε-caprolactam and consists of more than 100 units of 6-aminohexanoate (Ahx). However, during the polymerization reaction, some molecules fail to polymerize and remain oligomers, whereas others undergo head-to-tail condensation to form cyclic oligomers. We have isolated bacterial strains that can degrade Ahx oligomers, which are by-products of nylon-6 production, and use these oligomers as their sole carbon and nitrogen source (2-6). Previous biochemical studies have revealed that three enzymes, NylABC, are responsible for the degradation of the by-products produced during nylon-6 manufacturing. The Ahx-cyclic dimer hydrolase (NylA; EC3.5.2.12), a member of the amidase signature (AS) hydrolase family, specifically hydrolyzes one of the two equivalent amide bonds in the Ahx-cyclic dimer, generating an Ahx linear dimer (7). The Ahx dimer hydrolase (NylB; EC3.5.1.46), a member of the penicillin-recognizing family of serine reactive hydrolases, hydrolyzes Ahx oligomers by an exo-type mode (8-12). The Ahx oligomer hydrolase (NylC; EC3.5.-.-) degrades Ahx-cyclic and -linear oligomers with a degree of polymerization greater than three by an endo-type mode (5, 13-15).

NylC has been found in Arthrobacter (pOAD2 plasmid-encoded NylC; NylC_{p2}), Agromyces (NylC_A) and Kocuria (NylC_K) (5). These enzymes are each encoded by a single gene that corresponds to a polypeptide chain of 355 amino acids. However, the post-translational cleavage of the nascent polypeptide between Asn266 and Thr267 generates a 27 kDa (α)-subunit and a 9 kDa (β)-subunit (5, 14). The autoprocessing of the inactive precursor to an active enzyme is a specific feature of the N-terminal nucleophile (N-tn) hydrolase family (16-33). NylC_A and NylC_K have 5 and 15 amino acid substitutions, respectively, relative to the NylC_{p2} sequence (supplemental Fig. S1), and both have 10-20°C higher thermostability than NylC_{p2} (5).

Recently, we reported the crystallization conditions of NylC_A that are suitable for x-ray crystallographic analyses (34). In this paper, we performed x-ray crystallographic analyses of NylC_A, examined its structural/evolutional relationship to proteins registered in the protein data bank (PDB), and estimated the residues responsible for the catalytic function and autoprocessing. We also analyzed the effect of amino acid mutations on the thermostability of NylC. Furthermore, we investigated the possibility of using the thermostable NylC mutant to degrade polymeric nylon-6 at high temperatures, which is expected to increase the reactivity of the polymer.

**EXPERIMENTAL PROCEDURE**

**DNA preparation and site-directed mutagenesis**

The plasmids pSKFC4 (NylC_{p2}), pSKRC4 (NylC_A), and pSKKC4 (NylC_K) contained 1.1 kb genes flanked by BamHI and PstI restriction sites, which were cloned into the expression vector pBluescript II SK(+) (Stratagene, La Jolla, CA.) (5). Escherichia coli JM109 competent cells were prepared by the CaCl₂ method (35) and stored at -80°C before use. To introduce the D36A, G111S, and NylC_{p2} mutations into the NylC_{p2} sequence, site-directed mutagenesis was performed using the PrimeSTAR mutagenesis kit (Takara Bio Inc., Japan) with the primers listed in supplemental Table S1. The plasmids containing the 1.1 kb fragments with mutated NylC_{p2} were isolated from transformed E. coli JM109 cells. DNA sequencing confirmed that
the desired mutations were introduced into the wild-type nylC<sub>p2</sub> sequence.

**Cultivation, enzyme purification and enzyme assay**

Cultivation and purification from *E. coli* clones were performed as previously reported (34). In the NylC activity assays, the enzyme solution (0.1 ml) was mixed with an Ahx-cyclic oligomer solution (0.9 ml, 4 mg ml<sup>-1</sup> Ahx-cyclic oligomer in 20 mM phosphate buffer, pH 7.3, 10% glycerol (buffer A)) and incubated at 30°C (standard assay condition). An increase in the concentration of the amino group was determined using trinitrobenzene sulfonic acid (TNBS) (5). Kinetic studies were performed under standard assay conditions, with the exception of the different Ahx-cyclic oligomer concentrations used.

**Nylon degradation tests**

Nylon-6 that was mechanically disintegrated to a powder was a generous gift from Toyobo Co. (Tsuruga, Japan). To analyze the particle sizes, the nylon sample was magnified 80-fold with a scanning electron microscope (Hitachi, model TM-1000, Japan). The diameter (D, maximum length) of 157 particles observed in five electron microphotographs was measured. The diameters ranged from 68 μm to 720 μm (average ± standard deviation, 270 ± 140 μm). To increase the reactivity of the nylon sample, the nylon-6 powder (10 mg) was autoclaved in plastic tubes (Eppendorf Co.) containing buffer A (180 μl) at 120°C for 20 min prior to the initiation of the enzyme reaction. Hydrolytic reactions were initiated by the addition of the thermostable NylC<sub>p2</sub>-G<sub>125</sub>Y<sup>30</sup>A<sub>36</sub>Q<sup>263</sup> mutant enzyme (1 mg ml<sup>-1</sup>, 20 μl) to each tube and incubated at 60°C for 2 h. The gas-cluster SIMS equipment (developed in the project “Development of System and Technology for Advanced Measurement and Analysis”, Japan Science and Technology Agency, 2006-2010) was used to analyze the reaction products (36). Enzyme reactions were performed in triplicate. The reaction products (containing both the insoluble and soluble fractions) from the first tube were spotted onto a silicon plate (1 cm<sup>2</sup> square plate). Mass-to-charge ratio (m/z) ranges of 5,000 to 35,000 and 0 to 1,500 were analyzed. The solid fractions (approximately 10 mg) that were recovered from the reaction products were washed with distilled water, lyophilized, and dissolved in trifluoroethanol (TFE; 0.2 ml). Subsequently, a fraction (0.02 ml) was spotted onto a silicon plate (1 cm<sup>2</sup> square plate). The m/z range of 1,500 to 15,000 was analyzed. The soluble fraction (1 μl) was spotted onto a thin layer plate and developed by a solvent mixture containing 1-propanol : water : ethylacetate : ammonia (24:12:4:1.3). The degradation products were detected by spraying the plate with a 0.2% ninhydrin solution (in n-butanol saturated with water) (5).

**Crystallographic analysis**

i) Crystallization and diffraction data collection

For analysis of NylC<sub>p2</sub>, plate-like crystals (0.8 x 0.4 x 0.3 mm) were obtained by the sitting-drop vapor-diffusion method. Droplets were prepared by mixing 2 μl of purified NylC<sub>p2</sub> solution (10 mg ml<sup>-1</sup> protein in buffer A) and 2 μl of reservoir solution (1.0 M sodium citrate as a precipitant in 0.1 M HEPES buffer (pH 7.5), 0.2 M NaCl) and were equilibrated against 100 μl of reservoir solution at 10°C for one week. The crystal belonged to the space group I222, with unit-cell parameters a = 155.86, b = 214.45, c = 478.80 Å (Table 1).

For native crystals, the crystals were soaked for 24 h in a cryoprotectant solution (1.0 M sodium citrate, 0.1 M HEPES (pH 7.5), 0.2 M NaCl, 25% glycerol). Heavy atom derivatives of NylC<sub>p2</sub> were prepared by soaking the crystals for 72 h in cryoprotectant solution containing 5 mM K<sub>2</sub>PtCl<sub>4</sub>. Cryo-cooling was performed by blowing cold nitrogen steam onto the crystals at 100 K.

The diffraction data sets were collected at the SPRing-8 (Hyogo, Japan) beamline BL38B1 equipped with a Rigaku Jupiter CCD detector system. The following parameters were chosen for data collection: wavelength, 1.0000 Å; crystal to detector distance, 180 mm; oscillation range per image, 0.5° (for native and Pt-derivative). Indexing, integration, and the scaling of reflections were performed using the HKL2000 program package (37). Diffraction data were collected from native NylC<sub>p2</sub> crystals and from the K<sub>2</sub>PtCl<sub>4</sub>-derivative to resolutions of 2.00 Å and 2.20 Å, respectively (Table 1).

ii) Phase determination, model building and crystallographic refinement

The NylC<sub>p2</sub> structure was determined by the
RESULTS AND DISCUSSION

Quaternary structure

The asymmetric unit contains fifteen molecules (molecules A-O), each comprised of an α and a β subunit. One molecule (O) is part of a tetramer in which the four heterodimers are related by crystallographic 222 symmetry. Two molecules in dimer (M/N) form another tetramer that is related by a crystallographic 2-fold axis, and the rest of the 12 molecules comprise three tetramers (A/B/C/D, E/F/G/H, and I/J/K/L) that are related by non-crystallographic 222 symmetries. The root mean square deviations (rmsd) of the superimposed Cα atoms for all 15 molecules were calculated to be within the range of 0.13Å to 0.38Å by secondary structure matching (SSM) (supplemental Table S2) (42), demonstrating that the overall structures are almost identical. Based on these results, we concluded that NylC adopts a doughnut-shaped quaternary structure in which four heterodimer molecules are mutually related by a perpendicular crystallographic and/or non-crystallographic 2-fold axis (Fig. 1). Contacts at the A/B and C/D interfaces (3,121 Å2) were observed to be more extensive than those at the B/C and A/D interfaces (1,451 Å2).

Subunit structure and function relationship with other N-tn family enzymes

Each heterodimer contains 10 helices, H1-H10, which are α-helices with the exception of H7 (310 helix), and 18 β-strands. The two subunits (α and β) obtained by intracleavage at Asn266/Thr267 are folded into a single domain, generating a stacked αβα core structure (Fig. 2A, supplemental Fig. S2). Namely, the central anti-parallel β-sheet composed of five β-strands (β3, β4a, β5, β6, β7) and another anti-parallel β-sheet composed of five β-strands (β4b, β12, β13, β16, β18) are packed against each other and connected by a common long β-strand (β4). These two sheets are flanked on one side by the α-helices H4 and H5 and on the other by α-helices H9 and H10. The αβα fold is typically conserved throughout the N-tn hydrolase superfamily (16-33). However, our data demonstrate that NylC differs from most N-tn hydrolases in the directionality and connectivity of its secondary structure elements.

To search for proteins that are evolutionary
related to NylC, we performed a homology search based on the NylC complex structure for PDB using the DALI (http://ekhidna.biocenter.helsinki.fi/dali/) (43). The following four proteins (including one hypothetical protein) that exhibited high z-scores (z-score > 14) were identified: L-aminopeptidase D-Ala-esterase/amidase from *Ochrobactrum anthropi* (DmpA; z-score = 26.3; PDB ID code: 1B65) (18), hypothetical D-aminopeptidase (z-score = 25.9; PDB ID code, 2DRH), β-peptidyl aminoesterase from *Sphingosinicella xenopeptidilytica* (BapA; z-score = 25.2; PDB ID code: 3N33) (20), and ornithine acetyltransferase (OAT; z-score = 14.1; PDB ID code: 1VZ6) (21) (supplemental Table S3). In contrast, the DALI z-scores of the other proteins in the PDB were determined to be below 8.

DmpA is an aminopeptidase that hydrolyzes peptides, with a preference for N-terminal residues in an L-configuration (L-Ala-Gly-Gly), and also amide or ester derivatives of D-Ala (19). BapA hydrolyzes β-oligopeptides and mixed β/α-oligopeptides with a β-amino acid residue at the N-terminus (20). OAT is involved in the arginine biosynthetic pathway because it catalyzes the transfer of an acetyl group from N-acetylornithine to glutamate (21, 22). In contrast, NylC, a long β-helix and -linear oligomers (degree of polymerization > 3) but has no detectable activity with the 66 peptides tested (supplemental Table S4), including D,L-Ala-Gly-Gly (14, 15). Thus, NylC exhibits distinct substrate specificity from that of DmpA/BapA/OAT.

DmpA/OAT are considered to have a unique fold (designated as DOM-fold), which is different from that of most N-tn hydrolases (17). Based on fundamental topology differences, Cheng and Grishin proposed that the functional similarities between DmpA/OAT and the other N-tn hydrolases result from convergent evolution rather than from having a common evolutionary origin (17). The fold of NylC is similar to that of DmpA/BapA, and the structurally superimposable regions comprise 259 residues (DmpA) and 264 residues (BapA) (supplemental Table S3). The rmsd of the superimposed Cα atoms were calculated to be 2.0 Å (DmpA) and 2.0 Å (BapA) by SSM. However, the following major structural differences were found among the family enzymes:

i) In contrast to the single domain structure of NylC/DmpA/BapA, OAT is composed of two domains (supplemental Fig. S3C). NylC is superimposable with OAT-domain 1 located at positions 1-258. OAT-domain 2 is located at the C-terminal region and is composed of 124 residues that have a successive order of secondary structure (β10-H9-H10-H11-β11-β12-H12-β13-β14-H 13). DmpA and BapA are longer than NylC at the C-terminal region of the β-subunit by 34 and 30 residues, respectively (supplemental Fig. S4).

ii) Helix H8, which is located at the C-terminal region of the α-subunit, is unique in NylC. H8 and its linked loop region is flipped out to the surface of the adjacent molecules upon autoprocessing (Fig. 1C). In DmpA/OAT, the corresponding helix is absent. Furthermore, in BapA, the corresponding region (positions 236-245), which contains β14 and a loop, is folded within the same subunit molecule without flipping out to the adjacent molecule.

iii) The N-terminal 26 residues, including H1, generate unique structure specifically found in NylC. Moreover, in OAT, N-terminal 48 residues, including H1-H2-β1-β2, are absent (supplemental Fig. S4).

iv) A loop flanked by β-strand β18 and helix H10 in NylC (positions 317-333) spatially share different positions in Dmp (positions 299-318) and in BapA (positions 299-316). In OAT, the corresponding loop is absent, and β9 and H18 are combined with a single residue (Val235) at the junction (supplemental Fig. S4).

v) In NylC, a long β-strand (β4a, β4b) constitutes a part of the central two β-sheets (Fig. 2A). However, in DmpA/BapA/OAT, the corresponding β-strand is divided into two β-strands (e.g., β2, β3 in DmpA), which are connected by a short loop and constitute each β-sheet (supplemental Fig. S4).

**Residues responsible for autoprocessing and catalytic function**

In DmpA, autoprocessing occurs between Gly249 and Ser250. Additionally, the newly generated N-terminal Ser250 of the β-subunit plays the roles of both the nucleophile (hydroxyl group) and the general base (α-amino group) in catalytic reactions (18). In NylC, Thr267
participates in a hydrogen-bonding network with Asn219, Asp306 and Gly307 (backbone-N). The positions of these four residues are spatially similar to those of Ser250, Asn218, Ser288 and Gly289 (backbone-N) in DmpA, respectively (Fig. 2B). The catalytic nucleophile (Ser250) and the surrounding residues [Asn207, Ser288 and Gly289 (backbone-N)] are also conserved in BapA. These results indicate that Thr267 is most likely responsible for the catalytic function of NylC. Assuming that auto-proteolysis of the NylC precursor proceeds in manner similar to that proposed for other N-tn hydrolases (23-27), the reaction would be initiated by nucleophilic attack of Thr267-O, to Asn266-C, generating a tetrahedral intermediate. This intermediate would rearrange into an ester intermediate (N-O acyl shift) that is subsequently hydrolyzed by an adjacent water molecule, producing the active enzyme.

**Structural alterations induced by autoprocessing**

The poor electron density distribution in the carboxyl-terminal region of the α-subunit (position 261-267) of the active NylCα enzyme has prohibited the determination of a structural model. The C-terminal region of the α-subunit has not been identified by x-ray crystallographic analyses of DmpA/BapA/OAT (supplemental Fig. S4). In a three dimensional model of NylCα, the distance between Pro260-Cα and Thr267-Cα is estimated to be 32.6 Å (supplemental Fig. S2). The large distance suggests that the local structural alteration that occurs is accompanied by autoprocessing. Namely, the terminal region of the α-subunit, including helix H8 and Pro260, at each subunit interface flips out toward the adjacent molecules (Fig. 1C). We speculate that the structural alteration is important for generating the catalytic center responsible for the hydrolysis of incoming substrates (6-aminohexanoate-oligomers). Structural alterations induced by autoprocessing have been reported for other N-tn hydrolases (25).

**Effect of amino acid substitutions on protein stability**

Thermal denaturation experiments using circular dichroism (CD) showed that the $T_m$ of NylCα and NylCγ are 60°C and 67°C, respectively (Fig. 3, Table 2). These values are 8-15°C higher than the $T_m$ of NylCp2 (52°C). We have suggested that at least one among the five alterations (G111S, D122G, H130Y, L137A, V225M) in NylCα contributes to the increase in the thermostability of NylCp2. Moreover, at least one of the ten alterations (D36A, A41V, M50T, I60V, A62S, T230G, V231I, V257L, E263Q, G354A) in NylCκ is estimated to contribute to the further increase in thermostability (supplemental Fig. S1) (5). The three-dimensional structure of NylCα shows most amino acid residues that differ among the three NylC enzymes were found to be located at the interfaces between the subunits (Fig. 1B). To examine the effect of the amino acid substitutions on the thermostability of proteins, we individually replaced the residues in NylCp2 with the corresponding NylCα or NylCκ sequences.

A single amino acid mutation of either D122G or H130Y, which are located at the A/D interface, increased the $T_m$ of NylCp2 by 24°C and 11°C, respectively (Fig. 3, Table 2). However a single substitution of either G111S or L137A at the same interface decreases the stability by approximately 10°C. Even more drastic effects were observed with the combinations of these mutations. D122G/H130Y double mutations in NylCp2 enhanced the thermostability to 81°C. Far UV CD spectra showed very little change, even at 75°C, for the G122Y130 mutant, whereas the parental NylCp2 and NylCα enzymes denatured at 75°C (supplemental Fig. S5). The addition of two further mutations, D36A/E263Q derived from NylCκ, to the G122Y130 mutant enhanced the $T_m$ to 88°C (G122Y130A36Q263 mutant; Fig. 3). Notably, we observed that more than 90% of the enzyme activity was retained even after incubation of the G122Y130A36Q263 mutant for 30 min at 70°C. In contrast, an L137A mutation drastically decreased thermostability in the G122Y130 and S111G122Y130 mutants by 27°C. Similarly, a V225M single mutation decreased the protein stability and/or the expression level in cells because no protein was detected in the cell extracts that were prepared by expressing the M225-mutant NylCp2. However, the stabilization effect is altered by the combination of other amino acid alterations close to Met225 because the V225M mutation, in the context of the S111G122Y130A137 quadruple mutant, recapitulates the sequence of NylCα and improves its stability by 9°C. Additionally, the V225M...
mutation in the $G^{122}Y^{130}$ mutant improved the stability by 2°C (see $G^{122}Y^{130}M^{25}$). These results demonstrate that subunit interactions that generate the quaternary structure drastically affect the thermostability of the enzyme (47°C by five mutations; Fig. 3, Table 2).

**Molecular basis of protein stabilization**

Based on the three-dimensional structure of NylC, we estimate that the following structural effects occurring at the subunit interfaces A/D (D122G, H130Y, L137A, G111S and D36A) and A/B (V225M and E263Q) can cause changes in the thermostability of NylC.

**D122G:** In NylC, Lys159-NH$_3^+$ (in molecule A) is located 2.79 Å apart from Glu115-COO$^-$ (in molecule D) (Fig. 4A). Therefore, the electrostatic effect between the two residues will enhance subunit binding around Gly122 located on helix H4. However, in NylC$_{p2}$, the amino acid residue at position 122 is replaced with Asp, and the close proximity of the acidic residue Asp122 (close to Glu115) will reduce this stabilization effect.

**H130Y and L137A:** Tyr130 (in molecule A) is located at a loop region (Arg127-Ala135) between helix H4 and β-strand β7 (supplementary Fig. S4). Tyr130-O$_{H_2}$ forms a hydrogen-bond with Glu126-O$_{H_2}$ located at the end of helix H4 (Fig. 4B). Mutating Tyr130 to His may destroy the hydrogen-bonding and destabilize the loop. Therefore, a H130Y substitution in NylC$_{p2}$ will contribute to an increase in thermostability by stabilizing the loop region. In contrast, L137A substitution (in NylC$_{p2}$, $G^{122}Y^{130}$ and $S^{111}G^{122}Y^{130}$) occurring at the same loop region resulted in the drastic decrease in the protein stability (Fig. 3B). Therefore, hydrophobic effect by Leu137 should be involved in the stabilization of the loop region. Ala137/Leu137 affects the subunit interaction, cooperatively with Gly111/Ser111 (Fig. 4C).

**G111S:** Hydrophilic Ser111-O$_T$ (in molecule A) is surrounded by four hydrophobic residues [Tyr98, Ala137, Leu139 (in molecule D); Tyr112 (in molecule A)] (Fig. 4C). Therefore, substitution from Ser111 to Gly111 (aliphatic small residue) should improve the hydrophobic stabilization effect at A/D interface. In contrast, its reverse G111S substitution (in NylC$_{p2}$, $G^{122}Y^{130}$ and $G^{122}Y^{130}A^{137}$) actually reduces the thermotability (Fig. 3B).

**D36A:** Asp36 (in molecule D) is located at helix H2, which is close to Glu126 (in molecule A) (4.40 Å), whereas Asp36 is replaced with Ala36 in NylC$_K$ (Fig. 4B). Therefore, the D36A mutation will reduce electrostatic repulsion with Glu126. Actually, D36A substitution (in $G^{122}Y^{130}$ and $G^{122}Y^{130}Q^{263}$) improves the thermostability (Fig. 3B).

**V225M:** Met225 (in molecule A) does not directly contact with any adjacent subunits (Leu9-C$_{6}$ in molecule B is the nearest position; 5.29 Å). Met225 is close to Gln299 in the same subunit, which interacts with Arg296-N$_{C_6}$ (3.01 Å) (in molecule B) (Fig. 4D). Moreover, position of Gln299 should be stabilized by interaction with His245-N$_{C_6}$ (2.81 Å). Therefore, it is likely that amino acid substitution at position 225 indirectly affects the subunit interaction. Actually, V225M substitution (in $G^{122}Y^{130}$ and $S^{111}G^{122}Y^{130}A^{137}$) improves the thermostability (Fig. 3B), probably by generating a new interaction between Met-S$_{6}$ and Gln299-N$_{C_6}$ (3.56 Å) (Fig. 4D). However, it should be also noted that V225M substitution decreases the expression level and/or stability of NylC$_{p2}$ in cell, as described above. Therefore, total stabilization effects should be dependent on the combined interactions with the surrounding residues.

**E263Q:** Glu263 is located at the terminal region of the α-subunit (Val261-Asn266), which had a poor electron density distribution in the x-ray diffraction study of NylC$_{p2}$ (Fig. 1). In NylC$_K$, the acidic amino acid residue Glu263 is replaced with neutral Gln, suggesting that altering the electrostatic environment induced by the E263Q mutation assists in improving subunit interactions.

**Enzymatic hydrolysis of polymeric nylon-6**

Although the melting point of nylon-6 (220-225°C) is much higher than the thermostability of the enzyme, performing the reaction at a high temperature should have the following advantages for the hydrolysis of nylon.

1. Hydrogen bonding between the polymer chains is partially weakened, allowing the enzyme to attack the polymer chains that are exposed to the solvent.
2. The release of the cleaved fragments from the solid phase generates new sites for the subsequent reaction.

The mutated sites in the $G^{122}Y^{130}A^{36}Q^{263}$
quadruple mutant are located at the interface with other subunits and apart from catalytic Thr267 (e.g., 26.6 Å to Gly122 at Cα). Kinetic studies of the G122Y130A36Q263 mutant with the Ahx-cyclic oligomer determined that the $k_{\text{cat}}$ and $K_m$ values are 2.8 ± 0.11 s$^{-1}$ and 0.72 ± 0.06 mg ml$^{-1}$, respectively. Compared to the kinetic parameters of wild-type NylC, the G122Y130A36Q263 mutant (1.8 s$^{-1}$ ml mg$^{-1}$; $K_m = 3.7±0.27$ mg ml$^{-1}$) (5), these mutations decreased the turnover of the product but improved the affinity of the enzyme for the substrate. As a result, the calculated $k_{\text{cat}}/K_m$ of the G122Y130A36Q263 mutant (3.9 s$^{-1}$ ml mg$^{-1}$) is 2-fold higher than that of NylC$_{p2}$ (6.5 ± 0.29 s$^{-1}$; $K_m = 3.7±0.27$ mg ml$^{-1}$) (5), which is 2-fold higher than that of NylC$_{p2}$. Based on the high thermostability and increasing catalytic activity of the G122Y130A36Q263 mutant, we considered this mutant to be suitable for testing the hydrolysis of polymeric nylon-6.

To increase the efficiency of nylon degradation, pellets of nylon-6 plastic were mechanically disintegrated to powder (D = 0.27 ± 0.14 mm, see “Experimental Procedure”). The powder was resuspended in buffer A (50 mg ml$^{-1}$), and the suspension was pretreated at 120°C for 20 min. The enzyme reaction was performed using the G122Y130A36Q263 mutant (0.1 mg ml$^{-1}$) at 60°C for 2 h.

Secondary ion mass spectrometry (SIMS) measures the time of flight of the secondary ions generated by the bombardment of a sample with primary ion-beams and sensitively detects the alterations in mass size occurring at the surface of solid particles (36, 44). We used gas-cluster SIMS equipment, in which the primary ions are argon cluster ions with a kinetic energy per atom controlled to be lower than 4 eV. This low energy of primary ions reduces the internal cleavage of polymer molecules, increasing the detection of the intact ions (36, 44). We observed a wide peak with an m/z range of 10,000-25,000 (top peak: 14,500) for untreated nylon-6, but the major peak was shifted to a smaller range with an m/z range of 8,000-23,000 (top peak: 13,000) for the reaction products (Fig. 5A). Moreover, SIMS analyses of the reaction products (dissolved in TFE) revealed a new peak corresponding to an m/z range of 1,500-3,000 (top peak: 2,000; Fig. 5B). The presence of the specific fragments in the solid fraction demonstrated that NylC hydrolyzes nylon, but the fragments that were produced were still bound to polymer chains through hydrogen bonding (Fig. 6). These fragments corresponded to oligomers with 13-25 monomeric units, assuming an electric charge (z) = 1. In contrast, smaller fragments (<10 monomeric units) released from the solid fraction should be readily hydrolyzed to dimers and monomers (Fig. 5C). Consistent with this hypothesis, TLC analysis showed that the dimers and monomers are detected in the soluble fractions (Fig. 5D). Therefore, we confirmed that the dimers are converted to monomers by a subsequent NylB reaction.

The original nylon sample exhibited five major peaks (3K-7K in Fig. 5C), which are estimated to be potassium ion-bound forms, [HN(CH$_2$)$_3$CO]$_n$K$^+$ for Ahx-trimer to -heptamer (m/z = 378, 491, 604, 717, and 830), because the sample includes potassium ions in the reaction mixture and the cation-bound forms generally display a higher intensity than the unbound forms in the SIMS analysis (36). However, these peaks disappeared in the NylC-treated sample. Additionally, TLC analysis of the untreated nylon did not detect any spots by ninhydrin reaction. However, after NylC-treatment, spots indicative of dimers and monomers were produced (Fig. 5D). From these results, we conclude that the Ahx cyclic oligomers were attached to the nylon sample and that these compounds were hydrolyzed by NylC (Fig. 6).

**Potential use in industrial and environmental applications**

Because SIMS analyses of the reaction products demonstrate that the thermostable NylC hydrolyzes polymeric nylon-6, we propose that NylC should be designated as nylon hydrolase (or nylonase) (EC3.5.-.-). Notably, there is potential for the use of nylon hydrolysis in industrial and environmental applications, although the catalytic function and thermostability of NylC, as well as the pretreatment conditions of nylons for efficient hydrolysis, warrant further improvement.

**Tools for the evaluation of biodegradability of polyamides**: Ordinary biodegradability tests of polymers are performed in activated sludge (for one month) or in soil (for four months). Because the extent of hydrolysis of the polyamides by NylC is related to the biodegradability of the polyamide, a NylC reaction followed by SIMS analysis is appropriate for the prescreening of “biodegradable polyamides”.

**Improvement of the surface structures of...
**nylon fibers**: Suitable methods to improve the surface structures of nylon remain poorly developed. With NylC, the partial enzymatic hydrolysis of nylon surfaces can be used to change the smoothness of nylon fibers.

**Recycling of nylons**: Monomers (Ahx) obtained from nylon oligomers are aerobically metabolized in nylon oligomer-degrading strains (2-6). Suitable fermentation or biotransformation processes could enable the conversion of Ahx to other metabolites, such as organic acids or alcohols. Alternatively, Ahx may be a reusable reagent for the production of nylon-6 after conversion to \( \varepsilon \)-caprolactam by intramolecular dehydration. Therefore, the development of these processes could enable the recycling of nylons and decrease the environmental waste caused by the accumulation of man-made compounds.

**REFERENCES**


**FOOTNOTES**

* The atomic coordinates and structural factors for NylC₄ (PDB ID code: 3AXG) have been deposited in the Protein Data Bank (http://www.rcsb.org/).

This work was supported in part by a Grant-in-Aid for Scientific Research (Japan Society for Promotion of Science), and grants from the GCOE Program and the JAXA project. Nylon-6 was a generous gift from Toyobo Co. Ltd. (Tsuruga, Japan).

** These authors contribute equally to this study.

*** Abbreviations used: Ahx, 6-aminohexanoate; N-tn, N-terminal nucleophile; NylA, 6-aminohexanoate cyclic dimer hydrolase; NylB, 6-aminohexanoate-linear dimer hydrolase; NylC, 6-aminohexanoate oligomer hydrolase, or nylon hydrolase; rmsd, root mean square deviations; DmpA, L-aminopeptidase D-Ala-esterase/amidase; BapA, β-peptidyl aminopeptidase; OAT, ornithine acetyltransferase; SSM, secondary structure matching; TNBS, trinitrobenzenesulfonic acid; SIMS, secondary ion mass spectrometry.

**FIGURE LEGENDS**

Fig. 1. Stereoview of the quaternary structure of NylC₄. **A.** The quaternary structure is shown, with different colors highlighting the individual molecules A (green), B (blue), C (red) and D (yellow). The catalytic residue Thr267 (the N-terminus of the β-subunit) is shown as a space-filling model (red). **B.** An enlarged view of molecule A and its interfaces with the adjacent molecules B and D. Six residues selected for mutagenesis [Asp36 (Ala36 in NylC₄), Ser111, Gly122, Tyr130, Ala137 and Met225] are shown as space-filling models. Because of the poor electron density distribution of the C-terminal region in the α-subunit, including Glu263 (Gln263 in NylC₄), the adjacent Pro260 is shown as a space-filling model (magenta). **C.** Surface structure of NylC₄. The α-subunit and β-subunit in a single heterodimer (molecule A) are highlighted in dark green and light green, respectively. The other three heterodimers are shown in
different shades of grey.

Fig. 2. Stereoview of the subunit structure and catalytic center of NylC$_A$. A. The overall structure of the heterodimer (molecule A) is shown as a ribbon diagram. Ten helices (H1-H10) and eighteen β-strands (S1-S18) are colored in green and orange, respectively. H1-H6 and H8-H10 are α-helices. H7 is a 3$\_10$ helix. B. The structure around the catalytic residue Thr267 of NylC$_A$ (green) is superimposed onto the structure of DmpA (PDB ID code, 1B65; magenta) and BapA (PDB ID code, 3N33; orange). Possible hydrogen bonds in NylC$_A$ are indicated as dotted lines with the distances listed in angstroms.

Fig. 3. Thermostability of NylC mutants. A. Thermal transition curves of the various NylC mutant enzymes. CD measurements were performed at 220 nm from 25 to 95°C (1°C min$^{-1}$). The results are expressed as the mean residue molar ellipticity [θ]. Protein concentrations of 0.1 mg ml$^{-1}$ were used. B. The cumulative effects of amino acid mutations on the melting temperatures of denaturation ($T_m$) are shown.

Fig. 4. Possible interactions at the subunit interfaces. The possible interactions at the subunit interfaces around Gly122 (A), Asp36/Tyr130 (B), Ser111/Ala137 (C), and Met225 (D) are shown. Molecule A, molecule B, and molecule D in the quaternary structure are colored in green, blue, and yellow, respectively. Possible hydrogen bonds and contacts between two atoms are indicated as dotted lines with the distances listed in angstroms.

Fig. 5. Nylon degradation tests using argon-cluster SIMS and TLC. Nylon-6 powder (10 mg) was pretreated in triplicate in buffer A (180 µl) at 120°C for 20 min. The NylC$_{p2}$G$_{122}$Y$_{130}$A$_{36}$Q$_{263}$ mutant (1 mg ml$^{-1}$, 20 µl) was then added and incubated at 60°C for 2 h. The experiment was performed in triplicate. A. The reaction products (both the soluble and insoluble fractions) were spotted onto a silicon plate (1 cm$^2$). The m/z range of 5,000 to 35,000 was analyzed. Red line, pretreated nylon-6 at 120°C in buffer A; blue line, enzyme-treated nylon-6; black line, enzyme alone. B. The solid fraction was washed with distilled water, lyophilized, and dissolved in trifluoroethanol (TFE; 0.2 ml), and a fraction (0.02 ml) was spotted onto a silicon plate (1 cm$^2$). The m/z range of 1,500 to 15,000 was analyzed. C. The reaction products (both the soluble and insoluble fractions) were spotted onto a silicon plate (1 cm$^2$). The m/z range of 0 to 1,500 was analyzed. The positions of Ahx-monomer to -heptamer (marked by 1-7; m/z = 113, 226, 339, 452, 565, 678, 791) and those of the potassium bound forms (marked by 3K-7K) are shown. D. The soluble fractions (1 µl) were spotted onto a thin layer plate and developed, and the degradation products were detected by the ninhydrin reaction (5). Slot 1, 6-aminohexanoate; slot 2, 6-aminohexanoate-dimer; slot 3, pretreated nylon-6 at 120°C in buffer A; and slot 4, enzyme-treated nylon-6.

Fig. 6. The mode of nylon-6 degradation. The polymer chains are stabilized by hydrogen bonds with adjacent chains aligned in the reverse orientation (α-crystal) (1). Some chains constitute amorphous regions. The circles indicate the cyclic oligomers attached to the polymers. Long arrows indicate the direction of the polymer chains. Short arrows indicate cleavage by the enzyme. The 6-aminohexanoate-oligomers were converted to 6-aminohexanoate by the subsequent NylB reaction.
Table 1. (A)  Data Collection statistics

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<tr>
<th>Data collection</th>
<th>Native (NyLC₄)</th>
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<td>Space group</td>
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<td>I222</td>
</tr>
<tr>
<td>Unit cell (Å)</td>
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<tr>
<td>a</td>
<td>155.86</td>
<td>155.99</td>
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<tr>
<td>b</td>
<td>214.45</td>
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<td>c</td>
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<td>30 – 2.20 (2.28 – 2.20)</td>
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<td>Total reflections</td>
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<td>2,786,883</td>
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<td>Unique reflections (outer shell)</td>
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<td>398,011(36,644)</td>
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<td>Completeness (outer shell) (%)</td>
<td>94.3 (73.0)</td>
<td>99.0 (91.7)</td>
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<td>*Rmerge (outer shell) (%)</td>
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<td>&lt;I/σ(I)&gt; (outer shell)</td>
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<td>Multiplicity</td>
<td>3.6 (2.0)</td>
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*R = \sum |F_{o}\| - k |F_{c}\| (\sum |F_{o}\|)^{-1} \cdot k : scaling factor

Values in parentheses are for the outer resolution shell

Table 1. (B) Refinement statistics

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<th>Resolution range (outer shell) (Å)</th>
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<td>Rwork (outer shell) (%)</td>
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<td>Rfree (outer shell) (%)</td>
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<td>No. of water molecules</td>
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<td>Rms bond distances (Å)</td>
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<td>Rms bond angles (°)</td>
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Table 2. Effect of amino acid substitutions on thermostability

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<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
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<tr>
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<td>Single mutant</td>
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<td>D122G</td>
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<tr>
<td>H130Y</td>
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<td>L137A</td>
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<tr>
<td>V225M</td>
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<td>G122Y</td>
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<td>Triple mutant</td>
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<tr>
<td>G122Y H130Y D36A</td>
<td>D122G H130Y D36A</td>
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<td>D122G H130Y V225M</td>
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<td>S111G D122Y</td>
<td>G111S D122G H130Y</td>
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<td>Quadruple mutant</td>
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<tr>
<td>S111G D122Y H130Y L137A</td>
<td>G111S D122G H130Y L137A</td>
<td>51</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Nylon-6  \( H-[HN(CH_2)_5CO]_n-OH \)

\( \alpha \) -Crystal

Amorphous

Cyclic oligomer

\( \uparrow \uparrow \uparrow \)

release

\( \downarrow \) NyIC

release

\( \downarrow \) NyIB

6-Aminohexanoic acid (Ahx)  \( H_2N(CH_2)_5COOH \)

Fig. 6
The three-dimensional structure of nylon hydrolase and the mechanism of nylon-6 hydrolysis
Seiji Negoro, Naoki Shibata, Yusuke Tanaka, Kengo Yasuhira, Hiroshi Shibata, Haruka Hashimoto, Young-Ho Lee, Shohei Oshima, Ryuji Santa, Shohei Oshima, Kozo Mochiji, Yuji Goto, Takahisa Ikekami, Keisuke Nagai, Dai-ichiro Kato, Masahiro Takeo and Yoshiki Higuchi

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