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X-ray Crystallographic Analysis of 6-Aminohexanoate-Dimer Hydrolase

*MOLECULAR BASIS FOR THE BIRTH OF A NYLON OLIGOMER-DEGRADING ENZYME**

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6-Aminohexanoate-dimer hydrolase (EII), responsible for the degradation of nylon-6 industry by-products, and its analogous enzyme (EII′) that has only ~0.5% of the specific activity toward the 6-aminohexanoate-linear dimer, are encoded on plasmid pOAD2 of *Arthrobacter* sp. (formerly *Flavobacterium* sp.) KI72. Here, we report the three-dimensional structure of Hyb-24 (a hybrid between the EII and EII′ proteins; EII′-level activity) by x-ray crystallography at 1.8 Å resolution and refined to an *R*-factor and *R*-free of 18.5 and 20.3%, respectively. The fold adopted by the 392-amino acid polypeptide generated a two-domain structure that is similar to the folds of the penicillin-recognizing family of serine-reactive hydrolases, especially to those of D-alanyl-D-alanine-carboxypeptidase from *Streptomyces* and carboxylesterase from *Burkholderia*. Enzyme assay using purified enzymes revealed that EII and Hyb-24 possess hydrolytic activity for carboxyl esters with short acyl chains but no detectable activity for D-alanyl-D-alanine. In addition, on the basis of the spatial location and role of amino acid residues constituting the active sites of the nylon oligomer hydrolase, carboxylesterase, D-alanyl-D-alanine-peptidase, and β-lactamases, we conclude that the nylon oligomer hydrolase utilizes nucleophilic Ser^{112} as a common active site both for nylon oligomer-hydrolytic and esterolytic activities. However, it requires at least two additional amino acid residues (Asp^{181} and Asn^{266}) specific for nylon oligomer-hydrolytic activity. Here, we propose that amino acid replacements in the catalytic cleft of a preexisting esterase with the β-lactamase fold resulted in the evolution of the nylon oligomer hydrolase.

Microorganisms are believed to be highly adaptable toward environmental conditions. This can be elucidated from the observations that microorganisms capable of degrading unnatural synthetic compounds can be isolated relatively easily. Unnatural synthetic compounds include various chemicals such as endocrine disrupters and toxic compounds, which have unfavorable effects on living cells. A suitable system to enhance the biodegradability of these compounds is important from an environmental point of view. We have been studying the degradation of a by-product of nylon-6 manufacture (*i.e.* 6-aminohexanoate oligomers (namely nylon oligomers)) (Fig. 1), by *Flavobacterium* sp. KI72 as a model for studying the adaptation of microorganisms toward unnatural compounds (1, 2).[2] Three enzymes, 6-aminohexanoate-cyclic dimer hydrolase (3), 6-aminohexanoate-dimer hydrolase (EII)[3] (4), and endo-type 6-aminohexanoate-oligomer hydrolase (5), encoded on the plasmid pOAD2 (45,519 bp) (6) in strain KI72, were found to be responsible for the degradation of the nylon oligomers. It was also established that the EII-analogous protein (EII′) is located on a different part of the pOAD2 (7, 8). EII′ has 88% homology to EII (7) but has very low catalytic activity (1/200 of EII activity) toward the 6-aminohexanoate-linear dimer (Ald), suggesting that EII has evolved by gene duplication followed by base substitutions from its ancestral gene (8).

Enzyme assay using the purified enzyme revealed that EII is active on 6-aminohexanoate-linear oligomers from the dimer to the icosamer. It is more active on 6-aminohexanoyl-8-aminooctanoate (Ahx-Aoc) and 6-aminohexanoyl-aniline (Ahx-Ani) than Ald but is barely active on 4-aminobutyryl-6-aminohexanoate-Ahx or 8-aminooctanoyl-6-aminohexanoate (Aoc-Ahx) (Fig. 1) (1, 9). In addition, this enzyme has no detectable activity on the 6-aminohexanoate-cyclic dimer (substrate for 6-aminohexanoate-cyclic dimer hydrolase enzyme) (1, 3), 6-aminohexanoate-cyclic oligomers (degree of polymerization >3, substrates for the endo-type 6-aminohexanoate-oligomer hydrolase enzyme) (1, 5), or more than 60 kinds of various peptides tested such as L-alanyl-L-alanine (1, 4). Thus, the EII enzyme specifically recognizes amide compounds containing 6-aminohexanoate as the N-terminal residue in the substrate, but the recognition of the C-terminal residue in the substrate is not stringent.

Knowledge of the three-dimensional structures of the EII and EII′ enzymes allows us to study the catalytic mechanism and the evolution of these enzymes in comparison with proteins having the analogous three-dimensional structures. The EII enzyme was purified to homogeneity on SDS-PAGE. However, according to the light scattering diffraction pattern, the three-dimensional structure of the enzyme was still heterogeneous, and the purified enzyme gave no crystal formation under any

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The atomic coordinates and structure factors (code 1WYB) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[2] Strain KI72 was previously identified as *Flavobacterium* sp., since the strain is judged to be Gram-negative by the ordinary Gram staining method and produces a yellow pigment typical of *Flavobacterium* sp. However, we reinvestigated the phylogenic relationship of strain KI72 on the basis of the sequences of 16 S rDNA and concluded that the strain should be classified as *Arthrobacter* sp. (K. Yasuhira, A. Ohara, I. Kawamoto, M. Takeo, and S. Negoro, unpublished results).

[3] The abbreviations used are: EII, 6-aminohexanoate-dimer hydrolase; EII′, a protein with 88% homology to EII encoded on plasmid pOAD2; Ald, 6-aminohexanoate-linear dimer; Ahx, 6-aminohexanoate; Hyb-24, an EII/EII′ hybrid protein; Aoc, 8-aminooctanoate; Ani, aniline; MES, 4-morpholineethanesulfonic acid; DD, D-Ala-D-Ala; EstB, carboxylesterase from *Burkholderia*; r.m.s., root mean square; PBP, penicillin-binding protein.

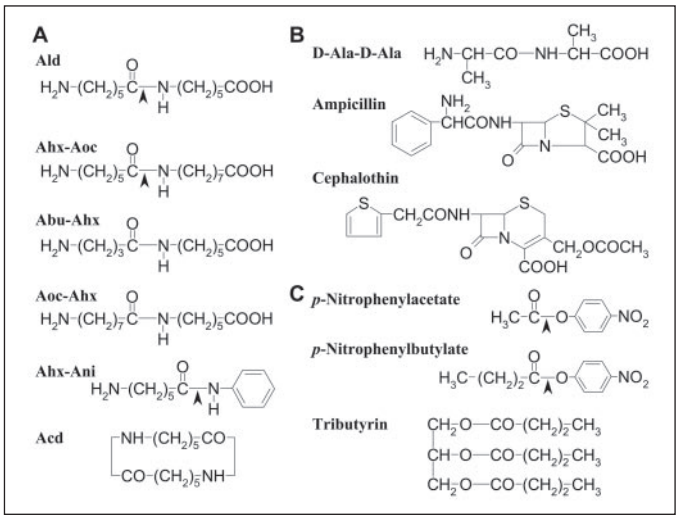

FIGURE 1. **Chemical formulas of various amide and ester compounds.** *A*, nylon oligomers: Ald, Ahx-Aoc, 4-aminobutyryl (*Abu*)-Ahx, Aoc-Ahx, Ahx-Ani, and 6-aminohexanoate-cyclic dimer (*Acd*). *B*, DD-peptide and β-lactams: D-Ala-D-Ala, ampicillin, and cephalotin. *C*, carboxylesters: *p*-nitrophenylacetate, *p*-nitrophenylbutyrate, and glyceryltributyrate (Tributyrin). The *arrows* indicate the positions that are effectively hydrolyzed.

crystallization conditions tested. In contrast, Hyb-24, which contains five amino acid replacements (T3A, P4R, T5S, S8Q, and D15G) in the EII′ protein, gave hexagonal-shaped crystals appropriate for x-ray crystallographic study.

In this paper, we performed x-ray crystallographic analysis of Hyb-24 and compared the protein fold with 5655 proteins in the Protein Data Bank. Moreover, we identified the amino acid residues responsible for catalytic function on the basis of the three-dimensional structures and discussed the evolution of nylon oligomer degrading enzymes.

EXPERIMENTAL PROCEDURES

Construction of a Hybrid Plasmid Expressing the Hyb-24 Protein

To construct plasmid pHY3, which expresses high levels of the EII′-type protein (Hyb-24), the 1,344-bp DNA fragment containing the *nylB* gene was initially amplified by PCR using *Ex Taq* DNA polymerase (Takara Co.). Two primers, NYL3 (5′-GCCGAGGCCATGGGCTACATCGATCTC-3′) and NYL2 (5′-CCACCGCGTCAGGCAGTCGCAGGATCCA-3′), which annealed to 81 bp upstream and 31 bp downstream of the *nylB* gene, respectively, were used for the PCR. The amplified fragment was ligated with pT7BlueT-Vector (Novagen), and plasmid pTA1 was obtained. The 1,441-bp EcoRI-HindIII fragment obtained from pTA1 was ligated with pKP1500 (10), which had been digested with EcoRI and HindIII, and plasmid pKT1 was obtained. To construct plasmid pHY3, the 1,212-bp PvuII-HindIII fragment containing the *nylB* gene in the plasmid pKT1 was replaced with the fragment containing the 1,212-bp PvuII-HindIII fragment containing the *nylB*′ region. The gene product (Hyb-24) included the five amino acid replacements in the EII′ sequence (*i.e.* T3A (from Thr (EII′ type) to Ala (EII type) at position 3), P4R, T5S, S8Q, and D15G) but had the EII′ level of activity.

Enzyme Purification

Hyb-24 was purified from *Escherichia coli* KP3998 cells (10) containing pHY3. Cell extracts obtained by ultrasonication were treated with successive column chromatography on anion exchange (Hi-Trap Q-Sepharose), gel filtration (Sephacryl S-200 HR), and anion exchange (Hi-Trap Q-Sepharose). The enzymes at the final stage were judged to be homogeneous from the SDS-PAGE and light scattering diffraction pattern.

Crystallographic Analysis

Crystallization and Data Collection of Diffraction—The purified Hyb-24 was crystallized by sitting drop vapor diffusion from 0.1 M MES buffer, pH 6.5, ammonium sulfate (2.0–2.2 M), lithium sulfate (0.1–0.2 M) at 10 °C to a final size of about 0.3 × 0.3 × 0.3 mm (2 ml of sample/2 ml of reservoir solution). Crystals belonging to the hexagonal space group $P3_221$ with unit cell parameters $a = 96.37$ Å and $c = 113.09$ Å were obtained in 2 days.

Heavy atom derivatives were prepared by soaking the crystals for 24 h in cryoprotectant solution containing 0.1 mM methylmercuric chloride. Cryocooling was performed by blowing cold nitrogen steam onto the crystals. Diffraction data sets of the frozen crystals were collected at 100 K using the Beamline BL44B2 (SPring-8, Hyogo, Japan) equipped with an area detector system (ADSC Quantum 210) at 1.8 Å resolution for both the native and derivative crystals. The following parameters were chosen for data collection: wavelength, 1.0000 Å; crystal to detector distance, 150 mm; oscillation range per image, 1°. Integration of reflections was performed using the HKL2000 program package.

Phase Determination, Model Building, and Crystallographic Refinement—The Hyb-24 structure was determined by the single wavelength anomalous diffraction method using a methylmercuric chloride derivative and refined against diffraction data extending to 1.8 Å resolution. Initial phase parameters were determined using the program SHARP (11). The electron density map was of sufficient quality to trace the entire molecule with ARP/wARP (available on the World Wide Web at www.embl-hamburg.de/ARP/) automatically except for two regions (*i.e.* amino acids at position 53–56 and 169–174). Region 53–56 was considered to be related to domain swapping, because the distance between the amino acids at positions 52 and 57 was estimated to be more than 35 Å, which was too far from the ordinary values. However, if domain swapping was assumed, the distance was estimated to be a more reasonable value of 12 Å. The initial model was then used for positional and B-factor refinement with the program CNS (13) with several cycles of manual model rebuilding by XFIT (14) to give an *R*-factor and *R*-free of 18.5 and 20.3%, respectively. The Ramachandran plot showed no residues in a disallowed region.

Preparation and Purification of the His-tagged EII Mutant Enzymes

To construct a plasmid expressing the His-tagged EII enzymes with Asp^{181} to Glu, Asn, His, and Lys substitutions, the 668-bp BglII-AatII fragment of Hyb-2E, Hyb-2N, Hyb-2H, and Hyb-2K (15) were combined with the 3,148-bp BglII-AatII fragment of pKT2 (a hybrid plasmid that expressed high levels of the EII gene from the *tac* promoter of plasmid pKP1500). To fuse the His-tagged region to the N terminus of each mutant enzyme, the 1,204-bp fragments were amplified by PCR using the primers FHisNYL-1 (5′-CGGAGAGCATGCTTGAACGCACGTTCCACCGGCCAGC-3′) and RHisEII-1 (5′-GGAGCGAAAGCTTCTACACTGCGTCGAGCGCGCGG-3′). The amplified fragments were expressed in *E. coli* JM109 (16) using the expression vector pQE-80L (Qiagen). The His-tagged enzymes were purified by conventional methods (16).

Enzyme Assay

To measure the Ald-hydrolytic activity, enzyme reactions were carried out in 20 mM phosphate buffer (pH 7.0) containing 10 mM Ald at 30 °C, and the reaction product, Ahx, was analyzed by reverse phase high pressure liquid chromatography (8). To measure the esterolytic

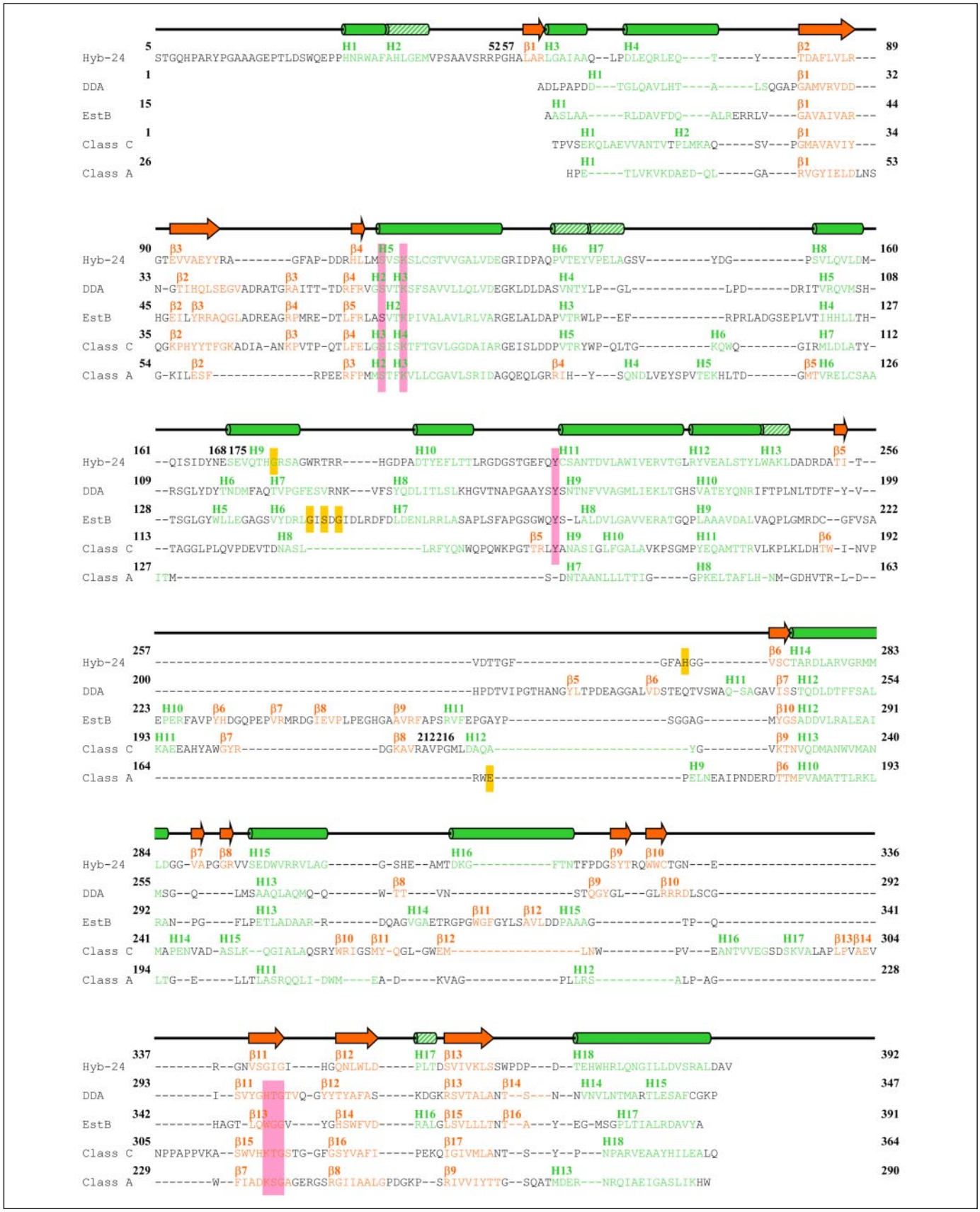

FIGURE 2. **Multiple three-dimensional alignment of Hyb-24, DD-peptidase (*DDA*; Protein Data Bank code 3PTE) from *Streptomyces* sp. (18), EstB carboxylesterase (Protein Data Bank code 1CI8) from *Burkholderia gladioli* (20), class C β-lactamases (Protein Data Bank code 1GCE) from *Enterobacter cloacae* (33), and class A β-lactamase (Protein Data Bank code 1BTL) from *E. coli* (26).** Helices and β-strands of Hyb-24 shown in Fig. 3*A* are illustrated at the *top* with *green cylinders* (α-helix), *green shaded cylinders* (310-helix), and *orange arrows* (β-strands). Multiple three-dimensional alignment was carried out using the method of secondary structure matching (48), and the secondary structures are shown

activity, reactions were carried out in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM *p*-nitrophenylacetate and *p*-nitrophenylbutyrate, and release of *p*-nitrophenol was monitored by absorbance at 400 nm ($e = 6{,}710\ M^{-1}\ cm^{-1}$).

Enzyme activity for Ald and D-alanyl-D-alanine (D-Ala-D-Ala) were measured qualitatively by TLC. 75 μl of the purified enzyme (EII, 0.1 and 1.5 mg/ml; Hyb-24, 1.5 mg/ml) was mixed with an equal volume of 20 mM Ald or 20 mM D-Ala-D-Ala. After the reactions were carried out at 30 °C, 25-μl aliquots were sequentially sampled, and the reactions were stopped by heating in boiling water for 3 min. Then the reaction mixtures (1 μl) were spotted onto a silica gel plate. The samples were developed by solvent mixture (1-propanol/water/ethyl acetate/ammonia = 24:12:4:1.3), and then degradation products were detected by spraying with 0.2% ninhydrin solution (in butanol saturated with water).

SDS-PAGE and Nucleotide Sequencing

The concentrations of samples were adjusted to $A_{280} = 10$, 20 μl of the sample was applied to SDS-polyacrylamide gel, and electrophoresis was carried out by conventional methods (16). Nucleotide sequences were determined by the dideoxy method (16) using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences).

RESULTS AND DISCUSSION

Overall Structure of Hyb-24 Protein

The Hyb-24 enzyme consists of a single polypeptide chain of 392 amino acid residues (Fig. 2) (6, 8). The overall structure of the molecule is divided into two domains, the α and α/β domains (Fig. 3*A*). The α domain contains eleven helices (H1, H2, and H5–H13) and three antiparallel β-strands (β_4, β_5, and β_6). The central helix (H5) is surrounded by six helices (H8 and H11–H15). The α/β domain consists of a central eight-stranded antiparallel β-sheet (β_1–β_3 and β_9–β_{13}) flanked by one long carboxyl-terminal helix (H18) and three other helices (H3, H4, and H16) on one face and two helices (H14 and H15) on the opposite face. In the electron density map, 415 water molecules were assigned and included in the final refined model.

Structural and Functional Comparison with Other Proteins in the Protein Data Bank

A homology search based on the Hyb-24 structure was carried out using the DALI program (17). Although the sequence identity between Hyb-24 and the proteins in the penicillin-recognizing family of serine-reactive hydrolases is low, ranging from 10 to 19% (Fig. 2 and TABLE ONE), their overall structures were very similar (Fig. 3). DD-peptidase (18, 19) and carboxylesterase (EstB) (20) possessed the highest and second highest Z-scores, respectively. In addition, class A β-lactamases (21–30) with relatively high Z-scores have been thoroughly studied in this family. Accordingly, the following experiments and discussions focus mainly on a comparison between the nylon oligomer hydrolases (EII, Hyb-24), DD-peptidase, carboxylesterase, and class A β-lactamase.

Comparison of Overall Structures

DD-peptidase—The structurally superimposable regions between Hyb-24 and DD-peptidase comprise 277 amino acid residues, and the r.m.s. deviations of the superimposed C_α atoms was calculated to be 2.8 Å. Major structural differences observed in Hyb-24 are as follows (Fig. 3*B*). (i) The 61 residues from the N terminus, including H1, H2, and β1, are present in Hyb-24. (ii) Helix H9 including Gly^{181} in Hyb-24 is divided into two helices (H6 and H7) in DD-peptidase. DD-peptidase has no corresponding residues at the position of Gly^{181} of Hyb-24, although it is located in the vicinity of the H7 of DD-peptidase. As described below, however, replacement of Gly^{181} in Hyb-24 by Asp highly increases the nylon oligomer-degrading activity, suggesting that Asp^{181} can be a substrate binding site in the nylon oligomer-degrading enzyme (Fig. 2) (15, 31). (iii) The regions, including β_5-β_6-H11 in DD-peptidase, are absent in Hyb-24.

EstB—The structurally superimposable regions between Hyb-24 and EstB comprise 264 amino acid residues, and the r.m.s. deviation of the superimposed C_α atoms was calculated to be 2.7 Å. Major structural differences observed in Hyb-24 are as follows (Fig. 3*C*). (i) The 62 residues from the N terminus, including H1, H2, and β_1, are present in Hyb-24. (ii) Helix H9, including the Gly^{181} residue in Hyb-24, is divided into two helices (H5 and H6) in EstB. (iii) One β-strand (β_5) is present, and 42 residues, including H10-β_6-β_7-β_8-β_9-H_{11}, are absent in EstB. EstB belongs to family VIII in eight families of esterases (32), according to the conserved sequence motifs and biological properties. A short esterase/lipase consensus sequence "Gly-*X*-Ser-*X*-Gly" found in the α/β hydrolase superfamily (32) is modified to "Trp-Arg-Thr-Arg-Arg" in Hyb-24 (Fig. 2).

Class A β-Lactamase—Comparison between the amino acid sequences revealed only 11% of strict identity between the Hyb-24 enzyme and class A β-lactamases (TABLE ONE). Although the total number of amino acids and number of helices and β-strands are quite different between the Hyb-24 and class A β-lactamase, their three-dimensional structures are similar (Fig. 3*D*). The structurally superimposable regions comprise 201 amino acids, and the r.m.s. deviation of the superimposed C_α atoms was 3.3 Å. Major differences observed in Hyb-24 are summarized as follows (Fig. 3*D*): (i) the presence of N-terminal 65 residues including H1, H2, β_1, and a part of H3; (ii) the presence of a large insertion including H9-H10, including Gly^{181}; (iii) insertion of 52 residues including H9-H10 between positions 129 and 130 in β-lactamase; (iv) the absence of the Ω loop, which contains the class A β-lactamase-specific "Glu-*X*-Glu-Leu-Asn" motif; (v) alteration of the sequence in the "KTG box" (Lys-Ser/Thr-Gly) located at β_7 in β-lactamase to Gly-Ile-Gly.

Other proteins in the penicillin-recognizing family of serine-reactive hydrolases exhibited similar folds with some variations in the width of the central β-sheet, class C β-lactamase (nine strands) (33–37) and class D β-lactamase (seven strands) (38, 39), although detailed comparisons were not carried out.

Comparison of Substrate Specificity

To examine the activity of the EII and Hyb-24 proteins on substrates recognized by penicillin-recognizing enzymes, we assayed the activity on D-Ala-D-Ala (DD-peptidase activity) (Fig. 4), and *p*-nitrophenylacetate (C2-ester) and *p*-nitrophenylbutyrate (C4-ester) (carboxylesterase activity) (TABLE TWO). TLC analyses demonstrated that no product (D-Ala) was obtained from D-Ala-D-Ala, even after continuing the reac-

as *green* (helix) or *orange* (β-strand) *letters*. The numbering of EstB esterase and class A β-lactamase is the same as the numbering registered in the Protein Data Bank (20–30). Some amino acid residues in conserved motif sequences are marked with a *pink color*: "Ser-X-X-Lys" β-lactamase motif (Ser^{112}/Lys^{115} (Hyb-24), Ser^{62}/Lys^{65} (DD-peptidase), Ser^{75}/Lys^{78} (EstB esterase), Ser^{64}/Lys^{67} (class C β-lactamase), and Ser^{70}/Lys^{73} (class A β-lactamase)); conserved Tyr (Tyr^{159} (DD peptidase), Tyr^{181} (EstB esterase), and Tyr^{150} (class C β-lactamase)); "Lys-Ser/Thr-Gly" (KTG box, class A β-lactamase, and class C β-lactamase); "Gly-*X*-Ser-*X*-Gly" esterase/lipase consensus sequence (EstB esterase). Amino acid residues essential for the Ald-hydrolytic activity (Gly^{181}/His^{266} in EII′ and Hyb-24; Asp^{181}/Asn^{266} in EII) and β-lactamase (Glu^{166} in class A β-lactamase) are marked with an *orange color*. Since the residues of the regions of 53–56 (Val-Asn-Ala-Pro) and 169–174 (Asp-Tyr-Val-Asp-Pro-Ala) of Hyb-24 were not able to fit in the electron density map due to poor electron density distributions, they were excluded from the three-dimensional alignment.

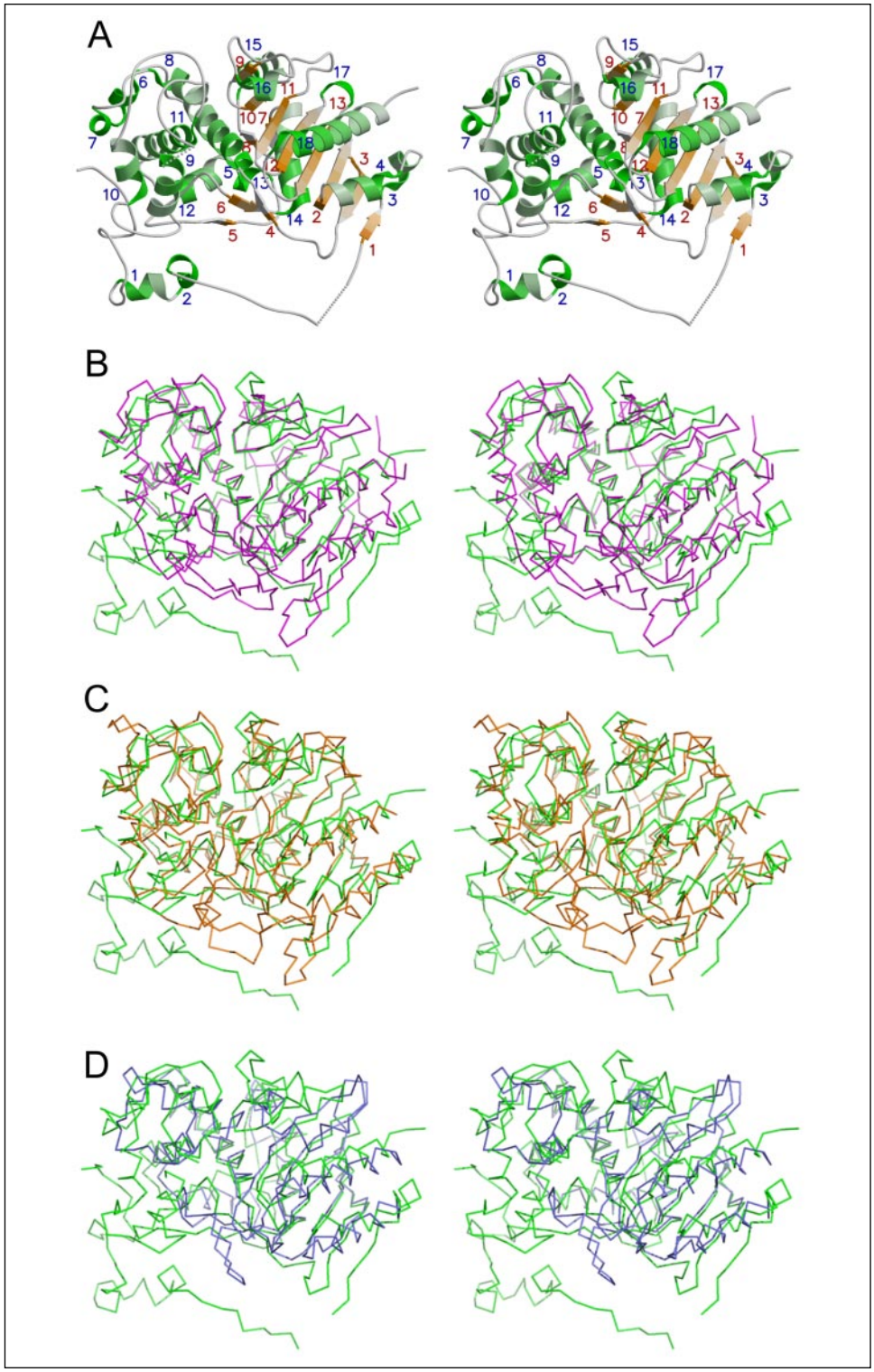

FIGURE 3. **Stereoviews of the overall structures of Hyb-24.** *A*, *ribbon diagram* of Hyb-24. α-helices and β-strands are *colored* in *green* and *orange*, respectively, with the *number* shown in Fig. 2. The *left portion* of the molecule is assigned as the α domain, and the *right portion* is assigned as the α/β domain. *B–D*, superimposition of Cα traces of Hyb-24 (*green*) with DD-peptidase (Protein Data Bank code 3PTE, *magenta*) (*B*), EstB carboxylesterase (Protein Data Bank code 1CI8, *orange*) (*C*), and class A β-lactamase (Protein Data Bank code 1BTL, *blue*) (*D*). Superimposition was carried out using the conventional methods (48). Figures were generated using MOLSCRIPT (49) and RASTER3D (12).

tion for 48 h using 0.75 mg/ml EII or Hyb-24 enzymes. Product (Ahx) was detected from Ald after only a 15-min reaction time using the wild-type EII enzyme (0.05 mg/ml). This suggests that the DD-peptidase activity in EII is less than 0.05% of the Ald-hydrolytic activity. Moreover, enzyme assay using purified EII demonstrated that the enzyme has no significant level of β-lactamase activity toward ampicillin or cephalothin (data not shown).

In contrast, the EII and Hyb-24 enzymes possessed hydrolytic activity toward the C2-ester (6.4 μmol/min (unit)/mg of protein (EII); 4.8 units/mg of protein (Hyb-24)) and lower activity toward the C4-ester (22–54% of the values toward the C2-ester) (TABLE TWO). Moreover, *E. coli* clones harboring the EII gene (*nylB*) or Hyb-24 gene (*nylB24*) produced clear halos on the LB-Tributyrin plate, suggesting that these enzymes hydrolyze the ester linkages in the glyceryl tributyrate (data not shown). Since EstB esterase hydrolyzes C4–C6 fatty acid esters (20), we have concluded that the EII and Hyb-24 enzymes are structurally and functionally related to EstB esterase.

TABLE ONE

Search for proteins with analogous folds using the program DALI

Z, Z-score (strength of structural similarity in S.D. above expected); r.m.s. deviation, positional root mean square deviation of superimposed C_α atoms in Angstroms; LALI, total number of equivalenced residues; LSEQ2, length of the entire chain of the equivalenced structure; %IDE, percentage of sequence identity over equivalenced positions.

Protein Data Bank code	Z	r.m.s. deviation	LALI	LSEQ2	%IDE	Protein
3PTE	26.7	2.8	277	347	16	DD-carboxypeptidase/transpeptidase
1CI8-A	24.1	2.7	264	377	19	EstB
1GCE-A	21.7	3.1	259	361	16	β-Lactamase (Cephalosporinase)
1EI5-A	20.0	3.0	256	518	16	D-Aminopeptidase (Dap)
1E25-A	14.3	2.8	199	278	15	Extended spectrum β-lactamase per-1 mutant
1BTL	13.3	3.3	201	263	11	β-Lactamase TEM-1
1SKF	10.8	3.1	178	262	15	DD-transpeptidase
1HD8-A	10.7	3.1	177	337	16	Penicillin-binding protein 5 mutant
1TVF-A	10.5	3.0	176	369	13	Penicillin-binding protein 4 (Pbp4)
1PMD	10.5	3.9	208	675	11	PBP2x from *Streptococcus pneumoniae*
1FOF-A	10.3	3.5	186	246	10	Class D β-lactamase Oxa-10 (Oxa-10)
1MWR-A	9.0	3.6	190	609	12	Penicillin-binding protein 2a (Saupbp2a) mutant

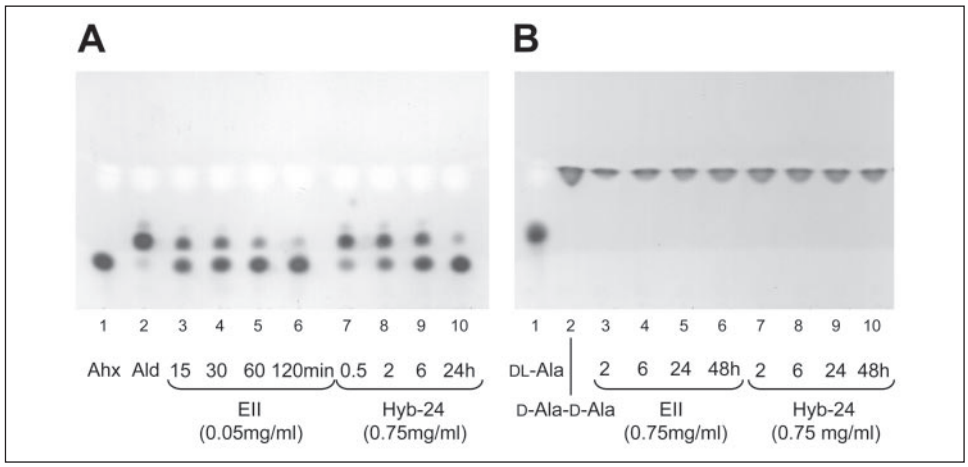

FIGURE 4. **Assay of Ald-hydrolytic (*A*) and DD-peptide-hydrolytic (*B*) activity by TLC.** *A*, *slot 1*, authentic Ahx; *slot 2*, authentic Ald; *slots 3–6*, reaction products after incubating 10 mM Ald with EII (0.05 mg/ml) at 30 °C for 15 min (*slot 3*), 30 min (*slot 4*), 1 h (*slot 5*), or 2 h (*slot 6*). *Slots 7–10*, reaction products after incubating 10 mM Ald with Hyb-24 (0.75 mg/ml) at 30 °C for 30 min (*slot 7*), 2 h (*slot 8*), 6 h (*slot 9*), and 24 h (*slot 10*). ***B***, *slot 1*, authentic DL-Ala; *slot 2*, authentic D-Ala-D-Ala; *slots 3–6*, reaction products after incubating 10 mM D-Ala-D-Ala with EII (0.75 mg/ml) at 30 °C for 2 h (*slot 3*), 6 h (*slot 4*), 24 h (*slot 5*), and 48 h (*slot 6*). *Slots 7–10*, reaction products after incubating 10 mM D-Ala-D-Ala with Hyb-24 (0.75 mg/ml) at 30 °C for 2 h (*slot 7*), 6 h (*slot 8*), 24 h (*slot 9*), and 48 h (*slot 10*). Reaction products were detected by spraying with ninhydrin. Details are described under "Experimental Procedures."

TABLE TWO

Ald-hydrolytic and esterolytic activity of the His-tagged Hyb-24 and EII mutant enzymes

The specific activity (μmol/min(unit)/mg of protein) and relative activity on the Ald, *p*-nitrophenylacetate (C2), and *p*-nitrophenylbutyrate (C4), expressed as percentages of the specific activity of the wild-type EII (in parentheses) are shown.

Enzyme	Ald-hydrolytic activity (relative activity)	Esterase activity (relative activity)	
		C2	C4
	units/mg protein	*units/mg protein*	
EII	4.16 (100)	6.39 (100)	1.44 (100)
EII-Glu181	0.035 (0.83)	2.10 (33)	0.26 (18)
EII-Asn181	0.15 (3.64)	7.56 (118)	1.23 (86)
EII-His181	0.00012 (0.003)	11.4 (178)	1.79 (125)
EII-Lys181	<0.00012 (<0.003)	13.1 (205)	1.94 (135)
Hyb-24	0.023 (0.55)	4.81 (75)	2.61 (182)

Active Site Amino Acid Residues

From the three-dimensional structure and functional analyses of the Hyb-24 protein, several amino acid residues in the cleft between the α and α/β domains were identified as the active sites.

Ser^{112}

The wild-type EII polypeptide includes 26 Ser residues. Previously, we found that EII activity was inhibited by the specific binding of diisopropylfluorophosphate to Ser^{112} (40). Moreover, site-directed

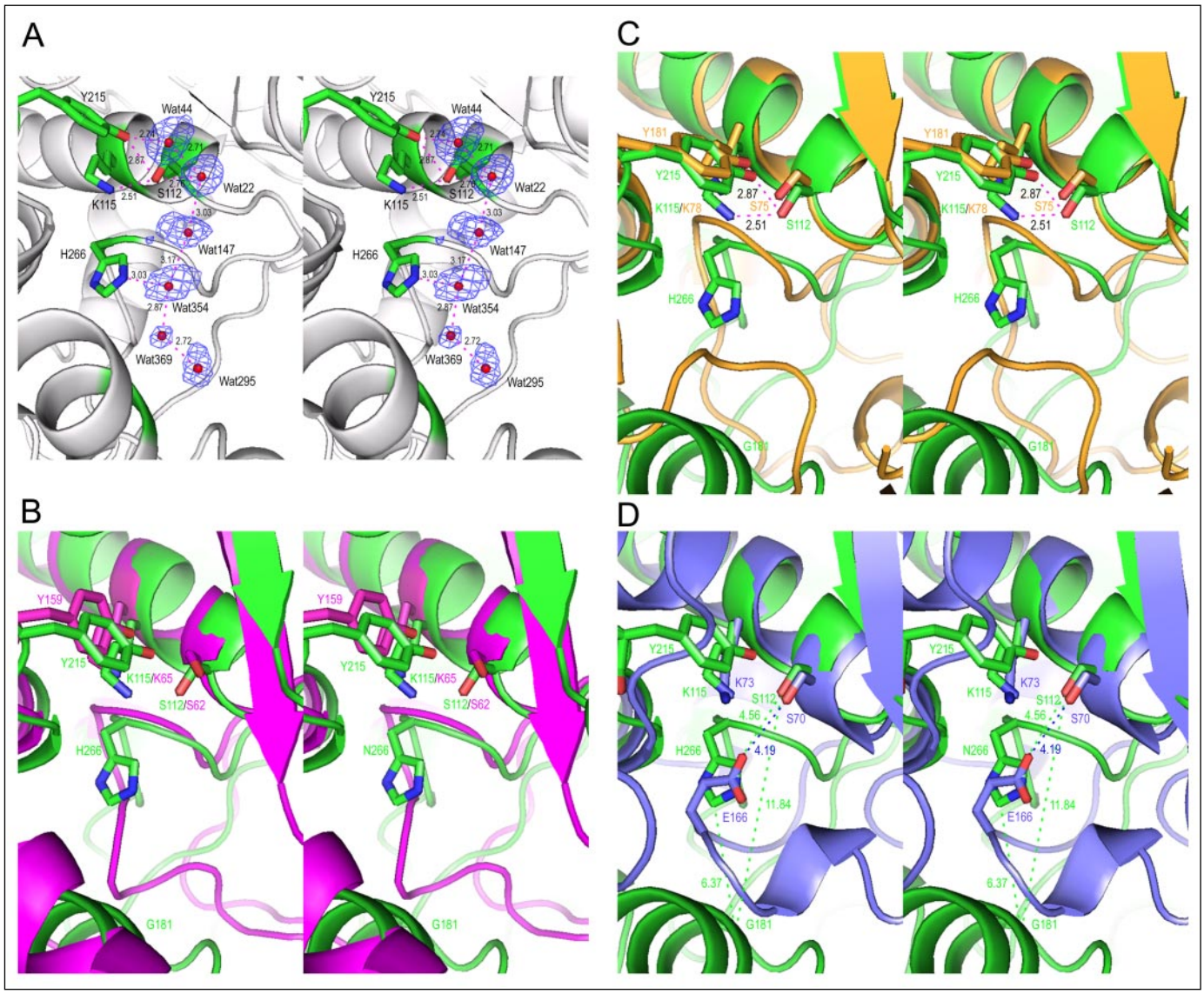

FIGURE 5. **Stereoviews of the catalytic cleft of Hyb-24.** *A*, the main-chain folding (*ribbon diagram*), side chain (*stick diagram*) of some residues (Ser^{112}, Lys^{115}, Tyr^{215}, His^{266}), and water oxygen atoms with their electron density maps are shown. Possible hydrogen bonds are indicated as *red dotted lines* with the distance in Angstroms. *B–D*, superimposition of the active site of Hyb-24 (*green*) with DD-peptidase (Protein Data Bank code 3PTE; *magenta*) (*B*), EstB carboxylesterase (Protein Data Bank code 1CI8, *orange*) (*C*), and class A β-lactamase (Protein Data Bank code 1BTL; *blue*) (*D*). Superimposition was carried out based on the transformation matrix generated by SSM (47). Figures were generated using PyMOL (available on the World Wide Web at www.pymol.org).

mutagenesis of Ser^{112} to Ala caused a drastic decrease in the enzyme activity to undetectable levels (40), suggesting the possible involvement of Ser^{112} in the catalysis. Moreover, the sequence motif common to the EII and EII′ proteins (*i.e.* Ser^{112}-Val-Ser-Lys^{115}, is located at the beginning of the α_5 helix. This motif is located at the structurally equivalent position in the penicillin-recognizing family of serine-reactive hydrolases (Fig. 5). From these results, we concluded that Ser^{112} acts as a nucleophile in the catalysis.

Tyr^{215} and Lys^{115}

The hydroxyl of the Ser^{112} probably forms an acyl intermediate with the substrate. The question is which residue acts as a general base. For class A β-lactamases, Lys^{73} in the "Ser-*X*-*X*-Lys motif" (24) or Glu^{166} in the Ω-loop (21, 23, 25, 28, 29) are candidates. To function as a general base, the pK_a of Lys^{73} should be decreased. However, two estimates of the pK_a values of Lys^{73} (*i.e.* $pK_a = 8.0-8.5$ (24) and $pK_a > 10$ (22)) lead to controversial conclusions that support or cast doubt on this hypothesis, respectively. In contrast, DD-peptidase, class C β-lactamase, and EstB esterase have no counterpart to Glu^{166} in the class A β-lactamase (Fig. 5). It is proposed that Tyr^{159} (DD-peptidase) (18), Tyr^{150} (class C β-lactamase) (37), and Tyr^{181} (EstB carboxylesterase) (20) are general bases and that Lys^{65} (DD-peptidase), Lys^{67} (class C β-lactamase), and Lys^{73} (EstB esterase) supply positive charges to stabilize the oxyanion (18, 20, 37). However, from the results of ^{13}C NMR spectroscopy using [^{13}C]Tyr-labeled class C β-lactamase, the pK_a value of the Tyr^{150} residue in class C β-lactamase of *Citrobacter freundii* is above 11, suggesting that Tyr^{150} does not directly participate in the activation of Ser^{64} as a general base (35). This suggests that positively charged Lys^{67} and Lys^{315} in the "KTG box" are involved in the reduction of the pK_a of Tyr^{150} (35).

In Hyb-24, N_ϵ of Lys^{115} and phenolic oxygen of Tyr^{215} are located 2.51 and 2.87 Å apart from the O_γ of Ser^{112}, respectively (Fig. 5, *A* and *C*). These spatial locations are similar to those of DD-peptidase (Lys^{65}/Tyr^{159}) (Fig. 5*B*) (18), EstB carboxylesterase (Lys^{78}/Tyr^{181}) (Fig. 5*C*) (20),

and class C β-lactamase (Lys^{67}/Tyr^{150}) (33). In addition, Lys^{315} in "KTG box" of class C β-lactamase was not conserved in Hyb-24 (Fig. 2).

From these considerations, Lys^{115} and Tyr^{215} are probably involved in maintaining the optimum electrostatic environment for the efficient catalytic activity in such a way that either one of these two residues functions as a general base or that both share the roles of promoting the acylation of Ser^{112}.

Asp^{181} and Asn^{266}

We have found that of the 46 amino acid alterations that differed between the EII and EII′ proteins, two amino acid replacements in the EII′ protein (*i.e.* Gly to Asp (EII-type) at position 181 (G181D) and His to Asn (EII-type) at position 266 (H266N)) are sufficient to increase the Ald-hydrolytic activity back to the level of the parental EII enzyme. The other 44 amino acid alterations have no significant effect on the increase of the activity (41). Moreover, we confirmed that a single alteration in Hyb-24 from Gly^{181} located at H9 to Asp increased the Ald-hydrolytic activity 11 times.[4] Therefore, it can be postulated that Asp^{181} has a similar role to Glu^{166} in class A β-lactamase (TEM-1). This structural alteration is apparently similar to that between the class A β-lactamase and penicillin-binding proteins (PBPs).

The class A β-lactamases and PBPs react with β-lactams to form acyl enzymes. The stability of the PBP acyl enzymes results in the inhibition of transpeptidase function (42). However, the deacylation of the β-lactamases is extremely rapid, resulting in a high turnover of β-lactam hydrolysis. This hydrolytic activity is due to the involvement of an acidic amino acid residue (Glu^{166}), which is absent in PBP (43). Actually, it was reported that a single amino acid alteration from Phe^{450} to Asp in PBP2x, which occupies the same spatial location as Glu^{166} in class A β-lactamase, confers increased resistance to cephalosporins and the deacylation rate of the PBP-acyl enzyme (42). A mutant enzyme, which has a 10^5-fold increase in the cefotaxime deacylation rate but conserved DD-peptidase activity, has been isolated (44). However, there are two major differences between the class A β-lactamase and the nylon oligomer hydrolase. (i) In class A β-lactamase (Protein Data Bank code 1BTL), the distance between O_γ of Ser^{70} and $O_{\epsilon 2}$ of Glu^{166} is 4.19 Å, and so-called "hydrolytic water" forms a bridge between the two molecules by hydrogen bonding (Fig. 5*D*). Moreover, this network is believed to be responsible for the β-lactam hydrolysis (23, 25, 28). In Hyb-24, however, the distance between C_α of Gly^{181} and the O_γ of Ser^{112} is 11.84 Å (Fig. 5*D*), which is larger than the distance (8.54 Å) between O_γ of Ser^{70} and C_α of Glu^{166} in the class A β-lactamase (Fig. 5*D*) (26, 28). (ii) G181D mutation is not sufficient to increase the activity to the level of the wild-type EII enzyme, and combination with another mutation H266N (located close to Ser^{112} (distance between His^{266}-$C_{\delta 2}$ and Ser^{112}-O_γ, 4.56 Å; see Fig. 5*D*)) is needed to increase the Ald-hydrolytic activity to the level of the wild-type EII (41).

Effect of Amino Acid Alterations at Position 181 on the Nylon Oligomer Hydrolytic and Esterolytic Activity

To examine the effects of mutation at position 181 in the wild-type EII enzyme, we replaced Asp^{181} with Asn, Glu, His, and Lys by site-directed mutagenesis and fused the mutated genes downstream of the His-tagged region in a vector plasmid pQE-80L. After purification on a nickel-nitrilotriacetic acid-agarose column, the purity of the enzymes was confirmed by SDS-PAGE. To examine the enzyme function, enzyme activity toward a nylon oligomer (Ald) and carboxyl esters was assayed (TABLE TWO). A single amino acid substitution at position 181 resulted in the drastic decrease in the Ald-hydrolytic activity, especially in the Lys^{181} mutant (<0.003% of the activity of the EII enzyme). The enzyme activity on *p*-nitrophenyl esters varied only in the range from 33 to 205% (*p*-nitrophenylacetate (C2)) and from 18 to 135% (*p*-nitrophenylbutyrate (C4)) among the mutant enzymes (TABLE TWO). Thus, the significant difference in the activity profiles raised a question as to how the EII enzyme discriminates between the esterolytic and nylon oligomer hydrolytic activity.

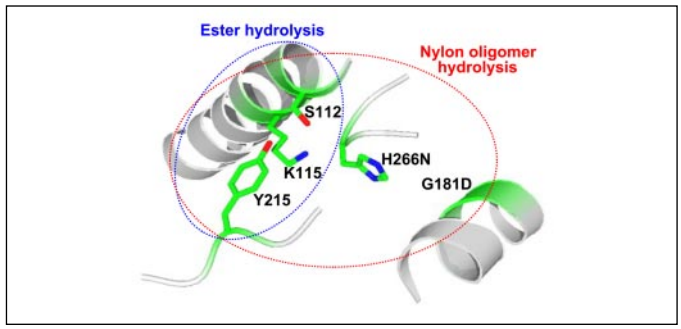

FIGURE 6. **Proposed amino acid residues constituting the catalytic center of nylon oligomer hydrolase (*red circle*) and ester hydrolase (*blue circle*).**

At the acylation step, as stated above, the hydroxyl of Ser^{112} probably acts as a nucleophile and attacks the ester-carbonyl and amide-carbonyl of the substrates. The subsequent deacylation step has to involve the attack of the acyl enzyme intermediate by a water molecule. Since EstB esterase has no counterparts to Glu^{166} in class A β-lactamase, water molecules from the solvent are considered to function as a general base for the hydrolysis of the acyl intermediate (20). Since the spatial location of the residues of Ser^{112}, Lys^{115}, and Tyr^{215} in the nylon oligomer hydrolase (Hyb-24) is very similar to that of EstB esterase (Ser^{75}, Lys^{78}, and Tyr^{181} in Fig. 5*C*) and since the esterolytic activity of EII is not so much affected by mutations at position 181, water molecules from the solvent are considered to function as a general base for the hydrolysis of the acyl intermediate. In contrast, the Ald-hydrolytic activity of EII is highly affected by substitutions at position 181, especially by substitutions to the basic amino acids (TABLE TWO). Moreover, the activity of the EII′-type enzyme is enhanced ~10-fold by the G181D substitution and ~200-fold by the G181D/H266N double substitutions (41). These results suggest that the nylon oligomer hydrolase utilizes Ser^{112}/Lys^{115}/Tyr^{215} as common active sites, both for Ald-hydrolytic and esterolytic activity, but requires at least two additional amino acid residues (Asp^{181}/Asn^{266}), specific for Ald-hydrolytic activity (Fig. 6).

Evolutionary Implication

We have previously proposed that the nylon oligomer hydrolase (EII) evolved by gene duplication from the common antecedent of EII and cryptic EII′ proteins located on the same plasmid (8). However, the following two hypotheses have been proposed. (i) The EII enzyme is specified by an alternative open reading frame from a preexisting coding sequence that originally specified a 472-residue-long Arg-rich protein and a frameshift mutation in the ancestral gene, creating a gene responsible for nylon oligomer hydrolysis (45). (ii) There is a special mechanism for protecting a nonstop frame, namely a long stretch of sequence without chain-terminating base triplets, from mutations that generate the stop codons on the antisense strand, and such a mechanism enables the nonstop frame to evolve into a new functional gene (46).

Recently, through directed evolution from Hyb-24 using PCR random mutagenesis and selection for Ald-hydrolase activity, we found that of seven clones that were enhanced in Ald-hydrolytic activity, three

[4] T. Ohki, Y. Wakitani, M. Takeo, N. Shibata, Y. Higuchi, and S. Negoro, manuscript in preparation.

clones contained the G181D mutations in common.[4] This suggests that the G181D mutation is preferentially selected during the evolution of the nylon oligomer hydrolase, when Ald-degradation has advantages in the selection.

Ser^{112} and Lys^{115} were conserved in all serine β-lactamases, and Tyr^{215} was conserved in EstB esterase, DD-peptidase, class C β-lactamase, and 6-aminohexanoate-dimer hydrolase (EII and EII′), suggesting that these residues have been conserved during the evolution. In contrast, amino acid residues corresponding to Gly^{181} in Hyb-24 have been diversified (Thr^{123} (DD-peptidase), Val^{142} (EstB esterase), Asp^{127} (class C β-lactamase)). In the case of class A β-lactamase, no amino acid residue can be aligned to Gly^{181} in Hyb-24. Similarly, amino acid residues corresponding to His^{266} in Hyb-24 have also been diversified (Gln^{229} (DD-peptidase), Gly^{274} (EstB esterase), Pro^{167} (class A β-lactamase)), and no amino acid residue can be aligned in class C β-lactamase (Fig. 1). However, because of the lack of structural similarities among the five enzymes, it has been impossible to do precise three-dimensional alignment at the regions 162–213 and 258–267 of Hyb-24. These results indicate that the G181D and H266N are amino acid alterations specific for the increase of nylon oligomer hydrolysis. Thus, the nylon oligomer-degrading enzyme (EII) is considered to have evolved from preexisting esterases with β-lactamase folds.

The structurally related proteins in the penicillin-recognizing family of serine-reactive hydrolases catalyze different distinct reactions (*i.e.* DD-transpeptidation, DD-peptide hydrolysis, β-lactam hydrolysis, and carboxyl ester hydrolysis). This illustrates how new enzyme functions evolve from a common ancestor while retaining the same basic fold. The present studies suggest a strategy to create new enzymes active toward various amide and ester compounds from an enzyme having "Ser-*X*-*X*-Lys" as a common active center.

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