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Enzymatic synthesis of nylon-6 units in organic solvents containing low concentrations of water

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ABSTRACT

NylB′ carboxylesterase, which is 88% homologous to functional 6-aminohexanoate-dimer hydrolase (NylB) from *Arthrobacter* sp., possesses trace synthetic activity [0.0004 μmol min^{-1} mg^{-1} (U/mg)] from 6-aminohexanoate (Ahx) to its oligomers in 90% *tert*-butyl alcohol. The synthetic activity and the ratio of the synthetic activity to the hydrolytic activity were significantly affected by amino acid substitutions at positions 181, 266 and 370. The synthetic activity was enhanced to 2.7 U/mg by G181D-H266N substitutions, and the activity was further enhanced in the G181D-H266N-D370Y triple mutant to a level approximately 10^4-fold greater than the parental carboxylesterase form (3.4 U/mg), which was nearly equal to the ordinary hydrolytic activity in water (type *A*-mutants). Type *A*-mutants possessed more than 50% of the 6-aminohexanoate-linear dimer (Ald)-hydrolytic activity at 0–70% *tert*-butyl alcohol, but the synthetic reaction became predominant at 85–90% *tert*-butyl alcohol. In contrast, type *B*-mutants (G181E-H266N and G181N-H266N) possessed quite low levels of Ald-hydrolytic activity (<0.01 U/mg) at 0–70% *tert*-butyl alcohol. However, both the hydrolytic and synthetic activities were enhanced at higher concentrations, and the maximum activity was obtained at 90% *tert*-butyl alcohol for both hydrolysis and synthesis. In a type *C*-mutant (R187S-F264C-D370Y), the Ald-hydrolytic activity was enhanced to approximately 80-fold that of the parental carboxylesterase, but the mutant barely demonstrated any synthetic activity. On the basis of the three-dimensional structure of the Ald-bound enzyme and a kinetic study for typical mutant enzymes, we propose that the efficient enzymatic syntheses of nylon-6 units were achieved by (i) stable binding of the 1st-Ahx at the N-terminal region with Asp181, (ii) interaction of the 2nd-Ahx at the C-terminal region with Tyr370, and (iii) motion of Tyr170 that generated a closed form in the catalytic center of Ald hydrolase.

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1. Introduction

Enzymatic catalysis in organic solvents has been recognized as an effective tool to produce various esters and peptides [1]. In reactions such as RCOOH + H_2NR' (HO-R') ⇄ RCO-NHR' (RCO-OR') + H_2O, it is possible to shift the thermodynamic equilibrium of the reaction toward product formation rather than hydrolysis by limiting the water concentration. Various attempts to produce fatty acid esters, sugar esters, and polyesters in organic solvents have been studied using the reverse reaction of hydrolysis or trans-esterification reactions, using lipases [2–8]. Generally, enzymatic synthesis of amides is less efficient than that of esters, although peptide synthesis using proteinases (such as pepsin, subtilisin, trypsin, α-chymotrypsin, and thermolysin) in organic solvents has been achieved [9–14].

In contrast to the extensive study of esters/peptides-syntheses, the production of unnatural amides by enzymatic reactions has scarcely been studied thus far. In the chemical processes that condense hexamethylenediamine and adipic acid directly (nylon 6-6 production) or ring-cleavage polymerization of ε-caprolactam (nylon-6 production), reactions at high temperature (220–300 °C) for >10 h are required to complete these polymerization reactions. In the chemical synthesis of peptides, highly reactive carboxylesters are prepared prior to the condensation reaction with the amino group to enhance the reactivity of inactive carboxyl groups with free amino groups at a lower temperature. Otherwise, condensation-reagents such as carbodiimide are generally

Abbreviations: Ahx, 6-aminohexanoate; Ald, 6-aminohexanoate-linear dimer; NylB, 6-aminohexanoate-dimer hydrolase; NylB′, a protein with 88% homology to NylB encoded by the plasmid pOAD2; Hyb-24, a NylB/NylB′ hybrid protein.

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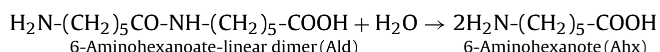
used [15–17]. However, the byproducts formed in the chemical processes are environmentally undesirable. Concerning the enzymatic system responsible for the metabolism of unnatural amide compounds, we have been studying the degradation of a byproduct from the manufacture of nylon-6, 6-aminohexanoate (Ahx)-oligomer (or nylon oligomer), by *Arthrobacter* sp. strain KI72 [18,19]. We found that the Ahx-linear dimer (Ald) is hydrolyzed by Ald hydrolase (NylB) according to the following reaction.



$$\underset{\text{6-Aminohexanoate-linear dimer (Ald)}}{H_2N\text{-}(CH_2)_5\text{-}CO\text{-}NH\text{-}(CH_2)_5\text{-}COOH} + H_2O \rightarrow \underset{\text{6-Aminohexanote (Ahx)}}{2H_2N\text{-}(CH_2)_5\text{-}COOH}$$

Biochemical study revealed that strain KI72 also produces the NylB′ protein, which is 88% homologous to NylB, and that NylB′ possesses approximately 0.5% of the level of the Ald-hydrolytic activity of NylB [20]. However, NylB′ is classified rather as a carboxylesterase, as the enzyme favors carboxylesters with short acyl chains as substrates [21–26]. A NylB/NylB′-hybrid (Hyb-24), which was suitable for x-ray crystallographic analysis, contained five amino acid replacements (T3A, P4R, T5S, S8Q, and D15G) in the NylB′ protein and has been shown to possess levels of Ald-hydrolytic activity that are similar to NylB′ [21,22] (Fig. 1). On the basis of their three-dimensional structures, NylB/NylB′ are classified as members of a penicillin-recognizing family of serine-reactive hydrolases, and they utilize Ser112-Lys115-Tyr215 residues as catalytic triads [22,23]. Two substitutions (G181D and H266N) in Hyb-24 have been shown to be sufficient to increase the level of the Ald-hydrolytic activity to that of the wild-type NylB enzyme (Fig. 1, see Hyb-24DN). In Hyb-24DN, Asp181-COO^- stabilizes the substrate binding by electrostatic interactions with Ald-NH_3^+, while Asn266 makes suitable contacts with Ald and improves the electrostatic environment in the N-terminal region of Ald [23,24]. Moreover, the D370Y substitution in Hyb-24DN, which stabilizes the C-terminal region of Ald, improves the k_{cat}/K_m value of Hyb-24DN 5-fold by stabilizing the binding at the N-terminal and C-terminal regions of the substrate [24]. Recently, we found that, by using PCR-based directed evolution to optimize Ald-hydrolytic activity from the common carboxylesterase (Hyb-24), there existed an alternative conformation involving R187S-F264C-D370Y triple substitutions, and that the activity was enhanced up to 80-fold compared to the parental Hyb-24 in the directedly evolved Hyb-S4M94 enzyme [25,26].

In most Ser-reactive hydrolases, the catalytic sites are exposed to solvent [27–30]. However, in nylon-oligomer hydrolase (Hyb-24DN and its related enzymes), Ald-binding induces a large movement in the loop region (Asn167-Val177) and a flip-flop of Tyr170, thereby resulting in the transition from an open to a closed form. This induced fit motion removes water molecules from the catalytic centers at the acylation stage [23]. In the dehydration reaction required for amide synthesis, generation of a hydrophobic environment in the catalytic center is expected to provide a suitable environment for the functioning of the synthetic reaction. Thus, the nylon-oligomer degrading enzymes provide us with a suitable system to study the biochemical basis of amide-synthetic reactions.

In this study, we examined the possibility of producing unnatural amide compounds that contain a nylon-6 unit by using various NylB/NylB′ mutants, and we have analyzed the structural requirements for effective amide-synthetic reactions.

2. Materials and methods

2.1. Enzymes, plasmids and site-directed mutagenesis

The mutant enzymes and plasmids used in this study are listed in Table 1 [21–26]. To obtain Hyb-24EN (Hyb-24 having G181E-H266N substitutions), Hyb-24NN (Hyb-24 having G181N-H266N substitutions) and Hyb-24NY (Hyb-24 having H266N-D370Y substitutions), we carried out site-directed mutagenesis using a PCR-based "modification of restriction-site (MR)" method [31], using the Hyb-24N gene (Hyb-24 having a H266N substitution) as the template DNA and the specific primers shown in Table 2.

	Enzyme	Residues (170 181 266 370)	Enzyme activity (U/mg): Hydrolysis, Assay H_A	Hydrolysis, Assay H_B	Synthesis, Assay S	Activity ratio (Assay S / Assay H_A)
	Hyb-24	YG H D	0.023	0.0002	0.0004	0.017
A	Hyb-24D	D H D	0.47	0.17	0.49	1.1
	Hyb-24DN	D N D	3.53	0.61	2.72	0.77
	Hyb-24DY	D H Y	2.21	1.27	1.36	0.61
	Hyb-24DNY	D N Y	3.60	1.32	3.41	0.95
	Hyb24FDN	FD N D	0.050	<0.001	0.050	1.0
B	Hyb-24NN	N N D	0.14	0.24	1.12	8.0
	Hyb-24EN	E N D	0.011	0.21	0.40	36.4
C	Hyb-24Y	G H Y	0.19	<0.001	<0.001	<0.005
	Hyb-24NY	G N Y	0.79	0.11	<0.001	<0.001
	Hyb-S4M94	GS CH Y (187 264)	1.86	0.89	0.008	0.004
D	Hyb-24HN	H N D	<0.001	<0.001	<0.001	-
	Hyb-24KN	K N D	<0.001	<0.001	<0.001	-

Fig. 1. The effect of amino acid substitutions on enzyme activities. Hyb-24 is a NylB/NylB′ hybrid protein constructed from conserved *Pvu*II sites located 24 amino acid residues downstream of the initiation codons and contains five amino acid replacements (T3A, P4R, T5S, S8Q, and D15G) in the NylB′ protein. Open boxes and shaded boxes represent the NylB′ and NylB regions, respectively. Amino acid residues substituted in the mutant enzymes are shown as *one-letter* codes in the open boxes. The synthesis of Ahx to Ald (assay S) and the hydrolysis of Ald to Ahx (assay H_B) were assayed in 90% *tert*-butyl alcohol/10% buffer A using purified enzymes. The Ald-hydrolytic activities of the His-tagged purified enzymes (Hyb-24NN, Hyb-24EN and Hyb-24NY) were assayed in buffer A using 10 mM Ald (assay H_A). The hydrolytic activities of the other His-tagged enzymes have been previously measured using "assay H_A" [22–26]. On the basis of the synthesis/hydrolysis activity ratio (assay S/assay H_A), the Hyb-24 mutants were classified as type *A* (ratio = 0.6–1.1), type *B* (ratio = 8–36), or type *C* (ratio < 0.01). Mutants that possessed neither detectable synthetic activity nor hydrolytic activity were classified as type *D*.

2.2. Enzyme purification and protein concentration

The mutant enzymes were expressed in *Escherichia coli* KP3998 using the pKP1500 vector and purified as described previously [21,22]. In summary, the cells (grown in a 250 ml culture of Terrific broth medium containing 25 mg of ampicillin) were harvested by centrifugation, washed with buffer A (composition: 20 mM phosphate buffer containing 10% glycerol, pH 7.3), and resuspended in 60 ml buffer A. The cells were lysed by sonication, the lysate was centrifuged, and the supernatant was loaded onto a Hi-Trap Q-Sepharose (GE Healthcare Bio-Science AB, Uppsala, Sweden) column (5 ml) equilibrated with buffer A. After washing with buffer A, the enzyme was eluted with a total of 300 ml of the same buffer containing a linear NaCl gradient from 0 to 0.5 M. The fractions containing the enzyme were pooled and concentrated to a volume of

Table 1
Enzymes and plasmids.

Abbreviations	Characteristics	Reference
Hyb-24	NylB (Wild-type 6-aminohexanoate-linear dimer (Ald) hydrolase from *Arthrobacter* sp. KI72)/NylB′ (Wild-type carboxylesterase with the β-lactamase fold from *Arthrobacter* sp. KI72, 88% homology to NylB), a hybrid protein constructed from conserved *Pvu*II sites located 24-amino acid residues downstream of the initiation codons (NylB′ containing T3A/P4R/T5S/S8Q/D15G substitutions)	[21,22]
Hyb-24D	Hyb-24 containing G181D substitution	[25]
Hyb-24N	Hyb-24 containing H266N substitution	[24]
Hyb-24Y	Hyb-24 containing D370Y substitution	[25]
Hyb-24DN	Hyb-24 containing G181D/H266N substitutions	[23]
Hyb-24DY	Hyb-24 containing G181D/D370Y substitutions	[25]
Hyb-24NY	Hyb-24 containing H266N/D370Y substitutions	This study
Hyb-24DNY	Hyb-24 containing G181D/H266N/D370Y substitutions	[24]
Hyb-24FDN	Hyb-24 containing Y170F/G181D/H266N substitutions	[23]
Hyb-24EN	Hyb-24 containing G181E/H266N substitutions	This study
Hyb-24HN	Hyb-24 containing G181H/H266N substitutions	[22]
Hyb-24KN	Hyb-24 containing G181K/H266N substitutions	[22]
Hyb-24NN	Hyb-24 containing G181N/H266N substitutions	This study
Hyb-S4M94	An Ald-hydrolytic activity-enhanced mutant obtained from Hyb-24 by 4 cycles of mutagenic PCR/DNA-shuffling (Hyb-24 containing A61V/A124V/R187S/F264C/G291R/G338A/D370Y substitutions)	[26]
pHY3	A hybrid plasmid composed of the vector pKP1500 and a 1.4-kb *Eco*RI/*Hind*III fragment containing the Hyb-24 gene	[21,22]
pS4M94	A hybrid plasmid composed of the vector pKP1500 and a 1.4-kb *Eco*RI/*Hind*III fragment containing the Hyb-S4M94 gene	[26]

1 ml using a Centriprep YM-10 (Millipore Inc.). The concentrated enzyme was applied onto a Sephacryl S-200 High-Resolution gel-filtration (GE Healthcare Bio-Science AB, Uppsala, Sweden) column that had been equilibrated with buffer A. The same buffer was used to elute the enzyme. The fractions containing the Hyb-24 related enzyme (approximately 1 ml) were pooled, and the enzyme solution was again applied onto a Hi-Trap Q-Sepharose column. The enzyme was eluted as described above. All purification and concentration steps were carried out at 4 °C. Typically, a yield of 10 mg of enzyme per 250 ml of culture was achieved.

For Hyb-24NN, Hyb-24EN and Hyb-24NY, a His-tagged region was fused to the N-terminus of the mutant enzymes using the pQE-80L vector (Qiagen GmbH, Hilden, Germany). The His-tagged enzymes were expressed in *E. coli* JM109 and purified to homogeneity by conventional methods [22].

The protein concentrations were assayed using the Lowry-Folin method.

2.3. Enzymatic synthesis and hydrolysis of Ahx oligomers

Synthetic activities were assayed at 30 °C using 10 mM Ahx (Nakarai tesque, Kyoto, Japan) in 90% *tert*-butyl alcohol containing 10% buffer A (Fig. 2A). For the qualitative detection of synthetic activity, the samples were developed using a solvent mixture (1-propanol:water:ethylacetate:ammonia = 24:12:4:1.3), and the degradation products were detected by spraying with 0.2% ninhydrin solution [22]. For quantitative assays, 10 μl aliquots were sequentially sampled, fractionated using a C_{18} reverse phase HPLC column (TSK-GEL ODS-80Ts, TOSOH Co., Tokyo, Japan), and the increase in Ald was monitored by following the absorbance at 210 nm (hereafter designated as “assay S”). To study the kinetics of the synthetic reaction, we assayed the enzyme activity using “assay S”, except that various concentrations of Ahx were used. Kinetic parameters (k_{cat} and K_m values) were evaluated by directly fitting the Michaelis–Menten equation to the data using GraphPad prism, version 5.01 (GraphPad, San Diego, CA USA). The k_{cat} values were expressed as the turnover numbers per subunit (M_r of the subunit: 42,000). As 10 mM Ahx generates 5 mM Ald stoichiometrically, the reverse reaction (*i.e.*, the hydrolysis of Ald) in 90% *tert*-butyl alcohol/10% buffer A was performed using 5 mM Ald (this was chemically synthesized in our laboratory), and decreases in the levels of Ald were quantified using HPLC (designated hereafter as “assay H_B”).

We have confirmed that adding the histidine tag to the N-terminus of Hyb-24-related enzymes barely affected the original Ald-hydrolytic activity. The hydrolytic activity of His-tagged purified enzymes (except for Hyb-24NN, Hyb-24EN and Hyb-24NY) has been identified in water (buffer A without organic solvent) for 10 mM Ald at 30 °C (assay H_A) [22–26]. For Hyb-24NN, Hyb-24EN and Hyb-24NY, the hydrolytic activity was similarly assayed using the purified His-tagged enzymes using “assay H_A”. We used the activity ratio (assay S/assay H_A) as a parameter to describe the amide-synthetic ability and classification of the mutants (types *A–D*).

One unit of enzyme activity was defined as the amount of enzyme capable of catalyzing the synthesis (assay S) or hydrolysis (assay H_A and H_B) of 1 μmol of amide linkage in 1 min.

2.4. The three-dimensional model of the protein structure

The structure of the Hyb-24DNY-A^{112}/Ald complex (PDB ID code: 2ZMA) [24] has previously been determined. The figure showing the three-dimensional model was generated using the “MolFeat” program (ver. 3.6, FiatLux Co., Tokyo, Japan).

Table 2
Primer DNA used for site-directed mutagenesis of the Hyb-24N gene.

Mutation	Primer	Sequence
G181E	RGmutE1	5′-AGCCGGCCGAGCGTTCGTGGGTCTGCACC-3′
G181N	RGmutN1	5′-AGCCGGCCGAGCGGTTGTGGGTCTGCACC-3′
D370Y	RDmutY1	5′-GTGTAGGGATCGGGCCACG-3′

Site-directed mutagenesis was carried by a “modification of restriction-site (MR)” method [31]. Mutated sites in the primer sequences are underlined.

3. Results

3.1. Optimization of the solvent system for efficient amide synthesis

To achieve an efficient condensation reaction between free amino- and carboxyl-group in organic solvents, the choice of a suit-

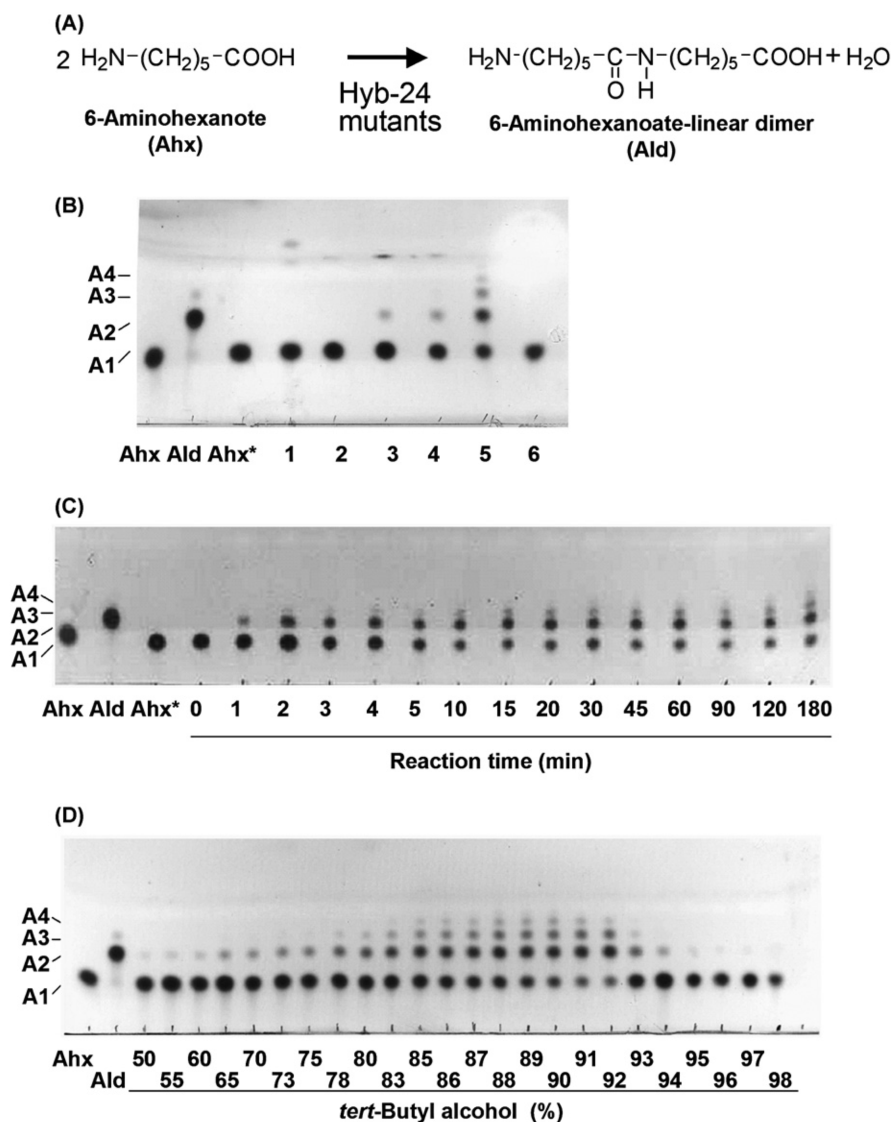


Fig. 2. TLC analysis of the amide synthesis catalyzed by Hyb-24DN. (A) The reaction scheme of Ald synthesis. (B) Enzyme reactions were performed in 90% solvent/10% buffer A using Hyb-24DN (0.3 mg/ml) and 10 mM Ahx, and the synthesized products were analyzed by TLC. Slot 1, methyl alcohol; slot 2, ethyl alcohol; slot 3, *n*-propyl alcohol; slot 4, *n*-butyl alcohol; slot 5, *tert*-butyl alcohol; slot 6, dimethylsulfoxide. (C) Enzyme reactions were performed in 90% *tert*-butyl alcohol using Hyb-24DN (0.3 mg/ml) and 10 mM Ahx. Reaction mixture (1 μl aliquot) was sequentially removed and spotted onto a TLC plate. (D) Enzyme reactions were performed in various concentrations (50–98%) of *tert*-butyl alcohol dissolved in buffer A using Hyb-24DN (0.3 mg/ml) and 10 mM Ahx. After a 1 h reaction, the reaction mixture (1 μl aliquot) was spotted onto a TLC plate. Authentic Ahx and Ald (10 mM each) were spotted as controls. Ahx (10 mM) was dissolved in 90% *tert*-butyl alcohol and analyzed to determine the effect of the solvent in the sample on the mobility of Ahx (slot Ahx*). The positions of the spots for Ahx (A1), Ald (A2), Ahx-trimer (A3), and Ahx-tetramer (A4) are shown in panels B–D.

able solvent is often critical. Although the optimum pH for the hydrolytic reaction of NylB is known to be pH 8–9 [32,33], the enzyme activity is rather stable in 20 mM phosphate buffer containing 10% glycerol at pH 7.3 (buffer A). Therefore, to check the suitability of a potential solvent system, we analyzed the conversion of Ahx to synthesized product using Hyb-24DN (0.3 mg/ml) in various organic solvents (30 kinds) that were dissolved or emulsified in buffer A (Fig. 2).

TLC analysis of the reaction products revealed that synthesized products were identified in reaction mixtures that contained 90% of propyl alcohol (*n*-, *iso*-), butyl alcohol (*n*-, *iso*-, *tert*-), or pentyl alcohol (*n*-, *iso*-) (Fig. 2B). In the reaction mixture containing *tert*-butyl alcohol, Ald was formed within a 1 min reaction, whereas linear Ahx trimers and tetramers were formed sequentially (Fig. 2C), thereby demonstrating that the oligomers were synthesized by exo-type modes. However, no detectable amounts of products were identified for 23 kinds of solvent systems, including mixtures that contained 90% of methanol, ethanol, 2-methoxyethanol, acetonitrile, glycerin, acetone, ethyleneglycol, diethyleneglycol, triethyleneglycol, polyethyleneglycol, dimethylsulfoxide, 2-ethylhexyl alcohol, *n*-octyl alcohol, benzyl alcohol, *n*-hexane, *n*-heptane, chlorobenzene, methyl-*iso*-butylketone, di-*iso*-butylketone, ethylenedichloride, decahydronaphthalene, dioctylphthalate and di-*n*-butylbsebacate. We found that the addition of these solvents to the enzyme solution resulted in precipitation of the enzyme, thereby eliminating functional enzymatic activity from the solution. To examine the concentration dependence of *tert*-butyl alcohol on the enzyme activity, we performed the synthetic reaction with Hyb-24DN (0.3 mg/ml) in various concentrations of *tert*-butyl alcohol. We found that efficient product formation was achieved within the concentration range of 85–92% alcohol, but abruptly decreased at higher concentrations (especially at >93%) (Fig. 2D).

The initial rates of synthetic activity of Hyb-24DN (0.01 mg/ml) were determined by quantifying the increase of Ald using HPLC

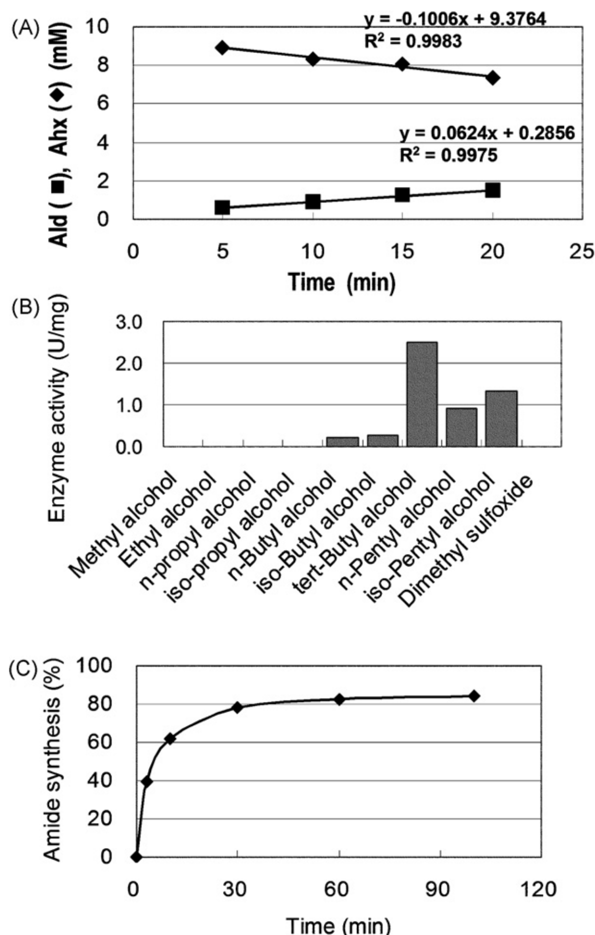


Fig. 3. HPLC analysis of amide synthesis catalyzed by Hyb-24DN. (A) The enzyme reaction was carried out in 90% *tert*-butyl alcohol/10% buffer A using Hyb-24DN (0.01 mg/ml) and 10 mM Ahx, and the initial rates of the synthetic reactions were quantified by the increase of Ald (■) (assay S) and by the decrease of Ahx (♦). (B) The initial rates of the synthetic reactions in 90% organic solvent/10% buffer A using Hyb-24DN (0.01 mg/ml) and 10 mM Ahx were quantified by the increase of Ald. (C) The enzyme reaction was carried out using Hyb-24DN (0.3 mg/ml) in 90% *tert*-butyl alcohol/10% buffer A using 10 mM Ahx, and the remaining Ahx in the reaction mixture was quantified using HPLC. Amide synthesis was expressed as the percentage of the amount of incorporated Ahx divided by the initial amount of Ahx.

(Fig. 3A). The observed reaction rates [2.72 μmol min^{-1} mg^{-1} (U/mg)] that were found in 90% *tert*-butyl alcohol agreed well with the initial rates identified by the decrease of Ahx (2.55 U/mg). Among the solvent systems using the three isomers of butyl alcohol (90% each), the highest activity was found when using *tert*-butyl alcohol. Only approximately 10% of the enzymatic activity was identified when using *n*- and *iso*-butyl alcohol. Similarly, 40–50% of the activity was found in *n*- and *iso*-pentyl alcohol (Fig. 3B). At 85% *tert*-butyl alcohol, the initial rates of the synthetic reaction exceeded the rates of the hydrolytic reaction, but the activity drastically decreased at 95% *tert*-butyl alcohol, due to precipitation of the enzyme (Fig. 4A).

In 90% *tert*-butyl alcohol, the maximum yield (approximately 82%) was obtained within 30 min using Hyb-24DN (0.3 mg/ml), and this conversion ratio was maintained stably even after extending the reaction time to 100 min (Fig. 3C). Moreover, the solvent (90% *tert*-butyl alcohol) was mixed homogeneously with water and did not form a biphasic layer. On the basis of these findings, we established 90% *tert*-butyl alcohol/10% buffer A as the solvent system for the standard assay for the synthetic reaction (designated "assay S").

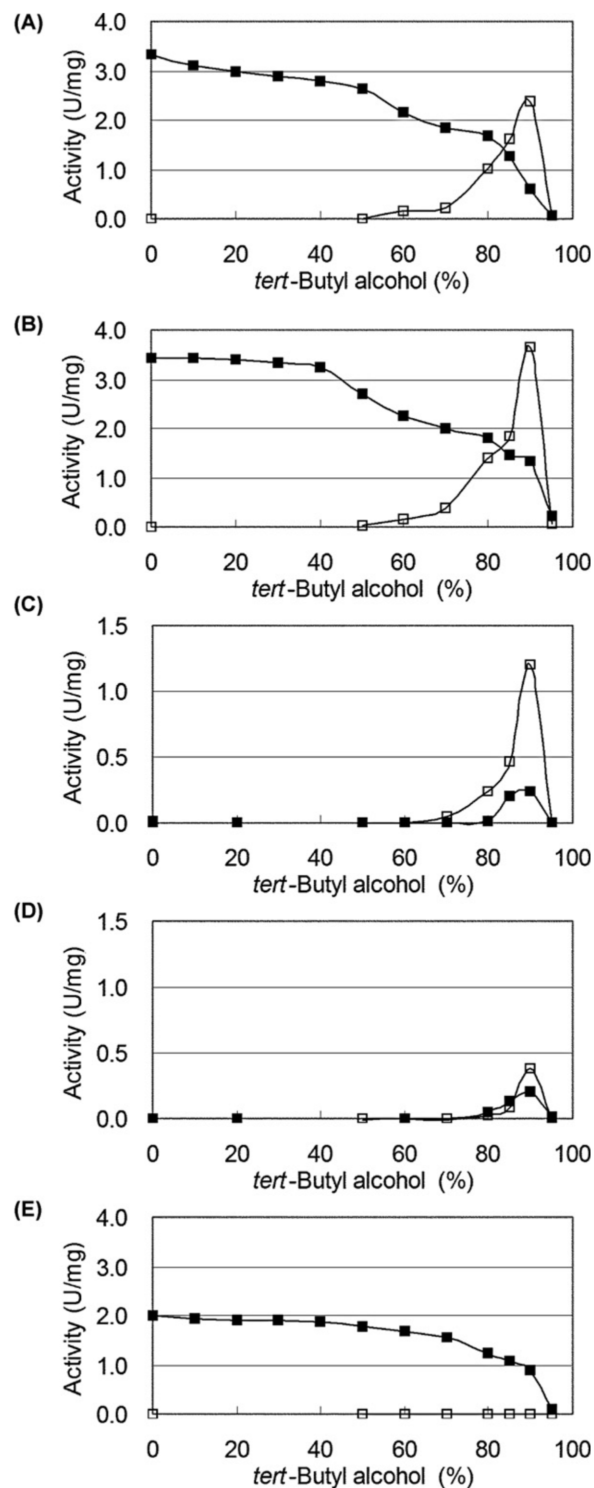


Fig. 4. The effect of the concentration of *tert*-butyl alcohol on the initial rates of the synthetic and hydrolytic reactions. Enzyme reactions were performed in various concentrations of *tert*-butyl alcohol dissolved in buffer A using Hyb-24 mutants (A–E) and 10 mM Ahx, and the initial rate of the synthetic reactions were quantified by the increase of Ald (□). Enzyme reactions were performed in various concentrations of *tert*-butyl alcohol using Hyb-24 mutants (A–E) and 5 mM Ald, and the initial rate of the hydrolytic reactions were quantified by the decrease of Ald (■). (A) Hyb-24DN, (B) Hyb-24DNY, (C) Hyb-24NN, (D) Hyb-24EN, and (E) Hyb-S4M94. The hydrolytic and synthetic activity at each *tert*-butyl alcohol concentration is also shown in supplementary Tables S1 and S2, respectively.

3.2. The effect of amino acid alterations on the amide-synthetic activity

On the basis of the three-dimensional structures of Hyb-24 and its mutants, we have proposed that (i) Asp181-COO^- stabilizes the binding of substrate through electrostatic interactions at the N-terminal region of Ald, (ii) Asn266 makes suitable contacts with Ald and improves the electrostatic environment, and (iii) Tyr370 stabilizes the binding of Ald through hydrogen-bonding/hydrophobic interactions at the C-terminal region of Ald [22–26]. Therefore, we first examined the amide-synthetic activity for enzymes having mutations at positions 181, 266 and/or 370 (Fig. 1). As expected, trace synthetic activity (0.0004 U/mg) that was originally retained in the parental Hyb-24 enzyme was significantly affected by substitutions at these substrate-binding sites. Interestingly, as described below, the ratio of the synthetic activity (in 90% *tert*-butyl alcohol) to the hydrolytic activity (in water) (*i.e.*, assay S/assay H_A) varied drastically among the mutants. From the ratio, we classified the mutant enzymes as follows: ratio = 0.6–1.1 (type *A*); 8–36 (type *B*); <0.01 (type *C*). Mutants that possessed neither synthetic nor hydrolytic activity were classified as type *D*.

Type *A*: G181D, H266N and D370Y substitutions in Hyb-24 increased the synthetic activity additively up to 3.4 U/mg (approximately 10^4-fold increase from Hyb-24). It should be noted that the synthetic/hydrolytic activity ratios were observed consistently to be between 0.77 and 1.1 among the Hyb-24D, Hyb-24DN and Hyb-24DNY mutants, thereby demonstrating that the level of synthetic activity roughly correlated with that of the hydrolytic activity. Generally, the hydrolytic activity in the organic solvent/water mixed system was expected to be lower than the activity in water. We found that type *A*-mutants possessed more than 50% of the Ald-hydrolytic activity in 0–70% *tert*-butyl alcohol, but the synthetic reaction became predominant at 85–90% *tert*-butyl alcohol (Fig. 4A and B). Hyb-24FDN (Hyb-24DN containing a Y170F substitution) decreased both the synthetic and hydrolytic activity to approximately 2% of Hyb-24DN (0.05 U/mg), and the synthetic/hydrolytic activity ratio was maintained at 1.0 (Fig. 1). This suggested that Tyr170 plays an important role not only in the hydrolysis of amides, but also in synthesis.

Type *B*: A single D181E and D181N substitution in Hyb-24DN decreased the hydrolytic activity to 0.3% and 3.9% of the level of Hyb-24DN, respectively (Fig. 1). However, the mutants still possessed 17% and 51% of the level of the synthetic activity of Hyb-24DN, respectively. Interestingly, type *B*-mutants (Glu181- and Asn181-mutants) possessed quite low levels of Ald-hydrolytic activity (<0.01 U/mg) at <70% *tert*-butyl alcohol (Fig. 4C and D). However, both the hydrolytic and synthetic activities were enhanced at higher concentrations, and the maximum activity was obtained at 90% *tert*-butyl alcohol both for hydrolysis and synthesis. In addition, it should be noted that the synthetic activity was higher than the hydrolytic activity even at 70% *tert*-butyl alcohol, although the specific synthetic activity was lower than the activity of Hyb-24DN and Hyb-24DNY.

Type *C*: The hydrolytic activity of Hyb-24 (in water) was enhanced 8-fold in the Hyb-24Y mutant by a single D370Y substitution, and this has been demonstrated to improve Ald-binding at the C-terminal region of Ald [25]. The activity was further enhanced 10-fold in the Hyb-S4M94 mutant [26]. Furthermore, we have identified that efficient Ald-binding was due to Ser187-Cys264 (these are stabilizing at the N-terminal region) and Tyr370 (this is stabilizing at the C-terminal region) [26]. Moreover, the enzyme assay of a newly constructed Hyb-24NY mutant (Hyb-24 having H266N-D370Y substitutions) demonstrated that the presence of Asn266, which was an effective Ald-binding residue in Hyb-24DN, increased the Ald-hydrolytic activity of Hyb-24Y approximately 4-fold (Fig. 1). However, all Gly181-enzymes (Hyb-24Y, Hyb-24NY and Hyb-S4M94) possessed hardly any synthetic activity in 90% *tert*-butyl alcohol (<0.01 U/mg using "assay S"). In addition, enzyme assays conducted using various concentrations of *tert*-butyl alcohol revealed that the Ald-hydrolytic activity of Hyb-S4M94 in 90% *tert*-butyl alcohol was >50% of the activity in water, whereas the synthetic activity was barely detectable in any concentration of *tert*-butyl alcohol (Figs. 1 and 4E).

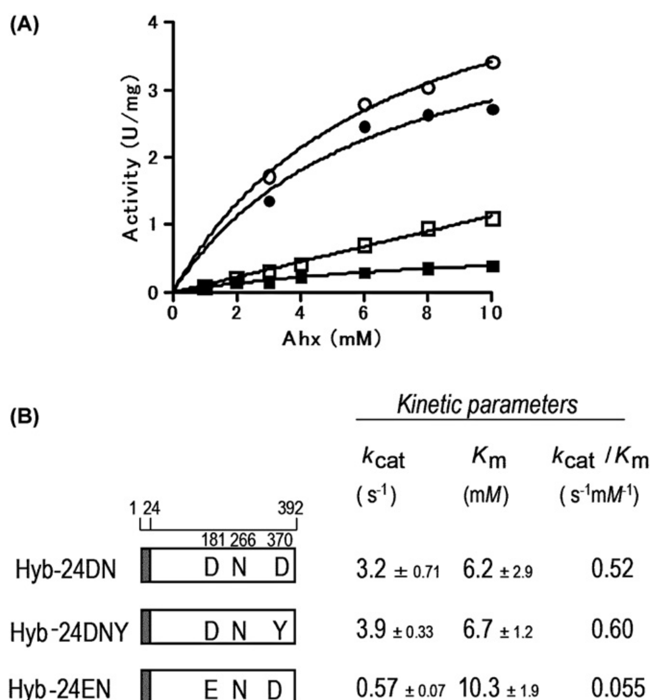


Fig. 5. The effect of the concentration of Ahx on the synthetic activity. (A) The Ald-synthetic activity was assayed using the purified enzymes using "assay S", except that various concentrations of Ahx were used. (○) Hyb-24DNY, (●) Hyb-24DN, (■) Hyb-24EN, and (□) Hyb-24NN. (B) The kinetic parameters (the k_{cat} and K_m values) were evaluated by fitting the data to the Michaelis–Menten equation directly using the program "GraphPad prism, version 5.01" (GraphPad, San Diego, CA, USA). The k_{cat} values are expressed as the turnover numbers per subunit (the M_r of the subunit = 42,000).

Type *D*: The amino acid substitutions to a basic amino acid residue at position 181 in Hyb-24DN (*i.e.*, His181, Lys181) decreased Ald-hydrolytic activity drastically to an undetectable level (<0.1% of the level of Hyb-24DN). These mutations also resulted in elimination of the amide-synthetic activity.

3.3. Kinetic study

We found that enzymatic amide synthesis in 90% *tert*-butyl alcohol follows Michaelis–Menten kinetics for the type *A* mutants (Hyb-24DN, Hyb-24DNY), although it was only possible to perform kinetic studies at concentrations of Ahx lower than 10 mM due to the limited solubility of Ahx in 90% *tert*-butyl alcohol (Fig. 5). The k_{cat}/K_m value of Hyb-24DN (0.52 s^{-1} mM^{-1}) was improved by D370Y substitution in Hyb-24DNY (0.60 s^{-1} mM^{-1}). In Hyb-24EN (*i.e.*, the Glu181-enzyme, a type *B* mutant), high affinity for Ahx was retained (K_m = 10.3 mM), while the k_{cat} was approximately 18% that of Hyb-24DN (Fig. 5B). In contrast, in Hyb-24NN (*i.e.*, the Asn181-enzyme, another type *B* mutant), the synthetic activity increased linearly between 1.0 and 10 mM Ahx, and therefore, the K_m value was estimated to be much higher than the saturated Ahx concentration (Fig. 5A). For the types *C* and *D* mutants, it was not possible to measure the K_m and k_{cat} values for the synthetic reactions due to their low activities. On the basis of the three-dimensional structures of the enzyme/Ald complex, we will discuss the structural

requirements for efficient amide synthesis and the relationship between the synthetic and hydrolytic activities.

4. Discussion

In the thermodynamic-controlled amide/ester synthesis, the water content should ordinarily be maintained at a level lower than 0.5% to shift the reaction toward synthesis rather than hydrolysis. The optimal water content will depend on the stability of the protein and the distribution of the water in the organic solvent [9]. However, we found that the synthetic reaction of Hyb-24DN and its mutants became even more favorable in the presence of 15–30% water (Fig. 4). As the catalytic process should pass through the same transition state in both the hydrolytic and synthetic reactions, the rate of the synthetic reaction is relevant to the mechanism of the hydrolytic reaction. If this catalytic process is conserved among the series of Hyb-24 mutants, the synthetic/hydrolytic activity ratio should be very similar among the mutant enzymes. However, we found that a small number of amino acid alterations in the 6-aminohexanoate-dimer hydrolase (Hyb-24DN) affected the synthetic/hydrolytic activity ratios significantly under standard assay conditions [*i.e.*, the hydrolysis in water assay (assay H_A), the hydrolysis in 90% *tert*-butyl alcohol assay (assay H_B), and the synthesis in 90% *tert*-butyl alcohol assay (assay S)].

In type *A*-mutants (Asp181-enzymes), G181D-H266N-D370Y substitutions in the parental enzyme (Hyb-24) cumulatively increased both the synthetic activity (in 90% *tert*-butyl alcohol) (assay S) and the normal hydrolytic activity (*i.e.*, in water, assay H_A), and the ratio was maintained between 0.6 and 1.1. We estimate that the 1st-Ahx-COOH (*i.e.*, the N-terminal Ahx-moiety in Ald) and the 2nd-Ahx-NH_2 (*i.e.*, the C-terminal Ahx-moiety in Ald) are localized close to the Ser112-Lys115-Tyr215 catalytic triads during the dynamic motion of the enzyme complex (Fig. 6A and B). Tyr170 appears to contribute to both the amide-synthetic reaction and the Ald-hydrolytic reaction [23], as the Y170F substitution in Hyb-24DN decreased the activity to approximately 2% of the level of Hyb-24DN (see Fig. 1, Hyb-24FDN). In the type *D*-mutants (*i.e.*, the Lys181/His181-enzymes), both the synthetic and hydrolytic activities were found to be lower than the detection limit (<0.01% the activity of Hyb-24DN). Therefore, electrostatic repulsion of the positive charges at Lys181-NH_3^+ and His181-imidazole N^+ should drastically diminish the substrate-binding ability. We estimate that stable binding of the 1st-Ahx was achieved primarily by an electrostatic stabilization effect from Asp181-COO^-, even in a solvent system containing 10% water. Moreover, in addition to the effect at position 181, interaction with Asn266 and Tyr370 should enhance the substrate-binding ability cumulatively, resulting in an increase in the synthetic and hydrolytic activities (Fig. 6A and B). However, it should be noted that the D370Y substitution has a more moderate effect on the synthetic activity than on the hydrolytic activity. That is, the kinetic study revealed that for the hydrolytic reaction in water, the D370Y substitution improves the Ald-binding of Hyb-24DN (K_m = 27 mM) approximately 14-fold (K_m = 2.0 mM for Hyb-24DNY) and improves the k_{cat}/K_m value of Hyb-24DN (0.34 s^{-1} mM^{-1}) approximately 5-fold (k_{cat}/K_m = 1.58 s^{-1} mM^{-1} in Hyb-24DNY) [24]. In contrast, for the synthetic reactions in 90% *tert*-butyl alcohol, the D370Y substitution hardly affects the K_m value for Ahx (6.2–6.7 mM), and it increases the k_{cat}/K_m by only 1.2-fold (Fig. 5). This result demonstrates that for the hydrolytic reaction, the substrate binding was enhanced by dual effects for a single Ald molecule both at the C- and N-terminal regions of the substrate, whereas for the synthetic reaction, Asp181 and Tyr370 stabilize the 1st-Ahx and 2nd-Ahx independently, and the cooperative effect is not estimated to be as significant.

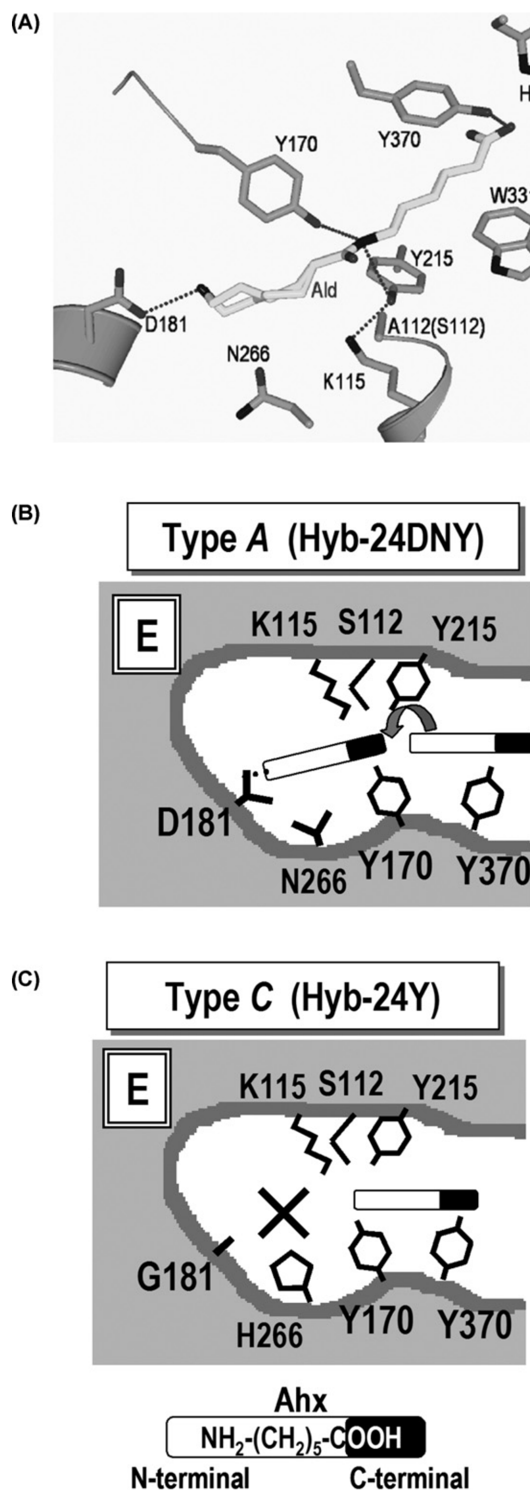


Fig. 6. Enzyme–substrate interaction at the catalytic cleft of nylon-oligomer hydrolase. (A) The structure of the Hyb-24DNY-A^{112}/Ald complex (PDB ID code: 2ZMA) identified by X-ray crystallography [24]. Figures showing the three-dimensional models were generated with the "MolFeat" program (ver. 3.6, FiatLux Co., Tokyo, Japan). (B) A model showing the catalytic centers of Hyb-24DNY. The 1st-Ahx is maintained stably by an interaction with Asp181-COO^-, and the close positioning of the 2nd-Ahx-NH_2 to the 1st-Ahx-COOH results in a condensation reaction. (C) A model showing the catalytic centers of Hyb-24Y. Inefficient binding of the 1st-Ahx (marked by an X) resulted in the inability of the enzyme to catalyze the amide-synthetic reaction.

The type *B*-mutants (*i.e.*, the Glu181/Asn181-enzymes) hardly possessed any synthetic or hydrolytic activities in 0–70% *tert*-butyl alcohol (Fig. 4C and D). However, both activities were significantly increased at higher *tert*-butyl alcohol concentrations. Therefore, we estimated that the increased hydrolytic activity in organic solvent is correlated with the appearance of the synthetic activity. A single D181N substitution in an Asp181-enzyme (Hyb-2) has also been shown to decrease the affinity of the enzyme for Ald (K_m = 80 mM) [34]. Similarly, as stated above, we estimate that the K_m value is much higher than the saturated Ahx concentration in 90% *tert*-butyl alcohol, as the synthetic activity increased linearly with Ahx concentrations in the range of 1.0–10 mM (Fig. 5). The low affinity of Ahx in the Asn181-enzyme suggests that the electrostatic effect is still predominant in reaction mixtures composed of 90% *tert*-butyl alcohol, although the effects of the negative charge of Asp-COO^- would be weakened with increasing organic solvent concentration. In addition, because the Hyb-24NN possesses a high level of synthetic activity (1.1 U/mg) using "assay S", the enzyme should possess a high k_{cat} value for the synthetic reaction in 90% *tert*-butyl alcohol. Therefore, minor structural alterations induced by the solvent suitably improve the positioning of the Ahx molecule against the catalytic residues.

We have previously reported that a D181E substitution in the NylB-type protein (Hyb-2; Asp181-enzyme) decreases the affinity of the enzyme for Ald (K_m = 220 mM) [34]. In the Glu181-enzyme, the longer side chain (compared to Asp) sterically hinders Ald-binding and led to incorrect positioning of the amide linkage of Ald against the catalytic residues. In contrast to the low hydrolytic activity of Hyb-24EN in water (assay H_A), increased Ald-hydrolytic activity in 90% *tert*-butyl alcohol (assay H_B) may imply that a minor structural alteration in the catalytic cleft induced by organic solvent allows for the effective binding of Ald. In addition, the kinetic study of the synthetic reaction demonstrates that Hyb-24EN possesses a relatively high affinity for Ahx (K_m = *ca.* 10 mM), suggesting that the enzyme holds Ahx stably in the catalytic cleft, although the k_{cat} is 18% that of Hyb-24DN (Fig. 5).

In the type *C*-mutants (*i.e.*, the Gly181-enzymes), the Ald-hydrolytic activity was cumulatively enhanced by approximately 8-fold (Hyb-24Y), 34-fold (Hyb-24NY) and 80-fold (Hyb-S4M94) as compared with the parental carboxylesterase (Hyb-24), while retaining the common Gly181/Tyr370 residues. However, these mutants hardly possessed any synthetic activity at any concentration of *tert*-butyl alcohol (Figs. 1 and 4E). We suggest therefore, that inefficient binding of the 1st-Ahx maintains the enzyme structure in an open form and that the 2nd-Ahx is less likely to be bound to the enzyme despite the presence of an effective Ald-binding residue (Tyr370) interacting at the C-terminal region of the substrate (Fig. 6C). These results demonstrate that, even if a catalytic function is optimized for the forward reaction (hydrolysis), the selected structure may not be suitable for the reverse reaction (synthesis).

From the structural and functional analyses of the Hyb-24 mutants, we have summarized the enzyme/substrate interactions responsible for efficient synthesis of nylon-6 units as follows:

(i) The 1st-Ahx binding is facilitated by an interaction with Asp181. Asn266 increases the 1st-Ahx-binding cooperatively with Asp181.
(ii) A 2nd-Ahx is stabilized by Tyr370. Movement of the flexible loop region and rotation of Tyr170 generate the hydrophobic environment suitable for the synthetic reaction.
(iii) The synthetic reaction proceeds by catalytic functions involving Ser112-Lys115-Tyr215. The catalysis is enhanced by the cooperative functioning of Tyr170.

5. Conclusions

We have shown that nylon-oligomer hydrolase efficiently catalyzes a direct condensation reaction between a free amino-group and a carboxyl-group to generate nylon-6 units. We propose that the hydrophobic environment generated in the closed form of the enzyme potentially provides a suitable environment for synthesizing amide compounds at a high efficiency, and this is preferable in terms of industrial production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.02.006.

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