Significant enhancement in the binding of *p*-nitrophenyl-β-D-xylobioside by the E128H mutant F/10 xylanase from *Streptomyces olivaceoviridis* E-86

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Abstract Mutagenesis studies were carried out to examine the effects of replacement of either the nucleophile Glu-236 or the acid/base Glu-128 residue of the F/10 xylanase by a His residue. To our surprise, the affinity for the *p*-nitrophenyl-β-D-xylobioside substrate was increased by 10^3-fold in the case of the mutant E128H enzyme compared with that of the wild-type F/10 xylanase. The catalytic activity of the mutant enzymes was low, despite the fact that the distance between the nucleophilic atom (an oxygen in the native xylanase and a nitrogen in the mutant) and the α-carbon was barely changed. Thus, the alteration of the acid/base functionality (Glu-128 to His mutation) provided a significantly favorable interaction within the E128H enzyme/substrate complex in the ground state, accompanying a reduction in the stabilization effect in the transition state.

Key words: Site-directed mutagenesis; Enhanced binding; *Streptomyces olivaceoviridis*; Family F/10 xylanase; Double displacement mechanism

1. Introduction

β-Xylanase (EC 3.2.1.8) hydrolyzes β-1,4-glycosidic linkages within the xylan backbone to yield short chain xylo-oligosaccharides of varying length. β-Xylanases have been classified into two families of glycanases (F/10 and G/11) on the basis of amino acid sequence similarities among catalytic domains [1,2]. The two families of xylanases differ in the patterns of cleavage of various heteroxylans [3]. For example, members of the F/10 family of xylanases cleave arabinoxylans at the β-1,4-linkages at non-reducing ends of arabinofuranose-blanched xylopyranoses but the members of the G/11 family cannot do so [4]. It is, therefore, of interest to characterize and compare the reaction mechanisms and structure-function relationships of these enzymes.

The mechanism of action of glycosidases is of considerable current interest [5–7]. Glycosidases catalyze the hydrolysis of glycosidic bonds by two distinct but somewhat similar mechanisms [8]. Both types of reaction proceed via an oxocarbonium ion-like transition state and, in both cases, a pair of carboxylic acids functions as catalytic residues [9]. In retaining glycosidases such as F/10 xylanase (FXYN) (their products have retention of configuration because of formation of a glycosyl-enzyme intermediate: see Fig. 1a), one carboxylic acid functions as a nucleophile and the other as an acid/base catalyst, with the average distance between the two essential carboxylic acids being about 5.5 Å (the distance between Glu-236 and Glu-128 in Fig. 1a). During the first step, a proton is transferred from the acid catalyst (Glu-128), with accompanying nucleophilic attack (by Glu-236) at the anomeric carbon, to yield a glycosyl-enzyme intermediate (glycosylation step). During the second step, the newly generated base catalyst (Glu-128) removes a proton from water in a concerted process while the water molecule attacks the anomeric center of the glycosyl-enzyme intermediate (deglycosylation step).

In inverting enzymes (their products have inversion of configuration because of a single S_N2 attack by a water molecule without formation of the glycosyl-enzyme intermediate), one carboxylic acid functions as a general acid and the other carboxylic acid functions as a general base, with the distance between them being more than 10 Å. Thus, a nucleophilic water molecule can be accommodated between the two essential carboxylic acids. At the catalytic site of T4 lysozyme, the substitution of the catalytically ineffective Thr-26 residue by His altered the catalytic mechanism from inverting to retaining [10]. In attempts to elucidate the structure-function relationships in xylanases, both the effect of the distance between the two catalytic residues and the effects of the pK_a values of these residues have been examined [5,6,11,12]. However, to our knowledge, no attempts have been made to replace either of the two essential carboxylic acids by a His residue, which has the potential to act as a good nucleophile, as demonstrated in the T4 lysozyme [10], and also to act as a good acid/base catalyst [13,14].

FXYN (45 kDa) from *Streptomyces olivaceoviridis* E-86 was purified and the substrate specificity of this enzyme was previously well characterized [15–19]. We recently succeeded in crystallizing the intact FXYN [20] and also in isolating the

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gene for the xylanase [21]. Here, we describe analysis of the wild-type (wt) FXYN and of His-containing mutants to examine the potentiality of the His residue as a nucleophile and/or an acid/base catalyst since the distance between the two potentially catalytic residues appeared to be similar to that of the wt FXYN (Fig. 1b). The results of site-directed mutagenesis indicated that, unexpectedly, the affinity for the *p*-nitrophenyl-β-D-xylobioside (pNP-X_2) substrate was increased by 10^3-fold in the case of the mutant E128H enzyme compared with that of the wt FXYN, although a His residue at the catalytic site of FXYN could not properly act as a nucleophile.

2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutagenesis was performed by the improved megaprimer PCR mutagenesis strategy that was originally described by Séraphin et al. [22]. The primers used to replace Glu-128 and Glu-236 by His were 5′-CGT CGT GAA CCA AGC CTT CTC GG-3′ and 5′-GGC CAT CAC CCA ACT CGA CAT-3′ (the substituted nucleotides are underlined), respectively. These oligonucleotide primers were synthesized by a DNA/RNA synthesizer (model 394: PE Applied Biosystems, Foster City, CA, USA). The first PCR was performed in a final volume of 25 μl with 2 ng of template DNA, namely, pCR*fxyn* (cleaved with *Nru*I for E128H FXYN and *Pst*I for E236H FXYN), 50 pmol of each primer and 5 nmol dNTPs. Each of 20 amplification cycles consisted of denaturation at 98°C for 1 min and annealing and primer extension at 65°C for 5 min. After ethanol precipitation, the amplified DNA was treated with T4 DNA polymerase (TAKARA Shuzo, Kyoto, Japan). A second PCR was performed with 2 ng of template DNA, namely, pCR*fxyn* (cleaved with *Pst*I for E128H FXYN and *Bgl*II for E236H FXYN), 0.5 μl of the product of the first PCR that was blunt-ended by T4 DNA polymerase, 50 pmol of 5′-primer and 5 nmol dNTPs. Each of 20 amplification cycles included denaturation at 98°C for 1 min and annealing and primer extension at 72°C for 5 min. A 10 min incubation at 72°C followed the final amplification cycle. Amplified DNA fragments were subcloned into pCR2.1 using an Original TA cloning kit (Invitrogen, Carlsbad, CA, USA) to yield pCRE128H*fxyn* and pCRE236H*fxyn* and the sequences of inserts were confirmed by nucleotide sequencing with an automated DNA sequencer (model 373A: PE Applied Biosystems).

2.2. Production of enzymes in Escherichia coli

For expression in *E. coli* and purification of the wt FXYN and the two mutants using the QIAexpress system (QIAGEN GmbH, Hilden, Germany), each gene was inserted individually into the pQE60 vector (to yield pQE*fxyn*, pQEE128H*fxyn*, pQEE236H*fxyn*, respectively). The enzymes were expressed as fusion proteins that consisted of each FXYN plus a carboxyl-terminal tag with six histidines. The plasmid was used to transform *E. coli* JM109 and transformant was cultivated in 1 l of LB medium that contained ampicillin (0.1 mg/ml) at 37°C, with shaking, to an optical density at 600 nm of 0.6. After addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 2 mM, the culture was incubated at 37°C, with agitation, for 4 h. The cells were harvested and lyzed and the cell lysate was then loaded on a HisTrap chelating column (Pharmacia, Uppsala, Sweden). The bound enzyme was eluted with 200 mM imidazole in 20 mM phosphate buffer (pH 7.0). The elution of the enzyme was monitored by SDS-PAGE [23]. A single peak of enzyme was detected and corresponding fractions were pooled and concentrated with Centriprep (Amicon, Beverly, MA, USA) to 1.0 ml. The concentrated solution was loaded on a HiTrap desalting column (Pharmacia) in order to remove the imidazole.

2.3. Circular dichroism

The circular dichroism of the wt FXYN and two mutants were obtained using a CD spectrophotometer (model J-720: JASCO, Tokyo, Japan) at room temperature. The protein concentration of each enzyme was 0.5 mg/ml. All data were averaged from five acquisitions between 250 and 190 nm at a scan rate of 20 nm/min.

2.4. Steady-state kinetic studies

pNP-X_2 and *o*-nitrophenyl-β-D-xylobioside (oNP-X_2) were synthesized from a xylobiose, which was purified from xylobiose mixture (SUNTORY, Osaka, Japan), as described by Takeo et al. [24]. The final concentrations of wt FXYN and its mutants were 0.0005 and 0.01 mg/ml, respectively. Higher concentrations of these enzymes were used for His mutants because of their low activities. Steady-state kinetic parameters were determined as described by Lawson et al. [6] with pNP-X_2 as the substrate. The substrate, at various concentrations, in 25% McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4, pH 4.75 or pH 7.0) that contained 0.05% bovine serum albumin (BSA) was incubated at 45°C for 10 min and then 50 μl of enzyme solution was added. The amount of *p*-nitrophenol released was determined by monitoring the absorbance at 400 nm with a spectrometer (DU-7400: Beckman, Palo Alto, CA, USA). For determinations of k_{cat} and K_m, the concentration of substrate was adjusted such that two sets of concentrations were above the estimated K_m, one was approximately equal to the K_m and two other sets were below the K_m value, as described previously [14,25].

2.5. pH stability of the enzymes

The pH stability of E128H FXYN was determined by measuring the residual activity of the enzyme that was kept in a solution with a fixed pH. The E128H FXYN was warmed in a buffer containing 1% BSA (at a pH value ranging from 3 to 10) at 30°C for 1 h. 20 μl of the treated enzymes was then added to the pre-heated 180 μl of a reaction solution containing 0.5 mM pNP-X_2 and 0.05% BSA in 25% McIlvaine buffer (pH 4.75) at 45°C. After 1 h incubation, 200 μl of 0.2 mM Na_2CO_3 was added to stop the reaction. The amount of *p*-nitrophenol released was determined as described above.

2.6. pH-dependence of k_{cat}

The pH-dependencies of k_{cat} for the wt FXYN and E128H FXYN were determined as described previously [6]. Because of the higher solubility of oNP-X_2 than pNP-X_2, the former was used in the study of pH-dependency instead of pNP-X_2. Saturating concentrations of oNP-X_2 (0.1 mM, about $35K_m$ for the E128H FXYN, and 10 mM, about $5K_m$ for the wt FXYN) in 25% McIlvaine buffer containing 0.05% BSA (at a pH value ranging from 3.0 to 8.0) were equilibrated at 37°C for 10 min and, then, reactions were initiated by the addition of enzyme (the final concentrations of wt FXYN and E128H FXYN were 0.0005 and 0.01 mg/ml, respectively). The amount of *o*-nitrophenol released was determined by monitoring the absorbance at 400 nm with a spectrometer (DU-7400: Beckman). Values of k_{cat} were then calculated from initial rates of hydrolysis. The pH value of each reaction mixture was re-checked after each measurement.

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Fig. 1. (a) Reaction mechanism of retaining (non-inverting) β-1,4-glycanases. The numbered catalytic residues are those of FXYN from *S. oliviaceoviridis*. During the first step, a proton is transferred from the acid catalyst (Glu-128), with accompanying nucleophilic attack at the anomeric carbon, and the nucleophile (Glu-236) participates in the formation of a glycosyl-enzyme intermediate (glycosylation step). During the second step, the base catalyst (Glu-128) removes a proton from water and the water attacks the anomeric carbon of the glycosyl-enzyme intermediate (deglycosylation step). This reaction proceeds via oxocarbonium ion-like transition states. (b) The hypothetical mechanism of action of mutant proteins E128H FXYN and/or E236H FXYN. Catalytic residues, such as a nucleophile (Glu-236) and an acid/base (Glu-128), within the active sites of xylanases have been replaced in the past by other potentially functional residues, such as Cys, IAA-Cys and Asp [6,12]. However, no previous attempts have been made to examine whether His might be able to replace the function of either a nucleophile (Glu-236) or an acid/base (Glu-128). As shown here, His has the potential to act as a nucleophile and an acid/base. It is to be emphasized that the distance, which is believed to be the most important determinant in catalysis, between the nucleophile and the acid/base remains unchanged after insertion of His in the place of Glu because the attacking atom remains at the ε position.

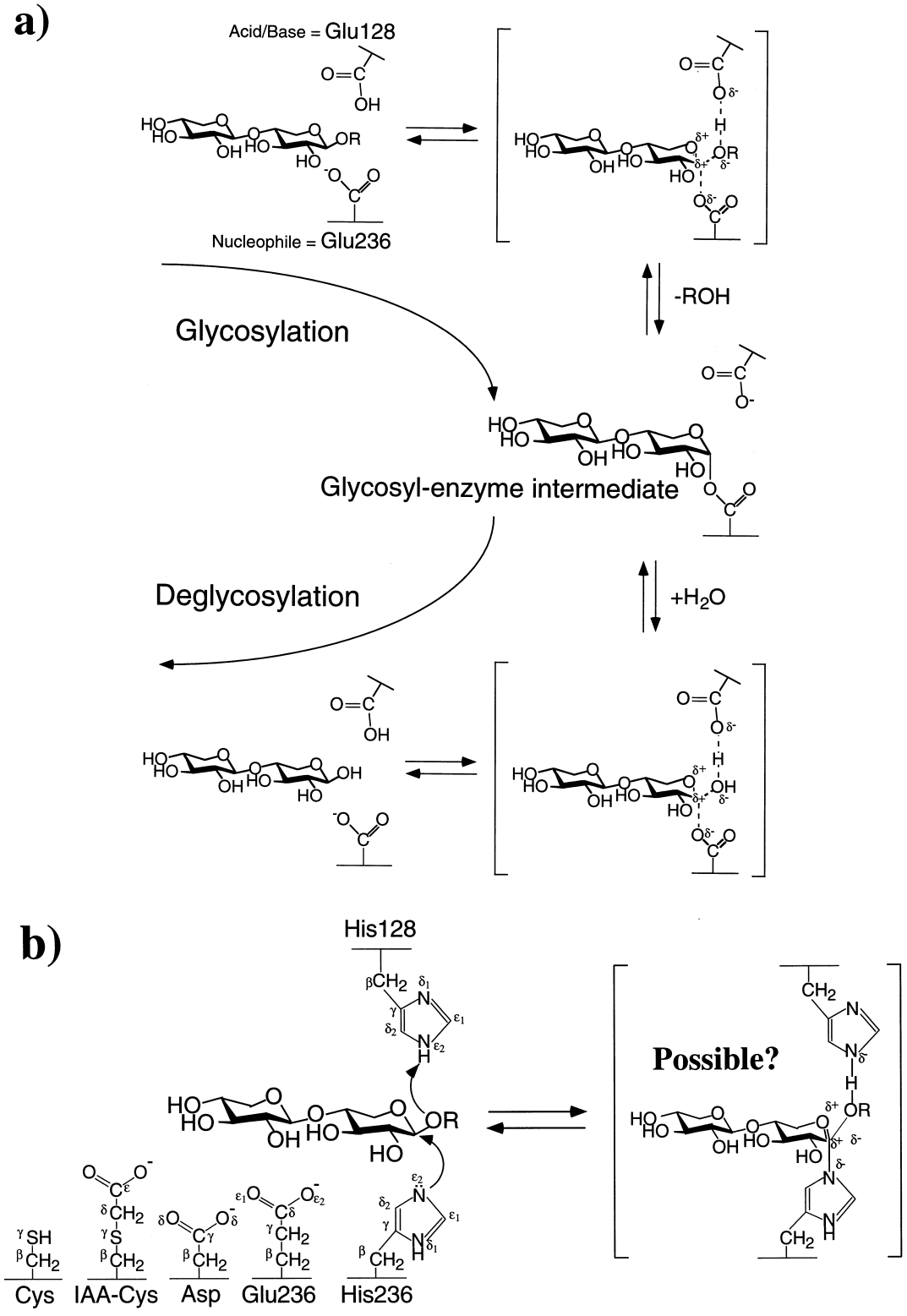
2.7. Effects of sodium azide on reaction rates

Reaction rates for wt FXYN and E128H FXYN were determined in the presence of sodium azide under the following conditions. 50 μl of the enzyme solution (0.1 mg/ml) was added to the pre-heated 450 μl of the 25% McIlvaine buffer (pH 7.0) solution that contained pNP-X_2 (0.1 mM), 0.05% BSA and various concentrations of sodium azide (0–200 mM). The amount of *p*-nitrophenol released was determined as described above.

3. Results

3.1. Site-directed mutagenesis and characterization of His mutants

As shown in Fig. 1a, two glutamic acids, Glu-128 and Glu-236, are located at the catalytic center and these carboxylic acids act, respectively, as the acid/base and the nucleophile in



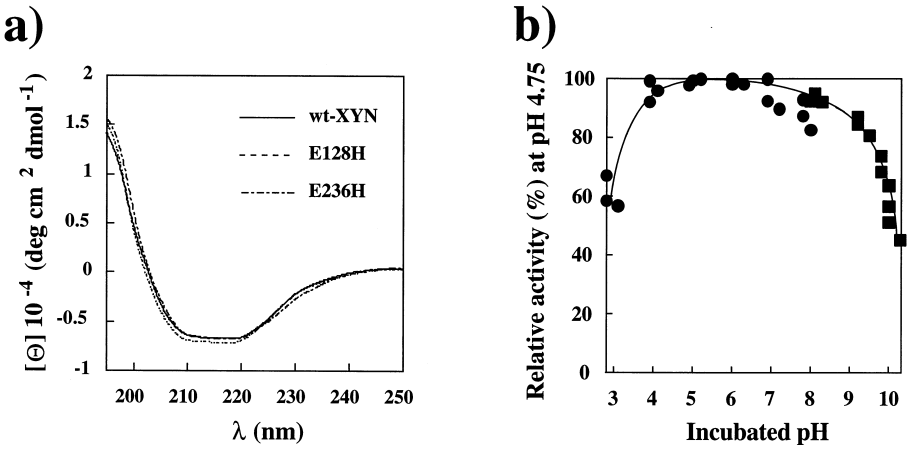


Fig. 2. (a) Circular dichroism spectra of wt (solid line) and His mutant (dotted lines) FXYNs. (b) The remaining activity at pH 4.75 of E128H FXYN mutant enzyme that was incubated at 30°C for 1 h at given pH values.

the double displacement mechanism. In a previous study, it was demonstrated that, in an engineered T4 lysozyme, a newly introduced His was able to function as a nucleophile, with a change from the original inverting mechanism to a double displacement, retaining mechanism [10]. Since His can potentially act as a good nucleophile and a good acid/base catalyst, we investigated the effect of replacement of Glu-128 and Glu-236, separately, by His. Site-directed mutagenesis was performed by the improved megaprimer PCR method, as described in Section 2. Both His mutants were easily purified by HisTrap chelating columns and, based on a SDS-PAGE analysis, the estimated molecular weight of each mutant was confirmed to be the same as that of the wt FXYN. Comparison of both mutants with the wt FXYN by circular dichroism measurements in the far-UV regions did not reveal any significant difference, indicating that the isosteric mutation did not modify the fold of the enzyme (Fig. 2a). Moreover, the stability at a given pH of the E128H mutant did not change significantly from that of wt FXYN (Fig. 2b).

3.2. Steady-state kinetic studies

The His mutants failed to hydrolyze Remazol Brilliant blue xylan, whereas pNP-X_2 (2 mM) could be hydrolyzed by these mutants. We, therefore, determined kinetic parameters, namely, K_m and k_{cat} of wt FXYN and His mutants with pNP-X_2. These measurements were made at physiological pH 7.0 and also at pH 4.75, at which the mutant enzyme (E128H) had the optimal activity (Fig. 3a), and the determined parameters by the Eadie-Hofstee plots (Fig. 3b, c) are summarized in Table 1. The k_{cat} value of the mutant enzyme (E128H FXYN) was at least 10^4-fold lower than that of the wt FXYN, indicating that the functional groups were not properly oriented in the transition state of the reaction. By contrast, at the physiological pH 7.0, the K_m value of E128H FXYN (0.0028 mM) was reduced by 10^3-fold compared with that of the wt FXYN (2.2 mM). To our surprise, the E128H mutation was favorable for the binding of pNP-X_2, whereas the bound pNP-X_2 was hydrolyzed at a much lower rate. Since the activity of E236H FXYN was much lower than that of E128H, kinetic parameters of the former could not be obtained, indicating that His-236 apparently could not act as a nucleophile nor as a general base to activate a nucleophilic water molecule.

Since the E128H mutant had a significantly favorable binding interaction with pNP-X_2, the mutant could compete more favorably with wt FXYN under the conditions of low concentrations of the substrate (Fig. 4). Specifically, at the substrate concentration of 3 μM, wt FXYN was only 50-fold more active than E128H, whereas, at the substrate concentration of 2 mM, the activity difference was larger than 10^4.

Addition of azide to the reaction mixture enhanced the hydrolysis of pNP-X_2 by the E128H mutant (Fig. 5), whereas in the case of wt FXYN, the addition of azide did not change the rate of hydrolysis (data not shown: see also [6]). Kinetic parameters of E128H in the presence of 100 mM sodium azide are listed in Table 2.

Table 1
Kinetic paramaters of wild-type and His mutant FXYNs from *S. olivaceoviridis* with pNP-xylobioside as substrate

	wt		E128H		E236H
	pH 4.75	pH 7.0	pH 4.75	pH 7.0	
K_m (mM)[a]	1.1	2.2	6.6×10^{-2}	2.8×10^{-3}	–[d]
k_{cat} (min^{-1})[b]	5.5×10^3	3.4×10^3	0.54	0.13	–
k_{cat}/K_m	4.9×10^3	1.6×10^3	8.2	46.4	–
$\Delta(\Delta G)$ (kJ/mol)[c]	–	–	30.8	34.0	–

[a] K_m values are in mM pNP-xylobioside.
[b] k_{cat} values are in mol of product/mol of enzyme/min.
[c] From the equation $\Delta(\Delta G) = -RT\ln((k_{cat})_{mutant}/(k_{cat})_{wt})$.
[d] Parameter not determined.

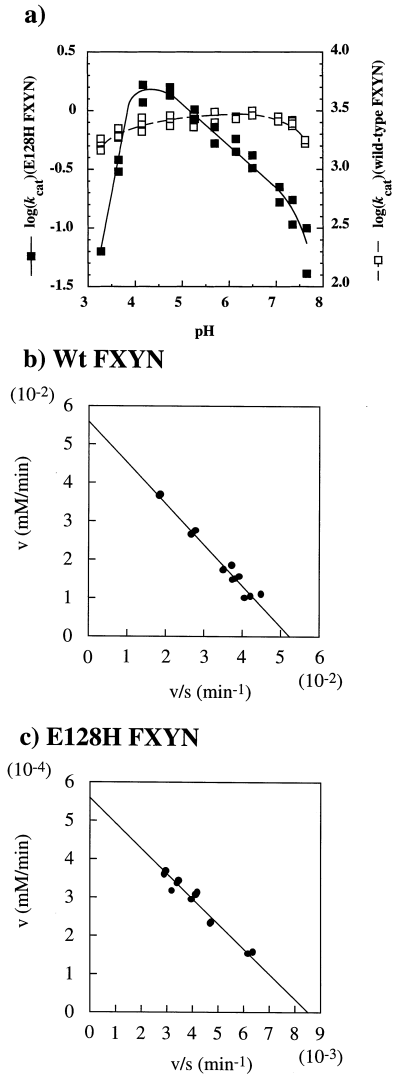


Fig. 3. (a) Dependence upon pH of k_{cat} values for the hydrolysis of oNP-X_2 by the wt FXYN (dashed line) and the mutant E128H FXYN (solid line). Plots of *v* against *v/s* (Eadie-Hofstee plot) of wt FXYN (b) and E128H FXYN (c).

4. Discussion

Using the cloned *fxyn* gene [21], we altered the catalytic site of the xylanase and characterized two mutant enzymes. In wt FXYN, two glutamate residues, conserved in all members of the F/10 and G/11 families, are located within the cavity that forms the active site, with the carboxyl groups at a distance of 5.5 Å from one another (Fig. 1a). The effect of the distance between the two catalytic carboxylic acids was examined by Lawson et al. [6,12]. An increase in the distance by a mutation that altered the acid/base functionality (Glu-172-Asp: mutation of Glu-172 to Asp-172) resulted in a 400-fold decrease in the k_{cat} for hydrolysis of xylan [6]. Altering the length of the acid/base catalyst had a less detrimental effect on the hydrolysis of aryl xylobiosides, with k_{cat}/K_m values being reduced only 3–23-fold relative to the wt enzyme. By contrast, shortening of the nucleophilic acid chain (Glu-78-Asp) decreased values of k_{cat}/K_m at least 1600-fold for aryl xylobiosides and much more for xylan. An increase in length (Glu-78-IAA-Cys: see Fig. 1b for the structure of the IAA derivative) decreased the values of k_{cat}/K_m for the aryl xylobiosides by only 16–100-fold [6,12]. Thus, it appeared that the positional requirements for proton transfer are less rigid than those for formation of a carbon-oxygen bond, namely, for nucleophilic attack [6].

A recent study by Kuroki et al. [10] revealed that, at the active site of T4 lysozyme, His can be successfully employed as the nucleophile that attacks the anomeric center. However, in previous analyses of potential nucleophiles at the active site of xylanases, His was never tested as a nucleophile or as an acid/base catalyst. Therefore, we created two mutant proteins, Glu-236-His and Glu-128His (Fig. 1b), and examined their catalytic activities (Table 1). We reasoned that, if the distance between the two essential catalytic residues was to be the most important factor in maintaining the catalytic machinery [11,12], both Glu-236-His and Glu-128-His would have a catalytic activity comparable to that of wt FXYN since the distance appeared unchanged after mutagenesis (the N-nucleophile is at the ε position, as is the O-nucleophile in the wt xylanase: Fig. 1b). There seems to be quite a lot of room at the active site or at least enough room for the IAA-Cys side chain to fold down and adopt a position in which the carboxylate can function efficiently as a nucleophile [12]. Despite

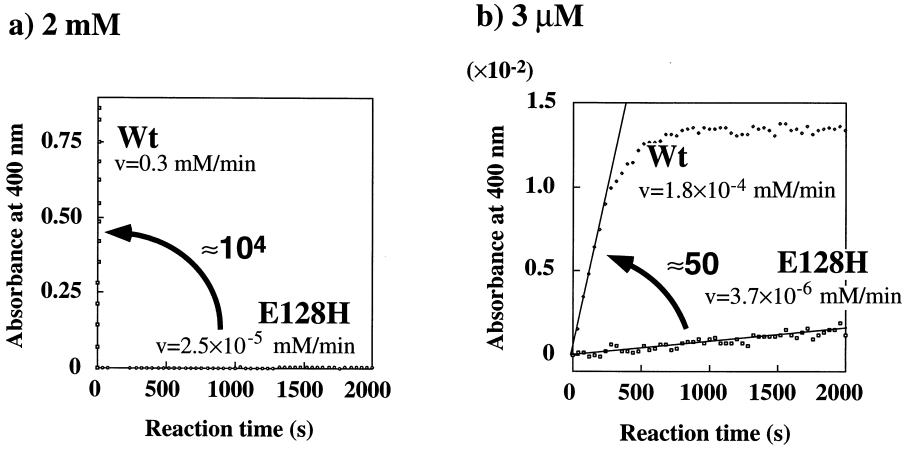


Fig. 4. Difference between hydrolysis rates of wt FXYN and E128H FXYN in the presence of either 2 mM of pNP-X_2 (a) or 3 μM of pNP-X_2 (b).

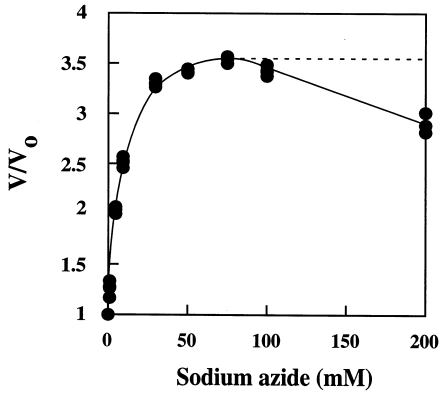


Fig. 5. Effect of azide on the hydrolysis rate of pNP-X_2 by E128H FXYN.

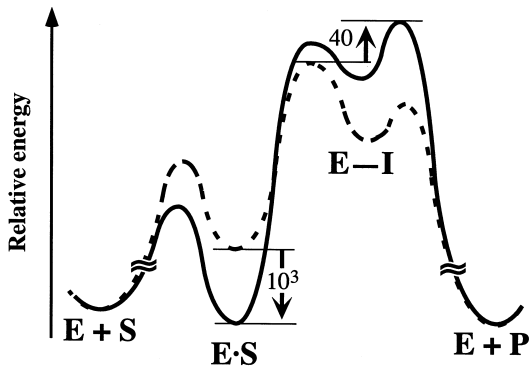


Fig. 6. Schematic representation of the relative energies for reactions catalyzed by wt FXYN (broken line) and E128H FXYN (solid line).

these considerations, the k_{cat} values of the mutant proteins decreased dramatically (at least 10^4-fold decrease in the case of E128H FXYN and to a greater extent in the case of E236H FXYN), indicating that the functional groups were not properly oriented in the transition state of the reaction (Table 1).

Nevertheless, to our surprise, the affinity for the pNP-X_2 substrate was increased by 10^3-fold in the case of the E128H enzyme compared with that of wt FXYN. Thus, the alteration of the acid/base functionality (Glu-128 to His mutation) provided a significantly favorable interaction within the E128H enzyme/substrate complex in the ground state, accompanying a reduction in the stabilization effect in the transition state, as demonstrated in Fig. 4 and Table 1. Observations that the addition of azide enhanced the E128H-catalyzed hydrolysis of pNP-X_2 (Fig. 5) but not the wt FXYN-catalyzed reactions [6] clearly demonstrated that the rate-limiting step had changed by the E128H mutation from the rate-limiting glycosylation step (for wt FXYN) to the rate-limiting deglycosylation. The overall effect on the energy diagram by the E128H mutation is schematically illustrated in Fig. 6.

Catalysis in xylanase-mediated reactions appears to be more complicated than we anticipated. We need to ask ourselves why His appears to act as a good nucleophile in T4 lysozyme-catalyzed reactions while it does not do so in xylanase-catalyzed reactions, even when the distance between the nucleophilic atom and the α-carbon is unchanged. In addition, His did not function properly as an acid/base catalyst in our system, even though positional requirements for proton transfer were not expected to be so strong [6]. It is to be noted that, in another system, artificially introduced His functioned as an acid/base catalyst within a newly created catalytic triad that was similar to those in serine proteases and, as a result, cyclophilin was successfully engineered to yield a proline-specific endopeptidase [26]. We cannot exclude the possibility that, in (mutant) xylanase-mediated reactions, the distance, between the two essential catalytic residues, at least by itself is not the determinant in maintaining the catalytic machinery.

Table 2
Kinetic paramaters of His mutant FXYNs from *S. olivaceoviridis* with or without sodium azide

Sodium azide (mM)	E128H	
	0	100
K_m (mM)[a]	2.8×10^{-3}	1.6×10^{-2}
k_{cat} (min^{-1})[b]	0.13	0.49
k_{cat}/K_m	46.4	30.6

[a] K_m values are in mM pNP-xylobioside.
[b] k_{cat} values are in mol of product/mol of enzyme/min.

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