Identification of amino acid residues critical for catalysis and stability in *Aspergillus niger* family 1 pectin lyase A

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Site-directed-mutagenesis studies were performed on family 1 pectin lyase A (PL1A) from *Aspergillus niger* to gain insight into the reaction mechanism for the pectin lyase-catalysed β-elimination cleavage of methylesterified polygalacturonic acid and to stabilize the enzyme at slightly basic pH. On the basis of the three-dimensional structures of PL1A [Mayans, Scott, Connerton, Gravesen, Benen, Visser, Pickersgill and Jenkins (1997) Structure **5**, 677–689] and the modelled enzyme–substrate complex of PL1B [Herron, Benen, Scavetta, Visser and Jurnak (2000) Proc. Natl. Acad. Sci. U.S.A. **97**, 8762–8769], Asp^{154}, Arg^{176}, Arg^{236} and Lys^{239} were mutagenized. Substituting Arg^{236} with alanine or lysine rendered the enzyme completely inactive, and mutagenesis of Arg^{176} and Lys^{239} severely affected catalysis. The $Asp^{154} \rightarrow$ Arg and $Asp^{154} \rightarrow$ Glu mutant enzymes were only moderately impaired in respect of catalysis. The results strongly indicate that Arg^{236}, which is sandwiched between Arg^{176} and Lys^{239}, would initiate the reaction upon enzyme–substrate interaction, through the abstraction of the proton at C^5 of the galacturonopyranose ring. The positively charged residues Arg^{176} and Lys^{239} are responsible for lowering the pK_a of Arg^{236}. Arg^{176} and Lys^{239} are maintained in a charged state by interacting with Asp^{154} or bulk solvent respectively. The deprotonation of the Asp^{186}–Asp^{221} pair was proposed to be responsible for a pH-driven conformational change of PL1A [Mayans, Scott, Connerton, Gravesen, Benen, Visser, Pickersgill and Jenkins (1997) Structure **5**, 677–689]. Substitution of Asp^{186} and Asp^{221} by Asn^{186} and Asn^{221} was expected to stabilize the enzyme. However, the $Asp^{186} \rightarrow Asn/Asp^{221} \rightarrow$ Asn enzyme appeared less stable than the wild-type enzyme, even at pH 6.0, as evidenced by fluorescence studies. This demonstrates that the pH-dependent conformational change is not driven by deprotonation of the Asp^{186}–Asp^{221} pair.

Key words: conformation, fluorescence, mutagenesis, subsite, three-dimensional structure.

INTRODUCTION

Pectin lyases (EC 4.2.2.10) are enzymes that catalyse the degradation of pectin, a main structural polysaccharide of the primary cell wall and middle lamella of higher plants [1,2]. Pectin is a methylesterified form of polygalacturonic acid. The enzymes utilize a β-elimination reaction to cleave the α-D-(1 $\rightarrow$ 4) glycosidic bond between galacturonosyl residues in the homogalacturonan (the so-called 'smooth') part of the pectin molecule, resulting in the formation of a C^4–C^5 double bond at the non-reducing end of the cleaved polysaccharide [3]. Pectin lyases belong to the family of polysaccharide lyases of which currently 12 families are known [4]. These families have been grouped according to their primary sequence alignments, and the family number is nowadays included in their name, i.e. family 1 PLA will be designated PL1A when appropriate.

The crystal structures of seven of these enzymes have recently been solved, including two *Aspergillus niger* pectin lyases, namely PL1A [5] and PL1B [6], and five pectate lyases, namely *Erwinia chrysanthemi* Pel1C [7] and Pel1E [8], *Bacillus subtilis* Pel1 [9], *Bacillus*. sp. KSM3-P15 Pel-15 [10] and *Pseudomonas fluorescens* subsp. *cellulosa* PL10A [11]. The family 1 and 3 structures share the same topology, namely that of a parallel β-helix wound into a large right-handed coil,whereas PL10A is predominantly an α-helical enzyme. For Pel1C the structure of an enzyme–substrate complex was recently solved [12] that served to model the enzyme–substrate complex of PL1B [13]. For PL1B as well as Pel1C it was shown that an arginine residue could well serve as the base that initiates the β-elimination reaction. However, no direct evidence has been obtained yet on the role of other positively charged residues, such as Arg^{176} and Lys^{239}, which are potentially involved in catalysis in the pectin lyases. Furthermore, PL1A is subject to a pH-dependent structural change, as was shown by Mayans et al. [5], who solved structures at pH 6.5 and 8.5. This structural change was attributed to the ionization of Asp^{186}, which is in close contact with Asp^{221} in the pH 6.5 structure, but that emerged from the structure at pH 8.5 due to repelling by Asp^{221}. To test this hypothesis and to render the enzyme more stable, Asp^{186} and Asp^{221} were subjected to mutagenesis.

In the present paper we report on the characterization of wild-type and mutated PL1A forms, namely D154E/A, R176A/D/K, R236A/K, K239N, D186N, D221N and D186N/D221N (the letters represent amino acids in one-letter code and the numbers are the positions of the amino acids mutated) and discuss their role in catalysis and stability.

MATERIALS AND METHODS

DNA manipulations

Standard cloning experiments were performed in *Escherichia coli* DH5α [14]. DNA manipulations were carried out using standard

Abbreviations used: D154E (etc.), $Asp^{154} \rightarrow$ Glu (etc.); DM, degree of methylesterification; FPME, fungal (*Aspergillus niger*) pectin methylesterase; Gal*p*A, galacturonopyranose; PL1A, family 1 pectin lyase A; PPME, plant (orange) pectin methylesterase.

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protocols [15]. Restriction enzymes were used as described by the supplier (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, U.S.A.). Nucleotide sequences were determined using a Cy5™ AutoCycle Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) with universal and reverse primers or gene-specific primers. The reaction mixtures were analysed with an ALFred™ DNA sequencer (Pharmacia Biotech). Computer analysis was done using the program GeneRunner (Hastings Software, Hastings, NY, U.S.A.).

Construction of promoter–gene fusion

The cloning of the *A. niger* N400 *pel*A gene encoding PL1A has been described previously [16]. A translational promoter gene fusion was constructed using the pyruvate kinase promoter [17]. The promoter gene fusion, *pki–pel*A, was constructed using plasmid pGW820 [16] as a template in a PCR reaction. For this reaction oligonucleotides pelAF1 (5′-ACTTCACC**ATGCAT**T-ACTCTACTTTCAGG-3′) and pelAF2 (5′-CTCATCGATAG-TGTCGGG-3′) were used. An *Nsi*I site was introduced at the start of the *pel*A gene (region shown in **bold** in pelAF1 above). A 60 bp *Nsi*I–*Pst*I fragment of the PCR product was cloned into the plasmid pPROM-H [18] digested with the same enzymes, resulting in the plasmid *pki–pel*A1. The *Pst*I–*Hin*dIII fragment containing the remainder of the *pel*A gene was isolated from plasmid pGW820 and cloned into *Pst*I–*Hin*dIII-digested *pki–pel*A1, yielding plasmid pIM3500.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Altered Sites II kit (Promega, Madison, WI, U.S.A.) and synthetic oligonucleotides (Isogen, Maarsen, The Netherlands). The procedure was performed as previously described [19]. The *pki–pel*A promoter–gene fusion was excised from the pUC18-based plasmid pIM3500 using restriction endonucleases *Bam*HI and *Hin*dIII and ligated into *Bam*HI- and *Hin*dIII-digested pALTER I, resulting in plasmid pIM3501. Following mutagenesis, plasmid DNA was isolated and sequenced to confirm the desired mutations and to check the gene for undesired mutations. Those plasmids showing the correct sequence and the expected mutation were used to transform *A. niger* strain NW188 (*csp*A1, *pyr*A6, *leu*-13, *prt*F28) a derivative of *A. niger* N400 (CBS.120.49). Transformations were carried out as described in [20].

Culture conditions and enzyme purification

Wild-type and mutant PL1A-producing multicopy transformants were selected by growing individual transformants in minimal medium as described in [18]. Large-scale cultivation of transformants producing (mutant) PL1A was performed as outlined in [18] in multiple 300 ml batches in 1-litre Erlenmeyer flasks incubated in an orbital shaker (250 rev./min) at 30 °C.

Wild-type and mutant PL1A were purified from the culture medium of *A. niger pki–pel*A multicopy transformants. After removing the mycelium by filtration over nylon gauze, the culture medium was adjusted to pH 6.0, and 100 g (wet weight) of DEAE Streamline (Pharmacia) was added and stirred for 2 h. Next the matrix was recovered in a column and washed with 20 mM piperazine, pH 6.0. The proteins were eluted with 20 mM piperazine, pH 6.0, containing 1 M NaCl. The protein containing fractions were pooled and dialysed against 20 mM piperazine, pH 6.0. The dialysate was loaded on to Source 30 Q (15.5 ml bed vol.; Pharmacia) equilibrated in the same buffer. PL1A and mutated forms thereof were eluted with a 0–0.5 M NaCl linear gradient in the same buffer. Fractions containing purified PL1A were collected, pooled and dialysed against 20 mM sodium phosphate buffer containing 0.2 M NaCl, pH 6.0, and stored at −20 °C.

The purity of the (mutant) enzymes was confirmed by SDS/PAGE and Coomassie Brilliant Blue R250 staining. The concentration of purified PL1A protein was determined by measuring A_{280} and using a molar absorption coefficient (ϵ_{280}) of 37937 $M^{-1} \cdot cm^{-1}$ (calculated from the tryptophan, tyrosine and cysteine content) [21].

Enzyme assay and determination of kinetic parameters and pH optimum

Standard PL1A assays were carried out in triplicate using McIlvaine buffer, pH 6.0, containing 3 mg/ml E81 citrus pectin (a regular commercial pectin), provided by Danisco Cultor, Copenhagen, Denmark, with 81 % methyl esterification, in a total volume of 1.0 ml. The partially methylesterified substrates were kindly provided by Dr. Hans Christian Buchholt, Danisco Ingredients, Brabrand, Denmark, and consisted of two series. For the two series, citrus E81 pectin served as the starting material. For one series E81 was de-esterified by a fungal (*Aspergillus* sp.) pectin methylesterase (FPME), resulting in a random removal of methyl esters [F43 and F58, the number indicating the degree of methyl esterification (DM)]. For the second series, E81 was treated with a plant (orange) pectin methylesterase (PPME), yielding a blockwise distribution of methyl esters (P41 and P60). The assay mixture was equilibrated at 30 °C and the reaction was started by the addition of 20 μl of enzyme solution. Depending on the specific activity of the (mutated) enzyme, the concentration of each enzyme solution was adjusted to be able to accurately determine the initial rate. The activity was determined by measuring the increase of absorbance at 235 nm ($\epsilon_{235} = 5200\ M^{-1} \cdot cm^{-1}$) as a result of the formation of $\Delta^{4,5}$-unsaturated products; 1 unit of enzyme activity is defined as 1 μmol of $\Delta^{4,5}$-unsaturated product formed/min. The kinetic traces were corrected for spontaneous chemical β-elimination. K_m and V_{max} values were determined by fitting the data to the Michaelis–Menten equation. Triplicate initial-rate measurements were performed in the same way as described for the standard assay using McIlvaine buffers (pH 5.0, 6.0 and 8.0); only the pectin concentration was varied from 0.5 to 7 $mg \cdot ml^{-1}$. For the determination of the pH optimum, incubations were carried out as for the standard enzyme assay using 3 $mg \cdot ml^{-1}$ E81 lime (*Citrus aurantifolia*) pectin in McIlvaine buffers ranging from pH 4.0 to pH 8.0. Throughout the ionic strengths of the McIlvaine buffers were adjusted to $I = 0.5$ using KCl.

Fluorescence studies

Fluorescence studies were carried out using a thermostatically controlled Hitachi 4500 fluorescence spectrophotometer. Emission spectra were recorded from 295 to 360 nm (scan speed 1 nm/s). The excitation wavelength was 278 nm. Emission and excitation bandwidths were 2.5 nm. Enzymes at 0.1 mg/ml were incubated at 25 °C in 20 mM sodium phosphate buffer/20 mM NaCl, at pH 6.0 and 8.0.

CD

CD spectra of enzymes at 0.1 mg/ml in 20 mM sodium phosphate buffer/20 mM NaCl, pH 6.0 or 8.0, were recorded at 25 °C using a thermostatically controlled Jasco J-715 spectropolarimeter (Jasco Corporation, Tokyo, Japan). Spectra (20 millidegree sensitivity) ranged from 260 to 190 nm and were recorded at 20 nm/min, with a 0.1 nm step size and 1.0 nm bandwidth.

RESULTS AND DISCUSSION

Purification of wild-type and mutated PL1A

The enzymes were purified to homogeneity by anion-exchange chromatography (FPLC) and analysed by SDS/PAGE to check for purity (results not shown). During the last step of the purification procedure of the PL1A wild-type and mutant enzymes, two peaks were observed in the chromatogram, which were pooled separately. The pool eluted first (at 320 mM NaCl), and constituting the majority of the PL1A protein, migrated slightly higher in the gel than the second pool (eluted at 360 mM NaCl). For enzyme N109A (no N-glycosylation), only one peak was observed in the chromatogram, and it co-migrated with the second pool of the wild-type enzyme. This indicates that the N-glycosylation changes the chromatographic behaviour and that the majority of the protein is N-glycosylated. Pool 1 represented an apparent molecular mass of 45 kDa, whereas this was 43 kDa for pool 2. Both pools migrated slightly higher in the gel than expected for the mature PL1A (37.9 kDa). Apart from the PL1A protein, no other proteins were visible on the gel.

In a standard assay, wild-type PL1A pool 1 (81.1 units/mg) was slightly more active than pool 2 (70.0 units/mg). For further experimentation, including the mutated enzymes, only pool 1 was used.

Kinetic parameters, specific activities and pH optima of wild-type and mutated forms of PL1A

In Table 1 the kinetic parameters for the wild-type enzyme and the mutated forms are presented. For the wild-type enzyme, in addition to the standard substrate E81, substrates P60 and F58 were used to determine the kinetic parameters, whereas for substrates P41 and F43 only the specific activity was determined.

Table 1 Kinetic parameters of wild-type and mutated pectin lyase A

Kinetic parameters were determined in McIlvaine buffer at 30 °C at the pH indicated. Lime pectin E81 was used as a substrate.

Enzyme	pH	V_{max} (units/mg)	K_m (mg/ml)
Wild-type	5.0	41.7 ± 0.8	0.5 ± 0.1
	6.0	152.7 ± 8.1	2.2 ± 0.3
	8.0	108.4 ± 21.0	3.2 ± 1.4
N109A	6.0	118.1 ± 2.6	1.9 ± 0.1
D154A	6.0	57.7 ± 1.5	2.0 ± 0.2
D154E	6.0	78.3 ± 2.5	1.1 ± 0.1
R176A	6.0	0.8 ± 0.03	1.7 ± 0.2
R176K	6.0	0.4 ± 0.01	4.3 ± 0.1
R176D	6.0	0.3 ± 0.03	3.8 ± 0.3
R236A	6.0	0	n.a.†
R236K	6.0	0	n.a.
K239N	6.0	1.0 ± 0.01	0.1 ± 0.01
D186N	5.0	94.4 ± 1.3	1.2 ± 0.1
	6.0	50.0 ± 3.0	2.2 ± 0.4
	8.0	41.6 ± 1.3*	> 7
D221N	5.0	18.8 ± 1.7*	> 7
	6.0	7.3 ± 0.2	2.0 ± 0.1
	8.0	34.8 ± 1.9*	> 7
D186N/D221N	5.0	5.4 ± 0.4*	> 7
	6.0	3.2 ± 0.2	2.4 ± 0.3
	8.0	9.2 ± 1.9*	> 7

* Specific activity at the highest substrate concentration used (7.0 mg/ml); see also the text.
† n.a., not applicable.

For the mutant enzymes the kinetic parameters were only determined using E81. Since the mutations were not expected to change the binding of the substrate directly, the analyses using other substrates were considered not relevant.

The wild-type enzyme shows its highest specific activity using E81, whereas V_{max} is virtually the same with E81 and P60 (V_{max} = 161.0 ± 4.9 units/mg; K_m = 4.4 ± 0.2 mg/ml). The similar V_{max} can easily be explained by the fact that the P60 substrate is intrinsically the same as E81 with respect to distribution of methyl esters, since the PPME used to de-esterify E81 to DM 60 generates blocks of non-esterified galacturonopyranose (Gal*p*A) residues. This mode of action of the PPME thus only decreases the actual concentration of stretches of methylesterified residues, the preferred substrate of PL1A, which is reflected by a decreased specific activity and increased K_m for P60. Using F58, de-esterified by an FPME that randomly attacks the substrate and thus changes the distribution of methyl esters, resulted not only in an increased K_m (7.1 ± 0.3 mg/ml), but a decreased V_{max} (129.5 ± 3.3 units/mg) as well. This is also reflected by a lower specific activity than was observed for P60. For substrates P41 and F43, with specific activities of 47.0 ± 1.5 and 11.3 ± 0.4 units/mg^{-1} respectively, the same rationale applies. These results show that PL1A indeed prefers high-DM pectin. The results presented for the wild-type enzyme also show that optimal activity is displayed at pH 6.0 (see also Figure 1). When compared with all pectate lyases and PL1B, which are optimally active between pH 8 and 10, the pH optimum of PL1A is quite low.

Identification of catalytic residues and implications for the reaction mechanism

Herron and co-workers [13] mutagenized two strictly conserved charged residues in the pectic lyase superfamily in the highly similar pectin lyase B (PL1B) from *A. niger*. These residues are Asp^{154} and Arg^{236}. Analysis of the catalytic properties of PL1B D154E and D154N, and PL1B R236K and R236Q demonstrated that Asp^{154} is not important, whereas Arg^{236} plays a critical role in catalysis. Mutagenesis of Arg^{236} in PL1A confirms the critical role of this residue (Table 1). PL1A enzymes D154E and D154A show that this residue is indeed not important for catalysis *per se*, but rather that the engineering of an uncharged residue [Asn in PL1B [13]; Ala in PL1A (Table 1)] affects catalysis more than a conservative change. This phenomenon can be explained by the fact that Asp^{154} interacts directly with Arg^{176}, a positively charged residue in the pectin lyases that is in an equivalent position to the catalytically important Ca^{2+} ion in pectate lyases, and hence stabilizes the charge and position of Arg^{176} [5,6]. The importance of Arg^{176} for catalysis is demonstrated by the observation that enzymes R176A, R176D and R176K became almost inactive (Table 1).

It has been pointed out that the actual proton abstraction from C^5 of the galacturonic acid unit at the potential newly formed non-reducing end in PL1C most likely occurs via Arg^{218} [12,13]. The counterpart of Arg^{218} in PL1C is Arg^{236} in PL1A and PL1B. The modelled enzyme–substrate complex of PL1B shows a proper alignment of Arg^{236} to the relevant galacturonic acid unit to carry out this initial proton abstraction [13], and the results from mutagenesis of Arg^{236} in PL1A and PL1B are in agreement with the proposed role. Since the pH optimum of PL1A is at pH 6.0, a strong lowering of the pK_a of both the C^5 proton and Arg^{236} is required, as the pK_a of an arginine residue is normally about 12, whereas the pK_a of the C^5 proton is even higher. Lowering the pK_a of both groups requires shielding from the solvent, lowering the dielectric constant and increasing the basic nature of the environment. In the enzyme the region

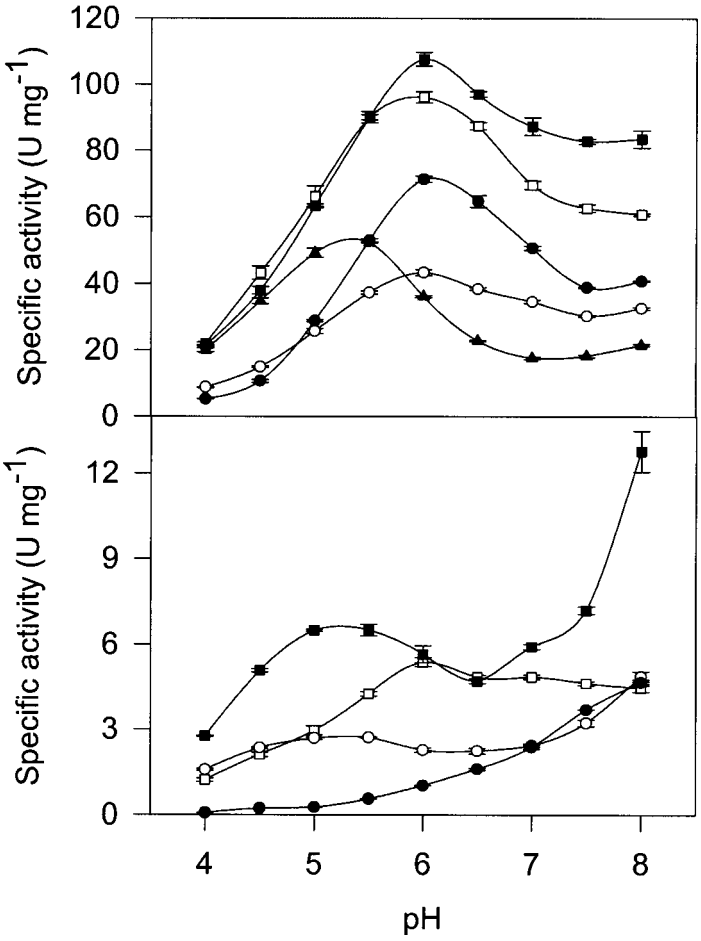

Figure 1 pH optima for wild-type and mutated forms of PL1A

The pH optima were determined using McIlvaine buffers of constant ionic strength at 30 °C. E81 pectin (3 mg/ml) was used as substrate. Upper panel, enzymes with high activity: ■, WT; □, N109A; ●, D154E; ○, D154A; and ▲, D186N. Lower panel, enzymes with low activity: ■, D221N; □, R176A; ●, K239N; ○, D186N/D221N. Abbreviation: U, unit.

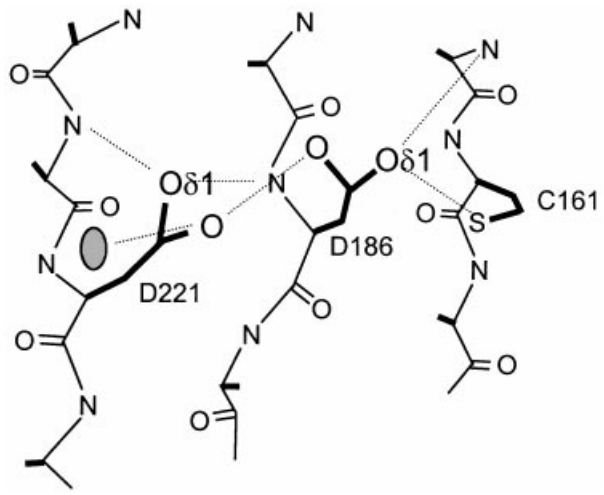

Figure 2 Schematic drawing of the contacts of Asp[186] and Asp[221]

Relevant parts of the rungs of the parallel β-helix of PL1A are shown. The view is from within the helix. (Parts of) the side chains are depicted with heavy lines. A water molecule [H_2O-3 in the Protein Data Bank (PDB) code] buried inside the helix next to Asp[221] is indicated by a grey oval. Hydrogen bonds are indicated with dotted lines. The diagram was drawn on the basis of the PDB structure 1IDJ [5].

surrounding Arg[236] is highly apolar as a result of the presence of several tryptophan and tyrosine residues (Trp[66], Trp[81], Trp[151] and Trp[212]; Tyr[211] and Tyr[215]), thus complying with a low dielectric constant. Almost on opposing sides of Arg[236] two basic residues are found with their guanidinium groups only 0.45 nm (4.5 Å) from the Arg[236] guanidinium group, namely Arg[176], which is maintained in a charged form by interacting with the conserved Asp[154], and Lys[239], which is solvent-accessible and which is therefore likely charged. In combination, these two basic residues may decrease the pK_a of Arg[236]. Furthermore, in the uncomplexed enzyme the guanidinium moiety of Arg[236] is to a large extent buried and only N^{η}-2 is partly accessible to bulk solvent. Binding of the substrate would completely shield Arg[236] from the solvent, giving rise to a further decrease of the pK_a. The decrease of the pK_a of Arg[236] upon substrate binding will be accompanied by an increase of the acidity of the C^5 proton, as it will encounter the same environment as Arg[236].

Testing the hypothesis of the apolar environment by mutagenizing Trp[212], which is closest to Arg[236], was unsuccessful, since the enzyme, which is normally secreted, could not be detected in the culture fluid. However, tiny amounts of protein could be detected in the cell-free extract using anti-PL1A antibodies. This indicates that the protein either is not secreted or is not stable when secreted. A similar observation was made previously with a polygalacturonase where Tyr[283] was mutated [22].

By mutagenizing Lys[239] into asparagine (K239N) the effect on the basic environment was probed. Enzyme K239N shows a considerable loss of activity, similar to that shown by enzymes R176K or R176A, which supports the idea of a similar functionality, namely decreasing the pK_a of Arg[236]. This is also supported by the observation that the specific activity of K239N increased with rising pH, whereas for the wild-type enzyme a reverse effect was observed. However, the possibility cannot be ruled out that, in enzyme K239N, Arg[236] has become somewhat solvent-accessible in the substrate-complexed situation. This would in itself result in a decreased activity.

pH-driven conformational changes in PL1A

Mayans and co-workers [5] solved the three-dimensional structures of *A. niger* PL1A at pH 6.5 (strain N400) and pH 8.5 (strain 4M-147). A major difference observed, apart from the fact that it concerned two isoforms that differ in respect of nine amino acid residues, is a conformational change of the loop formed by residues 182–187, which is remote from the catalytic residues. In the pH 6.5 structure, Asp[186] and Asp[221] face into the β-helical core of the enzyme (Figure 2). They are in close proximity [0.272 nm (2.72 Å)], thus sharing a proton. In the pH 8.5 structure Asp[186] has moved completely outside the core, whereas Asp[221] remains in the core. The movement of Asp[186], and, as a result, the region from residues 182 to 187, was attributed to repelling of Asp[186] as a consequence of deprotonation of the two aspartic acid residues which results in a change of the substrate-binding cleft. The position originally taken by Asp[186] at pH 6.5 becomes occupied by Thr[183] at pH 8.5, which previously was solvent-exposed. The movement of Asp[186] results in a change of the architecture of the substrate-binding cleft [5]. This changed conformation was proposed to be the

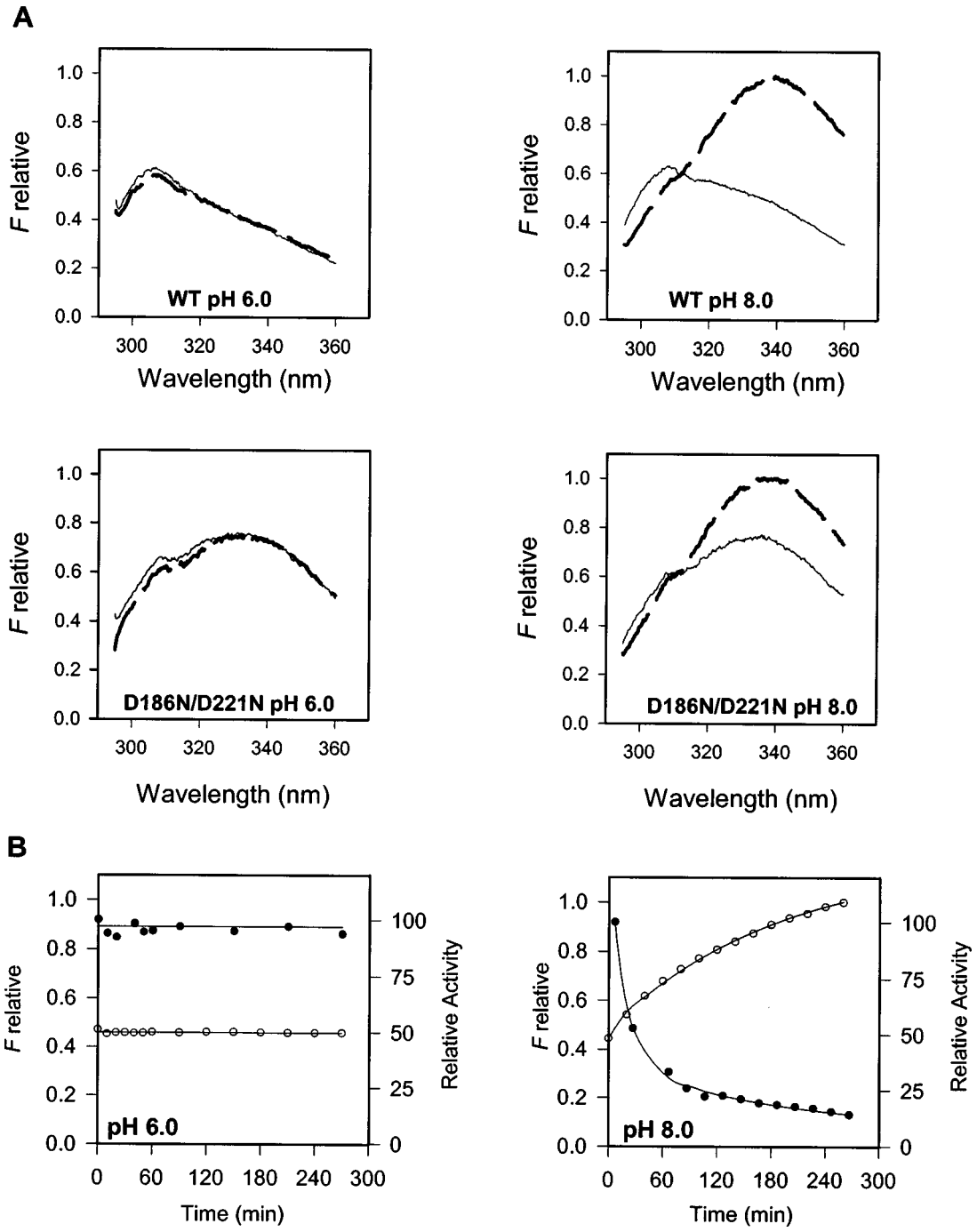

Figure 3 Relative fluorescence emission spectra for PL1A wild-type and mutant D186N/D221N

(**A**) The initial and final relative fluorescence emission spectra of a 240 min incubation of the enzymes at the pH values indicated. The final spectrum is indicated by a bold broken line. Spectra for each enzyme were normalized to the 240 min spectrum at pH 8.0. Abbreviation: WT, wild-type. (**B**) Time- and pH-dependent relative fluorescence and activity changes of wild-type PL1A. For the relative fluorescence changes (○) the relative quantum yield at the emission maximum (340 nm) is plotted. The quantum yield was normalized to the final yield at $t = 270$ min at pH 8.0. Aliquots of the incubation mixture were assayed for activity and the results are plotted as relative activity (●) where $t = 0$ min was set at 100%.

major cause of the decreased activity above pH 7.0, since the catalytic residue does not ionize at this pH.

In order to gain more insight into the process of the conformational change, enzymes D186N, D221N and D186N/D221N were prepared and characterized. Replacement of the aspartic acid by asparagine residues was chosen to minimize structural changes and, meanwhile, avoid repelling forces due to deprotonation. The kinetic parameters and specific activities of the enzymes are listed in Table 1. The pH optima are presented in Figure 1.

When compared with the wild-type enzyme, V_{max} for enzyme D186N is higher at pH 5.0 but lower at pH 6.0. However, at

pH 8.0, V_{max} could not be determined, since, for the substrate range used (up to 7 mg/ml), a linear increase of V_{app} was observed which precluded fitting of the data to the Michaelis–Menten equation. At 7 mg/ml, V_{app} was 41.6 units/mg. For enzyme D221N the effect on activity was more pronounced than in the case of D186N. In common with the situation with enzyme D186N, V_{max} could not be determined accurately at pH 8.0 and also at pH 5.0. Surprisingly, enzyme D221N is more active at pH 8.0 than at pH 6.0. The double mutant D186N/D221N shows a further reduction in activity and is also more active at pH 8.0 than at pH 5.0 or pH 6.0. Again, V_{max} values could not be determined at pH 5.0 and pH 8.0. From the effect of the mutation on catalysis, it can be inferred that residues Asp^{186} and Asp^{221} have important roles in catalysis by maintaining the proper conformation of the enzyme. Furthermore, at pH 8.0, the administration of substrate to the enzymes D221N and D186N/D221N apparently reverses the conformational change, as evidenced by the fact that the activity at 7 mg/ml substrate is higher than V_{max} at pH 6.0.

As the substrate-binding cleft contains many tryptophan and tyrosine residues, fluorescence spectroscopy was applied to monitor conformational changes and to assess the contribution of each residue to the structural integrity of the enzyme. In Figure 3(A), fluorescence emission spectra of the wild-type enzyme and enzyme D186N/D221N are presented as recorded at pH 6.0 and pH 8.0. For enzymes D186N and D221N the fluorescence emission spectra are qualitatively similar to those for the wild-type enzyme and are therefore not shown. For all four enzymes, changes in the emission spectra as a function of time are very small at pH 6.0, whereas large changes occurred at pH 8.0. Samples were taken from the incubation mixtures and assayed for activity. To avoid 'renaturation' due to incubation at pH 6.0, the assay was carried out at the suboptimal pH value 7.5. The results for the wild-type enzyme are presented in Figure 3(B). The results for the enzymes D186N and D221N are similar and are therefore not shown. For enzyme D186N/D221N the activity in the suboptimal assay was too low to be determined accurately and is therefore not included in Figure 3(B). At pH 6.0 the wild-type enzyme and enzymes D186N and D221N remained fully active during the entire time course of the experiment. However, at pH 8.0 a rapid decrease in activity occurred that coincided with a significant increase of the emission at 340 nm (see, in addition, Figure 3A). The activity curves for the wild-type enzyme and enzyme D186N show that the inactivation of the enzymes proceeds via at least two processes. This is supported by the fact that, in the compilation of emission spectra, no isosbestic points were observed (results not shown). In independent repetitions of the experiment it turned out that the administration of NaCl (up to 200 mM) resulted in a partial restoration of the emission spectra towards the active state. A similar phenomenon – activation of an enzyme preparation by NaCl – was described previously for the highly homologous PL1B [23]. To investigate whether the changes in the fluorescence emission spectra are a result of conformational changes, an identical experimental set-up using wild-type enzyme was followed by CD measurements (results not shown). This experiment revealed a significant change of the CD spectra for the wild-type enzyme at pH 8.0, whereas no changes were observed at pH 6.0, demonstrating that the changes in the fluorescence spectra indeed reflect conformational changes.

The emission spectra for enzyme D186N/D221N at pH 6.0 changed minimally. However, the starting spectrum already revealed that the enzyme is in a different, 'partially denatured', conformation compared with the other two mutant enzymes and the wild-type enzyme.

Since the origin of the conformational change was proposed to reside in the deprotonation of the Asp^{186}–Asp^{221} pair [5], it is expected that the change would not occur when residues are engineered that do not repel. Thus, enzyme D186N/D221N should be most stable. However, this enzyme appears to be the least stable, and, even at pH 6.0, it adopts an inactive configuration, as evidenced by the fluorescence spectra. This strongly suggests that the driving force for the conformational change resides outside residues Asp^{186} and Asp^{221} and that these residues in fact keep the integrity of the structure intact. This is corroborated by the fact that, in PL1B, Asp^{186} and Asp^{221} are conserved and take identical positions, whereas this enzyme is perfectly stable and active at pH 8.5, suggesting that the driving force in PL1B is smaller or absent.

In Figure 2 a schematic drawing of the contacts of Asp^{186} and Asp^{221} is presented. In enzyme D186N as well as D221N, $O^{\delta 2}$ is replaced by N^{δ}. N^{δ} of Asn^{186} (enzyme D186N) can still be a donor to $O^{\delta 2}$ of Asp^{221}, especially when Asp^{221} is deprotonated. Likewise, in enzyme D221N, N^{δ} of Asn^{221} can still be a donor to $O^{\delta 2}$ of Asp^{186}. The interactions with other amino acids via $O^{\delta 1}$ of the aspartic acid residues can be maintained by $O^{\delta 1}$ of the asparagine residues engineered. In both cases there is no obvious reason for the enzymes to become less stable with increasing pH, unless the driving force originates somewhere else. The fact that they become more unstable when the driving force is applied may originate from the fact that the hydrogen bonds formed between Asn^{186} and Asp^{221} or Asp^{186} and Asn^{221} are less strong than when they share a proton, as is the case for the wild-type enzyme. The studies presented here show that enzyme D186N is more stable than D221N. However, at present we cannot explain why enzyme D186N is more stable than enzyme D221N, nor can we identify the origin of the driving force for the pH-driven conformational change.

The studies described here present evidence that Arg^{236} is the catalytic base that initiates the β-elimination reaction carried out by pectin lyases. To carry out this unusual function, Arg^{236} is flanked by Arg^{176} and Lys^{239}, which lower the pK_a of the guanidinium group of Arg^{236}. Arg^{176} is maintained in a protonated state by the neighbouring Asp^{154}, and Lys^{239} remains charged as a result of bulk solvent accessibility. The pH-driven conformational change, which results in a rather low pH optimum for PL1A, is not a result of deprotonation of the Asp^{186}–Asp^{221} pair. Rather, the Asp^{186}–Asp^{221} pair is involved in maintaining the structural integrity that is forced apart at higher pH by an as-yet-unidentified trigger.

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