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Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.275.1.691

Publication date:
2000

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 1, Issue of January 7, pp. 691–696, 2000

Printed in U.S.A.

The Active Site Topology of *Aspergillus niger* Endopolygalacturonase II as Studied by Site-directed Mutagenesis*

(Received for publication, April 6, 1999, and in revised form, August 18, 1999)

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Strictly conserved charged residues among polygalacturonases (Asp-180, Asp-201, Asp-202, His-223, Arg-256, and Lys-258) were subjected to site-directed mutagenesis in *Aspergillus niger* endopolygalacturonase II. Specific activity, product progression, and kinetic parameters (K_m and V_{max}) were determined on polygalacturonic acid for the purified mutated enzymes, and bond cleavage frequencies on oligogalacturonates were calculated. Depending on their specific activity, the mutated endopolygalacturonases II were grouped into three classes. The mutant enzymes displayed bond cleavage frequencies on penta- and/or hexagalacturonate different from the wild type endopolygalacturonase II. Based on the biochemical characterization of endopolygalacturonase II mutants together with the three-dimensional structure of the wild type enzyme, we suggest that the mutated residues are involved in either primarily substrate binding (Arg-256 and Lys-258) or maintaining the proper ionization state of a catalytic residue (His-223). The individual roles of Asp-180, Asp-201, and Asp-202 in catalysis are discussed. The active site topology is different from the one commonly found in inverting glycosyl hydrolases.

Pectic polysaccharides are among the most complex plant cell wall polysaccharides. In the homogalacturonan part, the so-called smooth regions, the 1,4-α-D-galacturonic acid backbone is partly esterified. These smooth regions are interspersed by the rhamnogalacturonan parts consisting of repeating stretches of 1,2-α-L-rhamnose-1,4-α-D-galacturonic acid dimers. Other sugar residues can be attached to the rhamnose residues (1). Because of this complexity, a wide range of enzymes, the so-called pectinases, is necessary for the complete degradation of pectic substances. Two main classes of depolymerizing enzymes act on these polysaccharides: the hydrolases (endopolygalacturonases and rhamnogalacturonases) and the lyases (pectin lyase, pectate lyase, and rhamnogalacturonan lyase).

Endopolygalacturonases (PGs; EC 3.2.1.15)[1] catalyze the random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectates. They have been isolated from a variety of organisms (eukaryotae and prokaryotae). Furthermore, over 40 genes encoding PGs have been cloned and sequenced. The corresponding enzymes have been grouped in family 28 of the general classification of glycosyl hydrolases based on amino acid sequence similarities (2, 3).

The gene encoding the endopolygalacturonase II (PGII) from *Aspergillus niger* has been previously cloned, sequenced, and expressed in *A. niger* (4). The enzyme hydrolyses the glycosidic linkages with inversion of configuration (5). Recently, PGII was extensively characterized with respect to activity on polygalacturonic acid, mode of action, and kinetics on oligogalacturonates (6).

Two different mechanisms have been identified for glycosyl hydrolases: one resulting in retention and the other in inversion of the configuration at the anomeric carbon of the scissile bond (7, 8). Despite this difference, in most glycosidases two residues are directly involved in catalysis: a nucleophile and a proton donor. The average distance between the two catalytic residues has been shown to be about 5.5 Å in retaining glycosidases and about 9.5 Å in inverting enzymes, irrespective of whether α- or β-glycosidic bonds are hydrolyzed (9, 10). Moreover, crystallographic studies revealed that the catalytic amino acids are always aspartates and/or glutamates (11). However, site-directed mutagenesis experiments remain important in the identification of amino acids involved in catalysis.

Of the family 28 enzymes, the rhamnogalacturonase A from *Aspergillus aculeatus* and the polygalacturonase from *Erwinia caratovora* are the only members for which three-dimensional structures have been described (12, 13). Even though the two enzymes do not act on the same region of the pectic molecule, their structures, as well as the structure of *A. niger* PGII,[2] do indeed show similar topologies, and many of the conserved residues throughout family 28 are located in the active sites of rhamnogalacturonase A and the polygalacturonases.

There are two aspartate residues strictly conserved within family 28, which could have catalytic roles. Moreover, other charged amino acids conserved among polygalacturonases are likely to play important roles such as maintaining the structure of the enzyme and establishing hydrogen bonding and hydrophobic interactions between the enzyme and the substrate.

For a better understanding of the mode of action and function of *A. niger* PGII in the degradation of pectins, site-directed mutagenesis has been carried out on the six charged amino acids (Asp-180, Asp-201, Asp-202, His-223, Arg-256, and Lys-258; sequence numbers according to PGII) that are conserved among polygalacturonases. Based on our results, we propose two aspartic acids to act together to activate the water,

* This work was supported by European Community Grant ERB-BIOCT960685. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PG, polygalacturonase; BCF, bond cleavage frequency; Gal*p*A, galacturonic acid; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; HPLC, high pressure liquid chromatography.

[2] Y. van Santen and B. W. Dijkstra, unpublished results.

whereas a third aspartic acid serves as the general acid in PGII. The corresponding residues in the polygalacturonase from *E. caratovora* were identified in the recently solved crystal structure (13).

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The strains of *Escherichia coli* used in this study were JM109 (14) and *E. coli* BMH 71-18 *mut*S ([*mut*S::Tn10] Δ(*lac-pro*) *thi supE* [F′ *proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*]). The *A. niger* strain used was NW188 (*pyr*A6, *prt*F28, *gox*C17, *leu*A1), which is derived from *A. niger* N400 (CBS 120.49). The original *prt*F28 mutation was described previously (15). *E. coli* strains containing recombinant plasmids were cultured in LB supplemented with 80 μg/ml ampicillin at 37 °C. *A. niger* co-transformants containing the desired recombinant plasmids were cultured in a minimal medium containing 0.15% (w/v) KH_2PO_4, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.4% (w/v) NH_4Cl (pH 6.0) supplemented with 0.1% (w/v) yeast extract, trace metal solution (16), 3% (w/v) fructose, and 0.02% (w/v) leucine.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the Altered Sites II kit (Promega, Madison, WI) and synthetic oligonucleotides (Isogen, Maarsen, The Netherlands). Reactions were done according to the supplier recommendations with the following modifications. First, single-stranded DNA of the *pki-pga*II promoter gene fusion cloned into pAlter I was used as a template instead of double-stranded DNA. The *pki-pga*II promoter gene fusion was recovered as an *Eco*RI-*Hin*dIII insert from pIM3710 (6) and cloned into *Eco*RI-*Hin*dIII restricted pAlter I. Second, following *E. coli* BMH 71-18 *mut*S cells transformation with the mutagenesis mixture, plasmids were isolated instead of phagemids. *E. coli* JM109 competent cells were transformed with the potential mutated plasmids. To establish the expected mutation and to check for undesired mutations, the complete mutated *pga*II gene was sequenced. *A. niger* NW156 was then co-transformed with the plasmids showing the expected mutations and pGW635 carrying the *pyr*A gene as a selection marker (17). Mutated PGII producers were selected the same way as described for *pga*E (18). Appropriate mutations were generated by using synthetic oligonucleotides specifying the desired change (sequences available upon request).

Enzyme Purification—Mutated forms of PGII were purified to homogeneity as follows. Cultures of appropriate strains of *A. niger* were grown at 30 °C in 1-liter flasks containing 300 ml of minimal medium supplemented as described above. After 18–20 h of cultivation, the mycelium was removed by filtration over nylon gauze, the culture medium was diluted twice with distilled water, adjusted to pH 3.8 and applied onto a Streamline SP column (Amersham Pharmacia Biotech) previously equilibrated with 10 mM sodium citrate buffer (pH 3.8). After washing with the same buffer, the proteins were eluted with 10 mM sodium citrate buffer (pH 3.8) containing 1 M NaCl. The protein containing fractions were identified by measuring the absorbance at 280 nm, pooled, dialyzed overnight against 10 mM sodium citrate (pH 3.8), and loaded onto a Source S column (bed volume 11 ml, Amersham Pharmacia Biotech) equilibrated with the same buffer. Mutated forms of PGII were eluted with a 0–0.5 M NaCl gradient. Elution was monitored by measuring the absorbance at 280 nm, and the fractions containing pure PGII were pooled, dialyzed overnight against 50 mM sodium acetate (pH 4.2) and stored at +4 °C. The purity of the enzymes was evaluated by SDS-polyacrylamide gel electrophoresis analysis and Coomassie Brilliant Blue staining. Protein concentration of the purified PGII was determined by measuring A_{280} using a molar extinction coefficient of 48590 M^{-1} cm^{-1}.

Enzyme Assay—Standard PG activity assays were carried out using 50 mM sodium acetate buffer (pH 4.2), containing 0.25% (w/v) polygalacturonic acid (U. S. Biochemical Corp., Cleveland, OH) in a total volume of 0.5 ml. The assay buffer was equilibrated at 30 °C, and the reaction was initiated by the addition of 20 μl of enzyme solution in the same buffer at a concentration with which the reaction rates were linear over the selected time course. The amount of reducing end groups liberated after different incubation times was measured as described (19). In addition, the polygalacturonase activity was also measured in 20 mM methyl-piperazine/HCl and McIlvaine buffers (pH 4.2) (20) to study the ionic strength dependence of each of the mutated forms of PGII.

Determination of Kinetic Parameters on Polygalacturonic Acid—Initial rate measurements were made in 50 mM sodium acetate buffer (pH 4.2) at several substrate concentrations. The reaction was initiated by the addition of enzyme and monitored as for the standard PG assay. Values of K_m and V_{max} were obtained by nonlinear regression analysis using the program Sigmaplot.

Determination of the pH Optimum—Incubations were carried out as for the standard enzyme assay on 0.25% (w/v) polygalacturonic acid using McIlvaine buffers ranging from pH 2.5 to pH 6. For the K258N mutant, the reactions were carried out in pH adjusted distilled water containing 50 mM NaCl, and the pH of the reaction mixture was carefully measured at the end of the reaction.

HPLC Analysis of the Hydrolysis Products of Oligogalacturonates—Oligogalacturonates were isolated as described by Kester and Visser (21). Determination of bond cleavage frequencies (BCFs) for each mutated form of PGII was performed on 0.5 mM oligomers of different degree of polymerization (n = 3–6) at 30 °C. After different incubation times (0–30 min), the enzymatic hydrolysis was stopped, and the reaction products were analyzed and quantified by HPAEC-PAD as described previously (18).

HPLC Analysis of the Products Distribution after the Hydrolysis of Polygalacturonic Acid—Each mutant enzyme was incubated with 1 ml of 1% (w/v) polygalacturonic acid in 50 mM sodium acetate buffer (pH 4.2) at 30 °C. After different incubation times (0–24 h), the enzymatic hydrolysis was stopped as described (18), and the solution was diluted three times with water prior to HPAEC-PAD analysis as described (6).

RESULTS

Expression and Purification of the Enzymes—Asp-180, Asp-201, Asp-202, His-223, Arg-256, and Lys-258 were changed to investigate their role in catalysis by PGII (Table I). The amino acids introduced were chosen such as to minimize the risk of disturbance of the overall structure of the enzyme. The locations of the mutated residues are depicted in Figs. 1 and 2.

The enzymes were purified to homogeneity by cation-exchange chromatography (fast protein liquid chromatography) and analyzed by SDS-polyacrylamide gel electrophoresis to check for purity. Each protein ran as a single band (of about 38 kDa) at the same position as the native enzyme, indicating no differences in the glycosylation between the mutated and the wild type PGII (data not shown).

Specific Activity—The specific activity of each mutated enzyme was determined in a standard assay and compared with the specific activity of the wild type enzyme (Table I). The data presented show that all mutations affect the PG activity. The examination of the specific activities obtained in 50 mM sodium acetate buffer (pH 4.2) reveals three different classes of enzymes. The first class comprises the most affected mutated forms of PGII (D180(A/E), D201(E/N), D202N, D180E/D201E, and H223Q) for which the recorded activity is at least 10^4 fold lower than that of the wild type enzyme. A second class of enzymes is composed of D180N, D202E, H223A, H223S, H223C, and K258N that retain between 0.01 and 1% of the wild type activity. Finally, R256Q retained 6.5% of the wild type activity.

The wild type enzyme and most of the mutated forms of PGII appeared to be insensitive to the ionic strength of the reaction medium because their specific activities were not influenced by the buffer used (Table I). However, two PGII mutants showed a distinct behavior. R256Q was 2.5 times more active in 20 mM methyl-piperazine/HCl buffer than in the two other buffers. K258N was also particular because it was found to be inactive in McIlvaine buffer, sodium citrate, and di-sodium hydrogen phosphate (the two components of this buffer) both behaving as strong inhibitors of the enzyme (data not shown).

It should be noted that although the overall effect of each mutation is clear, the nature of the amino acid introduced also influenced the remaining specific activity of the different mutated forms of PGII (Table I). The most noticeable effect was observed within the different His-223 mutated proteins of which the specific activities in sodium acetate buffer range from 0.2 unit mg^{-1} (for H223Q) to 21.1 unit mg^{-1} (for H223C). Asp-202 mutated proteins were also affected, because D202E was 50–100 times more active than D202N in sodium acetate buffer and McIlvaine buffer, respectively. D180N was 6 times more active than the other Asp-180 mutated proteins in sodium

TABLE I

Specific activities and kinetic parameters of mutated endopolygalacturonase II

Specific activities of PGII mutants were determined in 50 mM sodium acetate buffer (NaAc) (pH 4.2), 20 mM methyl-piperazine/HCl buffer (MePIP) (pH 4.2), and McIlvaine buffer (McIlv) (pH 4.2). Kinetic parameters were determined in 50 mM sodium acetate buffer (pH 4.2). pH optima (pH opt) were determined using McIlvaine buffers. Polygalacturonic acid was used as a substrate. For specific activities 2.5 mg ml^{-1} polygalacturonic acid was used. Temperature throughout 30 °C. $\Delta\Delta G$ values signify the transition state energy difference between wild type and mutant enzyme as calculated from the kinetic parameters. ND, not determined.

Enzyme	pH opt (McIlv)	Specific activities			Kinetic parameters (NaAc)		$\Delta\Delta G$
		NaAc	MePIP	McIlv	V_{max}	K_m	
		units mg^{-1}	*units* mg^{-1}	*units* mg^{-1}	*units* mg^{-1}	*mg* ml^{-1}	*kJ* mol^{-1}
Wild type	4.2	2000	2270	2140	2000	<0.15	0
D180A	4.1	0.18	0.28	0.27	0.17	0.15	22.5
D180E	4.2	0.15	0.20	0.14	0.24	0.3	23.3
D180N	3.9	0.95	1.25	0.95	1.4	1.5	23.0
D201E	4.2	0.05	0.06	0.04	0.04	0.3	27.6
D201N	3.9	0.15	0.38	0.22	0.19	0.3	23.9
D202E	4.2	9.4	15.4	10.2	12.7	0.7	15.9
D202N	4.1	0.17	0.26	0.12	0.3	1.5	26.7
D180E/D201E	4.2	0.04	0.01	0.02	0.04	<0.15	26.0
H223A	4.1	ND	ND	ND	10.0	0.15	15.6
H223C	3.8	21.1	18.9	18.0	21.5	0.80	14.9
H223Q	3.9	0.19	0.38	0.21	0.36	1.1	25.5
H223S	4.1	1.68	1.65	1.2	1.7	1.5	22.5
R256Q	3.8	130	326	129	278	1.7	10.6
K258N	3.8[a]	12.7	8.9	0.00	16.2	2.8	18.6

[a] Determined in water.

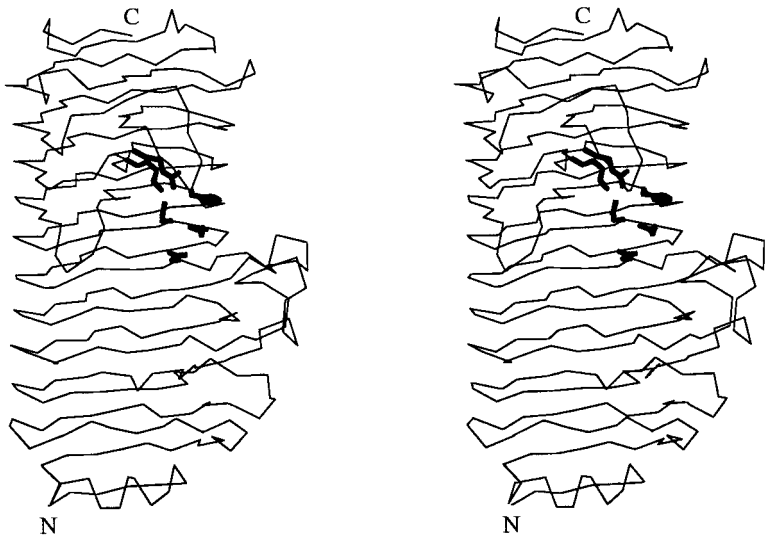

FIG. 1. **Stereofigure of the Cα of *A. niger* endopolygalacturonase II with side chains of mutated residues (*bold lines*).** This figure was rendered using MOLSCRIPT (30).

acetate, but the specific activities of all the Asp-180 mutated forms of PGII in McIlvaine buffer were comparable. Finally, the amino acid introduced to replace Asp-201 only played a minor role because the specific activities between D201E and D201N only varied by a factor of 3–5, depending on which buffer was used. The reason why the nature of the amino acid introduced leads to a difference in the remaining specific activity of the enzyme is still unclear, but detailed crystallographic studies may provide further insights.

Kinetic Parameters—The kinetic parameters K_m and V_{max} for the hydrolysis of polygalacturonic acid in 50 mM sodium acetate buffer (pH 4.2) were determined for the wild type (6) and the mutated enzymes by measuring the initial reaction rates at different substrate concentrations. The results are listed in Table I.

D180(A/E), D201(E/N), D180E/D201E, and H223A displayed values of K_m (between 0.15 and 0.3 mg ml^{-1}) in the same range as the K_m calculated for the wild type enzyme (<0.15 mg ml^{-1}). All the other mutated forms of PGII exhibited higher values of K_m, ranging from 0.7 mg ml^{-1} for D202E to 2.8 mg ml^{-1} in the case of K258N.

As already observed with the specific activities, the effect on K_m appears to depend on the side chain engineered as well as on the residue replaced. For instance, mutagenesis of His-223 resulted in enzymes with K_m values ranging from 0.15 mg ml^{-1} (H223A) to 1.5 mg ml^{-1} (H223S). Changes in K_m values were also observed among the different Asp-180 mutated forms of PGII. The only exceptions were enzymes D201E and D201N for which the K_m was found to be 0.3 mg ml^{-1} in both cases.

pH Optimum—The pH optima for the activity on polygalacturonic acid were determined using McIlvaine buffers in the pH range from 2.5 to 6.0, except for K258N. As this enzyme was not active in McIlvaine buffers, its activity was measured in pH adjusted nonbuffered solutions. The pH optima of the mutated enzymes varied only slightly between pH 3.8 and pH 4.1. Thus, the mutations only produced a minimal effect on the pH optimum of the mutated forms of PGII.

Bond Cleavage Frequencies on Oligogalacturonates—By studying the hydrolysis reactions of the wild type PGII on reduced and nonreduced oligogalacturonates, it was previously shown that the cleavage of the glycosidic bond in the oligomers occurs from the reducing end (6). In a similar way, the cleavage patterns for each mutated form of the polygalacturonase were determined using (Gal*p*A)$_{3-6}$ as substrates. The hydrolysis re-

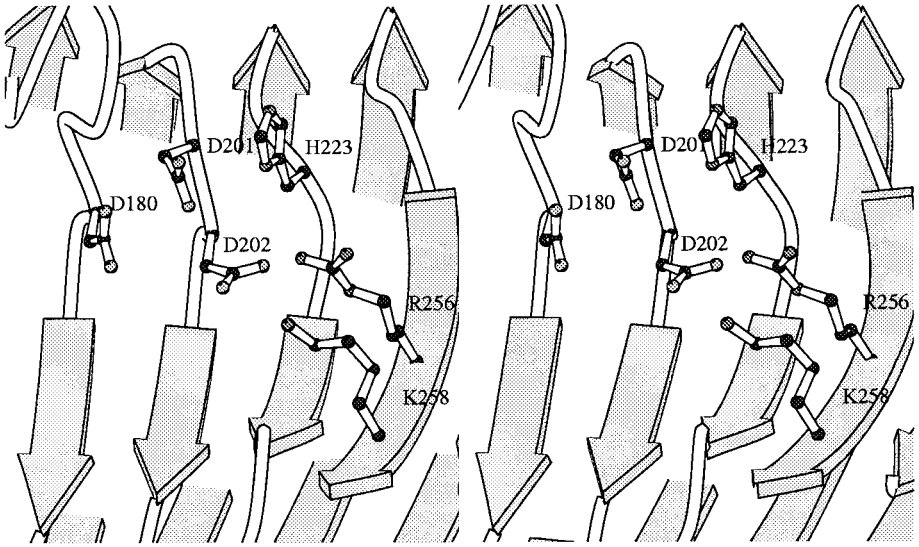

FIG. 2. **Stereofigure showing the location of the mutated residues in the active site.** This figure was rendered using MOLSCRIPT (30).

actions were conducted in 50 mM sodium acetate buffer (pH 4.2) at an oligomer concentration of 0.5 mM. The BCFs thus calculated are presented in Table II.

None of the enzymes, including the wild type PGII, appeared to hydrolyze (Gal*p*A)$_3$ under these conditions. The hydrolysis of (Gal*p*A)$_4$ by each enzyme resulted only in (Gal*p*A)$_1$ and (Gal*p*A)$_3$, as already observed for the wild type PGII (6). A minor production (<0.5%) of (Gal*p*A)$_2$ was, however, observed in some cases, when the reaction was allowed to proceed beyond 50% of conversion of the substrate.

Benen *et al.* (6) showed that the hydrolysis of (Gal*p*A)$_5$ by native polygalacturonase II produced (Gal*p*A)$_1$ and (Gal*p*A)$_4$ with a BCF of 67%, whereas the formation of (Gal*p*A)$_2$ and (Gal*p*A)$_3$ occurred with a BCF of 33%. For the majority of the mutated forms of PGII (D180(A/N), D201(E/N), D202N, D180E/D201E, H223A, and H223S), the observed BCFs of the pentamer are comparable with those of native PGII. D180E, D202E, H223Q, R256Q, and K258N did not show any preference for the linkage hydrolyzed because the cleavage at the first and second glycosidic bond counting from the reducing end occurred with the same frequency. H223C displayed the most striking effect compared with the wild type enzyme because this enzyme hydrolyzed (Gal*p*A)$_5$ preferentially producing (Gal*p*A)$_2$ and (Gal*p*A)$_3$ (BCF of 80%, compared with 33% for the native enzyme).

The hydrolysis of (Gal*p*A)$_6$ revealed stronger effects on the BCFs because none of the mutated enzymes displayed an hydrolysis pattern comparable with that of the wild type PGII (Table II). Each mutated form of PGII exhibited a shift in BCFs in favor of the second and/or third linkage, counting from the reducing end. The most remarkable effect was once again observed with H223C, which hydrolyzed the hexamer producing (Gal*p*A)$_4$ and (Gal*p*A)$_2$ with a BCF of 84%, and only attacked the first glycosidic bond sporadically (BCF of 2%). For the majority of the other mutated enzymes, the cleavage at the first glycosidic linkage also occurred less often than in the case of the wild type PGII, favoring the attack of the second glycosidic bond, whereas the BCF at the third bond was unchanged. D180A, H223S, R256Q, and K258N were the only enzymes to display a clear increase in the hydrolysis of the hexamer into (Gal*p*A)$_3$, even if the release of (Gal*p*A)$_2$ and (Gal*p*A)$_4$ remained predominant as is the case for the native polygalacturonase II.

The shift in BCFs for the mutated enzymes was also reflected in the product progression curves during the hydrolysis of polygalacturonic acid. For the wild type enzyme a product progression curve typical for endo-acting enzymes was observed (6). The mutated enzymes accumulated higher oligomers ($n > 5$) in a way similar to that of the wild type PGII. However, the rate of accumulation of smaller oligomers ($n < 5$) was different depending on the enzyme. Enzymes D180(A/E/N), D201(E/N), and D202N showed only small differences compared with the wild type enzyme with respect to the ratio of oligomers formed. More striking differences were observed with H223(A/S/C), D202E, R256Q, and K258N, where a strong accumulation of (Gal*p*A)$_2$ was monitored (not shown). These data correlate with the observed changes in the BCFs on penta- and hexaoligosaccharides.

DISCUSSION

The data presented in this paper describe the biochemical characterization of several site-specific mutants of PGII from *A. niger*. The primary objective of this study was to investigate the role of the highly conserved residues in the active site of PGII.

General Considerations—All the residues mutated appeared to be very critical for catalysis. For each residue, except for His-223, a counterpart is present in the rhamnogalacturonase A from *A. aculeatus* (12). The basic difference between the polygalacturonases and the rhamnogalacturonases resides in their substrate specificity. Whereas polygalacturonases hydrolyze the α-1,4 glycosidic linkage between galacturonate residues, rhamnogalacturonases hydrolyze the α-1,2 glycosidic linkage between galacturonate and rhamnose. The common part of the substrate, the galacturonate moiety, will be accommodated at subsite -1. This subsite -1 is expected to display the highest sequence conservation. As a consequence, residues Asp-180, Asp-201, Asp-202, Arg-256, and Lys-258 should constitute residues of subsite -1 and the catalytic machinery between subsites -1 and $+1$. It is therefore assumed that the mutations do not affect subsites -4, -3, -2, $+2$, and $+3$.

The bond cleavage frequencies for a particular (mutated) enzyme do not necessarily reflect the real binding energy distribution over the subsites covered because the rates of hydrolysis of each particular binding mode may be different (22). Indeed, the bond cleavage frequencies only take productive complexes into account, thus the substrate must always cover subsites -1 and $+1$. Therefore any change of affinity at subsites -1 or $+1$ would affect any binding mode covering subsites -1 and $+1$ with the same ΔG change. However, changes in BCFs were observed that could be explained by the following. At subsite -1, the substrate moiety is generally thought to be bound in a particular distorted configuration to facilitate the

TABLE II

Bond cleavage frequencies for wild type and mutated endopolygalacturonase II

Assay conditions: 0.5 mM oligogalacturonates were incubated with (mutated) endopolygalacturonase II in 0.5 ml of 50 mM sodium acetate (pH 4.2). At timed intervals 50-μl aliquots were withdrawn and mixed with 50 μl of stopmix (2.0 mM Tris, 50 mM NaOH) to raise the pH to 8.3–8.5. Products were analyzed and quantified by HPAEC-PAD as described under "Experimental Procedures." The bold type indicates the reducing end. Bond cleavage frequencies are given in percentages. Gn signifies $(GalpA)_n$.

Enzyme	Gn											
	5			G	—	G	—	G	—	G	—	**G**
Wild type									33		67	
D180A									30		70	
D180E									50		50	
D180N									36		64	
D201E									39		61	
D201N									32		68	
D202E									52		48	
D202N									30		70	
D180E/D201E									35		65	
H223A									27		73	
H223C									80		20	
H223Q									48		52	
H223S									28		72	
R256Q									50		50	
K258N									52		48	
	6	G	—	G	—	G	—	G	—	G	—	**G**
Wild type							8		57		35	
D180A							20		58		22	
D180E							9		73		18	
D180N							10		68		22	
D201E							9		76		15	
D201N							11		65		24	
D202E							9		81		10	
D202N							11		62		27	
D180E/D201E							11		63		26	
H223A							10		65		25	
H223C							14		84		2	
H223Q							6		72		22	
H223S							16		64		20	
R256Q							15		74		11	
K258N							18		70		12	

glycosidic bond cleavage (31), which results in a net negative affinity at this subsite. To compensate for this negative affinity and to properly align the scissile bond, subsite +1 binds the substrate with high affinity and thus allows effective catalysis. Any decrease of the affinity at subsite +1 may not allow for compensation for the negative affinity at subsite −1, and this would therefore result in less effective catalysis. Only by invoking subsite +2 would there be enough binding energy to compensate for the low affinity at subsite −1, and hence this would result in a shift of the BCFs. For affinity changes at subsite −1, the reasoning follows the same lines. Any change at this site involved in the binding of the substrate would decrease the rate of hydrolysis, because the ideal distortion will be changed. To compensate for this and to provide enough strain on the scissile bond to allow an effective catalysis, additional binding at subsite +2 would be required. Thus, the bond cleavage frequencies become meaningful when combining both the affinity and the rate of hydrolysis in terms of effectiveness.

Importance of Arg-256 and Lys-258—To evaluate the role of these two residues, they were substituted for glutamine and asparagine respectively, and the biochemical properties of the resultant proteins were analyzed. Among all the mutated enzymes studied, R256Q and K258N displayed the highest specific activities and highest K_m values on polygalacturonic acid. Despite the high K_m values, the mutated enzymes revealed the smallest effect on the transition state energy. It should be noted that for the calculation of the transition state energy for the wild type enzyme a K_m value of 0.15 mg ml^{-1} was used, which may result in an underestimation of the effect of the mutations on the transition state energy. In the crystal structure Arg-256 and Lys-258 are about 4 Å apart. This suggests that the residues may interact with adjacent galacturonate residues, occupying subsite −1 (Lys-258) and +1 (Arg-256). This is corroborated by the transition state energy difference, which is for both mutants in the order of an ionogenic bond. Furthermore, mutant K258N was severely inhibited by the negatively charged ions citrate and phosphate. Also, both mutations led to enzymes with a mode of action on oligogalacturonates clearly different from the wild type PGII, which is compatible with a mutation at subsite −1 or +1. It can easily be envisaged that the absence of the interaction between the substrate and residue Arg-256 or Lys-258 will change the geometry of the ideal distortion of the substrate, which will result in the observed decreased V_{max}. Taken together, these data strongly suggest that Arg-256 and Lys-258 are primarily involved in the interactions with the substrate.

Role of His-223—The (in)direct involvement of a histidine residue in the activity of PGII has been proposed several times on the basis of chemical modifications (23–25) and site-directed mutagenesis (26). However, there is no example of such a residue being one of the catalytic amino acids in glycosyl hydrolases. In addition, although this histidine is strictly conserved among the family 28 polygalacturonases, it is not present in the rhamnogalacturonase A from *A. aculeatus*, which belongs to the same family. As the catalytic residues are always strictly conserved within a family, this latter point definitely rules out His-223 to be the proton donor in the reaction catalyzed by PGII. The remaining activity of the His-223 mutated enzymes was extremely dependent on the nature of the amino acid introduced, which is in contradiction with His-223 being a catalytic residue. Inspection of the His-223 mutated enzymes revealed that all enzymes were severely affected in catalysis but that the bond cleavage frequencies did not change dramatically for enzymes H223A and H223S, when a small residue is

engineered. This implies that the relative effectiveness of the wild type enzyme and enzymes H223A and H223S has not changed, and thus the disturbance at subsites −1 and +1 is minimal, which indicates that His-223 plays an indirect role in catalysis. This role may be to maintain the proper ionization state of a carboxylate involved in catalysis by sharing a proton. Based on the crystal structure,[2] this carboxylate could be Asp-201, which is the closest. The presumed role of His-223 is corroborated by the fact that enzyme H223C, whose sulfhydryl group is capable of sharing a proton, despite its interference with the substrate, still retains the highest activity. Moreover, the rhamnogalacturonases A and B from *A. niger*, which lack an His-223 counterpart have specific activities in the same range as the one found for enzyme H223C *viz*. 30–40 units mg^{-1} (27).

Importance of Asp-180, Asp-201, and Asp-202—Polygalacturonases have been shown to hydrolyze glycosidic bonds with an inverting mechanism (5) that requires two carboxylic groups at a distance of 9–9.5 Å from each other (9, 10). Sequence alignment of polygalacturonases revealed three strictly conserved Asp residues among these enzymes (Asp-180, Asp-201, and Asp-202). However, in the rhamnogalacturonase that belongs to the same family, Asp-202 is replaced by a Glu. Our data show that the mutation of each of these residues led to a dramatic decrease of the specific activity of PGII. All the mutated enzymes indeed retained between 0.002 and 0.07% of the wild type activity, except D202E, for which the remaining activity was 0.64%.

To understand the role of the acidic residues, we compared the PGII structure[2] with the similar phage 22 tailspike rhamnosidase (28). This latter enzyme also shows a β-helix fold and, moreover, has three acidic residues in the active site (Glu-359, Asp-392, and Asp-395) in a geometry similar to that of the polygalacturonase and rhamnogalacturonase. In accordance with our results, mutagenesis of those residues also resulted in severe reduction of enzymatic activity in each case (29). Comparison of the rhamnosidase structures with and without the *Salmonella typhy 253Ty* nonasaccharide bound revealed that the water bound between Glu-359 and Asp-395 (Asp-180 and Asp-202 in endopolygalacturonase II) is in an ideal position for nucleophilic attack in an inverting mechanism. Asp-392 (Asp-201 in endopolygalacturonase II), at hydrogen bonding distance from the O-1 atom of the rhamnose at subsite −1, may be the general acid. In PGII, the structure indeed revealed that a water molecule is bound between Asp-180 and Asp-202. Moreover, a galacturonate residue could be modelled into the structure of PGII based on the superposition of the catalytically important oxygen atoms resembling the position of the rhamnose in the rhamnosidase.[2]

Based on our results and in analogy to the phage 22 tailspike rhamnosidase, we propose that Asp-180, with the assistance of Asp-202, acts as a base to activate the bound water molecule. The fact that the mutation of Asp-180 into either Ala or Gln or Glu leads to an inactive enzyme supports the possibility that this amino acid is much more critical for the catalysis than Asp-202. Indeed, the mutation of Asp-202 into Glu (a conservative change that retains the anionic property) leads to an enzyme that displays the highest activity among the Asp mutants.

Asp-201 would then be the general acid that protonates the product when it departs. Three arguments are in favor of this proposal: (i) the mutation of this residue led to an inactive enzyme; (ii) its replacement revealed the smallest effect on the BCFs on oligogalacturonates, which suggests that Asp-201 does not directly interact with the substrate; and (iii) His-223, which is important for catalysis most likely shares a proton with Asp-201, allowing this latter amino acid to be in the proper ionization state to protonate the product. These three aspartic residues in the polygalacturonase from *E. caratovora* were identified in the recently solved crystal structure, and Pickersgill *et al.* (13) the authors also proposed that the amino acids corresponding to Asp-180 and Asp-201 in PGII are directly involved in catalysis.

A careful inspection of both rhamnogalacturonase and polygalacturonase structures (12, 13) revealed that the three acidic residues are very close to each other and that the distance in PGII between Asp-180 and Asp-201 (4.1 Å), Asp-201 and Asp-202 (4.9 Å), and Asp-180 and Asp-202 (5.7 Å) is not compatible with an inverting mechanism. Our data and the data obtained for the phage 22 tail spike rhamnosidase strongly indicate that nucleophilic attack and protonation can occur from the same side of the glycosidic bond in α-linked carbohydrates. It can therefore be stated that family 28 glycosyl hydrolases diverge with respect to their active site configuration from the generally observed active site architecture found in inverting enzymes.

To firmly establish the exact role of the individual amino acids, it is important to obtain an enzyme-substrate complex with polygalacturonase II. The mutant D180E/D201E prepared in this study would be the protein of choice for the study of the enzyme-substrate complex structure.

REFERENCES

1. Bacic, A., Harris, P. J., and Stone (1988) *The Biochemistry of Plants: Carbohydrates*, Vol. 14, pp. 297–369, Academic Press, London
2. Henrissat, B. (1991) *Biochem. J.* **280,** 309–316
3. Henrissat, B., and Bairoch, A. (1993) *Biochem. J.* **293,** 781–788
4. Bussink, H. J. D., Kester, H. C. M., and Visser, J. (1990) *FEBS Lett.* **273,** 127–130
5. Biely, P., Benen, J. A. E., Heinrichová, K., Kester, H. C. M., and Visser, J. (1996) *FEBS Lett.* **382,** 249–255
6. Benen, J. A. E., Kester, H. C. M., and Visser, J. (1999) *Eur. J. Biochem.* **259,** 577–585
7. Koshland, D. E. (1953) *Biol. Rev., Cambridge Phil. Soc.* **28,** 416–436
8. Sinnott, M. L. (1990) *Chem. Rev.* **90,** 1171–1202
9. White, A., Withers, S. G., Gilkes, N. R., and Rose, D. R. (1994) *Biochemistry* **33,** 12546–12552
10. McCarter, J., and Withers, S. G. (1994) *Curr. Opin. Struct. Biol.* **4,** 885–892
11. Davies, G., and Henrissat, B. (1995) *Structure* **3,** 853–859
12. Petersen, T. N., Kauppinen, S., and Larsen, S. (1997) *Structure* **5,** 533–544
13. Pickersgill, R. Smith, D., Worboys, K., and Jenkins, J. (1998) *J. Biol. Chem.* **273,** 24660–24664
14. Yanisch-Perron, C., Viera, J., and Messing, J. (1985) *Gene* (*Amst.*) **33,** 103–119
15. van den Hombergh, J. P. T. W., van de Vondervoort, P. J. I., van der Heijden, N. C. B. A., and Visser, J. (1995) *Curr. Genet.* **28,** 299–308
16. Vishniac, W., and Santer, M (1957) *Bacteriol. Rev.* **21,** 195–213
17. Kusters-van Someren, M. A., Harmsen, J. A. M., Kester, H. C. M., and Visser, J. (1991) *Curr. Genet.* **20,** 293–299
18. Parenicová, L., Benen, J. A. E., Kester, H. C. M., and Visser, J. (1998) *Eur. J. Biochem.* **251,** 72–80
19. Stephens, B. G., Felkel Jr, H. J., and Spinelli, W. M. (1974) *Anal. Chem.* **46,** 692–696
20. Elving, P. J., Markowitz, J. M., and Rosenthal, I. (1956) *Anal. Chem.* **28,** 1179–1180
21. Kester, H. C. M., and Visser, J. (1990) *Biotechnol. Appl. Biochem.* **12,** 150–160
22. Thoma, J. A., Rao, G. V. K., Brothers, C., Spradlin, J., and Li, H. L. (1971) *J. Biol. Chem.* **246,** 5621–5635
23. Cooke, R. D., Ferber, C. E. M., and Kanagasabapathy, L. (1976) *Biochim. Biophys. Acta* **452,** 440–451
24. Rexová-Benková, L., and Mracková, M. (1978) *Biochim. Biophys. Acta* **523,** 162–169
25. Rao, M. N., Kembhavi, A. A., and Pant, A. (1996) *Biochim. Biophys. Acta* **1296,** 167–173
26. Caprari, C., Mattei, B., Basile, M. L., Salvi, G., Crescenzi, V., De Lorenzo, G., and Cervone, F. (1996) *Mol. Plant-Microbe Interact.* **9,** 617–624
27. Suykerbuyk, M. E. G., Kester, H. C. M., Schaap, P. J., Stam, H., Musters, W., and Visser, J. (1997) *Appl. Environm. Microbiol.* **63,** 2507–2515
28. Steinbacher, S., Baxa, U., Miller, S., Weintraub, A., Seckler, R., and Huber, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10584–10588
29. Baxa, U., Steinbacher, S., Miller, S., Weintraub, A., Huber, R., and Seckler, R. (1996) *Biophys. J.* **71,** 2040–2048
30. Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24,** 946–950
31. Sulzenbachek, G., Driguez, H., Henrissat, B., Scülein, M., and Davies, G. J. (1996) *Biochemistry* **35,** 15280–15287