Active Site and Oligosaccharide Recognition Residues of Peptide-\(N^\text{acetylated}\)-\(\beta\)-\(\text{d}-\text{glucosaminyl}\)asparagine Amidase F*

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Peter Kühn\(^\text{¶}§\), Chudi Guan\(\text{¶}\), Tao Cui\(\text{¶}\), Anthony L. Tarentino\(\text{¶}\), Thomas H. Plummer, J. r. t.\(\text{¶}\), and Patrick Van Roey\(\text{¶}\)***

From the Wadsworth Center, New York State Department of Health, Albany, New York 12201, the \(\text{§}\)Physics Department, University at Albany, Albany, New York 12222, \(\text{\¶}\)New England Biolabs, Inc., Beverly, Massachusetts 01915, and the \(\text{¶}\)Biomedical Sciences Department, School of Public Health, University at Albany, Albany, New York 12201

Crystallographic analysis and site-directed mutagenesis have been used to identify the catalytic and oligosaccharide recognition residues of peptide-\(N^\text{acetylated}\)-\(\beta\)-\(\text{d}-\text{glucosaminyl}\)asparagine amidase F (PNGase F), an amidohydrolase that removes intact asparagine-linked oligosaccharides from glycoproteins and glycopeptides. Mutagenesis has shown that three acidic residues, Asp-60, Glu-206, and Glu-118, that are located in a cleft at the interface between the two domains of the protein are essential for activity. The D60N mutant has no detectable activity, while E206Q and E118Q have less than 0.01 and 0.1% of the wild-type activity, respectively. Crystallographic analysis, at 2.0-Å resolution, of the complex of the wild-type enzyme with the product, \(N, N'-\text{diacetylchitobiose}\), shows that Asp-60 is in direct contact with the substrate at the cleavage site, while Glu-206 makes contact through a bridging water molecule. This indicates that Asp-60 is the primary catalytic residue, while Glu-206 probably is important for stabilization of reaction intermediates. Glu-118 forms a hydrogen bond with \(O_\text{e}\) of the second N-acetylglucosamine residue of the substrate and the low activity of the E118Q mutant results from its reduced ability to bind the oligosaccharide. This analysis also suggests that the mechanism of action of PNGase F differs from those of \(\text{\L}-\text{asparaginase}\) and glycosylasparaginase, which involve a threonine residue as the nucleophile.

Peptide-\(N^\text{acetylated}\)-\(\beta\)-\(\text{d}-\text{glucosaminyl}\)asparagine amidase F (PNGase F, \(\text{\F}\)peptide N-glucanase) is a 34.8-kDa amidohydrolase secreted by Flavobacterium meningosepticum (1). The enzyme degrades the \(\beta\)-asparagylglucosamine bond of asparagine-linked oligosaccharides, converting the asparagine residue to an aspartic acid (2). The 1-amino-oligosaccharide product spontaneously hydrolyzes to ammonia and the intact oligosaccharide chain (3). The enzyme has a broad substrate specificity, but both the amino and carboxyl groups of the asparagine residue have to be in peptide linkage, while the oligosaccharide must consist at least of the \(N, N'-\text{diacetylchitobiose}\), GlcNAc\(\text{\text{-}3}\)GlcNAc. The enzyme is highly sensitive to modifications of this core; an \(\alpha-1\rightarrow3\)-fucose substituent on the asparagine-proximal, or reducing end, GlcNAc totally blocks PNGase F activity (4), but an \(\alpha-1\rightarrow6\)-fucose substituent has no effect. The enzyme is extensively used as a biochemical tool for the study and analysis of glycoproteins (5), including the analysis of oligosaccharides as well as the deglycosylation of glycoproteins for structural analysis.

Two crystal structures of PNGase F, in different crystal forms, were recently published (6, 7). The molecular structure, shown in Fig. 1, consists of two eight-stranded antiparallel \(\beta\)-sandwiches that lie side-by-side so that the interface runs the full length of the \(\beta\)-sheets. Very little information regarding the mechanism of action of the enzyme was available when the structures were determined, and the assignment of a possible active site was based primarily on the observation of surface features of the molecule, led to the identification of three possible substrate binding sites (6): a bowl on one face of the molecule that contains a group of residues that somewhat resemble the active site of \(\text{\L}-\text{asparaginase}\), a shallow S-shaped cleft on the opposite face of the molecule that contains a number of acidic residues and threonine residues, and a deep cleft at the interface between the two domains at one end of the molecule. This cleft, formed by the loops connecting \(\beta\)-strands between the \(\beta\)-sheets contains several acidic residues and serines, as well as many aromatic residues. Most remarkable is the presence of five tryptophan residues.

In this paper we describe the results of site-directed mutagenesis studies, resulting in the identification of the active site area, and of the crystallographic analysis of the complex of the enzyme with the product disaccharide \(N, N'-\text{diacetylchitobiose}\). These studies lead to the unambiguous identification of the catalytic residues and the detailed analysis of the oligosaccharide recognition site.

MATERIALS AND METHODS

Site-directed Mutagenesis—The coding sequence of PNGase F was cloned into pLITMUS 29 (New England Biolabs). Site-directed mutagenesis was performed following the Kunke protocol (11), using 39-base pair oligonucleotides bearing the site of mutation at positions 22–24. All substitutions were confirmed by DNA sequencing at the mutation site in the pLITMUS 29 vector and again by sequencing of the full PNGase F gene in the pMAL p2 construct. Single colonies were picked until at least two independent clones could be derived that

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The atomic coordinates (code 1PNF) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

** To whom correspondence should be addressed: Wadsworth Center, New York State Dept. of Health, Empire State Plaza, P. O. Box 509, Albany, NY 12201-0509. Tel.: 518-474-1444; Fax: 518-474-7992; E-mail: vanroey@wadsworth.org.

The abbreviations used are: PNGase, peptide-\(N^\text{acetylated}\)-\(\beta\)-\(\text{d}-\text{glucosaminyl}\)asparagine amidase; HPLC, high performance liquid chromatography.
Fig. 1. Stereo ribbon diagram showing the α-carbon tracing of the three-dimensional structure of PNGase F with N,N′-diacetylchitobiose in the substrate binding cleft. The molecule consists of two nearly identical eight-stranded antiparallel β-barrel domains with jelly roll motifs. Side chains of representative residues in the three areas initially identified as possible active sites are shown: Thr-101, lower back; Thr-72, lower front; Glu-118, top front; and, Asp-60 and Glu-206, top rear. The figure was prepared with program MOLscript (21).

Table I

| Location          | Mutant | Relative activity |
|-------------------|--------|-------------------|
| Bowl (back)       | T101G  | 50                |
|                   | T101A  | 50                |
|                   | D99N   | 100               |
|                   | D102N  | >30               |
| S-cleft (front)   | T71A   | 100               |
|                   | T72A   | 100               |
| Interdomain cleft (top) |          |                    |
| Front end         | E118Q  | 0.1               |
|                   | S154A  | 100               |
|                   | S155A  | 100               |
|                   | D157N  | 100               |
| Back end          | D60N   | Not detectable    |
|                   | D60E   | 0.1               |
|                   | Y85F   | >10               |
|                   | E206Q  | <0.01             |
|                   | E206D  | 0.01              |

latter residue was considered to be the most likely candidate for the acidic residue mutants. Indeed, the two mutants of residues in the S-shaped cleft, T71A and T72A, are fully active. Therefore, these two areas were eliminated from further consideration. The cleft at the interface between the two domains at the top end of the molecule has two areas of interest that are about 12 Å apart opposite ends of the cleft. Both areas include acidic residues as well as other polar residues that could be involved in catalysis or substrate recognition. One group includes Glu-118, Ser-154, Ser-155, and Asp-157. The E118Q mutant has greatly reduced activity, but the S154A, S155A, and D157N mutants are fully active, indicating that only the glutamic acid directly interacts with the substrate. At the other end of the cleft, Asp-60 and Glu-206 are in hydrogen bonding contact with each other via a very tightly bound water molecule. This water molecule also makes additional contacts with Tyr-85 and Arg-248. When the acidic residues were mutated, the D60N mutant was found to have absolutely no activity. The activity of E206D was extremely low. To further evaluate this site, three additional mutants were prepared: D60E, E206Q, and Y85F. The very low activity levels of the acidic residue mutants confirm their role in catalysis while suggesting strict geometric requirements. The hydroxyl group of Tyr-85, however, is not of great importance in view of the relatively high activity of the Y85F mutant. No other hydroxyl groups are present in the active site cleft. Therefore, the mechanism of action of PNGase F, unlike those of l-asparaginase and glycosylasparaginase, does not appear to involve a hydroxyl group as the nucleophile.
in E. coli, purified in large quantities, and crystallized. All three crystallized in the same space group under nearly identical conditions as the wild-type enzyme (16). The crystallographic analysis showed that their structures are essentially identical to that of the wild-type enzyme, confirming that the reduced activities are caused by diminished catalytic functionality or impaired substrate binding, not by altered protein conformation.2

X-ray Crystallographic Analysis of the N,N'-Diacetylchitobiose Complex—PNGase F hydrolyzes glycopeptides containing the minimal oligosaccharide moiety N,N'-diacetylchitobiose, GlcNAcβ1→4GlcNAc. The initial oligosaccharide product of the PNGase F hydrolysis contains the 1-amino-N,N'-diacetylchitobiose core, which spontaneously degrades to N,N'-diacetylchitobiose. This compound was tested as a potential inhibitor against the standard PNGase F substrate, dansyl-Leu-Ala-Asn(CHO)-dansyl-AeCys-Ser using an HPLC assay (12). N,N'-Diacetylchitobiose inhibited substrate hydrolysis by 36% at a 12.5-fold molar excess over substrate but by only 50% at a 100-fold excess. Even though the inhibitory activity of the disaccharide was relatively poor, co-crystallization experiments were conducted, using a 30:1 molar ratio of N,N'-diacetylchitobiose to PNGase F.

The crystals of the complex were grown under identical conditions and belong to the same space group as those of the uncomplexed protein. The structure of the complex of the wild-type enzyme has been fully refined to an R value of 0.197 at 2.0-Å resolution. Fig. 2 shows the electron density of the disaccharide in the refined structure. The protein conformation is essentially unaffected by the binding of the ligand. The least-squares fit of the main chain atoms of all but the four N-terminal residues has a root mean square deviation of 0.33 Å and a maximum deviation of 1.30 Å. All of the largest deviations are in loops that are far removed from the binding cleft. The main difference in the cleft is a slight change in the orientation of the side chain of Trp-191, with a shift in the

FIG. 2. Stereodiagram showing the electron density for the N,N'-diacetylchitobiose in the final 2F_{o} − F_{c} map, contoured at 1.5 σ of the map.

FIG. 3. Detailed image of the interactions of N,N'-diacetylchitobiose with PNGase F showing the hydrogen bonding interactions between the disaccharide, water molecules, and the protein. Aromatic residues also make important contacts with the substrate. Trp-191 is positioned nearly perpendicular to the disaccharide and forms a hydrogen bonding contact rather than a hydrophobic interaction. Trp-120 forms a hydrogen bond with O_{6} but also appears to be positioned correctly to be able to make a hydrophobic contact with the next residue, the first mannose, in the intact substrate. The figure was prepared with the program SECTOR (22).

2 Details of the crystallographic analysis of the mutant enzymes and their complexes will be published elsewhere (Van Roey, manuscript in preparation).
position of N$_a$ by 0.95 Å. The average thermal parameters for the protein molecule are significantly lower in the complex: 18.4 Å$^2$ and 19.5 Å$^2$ for the main chain and side chain atoms in the complex, compared with 23.2 and 25.4 Å$^2$ in the uncomplexed structure. Interestingly, the change in the thermal parameters is uniformly distributed over the molecule and does not result from large changes in the binding cleft area. The N,N’-diacetylchitobiose molecule has an average thermal parameter of 16.6 Å$^2$ with a range from 11.0 to 26.0 Å$^2$. The N-acetyl group of the first GlcNAc is most deeply buried into the protein molecule and corresponds to the lowest thermal parameters.

In solution, N,N’-diacetylchitobiose exists in an equilibrium state containing a mixture of β- and α-configurations of the O$_1$-hydroxyl group at the reducing end. In the wild-type PNGase F complex structure, only the α-conformation is observed, despite the fact that the oligosaccharide moiety attached to the N$_a$ of the asparagine is in the β-conformation. The glycosidic link between the GlcNAc residues is in an extended conformation with torsion angles, $\phi$ (O$_2$-C$_1$-O$_4$-C$_4$) = −85° and $\psi$ (C$_1$-O$_4$-C$_4$-C$_5$) = −123°. These angles are well within the range of those observed for other β-1→4-linked disaccharides in complexes with lectins (17, 18) but are most similar to those seen in complexes of lysozyme with GlcNAc multimers (19, 20).

Fig. 3 shows the location and orientation of the N,N’-diacetylchitobiose molecule in the cleft of the enzyme. The molecule is inserted edgewise into the cleft and spans the distance between the two groups of acidic residues in the cleft, with O$_1$ of the reducing end GlcNAc residue forming hydrogen bonds with Asp-60 and the water molecule, Wat$^{346}$. Fig. 4 shows a schematic diagram of all important intermolecular contacts. The first GlcNAc residue makes extensive contacts with the protein, although several are mediated by tightly bound water molecules. One interesting feature, shown in Fig. 5, is the location of the N-acetyl group, which extends into a deep hole in the interface between the two domains of the protein. The contacts of the second GlcNAc are much weaker and only O$_6$ and O$_7$ are involved in direct hydrogen bonds with the protein. On the reducing end, Asp-60 forms hydrogen bonds with O$_6$ of the N,N’-diacetylchitobiose on one side and with Wat$^{346}$ on the other side. Glu-206 does not make any direct contacts with the disaccharide. The position and orientation of the disaccharide in the cleft easily explains the inability of the enzyme to process substrates with an O$_6$ substitution; O$_6$ is buried and inaccessible while O$_6$ is fully exposed to the solvent.

The solvent-accessible surface of the protein is reduced by less than 200 Å$^2$ in the complex: 12,395 Å$^2$ for the protein in the complex versus 12,587 Å$^2$ in the uncomplexed structure. This is explained in part by the location of the molecule toward one side of the cleft, making close contacts with many residues of the N-terminal domain but allowing for a layer of water molecules to form bridging contacts with the residues of C-terminal domain. Many of these water molecules, including Wat$^{346}$, Wat$^{75}$, and Wat$^{346}$, are in nearly identical positions in the uncomplexed and complex structures. Of all the water molecules involved in contacts with the protein and the disaccharide, only Wat$^{346}$ and Wat$^{346}$ have no water molecule within 0.5 Å in the uncomplexed structure. However, all water molecules that only make contacts with the N,N’-diacetylchitobiose molecule are disordered in the uncomplexed structure.

**Fig. 4.** Schematic diagram showing the intermolecular hydrogen bonding contacts between PNGase F, N,N’- diacetylchitobiose and water molecules. Protein residues are indicated with single-letter amino acid code and sequence number in rectangular boxes, water molecules are indicated by a number, corresponding to their number in the file deposited with the Protein Data Bank. The reducing end GlcNAc residue is on the left. Hydrogen bonding distances, in Å, are shown in italics. Note that Wat$^{346}$ (349) is present twice, once in contact with O$_5$ and once with Arg-61.

**Fig. 5.** GRASP-image (23) showing the molecular envelope of PNGase F, cut-off through the oligosaccharide binding cleft to show the penetration of the N-acetyl group of the first GlcNAc residue into the cavity in the core of the molecule. The carbonyl oxygen makes hydrogen bonds with two water molecules in the cavity, while the methyl group is in close proximity to a hydrophobic area, primarily formed by the Ile-82 side chain. The protein surface is colored according to its electrostatic potential. Potentials greater than 9 T are blue, those less than −22 T are red, and neutral ones are white.
DISCUSSION

Site-directed mutagenesis experiments were used to determine that the active site of PNGase F is located in the cleft at the interface between the two domains of the molecule and showed that three acidic residues, Asp-60, Glu-118, and Glu-206, are essential for activity.

The combination of this information with the crystal structure analysis of the complex with N,N'-diacetylchitobiose clearly reveals the distinct roles of these residues in the mechanism of action of the enzyme. Mutagenesis of Asp-60 to the corresponding asparagine results in total loss of activity. This mechanism of action of the enzyme. Mutagenesis of Asp-60 to the corresponding asparagine results in total loss of activity. This will be focused on the analysis of the interaction of the enzyme with the peptide component of the substrate.

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REFERENCES

1. Tarentino, A. L., Gomez, C. M., and Plummer, T. H., J. r. (1985) Biochemistry 24, 4663–4671
2. Plummer, T. H., J. r., Elder, J. H., Alexander, S., Phelan, A. W., and Tarentino, A. L., (1984) J. Biol. Chem. 259, 10700–10704
3. Rasmussen, J. R., Davis, J., Risley, J. M., and VanEtten, R. L. (1992) J. Am. Chem. Soc. 114, 1124–1126
4. Tarentino, A. L., and Plummer, T. H., J. r. (1993) Trends Glycosc. Glycotechnol. 5, 163–170
5. Tretter, V., Altmann, F., and März, L. (1991) Eur. J. Biochem. 199, 647–652
6. Kuhn, P., Tarentino, A. L., Plummer, T. H., J. r., and Van Roey, P. (1994) Biochemistry 33, 11699–11706
7. Norris, G. E., Stillman, T. J., Anderson, B. F., and Baker, E. N. (1994) Structure 2, 1049–1059.
8. Swain, A. L., Jaskowsk, M., Hossen, D., Rass, J. K. M., and Wlodawer, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1474–1478
9. Tollerud, O. K., and Aronson, N. N., J. r. (1992) Biochem. J. 282, 891–897
10. Tarentino, A. L., and Plummer, T. H., J. r. (1993) Biochem. Biophys. Res. Commun. 197, 179–186
11. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
12. Tarentino, A. L., and Plummer, T. H., J. r. (1994) Methods Enzymol. 230, 44–57
13. Brünger, A. T., Kuriyan, J., and Karplus, M. (1987) Science 235, 458–460
14. Sack, J. (1988) J. Mol. Graphics 6, 244–245
15. Laskowski, R. A., McArthur, M. W., Moss, M. W., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 157–159
16. Kuhn, P., Tarentino, A. L., Plummer, T. H., J. r., and Van Roey, P. (1994) J. Mol. Biol. 241, 622–623
17. Bourne, Y., Mazurier, J., Legrand, D., Rougé, P., Montereuil, J., Spik, G., and Cambillau, C. (1994) Structure 2, 209–219
18. Bourne, Y., Bigliani, B., Liao, D.-J., Stredder, G., Cantau, P., Herzberg, O., Feizi, T., and Cambillau, C. (1994) Nature Struct. Biol. 1, 863–845
19. Cheetham, J. C., Artyomov, P. J., and Phillips, D. C. (1992) J. Mol. Biol. 224, 613–620
20. Hadfield, A. T., Harvey, D. J., Archer, D. B., MacKenzie, D. A., Jenes, D. J., Radford, S. E., Lowe, G., Dobson, C. M., and Johnson, L. N. (1994) J. Mol. Biol. 243, 856–872
21. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
22. Evans, S. E. (1993) J. Mol. Graphics 11, 134–138
23. Nicholls A., Sharp, K. A., and Honig, B. H. (1991) Proteins 11, 281–296
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