Secondary structure and NMR assignments of *Bacillus circulans* xylanase

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Abstract

Bacillus circulans xylanase (BCX) is a member of the family of low molecular weight endo- β -(1,4)-xylanases. The main-chain ^1H , ^{13}C , and ^{15}N resonances of this 20.4-kDa enzyme were assigned using heteronuclear NMR experiments recorded on a combination of selectively and uniformly labeled protein samples. Using chemical shift, NOE, J coupling, and amide hydrogen exchange information, 14 β -strands, arranged in a network of three β -sheets, and a single α -helix were identified in BCX. The NMR-derived secondary structure and β -sheet topology agree closely with that observed in the crystal structure of this protein. The H^{N} of Ile 118 has a strongly upfield-shifted resonance at 4.03 ppm, indicative of a potential amide–aromatic hydrogen bond to the indole ring of Trp 71. This interaction, which is conserved in all low molecular weight xylanases of known structure, may play an important role in establishing the active site conformation of these enzymes. Following hen egg white and bacteriophage T4 lysozymes, *B. circulans* xylanase represents the third family of β -glycanases for which extensive NMR assignments have been reported. These assignments provide the background for detailed studies of the mechanism of carbohydrate recognition and hydrolysis by this bacterial xylanase.

Keywords: amide–aromatic hydrogen bond; β -glycanase; chemical shift index; NMR assignments; secondary structure; xylanase

Xylans are abundant structural heteropolysaccharides, formed from a backbone of β -(1,4) linked xylopyranosyl residues, that are found in plant cell walls (Coughlan & Hazlewood, 1993). The degradation of these complex polysaccharides by bacteria and fungi requires the synergistic action of endo- β -(1,4)-xylanases to hydrolyze the xylan backbone, β -xylosidases to further cleave the product xylo-oligomers, and a series of accessory debranching enzymes (Hazlewood & Gilbert, 1993). Xylanolytic enzymes have many applications, including the clarification of juices and wines, the saccharification of agricultural and forestry wastes for fermentation to fuels, the improvement of the nutritional value of agricultural silage, and the conversion of xy-

lans to simple xylose derivatives used, for example, as sweeteners (Coughlan & Hazlewood, 1993; Hazlewood & Gilbert, 1993). Also, the addition of cellulase-free xylanases during the bleaching process of kraft pulp reduces the environmental impact from the chlorine derivatives currently used to whiten paper (Wong & Saddler, 1992). Accordingly, xylanases remain the focus of research aimed both at understanding the mechanisms by which they bind and hydrolyze xylan, and at exploiting these enzymes for their effective use in biotechnology.

Bacillus circulans xylanase is a member of the family 11 or G low molecular weight endo- β -(1,4)-xylanases (Paice et al., 1986; Gilkes et al., 1991; Gebler et al., 1992; Henrissat & Jiroch, 1993; Davies & Henrissat, 1995). This family of enzymes preferentially hydrolyzes xylose polymers ($n > 5$ –7), ultimately yielding xylobiose and xylotriose (Bray & Clarke, 1992). An investigation of the stereochemical course of the reaction catalyzed by BCX demonstrated that hydrolysis proceeds with retention of the anomeric configuration (Gebler et al., 1992). Combined with the observation that the enzymes also catalyze transglycosylation, this shows that hydrolysis follows a double-displacement mechanism in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed through oxocarbenium ion-like transition states. This mechanism, com-

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Abbreviations: BCX, *Bacillus circulans* xylanase; CSI, chemical shift index; CT, constant-time; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; d^3 -acetate, ^2H -[2,2,2]-acetate; HSQC, heteronuclear single quantum correlation; INEPT, insensitive nuclei enhanced by polarization transfer; MES, 2-[N-morpholino]ethane-sulfonic acid; pH^* , pH meter reading uncorrected for isotope effects; TSP, 3-(trimethylsilyl)propionate.

mon to "retaining" glycosidases, involves two active site carboxyl groups, one serving as an acid-base catalyst and the second as a nucleophile (Sinnott, 1990). The catalytic residues in BCX have been identified as Glu 78 and Glu 172, respectively, using both mutagenesis and mechanism-based inhibitors (Wakarchuk et al., 1992, 1994a; Miao et al., 1994).

The crystal structures of the homologous xylanases from *B. circulans*, *B. pumilis*, *Trichoderma harzianum*, and *T. reesei* have been determined (Okada et al., 1989; Campbell et al., 1993; Törrönen et al., 1994; Wakarchuk et al., 1994a; Törrönen & Rouvinen, 1995).⁴ These proteins have the same general fold, yet there are clearly major differences in their catalytic activities, specificities, pH optima, and thermostabilities (Wakarchuk et al., 1992, 1994a and references within). Complexes of a catalytically incompetent BCX mutant with xyloetraose (Wakarchuk et al., 1994a) and *T. reesei* xylanase II with partially hydrolyzed oat spelt xylan (Törrönen & Rouvinen, 1995) have also been characterized crystallographically. In both cases, the bound sugars were disordered, thus precluding a complete description of the interactions leading to substrate binding and hydrolysis. However, detailed kinetic studies of several active site mutants have helped define the enzymatic mechanism of BCX (Wakarchuk et al., 1994a). Significant progress has also been made toward engineering higher thermostability in this protein (Wakarchuk et al., 1994b). Still, to direct further protein engineering efforts aimed at enhancing catalysis for biotechnological applications, a detailed description of the substrate-protein interactions is essential.

The goal of our research is to investigate the basis of carbohydrate recognition by BCX and the mechanism of xylan hydrolysis using a combined approach of NMR spectroscopy and mutagenesis. In addition to providing structural information, NMR offers the possibility to investigate the role of protein dynamics in substrate binding and to dissect the electrostatic (pH) dependence of hydrolysis. To date, hen egg white and T4 phage lysozymes (or closely related homologues) are the only β -glycanases for which NMR has been used for detailed structure-function studies (Redfield & Dobson, 1988; McIntosh et al., 1990). As a first step toward these goals, we have assigned the main-chain and selected side-chain ^1H , ^{13}C , and ^{15}N resonances of isotopically labeled BCX. In parallel, we have also determined the secondary structure of this protein using chemical shift, NOE, and J-coupling information, combined with amide hydrogen exchange measurements. Consistent with crystallographic studies, BCX is composed of 14 β -strands, arranged in a network of three β -sheets, and a single α -helix. A potential amide-aromatic hydrogen bond that may contribute to the structure of the active site cleft of this enzyme was also identified.

Results

The NMR spectra obtained from BCX are generally of superior quality to those of most proteins in the 20-kDa molecular weight range. This is exemplified by the ^1H - ^{15}N HSQC spectrum of BCX in which essentially all the peaks from the 178 non-proline residues in the protein are clearly resolved (Fig. 1). This excellent spectral dispersion is attributed in part to the extensive β -sheet

secondary structure of BCX and to its high content of aromatic residues (4 phenylalanines, 11 tryptophans, and 15 tyrosines).

BCX is a stable thiol-free protein with a basic isoelectric point, for which good quality NMR spectra can be obtained from pH $\sim$ 3–8 at 30 °C. During the initial screening of BCX, we noted a deterioration in the quality of its spectra with either increasing ionic strength or protein concentration, possibly due to limited association or aggregation. The experimental conditions of 0.8 mM xylanase in 25 mM acetate buffer at pH 5.8 and 30 °C were chosen as an optimal compromise for maintaining good signal-to-noise and narrow resonance line widths. The enzyme is maximally active near this pH, and thus these conditions match those used for enzymatic studies of BCX (Wakarchuk et al., 1992, 1994a).

The apparent molecular weight of BCX was determined to be 18.0 kDa by equilibrium sedimentation studies (50 μM protein in 100 mM NaCl, 10 mM MES, pH 6.0, 20 °C), with no evidence of higher-order association at this protein concentration. The molecular weight of the unlabeled protein is $20,396 \pm 2$ amu as measured by electrospray mass spectroscopy. This latter value is in exact agreement with that expected from the sequence of BCX after posttranslational cleavage of the N-terminal methionine introduced by the plasmid expression system (Paice et al., 1986). The lower molecular weight determined by equilibrium sedimentation may result from the assumption of an average partial specific volume of $0.73 \text{ cm}^3/\text{gm}$. We note that use of a partial specific volume of $0.70 \text{ cm}^3/\text{gm}$, as calculated from the amino acid composition of BCX, would lead to an even lower apparent molecular weight of 16.2 kDa, whereas a value of $0.76 \text{ cm}^3/\text{gm}$ would be required to produce the true molecular weight of the protein from the observed equilibrium sedimentation data (Zamyatnin, 1984).

Velocity sedimentation measurement of the protein under the same conditions used for the equilibrium studies yielded an intrinsic sedimentation coefficient ($S_{20,w}^\circ$) of 2.22 S and a calculated Stokes radius of 18.9 Å (Tanford, 1961). This is consistent with the crystal structure of BCX, in which the protein is essentially spherical with a nonhydrated radius of $\sim$ 18 Å. Based on a preliminary analysis of ^{15}N relaxation data, the isotropic rotational correlation time of BCX is 8.4 ns under the conditions employed for the current NMR studies (data not shown). This correlation time is longer than that expected for a sphere of the above Stokes radius, unless an additional hydration shell of 3 Å is invoked (Venable & Pastor, 1988). The difference in radii determined by ultracentrifugation and NMR relaxation measurements may result from the higher protein concentration (0.8 mM) used for the latter analysis. Nevertheless, the sedimentation coefficient and rotational correlation time measured for BCX are slightly lower than those characteristic of proteins in the same molecular weight range (Tanford, 1961). This shows that BCX has a very compact globular structure in solution, resulting in its favorable relaxation behavior for characterization by NMR spectroscopy.

Main-chain resonance assignments

We have assigned the resonances from the main-chain ^1H , ^{13}C , and ^{15}N in BCX using a combination of two- and three-dimensional heteronuclear NMR experiments (Table 1). The ^1H - ^{15}N spectrum of BCX, with one peak expected per residue, excluding the N-terminus and the six prolines, served as the start-

⁴ The Brookhaven Protein Data Bank codes for the proteins are: *B. circulans* xylanase (1XNB), *T. harzianum* xylanase (1XND), *T. reesei* xylanase I (1XYN), and *T. reesei* xylanase II (1XYO and 1XYP). Coordinates for *B. pumilis* xylanase have not been released.

Fig. 1. ^1H - ^{15}N HSQC spectrum of uniformly $^{13}\text{C}/^{15}\text{N}$ -enriched BCX at pH 5.8 and 30 °C. The two cross peak within boxes correspond to amides with ^{15}N shifts that are outside the spectral window and are thus aliased and inverted in the spectrum. Ile 118, with an H^{N} shift of 4.03 ppm and ^{15}N shift of 113.6 ppm, is not shown. Assignments of 8 of the 11 tryptophan indole $^{15}\text{NH}^{\text{H}}$ cross peaks are labeled within parentheses, whereas the remaining three unassigned cross peaks are denoted by asterisks (*). The $^{15}\text{NH}^{\text{H}}$ peaks from five of the seven arginine side chains are aliased within the spectrum, but not assigned. The NH_2 groups from the Asn and Gln side chains were not observed due to incorporation of a refocused INEPT sequence, tuned for AX spin systems, followed by WALTZ-16 ^1H decoupling during t_1 into the sensitivity-enhanced gradient HSQC sequence. The amide ^{15}N were decoupled from the neighboring $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}'$ nuclei by the application of a hard 180° pulse applied at 114 ppm in ^{13}C during t_1 .

ing point for the assignments (Fig. 1). However, even with the excellent dispersion, the analysis of this spectrum was a challenging task due to the complexity arising from the large number of residues in xylanase. To simplify the assignment protocol, we initially recorded the HSQC spectra of BCX selectively labeled with α - ^{15}N -valine, -tyrosine, -aspartic acid and -asparagine, -glycine, or -glycine and -serine. We chose these residues based on their relative abundance in BCX and the availability of inexpensive α - ^{15}N amino acids. The asparagine and serine were labeled by metabolic conversion of the precursor ^{15}N -aspartic acid or -glycine, respectively, from the medium during xylanase expression in *Escherichia coli* (McIntosh & Dahlquist, 1990). These selectively labeled amino acids, which comprised 52% of the protein sequence, provided unambiguous reference points for the specific resonance assignments.

Although $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ chemical shifts are diagnostic of amino acid type and thus facilitate the mapping of spin systems to the primary sequence of a protein (Richarz & Wüthrich, 1978; Oh et al., 1988; Meadows et al., 1994), the use of selective la-

bels greatly increased the speed and confidence with which the spectrum of BCX was assigned manually. For example, the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ of tyrosine, phenylalanine, asparagine, and aspartic acid have similar chemical shift ranges in proteins, and thus the 15 Tyr and 25 Asp/Asn were distinguished immediately based on the selective labeling. Similarly, serine and threonine both generally have $^{13}\text{C}^{\beta}$ resonances downfield of their $^{13}\text{C}^{\alpha}$ resonances. Hence, 18 serine residues were differentiated unambiguously from the 25 threonine residues based on the HSQC spectrum of BCX labeled with the former amino acid. The $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ chemical shifts of valine and proline are also very similar in proteins. Because the assignment method outlined below is interrupted at the proline residues, it was very useful to determine unambiguously, from the spectrum of BCX labeled with ^{15}N -valine, whether a given residue at an apparent interruption point was a proline or a valine. Although this assignment strategy did require several samples of labeled protein, the total time and financial cost was low due to the good expression (~30 mg/L media) and one-column purification of BCX.

Table 1. Main-chain ^1H , ^{13}C , and ^{15}N resonance assignments for *Bacillus circulans* xylanase (pH 5.8 and 30 °C)^a

Residue	^{15}N ($^1\text{H}^\text{N}$)	$^{13}\text{C}^\alpha$ ($^1\text{H}^\alpha$)	$^{13}\text{C}^\beta$ ($^1\text{H}^\beta$)	$^{13}\text{C}'$	Residue	^{15}N ($^1\text{H}^\text{N}$)	$^{13}\text{C}^\alpha$ ($^1\text{H}^\alpha$)	$^{13}\text{C}^\beta$ ($^1\text{H}^\beta$)	$^{13}\text{C}'$
A1					N63	124.6 (8.91)	53.8 (5.08)	38.0 (2.81, 3.93)	176.5
S2		59.0	63.8	174.9	G64	118.9 (8.04)	46.4 (3.75, 5.00)		171.2
T3	116.3 (8.37)	61.7 (3.37)	69.7 (0.53)	174.8	Y65	119.5 (9.68)	54.1 (5.85)	42.6 (3.09, 3.51)	173.6
D4	120.4 (8.02)	53.6 (4.76)	42.3 (2.49, 3.13)	175.1	L66	127.8 (7.95)	54.2 (4.70)	40.7	175.7
Y5	122.2 (7.84)	58.4 (4.71)	42.3 (1.95, 2.49)	173.3	T67	123.0 (8.95)	58.1 (5.71)	72.6 (3.94)	172.3
W6	133.6 (7.40)	54.6 (4.93)	32.9 (2.77)	171.3	L68	135.0 (9.31)	56.1 (5.01)	43.3 (1.77)	177.7
Q7	128.9 (8.13)	52.6 (3.96)	30.0 (1.26)	172.2	Y69	134.3 (9.62)	56.5 (5.04)	43.4 (2.30, 2.50)	172.5
N8	125.4 (7.98)	52.6 (4.40)	37.3 (2.23)	173.0	G70	113.4 (6.40)	45.6 (3.06, 3.46)		168.8
W9	127.5 (8.60)	57.5 (4.69)	31.5 (2.98)	174.0	W71	111.9 (6.11)	55.1 (5.47)	35.4 (1.90, 2.71)	169.8
T10	120.9 (7.10)	57.8 (4.20)	70.5 (3.97)	171.0	T72	113.8 (9.13)	60.7 (4.47)	72.9 (3.66)	172.0
D11	124.1 (7.50)	40.2 (4.06)	53.7 (2.69, 3.20)	177.3	R73	118.0 (8.87)	55.4 (4.11)	33.4 (1.31)	176.1
G12	110.6 (8.44)	44.8 (3.54, 4.28)		175.3	S74	111.6 (8.57)	57.8 (3.85)	62.0	172.8
G13	111.0 (8.47)	45.1 (3.54, 4.11)		175.3	P75		63.2 (4.44)	34.8 (2.28)	174.6
G14	113.8 (8.48)	46.4 (3.67, 3.79)		173.2	L76	120.4 (7.76)	54.9 (4.98)	42.6 (1.98)	175.1
I15	122.7 (8.18)	60.2 (4.23)	40.9 (1.67)	174.9	I77	131.1 (8.10)	59.1 (4.44)	41.2 (1.46)	173.3
V16	128.8 (8.46)	62.5 (3.73)	33.1 (1.75)	173.4	E78	130.2 (7.42)	52.9 (3.35)	32.1 (0.88)	173.7
N17	127.8 (8.75)	52.1 (5.04)	38.7 (2.72, 2.90)	173.6	Y79	124.0 (7.71)	54.6 (5.54)	42.5 (1.95, 3.27)	171.9
A18	131.4 (8.79)	50.1 (5.38)	22.1 (1.51)	176.6	Y80	115.9 (8.15)	57.0 (5.28)	44.6 (2.15, 2.87)	177.8
V19	126.1 (9.40)	62.4 (4.03)	34.0 (2.17)	174.4	V81	121.4 (8.08)	62.7 (4.88)	32.2 (2.03)	174.5
N20	127.6 (8.35)	51.8 (3.48)	35.6 (2.40)	175.2	V82	128.2 (9.63)	62.9 (4.32)	33.9 (1.27)	175.6
G21	114.7 (7.23)	44.2 (3.62, 4.13)		172.9	D83	129.9 (8.40)	54.9 (5.68)	43.6 (2.30, 2.45)	176.5
S22	114.3 (8.30)	58.8 (4.20)	63.8 (3.80)	177.0	S84	109.6 (8.23)	57.6 (4.20)	57.6 (3.74)	172.6
G23	115.1 (8.90)	47.5 (3.80, 3.89)		176.0	W85	120.9 (7.74)	57.3 (6.59)	29.4 (3.83, 3.99)	175.2
G24	117.4 (9.30)	46.1 (4.78, 4.40)		175.4	G86	111.9 (9.34)	45.1 (4.78)		175.3
N25	120.5 (8.18)	54.4 (5.70)	42.9 (2.75, 2.96)	175.8	T87	118.9 (8.05)	63.5 (4.25)	69.0 (3.84)	175.3
Y26	126.3 (9.74)	57.8 (5.05)	42.9 (3.03, 3.71)	171.0	Y88	123.6 (8.18)	56.7 (4.83)	40.9 (2.76, 2.95)	173.0
S27	113.0 (8.75)	56.7 (5.24)	66.6 (3.95, 4.20)	173.4	R89	133.8 (8.07)	51.7 (3.11)	30.3	171.8
V28	120.6 (9.18)	61.2 (5.22)	34.9 (2.49)	174.2	P90		63.0 (3.40)	63.1 (1.32)	173.6
N29	127.7 (8.48)	53.3 (5.53)	41.8 (2.74)	173.0	T91	106.7 (6.03)	58.7 (2.76)	72.0 (3.26)	175.3
W30	126.9 (9.16)	55.9 (5.59)	33.0 (2.30, 2.98)	175.4	G92	108.5 (5.22)	46.1 (3.22, 3.87)		172.6
S31	117.7 (9.23)	57.5 (4.84)	65.1 (3.64)	172.7	T93	125.7 (9.02)	64.2 (4.43)	68.6 (4.35)	174.5
N32	127.5 (8.32)	54.4 (4.26)	37.7 (2.65, 2.86)	174.7	Y94	132.0 (9.09)	60.0 (4.51)	39.2 (2.94, 3.27)	176.2
T33	107.5 (8.33)	63.2 (4.06)	71.5 (4.56)	178.8	K95	125.7 (8.47)	53.9 (4.22)	33.5 (1.58)	175.4
G34	107.4 (7.61)	46.5 (4.38)		175.1	G96	104.6 (5.61)	44.9 (3.48, 3.75)		167.0
N35	129.2 (9.68)	52.6 (5.59)	42.0 (2.84)	172.6	T97	110.3 (8.13)	60.2 (5.62)	74.3 (3.95)	174.0
F36	121.0 (7.76)	67.1 (5.01)	41.8 (2.89, 3.18)	177.3	V98	121.0 (9.29)	59.7 (4.50)	34.8 (1.78)	172.3
V37	121.3 (9.16)	62.9 (4.22)	35.5 (1.85)	173.6	K99	130.3 (8.51)	54.9 (5.29)	33.0 (1.65, 1.72)	176.2
V38	128.5 (8.60)	58.7 (4.56)	36.7 (1.63)	175.1	S100	118.0 (8.75)	59.4 (4.34)	64.4 (3.08, 3.60)	174.7
G39	110.1 (8.40)	45.8 (3.07, 3.60)		170.0	D101	125.6 (9.66)	55.8 (4.33)	41.7 (2.99)	176.5
K40	117.0 (6.44)	54.8 (5.40)	38.7 (1.74)	175.7	G102	106.9 (8.84)	46.1 (4.69)		173.8
G41	109.6 (7.93)	47.5 (2.58, 4.57)		170.5	G103	110.4 (8.23)	43.4 (3.60, 4.75)		172.4
W42	131.5 (9.61)	59.9 (5.39)	32.6 (2.79, 3.45)	175.1	T104	117.5 (8.06)	62.7 (4.86)	70.0 (3.79)	172.7
T43	125.0 (10.24)	66.2 (3.32)	69.7 (4.50)	174.3	Y105	127.4 (9.81)	56.1 (4.95)	39.6 (2.71, 3.18)	175.0
T44	115.7 (8.50)	60.6 (4.90)	70.3 (4.99)	175.1	D106	123.2 (8.81)	54.5 (5.16)	44.9 (2.08, 2.38)	174.3
G45	111.4 (8.18)	46.0 (3.56, 4.14)		174.0	I107	122.9 (8.45)	59.6 (4.97)	39.4 (1.23)	175.0
S46	111.0 (7.46)	52.3 (3.87)	65.7	173.8	Y108	124.2 (9.19)	54.8 (5.87)	43.4 (2.16, 2.48)	175.4
P47		63.0 (4.06)	31.4 (1.28, 1.90)	173.7	T109	110.0 (8.36)	60.0 (5.76)	71.8 (4.34)	174.5
F48	114.8 (7.19)	55.7 (4.53)	39.4 (2.39)	175.9	T110	115.8 (8.84)	61.7 (4.71)	73.1 (4.45)	173.3
R49	123.2 (6.58)	57.1 (3.86)	31.6 (1.04, 1.43)	176.0	T111	118.4 (8.49)	62.4 (4.59)	71.4 (3.62)	172.8
T50	126.0 (8.57)	62.6 (4.66)	69.3 (3.98)	173.2	R112	127.1 (8.32)	53.6 (4.27)	30.9 (1.05, 1.23)	173.2
I51	130.3 (8.70)	62.4 (4.00)	40.1 (1.89)	174.3	Y113	121.0 (8.16)	56.7 (4.64)	40.7 (2.64, 2.79)	175.4
N52	127.7 (8.77)	51.1 (5.16)	40.0 (0.86, .60)	172.5	N114	122.3 (8.01)	54.1 (3.77)	37.2 (2.61, 2.81)	173.4
Y53	116.9 (8.61)	56.9 (5.53)	41.1 (2.86)	172.6	A115	124.1 (9.31)	50.1 (5.04)	22.0	174.8
N54	118.1 (8.91)	53.1 (4.75)	40.9 (2.98)	176.3	P116		63.0 (4.68)	31.4 (2.26)	173.8
A55	134.0 (9.67)	50.3 (5.48)	18.2 (1.53)	175.2	S117	116.8 (8.64)	56.5 (5.23)	68.2	172.7
G56	117.5 (8.32)	47.1 (3.96)		175.4	I118	113.6 (4.03)	63.1 (3.38)	36.4 (−0.34)	174.7
V57	118.9 (8.20)	62.5 (4.15)	35.8 (1.79)	175.4	D119	120.9 (7.21)	54.1 (4.69)	42.1 (2.07, 2.69)	171.9
W58	129.1 (8.99)	56.3 (5.30)	31.0 (3.15, 3.34)	172.4	G120	109.5 (7.21)	45.1 (3.70, 3.85)		172.3
A59	129.6 (8.05)	49.7 (4.57)	18.4 (0.99)	174.0	D121	119.3 (8.11)	55.3 (4.46)	41.5 (2.54)	175.7
P60		63.6 (4.11)	31.2 (2.04)	175.8	R122	121.8 (8.26)	55.4 (4.07)	30.7	174.9
N61	126.5 (9.31)	52.0 (4.85)	37.5 (2.32, 2.65)	176.1	T123	118.2 (9.03)	60.6 (4.78)	72.4 (4.14)	172.6
G62	110.3 (8.13)	44.4 (3.91, 4.68)		172.3	T124	119.6 (8.30)	60.7 (5.29)	71.7 (3.98)	173.7

(continued)

Table 1. *Continued*

Residue	^{15}N ($^1\text{H}^{\text{N}}$)	$^{13}\text{C}^{\alpha}$ ($^1\text{H}^{\alpha}$)	$^{13}\text{C}^{\beta}$ ($^1\text{H}^{\beta}$)	$^{13}\text{C}'$	Residue	^{15}N ($^1\text{H}^{\text{N}}$)	$^{13}\text{C}^{\alpha}$ ($^1\text{H}^{\alpha}$)	$^{13}\text{C}^{\beta}$ ($^1\text{H}^{\beta}$)	$^{13}\text{C}'$
F125	123.6 (8.16)	56.0 (4.94)	39.7 (3.00, 3.18)	172.5	H156	119.7 (7.53)	55.6 (4.74)	28.9 (2.78, 3.40)	174.5
T126	118.0 (7.48)	62.4 (4.63)	70.4 (3.76)	175.7	G157	108.9 (7.85)	46.2 (3.79, 4.00)		173.8
Q127	122.6 (8.97)	53.0 (5.57)	31.3 (1.19)	175.7	M158	123.0 (7.81)	54.2 (3.95)	32.7 (1.49, 1.62)	173.5
Y128	123.4 (8.08)	51.9 (4.90)	37.7 (1.12, 1.95)	173.8	N159	120.9 (8.08)	53.2 (4.66)	39.8 (2.65)	176.8
W129	119.1 (8.64)	56.1 (5.38)	32.5 (2.95, 3.12)	177.3	L160	129.9 (9.14)	54.5 (4.18)	43.3 (1.77, 1.87)	176.3
S130	121.4 (9.13)	58.4 (5.76)	66.7 (3.79, 4.09)	175.0	G161	106.8 (7.08)	45.5 (3.52, 4.14)		172.4
V131	125.6 (10.28)	61.5 (4.88)	35.1 (2.13)	175.9	S162	110.6 (7.46)	58.7 (4.35)	64.6 (3.91, 4.03)	174.8
R132	137.6 (9.07)	58.9 (3.64)	30.0 (1.95, 2.07)	177.7	N163	122.5 (7.75)	51.8 (5.10)	40.2 (2.50)	174.3
Q133	123.6 (9.06)	60.3 (3.93)	27.9 (2.03)	177.1	W164	129.4 (9.08)	59.9 (3.85)	29.5 (3.06, 3.20)	175.4
S134	107.4 (7.51)	59.2 (4.65)	71.8 (3.91)	174.4	A165	128.2 (7.36)	49.9 (4.78)	18.0 (1.06)	175.4
K135	126.0 (8.59)	58.7 (3.41)	32.5 (1.69)	176.9	Y166	117.0 (6.12)	57.9 (4.41)	38.4 (1.95, 2.37)	175.5
R136	128.1 (8.99)	55.3 (5.61)	33.0	175.4	Q167	124.1 (8.61)	55.3 (4.99)	34.9	174.4
P137		63.8 (4.49)	32.0 (2.43)	177.7	V168	120.9 (9.99)	59.2 (4.89)	36.8 (1.66)	173.3
T138	115.4 (8.51)	60.7 (4.21)	70.3 (3.83)	175.3	M169	124.1 (8.52)	54.9 (5.37)	32.1 (1.47, 2.43)	175.7
G139	114.9 (9.08)	45.6 (3.74, 4.48)		174.1	A170	135.8 (9.83)	52.3 (5.25)	23.4 (1.12)	176.2
S140	114.3 (7.47)	65.4 (4.70)	56.3 (3.74)	173.4	T171	115.5 (9.14)	62.6 (4.94)	72.0 (4.03)	172.4
N141	123.2 (8.74)	54.6 (4.56)	37.0 (2.88, 3.05)	174.4	E172	127.3 (8.69)	52.8 (4.46)	31.8 (2.28)	174.0
A142	131.2 (9.40)	51.0 (4.43)	21.0 (0.57)	175.9	G173	110.0 (6.01)	44.5 (2.93, 4.39)		171.3
T143	113.0 (7.30)	61.0 (5.13)	72.4 (3.71)	175.6	Y174	118.5 (7.03)	56.7 (5.00)	40.4 (2.52, 2.83)	174.2
I144	126.4 (9.54)	61.3 (4.36)	40.9 (1.74)	175.4	Q175	124.4 (7.62)	56.5 (4.44)	25.9 (1.63)	176.2
T145	132.8 (10.58)	61.6 (4.27)	66.2	175.9	S176	112.3 (7.63)	56.4 (5.24)	68.3 (4.23)	171.8
F146	129.5 (7.60)	60.2 (4.14)	39.3 (2.82, 3.20)	174.6	S177	112.3 (7.58)	56.4 (4.67)	67.1 (3.53, 3.97)	170.7
T147	115.6 (8.96)	61.4 (3.62)	62.4 (3.82)	176.6	G178	108.7 (7.54)	44.8 (2.70, 3.23)		170.7
N148	119.5 (7.25)	55.2 (4.24)	36.0 (2.19, 2.79)	178.8	S179	113.3 (6.30)	57.6 (5.31)	66.8 (3.53, 3.97)	172.6
H149	123.3 (6.70)	59.7 (3.09)	29.7 (1.40, 2.20)	174.5	S180	117.6 (9.50)	56.9 (5.07)	67.2 (4.67)	172.8
V150	118.7 (7.17)	66.7 (2.69)	31.4 (0.64)	179.6	N181	126.6 (9.16)	55.1 (5.04)	41.1 (2.61, 2.79)	173.9
N151	117.7 (7.98)	56.2 (4.25)	37.7 (2.61)	177.7	V182	126.8 (8.60)	61.1 (4.91)	36.2 (1.66)	174.3
A152	126.3 (7.36)	55.0 (4.14)	18.4 (1.33)	181.1	T183	124.0 (8.54)	62.2 (5.10)	70.7 (4.24)	173.1
W153	123.7 (9.24)	58.6 (4.51)	27.2 (3.23, 3.34)	180.1	V184	130.1 (8.52)	62.0 (5.08)	35.6 (2.07)	174.3
K154	123.3 (8.33)	60.6 (3.54)	32.0 (1.95)	180.2	W185	133.9 (9.51)	60.1 (4.88)	30.2	
S155	117.6 (7.79)	61.2 (4.16)	62.7 (3.82)	174.4					

^a Chemical shifts for ^1H , ^{13}C , and ^{15}N , are given in ppm.

The next step of the assignment protocol involved the use of three-dimensional triple-resonance NMR experiments to provide scalar correlations between main-chain ^1H , ^{15}N , and ^{13}C nuclei in BCX (Ikura et al., 1990). The HNCACB and CBCA(CO)NH experiments were used to link sequentially the backbone H^{N} and ^{15}N resonances of each residue to the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ resonances from its own side chain and from the side chain of the preceding amino acid (Fig. 2; Grzesiek & Bax, 1992; Wittekind & Mueller, 1993). The HNCACB spectrum of BCX had sufficient signal-to-noise such that both the inter- and intraresidue connections between the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ and the amide H^{N} of almost every amino acid in the protein were observed. Additionally, the peaks from the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ were differentiated readily by their opposite signs. In principle, this provided enough information to assign extensively the resonances from the main-chain nuclei of BCX. Still, the CBCA(CO)NH spectrum, which contains only interresidue correlations, was important for distinguishing the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ resonances of an amino acid from those of its preceding neighbor, as well as for identifying possible chemical shift degeneracies.

One difficulty encountered with the resonance assignments of BCX arose from the high number of glycine residues in the pro-

tein. This was compounded by the fact that several of these residues were either adjacent or separated by a single amino acid in the sequence of the protein. At each of the 25 Gly residues, the sequential assignments from the HNCACB experiment were based only on the $^{13}\text{C}^{\alpha}$ resonances, for which there was often significant chemical shift overlap. Therefore, the assignment protocol relied heavily on the CBCA(CO)NH experiment to identify the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ resonances of the residues preceding glycines in BCX. In the cases where this information was still insufficient to assign unambiguously the glycine spin systems, the proton chemical shifts obtained from the CBCA(CO)NH, $\text{H}(\text{CCO})\text{NH}$, and HSQC-TOCSY experiments were also used.

Using the powerful HNCACB and CBCA(CO)NH experiments, in combination with selective isotope labeling, we were able to assign specifically all the main-chain H^{N} , ^{15}N , $^{13}\text{C}^{\alpha}$, and $^{13}\text{C}^{\beta}$ resonances of this 20.4-kDa protein, with the exception of the two amino terminal residues, Ala 1 and Ser 2. The $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ resonances of Ser 2 were identified from the correlations with the amide of Thr 3. The amide ^{15}NH resonances of this residue were not observed in the spectra of BCX, thus precluding the assignment of the carbon resonances of Ala 1. Line broadening due to conformational averaging of this region

Fig. 2. Assignment of the main-chain resonances of BCX using the HNCACB and CBCA(CO)NH experiments. The spectra are presented as pairs of strip plots taken from the ^1H - ^{13}C (ω_3 - ω_1) planes of the three-dimensional HNCACB (right) and CBCA(CO)NH (left) data sets at the ^{15}N frequencies of the indicated residues. Cross peaks in the HNCACB strips arising from intrasidue correlations to $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ nuclei are labeled α and β , respectively. Cross peaks to the $^{13}\text{C}^\beta$ are inverted relative to those to the $^{13}\text{C}^\alpha$ in the HNCACB, but are plotted without distinction of sign (Wittekind & Mueller, 1993). As exemplified with Tyr 166, the HNCACB provides correlations between the H^N and ^{15}N of residue i (Y166) and the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ of both residues i (Y166) and $i-1$ (A165), whereas the CBCA(CO)NH provides only the interresidue connections to $i-1$ (A165).

of BCX or base-catalyzed hydrogen exchange accelerated by the adjacent positively charged amino terminus (Bai et al., 1993) are possible reasons for the absence of detectable amide resonances from Ser 2. However, rapid amide hydrogen exchange is not expected to cause significant line broadening at pH 5.8, because the water magnetization is only minimally perturbed in the experiments employing pulsed field gradients and selective "flip-back" pulses (Grzesiek & Bax, 1993).

Once the backbone amide assignments were completed, the carbonyl C' resonances were identified in a straightforward fashion in the HNCO experiment. This correlates the H^N and ^{15}N of a non-proline residue to the $^{13}\text{C}'$ of its preceding neighbor (Ikura et al., 1990). The H^α resonances were then identified using the CBCACO(CA)HA experiment, which links the H^α , C' , and $^{13}\text{C}^\alpha/^{13}\text{C}^\beta$ resonances of each non-glycine residue (Kay, 1993). Figure 3 exemplifies the excellent dispersion seen also for the H^α and C' resonances in BCX. The glycine signals were not detected in this experiment due to the optimization of a refocused INEPT module within the pulse sequence for AX spin systems. This CBCACO(CA)HA experiment was also used to assign the $^{13}\text{C}'$ resonances of the residues preceding the six prolines in BCX that obviously lack H^N protons required for detection in the HNCO experiment. The combination of the HNCO and CBCACO(CA)HA experiments also provided an independent two-step pathway from the H^N and ^{15}N resonances of a residue to the $^{13}\text{C}'$ of its preceding neighbor and then to

the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ of that residue. This was useful to confirm the assignments deduced initially from the HNCACB and CBCA(CO)NH experiments.

Finally, the assignments of the side-chain protons in BCX were obtained with the H(CCO)NH and HSQC-TOCSY experiments recorded with $^{15}\text{N}/^{13}\text{C}$ -labeled BCX and ^{15}N -labeled BCX, respectively. The H(CCO)NH correlates the H^N and ^{15}N resonances of a residue with the proton resonances of the preceding amino acid, obtained via transfer of magnetization along its ^{13}C aliphatic side chain (Grzesiek et al., 1993). The simpler HSQC-TOCSY shows only intrasidue proton-proton correlations, resolved by the amide ^{15}N shift. Similar proton assignments can be obtained from the HCCH-TOCSY experiment, yet this former approach has the distinct advantage of spectral resolution based on the amide H^N and ^{15}N shifts of a protein. Using the H(CCO)NH and HSQC-TOCSY spectra, we confirmed the H^α assignments of BCX and identified the H^β and additional side-chains resonances of this protein. This was particularly important for the 24 glycine residues for which H^α signals could not be identified by the CBCACO(CA)HA experiment. The complementary H(CCO)NH and HSQC-TOCSY experiments were useful to resolve ambiguities in the limited number of cases of residues with overlapping ^{15}N and H^N chemical shifts. In addition, this provided yet a third method to confirm the sequential assignments of BCX. We have not completed the assignments of the remaining side-chain ^{13}C resonances at this

Fig. 3. The ω_3 - ω_2 face of the CBCACO(CA)HA spectrum of BCX, showing the excellent dispersion resulting from the intra-residue H^α and $^{13}C'$ correlations. Data were recorded in a two-dimensional mode and a first-order phase shift was applied to the interferogram before Fourier transformation to shift the center of the spectrum for clearer presentation (Kay, 1993). The two peaks marked by an asterisk (*) are aliased and inverted in the $^{13}C'$ dimension.

stage of the analysis, yet it would be straightforward using the C(CO)HN or HCCH-TOCSY experiments (Grzesiek et al., 1993).

Side-chain ^{15}NH assignments

In addition to the backbone amides, the 1H - ^{15}N HSQC spectrum of BCX contains peaks from the primary amides of asparagine and glutamine, the indole rings of tryptophans, and the side chains of arginine and histidine. Although primary amides can be assigned readily using the HNCACB experiment published originally (Wittekind & Mueller, 1993), they are not detected in the modified sensitivity-enhanced gradient version of the experiment due to its optimization for AX spin systems. Therefore, we did not complete the assignment of the asparagine and glutamine side-chain amides of BCX at this stage of the analysis. At pH 5.8, five of the seven arginine $^{15}N^H$ groups were detected in the HSQC spectrum of the BCX, whereas at pH 4, all seven were observed (not shown). The arginine $^{15}N^H$ groups were not assigned in this study. The imidazole side chain

of His 149 is entirely buried within BCX, existing in the neutral N^H tautomeric form at all pH's under which the protein is folded (L.A. Plesniak, W.W. Wakarchuk, & L.P. McIntosh, in prep.). The $^{15}N^H$ group of this histidine does not undergo fast exchange with water and is observed in the HSQC spectrum of BCX at 168.2 ppm and 12.2 ppm. In contrast, His 156 is exposed to the solvent and thus the nitrogen-bound protons are not observable.

The $^{15}N^H$ groups of the 11 tryptophan indole rings in BCX are clearly resolved in the HSQC spectrum shown in Figure 1. Because tryptophans are unusually abundant in this protein and play an important role in xylan recognition (Wakarchuk et al., 1994a), we were particularly interested in completing their assignments. In previous protein NMR studies, these indole resonances have been assigned based on NOE correlations (Wüthrich, 1986). To avoid possible ambiguities associated with this approach, we used the $(H\beta)C\beta(C\gamma C\delta)H\delta$ experiment to link the $H^{\delta 1}$ of the tryptophan rings in uniformly $^{13}C/^{15}N$ -labeled BCX to their corresponding $^{13}C\beta$ via scalar couplings (Fig. 4; Yamazaki et al., 1993). From the main-chain assignments dis-

Fig. 4. A portion of the $(H\beta)C\beta(C\gamma C\delta)H\delta$ spectrum of BCX showing correlations between the $^{13}C^{\beta}$ and $H^{\delta 1}$ or $H^{\delta 2}$ resonances of the 11 tryptophan and 2 histidine side chains, respectively. Only eight tryptophan cross peaks are specifically assigned. Correlations for the tyrosine and phenylalanine residues occur downfield in the $^{13}C^{\beta}$ dimension and are not shown.

cussed above, this provided immediately the identification of eight tryptophan $H^{\delta 1}$ resonances, as well as the $H^{\delta 2}$ resonances of His 149 and 156. Unfortunately, the $^{13}C^{\beta}$ of Trp 6, 30, and 42, as well as two of the three $H^{\delta 1}$'s of these residues, were nearly degenerate, thus precluding their assignment based upon this data alone. The $H^{\delta 1}$'s were then linked to the neighboring ^{15}N -bonded $H^{\epsilon 1}$'s using the ^{15}N -resolved TOCSY-HSQC spectrum of uniformly ^{15}N -labeled BCX. The assignments were further supported using the HMQC-J experiment reprocessed with 90° zero-order phase shifts in ω_1 and ω_2 to yield the two-dimensional HMQC-COSY spectrum (Foreman-Kay et al., 1990). In this latter spectrum, the $H^{\delta 1}$ resonances are resolved by the corresponding $^{15}N^{\epsilon 1}$ shift of the tryptophan ring. Lastly, the $^{13}C^{\delta 1}$ resonances of the tryptophans were identified readily from a constant-time 1H - ^{13}C HSQC spectrum of BCX due to their inverted sign relative to most peaks from the aromatic carbons with two carbon neighbors (not shown; Santoro & King, 1992). The resulting assignments of eight tryptophan residues are summarized in Table 2 and Figures 1 and 4.

Secondary structure analysis

The secondary structure of BCX is composed of 14 β -strands and a single α -helix. These structural elements were determined based on a consensus of four NMR approaches, as summarized in Figures 5, 6, and 7.

Secondary chemical shifts

A well-established correlation exists between secondary structure and the deviations of the H^{α} , $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{13}C'$ chemical shifts of an amino acid in folded protein from its random coil values (Spera & Bax, 1991; Wishart et al., 1992; Wishart & Sykes, 1994). Residues in β -strands show positive (downfield) H^{α} and $^{13}C^{\beta}$ and negative (upfield) $^{13}C^{\alpha}$ and $^{13}C'$ structure-dependent chemical shift deviations ("secondary shifts"), whereas those in α -helices show the opposite pattern, albeit with less pronounced $^{13}C^{\beta}$ shifts.

To identify α -helices and β -strands, the H^{α} , $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{13}C'$ chemical shifts were examined in two ways. First, the secondary shifts of each residue were calculated as the differences between the observed chemical shifts from the corresponding "random coil" values (Fig. 5; Wishart et al., 1992; Wishart & Sykes, 1994). Information regarding the secondary structure of BCX was then deduced manually from the observed patterns of secondary shifts, using the above-stated criteria. This interactive approach was particularly important when deviations from regular patterns of secondary shifts were observed due to β -bulges (e.g., Ala 55 or Tyr 166) or at sites where the pairings between β -strands change (e.g., Leu 68).

Second, these data were treated using the chemical shift index algorithm developed by Wishart and Sykes (1994). This computerized method involves assigning a ternary index of +1, 0, or -1 for the H^{α} , $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{13}C'$ of each residue

Table 2. Assignment of the tryptophan side-chain resonances of *Bacillus circulans* xylanase

Tryptophan ^a	¹³ C ^β (ppm) ^b	¹ H ^{δ1} (ppm) ^c	¹³ C ^{δ1} (ppm) ^d	¹ H ^{ε1} (ppm) ^e	¹⁵ N ^{ε1} (ppm) ^e
9	31.5	7.27	127.3	10.02	132.2
58	31.0	7.30	128.1	8.99	127.4
71	35.4	5.56	126.2	8.89	130.8
85	29.4	6.86	127.5	10.41	133.3
129	32.5	6.65	125.1	9.82	133.7
153	27.2	6.47	122.4	9.62	127.5
164	29.5	7.14	126.1	9.77	130.9
185	30.2	7.17	127.8	10.07	131.3

^a Tryptophans 6, 30, and 42 were not assigned due to spectral overlap. These three tryptophans have ¹⁵N^{ε1}-H^{ε1} peaks at (132.9 ppm, 10.22 ppm), (131.3, 9.55), and (129.7, 10.08), and ¹³C^{δ1}-H^{δ1} peaks at (130.8, 7.33), (127.9, 6.99), and (126.9, 7.32).

^b Taken from Table 1.

^c Taken from the (Hβ)Cβ(CγCδ)Hδ spectrum presented in Figure 4.

^d The ¹³C^{δ1} assignments are from a ¹H-¹³C CT-HSQC spectrum of the aromatic region of BCX. The ¹³C^{δ1}-H^{δ1}, which have only one neighboring ¹³C, were readily identified by their inverted sign relative to the majority of aromatic carbons with an even number of ¹³C neighbors (not shown).

^e Taken from the ¹H-¹⁵N HSQC spectrum of BCX presented in Figure 1.

based upon the observed secondary shifts. Next, for each of the four individual chemical shift parameters, a residue is assigned as being in a helix, strand, or coil using empirical rules regarding the patterns of neighboring group secondary shifts. Finally, a consensus secondary structure is reported as a direct average of the secondary structures deduced from the analyses of the H^α, ¹³C^α, ¹³C^β, and ¹³C^γ chemical shifts for each individual residue. The secondary shift thresholds used to assign the ternary indices are relatively narrow (e.g., ±0.5 ppm for ¹³C^α or ¹³C^β) and thus it was important to include a correction term of +0.15 ppm to account for the difference between DSS and TSP as a ¹³C reference. Using this automated approach, we found only ~70% agreement with the secondary structure (helix, strand, or coil) deduced from a combined approach of chemical shift, J coupling, NOE, and amide hydrogen exchange information (Fig. 5).

Obvious errors arising from the automated CSI algorithm, including the omission of β-strand I.1 and part of strand I.3, and the assignment of Ser 22–Gly 23 and Arg 132–Lys 135 to helical conformations. These latter residues comprise a β-turn in sheet I and an irregular or coil region, respectively. These discrepancies arose in part from defining the consensus structure as a direct average of the structures assigned first by the individual secondary chemical shifts. This resulted, for example, in the prediction of a two-residue helix formed by Ser 22–Gly 23. As an alternate tact, we determined a consensus CSI structure by first averaging the ternary indices defined for each of H^α, ¹³C^α, ¹³C^β, and ¹³C^γ, followed by assignment of the structure type based on the neighbor rules outlined by Wishart and Sykes (1994). An improved agreement of ~80% between the CSI prediction and the secondary structure of BCX defined by all NMR methods was observed using this modified algorithm (Fig. 5). Importantly, only a single helical region was identified correctly in BCX, and residues 15–19 were classified as β-strand I.1.

Overall, the two algorithms for defining the secondary structure of BCX using a consensus of chemical shift information were in agreement, differing mainly at the boundaries of the β-strands and α-helix. Only one small three-residue β-strand, II.4, was not

detected by either CSI method, whereas strands II.2 and I.1 were identified only by the first or second algorithm, respectively. Interestingly, BCX is rich in β-sheet structure, yet the CSI approach was overly conservative in assigning residues, particularly in the N-terminal portion of the protein, to this type of secondary structure. This may reflect subtle effects due to the unusually flat nature of sheets I and II (Campbell et al., 1993), or to the sensitivity of chemical shifts to additional structural features including bulges and strand cross-overs.

³J_{NH-Hα} scalar coupling

The secondary structures of proteins are characterized by regular patterns of backbone dihedral angles, and therefore by regular patterns of ϕ-dependent ³J_{NH-Hα} coupling constants. Specifically, residues in helical conformations have ³J_{NH-Hα} couplings on the order of 4–6 Hz, whereas those in extended conformations show couplings larger than 7 Hz (Wüthrich, 1986). Using the HMQC-J experiment, we were able to measure quantitatively ³J_{NH-Hα} couplings greater than 5.6 Hz for 103 nonglycine residues in BCX (Fig. 5). Due to the apparent ¹H-¹⁵N multiple quantum line widths of ~10 Hz for this protein, no splitting in ω1 was observed for an additional 24 residues, for which we have conservatively assigned ³J_{NH-Hα} < 6. Coupling information was not determined for the remainder of the protein, due either to spectral overlap or low signal-to-noise, possibly resulting from solvent suppression by presaturation.

Quantitatively, the measured ³J_{NH-Hα} coupling constants agreed well with those calculated from the crystallographically determined ϕ dihedral angles of BCX using the Karplus equation parameterized as ³J = 6.51 cos²(ϕ–60°) – 1.76 cos(ϕ–60°) + 1.60 (Vuister & Bax, 1993). This comparison includes three of the six non-glycine residues (Ser 74, Asn 114, and Asp 121) with ϕ angles between +40° and +60°. The average difference between the observed and calculated ³J_{NH-Hα} values is –0.7 Hz with a standard deviation of 1 Hz. This difference may reflect a systematic error in the correction of the observed peak splittings in the HMQC-J experiment necessary to obtain the actual

Fig. 5. Summary of the NMR evidence used to derive the secondary structure of BCX. Locations of the β -strands and the α -helix are marked by solid arrows and a coil, respectively. HX: Amide hydrogen-deuterium exchange measured after transfer of the protein to D₂O buffer at pH* 6.1 and 30 °C. Filled circles indicate slow amide exchange ($\tau > 500$ min), half-filled circles indicate intermediate exchange ($\tau \sim 300$ min), and open circles indicate that the ^{15}NH of the residue was observed only in the first HSQC spectrum recorded after transfer to D₂O buffer and thus the exchange rate could not be accurately measured ($\tau \sim 30$ min). An asterisk (*) indicates fast exchange ($\tau < 30$ min) and a blank denotes residues for which spectral overlap precluded measurement of the exchange kinetics. J: Backbone $^3J_{\text{NH-H}\alpha}$ coupling constants measured using the HMQC-J experiment. The coupling constants in Hz are rounded to the nearest integer, and two-digit numbers are italicized. Amides with $^3J_{\text{NH-H}\alpha} < 6$ Hz are marked by (*). CSI: The locations of residues assigned to β -stands and α -helices according to the chemical shift index are shown by a box above or below the horizontal line, respectively (Wishart & Sykes, 1994). Downward-left hatching denotes the structure taken as a consensus after the complete CSI analysis for each individual chemical shift parameter (according to the published algorithm), whereas downward-right hatching denotes the secondary structure identified after averaging of the four chemical shift indices for each residue (see the Results). H α : The H α chemical shifts are presented in the form of a ternary chemical shift index, where +1 indicates that $(\delta_{\text{obs}} - \delta_{\text{random coil}}) > +0.1$ ppm, -1 indicates that $(\delta_{\text{obs}} - \delta_{\text{random coil}}) < -0.1$ ppm, and 0 indicates that the difference in chemical shifts lies within these ranges (Wishart et al., 1992). The average H α shift was used for the glycine residues. The main-chain $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and $^{13}\text{C}'$ chemical shifts are plotted as the difference in ppm from the corresponding random coil values $(\delta_{\text{obs}} - \delta_{\text{random coil}})$, tabulated by Wishart and Sykes (1994). The small difference due to the use of DSS as the ^{13}C shift reference for the BCX data as compared to TSP for the random coil chemical shifts was neglected for this figure, but included in the CSI analysis ($\delta_{\text{obs,DSS}} + 0.15 \text{ ppm} = \delta_{\text{obs,TSP}}$).

$^3J_{\text{NH-H}\alpha}$ coupling constants (Kay & Bax, 1990). However, we did not investigate this further by using alternative approaches, such as the HNHA (Vuister & Bax, 1993) or HSQC-J experiments (Billeter et al., 1992), to re-measure these vicinal cou-

pling constants. The largest deviations were observed for Tyr 5 (-2.5 Hz), Trp 6 (-1.9), Trp 9 (-1.5), Thr 10 (-1.6), Val 19 (-1.6), Tyr 26 (-1.5), Ser 27 (-2.3), Trp 58 (-2.1), Asn 61 (-1.6), Asp 83 (-2.1), Ser 100 (-1.6), Thr 110 (-1.5), Asp 119

Fig. 6. Schematic representation of the three β -sheets identified in BCX. **A:** Sheet I. **B:** Sheet II. **C:** Sheet III. The NOEs between H^N and H^α observed in the NOESY-HSQC spectrum are indicated by arrows. The amide protons with slow or intermediate hydrogen-deuterium exchange kinetics are boxed, those observed only in the first HSQC spectrum recorded after exchange into D_2O ($\tau \sim 30$ min) are circled. Gly 56 and Tyr 166 form β -bulges and are indicated by the zig-zag lines between Ala 55 and Val 57 and A165 and Q167, respectively.

Fig. 7. Topology of BCX determined by NMR. The shaded arrows represent β -strands and the cylinder denotes the α -helix. The three β -sheets are defined as I, II, and III, and the individual strands within each sheet are numbered according to their order in the sequence of the protein. The arrows and box are scaled approximately according to the number of residues in each β -strand or α -helix, respectively, and the arrows are aligned according to their pairings in the β -sheets.

(+2.7), Ser 162 (−1.5), and Ser 177 (−1.5 Hz). The first four of these residues lie in the N-terminal β -strand (II.1) on the edge of sheet II of BCX, whereas residues 19, 26, 27, 58, 61, and 177 lie across sheet I, and residues 100, 119, and 162 occur in loop regions linking several β -strands in sheet III (Fig. 6). It is possible that these larger deviations between the observed and calculated $^3J_{\text{NH-H}\alpha}$ coupling constants reflect small structural or dynamic differences between BCX in solution and in the crystalline state.

Qualitatively, the patterns of $^3J_{\text{NH-H}\alpha}$ coupling constants facilitated the identification of the 14 β -strands and one α -helix in BCX. Except for Tyr 26, Val 82, and Gln 167, all residues identified in β -strands had measurable couplings ≥ 7 Hz. In contrast, with the exception of His 149, all the residues in the single α -helix in BCX (Phe 146–Ser 155) showed no measurable splitting in the HMQC-J spectra and thus have $^3J_{\text{NH-H}\alpha} < 6$ Hz. Furthermore, the ends of the β -strands and α -helix were generally identified based upon distinct changes in the patterns of backbone coupling constants.

Sequential and short-range NOE correlations

Regular α -helical, parallel, and antiparallel β -sheets and turns show characteristic NOE patterns between H^{N} , H^{α} , and H^{β} protons (Wüthrich, 1986; Englander & Wand, 1987). For example, residues in the extended conformations, such as β -strands, are identified by relatively intense $\text{H}_i^{\alpha}\text{--H}_{i+1}^{\text{N}}$ NOEs and the absence of sequential $\text{H}_i^{\text{N}}\text{--H}_{i+1}^{\text{N}}$ NOEs. Xylanase is almost entirely composed of β -sheet structures, and therefore strong NOE connectivities between H_i^{α} and $\text{H}_{i+1}^{\text{N}}$ were ubiquitous in its three-dimensional $^1\text{H}\text{--}^{15}\text{N}$ NOESY-HSQC spectrum. Indeed, with the exception of the α -helical region, the interresidue $\text{H}_i^{\alpha}\text{--H}_{i+1}^{\text{N}}$ NOE was generally one of the strongest crosspeaks observed for each residue in this protein. In contrast, for residues 146–155 in the α -helix, the $\text{H}_i^{\alpha}\text{--H}_{i+1}^{\text{N}}$ NOEs were weak, whereas the diagnostic $\text{H}_i^{\text{N}}\text{--H}_{i+1}^{\text{N}}$ NOEs were uniformly observed (data not shown). In all cases, the observed NOEs used to define the secondary structure of BCX were consistent with interproton distances defined by the crystal structure of this protein.

A primary criteria for the identification of the β -strands in BCX lay with the pairing of these strands into parallel or antiparallel β -sheets using NOE restraints (Fig. 6). Residues in extended structures may show patterns of intraresidue NOEs and J-couplings similar to those diagnostic of β -strands. However, those existing in β -sheets are distinguished by the cross-strand NOEs between main-chain H^{N} and H^{α} protons. The $\text{H}_i^{\alpha}\text{--H}_j^{\alpha}$ NOEs, which are highly diagnostic of antiparallel β -sheets, are not observed in a $^1\text{H}\text{--}^{15}\text{N}$ NOESY-HSQC spectrum. Thus, we relied on the observation of $\text{H}_i^{\text{N}}\text{--H}_j^{\text{N}}$ and $\text{H}_i^{\text{N}}\text{--H}_j^{\alpha}$ NOEs to identify the β -sheets in BCX. Based on 74 interstrand NOEs, we identified 14 β -strands in BCX, associated to form a network of three major β -sheets. Two strands (III.2 and III.5) are parallel, whereas the remaining are aligned antiparallel.

Also illustrated in Figure 6 are several sequential $\text{H}_i^{\text{N}}\text{--H}_{i+1}^{\text{N}}$ NOEs that served to identify the ends of the β -strands and the beginnings of turns or the α -helix in BCX. For example, strand 3 of sheet I ends at Gly 62 and strand 3 of sheet II begins at Gly 64. These two strands are not continuous, as evident by two sequential NOEs, $\text{H}_{62}^{\text{N}}\text{--H}_{63}^{\text{N}}$ and $\text{H}_{63}^{\text{N}}\text{--H}_{64}^{\text{N}}$, that interrupt the regular NOE pattern characteristic of antiparallel β -sheets. These two β -strands are also paired to additional β -strands that are well separated along the primary sequence of the protein. As a second example, the carboxyl terminus of strand 4 of sheet III is delineated by the $\text{H}_{114}^{\text{N}}\text{--H}_{115}^{\text{N}}$ NOE.

Amide hydrogen exchange

The hydrogen–deuterium exchange rates of the individual amides in BCX were measured by recording a series of $^1\text{H}\text{--}^{15}\text{N}$ HSQC spectra after transfer of the protein into D_2O buffer at pH* 6.1 and 30 °C. As summarized in Figure 5, amides were divided into four categories based upon their exchange rates. (1) Fifty-four amides exchanged rapidly and were not detected, whereas (2) 27 amide protons were observed only in the first HSQC spectrum. The exchange rates of these latter amide protons were estimated to be $\sim 5 \times 10^{-4} \text{ s}^{-1}$ (or $\tau \sim 30$ min). (3) Five amides showed slow yet complete exchange over the day of the experiment, corresponding to exchange rates of $\sim 5 \times 10^{-5} \text{ s}^{-1}$ (or $\tau \sim 300$ min) when fit to a single exponential decay. (4) Eighty-six amide protons in BCX did not exchange completely and thus have lifetimes longer than ~ 500 min. These later

two groups of slowly exchanging amides in BCX are protected by at least 10^4 – 10^5 fold relative to an amide in an unstructured polypeptide (Bai et al., 1993).

α -Helices and β -sheets in proteins are characterized by patterns of backbone hydrogen bonding. Evidence for this hydrogen bonding is provided by the observation of significant protection of an amide H^N from exchange with the solvent water. All the amides identified within the β -sheets and α -helix of BCX, which were expected to form the hydrogen bonds diagnostic of these secondary structures, showed significant protection ($\tau > 300$ min) from hydrogen–deuterium exchange (Figs. 5, 6). Conversely, the amides on the ends of the β -strands and the α -helix, as well as the outside edges of the β -sheets, exchanged rapidly with the solvent, providing further criteria for defining the termini of these structural elements.

It is interesting that residues 165–185 all have lifetimes for amide hydrogen exchange greater than 500 min, except for those in the break between β strands II.5 and I.5. These C-terminal residues form two β -strands located in the interiors of β -sheets I and II, and thus are strongly protected from exchange due to their involvement in stable hydrogen bonds. The extreme C-terminus of BCX is clearly well structured. In contrast, the first four N-terminal residues of the protein are not significantly protected from amide hydrogen exchange.

β -Sheet structure of BCX

The 14 β -strands identified in BCX were aligned based on cross-strand $H_i^N-H_j^N$ and $H_i^N-H_j^C$ NOEs to form a network of three β -sheets (Figs. 6, 7). We have followed the nomenclature of Campbell et al. (1993), identifying these sheets as I, II, and III, and their component strands by Arabic numbers according to their order within the sequence of the protein. Sheet I is clearly distinct, whereas sheets II and III share two common β -strands (II.5/III.6 and II.3/III.1). Campbell et al. (1993) describe each of these long strands in the crystal structure of BCX as divided into two β -strands due to their pronounced twist near the active site of the protein. However, based on the above-stated NMR criteria, both strands appear continuous, leading to the topological description of BCX shown in Figure 7. An analysis of the crystallographic coordinates of BCX using the secondary structure algorithm DSSP supports this description (Kabsch & Sander, 1983). Törrönen and Rouvinen (1995) also defined the strands in the homologous *T. reesei* xylanases I and II in this fashion, yet classified sheets II and III as a single extensive β -sheet. The variation in nomenclature simply reflects the different criteria used for defining the structures of proteins and does not imply any real conformational differences.

Sheet I is composed of five antiparallel β -strands (Fig. 6A). This sheet, which forms one face of the BCX molecule, is largely amphipathic in nature. Interestingly, the only polar residues found within sheet I are asparagine, serine, and threonine. A second notable feature of this sheet is a β -bulge involving Gly 56. The insertion of this residue was identified readily by the change in the register of strands I.3 and I.5 with NOEs observed between Asn 54 and Thr 183 and between Val 57 and Asn 181. Secondary $^{13}C^\alpha$ and $^{13}C'$ shifts and amide hydrogen exchange kinetics highlight further the deviation of Gly 56 from a regular extended conformation (Fig. 5).

Sheet II, also composed of five antiparallel β -strands, is relatively nonpolar due to an abundance of aromatic residues

(Fig. 6B). This sheet forms one wall of the active site cleft of the enzyme, with the catalytic acid/base Glu 172 located in strand II.5 (Campbell et al., 1993). A second β -bulge, formed by Tyr 166, results in the twist of strand II.5 along the active site as it continues to become III.6. This bulge is evident from the small $^3J_{NH-H\alpha}$ coupling constant, small secondary $^{13}C^\alpha$ and $^{13}C'$ shifts, and fast amide hydrogen exchange kinetics observed for Tyr 166 (Fig. 5).

Sheet III contains six β -strands, with III.2 and III.5 aligned to form the only parallel β -strands in BCX (Fig. 6C). One edge of sheet III is nonpolar, whereas the other contains an excess of polar threonine residues. The nonpolar edge, with the catalytic nucleophile Glu 78 in strand III.2, contributes to the active site cleft, whereas the hydrophilic region of the sheet lies along the surface of the protein molecule. Based on the crystallographic structure of BCX, the peptide bond linking Ser 74 and Pro 75 in the turn between strands III.1 and III.2 adopts a *cis* conformation. Although we have not conducted a complete analysis of these residues, the identification of a strong NOE between the H^α of Pro 75 and the amide H^N of Ser 74 is consistent with this conformation.

Discussion

At the inception of this study, the only known structure of a xylanase was that reported preliminarily for the enzyme from *B. pumilis*. Since then, the high-resolution crystal structures of *B. circulans* xylanase, *T. harzianum* xylanase, and *T. reesei* xylanase I and II have been determined. These proteins are homologous in sequence and conformation. As described by Törrönen et al. (1994), the general fold of the xylanases resembles that of a partially closed right-hand, with the active site “palm” formed by the cleft between the “fingers” and “thumb.” This structure can be envisioned readily from Figure 7 by folding sheet I below sheet II to form the “fingers” and sheet III above sheet II to complete the “palm” and “thumb.”

In parallel with the crystallographic analysis of BCX, we have used NMR to characterize this enzyme in solution. Based on chemical shift, NOE, J-coupling, and amide hydrogen exchange data, 14 β -strands and a single α -helix were identified as the secondary structural elements of BCX. The locations of the strands and helices along the sequence of the protein agree closely with that observed in the crystal structure (Fig. 5). As discussed previously, minor differences between the reported boundaries of the helices and sheets are attributed to differences in the criteria used to define the secondary structure of this protein. More importantly, the pairing of the 14 strands using NOE restraints to form three β -sheets (Figs. 6, 7) corresponds exactly with the topological arrangement of these strands found in the crystal structure of BCX. Although not unexpected, this shows that the global fold of the protein in solution must resemble closely that established for the crystalline state.

Active site residues

Glutamic acids 78 and 172, which are conserved in all G family xylanases, have been identified as the catalytic carboxyl groups in BCX. Site-directed mutation of either of these residues severely cripples the enzyme, thus confirming their intimate roles in catalysis (Wakarchuk et al., 1992, 1994a). Withers and co-workers (Miao et al., 1994) have identified Glu 78 as the nucleophile in

BCX using a mechanism-based inhibitor, 2',4'-dinitrophenyl 2-deoxy-2-chloro- β -xylobioside, that forms a stable covalent adduct with this carboxyl group. Accordingly, Glu 172 is the acid-base catalyst. Similar results were reported for *B. pumilis* xylanase (Ko et al., 1992). Consistent with their catalytic roles, the pK_a 's of Glu 78 and Glu 172 are 4.6 and 6.7, respectively. These values were determined by using ^{13}C -NMR to monitor the pH titration of BCX labeled selectively with δ - ^{13}C -glutamic acid (L.P. McIntosh et al., in prep.; see also Davoodi et al., 1995).

As illustrated in Figure 7, Glu 78 is found near the N-terminal end of strand III.2 and Glu 172 near the C-terminal end of strand II.5. The folding of the β -sheets to form the "palm" of BCX orients the two residues on opposite sides of the active site cleft, in position to hydrolyze the glycosidic bond of an intervening xylan substrate. Many aromatic side chains, including Trp 9, Tyr 69, Tyr 80, and Tyr 166, lie on the same faces of the β -sheets as do the catalytic glutamic acid residues, and must also be exposed within the active site cleft (Fig. 6). This is consistent with mutagenesis studies showing that these groups are important for the activity of BCX, and with the general observation that carbohydrate binding sites are often rich in aromatic residues (Vyas, 1991; Wakarchuk et al., 1994a).

One end of the active site of the xylanases is partially closed by a long extended loop (or "chord;" Törrönen et al., 1994) linking β -strands II.4 and III.3. This eight-residue segment, spanning from Tyr 87 to Tyr 94, appears very exposed yet well-defined in the crystal structure of BCX. As summarized in Figure 5, the residues in this loop do not adopt any regular secondary structure and are not protected significantly from amide hydrogen exchange. However, the ^1H line widths of these amino acids are very similar to those from the bulk of the protein, indicating that the loop does not exhibit conformational flexibility as expected for a disordered segment of polypeptide chain. Preliminary ^{15}N relaxation measurements are consistent with this conclusion (not shown). These observations suggest that the exposed loop region has a well-defined conformation in order to play an important role in maintaining the structure of the active site of BCX.

Amide-aromatic interaction

A second unusual feature of the active site of BCX involves the loop or "thumb" that joins strands III.4 and III.5. This nine-residue segment folds back into the active site of the protein such that Ile 118 contacts Trp 71 in strand III.1 of the same β -sheet. The close proximity of the Ile to an aromatic ring is evident by the strongly upfield-shifted H^{N} (4.03 ppm) and H^{β} (−0.34 ppm) resonances assigned to this residue. The ring was identified unambiguously as that of Trp 71 based on several NOEs between the H^{N} of Ile 118 and the indole protons of this aromatic residue and between the $\text{H}^{\epsilon 1}$ of Trp 71 and the distinct aliphatic side-chain protons of Ile 118.

Inspection of the crystal structure of BCX reveals that the H^{N} and H^{β} of Ile 118 lie directly over the indole ring of Trp 71 (Fig. 8). Furthermore, based on this structure, the distances between the center of the six-membered ring of Trp 71 and the amide H^{N} and N of Ile 118 are 3.0 and 3.8 Å, respectively. Together, the NMR and crystallographic data suggest that an amide-aromatic hydrogen bond exists between these two residues (Levitt & Perutz, 1988). Similar examples of highly upfield-

Fig. 8. A potential amide-aromatic hydrogen bond between the indole ring of Trp 81 and the backbone NH of Ile 118. This close contact results in the strongly upfield-shifted H^{N} and H^{β} resonances of Ile 118. For clarity, only the bonds linking the carbon and nitrogen atoms of the side chains of these two residues, as well as the carbonyl of Ser 116, are shown on the C^{α} trace of the backbone of BCX. The figure was generated from the crystallographic coordinates of *B. circulans* xylanase (1XNB), using the program SETOR (Evans, 1993).

shifted H^{N} resonances attributed to amide-aromatic hydrogen bonds have been reported for basic pancreatic trypsin inhibitor (Tüchsen & Woodward, 1987) and the cellulose-binding domain of *T. reesei* cellobiohydrolase I (Kraulis et al., 1989).

Hydrogen bonds between NH groups and aromatic rings were recognized initially from statistical analyses of protein structures (Burley & Petsko, 1986; Perutz et al., 1986). Theoretical calculations (Levitt & Perutz, 1988), further high-resolution structural studies of proteins (for example, Waksman et al., 1992), and detailed investigations of the interaction of water and ammonia with benzene (Suzuki et al., 1992; Rodham et al., 1993), support strongly the occurrence of these unconventional hydrogen bonds. However, the energetic contributions of these relatively weak interactions to the stability of proteins and protein-ligand complexes remains uncertain (Jamison et al., 1995). A more recent statistical survey has also indicated that NH-aromatic hydrogen bonds occur less frequently in proteins than thought initially (Mitchell et al., 1994). The question therefore arises as to the experimental criteria for identifying a hydrogen bond between an aromatic ring and an amide group.

We have assigned the interaction between Ile 118 and Trp 71 as a potential amide-aromatic hydrogen bond based on the examination of four possible criteria, namely NMR chemical shift perturbations, conformational parameters, energetic interactions (protection from amide hydrogen exchange), and structural conservation. First, relative to the random coil chemical shifts tabulated by Wüthrich (1986), the H^{N} and H^{β} resonances of Ile 118 are upfield shifted by approximately 4.2 and 2.2 ppm, respectively. Based on the crystallographic coordinates of BCX, the chemical shift perturbations due to induced aromatic ring currents are calculated to be 2.0 ppm for the H^{N} of Ile 118 and 2.7 ppm for the H^{β} (Ösapay & Case, 1991). Assuming that the result for the H^{β} reflects the accuracy of the calculations (and the structural coordinates), we conclude that the observed chemical shift perturbation of Ile 118 H^{N} is significantly greater than

that expected solely from the ring current of Trp 71. Additional deshielding of the amide proton of Ile 118 due to an aromatic hydrogen bond with the tryptophan side chain provides a plausible explanation for this discrepancy. Second, close analysis of the crystal structure of BCX reveals that the amide of Ile 118 lies toward the edge of the indole ring of Trp 71 ($r_{\text{HN-C}\eta 3} = 2.9 \text{ \AA}$ versus $r_{\text{HN-C}\delta 2} = 3.7 \text{ \AA}$) and that the NH bond is tilted with respect to its normal ($\theta_{\text{r-N-H}} = 30^\circ$, where r is central point of the six-membered ring). Although not optimal, the orientation of the amide and aromatic ring falls within the theoretical range for a favorable electrostatic interaction (Levitt & Perutz, 1988) and meets the structural criteria for a hydrogen bond defined by Mitchell et al. (1994). Third, although we have not investigated directly the energetic contribution of the interaction between Trp 71 and Ile 118 to the stability of BCX, we observed that the postulated hydrogen bond did not protect the amide proton of Ile 118 from exchange with the solvent (Fig. 5). However, under the conditions of these measurements, only those amides with exchange rates retarded by at least 10^3 -fold relative to an unstructured polypeptide were detected (Bai et al., 1993). Indeed, one would not expect Ile 118 to be protected significantly from exchange because the amide of this residue is partially exposed to the solvent and, based on theoretical analyses, an amide–aromatic hydrogen bond is, at best, only half as strong as a conventional hydrogen bond (Levitt & Perutz, 1988). More thorough hydrogen–deuterium exchange studies, possibly combined with site-directed mutagenesis of position 71, are required to determine the energetic consequences of the potential indole–amide hydrogen bond in BCX.

Finally, alignment of the sequences of 13 low molecular weight family 11 xylanases demonstrates that the tripeptides corresponding to Tyr 69–Gly 70–Trp 71 and Pro 116–Ser 117–Ile 118 of BCX are highly conserved, with the only reported substitution being Val or Leu at position 118 (Wakarchuk et al., 1994a). Moreover, the aromatic–amide interaction between Trp 71 and Ile 118 is conserved structurally in the xylanases from *B. circulans*, *T. harzianum*, and *T. reesei*. The loop containing Ile 118 forms the edge of the active site, whereas the indole N^{H} of Trp 71 participates in a network of hydrogen bonds that includes that catalytic nucleophilic Glu 78 (Wakarchuk et al., 1994a). It is reasonable to suggest that the conserved interaction between Trp 71 and Ile 118 is energetically favorable and plays a key role in establishing the precise conformation of the active sites of these xylanases, as required for the binding and hydrolysis of xylan.

Summary

We have assigned the main-chain resonances of *B. circulans* xylanase and defined the secondary structure and β -sheet topology of this protein using heteronuclear NMR experiments combined with selective and uniform isotopic labeling. Several interesting features, such as a potential amide–aromatic hydrogen bond that may contribute to the active site structure of BCX, were also identified. This NMR analysis provides the foundation necessary for on-going analyses of wild-type and mutant *B. circulans* xylanases, directed both at understanding the mechanisms by which this enzyme binds and hydrolyzes carbohydrate substrates, and at exploiting its activity for biotechnological applications.

Materials and methods

Sample preparation

The gene encoding BCX was cloned in a P_{tac} plasmid to allow efficient expression in *E. coli* (Sung et al., 1993). Uniformly ^{15}N -labeled protein was produced from the protrophic *E. coli* 594 strain cultured in M9 minimal media containing 1.0 g/L of 99% $(^{15}\text{NH}_4)_2\text{SO}_4$ (McIntosh & Dahlquist, 1990). Uniformly ^{15}N - and ^{13}C -enriched protein was produced from *E. coli* 594 grown in M9 minimal media containing 1.0 g/L 99% $^{15}\text{NH}_4\text{Cl}$ and 3.0 g/L 99% $[^{13}\text{C}_6]\text{-glucose}$. The backbone amides of the Tyr, Val, Gly, Gly/Ser, or Asp/Asn residues in BCX were selectively ^{15}N -labeled using *E. coli* DL39 cells, as described by McIntosh and Dahlquist (1990). The proteins were purified by cation exchange FPLC (Sung et al., 1993), and, in several cases, analyzed by electrospray mass spectroscopy to evaluate the extent of isotopic enrichment.

NMR measurements were made with $\sim 0.8 \text{ mM}$ protein in 25 mM $\text{d}_3\text{-acetate}$, 0.02% NaN_3 , 90% $\text{H}_2\text{O}/10\% \text{D}_2\text{O}$ at pH 5.8. A single $^{13}\text{C}/^{15}\text{N}$ -labeled BCX sample in this H_2O buffer was used for all triple-resonance NMR experiments.

NMR spectroscopy

Experiments were performed at 30°C on a Varian Unity 500 NMR equipped with three radio-frequency channels and a pulsed-field gradient accessory. Unless otherwise stated, the ^1H spectral widths were 6,250 Hz, with the transmitter set at the frequency of H_2O . The ^{15}N spectral widths were 1,575 Hz, centered at 121.4 ppm. The spectral width for the $^{13}\text{C}^{\alpha/\beta}$ dimension was 8,050 Hz, centered at 43 ppm, and for the backbone $^{13}\text{C}'$ carbonyl dimension, 1,150 Hz, centered at 173.6 ppm. The ^1H chemical shifts were referenced to water at 4.70 ppm, calibrated using an external DSS sample at 30°C . The ^{15}N chemical shifts were referenced to 2.9 M $^{15}\text{NH}_4\text{Cl}$ in 1 M HCl at 24.93 ppm (Levy & Lichter, 1979). The ^{13}C chemical shifts were referenced to the methyl carbon (0.00 ppm) of an external sample of DSS. Spectra were collected in the States-TPPI mode with the initial time point in each indirectly detected dimension set exactly to $1/(2 * \text{sw})$, such that a first-order phase correction of 180° is required after Fourier transformation and that aliased resonances are inverted relative to the those within the set spectral widths (Bax et al., 1991). For all experiments in which magnetization was detected on the amide H^{N} , sensitivity-enhanced pulsed-field gradient sequences were employed (Kay et al., 1992; Muhandiram & Kay, 1994), with selective “flip-back” pulses to return water magnetization to equilibrium before the acquisition of the NMR signal (Grzesiek & Bax, 1993; Kay et al., 1994; Zhang et al., 1994). The data from these experiments were manipulated as outlined by Muhandiram and Kay (1994) prior to processing.

The NMR data were analyzed using Felix v2.30 software (Biosym Technologies; San Diego, California). Generally, a Lorentzian-to-Gaussian transformation was applied in the directly detected ^1H dimension with -6 Hz line broadening and a maximum at 0.2 of the acquisition time. The residual signal from water was eliminated using a digital filter applied in the time domain (Marion et al., 1989). The indirectly detected dimensions were processed using squared sine bell apodization shifted by 70° . In dimensions where the data were collected in

a constant time mode, mirror image linear prediction (Zhu & Bax, 1992) was used to double the number of data points. Otherwise, linear prediction was used to extend the number of data points in the interferogram by approximately 30%.

Resonance assignments

The assignment of the backbone $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, ^{15}N , and H^N resonances were completed using sensitivity-enhanced versions of the three-dimensional HNCACB (Wittekind & Mueller, 1993) and CBCA(CO)NH (Grzesiek & Bax, 1992; Muhandiram & Kay, 1994) experiments. The HNCACB was acquired with 512, 39, and 45 complex points in t_3 (^1H), t_2 (^{15}N , constant time), and t_1 ($^{13}\text{C}^{\alpha,\beta}$), respectively. The CBCA(CO)NH experiment was collected with 512, 39, and 53 complex points in t_3 (^1H), t_2 (^{15}N , constant time), and t_1 ($^{13}\text{C}^{\alpha,\beta}$, constant time), respectively. For the assignment of the backbone carbonyl $^{13}\text{C}'$ and H^α , and confirmation of $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ assignments, the CBCACO(CA)HA (Kay, 1993) and sensitivity-enhanced HNCO (Ikura et al., 1990; Muhandiram & Kay, 1994; Kay et al., 1994) experiments were used. The HNCO experiment was collected as 512, 39, and 45 complex data points in t_3 (^1H), t_2 (^{15}N , constant time), and t_1 ($^{13}\text{C}'$), respectively. The CBCACO(CA)HA experiment was recorded as 512, 64, and 56 complex data points in t_3 (^1H), t_2 ($^{13}\text{C}'$), and t_1 ($^{13}\text{C}^{\alpha,\beta}$, constant time), respectively. For each of these four experiments, the final transformed data sets were 256, 128, and 128 real points in ω_3 , ω_2 , and ω_1 , respectively. The region upfield of the H_2O in ω_3 was discarded. However, because the H^N of Ile 118 in BCX resonates at 4.03 ppm, the data were reprocessed separately to retain a small region in ω_3 surrounding this peak.

To assign the side-chain proton resonances and to measure NOEs to amide H^N , sensitivity-enhanced versions of the H(CCO)NH (Grzesiek et al., 1993) and the TOCSY-HSQC and NOESY-HSQC (Fesik & Zuiderweg, 1990; Zhang et al., 1994) experiments were recorded with the samples of $^{13}\text{C}/^{15}\text{N}$ - and ^{15}N -labeled BCX, respectively. The H(CCO)NH spectrum was measured using 512, 39, and 64 complex data points in t_3 (H^N), t_2 (^{15}N , constant time), and t_1 (H), respectively, yielding a final transformed matrix of 256, 128, and 256 real points. The ^{13}C DIPSI mixing time was 12.6 ms at a field strength of 8.6 kHz. The spectral width in ω_1 ^1H dimension was 3,750 Hz, and thus the spectrum was shifted upfield by 2,080 Hz to encompass the side-chain protons by applying a first-order phase correction to the interferogram before Fourier transformation (Kay et al., 1989). The HSQC-TOCSY was acquired with a 55-ms ^1H DIPSI mixing time and 512, 64, and 128 complex data points in t_3 (H^N), t_2 (^{15}H), and t_1 (H), respectively. The NOESY-HSQC was recorded with a 125-ms mixing period and 512, 64, and 160 complex data points in t_3 (H^N), t_2 (^{15}H), and t_1 (H). Both data sets were processed to 256, 128, and 512 real points in ω_3 , ω_2 , and ω_1 , respectively.

The $\text{H}^{\delta 1}$ and $\text{H}^{\delta 2}$ of the tryptophan and histidine rings, respectively, were linked to their corresponding $^{13}\text{C}^\beta$ using the ($\text{H}\beta$) $\text{C}\beta$ ($\text{C}\gamma\delta$) $\text{H}\delta$ experiment (Yamazaki et al., 1993). The two-dimensional spectrum was recorded for the $^{13}\text{C}/^{15}\text{N}$ -enriched BCX with 512 and 32 complex points in t_2 (H^δ) and t_1 ($^{13}\text{C}^\beta$, constant time), and processed to 1,024 and 256 real points, respectively. The ^1H - ^{13}C HSQC spectrum of the labeled protein was recorded with a total constant time delay of 16.7 ms, based on a $^1J_{\text{CC}} \sim 60$ Hz for aromatic carbons (Santoro & King,

1992) and using pulsed-field gradients only to suppress the signal from water (Wider & Wüthrich, 1993). With this timing delay, the signals from the ^{13}C with a single ^{13}C neighbor are inverted relative to those bonded to an even number of ^{13}C nuclei.

$^3J_{\text{HN-H}\alpha}$ Coupling constants

The $^3J_{\text{HN-H}\alpha}$ coupling constants were measured using the HMQC-J experiment (Kay et al., 1989; Kay & Bax, 1990). The spectrum was acquired with a sample of ^{15}N -labeled BCX as 2,048 and 350 complex points in t_2 (H^N) and t_1 (^{15}N), respectively, giving a total acquisition time of 0.222 ms in t_1 . The data were processed using Lorentzian-to-Gaussian transformations with line broadening of -5 , -10 , or -15 Hz and an apodization maximum at 0.5 of the acquisition time (0.111 ms), and analyzed as described by Forman-Kay et al. (1990). The error in the measured coupling constants was estimated to be ± 0.5 Hz based on multiple analyses of data from several residues.

Amide hydrogen exchange

The rates of amide hydrogen-deuterium exchange in BCX were measured by rapidly transferring the ^{15}N -labeled protein into D_2O buffer, containing 25 mM d_3 -acetate and 0.02% NaN_3 at $\text{pH}^* 6.1$, using a Sephadex G-25 (medium grade) spin column in a table-top swinging bucket centrifuge (Anderson et al., 1993). After ~ 10 min of equilibration at 30°C , a series of sensitivity-enhanced gradient HSQC spectra, each requiring 60 min to collect, were measured over the period of a day. The rates of hydrogen exchange were determined based on the integrated volumes of the ^1H - ^{15}N crosspeaks in the transformed spectra.

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References

- Anderson DE, Lu J, McIntosh LP, Dahlquist FW. 1993. The folding, stability and dynamics of T4 lysozyme: A perspective using nuclear magnetic resonance. In: Gronenborn AM, Clore GM, eds. *NMR of proteins*. London: Macmillan Press. pp 258–304.
- Bai Y, Milne JS, Mayne L, Englander SW. 1993. Primary structure effects on peptide group hydrogen exchange. *Proteins Struct Funct Genet* 17:75–86.
- Bax A, Ikura M, Kay LE, Zhu G. 1991. Removal of F1 baseline distortion and optimization of folding in multidimensional NMR spectra. *J Magn Reson* 91:174–178.
- Billster M, Neri D, Otting G, Qian YQ, Wüthrich K. 1992. Precise vicinal coupling constants $^3J_{\text{HN}\alpha}$ in proteins from nonlinear fits of J-modulated ^{15}N - ^1H COSY experiments. *J Biomol NMR* 2:257–274.
- Bray MR, Clarke AJ. 1992. Action pattern of xylo-oligosaccharide hydro-

- lysis by *Schizophyllum commune* xylanase A. *Eur J Biochem* 204:191–196.
- Burley SK, Petsko GA. 1986. Amino–aromatic interactions in proteins. *FEBS Lett* 203:139–143.
- Campbell R, Rose D, Wakarchuk W, To R, Sung W, Yaguchi M. 1993. A comparison of the structures of the 20 kD xylanases from *Trichoderma harzianum* and *Bacillus circulans*. In: Suominen P, Reinikainen T, eds. *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulases and other hydrolases, Espoo, Finland, 1993*. Helsinki: Foundation for Biotechnological and Industrial Fermentation Research. pp 63–77.
- Coughlan MP, Hazlewood GP. 1993. β -1,4-D-Xylan-degrading enzyme systems: Biochemistry, molecular biology and applications. *Biotechnol Appl Biochem* 17:259–289.
- Davies G, Henrissat B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* 3:853–859.
- Davoodi J, Wakarchuk WW, Campbell RL, Carey PR, Surewicz WK. 1995. Abnormally high pK_a of an active site glutamic acid residue in *Bacillus circulans* xylanase. *Eur J Biochem* 232:839–843.
- Englander SW, Wand AJ. 1987. Main chain directed strategy for the assignment of 1H NMR spectra of proteins. *Biochemistry* 26:521–526.
- Evans S. 1993. SETOR: Hardware lighted three-dimensional solid model representations of macromolecules. *J Mol Graphics* 11:134–138.
- Fesik SW, Zuiderweg ER. 1990. Heteronuclear three-dimensional NMR spectroscopy of isotopically labelled biological macromolecules. *Q Rev Biophys* 23:97–131.
- Forman-Kay JD, Gronenborn AM, Kay LE, Wingfield PT, Clore GM. 1990. Studies on the solution conformation of human thioredoxin using heteronuclear ^{15}N - 1H nuclear magnetic resonance spectroscopy. *Biochemistry* 29:1566–1572.
- Gebler J, Gilkes NR, Claeysens M, Wilson DB, Béguin P, Wakarchuk WW, Kilburn DG, Miller RC Jr, Warren RAJ, Withers SG. 1992. Stereoselective hydrolysis catalyzed by related β -1,4-glucanases and β -1,4-xylanases. *J Biol Chem* 267:12559–12561.
- Gilkes NR, Henrissat B, Kilburn DG, Miller RC Jr, Warren RAJ. 1991. Domains in microbial β -1,4-glycanases: Sequence conservation, function, and enzyme families. *Microbiol Rev* 55:303–315.
- Grzesiek S, Anglister J, Bax A. 1993. Correlation of backbone amide and aliphatic side-chain resonances in $^{13}C/^{15}N$ proteins by isotropic mixing of ^{13}C magnetization. *J Magn Reson Series B* 101:114–119.
- Grzesiek S, Bax A. 1992. Correlating backbone amide and side chain resonances in larger proteins by multiple relayed triple resonance NMR. *J Am Chem Soc* 114:6291–6293.
- Grzesiek S, Bax A. 1993. The importance of not saturating H_2O in protein NMR. Application to sensitivity enhancement and NOE measurements. *J Am Chem Soc* 115:12593–12594.
- Hazlewood GP, Gilbert HJ. 1993. Molecular biology of hemicellulases. In: Coughlan MP, Hazlewood GP, eds. *Hemicellulose and hemicellulases*. London: Portland Press. pp 102–126.
- Henrissat B, Bairoch A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* 293:781–788.
- Ikura M, Kay LE, Bax A. 1990. A novel approach for sequential assignment of 1H , ^{13}C , and ^{15}N spectra of larger proteins: Heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin. *Biochemistry* 29:4659–4667.
- Jamison RS, Kakkad B, Ebert DH, Mewcomer ME, Ong DE. 1995. Test of the contribution of an amino–aromatic hydrogen bond to protein function. *Biochemistry* 34:11128–11132.
- Kabsch W, Sander C. 1983. Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22:2577–2637.
- Kay LE. 1993. Pulsed-field gradient-enhanced three-dimensional NMR experiment for correlating $^{13}C\alpha/\beta$, $^{13}C'$, and $^1H\alpha$ chemical shifts in uniformly ^{13}C labelled proteins dissolved in H_2O . *J Am Chem Soc* 115:2055–2057.
- Kay LE, Bax A. 1990. New methods for the measuring of $NH-C\alpha H$ coupling constants in ^{15}N -labelled proteins. *J Magn Reson* 86:110–126.
- Kay LE, Keifer P, Saarinen T. 1992. Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J Am Chem Soc* 114:10663–10665.
- Kay LE, Marion D, Bax A. 1989. Practical aspects of 3D heteronuclear NMR of proteins. *J Magn Reson* 84:72–84.
- Kay LE, Xu GY, Yamazaki T. 1994. Enhanced-sensitivity triple-resonance spectroscopy with minimal H_2O saturation. *J Magn Reson* 109:129–133.
- Ko EP, Akatsuka H, Moriyama H, Shinmyo A, Hata Y, Katsube Y, Urabe I, Okada H. 1992. Site-directed mutagenesis at aspartate and glutamate residues of xylanase from *Bacillus pumilus*. *Biochem J* 288:117–121.
- Kraulis PJ, Clore GM, Nilges M, Jones TA, Petterson G, Knowles J, Gronenborn AM. 1989. Determination of the three-dimensional solution structure of the C-terminal domain of cellobiohydrolase I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry–dynamical simulated annealing. *Biochemistry* 28:7241–7257.
- Levi M, Perutz MF. 1988. Aromatic rings act as hydrogen bond acceptors. *J Mol Biol* 201:751–754.
- Levy GC, Lichter RL. 1979. *Nitrogen-15 nuclear magnetic resonance spectroscopy*. New York: J. Wiley & Sons.
- Marion D, Ikura M, Bax A. 1989. Improved solvent suppression in one- and two-dimensional NMR spectra by convolution of time-domain data. *J Magn Reson* 84:425–430.
- McIntosh LP, Dahlquist FW. 1990. Biosynthetic incorporation of ^{15}N and ^{13}C for assignment and interpretation of nuclear magnetic resonance spectra of proteins. *Quart Rev Biophys* 23:1–38.
- McIntosh LP, Wand AJ, Lowry DF, Redfield AG, Dahlquist FW. 1990. Assignment of the backbone 1H and ^{15}N NMR resonances of bacteriophage T4 lysozyme. *Biochemistry* 29:6341–6362.
- Meadows R, Olejniczak E, Fesik S. 1994. A computer-based protocol for semiautomated assignments and 3D structure determination of proteins. *J Biomol NMR* 4:79–96.
- Miao S, Ziser L, Aebersold R, Withers SG. 1994. Identification of glutamic acid 78 as the active site nucleophile in *Bacillus subtilis* xylanase using electrospray tandem mass spectrometry. *Biochemistry* 33:7027–7032.
- Mitchell JBO, Nandi CL, McDonald IK, Thornton JM, Price SL. 1994. Amino/aromatic interactions in proteins: Is the evidence stacked against hydrogen bonding? *J Mol Biol* 239:315–331.
- Muhandiram D, Kay L. 1994. Gradient-enhanced triple-resonance three-dimensional NMR experiments with improved sensitivity. *J Magn Reson Ser B* 103:203–216.
- Oh BH, Westler WM, Darba P, Markley JL. 1988. Protein carbon-13 spin systems by a single two-dimensional nuclear magnetic resonance experiment. *Science* 240:908–911.
- Okada H. 1989. Tertiary structure of xylanase and estimation of active sites by site-directed mutagenesis. *Adv Prot Design* 12:81–86.
- Ösapay K, Case DA. 1991. A new analysis of proton chemical shifts in proteins. *J Am Chem Soc* 113:9436–9444.
- Paice MG, Bourbonnais R, Desrochers M, Jurasek L, Yaguchi M. 1986. A xylanase from *Bacillus subtilis*: Nucleotide sequence and comparison with *B. pumilus* gene. *Arch Microbiol* 144:201–206.
- Perutz MF, Fermi G, Abraham DJ, Poyart C, Bursaux E. 1986. Hemoglobin as a receptor of drugs and peptides: X-ray studies of the stereochemistry of binding. *J Am Chem Soc* 108:1064–1078.
- Redfield C, Dobson CM. 1988. Sequential 1H NMR assignments and secondary structure of hen egg white lysozyme in solution. *Biochemistry* 27:122–136.
- Richarz R, Wuthrich K. 1978. Carbon-13 NMR chemical shifts of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-L-Ala-OH. *Biopolymers* 17:2133–2141.
- Rodham DA, Suzuki S, Suenram RD, Lovas FJ, Dasgupta S, Goddard WA, Blake GA. 1993. Hydrogen bonding in the benzene–ammonia dimer. *Nature* 362:735–737.
- Santoro J, King GC. 1992. A constant-time 2D Overbordenhausen experiment for inverse correlation of isotopically enriched species. *J Magn Reson* 97:202–207.
- Sinnott ML. 1990. Catalytic mechanisms of enzymic glycosyl transfer. *Chem Rev* 90:1171–1202.
- Spera S, Bax A. 1991. An empirical correlation between protein backbone conformation and C^α and C^β ^{13}C NMR chemical shifts. *J Am Chem Soc* 113:5490–5492.
- Sung WL, Luk CK, Zahab DM, Wakarchuk W. 1993. Overexpression and purification of the *Bacillus subtilis* and *Bacillus circulans* xylanases in *Escherichia coli*. *Prot Express Purif* 4:200–216.
- Suzuki S, Green PG, Bumgarner RE, Dasgupta S, Goddard WA, Blake GA. 1992. Benzene forms hydrogen bonds with water. *Science* 257:942–945.
- Tanford C. 1961. *Physical chemistry of macromolecules*. New York: J. Wiley & Sons.
- Törrönen A, Harkki A, Rouvinen J. 1994. Three-dimensional structure of endo-1,4- β -xylanase II from *Trichoderma reesei*: Two conformational states in the active site. *EMBO J* 13:2943–2501.
- Törrönen A, Rouvinen J. 1995. Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. *Biochemistry* 34:847–856.
- Tüchsen E, Woodward C. 1987. Assignment of asparagine-44 side-chain primary amide 1H NMR resonances and the peptide amide N^1H resonances of glycine-37 in basic pancreatic trypsin inhibitor. *Biochemistry* 26:1918–1925.
- Venable R, Pastor RW. 1988. Frictional models for stochastic simulations of proteins. *Biopolymers* 27:1001–1014.

- Vuister G, Bax A. 1993. Quantitative J correlation: A new approach for measuring homonuclear three-bond $J_{(HN-HA)}$ coupling constants in ^{15}N -enriched proteins. *J Am Chem Soc* 115:7772-7777.
- Vyas NK. 1991. Atomic features of protein-carbohydrate interactions. *Curr Opin Struct Biol* 1:732-740.
- Wakarchuk WW, Campbell RL, Sung WL, Davoodi J, Yaguchi M. 1994a. Mutational and crystallographic analysis of the active site residues of the *Bacillus circulans* xylanase. *Protein Sci* 3:467-475.
- Wakarchuk WW, Methot N, Lanthier P, Sung W, Seligy V, Yaguchi M, To R, Campbell R, Rose D. 1992. The 20 kD xylanase of *Bacillus subtilis*: A structure/function analysis. In: Visser J et al., eds. *Xylan and xylanases*. Amsterdam: Elsevier Science B.V. pp 439-442.
- Wakarchuk WW, Sung WL, Campbell RL, Cunningham A, Watson DC, Yaguchi M. 1994b. Thermostabilization of the *Bacillus subtilis* xylanase by the introduction of disulfide bonds. *Protein Eng* 7:1379-1386.
- Waksman G, Komminos D, Robertson SC, Pant N, Baltimore D, Birge RB, Cowburn D, Hanafusa H, Mayer BJ, Overduin M, Resh MD, Rios CB, Silverman L, Kuriyan J. 1992. Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature* 358:646-653.
- Wider G, Wüthrich K. 1993. A simple experimental scheme using pulsed field gradients for coherence-pathway rejection and solvent suppression in phase-sensitive heteronuclear correlation spectra. *J Magn Reson* 102: 239-241.
- Wishart D, Sykes B, Richards FM. 1992. The chemical shift index: A fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* 31:1647-1651.
- Wishart D, Sykes B. 1994. The ^{13}C chemical-shift index: A simple method for the identification of protein secondary structure using ^{13}C chemical-shift data. *J Biomol NMR* 4:171-180.
- Wittekind M, Mueller L. 1993. HNCACB, a high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with the alpha- and beta-carbon resonances. *J Magn Reson Ser B* 101:201-205.
- Wong KKY, Saddler JN. 1992. *Trichoderma* xylanases, their properties and application. *Crit Rev Biotechnol* 12:413-435.
- Wüthrich K. 1986. *NMR of proteins and nucleic acids*. New York: J. Wiley & Sons.
- Yamazaki T, Foreman-Kay JD, Kay L. 1993. Two-dimensional NMR experiments for correlating $^{13}\text{C}^{\beta}$ and $^1\text{H}^{\delta/\epsilon}$ chemical shifts of aromatic residues in ^{13}C -labelled proteins via scalar couplings. *J Am Chem Soc* 115: 11054-11055.
- Zamyatnin AA. 1984. Amino acid, peptide, and protein volume in solution. *Annu Rev Biophys Bioeng* 13:145-165.
- Zhang O, Kay LE, Olivier JP, Foreman-Kay JD. 1994. Backbone ^1H and ^{15}N resonance assignments of the N-terminal SH3 domains of drk in folded and unfolded states using enhanced-sensitivity pulsed field gradient NMR techniques. *J Biomol NMR* 4:845-858.
- Zhu G, Bax A. 1992. Improved linear prediction of damped NMR signals using modified "forward-backward" linear prediction. *J Magn Reson* 100:202-207.