^{1}H NMR study of the interaction of *N,N′,N″*-triacetyl chitotriose with Ac-AMP2, a sugar binding antimicrobial protein isolated from *Amaranthus caudatus*

Patricia Verheyden[a,*], Jurgen Pletinckx[b], Dominique Maes[b], Henri A.M. Pepermans[c], Lode Wyns[b], Rudolph Willem[a], José C. Martins[a]

[a]*High Resolution NMR Centre, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussel, Belgium*
[b]*Laboratory for Ultrastructure, Institute for Molecular Biology, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint-Genesius-Rode, Belgium*
[c]*Unilever Research Laboratory, Olivier van Noortlaan 120, NL-3133AT Vlaardingen, The Netherlands*

Received 10 April 1995; revised version received 6 July 1995

Abstract The interaction between Ac-AMP2, a lectin-like small protein with antimicrobial and antifungal activity isolated from *Amaranthus caudatus*, and *N,N′,N″*-triacetyl chitotriose was studied using ^{1}H NMR spectroscopy. Changes in chemical shift and line width upon increasing concentration of *N,N′,N″*-triacetyl chitotriose to Ac-AMP2 solutions at pH 6.9 and 2.4 were used to determine the interaction site and the association constant K_a. The most pronounced shifts occur mainly in the C-terminal half of the sequence. They involve the aromatic residues Phe[18], Tyr[20] and Tyr[27] together with their surrounding residues, as well as the N-terminal Val-Gly-Glu segment. Several NOEs between Ac-AMP2 and the *N,N′,N″*-triacetyl chitotriose resonances are reported.

Key words: Ac-AMP2; *N,N′,N″*-triacetyl chitotriose; Protein–carbohydrate interaction; ^{1}H NMR; Lectin

1. Introduction

Ac-AMP2 is a small basic protein isolated from the seeds of amaranth (*Amaranthus caudatus*). It is characterised by potent antifungal and antimicrobial properties, which probably intervene to protect seeds or seedlings against micro organisms and fungi [1]. Although presently unknown, the mode of action is most probably related to the fact that Ac-AMP2 can bind to chitin, a polymer of β-1,4 linked *N*-acetyl-D-glucosamine. This binding, as measured by the adsorption behavior on chitin packed micro-columns, is reversible and pH controlled, with binding at neutral pH and dissociation at pH 2.8 or lower [1]. Both the chitin binding and the pH dependence make Ac-AMP2 functionally similar to the family of chitin-binding proteins. These include proteins such as basic chitinases as well as plant lectins made up of two (UDA) or four (WGA) homologous domains. These domains contain 40 to 45 residues, and are particularly rich in glycine and cysteine. The sequence is very similar to that of the protein hevein (isolated from rubber tree latex) which folds into a 'toxin-agglutinin fold' or 'hevein domain' [2]. Structure determination of hevein by ^{1}H NMR [3] and of WGA by X-ray diffraction [4] have shown that the WGA domains and hevein have a common structural fold. Although considerably shorter, Ac-AMP2 displays strong sequence homology with parts of hevein and the hevein-like domains (Fig. 1), including 6 of the 8 half cystines, making it the smallest member in the family of chitin binding proteins. In order to get a better understanding of the protein–carbohydrate interactions involved, detailed studies on Ac-AMP2 complexed to *N,N′,N″*-triacetyl chitotriose are presently under way by both NMR and X-ray diffraction methods.

The small size of Ac-AMP2 makes NMR particularly appropriate to study this protein–carbohydrate interaction and its effects both on the sugar and on the protein conformation. *N,N′,N″*-triacetyl chitotriose or GlcNAc-β(1–4)-GlcNAc-β(1–4)-GlcNAc was chosen as a model for chitine. We report on the first step of our study of the interaction which consisted of the determination of the binding affinity at neutral (6.9) and acidic (2.4) pH from 1D NMR. By monitoring the shifts induced by increasing amounts of *N,N′,N″*-triacetyl chitotriose in an Ac-AMP2 sample, the association constant K_a for the complex was determined. Residues involved in or affected by the binding are mapped onto the sequence. A location for the binding site on the protein is proposed on the basis of several intermolecular NOEs between *N,N′,N″*-triacetyl chitotriose and Ac-AMP2. Comparison with the results of similar studies recently performed on hevein shows that the protein–carbohydrate interactions are very similar in both systems [5].

2. Material and methods

Ac-AMP2 was isolated from seeds of *Amaranthus caudatus L.* (Hortiplan). 1 kg of seeds were ground in a coffee mill. The resulting meal was extracted overnight with 3 liters of a cold extraction buffer containing 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 2 mM EDTA, 1 mM PMSF. The homogenate was filtrated and clarified by centrifugation (40 min at 5000 × *g*). The extract was applied on a chitin column (technical grade, Sigma) that was previously washed with 1 N HCl, H_2O, 1 N NaOH, H_2O, 1 M NaCl and extraction buffer. Not specifically bound protein was washed off with 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, and AcAMP2 was eluted using a linear gradient of 100 mM–1 M KCl in the same buffer. The affinity purified protein was dialyzed against 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM NaCl. The dialysate was applied on a carboxymethyl-sepharose (fast flow) ion-exchange column and the two isoproteins were separated

*Corresponding author. Fax: (32) (2) 629.32.81.

Abbreviations: Ac-AMP2, anti-microbial peptide 2; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylene diaminetetraacetic acid; FID, free induction decay; GlcNAc, *N*-acetyl-D-glucosamine; HOHAHA, homonuclear Hartmann Hahn spectroscopy; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser spectroscopy; PMSF, α-toluenesulfonyl fluoride; TFA, trifluoroethanol; TPPI, time proportional phase increment; UDA, urtica dioica agglutinin; WGA, wheat germ agglutinin.

SSDI 0014-5793(95)00835-7

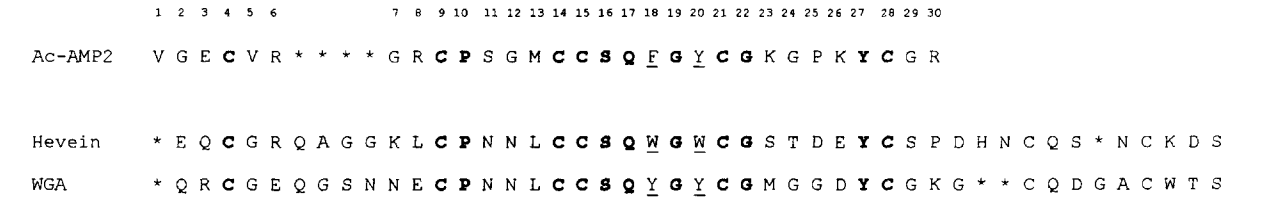

Fig. 1. Alignment of the amino acid sequences of Ac-AMP2, hevein and the A domain of wheat germ agglutinin. Conserved residues are in bold face, residues mutated to comparable aromatic ones are italic and underlined.

using a linear gradient of 100 mM–1 M NaCl in the same buffer. The collected protein was lyophilised.

All NMR samples were prepared from one batch of Ac-AMP2, obtained as described above. Samples were prepared in either H_2O/D_2O (9:1) or in D_2O and contained 50 μM NaN_3 and 20 mM Na_2HPO_4/KH_2PO_4 buffer at pH 6.9. Samples recorded at pH 2.4 were obtained by addition of small aliquots of TFA. pH values are not corrected for D_2O. The Ac-AMP2 concentration varied from 2 to 3 mM. Sequence specific resonance assignments were obtained in the usual way with standard 2D NMR techniques [6] and will be described elsewhere. Unless mentioned explicitly, the NMR spectra were recorded on a Bruker AMX500 equipped with an Eurotherm control unit, a digital lock and a BSMS unit. Chemical shifts are quoted relative to internal DSS. All 1D spectra were recorded with a spectral width of 6024 Hz and 16K data points, with 128 scans each. The temperature was either 288 K or 315 K except for the temperature study where the temperature was varied between 277 K and 320 K. Prior to Fourier transformation, the FID's were zero-filled to 32K data points and apodised with a squared sinebell with a shift of $\pi/2$. Baseline corrections were performed manually using the UXNMR software. Where necessary, presaturation was applied during the relaxation delay to eliminate the water resonance. To determine the chemical shifts of the Ac-AMP2 resonances with and without excess *N,N',N''*-triacetyl chitotriose, 2D HOHAHA [7] spectra were recorded using TPPI [8], collecting 512 FID's of 2K data points with 64 scans each. MLEV-17 mixing [9] bracketed between 2.5 ms trim pulses was applied for 60 ms using a 10 kHz field. The resulting dataset was zero-filled to 2K in t_1 and multiplied with a squared sinebell window shifted by $\pi/2$ in both dimensions.

To detect intermolecular contacts between *N,N',N''*-triacetyl chitotriose and Ac-AMP2, 2D NOESY spectra [10] with mixing times of 200 and 400 ms were recorded at 600 MHz on a Bruker AMX spectrometer on the sample which resulted from the titration at pH 6.9. To minimise the effects of the intense sugar resonances, special precautions were taken to obtain flat baselines using States-TPPI acquisition [11] and adjustment of preacquisition delay and receiver phase to eliminate the use of phase corrections [12].

The titrations were performed by adding small volumes of a concentrated *N,N',N''*-triacetyl chitotriose solution to an Ac-AMP2 solution. For sugar:protein ratios larger than unity, solid *N,N',N''*-triacetyl chitotriose was directly added to the NMR sample.

In order to optimise the accuracy of the K_a determination, we preferred to follow protein resonances as a function of *N,N',N''*-triacetyl chitotriose concentration since this enables accurate chemical shifts determination even at low sugar:protein ratios [13]. The dependence of the Ac-AMP2 chemical shift on *N,N',N''*-triacetyl chitotriose concentration was characterised from the following equation [13,14]:

$$(\nu_{obs} - \nu_{free}) = \frac{[PS]}{P_T}(\nu_{bound} - \nu_{free}) \quad (1)$$

P stands for the protein (Ac-AMP2), *S* for the sugar (*N,N',N''*-triacetyl chitotriose), and *PS* for the complex. ν_{bound} and ν_{free} are the resonance frequencies in the bound and free protein respectively, while ν_{obs} is the frequency actually observed for a given sugar concentration. [*PS*], the actual concentration of the complex in solution obeys the equation:

$$[PS] = \frac{1}{2}\{(P_T + S_T + K_a^{-1}) - \sqrt{[(P_T + S_T + K_a^{-1})^2 - 4P_TS_T]}\} \quad (2)$$

where S_T and P_T represent, respectively, the total sugar and the total protein concentration. K_a and ν_{bound} were obtained from the titration data by fitting equation (1) to the experimental data with a two parameter non-linear regression scheme as implemented in Sigmaplot 4.1 (Jandel Scientific). For all protein resonances used to determine K_a in this way, all fitted values of ν_{bound} approached satisfactorily those observed at high sugar:protein ratio.

3. Results and discussion

The suitability of ^1H NMR to study the protein–carbohydrate interaction was investigated by monitoring the effects caused by a two-fold excess of *N,N',N''*-triacetyl chitotriose on the spectral properties of the Ac-AMP2 resonances at 288 K and pH 6.9. As shown in Figs. 2a and b, this results in a significant line broadening of nearly all proton resonances and induces small to quite considerable shifts in their position with respect to free Ac-AMP2. In order to exclude this to result from non-specific aggregation between *N,N',N''*-triacetyl chitotriose and Ac-AMP2, the same experiment was repeated with sucrose. As evident from Fig. 2c, even a ten-fold excess of sucrose leaves the spectral characteristics of Ac-AMP2 unperturbed and thus provides evidence for the specific nature of its interaction with *N,N',N''*-triacetyl chitotriose.

The combined effect on the chemical shifts and line broadening categorises the interaction as being moderately fast on the NMR time scale [13,14]. This is confirmed by the dependence of the resonance line widths on the temperature as shown in Fig. 3. Lowering the temperature to 277 K further broadens the line width without generating slow exchange conditions. Only the Tyr^{27} *meta* proton resonance coalesces as it vanishes into the baseline. On the other hand the resonances narrow upon temperature increase, eventually yielding fast exchange conditions at 310 K or above. Such conditions make the interaction amenable to the determination of the equilibrium association constant K_a for binding of the sugar to the protein from the change in the resonance positions as a function of sugar concentration [13,14].

Since the most important shifts were observed for the aromatic resonances we titrated an Ac-AMP2 solution in D_2O with *N,N',N''*-triacetyl chitotriose at 315 K and pH 6.9 and followed the induced shifts with 1D NMR spectra (Fig. 4). In the absence of the residual water resonance the well-resolved $C^{\alpha}H$-resonances of Cys^9 and Cys^{21}, which also experience a considerable shift, could be followed as well (Fig. 4). From these $C^{\alpha}H$-resonances and three of the aromatic resonances the average association constant K_a was found to be $1000 \pm 440\ l \cdot Mol^{-1}$.

Since the binding of *N,N',N''*-triacetyl chitotriose to Ac-AMP2 depends on pH we repeated the titration experiment at pH 2.4, a pH lower to the one at which Ac-AMP2 elutes from

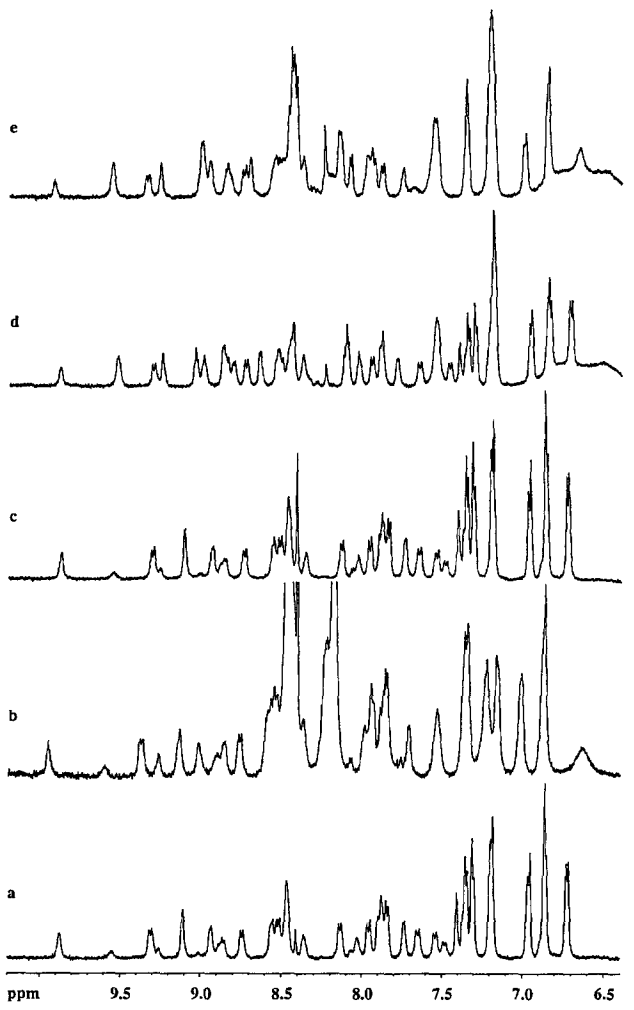

Fig. 2. Amide region of the 1D spectrum of 2.5 mM Ac-AMP2 in H_2O/D_2O (9/1) at pH 6.9 (a) without and (b) with a two-fold excess of *N,N′,N″*-triacetyl chitotriose. (c) With a ten-fold excess of sucrose. (d), (e). Same as (a) and (b) but at pH 2.4.

chitin packed columns. As deduced from Fig. 4b the initial slopes are smaller than at pH 6.9. The curves level off to identical values of $\Delta\delta$ but at a five-fold higher *N,N′,N″*-triacetyl chitotriose concentration. The K_a at pH 2.4 was derived from the same resonances and found to be $200 \pm 60\ l \cdot Mol^{-1}$. Thus changing from neutral to acidic pH results in a five-fold reduction of the affinity of Ac-AMP2 for *N,N′,N″*-triacetyl chitotriose.

To determine which residues are affected by the protein–carbohydrate interaction, the difference in chemical shift between free Ac-AMP2 and the complex has been determined using 2D HOHAHA spectra of Ac-AMP2 recorded without and with excess *N,N′,N″*-triacetyl chitotriose. When the number of induced shifts larger than 0.04 ppm per residue and the maximum shift per residue are mapped onto the sequence of Ac-AMP2 (Fig. 5), it is clear that several distinct regions of the protein are affected. The most pronounced shifts, both in size and number, occur mainly in the C-terminal half of the sequence. The aromatic residues Phe[18], Tyr[20] as well as their closest neighbors Ser[16], Glu[17], Gly[19], Cys[21] are among the most affected ones, followed by Tyr[27] and surrounding residues. At the N-terminal only the Val[1]-Gly[2]-Glu[3] residues are significantly affected. From Cys[4] to Gly[12], no pronounced shifts are apparent, indicating that these residues are not involved or influenced by the interaction. Interestingly, the multiplicity of the Cys[21] $C^{\alpha}H$ resonance changes from a pseudo-triplet (equal $^3J_{\alpha\beta}$ coupling constants) to a double doublet (two different $^3J_{\alpha\beta}$ coupling constants). This indicates a modification in the side chain orientation of Cys[21] and reveals a conformational change in the corresponding disulfide bridge upon *N,N′,N″*-triacetyl chitotriose binding.

Although chemical shift variations indicate residues influenced by the interaction, they do not distinguish residues involved in the binding site from those only affected by a conformational change. This is especially important in view of the presence of three aromatic residues, as slight changes in their side chain orientation can induce important resonance shifts on spatially nearby protons. 2D NOESY spectra recorded at 600 MHz reveal intermolecular NOE's between sugar rings 1 and 2 and Val[1], Phe[18] and Tyr[20] resonances as well as between the Nacetyl methyl group of the first or second sugar ring of

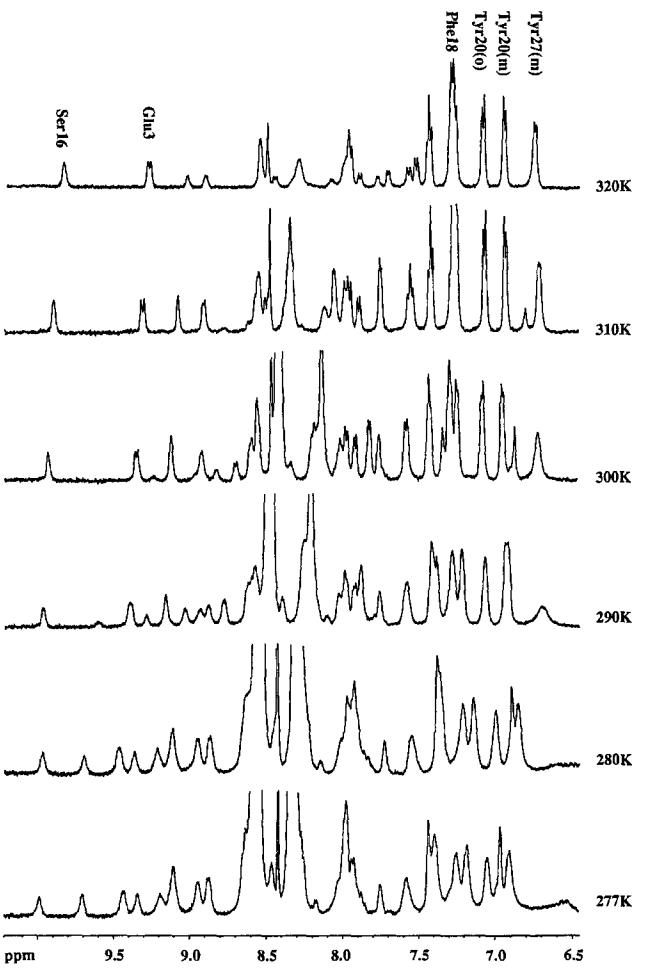

Fig. 3. Temperature study of 2.5 mM Ac-AMP2 in H_2O/D_2O (9/1) with a two-fold excess of *N,N′,N″*-triacetyl chitotriose at pH 6.9. Temperatures vary from 277 K to 320 K. Several NH resonances are attenuated or disappear at higher temperatures as a result of increasing saturation transfer with the presaturated solvent resonance. The sharp resonance at 8.4 p.p.m. corresponds to an impurity, unrelated to the molecules under study.

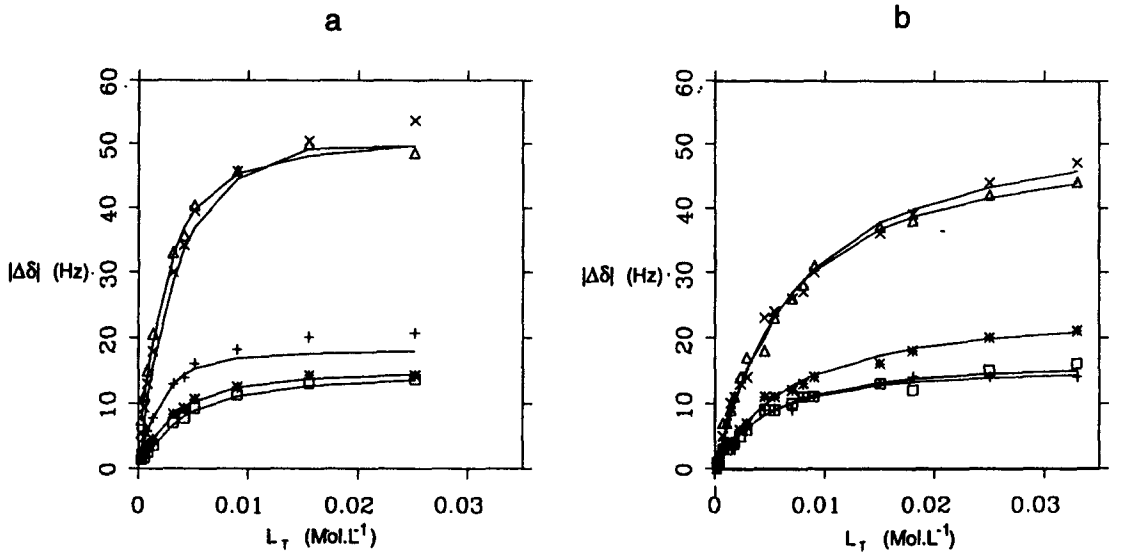

Fig. 4. Absolute values of the chemical shift changes of selected Ac-AMP2 resonances versus *N,N',N''*-triacetyl chitotriose concentration L_T at pH 6.9 (a) and at pH 2.4 (b). The data points indicate experimentally measured values, while the lines correspond to the best fit from which K_a was determined. (*: Tyr^{20} *ortho*; □: Tyr^{20} *meta*; x: Tyr^{27} *meta*; △: Cys^{21} $C^{\alpha}H$; +: Cys^{9} $C^{\alpha}H$).

N,N',N''-triacetyl chitotriose and Phe^{18}, Tyr^{20} and Tyr^{27} were observed (Fig. 6). The four clearly assignable NOE's indicate that the *ortho* and *meta* protons of Tyr^{20} are in close proximity of H3 in ring 1 and one H6 in ring 2 of *N,N',N''*-triacetyl chitotriose. Although severe overlap precludes the clear-cut assignment of the numerous other NOE's, they all indicate that both non-reducing rings are directly involved in binding with Ac-AMP2, and that the binding site on Ac-AMP2 consists of Val^{1}, Phe^{18}, Tyr^{20} and Tyr^{27}.

The most likely source for the pH dependence of the binding affinity is Glu^{3}. Although no intermolecular NOE's between this residue and *N,N',N''*-triacetyl chitotriose have been found, its carboxylic group becomes protonated upon decreasing the pH from 6.9 to 2.4 (as measured from characteristic chemical shift changes of its γCH_2 protons). Moreover NOE's between Glu^{3} and Tyr^{20} are present in native Ac-AMP2, indicating that Glu^{3} is close to the interaction site.

Ac-AMP2 shares sequence homology with parts of the sequence of hevein, and the hevein like domains in several plant lectins [1]. Both Ac-AMP2 and hevein are characterised by one binding site for chitine. For the interaction between hevein and

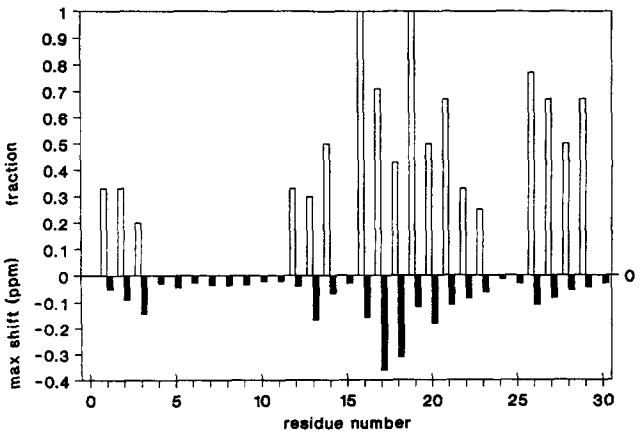

Fig. 5. Changes in the chemical shifts of Ac-AMP2 in the presence of excess *N,N',N''*-triacetyl chitotriose. Open bars represent the number of resolved proton resonances that experience a shift larger than 0.04 ppm upon complexation. The number was normalised with respect to the total number of resolved proton resonances for each residue to yield a fraction. Closed bars represent the absolute value of the largest shift (maxshift) that occurs within each residue.

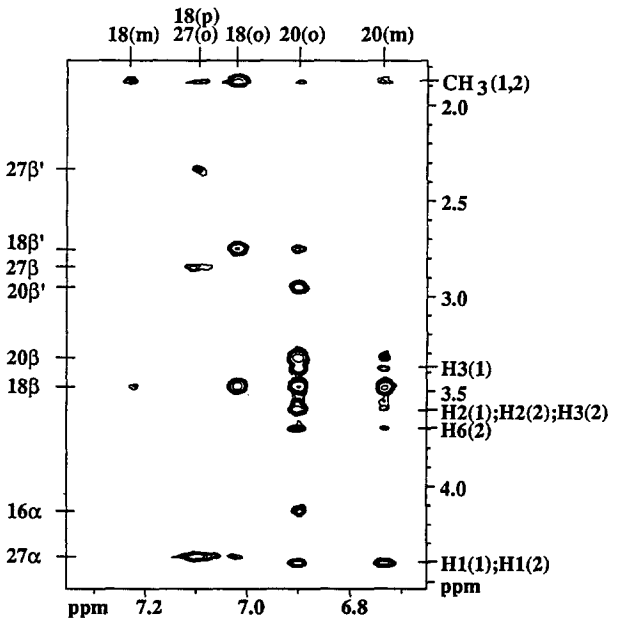

Fig. 6. Selected area of the 2D NOESY at 600 MHz (400 ms mixing time) at 310 K and pH 6.9 illustrating the intermolecular NOE contacts between Ac-AMP2 and excess *N,N',N''*-triacetyl chitotriose. The location of sugar and protein resonances are indicated on the right resp. left hand side of the spectrum. Numbers between parentheses indicate the corresponding sugar ring. Only the intermolecular contacts of the ortho respectively meta protons of Tyr^{20} with H3 in ring 1 and H6 in ring 2 can be assigned unambiguously.

N,N′,N″-triacetyl chitotriose, nearly identical observations are made as for the present case [5]. In hevein the two tryptophane residues at positions 21 and 23 and Tyr^{30} as well as Ser^{19} are implicated in *N,N′,N″*-triacetyl chitotriose binding. The K_a found for the *N,N′,N″*-triacetyl chitotriose Ac-AMP2 interaction is of the same order of magnitude as for hevein [5], albeit a factor 5 smaller. This may be caused by the differences in aromatic residue type (Fig. 1). When the Ac-AMP2 residues whose chemical shifts are most strongly affected by the interaction with *N,N′,N″*-triacetyl chitotriose (Fig. 5) are mapped onto the sequence alignment in Fig. 1, a clear pattern emerges. Indeed, all residues with significant shifts in Ac-AMP2 are located in a common Cys-Cys-Ser-Gln-Aro-Gly-Aro-Cys-Gly-$(Xxx)_4$-Tyr-Cys sequence (with Aro coding for either Tyr, Trp of Phe). In hevein and WGA, these residues are found in close spatial proximity and constitute the binding site for *N,N′,N″*-triacetyl chitotriose [4,6,15]. From our titration data and the observed intermolecular NOEs between the aromatic moieties of Phe^{18}, Tyr^{20} and Tyr^{27} with the *N,N′-N″*-triacetyl chitotriose resonances we therefore conclude that Ac-AMP2 has a similar sugar binding site. A more quantitative investigation of the interaction presented here is underway on the basis of a complete determination of the structure of Ac-AMP2 by NMR.

Acknowledgements: We thank Dr. J. Jiménez-Barbero for providing us data prior to publication and useful suggestions. P.V. is a senior research assistant and D.M. a research associate at the National Fund for Scientific Research of Belgium (NFWO). J.P. thanks the 'Vlaams Instituut voor de bevordering van het Wetenchappelijk-Technologisch onderzoek in de industrie' (IWT) for financial support. This work is supported by the Belgian 'Nationale Loterij' (Grant 9.0006.93), the Belgian 'Fonds voor Kollektief Fundamenteel Onderzoek' (Grant 2.0094.94), the VLAB project for biotechnology and by the Research Council of the Vrije Universiteit Brussel. The skillful help of A. Groenewegen (Unilever Research Laboratorium) is acknowledged.

References

[1] Broekaert, W.F., Mariën W., Terras, F.R.G., De Bolle, M.F.C., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S.B., Vanderleyden, J. and Cammue, B.P.A. (1992) Biochemistry 31, 4308–4314.
[2] Broekaert, W.F., Lee, H.I., Kush, A., Chua, C.H. and Raikel, N.V. (1990) Proc. Natl. Acad. Sci. USA 87, 7633–7637.
[3] Andersen, N.H., Cao, B., Rodriguez-Romereo, A. and Arreguin, B. (1993) Biochemistry, 32, 1407–1423.
[4] Wright, C.S. (1987) J. Mol. Biol. 194, 501–529.
[5] Asensio, J.L., Cañada, F.J., Bruix, M., Rodriguez-Romero, A., Jimenez-Barbero, J. (1995) Eur. J. Biochem. (in press).
[6] Wüthrich, K. (1986) NMR of proteins and nucleic acids, Wiley, New York.
[7] Braunschweiler, L. and Ernst, R.R. (1983) J. Magn. Reson. 53, 521–528.
[8] Marion, D. and Wüthrich, K. (1983) Biochem. Biophys. Res. Commun. 113. 967–974.
[9] Bax, A. and Davis, D.G. (1985) J. Magn. Reson. 65, 355–360.
[10] Macura, S., Huang, Y., Suter, D. and Ernst, R.R. (1981) J. Magn. Reson. 43, 259–281.
[11] Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) J. Magn. Reson. 85, 393–399.
[12] Zhu, G., Torchia, D.A. and Bax, A. (1993) J. Magn. Reson. A 105, 219–222.
[13] Feeney, J., Batchelor, J.G., Albrand, J.P. and Roberts, G.C.K. (1979) J. Magn. Reson. 33, 519–529.
[14] Lian L.Y. and Roberts, G.C.K. (1993) in NMR of Macromolecules, pp. 153–182, Oxford University Press, Oxford.
[15] Wright, C.S. (1990) J. Mol. Biol. 215, 635–651.