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Study of amide proton exchange in ^{15}N-enriched cryptogein using pH-dependent off-resonance ROESY-HSQC experiments

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Abstract A new pH-dependent off-resonance ROESY-HSQC experiment has been used to characterize the degree of protection of the amide protons of cryptogein, a protein of the elicitin family, against solvent exchange. The study of the pH dependence of solvent-shielded amide protons in this protein reveals that the helices have different levels of stability. Two of the five helices exhibit strong protection of amide hydrogens against exchange with the solvent. By contrast, greater flexibility is observed in the other three helices, particularly in the C-terminal helix. These results provide information on the dynamic features of the protein and are consistent with the RMSD for the backbone atoms of residues involved in helical structures. In addition, the question of the flexibility in a hydrophobic cavity made of conserved residues, which represent a plausible binding site, is addressed by this method.

Key words Off-resonance ROESY · Water-protein exchange · Cryptogein · Elicitin

Abbreviations *1D* One dimensional · *2D* Two dimensional · *BURP* Band-selective uniform response pure-phase · *DANTE* Delays alternating with nutations for tailored excitation · *HSQC* Heteronuclear single quantum correlation · *RMSD* Root mean square deviation · *NOE* Nuclear Overhauser effect · *ROE* Rotating frame Overhauser effect · *ROESY* Rotating frame Overhauser enhancement spectroscopy · *TPPI* Time proportional phase increment · *WATERGATE* Water suppression by gradient tailored excitation

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Introduction

Important information about protein structure and dynamics in proteins can be obtained from backbone amide hydrogen exchange rates (Rothemund et al. 1995; Englander et al. 1996). Generally, hydrogen exchange in folded proteins is considered to depend on local structural fluctuations in the native state or a cooperative global unfolding that expose buried and hydrogen-bonded amide protons, allowing for their exchange with solvent (Kim et al. 1993). Recently, a wealth of hydrogen exchange information has become available with the introduction of multidimensional, multinuclear NMR spectroscopy, which has permitted the measurements of exchange rate for individual amides in proteins.

The exchange peaks can be observed using two dimensional exchange spectroscopy (Macura et al. 1982) or water exchange filter experiments (Mori et al. 1994; Mori et al. 1996). Because of chemical shift overlap between the solvent and resonance lines from the macromolecule, the intramolecular NOEs and the exchange processes are distinguished with difficulty. Several methods have been used in order to distinguish the intramolecular NOEs and the solvent-proton exchange peaks. A comparison of NOESY and ROESY spectra was used to differentiate the two types of magnetization exchange (Otting and Wüthrich 1989). The cross relaxation effect can be suppressed by purging all signals from protons that are attached to ^{13}C in uniformly ^{15}N ^{13}C enriched proteins (Grzesiek and Bax 1993). A 1D NMR difference experiment (HYDRA) separates solvent-protein NOEs and intramolecular NOEs from the different diffusion properties of water and biomolecules (Moonen et al. 1992; Wider et al. 1996). We recently introduced the water-selective off-resonance ROESY experiment (Birlirakis et al. 1996) to study solvent-exchangeable amide protons. It is based on the extinction of dipolar relaxation along a tilted spin-lock axis. This experiment combines the advantage of efficient cancellation of intramolecular NOE and spin diffusion of exchanging peaks and clear definition of the mixing time in a pulse se-

quence that avoids radiation damping. A somewhat related approach was recently developed, where the NOE and ROE contributions are cancelled by use of a CLEAN mixing time (Hwang et al. 1997).

In this communication, we introduce a new pH-dependent 2D off-resonance ROESY-HSQC experiment devised to monitor exchange with the solvent in ^{15}N-labelled proteins and use this scheme to evaluate the cryptogein structure as solved by NMR and to monitor the flexibility of this protein. Cryptogein belongs to a family of proteins called elicitins which are secreted by *Phytophthora* fungi (Tercé-Laforgue et al. 1992). When applied to tobacco plants, elicitins exhibit a necrotic activity and induce protection against a subsequent inoculation of the phytopathogen *Phytophthora nicotianae* (Pernollet et al. 1993; O'Donohue et al. 1995). The solution structure of cryptogein was recently solved in our laboratory (Fefeu et al. 1996). Its structure reveals a new fold, with five helices and a double-stranded antiparallel β-sheet facing an Ω-loop. One edge of the β-sheet and the adjacent face of the Ω-loop form a hydrophobic cavity, made of conserved residues, which represents a plausible binding site. The five helices involve residues 5–18, 22–30, 44–51, 54–66 and 84–98 (called α1, α2, α3, α4 and α5 respectively). The Ω-loop made up of residues 33 to 42 links helices α2 and α3, the double-stranded antiparallel β-sheet consists of residues 73–75 and 80–82. The question of possible motions affecting the interaction between the β-sheet and the Ω-loop, favouring the anchoring of the protein to a receptor or a ligand, is of great interest.

Materials and methods

Samples were prepared by dissolving lyophilized ^{15}N labelled cryptogein in 0.3 mL of H_2O/D_2O (9:1) up to a final concentration of 1 mM. The pHs were adjusted by addition (small amounts) of HCl or NaOH.

All spectra were recorded at 318 K on a AMX600 Bruker spectrometer and data were processed using the GIFA software (Pons et al. 1996). The off-resonance ROESY-HSQC spectra were recorded with a mixing time of 114 ms, with 128 complex points in the indirect nitrogen dimension and 1024 real points in the acquisition dimension.

Results and discussion

Figure 1 shows the new 2D off-resonance ROESY-HSQC sequence used in the present study. It is based on the general scheme developed in our laboratory (Birlirakis et al. 1996). It consists of a first period designed to select only those protons that are in fast exchange with the solvent, chained with a 2D heteronuclear experiment that facilitates their attribution. In the first period, the water magnetization is excited by the selective 180° pulse and flipped to

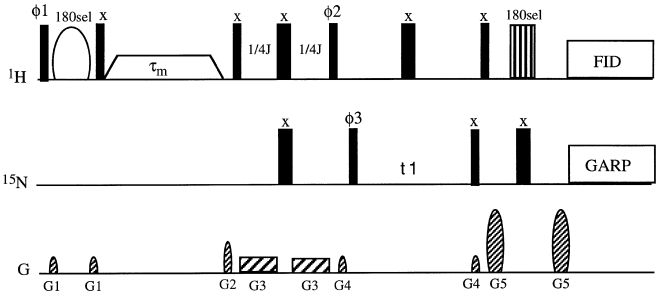

Fig. 1 2D off-resonance ROESY-HSQC sequence. Wide and narrow rectangles represent 180° and 90° hard pulses, respectively. The mixing time τm is 114 ms. The first 180° pulse (RE-BURP shaped DANTE sandwich) is selective at the water frequency (Roumestand et al. 1995) and is 12.7 ms. The solvent is suppressed using a WATERGATE pulse sequence. Quadrature detection along f_1 is obtained using the States TPPI scheme. G1, G2, G3, G4 and G5 are (0.9 ms · 1.92 G/cm), (1.1 ms · 4.32 G/cm), (2.5 ms · 1.44 G/cm), (0.7 ms · 2.40 G/cm) and (0.9 ms · 11.04 G/cm) respectively. Pulsed field gradients have a sinusoidal shape, except for G3 which are rectangular. The coherences are selected by phase cycling: ϕ1=2x, 4(–x), 2x; ϕ2=y; ϕ3=2x, 2(–x), 2x, 2(–x); Receptor=2x, 2(–x), 2x, 2(–x)

the longitudinal axis at the start of the mixing time τ_m. Radiation damping is avoided by using gradients before and after the selective 180° pulse. An off-resonance ROESY mixing time follows. To avoid a reduction of signal-to-noise ratio due to projection effects and distortion of antiphase cross peaks from the use of a square spin-lock pulse applied off-resonance, an off-resonance trapezoidal pulse is used during the mixing time. Through this spin-lock pulse, the longitudinal magnetization after the second 90° hard pulse is rotated adiabatically and aligned along the effective spin-lock axis at θ=35.5° to the z-axis, before being returned to the z axis. The off-resonance ROESY is followed by an HSQC-type experiment. The effective dipolar cross relaxation rate at θ=35.5° is equal to zero in the case of isotropic rotational motion of macromolecules, resulting in the observation of chemical exchange without interference from cross relaxation effects. Some very weak cross peaks could arise from the presence of significantly shorter local correlation times (Desvaux et al. 1994, 1995a, b). The observation of labile protons is only feasible when the inverse of the exchange rate with the solvent is on the order of the mixing time scale.

To gain more information on the exchange rates, the amide exchange was monitored as a function of pH. This is only possible because there is no change in chemical shifts in the 2D HSQC spectra in the 5.4–8.1 pH range, indicating no structural variation of cryptogein in that pH range. The amide proton exchange rates do, however, strongly depend on pH, higher pH values will usually markedly accelerate the exchange with the solvent because of base catalysis. In peptides, the amide proton exchange rate increases by more than two orders of magnitude when the pH increases from 5.4 to 8.1 (Wüthrich 1986). As a result, some protons will disappear at higher pH because the exchange of these amide protons with the solvent becomes fast compared to the inverse of the $^1J_{HN\text{-}15N}$ coupling con-

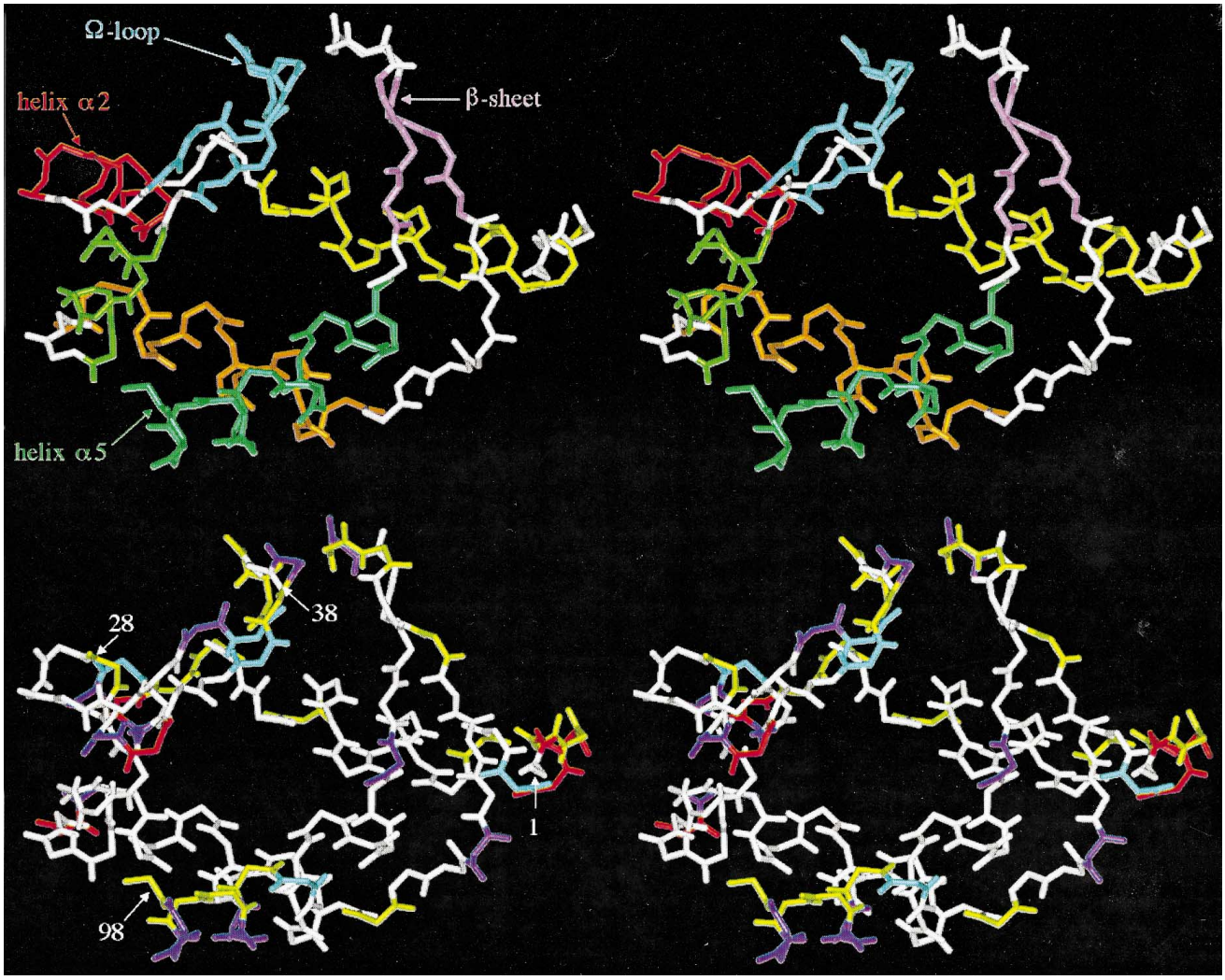

Fig. 2 Stereo view of β cryptogein. *Top* Plot of the backbone atoms Helices α1 to α5, the Ω-loop and the β-sheet are colored in yellow, red, pale green, orange, dark green, pink and pale blue, respectively. *Bottom* Plot of the backbone and H^N atoms. The residues that give rise to strong corss peaks at pH 5.4, weak cross peaks at pH 5.4, cross peaks at pH 6.4 and 8.1 are labelled in red, purple, blue and yellow, respectively. Residues 1, 28, 38 and 98 are labelled

stant value. Others will only be seen in the off-resonance ROESY-HSQC experiments run at higher pH values. This experiment thus allows an easy evaluation of the exchange over a broad rate range.

In addition, analysis of the build-up of the cross-peaks in the off-resonance ROESY-HSQC demonstrate that the cross peak intensities in the 114 ms mixing time spectra qualitatively reflect the exchange rate (Mori et al. 1996). The weakness of the exchange peaks reflects a more limited access to the solvent than for residues giving rise to peaks of stronger intensity.

The accessibility to the solvent of the amide protons of cryptogein was studied at 318 K. This high temperature was chosen in order to make the data directly comparable with the available cryptogein assignment and structure. Residues observed in the off-resonance ROESY-HSQC spectra at three pH values (5.4, 6.4 and 8.1) are listed in Table 1 and shown in Fig. 2, along with an evaluation of their intensities in the low pH spectrum. The amide protons protected from the solvent are expected to be either buried or involved in stable hydrogen bonds. Few cross-peaks in the off-resonance ROESY-HSQC experiment [5, 6, 22, 23, 34, 39 …] disappear at higher pH. Others are found only at higher pH. As expected exchangeable amide protons are found at the N-terminus of the protein (1–4, 1 exchanges are too fast to be detected at any pH) and in turn regions [20–21, 77–79]. One should note, however, that residues 3 and 4 belonging to the N-terminal part of the protein are observed only in the experiment at pH 8.1. This relative protection from the solvent for these two residues is probably due to the presence of a disulphide bond Cys3-Cys71 which locks the N-terminal region close to the β-sheet.

In the alpha helices, the non hydrogen-bonded N-terminal residues [5, 6, 7, 22, 23, 44, 45, 54, 55] are observed in the 2D off-resonance ROESY-HSQC spectra. Strong cross peaks found at pH 5.4 for residues 5, 6, 22, 44 and 54 reveal that these residues have the highest exchange rates. Weak cross peaks from the hydrogen-bonded amide

Table 1 Residues observed in the off-resonance ROESY-HSQC experiments at 318 K, at three different pH values (5.4, 6.4 and 8.1). The secondary structures are indicated

Spectrum pH 5.4	Spectrum pH 6.4	Spectrum pH 8.1
2		
		3
		4
5 (α1)		
6 (α1)	6 (α1)	
	7 (α1)	7 (α1)
		9 (α1)
		17 (α1)
		20 (turn)
		21 (turn)
22 (α2)	22 (α2)	
23 (α2)	23 (α2)	
	25 (α2)	
26 (α2)	26 (α2)	
		28 (α2)
34 (Ω-loop)	34 (Ω-loop)	
	35 (Ω-loop)	35 (Ω-loop)
		36 (Ω-loop)
		37 (Ω-loop)
39 (Ω-loop)	39 (Ω-loop)	
		40 (Ω-loop)
	41 (Ω-loop)	41 (Ω-loop)
44 (α3)	44 (α3)	
45 (α3)	45 (α3)	
54 (α4)		
55 (α4)	55 (α4)	
		67
70	70	
		74 (β-sheet)
77 (turn)	77 (turn)	
	78 (turn)	78 (turn)
		79 (turn)
83	83	
	90 (α5)	90 (α5)
		91 (α5)
92 (α5)	92 (α5)	
93 (α5)	93 (α5)	
		94 (α5)
		95 (α5)
96 (α5)	96 (α5)	
97 (α5)	97 (α5)	
		98 (α5)

protons of residues 92, 93, 96, 97 and 26 involved in helical regions are observed in the spectrum with a mixing time of 114 ms at pH 5.4. Additional residues involved in these two helices are found in experiments run at higher pHs (residues 90 and 25 at pH 6.4, and 91, 94, 95, 98, 28 at pH 8.1). Considerations of dynamic aspects of protein conformation have shown that access of water molecules can be made through a local transient unfolding (Englander et al. 1984, 1988, 1996). In order for these hydrogen-bonded amide hydrogens to exchange with water, the hydrogen bonds must be temporarily broken to expose the amide hydrogens to water. A high number of exchangable amide protons involved in the C-terminal end of helices α2 and α5 therefore implies a relatively high flexibility of these segments. Local unfolding of helix α1, but to a smaller extent than of helices α2 and α5, is reflected in the presence of exchangeable amide protons of residues 9 and 17 at pH 8.1. These results on the comparative stability of the helices in cryptogein show that the far higher RMSD values of helix α5 (RMSD for the backbone atoms of 18 structures from the averaged structure, calculated by averaging the coordinates of the atoms in the 18 cryptogein structures, are 0.34 ± 0.05 Å, 0.30 ± 0.06 Å, 0.24 ± 0.04 Å, 0.25 ± 0.04 Å and 0.45 ± 0.13 Å for helices α1, α2, α3, α4 and α5 respectively) is caused by flexibility of this last helix and not by a lack of NOE constraints. The amide protons involved in helices α3 and α4 are not observed in any off-resonance ROESY-HSQC experiments with the exception of residues located at the N-terminal ends. This indicates the presence of stable intramolecular hydrogen bonds, good solvent shielding and a high stability of these helices. This confirmed by the existence of a larger number of NOE constraints characteristic of helical structure found in these regions. In addition, it is worth noting that residues 26, 92, 93, 96 and 97 for which the weak cross peaks were observed at pH 5.4 are all positioned at the external side of helices α2 and α5 (with respect to the rest of the protein), whereas residues 25, 28 of helix α2 and 90, 91, 94, 95, 98 of helix α5 are located on the internal side and are less solvent exposed. These considerations could allow the orientation the helices around their axis during the reconstruction processes.

All the amide protons of the residues involved in the β-sheet and the Ω-loop are observed in at least one off-resonance ROESY-HSQC experiment except for the amide protons of residues 73, 75, 80, 81 and 82 participating in the β-sheet and the amide proton of residue 38 belonging to the Ω-loop. Residue pairs 73 and 82, and 75 and 80 are hydrogen-bonded and are protected from water even at pH 8.1, thus demonstrating the stability of the β-sheet. Residue 81 is at the bottom of a depression and is poorly accessible to the solvent. Except for residue 38, all the amide protons of residues 31–41 are accessible to the solvent, as expected for a loop. The amide proton of residue 38, protected from the solvent, was found to potentially form a hydrogen bond with the carbonyl oxygen of residue 34. This flexibility has to be paralleled with the observation of long distance NOEs between residues of the β-sheet and the Ω-loop and with the existence of a hydrogen bond between the hydroxyl proton of Ser 78 with the carbonyl atom of residue 38, as confirmed by the observation of the slow exchange of this proton with the solvent (Fefeu et al. 1996), which locks the β-sheet close to the Ω-loop. In summary, it follows from the present study that the interface between the Ω-loop and the β-sheet remains solvent accessible because of local fluctuations of the Ω-loop. These fluctuations probably allow the opening of the cavity and the give access to a potential ligand.

In summary, the pH dependence study of amide hydrogen exchange with the solvent, made possible by the off-resonance ROESY-HSQC experiments, allows the comparison of the stability of the secondary structures of cryptogein. The exposure to water of amide protons involved in α-helices regions is taken as a tool to estimate the stability of helices in cryptogein. The observation of a fast exchange with the solvent of several amide protons involved in helical structure indicates local fluctuations of these re-

gions. The evaluation of the exchange rates is also consistent with the known tertiary structure of cryptogein, giving information about the orientation of the helices around their axes with respect to the core of the protein. The flexibility of the Ω-loop substantiates the possibility that the hydrophobic cavity between the double-stranded antiparallel β-sheet and the Ω-loop represents a plausible binding site in the elicitin family.

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