Transferred nuclear Overhauser effect spectroscopy study of a peptide from the PapG pilus subunit bound by the *Escherichia coli* PapD chaperone

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Abstract Interaction of the *Escherichia coli* PapD chaperone with the synthetic peptide PapG308-314 (Thr–Met–Val–Leu–Ser–Phe–Pro), corresponding to the seven C-terminal residues of the PapG pilus subunit, was studied by transferred nuclear Overhauser effect (TRNOE) spectroscopy. The observation of cross-peaks corresponding to either intraresidue or sequential $C^{\alpha}H/NH$ and $C^{\beta}H/NH$ TRNOEs and the absence of sequential NH_i/NH_{i+1} TRNOEs indicate that the peptide binds to PapD in an extended conformation. In addition, line-broadening effects gave information of the peptide's mode of interaction with PapD. These observations were in excellent agreement with a recent crystal structure of a PapG peptide complexed with PapD.

Key words: P pili; PapD chaperone; Protein–peptide interaction; NMR; Transferred NOE

1. Introduction

Gram-negative bacteria have evolved filamentous polymeric protein structures called pili to mediate attachment to eukaryotic cells as an early event in bacterial infections [1]. A well-characterized example is uropathogenic *Escherichia coli* which express P pili that contain the adhesin PapG at the pilus tip. papG mediates binding to the disaccharide α-D-galactopyranosyl-(1–4)-β-D-galactopyranose [α-D-Gal-(1–4)-β-D-Gal] present in the globoseries of glycolipids on epithelial cells lining the urinary tract [2,3]. P pili are heteropolymeric protein structures consisting of a large number of subunits of six different types which make up a thick pilus rod joined to a thinner adhesive tip fibrillum [4,5]. P pili are assembled by the periplasmic chaperone PapD which is not itself incorporated into the pilus. PapD binds to each of the pilus subunit types as they are translocated across the cytoplasmic membrane and escorts them in assembly-competent, native-like conformations through the periplasm to outer membrane assembly sites [1]. X-ray crystallography revealed the PapD to consist of two immunoglobulin-like domains oriented in a boomerang shape such that a cleft is formed between the domains [6].

The PapD chaperone has been shown to bind to the conserved C-terminal part of the different pilus subunits [7,8]. Recently, synthetic peptides which were derived from the pilus subunit C-termini and ranged in size from seven to 19-mers were shown to be bound by PapD and to inhibit complex formation between PapD and the adhesin papG [8,9]. The crystal structure of PapD complexed with the most potent peptide, which corresponds to the 19 C-terminal residues of PapG (PapG296-314), was also determined and refined to 3.0 Å resolution [8]. The peptide bound in an extended conformation with its C-terminal residue (Pro^{314}) anchored within the interdomain cleft of PapD and forming a parallel β-strand interaction with strand G1 in the N-terminal domain of PapD. Within the crystal, the PapD-peptide β-sheet was extended even further as a result of non-crystallographic 2-fold symmetry that placed a second PapD-peptide complex adjacent to the first so that the two bound peptide chains interacted as antiparallel β-strands. This dimerization was proposed to be a consequence of crystal packing.

It can not be ruled out that the structure of the complex between papD and PapG296-314 was influenced of by the dimerization in the crystal. Therefore, we have now investigated the conformation of a peptide bound to PapD in solution, and the interactions in the complex, using TRNOE experiments [10–12]. The peptide PapG308-314 (Thr–Met–Val–Leu–Ser–Phe–Pro) was chosen for these studies since it appears to represent the minimal peptide bound by PapD [8,9]. In addition, the aggregation state of PapD in solution was analyzed with asymmetrical flow field-flow fractionation in the presence and absence of peptide.

2. Materials and methods

PapG308-314 was synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase strategy and purified as described in [9]. The asymmetrical flow field-flow fractionation experiment was carried out using a 0.1 mg/ml solution of PapD in PBS (pH = 7.4) with and without the peptides PapG296-314 and PapG308-314 in a 1:1 molar ratio. An experimental set-up as previously described was used [13].

Three identical peptide solutions were prepared for NMR spectroscopy by dissolving PapG308-314 in 50 mM phosphate buffer, pH 6.0,

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Abbreviations: Pap, pyelonephritis-associated pilus; TRNOE, transferred nuclear Overhauser effect; PBS, phosphate-buffered saline; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DIPSI, decoupling in the presence of scalar interactions

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containing 10% D_2O and 0.02% NaN_3 to a concentration of 0.5 mM. The low concentration was used due to the low solubility of the peptide. A stock solution of PapD in PBS (2 mg/ml), produced and purified as described in [6], was concentrated to 0.2 mM by centrifugation in an Ultrafree micro concentrator (cut-off = 5 kDa, Millipore, Bedford, MA) prior to dilution into one of the peptide solutions. The PapD concentration was 0.02 mM giving a peptide/protein ratio of 25:1. A control sample was prepared by dissolving lyophilized Calbindin D_{28k}, produced and purified as described in [14], into another of the peptide solutions to give a peptide/protein ratio of 10:1.

NMR spectra were obtained at 599.89 MHz on a Varian 600 Unity plus spectrometer. Phase sensitive NOESY [15,16], ROESY [17,18] and TOCSY [19] spectra were acquired at 27°C using the water resonance at 4.75 ppm as internal shift reference. Presaturation was used to attenuate the water signal except in the NOESY experiments where pulse field gradients were used to suppress the water signal. A WATERGATE-NOESY pulse sequence [20] was modified according to [21]. The NOESY spectra were acquired with mixing times of 20, 200 and 500 ms and the ROESY spectra were acquired with a continuous wave spin-lock mixing time of 200 ms. The TOCSY experiments were performed using the modification suggested by Rance [22] and the DIPSI-2 mixing sequence [23] with a spin-lock period of 90 ms. All spectra were recorded using the hypercomplex method [24] with 400 t_1 increments and 2048 complex data points in t_2. After zero filling the final size of the data matrixes were 1024·2048. Data were processed on a Sun Sparc workstation using the FELIX software (Biosym Technologies, San Diego, CA). One-dimensional spectra were acquired with 512 scans, 16 k complex data points and presaturation.

3. Results and discussion

Assignment of all the proton resonances in the free form of the PapG308-314 peptide was obtained using ROESY and TOCSY spectra (Table 1). Two forms of the peptide were observed originating from the *cis/trans* isomerization of the amide bond between Phe^{313} and Pro^{314}. The major form had a *trans* peptide bond and integration of the cross-peaks for Pro^{314} in the TOCSY spectrum showed a *cis*-content of 40%. *Cis/trans* isomerization in aqueous solution has also been observed for the longer PapG296-314 peptide with a similar *cis* content [25]. Apart from medium to weak intraresidue NOEs strong-intensity sequential $C^{\alpha}H_i/NH_{i+1}$ NOE connectivities were observed between adjacent residues along the whole peptide chain in the 200 ms ROESY spectrum (data not shown). No sequential NH_i/NH_{i+1} or medium- or long-range NOEs were observed for the free peptide. Altogether, these observations indicate that the backbone dihedral angles of the peptide are averaged but predominantly in the β (extended chain) region of φ, ψ space [26]. The absence of a preferred conformation is also indicated by the small deviations of the $C^{\alpha}H$-proton chemical shifts of PapG308-314 as compared to random-coil shifts of short peptides [27].

The NOESY spectrum of unbound PapG308-314 contains no cross-peaks since the correlation time of the peptide is such that $\omega\tau_c \approx 1$ resulting in NOEs close to zero (Fig. 1) [28]. On addition of PapD (MW ≈ 25 kDa and thus $\omega\tau_c \gg 1$) in substoichiometric amounts strong cross-peaks appear in the NOESY spectrum (Fig. 1). The much larger rate at which the NOEs develop when the ligand is in its bound state allows detection of the bound state NOEs under appropriate kinetic conditions. In a fast exchange situation, these NOEs are transferred into the free ligand population where they persist for a time governed by the relaxation time of the free ligand protons. Hence, observation of the interproton NOEs of the free ligand reveals the distance relationships of the bound ligand.

TRNOEs may also originate from non-specific binding of a ligand to a protein or as a result of an increase in the viscosity of the solution upon addition of the protein [12]. As a control a 200 ms NOESY spectrum was therefore acquired for a solution of PapG308-314 and the protein Calbindin D_{28k}, which has a similar molecular weight as PapD, using a peptide/protein ratio of 10:1. The spectrum did not show any cross-peaks indicating that the interaction between PapG308-314 and PapD is specific (data not shown).

The nature of the TRNOE experiment requires that the rate of exchange of the ligand between the free and bound states must be faster than the proton relaxation rate [12]. In the present study fast exchange has been obtained by the choice of peptide. If the rate of peptide association with PapD is diffusion limited (10^8 M^{-1} s^{-1}) and the dissociation constant $\sim 10^{-6}$ M, as measured for a modified PapG307-314 peptide (Soto G., personal communication), the off-rate is calculated to be about 100 s^{-1}. Since PapG308-314 is a weaker inhibitor than the 8-mer PapG307-314, the off-rate of PapG308-314 should be even higher, thus corresponding to the fast exchange regime.

No intermolecular TRNOEs between the ligand and the

Table 1
Chemical shifts for PapG308-314 in aqueous phosphate buffer, pH 6.0, at 27°C

Residue	Chemical shifts (ppm)				
	HN	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	Other
Thr^{308}	[a]	4.09	3.81	1.26	
Met^{309}	[a]	4.53	1.98, 2.04 (2.01)[b]	2.53[b]	2.08 ($C^{\varepsilon}H_3$)
Val^{310}	8.32	4.04 (4.05)	1.97	0.85, 0.90	
	8.33	4.07	2.00	0.90[b]	
Leu^{311}	8.32 (8.34)	4.33 (4.34)	1.43 (1.44), 1.56	1.56	0.83, 0.90 ($C^{\delta}H_3$)
	8.37 (8.38)	4.40	1.54, 1.61	1.61	0.86, 0.92 ($C^{\delta}H_3$)
Ser^{312}	7.99 (8.00)[c]	4.36 (4.37)[c]	3.66[b]		
	8.18	4.43	3.76[b]		
Phe^{313}	8.19	4.88	2.85, 3.20 (2.86, 3.21)		7.28, 7.33 (H-arom)
	8.01	4.65	2.93[b]		7.23, 7.35, 7.31 (H-arom)
Pro^{314}		4.24(4.25)	1.91, 2.20	1.97[b]	3.66 (3.67), 3.71 ($C^{\delta}H$)
		3.66	1.80, 1.85	1.67[b]	3.30, 3.47 ($C^{\delta}H$)

The chemical shifts for the *cis* form about the Phe–Pro amide bond are given on the second row. Numbers in parenthesis indicate the chemical shifts when PapD is present.
[a]Not observed due to fast amide proton exchange.
[b]Degeneracy has been assumed.
[c]Broad resonances, chemical shift determination uncertain.

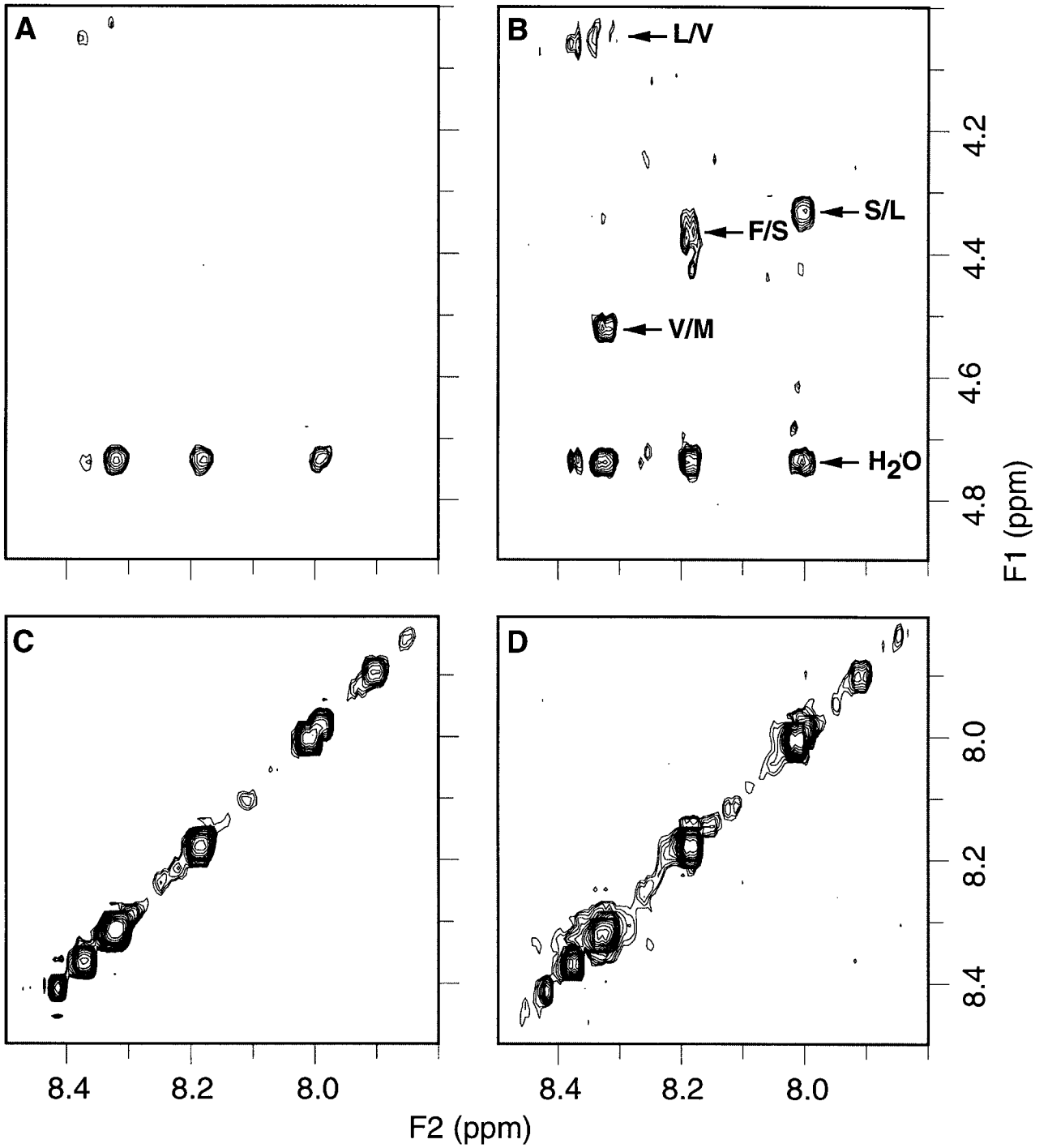

Fig. 1. A portion of the 200 ms NOESY spectrum of the PapG308-314 peptide alone (A,C) and in the presence of 20 μM PapD (B,D). The NH/$C^{\alpha}H$ (A,B) and NH/NH (C,D) regions are shown. Mainly TRNOEs corresponding to sequential $C^{\alpha}H_i/NH_{i+1}$ were observed (marked with letters of the corresponding amino acid in (B)) indicating that the peptide is bound by PapD in an extended conformation. All spectra are plotted at equal threshold. Pulse field gradients were used for water suppression and therefore exchange peaks to water are visible.

protein were observed in the spectra since the PapD concentration used in the experiment (0.02 mM) was too low to enable such observations. However, the low PapD concentration had the advantage that no cross-peaks from the protein that interfered with the resonances of the peptide were obtained. This simplified the interpretation of the peptide TRNOEs.

Both the *cis* and *trans* forms of the PapG308-314 peptide were observed to have TRNOEs indicating the ability of PapD to bind both forms. In the *trans* form strong sequential $C^{\alpha}H_i/NH_{i+1}$ TRNOEs were observed between Met^{309} through Phe^{313} and one $C^{\beta}H_i/NH_{i+1}$ TRNOE was observed between Ser^{312} and Phe^{313}. Weak intraresidue $C^{\alpha}H_i/NH_i$ TRNOEs were observed for Leu^{311} and Phe^{313}, and weak intraresidue $C^{\beta}H_i/NH_i$ TRNOEs were observed for Val^{310}, Ser^{312} and Phe^{313}. In addition, the characteristic NOEs between Phe^{313}

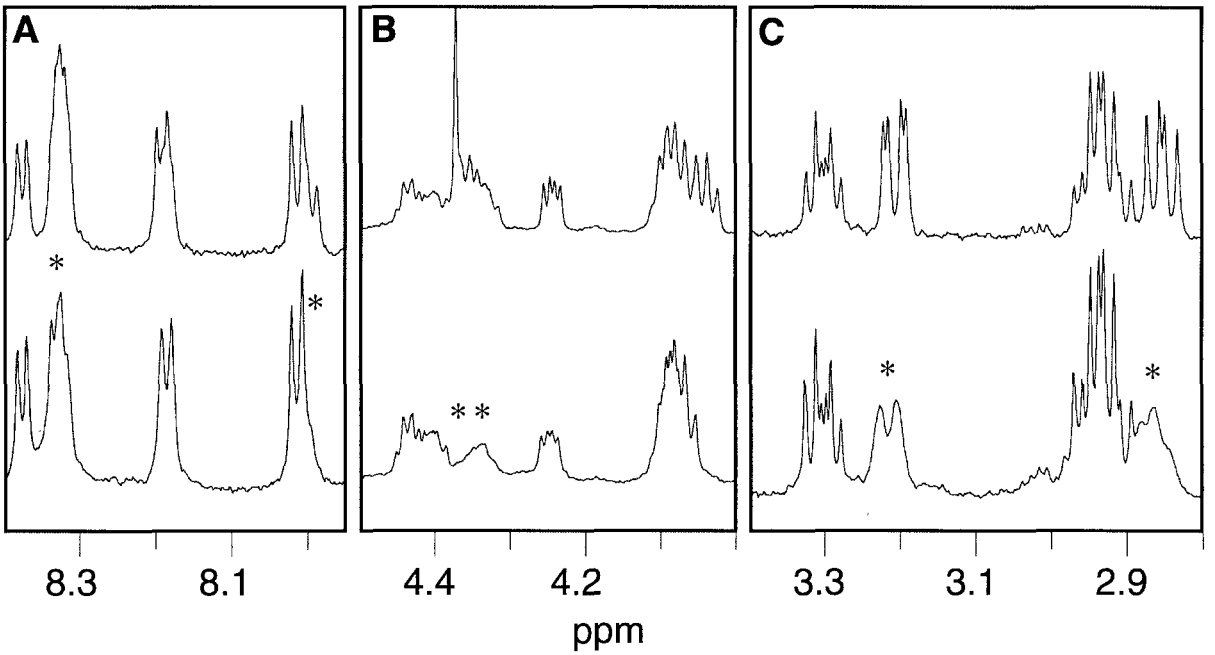

Fig. 2. Selected parts of a one-dimensional spectrum of the PapG308-314 peptide alone (upper) and in the presence of 20 μM PapD (lower). Stars indicate resonances that are broadened and shifted in the presence of PapD. Resonances for Leu^{311} NH and Ser^{312} NH are marked in (A), the Ser^{312} $C^{\alpha}H$ and Leu^{311} $C^{\alpha}H$ resonances are marked in (B) and resonances for the two $C^{\beta}H$ protons of Phe^{313} are marked in (C).

$C^{\alpha}H$ and the Pro^{314} $C^{\delta}H$ protons were observed. The *cis* form displayed sequential $C^{\alpha}H_i/NH_{i+1}$ TRNOEs between Leu^{311} through Phe^{313} and intraresidue $C^{\alpha}H_i/NH_i$ TRNOEs for Ser^{312} and Phe^{313}. In general, TRNOEs in the *cis* form were much weaker than corresponding TRNOEs in the *trans* form indicating a weaker binding and the *cis* form was therefore discarded from further analysis.

The absence of sequential NH_i/NH_{i+1} TRNOEs in the NOESY spectra suggests that the PapG308-314 peptide is in an extended conformation while bound by PapD. However, the absence of NH_i/NH_{i+1} cross-peaks could be the result of resonance broadening. For PapG308-314 only the amide proton resonances of Leu^{311} and Ser^{312} displayed broadening in the presence of PapD. These effects were too small to extensively affect any cross-peak intensity and in particular to completely suppress any sequential NH_i/NH_{i+1} TRNOEs. In α-helices sequential NH_i/NH_{i+1} NOEs are of equal or stronger intensity as intraresidue $C^{\alpha}H_i/NH_i$ NOEs in extended conformations

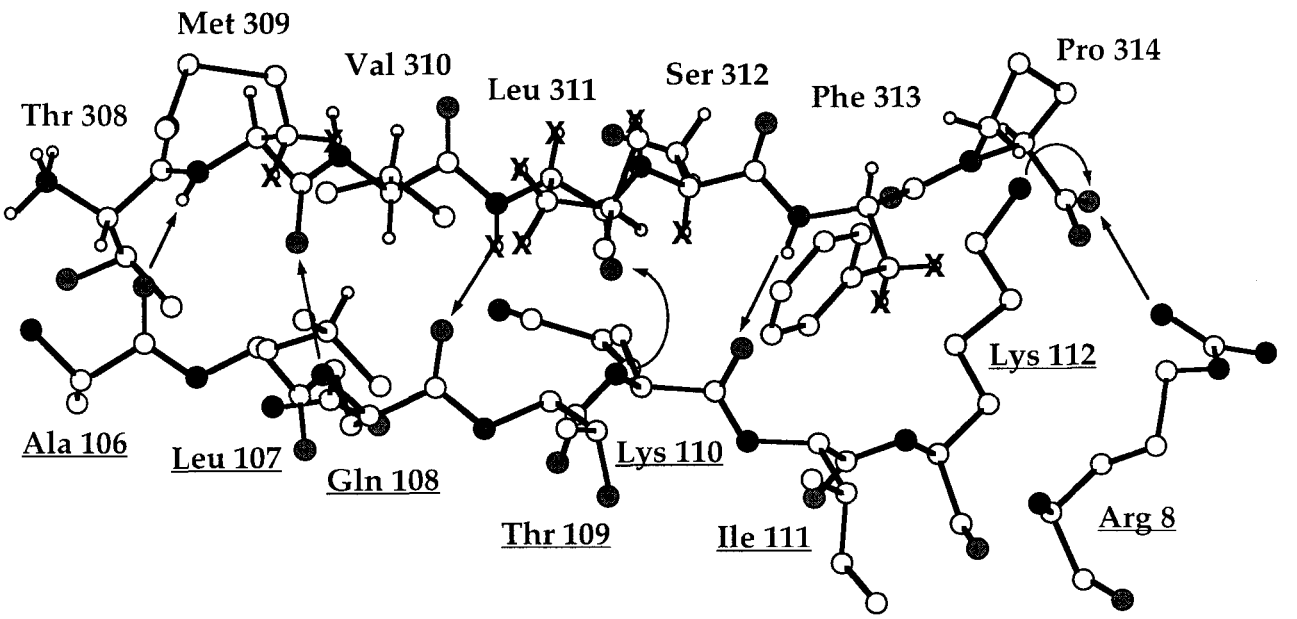

Fig. 3. Part of the crystal structure of the PapD/PapG296-314 complex [8]. The C-terminal seven amino acids of PapG296-314 that interact with Arg^8 and with residues Ala^{106}-Lys^{112} in the G1 β-strand of PapD are shown with intermolecular hydrogen bonds indicated with arrows. Resonances of hydrogen atoms which are broadened in the presence of PapD are marked with a cross. Only NH, $C^{\alpha}H$ and $C^{\beta}H$ hydrogen atoms are displayed. Oxygen atoms are black, whereas nitrogen and sulfur atoms are dark and light gray, respectively.

[29]. Despite the low peptide concentration used here weak intraresidue $C^{\alpha}H_i/NH_i$ cross-peaks were observed for Leu^{311} and Phe^{313} in the NOESY spectra (the $C^{\alpha}H_i/NH_i$ cross-peak for Val^{310} was not observed due to overlap and the one for Ser^{312} was absent) indicating that the absence of NH_i/NH_{i+1} cross-peaks is not due to insufficient S/N ratio. In addition, further support for the extended conformation of the peptide when bound by PapD is provided by the small size of the intraresidue $C^{\alpha}H_i/NH_i$ TRNOEs relative to the sequential $C^{\alpha}H_i/NH_{i+1}$ TRNOEs [26]. Altogether, this pattern with strong sequential $C^{\alpha}H_i/NH_{i+1}$ TRNOEs compared to weak or absent intraresidue $C^{\alpha}H_i/NH_i$ TRNOEs and the absence of sequential NH_i/NH_{i+1} TRNOEs indicates an extended conformation of the PapG308-314 peptide when bound by PapD. A conformation which is in agreement with the recently determined crystal structure of PapG296-314 complexed with PapD [8]. Additional TRNOEs observed in the spectra originate from intraresidue $C^{\alpha}H$ and side chain protons except for two medium range TRNOEs. The side chain methyl protons of Val^{310} and Leu^{311} show proximity to the $C^{\beta}H$ protons of Ser^{312} and the aromatic ring protons of Phe^{313}, respectively. This is also in agreement with an extended, β-strand structure for PapG308-314 in which the side chains of Val^{310} and Ser^{312} are on one side of the β-strand and those of Leu^{311} and Phe^{313} on the other.

Spin diffusion in large proteins, leading to intense cross-peaks even for protons which are not close in space, is a well known problem. The observed TRNOEs for PapG308-314 could therefore be affected by spin diffusion through protons of the protein. This type of indirect transfer of magnetization can be alleviated by keeping the fraction of bound peptide low [30] and a 25:1 ratio between PapG308-314 and PapD was therefore used here. Indirect transfer of magnetization appears with opposite sign from cross-peaks originating from direct transfer in the rotating frame and thus interfere destructively [12]. In the ROESY spectrum of PapD bound PapG308-314 no such effects could be observed for the sequential TRNOEs but the two medium range TRNOEs between the side chains of Val^{310} and Ser^{312} and between Leu^{311} and Phe^{313} were absent in the ROESY spectrum (data not shown). Therefore the observed medium range TRNOEs most likely arise from direct transfer in combination with some spin diffusion effects. Taking this into account, the intensities of the medium range TRNOEs corresponds well with the measured distances in the PapD-peptide crystal structure where the distances between the side chains of Val^{310} and Ser^{312} and between Leu^{311} and Phe^{313} are 4.5–4.9 Å and 3.8–7.2 Å, respectively. In addition, the occurrence of spin diffusion indicate closeness to a protein proton. In the crystal structure the side chain protons of Thr^{109} and Ile^{111} could act as such mediators for the NOE between Leu^{311} and Phe^{313} and the side chain of Lys^{110} could act as a mediator for the NOE between Val^{310} and Ser^{312}.

Further information on the mode of interaction of PapG308-314 with PapD was obtained from differential line-broadening and chemical shifts. Selected regions from one-dimensional spectra of the peptide–protein and free peptide solutions are displayed in Fig. 2. The $C^{\alpha}H$ resonances of Leu^{311} and Ser^{312} are considerably more broadened in the presence of PapD. Furthermore, the $C^{\beta}H$ resonances of Phe^{313}show a significant broadening as do the $C^{\beta}H$ protons of Leu^{311} and Met^{309}. Small effects could also be seen on the amide proton resonances of Ser^{312} and Leu^{311}. The $C^{\alpha}H$ resonances of Met^{309} and Phe^{313} could not be observed due to their closeness to the water resonance. The amide proton resonances of Thr^{308} and Met^{309} were absent in the spectra due to fast amide proton exchange. In addition, the cross-peaks involving Ser^{312} NH/$C^{\beta}H$, Ser^{312} $C^{\alpha}H/C^{\beta}H$ and Leu^{311} NH/$C^{\alpha}H$ were significantly broadened in a TOCSY spectrum of PapG308-314 in the presence of PapD (data not shown). The cross-peak between Ser^{312} NH and $C^{\alpha}H$ was absent, probably broadened beyond detection. Broadening of resonances is a consequence of large chemical shift differences between the free and bound form of the peptide. All of the above protons affected by broadening also showed chemical shift changes upon addition of PapD (Table 1). The broadening effects clearly indicate that the backbone of residues Leu^{311} and Ser^{312}, and the side chains of Met^{309}, Leu^{311} and Phe^{313} are mostly affected by contacts to the G1 strand of PapD. This is in perfect agreement with the orientation of the PapG296-314 peptide in the crystal structure [8] (Fig. 3).

Finally, the aggregation state of PapD in the presence and absence of peptides (PapG296-314 and PapG308-314) was investigated using asymmetrical flow field-flow fractionation [13]. Using this technique no dimers or higher aggregates could be detected for PapD in solution, confirming the dimer observed in the crystal is an artefact caused by crystal packing.

Based on TRNOE studies and differential line-broadening we have found that the peptide PapG308-314 binds to the chaperone PapD in an extended, β-strand like conformation in aqueous solution. Furthermore, the side chains of Met^{309}, Leu^{311} and Phe^{312} in the peptide make important contacts with PapD. Importantly, this is the conformation and the contacts found recently in the crystalline complex of PapD and the longer peptide PapG296-314. The crystalline complex contains PapD-peptide dimers, which could not be detected for complex formation in solution. We therefore conclude that dimerization has not substantially affected the conformation of the peptide and its interactions with PapD found in the crystal.

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