A peptide mimic of an antigenic loop of α -human chorionic gonadotropin hormone: solution structure and interaction with a llama V_{HH} domain

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The X-ray structure of a ternary complex between human chorionic gonadotropin hormone (hCG) and two Fvs recognizing its α and β subunits has been recently determined. The Fvs recognize the elongated hCG molecule by its two ends, one being the Leu-12–Cys-29 loop of the α subunit. We have designed and synthesized a 17-amino-acid peptide (named PepH14) derived from the sequence of this antigenic loop with the purpose of mimicking its three-dimensional structure and its affinity for antibodies. We have determined the solution structure of PepH14 by homonuclear NMR spectroscopy and derived distance restraints. Comparison of this structure with that of the corresponding antigenic loop of α -hCG reveals strong conformational similarities. In particular, the two pairs of residues that establish crucial contacts with the Fv fragment share the same conformation in PepH14 and in the authentic hormone loop. We propose a three-dimensional model of interaction of PepH14 with a llama V_{HH} (V_{HH} -H14) fragment cloned from a single-chain

llama immunoglobulin raised against α -hCG. This model has been constrained by the chemical shift variations of the H14 ^1HN and ^{15}N resonances monitored upon binding with PepH14. Mapping of the backbone chemical shift variations on the V_{HH} structure determined by NMR indicates that PepH14 binds to V_{HH} -H14 and forms a complex using the three complementary determining regions (CDRs). They define a shallow groove encompassing residues Thr-31, Ala-56, Tyr-59 and Trp-104 which have been shown to be in conformational exchange [Renisio, Pérez, Czisch, Guenneugues, Bornet, Frenken, Cambillau and Darbon (2002) *Proteins* **47**, 546–555] and also Phe-37 and Ala-50. This groove is close to the hydrophobic interface area observed between VH and VL domains in Fvs from classical antibodies, which explains the rather lateral binding of PepH14 on the V_{HH} .

Key words: antibody, camelids, NMR.

INTRODUCTION

Besides classical antibodies, camelids (e.g. camels, llamas and dromedaries) produce antibodies that are devoid of light chain and of the heavy chain CH1 domain [1,2]. Although their antigen combining site is borne only by a heavy chain variable domain, called V_{HH} , their affinities for antigens have been found to be similar to those of classical antibodies [3]. Camelids V_{HH} domains were demonstrated to bind proteins [4,5] as well as haptens [6,7], despite the lack of VL domain. To date, no example of a complex between a V_{HH} and a peptide has been documented. The V_{HH} -H14 is 117 residues long and has been cloned from a llama antibody raised against the human chorionic gonadotropin hormone (hCG) α subunit. The dissociation constant of V_{HH} -H14 towards hCG was found to be 3×10^{-7} M [8]. Both the crystal and the NMR three-dimensional structures of the free V_{HH} -H14 domain have been determined [8,9].

The hCG is a heterodimeric (subunits α and β) elongated glycoprotein involved in the maintenance of the corpus luteum in early pregnancy [10]. Much is already known about the three-dimensional structure of hCG [4,11]. It is a glycoprotein hormone structurally related to the follicle-stimulating, luteinizing and thyroid-stimulating hormone family. All the members of the family are heterodimers of α - and β -subunits

non-covalently associated [12,13]. The α -subunits of the four hormones are identical in a given species, whereas the β -subunits are different. Both the α - and β -subunits are necessary for binding to the receptor, whereas the β -subunit only determines the specific activity of the hormone [14].

A 3_{10} -type turn encompassing residues 20 to 23 and localized in a solvent-exposed loop of the α subunit was shown to be the main interaction component between the α -subunit and a Fv fragment [15]. Considering the α -hCG loop exposure to solvent and the shape of the Fv groove [15], this interaction has been related to a peptide/antibody recognition mode more than to a classical protein/antibody recognition mode [16]. In order to study a real peptide/ V_{HH} domain interaction, we have designed and synthesized a peptide (PepH14) whose sequence has been derived from the sequence of the hCG α subunit antigenic loop. The native sequence has been modified to ensure correct folding of the peptide with regard to the native loop while maintaining solubility. The residues known to be crucial for antibody recognition have been kept in place.

In this paper we report on the three-dimensional structure determination of the PepH14 synthetic peptide and demonstrate for the first time the capability of a V_{HH} domain as a peptide binder. The V_{HH} -H14 residues involved in the peptide binding have been identified and the restrained soft docking approach

Abbreviations used: α -hCG, α subunit of human chorionic gonadotropin hormone; CDR, complementary determining region; DQF-COSY, double-quantum-filtered correlation spectroscopy; HSQC, heteronuclear single-quantum correlation; nOe, nuclear Overhauser effect; RMSD, root-mean-square distance.

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has been used to assess an experiment-based model of the complex between the V_{HH} domain and PepH14.

MATERIALS AND METHODS

PepH14 modelling, synthesis and purification

The PepH14 peptide (see Figure 1) was obtained by chemical synthesis in solid phase. The analysis of the product was performed by HPLC and MS. The final product (25 mg) represents a purity greater than 95%. An amount of the peptide (18 mg) was dissolved in $H_2O/{}^2H_2O/DMSO$ (90:5:5, by vol.), at pH 3.0 (uncorrected for isotope effects) at a concentration of 10 mM. Recombinant uniformly ${}^{15}N$ -labelled V_{HH} -H14 was produced in *Saccharomyces cerevisiae* as described previously [17]. The V_{HH} -H14 sample concentration used for titration was 0.3 mM.

NMR spectroscopy

All NMR experiments were performed on a Bruker DRX500 spectrometer equipped with an HCN probe, and self-shielded triple axis gradients were used. Two-dimensional spectra were acquired using states-TPPI method [18] to achieve F1 quadrature detection.

For the resolution of the structure of PepH14, two series of spectra were recorded, at 283 K and 300 K, in order to solve signal overlaps. One double-quantum-filtered correlation spectroscopy (DQF-COSY) spectrum was recorded at 283 K for spin systems identification. Two total correlation spectroscopy (TOCSY) spectra with a spin lock value of 80 ms were recorded at the two above-mentioned temperatures, and two NOESY spectra (2048 complex points with 512 experiments) were recorded with mixing time values of 60 ms at 283 K, and 100 ms at 300 K.

To identify the residues of V_{HH} -H14 involved in the binding with pepH14, eight 1H - ${}^{15}N$ heteronuclear single-quantum correlation (HSQC) spectra were acquired at 300 K with 256 complex t1 points and 1024 complex t2 points. The spectral widths were 30 p.p.m. in the t1 dimension and 12 p.p.m. in the t2 dimension. The binding was monitored in a 10 mM sodium deuterated acetate buffer, pH 4.6. A first HSQC spectrum was recorded on V_{HH} -H14 alone (300 μ M). Seven 1H - ${}^{15}N$ HSQC spectra were then recorded with increasing amounts of PepH14 (PepH14 over V_{HH} -H14 molar ratio of 0.2, 0.5, 1, 2, 3, 4 and 5 respectively).

Data processing

Homonuclear and heteronuclear spectra were processed using UXNMR on a Silicon Graphic INDY R4000 workstation. The matrices were transformed to a final size of 2048 points in the acquisition dimension, and to 1024 points in the indirect dimension. The signal was multiplied by a shifted sine-bell window in both dimensions prior to Fourier transform, then a fifth-order polynomial baseline correction was applied. Proton chemical shifts were calibrated relative to H_2O (4.75 p.p.m. at 300 K and 4.91 p.p.m. at 283 K).

Spectral analyses were performed using the XEASY software [19] on a Silicon Graphic R10000 workstation. Identification of the amino acid spin systems and sequential assignment were performed by following the standard strategy described by Wüthrich [20].

Structure calculation of PepH14

Distance geometry calculations were performed with the variable function software DIANA 2.8 [21]. A preliminary set of 1000 structures was initiated, including only intra-residual and sequential upper-limit distances that were converted from peak volumes using the CALIBA routine of the DIANA package. From the first set of calculated structures, the 500 best (as determined by the value of the target function) were kept for a second round, including medium-range upper-limit distances, and the resulting 250 best for a third one, with the whole set of upper limit restraints. Finally, three runs of the REDAC strategy [21] were performed on the 100 best structures, and the 25 best structures were minimized under NMR restraints using the XPLOR 3.8 [22] minimization routine. The visual analysis of the structures was performed with TURBO software [23]. Quantitative analysis was performed with PROCHECK-NMR [24].

Docking calculations

Molecular interaction simulations were performed using the docking program BiGGER (<http://www.dq.fct.unl.pt/bioin/chemera>; Palma et al. [26]). This algorithm performs a complete and systematic search in the binding space of both molecules. A population of 1000 candidate protein-protein-docked geometries is generated and selected, based on the geometry complementarity and amino-acid pair-wise affinities between the two molecular surfaces. In this process, the algorithm enables implicit treatment of molecular flexibility. In a subsequent step, the putative docked structures are ranked using an interaction scoring function, which combines several interaction terms: geometric packing of the surfaces, electrostatic interactions, desolvation energy, and pair-wise propensities of the amino-acid side-chains to contact across the molecular interface. In the *ab initio* simulations, the entire molecular surface was searched using absolutely no additional information regarding the binding sites.

From the 1000 selected solutions, the NMR filtering followed the method described by Morelli et al. [25] and Palma et al. [26]. We have generated files corresponding to the NMR experiments where the information derived from the chemical shift variations upon the complex formation have been transformed into a list of amino acids with a distance filtering (cut off) chosen by the user (here 5 Å, where 1 Å = 0.1 nm). This information was provided by the chemical shift variations detected on the HSQC and the NOESY spectra.

From the HSQC experiments, 16 chemical shift variations monitored for the antibody have been injected to filter the potential solutions. The structure calculations have been done using the NMR structure of both the antibody [1] and the peptide (this study).

RESULTS

Design of PepH14

The sequence of the PepH14 peptide was derived from that of residues 12–29 in the loop of the hCG α -subunit (Figure 1), which is the main interacting part of this subunit, with a Fv from rabbit antibodies raised against it [15]. ELISA binding experiments have demonstrated that this peptide alone does not bind to the Fv. However, pepscan studies [27] starting from this peptide lead to a variant able to bind to Fv 3299 in ELISA experiments. Variants were identified with improved binding activity in pepscan ELISA through positional scanning, in which each of the amino acid residues in the synthetic peptide, covering residues

Table 1 Proton chemical shifts of PepH14

Residue	HN	H α	H β	Others
Asp 1	*	*	*	
Gln 2	8.903	4.380	2.342,*	C γ H ₂ 2.075, 1.977
Ile 3	8.492	4.112	1.813	C γ H ₂ 1.422, 1.173; C δ H ₃ 0.840
Asn 4	8.644	4.948	3.002, 2.728	
Pro 5	*	*	2.133,*	C γ H ₂ 1.898, 1.624; C δ H ₂ 3.853, 3.738
Phe 6	8.276	4.596	3.250, 3.080	C γ H ₂ 7.413; C ζ H ₂ 4.700
Thr 7	7.792	4.302	4.223	C γ H ₃ 1.200
Met 8	8.276	4.445	2.623, 2.519	C γ H ₂ 2.094, 2.016
Gln 9	8.384	4.609	2.401,*	C γ H ₂ 2.133, 1.911
Pro 10	*	*	2.310, 2.114	C γ H ₂ 2.009, 1.957; C δ H ₂ 3.811, 3.701
Gly 11	8.628	3.916,*		
Ala 12	8.072	4.308	1.395,*	
Ile 13	8.160	4.139	1.853	C γ H ₂ 1.200; C δ H ₃ 0.906
Val 14	8.244	4.112	2.042	C γ H ₃ 0.951
Leu 15	8.380	4.400	1.650,*	C γ H ₂ 1.572; C δ H ₃ 0.971, 0.893
Gln 16	8.384	4.328	2.382,*	C γ H ₂ 2.107, 1.964
Lys 17	8.564	4.348	1.938, 1.794	C γ H ₂ 1.689; C δ H ₂ 1.467; C ϵ H ₂ 2.989; N ϵ H ₂ 7.716

* Resonances that cannot be observed.

α -hCG	T12	L	Q	E15	N	P	F	F	S20	Q	P
pepscan	Q	D	W	I	N	P	F	T	M	Q	P
PepH14	D1	Q2	I3	N4	P5	F6	T7	M8	Q9	P10	
acc (α -hCG)	0	0	0	0	28	106	162	40	9	87	95
acc (PepH14)		0	112	45	100	17	131	0	138	6	76

α -hCG	G	A	P25	I	L	Q	C29
pepscan	Y	A	I	V	L	Q	V
PepH14	G11	A12	I13	V14	L15	Q16	K17
acc (α -hCG)	0	0	0	0	0	0	0
acc (PepH14)	0	0	23	7	0	42	146

Figure 1 Alignment of the sequences of the α -hCG antigenic loop with the pepsan proposal and the derived PepH14 sequence together with the matching numbers

12–29 of α -hCG, was replaced by each of the other 19 amino acids. The pepsan-derived peptide with the highest binding activity in ELISA (Figure 1) was too hydrophobic for solution studies. We therefore combined the original loop sequence with that of the pepsan peptide with the following rules: (i) keep unchanged the main interacting residues (Figure 1), (ii) keep as many pepsan peptides as possible, and (iii) replace peripheral apolar residues with polar or charged ones. The resulting sequence led to a soluble peptide that was amenable to structural studies (Figure 1).

Three-dimensional structure of PepH14

We have assigned all the spin systems present on the TOCSY spectrum and sequentially connected them using the NOESY spectrum (Table 1). The spin systems were connected using the H α /HN(*i*, *i* + 1) connections from residue 2 to residue 14 (Figures 2 and 3). The HN signal of D1 is missing because of a high exchange rate of the amide proton of the N-terminal residue. Leu-15 is connected to Val-14 only on the NOESY, with

Table 2 Structural statistics of the NMR solution structures of PepH14

1 cal = 4.184 J.

Parameter	Value
Number of restraints	
nOe	
Intraresidue	40
Sequential	30
Medium and long range	13
Average/residue	5
RMSD (Å)	
Backbone (C, Ca, N)	2.1 ($\pm$ 0.5)
All heavy atoms	3.2 ($\pm$ 0.5)
Energy (kcal/mol)	
Total	103.32
Bonds	7.40
van der Waals	−56.93
Dihedral	80.00
nOe	4.20

Figure 2 Sequential assignment of PepH14

H α -HN_{*i*+1} connections together with NH-NH_{*i*+1} connections are significant of a loop structure for the peptide.

a mixing time of 100 ms. Residues 6 to 9 and 11 to 12 are also connected by HN/HN nuclear Overhauser effects (nOes). The simultaneous observation of sequential H α /HN and HN/HN connectivities is evidence for a hairpin structure in the 6 to 12 region. Inter-sidechain nOes confirm the assignment. The final set of constraints contained 83 distance constraints removed from the NOESY spectra, leading to an average of five restraints per residue. At the end of the calculation, none of the upper limit distances were violated by more than 0.2 Å. The set of 25 best structures was subjected to a final run of 2000 steps of minimization in the XPLOR 3.8 software, leading to a global energy of about 420 kJ/mol (100 kcal/mol) (Table 2).

The final root mean square distance (RMSD) value obtained for all backbone atoms of the 17 best models was 2.1 Å, but this drops to 1.1 Å if one superimposes only the region from residues Glu-3 to Leu-15 (Figure 4A). The RMSD values clearly indicate that the structure of the central part of the peptide is well restrained while both ends are not. All the solutions have good non-bonded contacts and good covalent geometry, as shown by low values of XPLOR energy terms. The correlation with the experimental data shows no nOe-derived distance violation greater than 0.2 Å and the Ramachandran plot shows (in PROCHECK software nomenclature) 98 % of the residues in the most favoured and in the additional regions. We have generated and minimized an averaged structure of the peptide, which has been compared with the Leu-12–Cys-28 antigenic loop of hCG α -subunit

Figure 3 NOESY spectrum of PepH14

The spreading of amide signals from 6.8 to 9.0 p.p.m. is evidence for a stabilized structure in solution.

(Figure 4B). The conformation of the central part of the synthetic peptide is close to that of the hCG loop, the structure of the N- and C-termini diverging from the original conformation. This discrepancy between the synthetic peptide and the original loop comes from the lack of experimental constraints, indicating high mobility of both ends of the peptide. Moreover, the side chain conformation of three of the four crucial residues of α -hCG is conserved. The RMSD for those three residues is 0.23 Å for the backbone and 1.12 Å for all heavy atoms. Pro-5 side-chain conformation significantly differed in the two molecules. The trans-conformation of the culprit proline residue has been checked by a series of calculations to be the only conformation consistent with the experimental data.

H14 titration experiments

Superimpositions of ^1H - ^{15}N HSQC spectra, recorded on the V_{HH} -H14 domain alone (0.3 mM) and with increased amounts (1, 2, 3 and 5 equivalents) of synthetic peptide pinpoint the V_{HH} -H14 backbone amide groups affected by the peptide binding (Figure 5). The observed changes in the cross-peak chemical shifts indicates that the two molecules are in rapid exchange between the free and bound conformations. Changes in chemical shifts are observed throughout the protein but with different

values. We decided to consider as significant only the shifts larger than 0.03 p.p.m. for the NH protons (Figure 6A) and 0.25 p.p.m. for the ^{15}N (Figure 6B). The most significant shifts occurred in the three complementary determining regions (CDRs), as well as at the N- and C-terminal regions. Some residues belonging to the framework are also concerned. The largest shifts were observed for the N-terminal part (residues 2 to 6) and for residues 109–110. The Trp-107 side-chain NH proton also shows significant chemical shift variations. The amino acids identified in the interaction are mapped in red on to the V_{HH} -H14 in Figure 7(A).

Docking modelling

The newly described approach 'soft restrained docking' [25,26] has been applied to this complex, using the chemera package (<http://www.dq.fct.unl.pt/bioin/chemera>; Palma et al. [26]). The rigid-body approximation conditions necessary for this approach were fulfilled: the three-dimensional structure of both molecules are known, the dissociation constant is in the milli to micro molar range (see below) and the complex formation is in fast exchange in the NMR time-scale. The *ab initio* docking calculations were performed with the NMR structure of V_{HH} -H14 as target and PepH14 as the probe. The 1000 theoretical structures produced by BiGGER can be clustered into three

Figure 4 Solution structure of PepH14

(A) Superimposition of the 17 best solution structures. (B) superimposition of the best PepH14 structure (thick line) with the structure of the Leu-12–Cys-28 α -hCG loop [15] (thin line).

Figure 6 Chemical shift variations of V_{HH} -H14 amide proton (top) and amide nitrogen (bottom) upon binding of PepH14

The variations considered significant are above the grey region.

Figure 5 Superimposition of HSQC spectra of V_{HH} -H14 alone (black) and V_{HH} -H14 in the presence of five equivalents of PepH14 (red)

The chemical shifts of the residues affected by the presence of the peptide are shifted and have been used for BiGGER calculations.

Figure 7 Docking of PepH14 on to V_{HH} -H14

(A) Position of the residues (in red on the V_{HH} -H14 structure) affected upon binding of the peptide. (B) Twenty best positions of the centre of mass of the peptide, according to the BiGGER calculation using the HSQC data as a filter. (C) Molecular surface of V_{HH} -H14 exhibiting a groove formed by the three CDRs and a part of the framework of the molecule. (D) Model of the interaction between H14 and PepH14. (E) Complex between the VH part of the Fv with the whole hormone (only its antigenic loop is displayed). (F) Stereo view of the peptide in CPK.

families of solutions. After the NMR filtering only one cluster of solutions remains close to the residues of the CDRs (Figure 7B). We have subsequently derived a unique model extracted from the five best orientations of the peptide/ V_{HH} -H14 complex

(Figure 7D). The loop thought to be in interaction with the antibody is located between the three CDRs, whereas the rest of the peptide is in contact with the region just below the CDRs, both forming a groove into which the peptide can fit (Figure 7C).

Figure 8 Stereo view of the complex between Fv (black) with the antigenic loop of hCG (green) superimposed with V_{HH} -H14 (blue) in complex with PepH14 (red)

DISCUSSION

Peptide structure

The sequence of the PepH14 synthetic peptide was derived from the sequence of the authentic Leu-12–Cys-28 loop in α -hCG used as a template, by conserving the residues known to be crucial for the binding (Figure 1). A remarkable result is that such a small peptide (17 residues long), devoid of a stabilizing disulphide bridge, was found to be well folded in solution: the analysis of the set of calculated structures shows a good convergence for the overall three-dimensional conformations of PepH14. This indicates that this peptide is structurally stable, confirmed by the number of nOes (5 constraints per residue on average) collected with relatively short NOESY mixing times (60 ms and 100 ms).

V_{HH} -H14 combining site

The chemical shift variations observed upon binding of the synthetic peptide involve the three CDRs of V_{HH} -H14. Additional chemical shifts variations involve the N-terminus and some residues which belong to the inner core of the protein (residues 37, 46, 59, 94, 95, 104, 108 and 109). Some of these shifts may be due to a non-specific binding of a significant amount of the synthetic peptide near the N-terminus of V_{HH} -H14 or the binding of the peptide may induce some structural changes.

The dynamic studies of V_{HH} -H14 revealed that the region encompassing the three CDRs is susceptible to environmental changes [1]. We present, in this work, experimental evidence upon binding of the synthetic peptide, that these residues and others are involved in the interaction with hCG. This evidence strongly suggests that binding to PepH14 induces structural rearrangements outside the binding site. However, relaxation measurements of V_{HH} -H14 show a very rigid inner core of the protein, suggesting that such changes would be limited [1].

The docking of the ligand within the binding cavity of V_{HH} -H14 was investigated using the 'soft restrained docking' approach, which includes theoretical docking calculations filtered by NMR-derived titration data. The analysis of the obtained solutions has generated one single solution (Figure 7B).

In our model (Figure 6C), the peptide stacks against an area corresponding to the three CDRs and the residues Phe-37 and

Ala-50. An overall surface area of $640 \text{ \AA}^2$ of the V_{HH} is covered by the peptide. Part of this area, $290 \text{ \AA}^2$, belongs to the CDRs, while the rest, $350 \text{ \AA}^2$, belongs to framework residues. This accessible surface area value is between the values observed with haptens, approx. $300 \text{ \AA}^2$ [7,8] and those observed with proteins, between 800 and $1000 \text{ \AA}^2$. In the complex between the hCG α subunit and a Fv fragment, residues Phe-37 and Ala-50 belong to the interface between the VH and VL domains (Figure 7E). In Fvs, these residues are involved in the VH–VL interaction and cannot therefore be used for antigen recognition, except in the case of haptens, where the bottom of the cavity often interacts. In the case of V_{HH} , this hydrophobic surface is available for antigen recognition and might be used in some cases. For example, in the interaction of a dye hapten with its specific V_{HH} , the interaction involves framework residues and the hapten consequently occupies an upper-lateral position [8]. With proteins, two of the three crystallized complexes of V_{HH} with amylase have revealed the importance of framework residues for molecular recognition [5]. In the present case, the peptide also occupies an upper-lateral position on the V_{HH} , at a location normally occupied by the VL in the hCG–Fv complex (Figure 8). However, the anti-lysozyme or the anti-RR6 dye V_{HH} s complexes revealed a quite central interaction and involved only the CDRs [5,7]. V_{HH} domains adopt flexible binding behaviours, being able to recruit framework residues. This seems to be a peculiar feature of V_{HH} s in complex with haptens or proteins, in which numerous binding modes have already been deciphered in the few structures available. This might also be the case with peptides.

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