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Synthesis and Solution Characterization of a Porphyrin-CCK8 Conjugate

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Abstract: In this paper we report the synthesis and a detailed NMR solution characterization of a new CCK8 analogue and its indium(III) complex, PK-CCK8 and In-PK-CCK8. The new compounds contain a porphyrin moiety covalently bound through an amide bond to the side chain of a Lys residue introduced at the *N*-terminus of CCK8. A molecular dynamics simulation, based on the NMR structure of the complex between CCK8 and the *N*-terminal extracellular arm of the CCK_A receptor, is also reported. Both the NMR study and the molecular dynamics simulation indicate that the porphyrin–peptide conjugate might be able to bind to the CCK_A receptor model. The results of the molecular dynamics calculations show that the conformational features of the CCK8/CCK_A receptor model complex and of the PK-CCK8/CCK_A receptor-model complex are similar. This evidence supports the view that the introduction of the porphyrin-Lys moiety does not influence the mode of ligand binding to the CCK_A receptor model. The NMR structure of PK-CCK8 in DMSO consists of a well defined pseudo-helical *N*-terminal region, while the *C*-terminal region is flexible. Moreover, the absence of NOE contacts between the porphyrin and the peptide indicates that the macrocyclic ring is directed away from the peptide region involved in the binding with the receptor.

Keywords: CCK8 derivative; indium(III) complex; molecular dynamics; NMR solution conformation; porphyrin–peptide bioconjugate

INTRODUCTION

In the last few years an increasing interest has been noticed in the study of receptors for regulatory peptide hormones [1]. These receptors belong to the superfamily of G-protein coupled receptors (GPCRs) and are localized in the cell membrane. Most of them have been found overexpressed in tumours and thus they have been chosen as potential targets for tumour diagnosis. Among these, the two receptors for the peptide hormone cholecystokinin (CCK), CCK_A and CCK_B, have been intensively studied with the aim of characterizing the molecular basis of their interaction with the CCK peptide [2]. Most of the studies concern the binding mode of the *C*-terminal CCK octapeptide amide (CCK26–33 or CCK8), which displays high affinity for both receptors [3,4], even if the sulphated form of CCK8 (with a sulphate moiety on Tyr^{27} side-chain) is 1000-fold more active than the non-sulphated CCK8 in binding CCK_A. A detailed characterization of the interaction between CCK8 and the receptors is essential for development of CCK8 analogues bearing chelating agents able to coordinate with high stability radioactive metals for applications in cancer diagnosis by nuclear medicine techniques. Mutagenesis and photoaffinity cross-linking studies of the interaction of CCK8 with CCK_A [3,4] have led to contrasting conclusions on the residues involved: the

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differences are probably due to significant modifications of the system requested by the two different experimental approaches. Finally, the bimolecular complex of CCK8 with the *N*-terminal extracellular arm of CCK_A has been structurally characterized by high-resolution NMR and computational refinement [5]. The NMR structure of the complex suggests that CCK8 binds to CCK_A with the *C*-terminus within the seven-helical bundle of the GPCR and the *N*-terminus projecting out between the *trans*-membrane loops 1 and 7 and specifically interacting with the *N*-terminus of CCK_A. The structure of the complex also indicates that modifications on the *N*-terminal side of CCK8 obtained by introducing chelating agents and their metal complexes should not affect the interaction with CCK_A. The ^{111}In radiolabelled CCK8 analogue, [^{111}In-DOTA]CCK8, bearing the chelating agent tetraazacyclodecane tetraacetic acid (DOTA) at the *N*-terminus of CCK8, is now in preclinical evaluation for scintigraphy of human medullary thyroid cancers overexpressing CCK receptors [6].

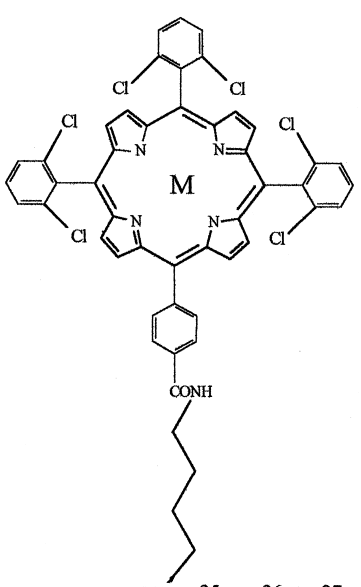
In this paper we report the synthesis and a detailed NMR solution characterization of a new CCK8 analogue and its indium(III) complex, PK-CCK8 and In-PK-CCK8. The new compounds contain a porphyrin moiety (P) covalently bound through an amide bond to the side chain of a Lys residue (K) introduced at the *N*-terminus of CCK8 (Figure 1). Moreover, the two Met residues of CCK8 are replaced by norleucine (Nle) residues to minimize synthetic problems. A molecular dynamics (MD) simulation, based on the NMR structure of the complex between CCK8 and the *N*-terminus extracellular arm of the CCK_A, is also reported. The major goal of our study was to verify if the presence of the bulky porphyrin substituent at the *N*-terminus of CCK8 would interfere with the conformation of the residues probably involved in receptor binding.

MATERIALS AND METHODS

Peptide–porphyrin Synthesis

Benzotriazol-1-yl-oxy-tris-pyrrolidino phosphonium hexafluorophosphate (PyBop), 1-hydroxybenzotriazole (HOBt), all the Fmoc-amino acid derivatives (Fmoc, 9-fluorenylmethyloxycarbonyl) and the Rink amide MBHA resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). All other chemicals were obtained from Aldrich (Milwaukee, WI, USA) and were used without further purification unless otherwise stated.

Solid-phase peptide synthesis was performed on a fully automated synthesizer Shimadzu SPPS-8 (Kyoto, Japan). Analytical RP-HPLC runs were carried out on a Shimadzu model 10A-LC apparatus using a Phenomenex (Torrance, CA, USA) C_{18} column, 4.6×250 mm, eluted with H_2O/0.1% trifluoroacetic



M = H_2 PK-CCK8
M = In(III) In-PK-CCK8

HLys^{25}Asp^{26}Tyr^{27}Met^{28}Gly^{29}Trp^{30}Met^{31}Asp^{32}Phe^{33}NH_2

Figure 1 Schematic representation of PK-CCK8 and its indium(III) complex. The numbering scheme 25→33 follows that of the full CCK peptide, 33-residue long, CCK8 being the *C*-terminal segment. Met^{28,31} are replaced by Nle residues.

acid (TFA) (A) and CH_3CN/0.1% TFA (B) linear gradients from 20% to 80% B over 40 min at 1 ml/min flow rate. Preparative RP-HPLC runs were carried out on a Waters (Milford, MA, USA) Delta Prep 4000 instrument equipped with a UV Lambda-Max model 481 detector using a Vydac (Hesperia, CA, USA) C_{18} column, 22×250 mm. A linear gradient from 20% to 80% B over 40 min at 20 ml/min flow rate was used. UV-vis spectra were recorded by using an UV-vis Jasco (Victoria, Canada) model 440 spectrophotometer with a cell of 1-cm path length.

5,10,15-Tris(2,6-dichlorophenyl)-20-(4-carboxyphenyl)porphyrin, (TDCMCPP-H_2) was synthesized as previously described [7]. The indium(III)-porphyrin complex, [TDCMCPP-In(III)], was obtained according to a previously described method [8]. A large excess of indium(III) chloride was added to a solution of TDCMCPP-H_2 in acetic acid containing 4% TFA. The reaction mixture was kept under reflux and monitored by UV-Vis spectroscopy. After 30 min the shift of the Soret band was completed. The solvent was removed under vacuum and the complex crystallized from diethyl ether. The yield for indium(III) insertion was higher than 98%. UV-Vis (DMF): λ_{max} (nm): 419 (Soret), 515, 540, 580.

Peptide synthesis was carried out in solid phase under standard conditions using the Fmoc protocol. The Rink-amide MBHA resin (0.54 mmol/g, 54 mmol scale, 0.100 g) was used. Double couplings were performed, adding each time four equivalents of *N*-protected amino acids activated by PyBop and HOBt and eight equivalents of *N*,*N*-diisopropylethylamine (DIPEA) in DMF, and stirring for 60 min. Fmoc-Lys(Mtt)-OH (Mtt, methyltrityl) was coupled at the *N*-terminus. When the peptide synthesis was complete, the Mtt Lys side-chain protecting group was removed by treatment with a TFA/triisopropylsilane/CH_2Cl_2 (1:5:94) mixture; then, TDCMCPP-H_2 or TDCMCPP-In(III) were coupled to the Lys ε-amino group using a 1.5 equivalent amount of porphyrin or indium(III)-porphyrin, PyBOP and HOBt and 3 equivalents of DIPEA in a single coupling. The reactions were carried out by stirring the mixtures in DMF for 2 h. Yields for porphyrin couplings, monitored by the Kaiser test, were in the range 92–95%. For deprotection and cleavage, the fully protected porphyrin–peptide resins were treated with TFA containing tri-isopropylsilane (2.0%), ethandithiole (2.5%) and water (1.5%). The porphyrin–peptide products precipitated at 0°C by adding diethyl ether dropwise. Purifications of the crude mixtures were carried out by RP-HPLC: PK-CCK8, $R_t = 37.2$; In-PK-CCK8, $R_t = 36.9$.

Mass spectra obtained on a MALDI-TOF Vojager-DE (Perseptive Biosystems, Foster City, CA, USA) apparatus confirm the product identities: PK-CCK8, MW = 2003 (calc. 2002); In(III)-PK-CCK8, MW = 2115 (calc. 2115).

Molecular Dynamics Simulations

All calculations and graphical analyses were run on a Silicon Graphics (Mountain View, CA, USA) O2 R10000 workstation. The package INSIGHT/DISCOVER (Biosym Technologies, San Diego, CA, USA) was used to perform energy minimization and molecular dynamics simulations (MD) *in vacuo* at 300 K, with the consistent valence force field (cvff) [9–11], setting pH 7 for all simulations. The steepest descent algorithm [12] was used in the early stages of refinement, whereas the quasi-Newton–Raphson algorithm [12] was used in the final stages.

The starting structure used in structural analysis and simulations was that of the complex CCK8/CCK_A receptor model obtained from the Protein Data Bank (Brookhaven National Laboratory, Upton, NY, USA) [5]. Computational conditions were chosen to avoid boundary effects [13].

In all simulations the coordinates of backbone atoms of CCK_A were kept fixed in position. The structure of the complex was minimized and the resulting structure was then used for subsequent MD simulations.

In the MD simulation, performed with a time step of 0.5 fs and a distance-dependent dielectric constant, at 300 K the system was equilibrated for 80 ps. After this first step, an additional 80 ps of simulation without rescaling was carried out, since energy conservation was observed and the average temperature remained essentially constant around the target values. Coordinates and velocities for the simulation were dumped to a disk every ten steps during the last 40 ps of the simulations.

NMR Spectroscopy and Structure Calculation

Nuclear magnetic resonance experiments were carried out on a Varian (Palo Alto, CA, USA) Unity 400 MHz spectrometer of the *Centro Interuniversitario di Ricerca su Peptidi Bioattivi*.

The solution for NMR measurements was prepared by dissolving ~ 2.2 mg of PK-CCK8 in 0.75 ml of DMSO-d_6 (Euriso-top SA, Saint Aubin, France, 99.96% relative isotopic abundance). All the spectra for the structure determination were acquired at 298 K and referenced to DMSO ($\delta = 2.5$

ppm). A series of monodimensional (1D) and TOCSY spectra were also acquired in the temperature range of 298–313 K for the measurements of amide proton temperature coefficients. The possible occurrence of aggregation was tested by measuring the chemical shifts in the 1D spectrum and analysing the cross-peak patterns in a NOESY spectrum recorded with a peptide concentration of 0.8 mM.

1D and bidimensional (2D) spectra were acquired with a spectral width of 6440 Hz. 2D experiments DQFCOSY [14], TOCSY [15], NOESY [16] and compensated ROESY [17,18] were recorded in the phase sensitive mode using the States–Haberkorn scheme. Sixty-four transients of 4 K were collected for each of the 512 increments; the data were zero filled to 1 K in $\omega 1$. Squared shifted sine-bell functions were applied in both dimensions prior to Fourier transformation.

TOCSY, NOESY and ROESY spectra were acquired with mixing times of 70, 200 and 180 ms, respectively. The spectra were processed with the software PROSA [19] and analysed with the software XEASY [20]. Cross-peak intensities in the NOESY spectrum were measured by volume integrations with the software XEASY. Measurements of $^{3}J_{HNH\alpha}$ coupling constants were obtained from 1D experiments and, after zero filling, from DQFCOSY.

The input for the structure calculations performed with the software DYANA [21] consisted of upper distance limits, derived from the NOESY cross-peaks volumes, and of dihedral angle constraints, derived from intraresidual and sequential NOEs.

The final round of DYANA structure calculations was started with 200 randomized conformers. The 20 conformers with the smallest residual target function were energy minimized with the package INSIGHT/DISCOVER (see above for experimental details) and were used to represent the solution structure of PK-CCK8. The program MOLMOL [22] has been used for rmsd calculations and for the preparation of the figures.

RESULTS AND DISCUSSION

The CCK8 peptide analogue functionalized with a porphyrin macrocycle (Figure 1) has been synthesized in the solid phase. The final step of the synthesis concerns the covalent binding of the porphyrin moiety to the ε-amino group of the Lys residue introduced as a spacer at the *N*-terminus of the CCK8 analogue. This reaction is very efficient: only 1.5 equivalents of the porphyrin derivative, with its carboxyl function activated by PyBOP and HOBt, are sufficient to give a high yield of the conjugated product in a single coupling. This result is in accordance with that already found for the coupling of the same porphyrin derivative to the Lys side chain in other peptide sequences [7]. Also, the coupling of the indium(III)-porphyrin complex occurs in high yield; unfortunately, some of the indium ion is lost during peptide cleveage and deprotection in the acid medium (TFA). Both the non-metallated PK-CCK8 and its indium complex, In(III)-PK-CCK8, are obtained in a pure form by HPLC treatment.

The porphyrin moiety was selected as the chelating agent for metals with potential application in nuclear medicine due to the fact that indium(III) complexes with tetrapyrrolic macrocycles are known to be stable *in vivo* against demetallation [6,8]. Moreover, it will be easy to prepare the radioactive compound ^{111}In(III)-PK-CCK8 using the synthetic procedure described here for the 'cold' isotope.

The choice of the best position for binding the chelating agent is based on the NMR structure of the complex between CCK8 and the receptor model formed by the *N*-terminal extracellular arm of CCK_A [5]. On these bases, modifications at the *N*-terminal side of CCK8, by introducing the porphyrin moiety and its indium(III) complex, should not affect the interaction with CCK_A.

To prove this hypothesis, we have first undertaken a molecular dynamics study of the PK-CCK8/CCK_A receptor-model complex, using as a template the NMR structure of the CCK8/CCK_A receptor-model complex [5]. Then, we have studied the solution conformation of the PK-CCK8 conjugate by NMR.

The MD simulations were carried out for the PK-CCK8/CCK_A receptor-model to obtain an indication of the conformational behaviour of the complex. The results for PK-CCK8 are summarized in Table 1. In Figure 2 the average structure for the complex, as obtained from MD simulations at 300 K, is reported. The root mean square deviation (RMS) from the NMR experimental data of the PK-CCK8 backbone atoms of the residues from Asp^{26} to Phe^{33}, as derived from MD simulations was 0.89 Å. The results of the MD calculations indicate that the conformational features of the CCK8/CCK_A receptor-model complex [5] and of the PK-CCK8/CCK_A receptor-model complex are very similar. This evidence underlines that the introduction of the

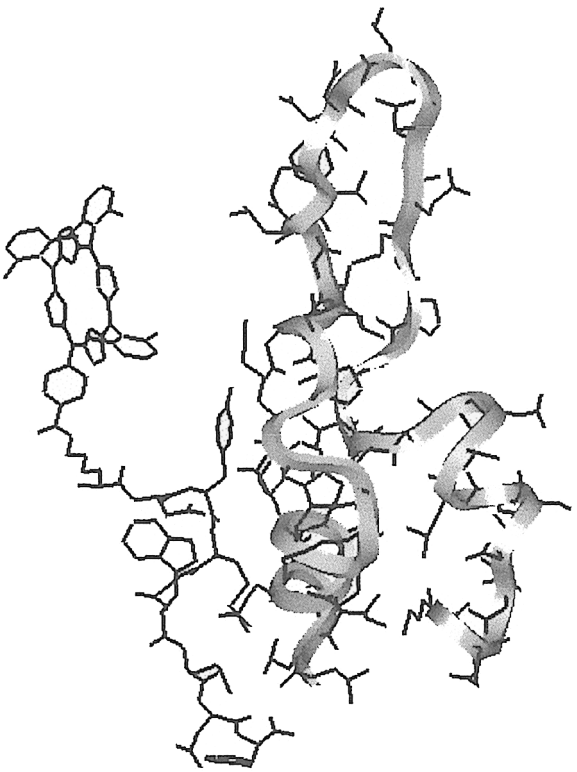


Figure 2 Average solution structure of the PK-CCK8/CCK_A receptor-model complex, as obtained from MD simulations *in vacuo* at 300 K.

Table 1 Average Model of PK-CCK8 in the PK-CCK8/CCK_A Receptor Complex as Obtained from MD Analysis *In Vacuo* at 300 K

Residue	ϕ	ψ
Lys^{25}		119.4
Asp^{26}	−92.0	167.5
Tyr^{27}	−69.0	−20.8
Nle^{28}	−114.8	92.2
Gly^{29}	−166.5	59.6
Trp^{30}	−127.7	−55.0
Nle^{31}	−145.1	141.9
Asp^{32}	−68.0	−59.1
Phe^{33}	−80.0	−36.1

porphyrin-Lys moiety does not influence the binding mechanism of the ligand to the CCK_A receptor model. Indeed, the Lys side-chain χ^1 and χ^2 dihedral angles are *g*-, *t*, respectively, that keep the porphyrin moiety spatially separated from the receptor. The MD results show that the complex is stabilized by a number of hydrophobic interactions involving Tyr^{27} and Nle^{31}. In addition, numerous Coulombic interactions between Asp^{26} and Tyr^{27} of the ligand and some residues of the CCK_A receptor model are found. These interactions are similar to those reported for the CCK8/CCK_A receptor-model complex [5].

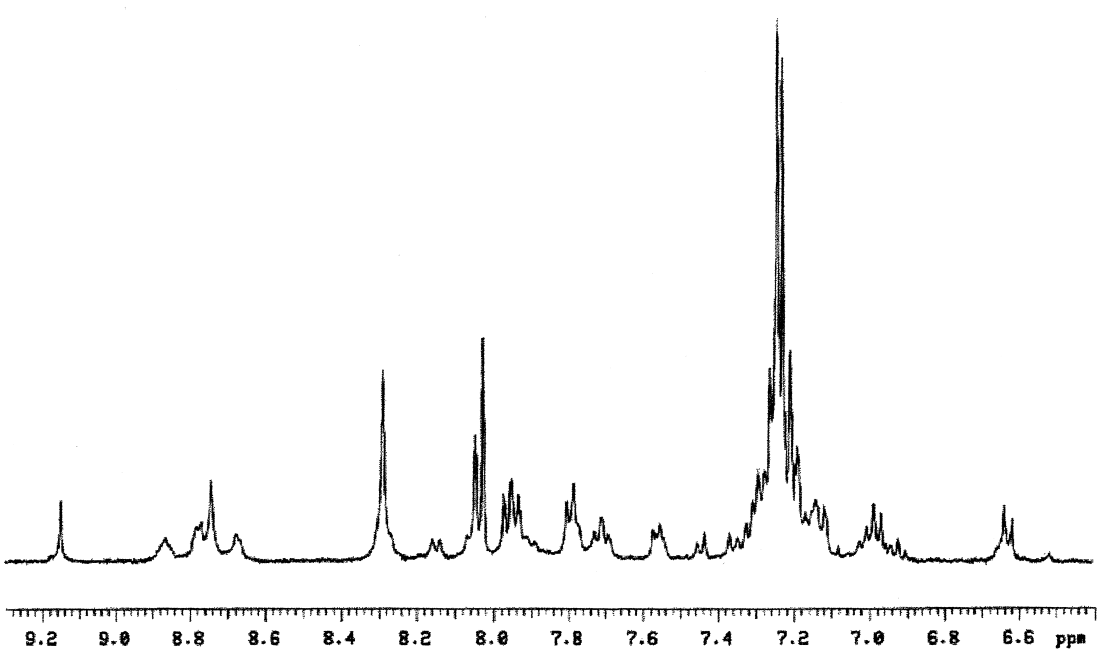


Figure 3 Expanded region of the 400 MHz ^{1}H-NMR spectrum of PK-CCK8 where the pyrrole and phenyl hydrogens are found.

The NMR study of PK-CCK8 has been undertaken to verify the possible occurrence of interactions between the peptide and the porphyrin ring and to compare the solution structure of PK-CCK8 with that adopted by CCK8 when complexed with the CCK_A receptor-model [5]. Considering that PK-CCK8 is an analogue of CCK8, it should be reasonable to carry out the NMR characterization in aqueous solution or in micelles as reported by Mierke and coworkers [5] for CCK8. Nevertheless, the presence of the porphyrin moiety covalently bound to the peptide strongly decreases the solubilization of PK-CCK8 in aqueous solution and in water containing micelles. Therefore, the solution characterization of PK-CCK8 was carried out in DMSO also because the high viscosity of this

Table 2 (a) ^{1}H Chemical Shifts (ppm), Amide Proton Temperature Coefficients (ppb/K) and $^3J_{NH\text{-}\alpha CH}$ (Hz) for the Peptide Region of PK-CCK8 in DMSO and (b) ^{1}H Chemical Shifts (ppm) of the Porphyrin Moiety in DMSO at 298 K

AA	NH	αCH	βCH	γCH	Others	$\Delta\delta/\Delta T$	$^3J_{NH\text{-}\alpha CH}$
(a)							
Lys[25]	7.55	4.05	1.65	1.46	δCH_2 1.65 εCH_2 3.42 εNH 8.87	–	7.8
Asp[26]	8.27	4.55	2.66, 2.48			−2.8	6.7
Tyr[27]	7.79	4.42	2.91, 2.72		2,6 H 6.98 3,5 H 6.64	−2.7	7.0
Nle[28]	7.89	4.18	1.59	1.47	δCH_2 1.19 εCH_3 0.80	−1.3	6.8
Gly[29]	7.93	3.66				−1.7	–
Trp[30]	7.94	4.56	3.12, 2.93		2 H 7.11 4 H 7.56 5 H 6.92 6 H 6.97 7 H 7.28 NH 10.73	−3.3	6.8
Nle[31]	8.06	4.16	1.46	1.46	δCH_2 1.16 εCH_3 0.77	−4.5	6.9
Asp[32]	8.15	4.49	2.64, 2.47			−3.8	7.8
Phe[33]	7.79	4.36	3.01, 2.83		Aromatics 7.2	−4.2	8.4

(b)

Protons	ppm
Pyrrole-H_3,H_4	8.78, 8.75
Carboxyphenyl-H_3,H_5	8.68
Carboxyphenyl-H_2,H_6	8.29
Dichlorophenyl-H_3,H_5	8.03
Dichlorophenyl-H_4	7.96

solvent is helpful in reducing the conformational flexibility of the peptide. A section of the ^{1}H-NMR spectrum of PK-CCK8 in DMSO is reported in Figure 3.

The porphyrin protons were assigned on the basis of previously reported results [7]. The ^{1}H-NMR assignment of the peptide chain resonances (Table 2) was carried out by means of TOCSY, NOESY and DQFCOSY experiments according to the standard procedures [23]. The resonance of the Lys^{25} εNH proton covalently linked to the porphyrin moiety appears as a downfield shifted triplet.

The temperature coefficients measured for the backbone amide protons and the $^3J_{\mathrm{NH}\text{-}\alpha\mathrm{CH}}$ coupling constants are reported in Table 2. Ninety-four NOE cross-peaks extracted from the NOESY spectrum, were assigned. The most significant sequential and medium-range NOEs are illustrated in Figure 4. It should be noted that no NOE contacts between the porphyrin ring protons and the peptide chain of the molecule were revealed, with the exception of those correlating the Lys^{25} εNH proton with the 2,6 and 3,5 protons of the adjacent phenyl ring. This observation clearly indicates that the porphyrin macrocycle does not lie close to the PK-CCK8 peptide moiety, thus confirming the results of the preliminary computational study. Moreover, the absence of NOEs involving porphyrin ring and peptide chain protons has allowed us to use only the peptide moiety of PK-CCK8 as a template for the structure calculations. The final input for DYANA calculations consisted of 63 (34 intraresidual, 24 sequential and five medium range) upper limit constraints and 21 angle constraints which were derived from the intraresidual and sequential NOEs and the coupling constants using the the grid search routine of the DYANA program. The 20 calculated conformers with the smallest residual target function were selected and energy minimized. No distance violations above 0.10 Å were observed. The superposition of the backbones of the 20 best DYANA conformers is shown in Figure 5. Clearly, the structure of PK-CCK8 in DMSO consists of a well-defined pseudo-helical *N*-terminal region (from residue 25 to 29), while the *C*-terminal end is flexible. The measured temperature coefficients are in agreement with the calculated structures. In Figure 6 a representative conformer of PK-CCK8 is superimposed with the structure of CCK8 in the complex with the CCK_A receptor [5]. The *N*-terminal segment of the two peptides adopt a very similar conformation. Interestingly, the side chains of PK-CCK8 Tyr^{27} and Nle^{28} residues nicely reproduce the spatial arrangement of the two corresponding residues in CCK8 which interact with the CCK_A receptor model.

On the contrary, the structure of PK-CCK8 is remarkably different from that reported by Temussi

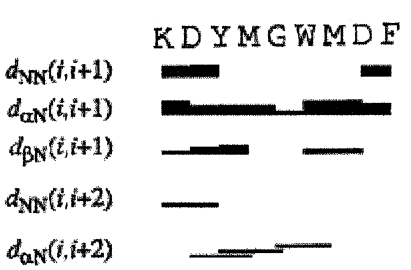


Figure 4 Diagram of the most significative sequential and medium-range NOEs.

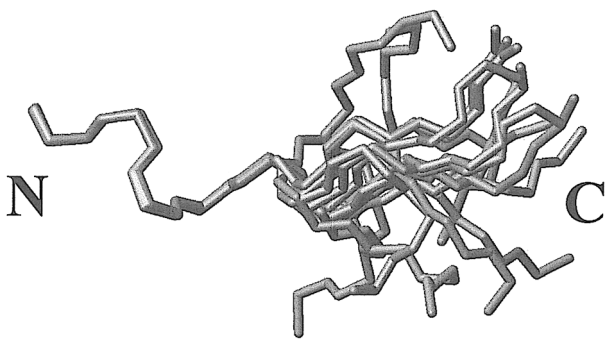


Figure 5 Backbone disposition of the 20 DYANA conformers of PK-CCK8 after the superposition of residues 1–5 (rmsd = 0.18 Å).

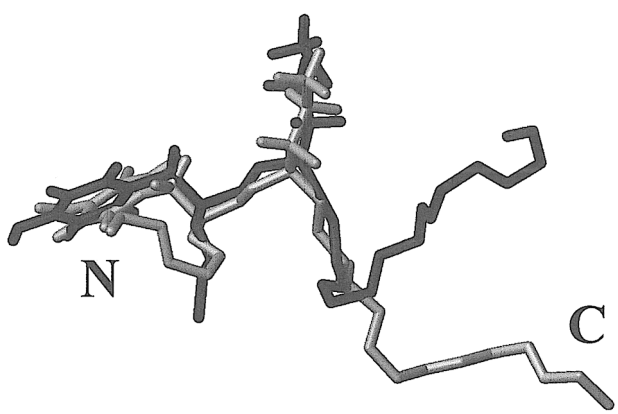


Figure 6 Superposition of one representative conformer of PK-CCK8 (in grey) with the CCK8 structure (in black) when complexed with the CCK_A receptor model [5]. The backbone and the side chains of residues 27 and 28 of both peptides are shown.

and coworkers [24] for the CCK9 peptide, a fully bioactive CCK_A ligand. It should be noted that the sequence of CCK9 differs from that of PK-CCK8 (residue 25 is Arg, residue 29 is Thr and Tyr^{27} is sulphated). Moreover, the NMR characterization of CCK9 was accomplished in a cryomixture (DMSO/H_2O, 80:20, at 278 K).

The NMR characterization of the indium complex In-PK-CCK8 was not performed. In any case, it is reasonable to assume that the In(III) ion complexed with the porphyrin moiety should not affect the conformation of the porphyrin–peptide conjugate.

CONCLUSIONS

In summary, the NMR study and the molecular dynamics simulation indicate that the presence of the bulky porphyrin substituent at the *N*-terminus of CCK8 does not interfere with the conformation of the residues supposedly involved in receptor binding. Therefore, the porphyrin–peptide conjugate might be able to bind to the CCK_A receptor model in same manner as the non-sulphated CCK8 peptide does.

Unfortunately, due to the very low water solubility of our porphyrin–peptide conjugate, we were unable to measure its binding constants both for the entire CCK_A receptor and for the 47-residue receptor-model. New CCK8 derivatives having a water solubilizing chelating moiety covalently bound to the *N*-terminus of CCK8 are currently in preparation. Their metal derivatives containing radioactive isotopes, such as ^{111}In(III), could be used to detect tissue expressing CCK_A receptors by nuclear medicine techniques.

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