Probing the Topological Arrangement of the N- and C-Terminal Residues of Bradykinin for Agonist Activity at the B1 Receptor†

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The conformational features of H-Lys-Arg-Ado-Ser-Pro-Phe-OH (Ado = 12-aminododecanoic acid), a des-Arg^9 analogue of Lys-bradykinin, have been determined by high-resolution NMR in the presence of a zwitterionic lipid environment. The analogue is the most active member of a series of analogues designed to probe the topological arrangement of the N- and C-termini required for agonistic activity at the B1 kinin receptor. A novel computational procedure for the utilization of NOE constraints from cis and trans configurational isomers is illustrated. Only with this computational methodology could the structural features of the N-terminus of the peptide be determined. Using radical-induced relaxation of the ^{1}H NMR signals, we measured the topological orientation of the peptide with respect to the zwitterionic lipid interface. The results indicate that the long, alkyl chain of the Ado amino acid imbeds into the lipid surface. The structural features of the C-terminus of the B1-selective analogue consist of a well-defined turn. Although removed from a standard β-turn, required for activity at the B2 kinin receptor, the topological orientation of the side chains of the des-Arg^9 compound are surprisingly similar to those previously observed for β-turn-containing bradykinin analogues. Therefore, we attribute the high B1 receptor selectivity, observed upon removal of Arg^9 from bradykinin, solely to the loss of a charged amino acid and not to altered structural features.

Introduction

Bradykinin is a nine-amino acid peptide hormone (BK; H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) involved in a variety of physiological and pathological processes, including inflammation, smooth muscle contraction, vasodilatation, increase of vascular permeability, generation of pain, and renal homeostasis.[1,2] Bradykinin is one of the kinin metabolites, together with kallidin (Lys-BK),[3,4] des-Arg^9-BK, and des-Arg^9-Lys-BK,[5] whose action is mediated by two G-protein coupled receptors, termed B1 and B2.[6,7] The two receptors share 36% sequence identity. The B2 kinin receptor is constitutively expressed, and its importance in mediating kinin function at physiological levels is well established.[8,9] Both BK and Lys-BK display high affinity for the B2 receptor, affinity which is drastically reduced with the removal of the C-terminal arginine. In contrast, the des-Arg^9 carboxypeptidase metabolites of BK and Lys-BK display high affinity for the B1 receptor (des-Arg^9-Lys-BK is the only natural kinin with subnanomolar affinity),[1] leading to extremely high receptor selectivity.[10] The expression of the B1 receptor is upregulated following tissue damage or inflammatory processes.[11] It has been suggested that the specific role of the receptor induction may be to amplify the tissue effects of kinins.[10] Due to these therapeutic consequences, considerable attention has been directed toward the study of the B1 receptor, aimed at understanding the mechanism of action and development of molecules to interfere with the inflammatory processes. The development of a structure−activity relationship for BK, BK fragments, and analogues targeted for the B1 receptor will contribute greatly to these efforts. In the absence of the structure of the binary ligand/receptor complex, conformational studies of BK analogues designed to probe specific requirements may be the only manner to proceed.

We have recently described a new class of pseudopeptide kinin analogues, where the central tetrapeptide Pro-Pro-Gly-Phe of BK and des-Arg^9-Lys-BK was replaced by alkyl spacers.[12] These analogues showed significant agonist activity selective for the B1 kinin receptor. In particular, the introduction of 12-aminododecanoic acid (Ado) in the central portion of des-Arg^9-Lys-BK gave rise to a pseudopeptide (H-Lys-Arg-NH-$(CH_2)_{11}$-CO-Ser-Pro-Phe-OH) which, despite the greatly simplified structure, has an EC_{50} value of 980 ± 190 nM, i.e., only 10-fold higher than the unmodified peptide des-Arg^9-Lys-BK (EC_{50} = 85 ± 25 nM).[12] Here, we described the conformational features of the Ado-containing analogue (H-Lys-Arg-NH-$(CH_2)_{11}$-CO-Ser-Pro-Phe-OH) in the presence of zwitterionic, lipid micelles used as a membrane mimetic.

† Abbreviations used: Ado, 12-aminodecanoic acid; BK, bradykinin; doxyl, 4,4-dimethyl-3-oxazolidinyloxy; DG, distance geometry; G-protein, guanine nucleotide-binding regulatory protein; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancements; ROESY, rotational Overhauser enhancement spectroscopy; DPC, dodecylphosphocholine; TOCSY, total correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy, NOESY, nuclear Overhauser enhancement spectroscopy.

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Table 1. Chemical Shifts (ppm) of the Ado Analogue in H_2O/DPC at 12 °C[a]

residue	HN	Hα	Hβ	Hγ	others
			Overlapped Resonances		
Lys^0		4.16	2.02	1.576, 1.81	Hδ: 3.10; Hϵ: 3.09
Arg^1	8.98	4.48	1.88	1.81	Hδ: 3.28; HNϵ: 7.82
Ado	8.62	H(12): 3.17, 3.38	H(11): 1.60		
			Trans Isomer		
Ado					H(4−10): 1.41; H(3): 1.70; H(2): 2.40
Ser^6	8.43	4.60	3.96		
Pro^7		4.59	1.65, 2.13	1.78, 1.33	Hδ: 3.55, 3.91
Phe^8	7.80	4.71	3.02, 3.42		H(2,6): 3.39; H(3,5): 7.35
			Cis Isomer		
Ado					H(4−10): 1.42; H(3): 1.68; H(2): 2.31
Ser^6	8.15	4.34	3.85		
Pro^7		4.81	2.19, 2.28	1.89, 1.31	Hδ: 3.36, 3.52
Phe^8	9.29	4.51	3.33		H(2,6): 7.39; H(3,5): 7.34

[a] The internal reference is tetramethylsilane (0.00 ppm).

The general consensus from conformational studies is that BK exists in many conformational states in aqueous solution,[13] while in alternative solvent systems (dimethyl sulfoxide, sodium dodecyl sulfate micelles, dioxane:water) there is a tendency for the C-terminus to adopt a turn-like structure.[14−20] A C-terminal β-turn was predicted to be energetically favorable from theoretical studies[21,22] and has been successfully utilized in the design of agonists and antagonists.[13,15−18] The highly potent BK antagonist Hoe 140 (Hoechst-140: H-D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9-OH) adopts a β-turn type II′ about D-Tic^7-Oic^8 in water/SDS-micelles,[23] in agreement with the important role for the C-terminal turn in the interaction with the B2 receptor.

Much less data is available on the structural requirements for binding to the B1 receptor. The results on a B1-specific antagonist (B-9858)[24] indicate the presence of a type II β-turn involving residues 2−5 (in a CD_3OH/H_2O solvent mixture). The presence of a β-turn at the C-terminus has not been ascertained and would require a shift in the sequence, due to the lack of the C-terminal arginine, the fourth residue ($i + 3$) in the turn observed for B2-selective BK analogues.

Both the B1 and B2 receptors belong to the family of G-protein coupled receptors, characterized by seven transmembrane hydrophobic helical segments. Computer-based molecular models of the receptors have been developed to assist in the characterization of the ligand/receptor interaction and modes of receptor activation.[25−27] The most likely mode of interaction of the ligand with the receptor is through a membrane associated pathway.[28−31] The theory proposes a favorable ligand/membrane interaction, leading to a high local concentration of the ligand at the lipid surface, as well as inducing the peptide to adopt the correct orientation and conformation for interaction with the receptor.[31−33] Through a lateral, two-dimensional diffusion process, the ligand interacts with the receptor, leading to binding and activation.[28−31] If this is the case, then the conformation of the ligand while associated with a membrane environment is recognized by the receptor and therefore is most important for the development of a structure−activity relationship. Micellar systems of dodecylphosphocholine (DPC) provide a zwitterionic lipid-surface, similar to membranes, which reorients rapidly in aqueous solutions, and therefore is suitable for standard high-resolution NMR methodologies.[34−38]

During the NMR-based structural investigation, a problem with overlapping signals from cis/trans isomerization about proline-7 (using the nomenclature of native BK) was encountered. The residues of the C-terminus had resolved ^1H resonances for the cis and trans isomers, and the structural features for both isomers could be ascertained from the NOEs. In contrast, the N-terminus produced only one set of NMR signals. In general, the NOEs for this region cannot be utilized because of the uncertainty of the contribution to the NOE intensity from each isomer (i.e., does the cis isomer contribute 25%? 50%? 100%?). Here we illustrate the utilization of a recently developed computational procedure to address this problem.[39] Using this refinement method, all of the measured NOEs can be used and the conformational features of the N-terminus, as well as the entire peptide, can be ascertained. From the structural features of this B1-selective analogue, a number of molecular descriptors, which may be important for B1 selectivity, are developed.

Results

The ^1H NMR spectra of the Ado-containing des-Arg^9-Lys-bradykinin analogue in the presence of DPC micelles revealed the presence of both cis and trans isomers in solution, which exchange slowly on the NMR time scale. The presence of exchange peaks in 2D-ROESY spectra (recorded with extended mixing times) verified the assignment of the NMR signals to cis/trans isomerization. The configuration about the Ser^6-Pro^7 amide bond was assigned by diagnostic $H^{\alpha}_i/H^{\delta}_{i+1}$ and $H^{\alpha}_i/H^{\alpha}_{i+1}$ cross-peaks for the trans and cis isomers, respectively. The relative intensities of the amide proton signals of the C-terminus indicate a trans:cis ratio of 57:43. The proton resonance assignments are given in Table 1.

The cis/trans isomerization produces resolved peaks only for the residues in close proximity to the proline residue, i.e., Ado(2−4)-Ser^6-Pro^7-Phe^8 (the methylene resonances of Ado, denoted in the parentheses, are numbered 2−12 according to the carbon to which they are attached). The proton signals for the N-terminus, Lys^0-Arg^1-Ado(5−11), are identical for the two configurational isomers.

From the NOESY spectrum, 47 and 38 NOEs between well-resolved proton signals for the trans and cis isomers, respectively, could be integrated. These NOEs

Table 2. Resolved NOEs of the Trans and Cis Isomers[a]

				distance (Å)	
residue 1	atom 1	residue 2	atom 2	trans	cis
Ado^3	H(4−10) 1,2	Ser^6	HN	3.82	3.99
Ado^3	H(3) 1,2	Ser^6	HN	3.85	3.98
Ado^3	H(2) 1,2	Ser^6	HN	3.18	3.22
Ado^3	H(2) 1,2	Ser^6	Hβ	4.93	
Ado^3	H(4−10) 1,2	Phe^8	HN		4.52
Ado^3	H(2) 1,2	Phe^8	H2,6		5.97
Ser^6	Hα	Ser^6	HN	2.52	2.58
Ser^6	Hβ1,2	Ser^6	HN	4.08	3.01
Ser^6	Hα	Pro^7	Hα		3.00
Ser^6	Hα	Pro^7	Hδ1	2.21	
Ser^6	Hα	Pro^7	Hδ2	2.21	
Ser^6	HN	Pro^7	Hδ1	2.77	
Ser^6	HN	Pro^7	Hδ2	3.19	
Ser^6	HN	Phe^8	HN		3.52
Ser^6	Hα	Phe^8	HN	3.50	2.30
Ser^6	Hβ1,2	Phe^8	HN	4.47	3.95
Ser^6	Hα	Phe^8	Hβ1,2		4.32
Ser^6	Hα	Phe^8	H2,6		5.06
Ser^6	Hβ1,2	Phe^8	H2,6	6.41	6.62
Pro^7	Hα	Pro^7	Hβ1	2.42	
Pro^7	Hα	Pro^7	Hβ2	2.53	
Pro^7	Hα	Pro^7	Hγ1	2.82	
Pro^7	Hα	Pro^7	Hγ2	2.81	
Pro^7	Hβ1	Pro^7	Hγ1	2.39	2.37
Pro^7	Hβ1	Pro^7	Hδ1	2.89	2.79
Pro^7	Hβ1	Pro^7	Hδ2	2.86	2.89
Pro^7	Hβ1	Pro^7	Hδ1		2.88
Pro^7	Hβ2	Pro^7	Hδ2	2.81	2.96
Pro^7	Hβ2	Pro^7	Hδ1	2.30	2.36
Pro^7	Hγ1	Pro^7	Hδ2	2.38	2.21
Pro^7	Hγ1	Pro^7	Hδ2		2.21
Pro^7	Hγ1	Pro^7	Hδ1	2.39	
Pro^7	Hα	Phe^8	HN	2.36	2.89
Pro^7	Hβ1	Phe^8	HN	3.31	3.09
Pro^7	Hβ2	Phe^8	HN	3.17	3.38
Pro^7	Hγ1	Phe^8	HN		3.78
Pro^7	Hγ2	Phe^8	HN	3.13	3.58
Pro^7	Hδ1	Phe^8	HN	3.29	3.55
Pro^7	Hδ2	Phe^8	HN	2.88	
Pro^7	Hα	Phe^8	H2,6	5.19	
Pro^7	Hγ2	Phe^8	H2,6	4.81	
Pro^7	Hδ1	Phe^8	H2,6	5.31	
Pro^7	Hδ2	Phe^8	H2,6	4.61	
Pro^7	Hγ2	Phe^8	H3,5		5.12
Pro^7	Hδ1	Phe^8	H3,5		4.73
Pro^7	Hδ2	Phe^8	H3,5	4.86	
Phe^8	Hα	Phe^8	HN	2.54	2.41
Phe^8	Hβ1	Phe^8	HN	2.48	3.1 (β1,2)
Phe^8	Hβ2	Phe^8	HN	2.65	
Phe^8	H2,6	Phe^8	HN	4.42	4.67
Phe^8	H3,5	Phe^8	HN	4.42	4.12
Phe^8	Hα	Phe^8	Hβ1	2.83	
Phe^8	Hα	Phe^8	Hβ2	2.63	
Phe^8	Hα	Phe^8	H2,6	3.33	
Phe^8	Hα	Phe^8	H3,5	3.97	3.97

[a] The reported distance corresponds to the upper bound utilized in the distance geometry calculations. The symbol Hβ1,2 is utilized for nonresolved methylene protons. For resolved methylene protons, the index 1 refers to the Pro^R and 2 to the Pro^S proton.

were converted to distances using the two-spin approximation and the protons of the δ methylene group of proline-7 as a reference. From the DG calculations and comparison with the molecular constitution of the peptide (holonomeric matrix), 44 and 35 (trans and cis, respectively) of these NOEs were judged structurally informative. The distances calculated for these informative NOEs are given in Table 2. In addition to the resolved NOEs, there are 36 NOEs between protons, listed in Table 3, which are overlapping in the cis and trans isomers. The location of the resolved and overlapping NOEs for the trans isomer is depicted in Figure 1. The unambiguous peaks, denoted by solid lines, are within the C-terminus of the peptide, covering Ado(2−4)-Ser^6-Pro^7-Phe^8. The overlapping NOEs, arising from both the cis and trans isomers, denoted by dashed lines, are concentrated in the N-terminus of the BK analogue.

The overlapping ensemble calculation was initiated with 100 structures, 57 and 43 in the trans and cis

Table 3. Approximate Intensity of the Cross-Peaks Reported for Overlapping NOEs[a]

residue 1	atom 1	residue 2	atom 2	intensity[a]
Lys^0	Hα	Lys^0	H1,2	m
Lys^0	Hα	Lys^0	Hγ1	s
Lys^0	Hα	Lys^0	Hγ2	s
Lys^0	Hγ2	Lys^0	Hϵ1,2	m
Lys^0	*Hα*	*Arg^1*	*Hβ1,2*	m
Lys^0	Hα	Arg^1	HN	s
Lys^0	Hβ1,2	Arg^1	HN	m
Lys^0	Hγ2	Arg^1	HN	m
Lys^0	Hα	Ado	HN	w
Lys^0	Hβ1,2	Ado	HN	w
Arg^1	Hα	Arg^1	HN	s
Arg^1	Hα	Arg^1	HNϵ	m
Arg^1	Hβ1,2	Arg^1	HN	m
Arg^1	Hγ1,2	Arg^1	HN	m
Arg^1	Hδ1,2	Arg^1	HN	w
Arg^1	*HNϵ*	*Arg^1*	*HN*	m
Arg^1	Hα	Arg^1	Hβ1,2	m
Arg^1	Hα	Arg^1	Hγ1,2	m
Arg^1	Hβ1,2	Arg^1	HNϵ	s
Arg^1	Hβ1,2	Arg^1	Hδ1,2	w
Arg^1	HN	Ado	HN	m
Arg^1	Hα	Ado	HN	s
Arg^1	Hβ1,2	Ado	HN	m
Arg^1	Hδ1,2	Ado	HN	w
Arg^1	*HNϵ*	*Ado*	*HN*	w
Arg^1	Hα	Ado	Hβ1,2	w
Arg^1	Hα	Ado	Hγ1,2	w
Arg^1	HN	Ado	Hγ1,2	w
Ado	Hα1	Ado	HN	s
Ado	Hα2	Ado	HN	s
Ado	Hγ1,2	Ado	HN	m
Ado	H(4−10)1,2	Ado	HN	m
Ado	H(12)1	Ado	H(10)1,2	m
Ado	H(12)2	Ado	H(10)1,2	m
Ado	H(12)1	Ado	H(10)1,2	m
Ado	H(12)2	Ado	H(10)1,2	m

[a] The symbol Hβ1,2 is utilized for nonresolved methylene protons. For resolved methylene protons, the index 1 refers to the Pro^R and 2 to the Pro^S proton. [b] s = strong, *m* = medium, w = weak.

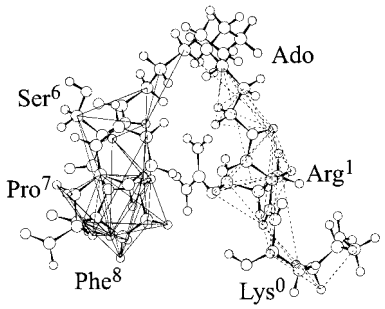

Figure 1. Representation of the NOEs observed for the trans configurational isomer of H-Lys-Arg-Ado-Ser-Pro-Phe-OH. The NOEs from well-resolved ^{1}H resonances which could be unambiguously assigned are indicated by solid lines. The overlapping NOEs arising from the unresolved resonances of the cis and trans isomers are shown as dashed lines. These unambiguous NOEs could only be applied in the structural refinement by the utilization of the overlapping ensemble calculation procedure described here.

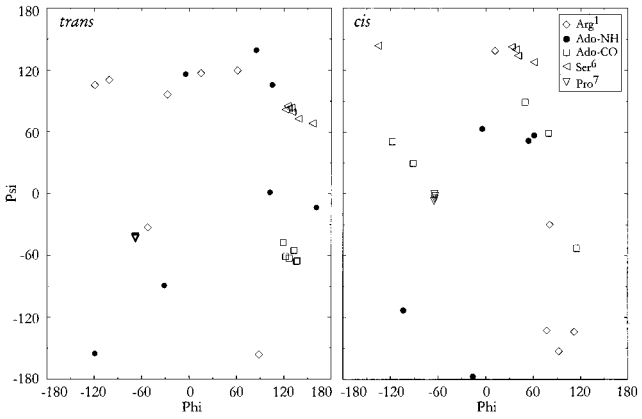

Figure 2. Ramachandran plots of the amino acids for the trans (left) and cis (right) configurational isomers of H-Lys-Arg-Ado-Ser-Pro-Phe-OH obtained from the overlapping ensemble calculations. The original ensemble of 100 structures was reduced to 12 members (7 trans, 5 cis).

orientation about the Ser^6-Pro^7 amide bond, respectively. These structures were generated following standard metric-matrix DG procedures that utilized only the unambiguous NOEs described above. This ensemble, containing the correct ratio of cis/trans isomers, was refined utilizing an ensemble based approach, in which the ensemble average of the trans isomers must fulfill the "trans NOEs" (i.e., those NOEs unambiguously assigned to the trans isomer), and the average over the cis isomers must fulfill the unambiguous "cis NOEs". The overlapping NOEs are applied to the entire ensemble (which has been properly weighted with the 57:43 ratio of cis:trans isomers).[39] The overlapping ensemble method allows for the utilization of the overlapped NOEs without any a priori assumption of conformation, after all the minor isomer, with the protons in question very close to each other, may contribute the majority (or all) of the cross-peak intensity.[39] After optimization, the ensemble was reduced in half (resulting in 28 trans, and 22 cis), by removing randomly chosen members of the ensemble. This reduced ensemble was then optimized against the NOEs. Since the penalty function for fulfillment of the NOEs did not increase, the reduced ensemble was maintained. In this manner, the size of the ensemble consistent with the NMR-derived observations is steadily decreased. This reduction procedure was carried out two more times, producing a final ensemble of 12 structures, with 7 trans and 5 cis isomers. The structural features of this final ensemble are described in detail.

The conformational features of the Lys-BK analogue are illustrated in the form of a Ramachandran map in Figure 2. For the trans isomers, the ϕ,ψ values for Ser^6, Pro^7, and the C-terminal portion of Ado, are tightly clustered (Figure 2A), indicating well-defined structure although not a standard γ- or β-turn. Calculation of dihedral angle order parameters[40] results in values of around 0.95 for the C-terminus, even for the side chain torsions, another indication of well-ordered structure. The effect of applying the overlapping NOEs can be observed in the clustering of the ψ dihedral angle of Arg^1 at 120°, most likely from the weak NOEs between the Lys^0 α and β to Ado$_{HN}$. The remainder of the N-terminus is less defined structurally as evidenced by dihedral angle order parameters below 0.6. Similar conclusions

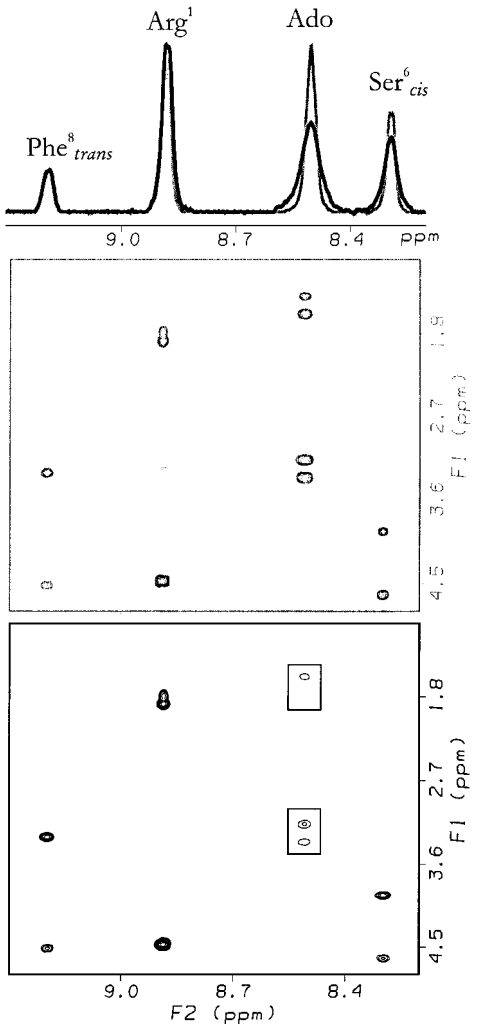

Figure 3. Illustration of the measurement of radical-induced relaxation of ^{1}H NMR signals. Expanded regions of ^{1}H-^{1}H TOCSY spectra are shown before (top) and after (bottom) addition of 5-doxylstearate. The one-dimensional spectrum illustrates the broadening of the some of the amide protons upon the addition of the nitroxide-containing detergent.

can be drawn concerning the conformational features of the cis configurational isomer. The C-terminus is well defined, but does not adopt a standard β- or γ-turn structure (Figure 2B).

The relative topological orientation of the molecule with respect to the lipid surface of the micelle was determined by monitoring the induced broadening of the NMR signals upon introduction of doxyl-sterates which readily incorporate into the micelle and place the radial at different depths within the lipid environment.[34,38,41] Upon the addition of 5-doxylstearate, the amide protons of Ado and Ser^6 broaden significantly while the signals of Lys^0 and Arg^1 are almost unaffected. Monitoring the intensity of the cross-peaks between amide and aliphatic protons in TOCSY spectra (illustrated in Figure 3) reveals the preference of the side chains of Pro^7 and Phe^8 and of the long backbone of Ado for the hydrophobic portion of the micelles.

Discussion

The NMR-based structural examination of peptides containing prolines (or other substituted amides) is often beset with the problem of NOEs originating from

overlapping signals of the cis and trans isomers. Here, we have illustrated the overlap-ensemble method to address the overlapping signals of Lys[0]-Arg[1]-Ado(5−12), a computational procedure which has previously only been shown with perfect, model-based data.[39] For the 36 NOE cross-peaks measured here for the overlapping signals, it is not clear what percentage of the NOE intensity is due from the cis isomer or the trans isomer (e.g., the trans configurational isomer may have the protons at a very long distance, with the cis isomer contributing 100% of the integrated NOE intensity). With standard refinement protocols, the overlapping NOEs could not be utilized because of this uncertainty. For both the cis and trans isomers, the utilization of the overlapping NOEs allowed for the identification of conformational order of the N-terminus, centered around Arg[1], which is important to probe the topological display of the peptide termini required for B1-selective activity.

With resolved signals for the cis and trans isomers and a large number of medium range NOEs, a higher degree of conformational order is observed for the C-terminus. In particular the cis isomer presents an αN-(i,i+2) NOE between Ser[6] and Phe[8], typical of a classic β-turn, together with some peculiar NOEs (e.g., Ser[6]$_{HN}$-Phe[8]$_{HN}$ and Ado(4)-Phe[8]$_{HN}$) suggesting an unusual bent conformation. The structure resulting from the calculations cannot be described as a standard β- or γ-turn. The average distances between the α-carbons, a measure often used for the characterization of turn-like structures, are 4.5 and 5.5 Å for Ser[6]-Phe[8] and Ado-Phe[8] (they would be 5.4 and 4.8 Å for a standard β-turn).

The trans isomer displays two weak NOEs in this region: Ser[6]$_{H\beta}$-Phe[8]$_{ring}$ and Ser[6]$_{H\beta}$-Phe[8]$_{HN}$. As a consequence the average distance between the α-carbons for Ser[6]-Phe[8] and Ado-Phe[8] is 6.0 and 9.4 Å, respectively, well removed from those of a standard β-turn. The structural features are not consistent with a γ-turn about the proline; the average distance between HN Phe[8] and CO Ser[6], which would form the seven-membered hydrogen bond of the turn, is over 5.4 Å.

The absence of a C-terminal β-turn, which is one of the conformational features of BK, is not too surprising. The turn in BK comprises residues Ser[6]-Pro[7]-Phe[8]-Arg[9]: with Arg[9] in the i + 3 position and proline in the highly favorable i + 1 position of the turn. The deletion of Arg[9] in the peptide examined here, a B1 receptor agonist, prevents the formation of such a turn and alters the conformational features of the C-terminus, features which are well defined by a large number of NOEs. It was previously shown that small mutations in the C-terminus sequence have significant consequences on binding and activation (Pro[7]/DPhe[7] and Phe[8]/Leu[8] produce antagonists for the B2 and B1 receptors, respectively).[1,42] In Figures 4 and 5, we compare the C-terminal region of BK with a type-I β-turn (as was observed for (Thr[6])-BK in a micellar environment, encompassing residues Thr[6]-Arg[9])[43] to the corresponding regions of the trans and cis configurational isomer. This representation illustrates the effect of the differing backbone conformations on the topological display of the side chains. For the trans isomer, the Ser[6] and Pro[7] superimpose almost perfectly, both considering the backbone and the side chain atoms (superposition not shown). In addition, as depicted in Figure 4, the overall topology

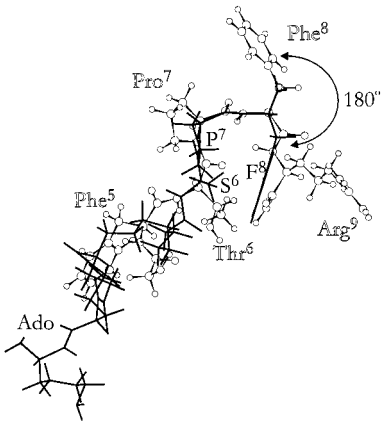

Figure 4. Comparison of the structural features of the C-termini of H-Lys-Arg-Ado-Ser-Pro-Phe-OH determined here (trans isomer; solid line) and the β-1 turn of Thr[6]-bradykinin, Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg (ball-and-stick representation), previously observed.[43] For clarity only Ado-Phe[8] of the Ado analogue (solid labels) and Phe[5]-Arg[9] of Thr[6]-bradykinin (open face labels) are displayed.

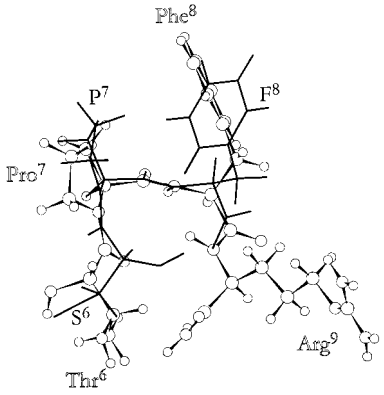

Figure 5. Comparison of the structural features of the C-termini of H-Lys-Arg-Ado-Ser-Pro-Phe-OH determined here (cis isomer; solid line) and the β-1 turn of Thr[6]-bradykinin, Arg-Pro-Pro-Gly-Phe-Thr-Pro-Phe-Arg (ball-and-stick representation), previously observed.[43] For clarity only Ser[6]-Phe[8] of the Ado analogue (solid labels) and Thr[6]-Arg[9] of Thr[6]-bradykinin (open face labels) are displayed. The carbonyl of Ser[6] of the Ado analogue and the Cα of Pro[7] of Thr[6]-BK are also labeled.

of the turn and the projection of the N-termini, with respect to the rest of the molecule, are preserved. The Phe[8] side chains could be matched up with a 180° rotation about ϕ of Phe[8], as indicated in the figure, a relatively minor modification for a C-terminal residue. The superposition with the cis isomer indicates a very good agreement both in the backbone and side chain orientation of the C-terminal residues, as illustrated in Figure 5. The two conformations diverge only at the level of the peptide bond between Ser/Thr[6] and Pro[7], as expected from the difference between the cis and trans configurations. This creates a shift in the superposition of one atom within the proline system, resulting in the placement of the α-carbon of Ser[6] (in the cis isomer) in place of the carbonyl. The similar topological arrangement of the side chain functionalities important for biological activity would suggest that the loss of affinity for the B2 receptor by the deletion of Arg[9] is due solely to the loss of the charge and not to an altered conformation of the C-terminus. It is interesting to note the selectivity of the B1 and B2 receptors has been proposed

to reside in a single amino acid located in the extracellular portion of the third transmembrane helix.[44] Introducing a Lys (found at this position in the B1 receptor) to replace Ser^{111} in the B2 receptor produces a receptor with B1-like selectivity, favoring des-Arg^9 analogues. According to this hypothesis, the presence of the positively charged side chain of Lys^{111} in the B2 receptor is sufficient for the decrease in affinity of BK (containing an Arg^9 residue).[44] The structural findings reported here are in accord with this hypothesis.

One of the aims of the study of this series of des-Arg^9-Lys-BK analogues was to probe the relative topological arrangement of the N- and C-terminal amino acids required for biological activity at the B1 receptor. The approach of replacing the central tetrapeptide of BK (Pro^2-Pro^3-Gly^4-Phe^5) with alkyl spacers was first illustrated by Kyle and co-workers in the design of analogues for the B2 receptor.[25,45] Recently, we synthesized a series of analogues varying the length of the spacer between Lys^0-Arg^1 and Ser^6-Pro^7-Phe^8.[12] The incorporation of shorter alkyl chains, including 6-aminohexanoic, 8-aminooctanoic acid, and 11-aminoundecanoic acid, produced analogues with no or greatly reduced activity.[12] If the distance between termini is an issue for biological activity, the backbone of the Ado residue can stretch to cover approximately 15 Å (N to CO). This should be contrasted with an average distance of 9.5 Å for the Pro-Pro-Gly-Phe tetrapeptide measured from our previous conformational study of (Thr^6)-BK in the presence of a membrane mimetic.[43] From a structural perspective, it is clear that the long alkyl chain of the Ado residue will introduce conformational freedom and flexibility between the N-terminal Lys^0-Arg^1 and C-terminal Ser^6-Pro^7-Phe^8. However, despite this flexibility there is a clustering of distances preferentially populated between the biologically important amino acids of the termini. The distances between the atoms of the side chains of Lys^0-Phe^8 and Arg^1-Phe^8 are depicted in Figure 6 for the reduced ensemble of 12 structures, 7 trans and 5 cis. These distances are representative of those obtained from the optimized ensemble of 100 structures (i.e., 13.7 ± 3.7 and 11.0 ± 3.4 Å for Lys^0-Phe^8 and Arg^1-Phe^8, respectively). The importance of these distances as molecular descriptors will await the collection of similar measures for additional analogues, both active and inactive.

Another issue that may be addressed by the study of the Ado-BK analogue regards the required hydrophobicity of the central tetrapeptide, residues 2−5, in particular with respect to the association of the peptide with the membrane and its topological arrangement. The relative orientation of the molecule with respect to the lipid surface of the micelle was determined by the monitoring of electron-radical-induced relaxation of the NMR signals. With the exception of the first lysine, and partially Arg^1, the peptide is in tight contact with the micelle; the long backbone of Ado is found to interdigitate into the lipid acting as a tethering point of the peptide to the membrane surface. The topological orientation obtained from these data is illustrated in Figure 7. This interaction with the membrane produces the surprisingly small range of distances between the termini, displayed in Figure 6. The interaction with the membrane surface provides the correct topological arrangement of the biologically important regions of the ligand for recognition by the receptor. We can hypothesize that the Ado residue is of sufficient length to interact with the membrane and optimize the distance between the peptide termini. The remaining molecules of this series, with shorter methylene linkers substituting the central tetrapeptide, though reasonably maintaining the membrane affinity, do not allow for the optimal distance between the Lys^0-Arg^1 and Ser^6-Pro^7-Phe^8 regions. The role of the central tetrapeptide in the native sequence, Pro-Pro-Gly-Phe, is therefore to anchor the peptide to the membrane environment and maintain the biologically important amino acids of the N- and C-termini at the correct distance and in the optimal topological orientation for interaction with the receptor. Ongoing efforts include the incorporation of different linkers to replace the central tetrapeptide which are more conformationally restrictive than Ado while maintaining the structural features described here. The analysis of these next-generation compounds will further refine the topological requirements for selective activity at the B1-kinin receptor.

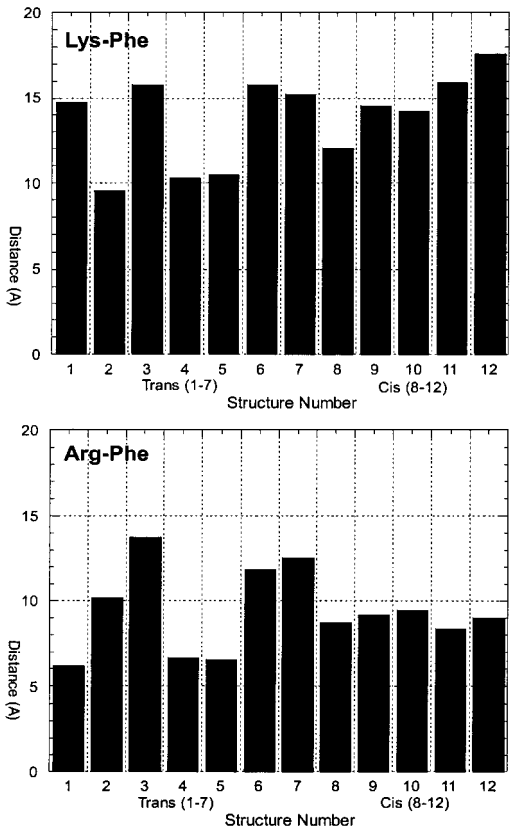

Figure 6. Distances between the side chains of Phe^8 (atom CG) and Arg^1 (atom NE) and Lys^0 (atom CE) observed for the trans and cis configurational isomers from the ensemble of 12 structures (7 trans, 5 cis) after optimization.

In summary we have illustrated the utilization of a novel computational procedure for the handling of NOEs from overlapping resonances from cis/trans isomerization. This structural information was vital to determine the relative topological arrangement of the termini of the ligand and to provide molecular descriptors for future design of B1-selective analogues. The high-resolution structure of the C-terminus indicates similar features for BK analogues previously studied and the Ado-containing des-Arg^9 peptide examined here. This

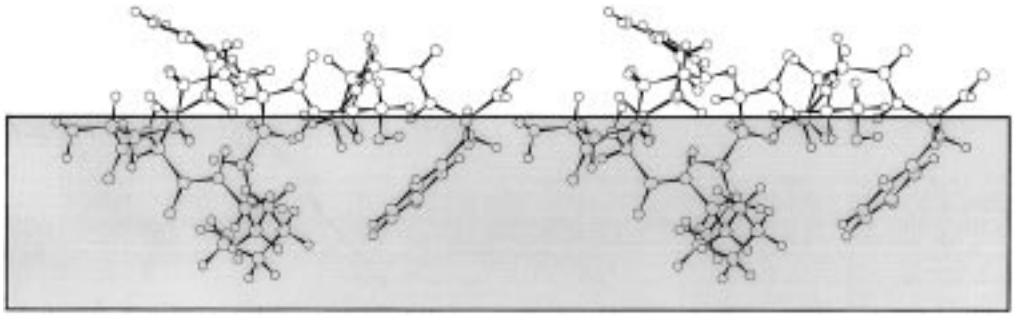

Figure 7. Illustration of the topological orientation of H-Lys-Arg-Ado-Ser-Pro-Phe-OH with respect to the lipid surface as determined by radical-induced relaxation of ^{1}H signals. One of the trans configurational isomers from the reduced ensemble is shown in ball-and-stick format with the shaded area indicating the lipid environment.

suggests that the receptor selectivity observed upon removal of the terminal arginine may be due solely to the loss of the positive charge.

Experimental Methods

Design and Synthesis. The design and synthesis of this series of bradykinin analogues has appeared elsewhere.[12]

Spectroscopic Investigations. The NMR experiments were carried out on a 4.9 mM sample (based on weight) in aqueous solution (90% H_2O−10% D_2O, Cambridge Isotopes) containing 173 mM DPC-d_{38} (98.6%, Cambridge Isotopes). The final pH of the solution was 4.5 (not corrected for isotope effect). Proton spectra were recorded on a Varian Unity 500 MHz spectrometer and processed using Varian VNMR software or *Felix* (Molecular Simulations, Inc.). Chemical shifts were calibrated with respect to internal tetramethylsilane.

For assignment of the spin systems, DQF−COSY,[46] TOCSY,[47,48] and NOESY[49,50] spectra were recorded in the phase-sensitive mode using the method from States.[51] A ROESY[52] spectrum with a mixing time of 200 ms was recorded in order to identify exchange phenomena from the cis/trans isomerization.[53] NOESY spectra were collected at 285−318 K with mixing times varying from 100 to 200 ms. Suppression of the solvent signal was achieved by continuous wave presaturation at low power level during the relaxation delay (1.2−2 s), and for NOESY experiments also during the mixing time. The typical spectral width was 5000 Hz in both dimensions, with 2048 data points in t_2 and 512 data points in t_1 and with 32−128 scans at each increment. Forward linear prediction to 1024 points and zero-filling to 2048 were applied to the incremented dimension. Gaussian apodization was used in both t_2 and t_1. The complete proton assignment is given in Table 1.

Cross-peak volumes from the 200 ms NOESY spectrum were obtained using *Felix* (Molecular Simulations, Inc.). The volumes were converted to distances using the isolated two-spin approximation and utilizing the cross-peaks between the two β-methylene protons of the prolines as a reference (1.78 Å), independently for each configurational isomer. No evidence of spin diffusion was observed up to a mixing time of 200 ms. The distances were adjusted by ±10% to produce the upper and lower distance restraints (Table 2). Pseudo atoms were used for aromatic protons and for methylene protons that could not be stereospecifically assigned, with the appropriate correction of the upper distance restraint following standard procedures.[54]

Radical-Induced Relaxation. 5-Doxylstearic acid was solubilized in methanol-d_4 to a final concentration of 53.3 mM. Aliquots of this solution were added to the solution of peptide and DPC to obtain 0.58 to 1.16 mM concentrations of the spin-label (spin-label:micelle concentration ratio of 1:5 to 1:2.5 approximately, for an aggregation number of 50 for DPC[34,41]). 1D and TOCSY experiments (mixing time 35 ms) were recorded under identical conditions before and after the addition of the doxylstearic acid. The intensities of cross-peaks involving both backbone (H^N-H^α) and side chain protons (H^α-H^β, H^γ-H^δ) were compared.

Structure Refinement. The metric-matrix DG calculations were carried out with a home-written program utilizing the random metrization algorithm of Havel.[55] Experimentally determined distances (obtained from the well-resolved NMR resonances of the C-terminus of the peptide) which were more restrictive than the geometric distance bounds (holonomic restraints) were added to create a distance matrix.[56] The structures were first embedded in four dimensions and refined following published procedures.[43,57] The resulting structures were then reduced to three-dimensions using metrization, and the optimization procedure was repeated. The 100 structures produced following this procedure did not utilize any of the NOEs of the N-terminus of the peptide.

Overlapping-Ensemble Calculations. In the standard ensemble method, the NOEs are treated separately from the holonomeric distances. Each member of the ensemble must fulfill the holonomeric distances and thereby maintain the correct geometry (e.g., bond lengths, angles) of the molecule. For the NOE restraints, the average over the ensemble is utilized. If the ensemble-averaged distance is too long, a restraining force to shorten the distance is applied to the entire ensemble.[58−61] To these two restraining functions, an additional penalty function was applied to force the ensemble to reproduce the volume of the overlapping cross-peak.[39] The ensemble-averaged distances for each isomer are converted to volumes using a reference cross-peak, between methylene protons, with a defined volume (using the isolated two-spin approximation):

$$\sigma_{ij} = \sigma_{\mathrm{ref}}(r_{ij}/r_{\mathrm{ref}})^6$$

A simple harmonic penalty function is then applied to minimize the differences between the ensemble-calculated volume and target volume. All distance geometry and ensemble-based refinement calculations were carried out on a Silicon Graphics Indy (R5000, 180 MHz) computer. The refinement of an ensemble of 100 structures of the peptide required approximately 2 CPU hours.

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