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Conformational studies of a benzodiazepine-like peptide in SDS micelles by circular dichroism, ^1H NMR and molecular dynamics simulation

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SUMMARY

The conformation of a benzodiazepine-like decapeptide corresponding to the YLGYLEQLLR fragment of a casein has been examined in a sodium dodecyl sulfate micellar medium using circular dichroism, two-dimensional ^1H NMR spectroscopy and restrained molecular dynamics simulation. The decapeptide adopts an amphipathic 3_{10} -helicoid structure in which the E⁶...R¹⁰ ionic bridge stabilizes the C-terminus.

INTRODUCTION

Many authors have evidenced that various enzymatic hydrolysates of milk proteins contain biologically active peptides [1-5], and some of these peptides have been shown to be active *in vivo* [6]. Recently, a tryptic casein hydrolysate has been found to decrease pentylentetrazole-induced convulsions in rats, to present benzodiazepine-like activity in a behavioral test revealing anxiolytic properties of drugs [7], and to have an appreciable affinity to the benzodiazepine site of the GABA_A receptor [8]. Only the YLGYLEQLLR fragment of this casein displays that activity.

New agonists without the secondary effects associated with long-term benzodiazepine treatment should be of greatest pharmacological interest. In

this context, the conformation of the decapeptide fragment is probably critical for the benzodiazepine-like activity, and we have investigated its structure in a sodium dodecylsulfate (SDS) micellar medium by circular dichroism (CD), energy minimisation (EM) and restrained molecular dynamics (MD) using nuclear Overhauser effects (NOEs) as geometrical constraints.

MATERIALS AND METHODS

Synthesis and binding tests

The decapeptide casein fragment was synthesized on /4-(oxymethyl)-phenyl-acetamidomethyl (PAM) resin (0.56 mmol/g substitution, Neosystem, Strasbourg, France), using Boc solid-phase peptide synthesis protocols on a Dupont (Wilmington, DE, U.S.A.) Coupler 250 peptide synthesizer. The peptide chain was assembled by sequen-

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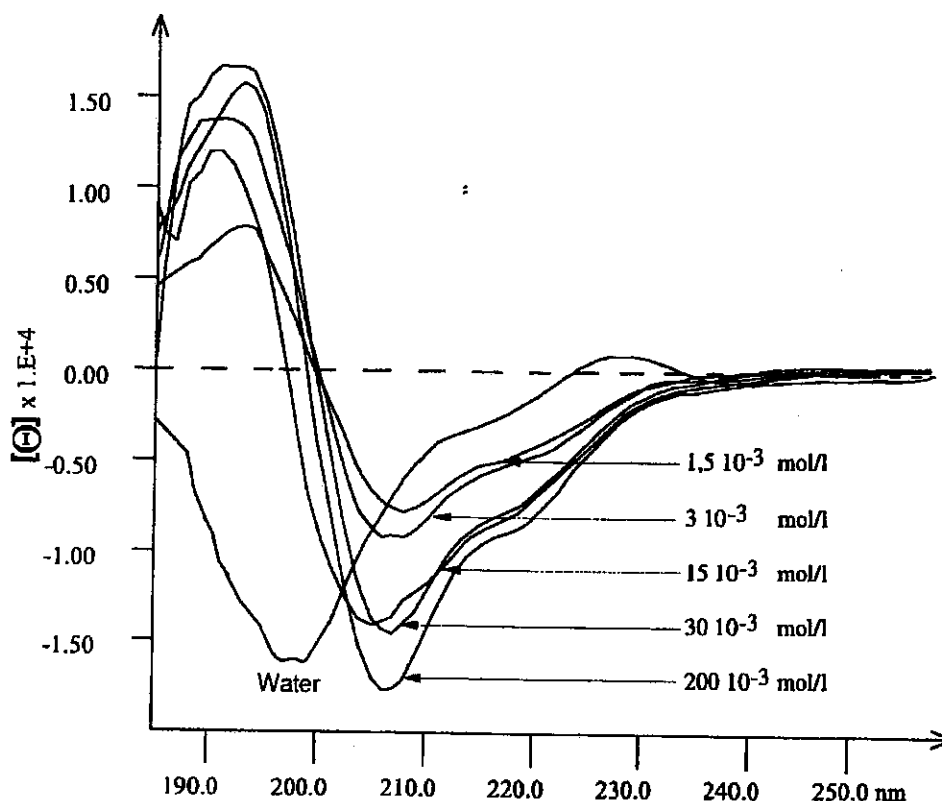


Fig. 1. Evolution of the CD spectrum for the casein fragment as a function of SDS concentration in water. The peptide concentration is 7.9×10^{-5} M in phosphate buffer (pH = 5.9).

tial coupling of the Boc-amino acids (3 equiv) in the presence of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 3 equiv) and diisopropylethylamine (DIEA, 9 equiv) in dimethylformamide/dichloromethane (1/3) mixture. Standard cleavage using trimethylsilyl trifluoromethane-sulfonate (TMSOTf) and trifluoroacetic acid (TFA) with thioanisole and 1,2-ethanedithiol as scavengers afforded the crude peptide, which was desalted on Sephadex G-25 and eluted with the quaternary solvent BuOH/pyridine/AcOH/H₂O (15:10:3:12). Reversed-phase HPLC was performed on a Merck (Darmstadt, Germany) L-6200 chromatograph coupled to a Jasco (Tokyo, Japan) 875 UV detector using a 1×25 cm Lichrocart packed with Lichrospher[®] WP300 RP-18 (5 μm) using a linear gradient of A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile, from 69% A to 39% A

over 40 min (4 mL min⁻¹, 214 nm). Matrix adsorption laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) was carried out on a Bruker (Rheinstetten, Germany) Protein TOF spectrometer ($M + 1 = 1268.48$ (calculated), 1268.27 (found)). The IC₅₀ value in binding assays to the GABA_A receptor in competition with [³H]flunitrazepam is 5.9×10^{-4} M.

CD and NMR spectroscopy

CD spectra were recorded at 25 °C on a Jobin Yvon (Longjumeau, France) CD6 spectrophotometer using a 1 mm path-length quartz cell. The decapeptide (7.9×10^{-5} M) was investigated in phosphate buffer (pH 5.9) with increasing SDS concentration up to 0.2 M. CD spectra are reported in ellipticity units per mole of peptide residue ($[\theta]_R$ in deg cm² dmol⁻¹).

NMR experiments were performed at 25 °C on the casein fragment (5 mM) in H₂O/D₂O (95:5) containing perdeuterated SDS-*d*₂₅ (250 mM), using a Bruker DRX-400 spectrometer and 3-(trimethylsilyl)propionic-2,2,3,3-*d*₄ acid, sodium salt (TMPS) as an internal standard. The 2D NOESY experiments were recorded with four mixing times (100, 200, 350 and 500 ms) in order to determine the mixing time interval where no spin diffusion occurs [9]. The WATERGATE sequence was used to suppress the water resonance and retain the exchangeable proton signals [10]. The interproton distances were calculated from the ratios of the cross-relaxation NOEs [9] and from the Y⁴-C^βH₂ interproton distance as a reference. A set of 39 backbone-backbone and 10 backbone-side chain constraints with interproton distances equal to the NOE distances ±0.5 Å were used for restrained MD calculations.

Molecular modeling

On the basis of the CD analysis and NOESY results (vide infra), the decapeptide casein fragment was built in the 3_{10} and α -helix form, and submitted to EM and MD calculations using the DISCOVER (Biosym, San Diego, CA, U.S.A.) program, and applying force constants of 12 and 4 kcal mol⁻¹ Å⁻² to the backbone-backbone and backbone-side chain constraints, respectively. Both calculations converged toward two quite similar conformers. In vacuo MD simulations of 0.1 ps each, using a six-step temperature elevation at 50, 100, 150, 200, 250 and 300 K, were run and followed by a 25 ps restrained MD run under temperature control. After thermal equilibration, a 320 ps restrained MD with weak coupling (0.1 ps) to the thermal bath was performed. The last 30 ps period of the MD simulation, which exhibited no substantial conformational changes, was used for conformational averaging and analysis. A 3r dielectric constant for Coulombic interactions and a double cutoff at 12.5 and 14 Å for all nonbonded interactions were applied.

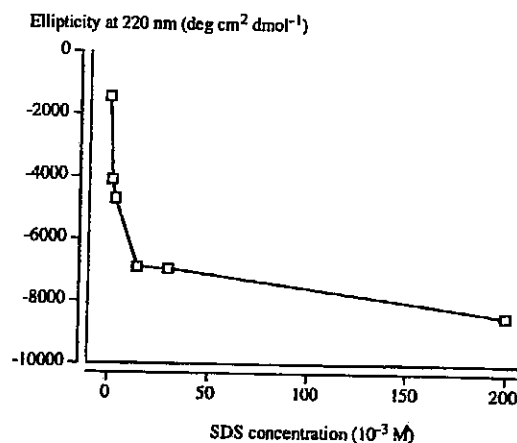


Fig. 2. Variation of the CD ellipticity at 220 nm as a function of SDS concentration in water.

RESULTS AND DISCUSSION

The dependence of the CD spectrum for the casein fragment on SDS concentration (Fig. 1) is typical of a transition from a random conformation in pure water (negative band at 198 nm) [11] to an ordered form presenting two negative bands at 208 and 220 nm. The ellipticity at 220 nm exhibits a rapid variation with SDS concentrations below the critical micellar concentration (about 8×10^{-3} M), and reaches a plateau above the SDS critical micellar concentration (Fig. 2). Under these conditions, the relative intensities of the two minima at 208 and 220 nm are in favor of a 3_{10} -helix rather than of an α -helix [12], although a certain percentage of the latter cannot be excluded. Similarly, the appearance of NOE connectivities on addition of SDS in water (Fig. 3) reflects the peptide-micelle interaction and the restricted flexibility of the peptide in this micellar medium. Due to proton resonance broadening in the presence of SDS, the vicinal coupling constants could not be determined.

The temperature coefficients (Table 1) reveal that the C-terminal NHs are less solvent-exposed than the N-terminal ones, and support the hypothesis of a helical structure. The R¹⁰-N^εH proton and one R¹⁰-N^ηH guanidinium proton are also solvent protected and most probably involved in a salt bridge with E⁶-C^δO₂⁻ and/or R¹⁰-CO₂⁻ [13]. The other three R¹⁰-guanidinium protons are in rapid exchange with wa-

TABLE 1
DIMENSIONS (Å, °) AND OCCURRENCE (%) OF THE HYDROGEN BONDS IN THE DECAPEPTIDE CASEIN FRAGMENT DURING THE LAST 30 PS OF RESTRAINED MD SIMULATIONS

Donor	Temperature coefficient (10 ⁻³ ppm/K)	Acceptor	Hydrogen bond type	N-H...O		Occurrence (%)
				Distance (Å)	Angle (°)	
L ² -NH	-5.1					
G ³ -NH	-6.4					
Y ⁴ -NH	-4.4					
L ⁵ -NH	-5.8					
E ⁶ -NH	-1.4	L ² -CO	i+4 → i	2.78	132	68
Q ⁷ -NH	-4.0	Y ⁴ -CO	i+3 → i	2.80	137	90
L ⁸ -NH	-2.1	L ⁵ -CO	i+3 → i	3.18	125	67
L ⁹ -NH	-2.9	E ⁶ -CO	i+3 → i	2.85	134	74
R ¹⁰ -NH	-3.3	E ⁶ -CO	i+4 → i	2.63	170	90
Q ⁷ -NH ₂	-4.1					
Q ⁷ -NH ₂	-4.2					
R ¹⁰ -N ^ε H	-1.8	R ¹⁰ -CO ₂ ⁻		2.82	144	98
R ¹⁰ -N ^η H	-1.6	E ⁶ -C ^δ O ₂ ⁻		2.95	114	75

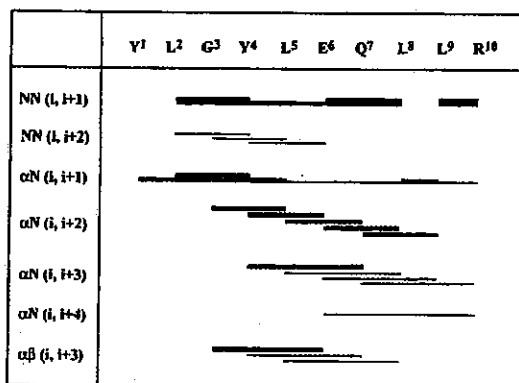


Fig. 3. ¹H/¹H NOE connectivities for the casein fragment in SDS aqueous solution. Strong, medium and weak intensity of the NOE cross peaks is indicated by the thickness of the lines.

ter and not visible at room temperature. The successive medium α N(i,i+2) cross peaks argue for the occurrence of a 3_{10} -helix spanning the Y⁴-L-E-Q-L-L⁹ sequence, in agreement with the small temperature coefficients for the last five C-terminal residues (Table 1). The weak α N(i,i+4) E⁶-C^αH/R¹⁰-NH NOE cross peak suggests that the R¹⁰ C-terminal residue could participate in an α -turn.

The time-averaged structure obtained from the 150 conformers in the last 30 ps MD calculations and subsequent EM is depicted in Fig. 4. Only two of the backbone-backbone and four of the backbone-side chain interproton distances are out of the margins by less than 0.45 Å, and the largest violations are observed for the latter. Such differences are commonly observed for oligopeptides which are known to adopt rather flexible structures, and especially in the orientation of their side chains [14]. The short N...O distances (Table 1) show three consecutive i+3 → i interactions, typical of a 3_{10} -helix encompassing the Y⁴-L-E-Q-L-L⁹ sequence, and two i+4 → i interactions, typical of an α -turn, involving the N-terminal L²-G-Y-L-E⁶ and C-terminal E⁶-Q-L-L-R¹⁰ sequences.

The C-terminus of the molecule exhibits the R¹⁰-N^εH to R¹⁰-CO₂⁻ and R¹⁰-N^ηH to E⁶-C^δO₂⁻ ionic interactions, in agreement with the small temperature coefficients pointed out for these guanidinium resonances. An ionic interaction between arginine guanidinium and glutamic carboxylate is actually known to be an important helix-promoting factor [17]. One notes the amphiphilic character of the resulting structure (Fig. 4), placing the Y¹, L², Y⁴, L⁵, L⁸ and L⁹

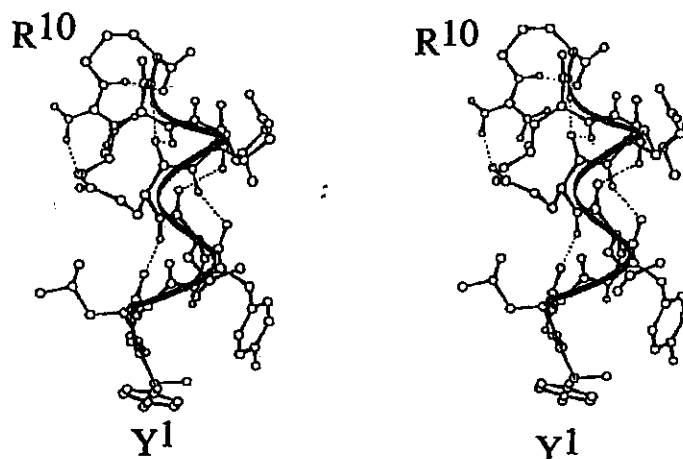


Fig. 4. Stereoview of the time-averaged structure from the last 30 ps of restrained MD simulation for the decapeptide casein fragment.

hydrophobic side chains on one side of the molecule, and the three E⁶, Q⁷ and R¹⁰ hydrophilic side chains on the opposite side. Such an amphiphilic structure allows its interaction with the apolar core of the micelles [15,16], which is a rough approximation of the cellular membrane in which the benzodiazepine receptor is anchored.

Although the affinity of the natural casein fragment to the GABA_A receptor is much lower than that of the benzodiazepines [8], their competitive recognition by the same receptor suggests that they should share common structural properties. Active benzodiazepines contain two aromatic rings, but only the benzo ring seems to be required for a good recognition by the receptor, as for the strong antagonist 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5a][1,4]benzodiazepine-3-carboxylic acid ethyl ester (Flumazenil) [18]. This observation suggests that one of the phenol rings could play the same role as the benzo ring in benzodiazepines. Experiments aiming at the determination of the essential residues will be reported elsewhere.

CONCLUSIONS

The conformation of the decapeptide casein fragment (YLGYLEQLLR) has been determined in the SDS-water micellar medium by combining 2D

NMR and restrained MD simulations. The time-averaged structure of the decapeptide is an amphiphilic 3_{10} -helix initiated and terminated by an α -turn. The ionic interactions between the R¹⁰-guanidinium and the R¹⁰- and E⁶-carboxylates illustrate the key role of the C-terminal arginine residue in the stabilization of the helix structure. The tyrosine phenol rings probably play a similar role to that of the aromatic rings in the benzodiazepines.

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