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High resolution ^1H NMR study of the solution structure of the S4 segment of the sodium channel protein

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A peptide (S4) of the rat brain sodium channel has been synthesized, studied by high-resolution NMR and its secondary structure modelled by distance geometry and restrained molecular dynamics techniques.

Na- channel; Peptide, S4-; NMR; Peptide structure

1. INTRODUCTION

The voltage-gated sodium channel is an integral membrane protein which fulfills a fundamental role in the generation and propagation of action potentials in most multicellular organisms. The functioning of the channel centres around the voltage and time dependence of its sodium conductance; this has been investigated extensively and the physiology of these processes is well understood.

Sequences of the sodium channel protein have recently become available for several species [1–5]. In each case the sodium channel molecule is predicted to be a large (over 1800 amino acids) single-chain polypeptide with four homologous domains. It has also been noted that these sequences have homology with a calcium channel protein [6] and this suggests that voltage-dependent ion channels represent a family of evolutionarily and structurally related proteins.

The amino acid sequences have led to various proposed models of sodium channel structure. With the exception of that of Kosower [7], they have considerable similarities. They predict that each homologous domain contains either 6 [4] or 8 [8–10] transmembrane α-helices, all of which are either hydrophobic or amphipathic except a segment designated S4 [4,9] or s_d [8], which contains a positively charged residue (i.e. an Arg or Lys) at almost every third position in its sequence. This segment is highly conserved betwen sodium channels of different species suggesting it has an important role to play in the mechanism of voltage-gated channels.

Most models have assumed that S4 is the voltage sensor of the sodium channel. The positive charges are envisaged to be evenly distributed about the face of an α-helix and ion paired with acidic residues on neighbouring transmembrane helices. In the 'sliding helix' model of sodium channel activation [9,11], membrane depolarisation causes the S4 helix to spiral towards the extracellular membrane surface; each 60° rotation causes a lateral movement of 4.5 Å. The net effect of the helix movement is to move one positive gating charge across the membrane for each S4 segment; this would explain the gating charge associated with sodium channel opening [12]. The movement of the helix could also induce a conformational change leading to sodium channel activation [11]. In the absence of structural data such models of S4's involvement in sodium channel activation are speculative although they are strongly supported by recent studies on the effect of site specific amino acid changes in the S4 segment [13].

In this work we assessed the models of S4 by synthesizing the S4 segment of the first internal repeat of the rat brain I and II sodium channels and examining its solution conformation by high-resolution NMR. We synthesized the sequence proposed for S4 by Noda et al. [1] with lysine as the 21st and final residue; it should be noted that the sequence proposed for S4 by Greenblatt et al. [8] ends four residues earlier (and thus contains only 17 residues), while that proposed by Guy and Seetharamulu [9] is the same length but begins and ends four residues earlier.

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2. MATERIALS AND METHODS

The 21 residue peptide Ala-Leu-Arg-Thr-Phe-Arg-Val-Leu-Arg-Ala-Leu-Lys-Thr-Ile-Ser-Val-Ile-Pro-Gly-Leu-Lys was synthesized on an Applied Biosystems model 430A synthesizer using *t*-butyloxycarbonyl (*t*-Boc) protection of the amine groups. The arginines were double coupled and the side chains blocked with mesitylene-2-sulphonyl. A phenylacetamidomethyl (PAM) linker was used between the carboxy-terminal amino acid and the polystyrene resin. The peptide was cleaved from the resin using trifluoromethanesulphonic acid and some impurities were removed on a G25-80 Sephadex (bead size 20–80 μm, fine) column (240 mm × 15 mm ID), eluting with 0.1 M ammonium acetate. The peptide was further purified on a semipreparative C18 reverse-phase HPLC column (300 mm × 8 mm) packed with Whatman Partisil-10 ODS-3 resin using 0.1% trifluoroacetic acid and acetonitrile as eluents. The eluate was monitored at 220 nm; one main peak was apparent and this was collected and passed through the HPLC column a second time to produce a product at least 95% pure. Amino acid analysis confirmed the composition. On lyophilisation a white powder was obtained. This was freely soluble in water but only soluble up to about 2 mM in methanol tending to form a gel at higher concentrations. In the NMR experiments described here, the peptide was dissolved in CF_3CD_2OH with approx. 11% water, or in CF_3CD_2OD plus D_2O. The concentration of S4 peptide ranged between 7 and 9 mM and the pH of the solutions was adjusted with HCl and NaOH (or DCl and NaOD) to lie between an uncorrected glass electrode reading (pH*) of 2.1 and 2.75. The sodium chloride concentration in the solutions was either 5.5 mM or approx. 0.1 M.

^{1}H NMR spectra with solvent OH suppression were recorded on a Bruker AM 500 spectrometer at 303 K as described previously [14]. Chemical shifts were referenced to the centre of the quartet due to the residual methylene resonance of CF_3CD_2OH at 3.88 ppm. One-dimensional spectra were recorded with a spectral width of 5000 Hz acquiring 32K, 16K, or 8K data points. The higher resolution spectra were used for measuring coupling constants (^3J).

Two-dimensional double-quantum-filtered COSY, RELAY COSY and NOESY spectra were acquired in the phase-sensitive mode with standard Bruker microprogrammes. The single-relay COSY experiments were recorded with a mixing time of 30 ms, and the double-relay experiment with mixing times of 30 ms and 40 ms. Mixing times of 200, 300, 400 and 450 ms were used for NOESY spectra.

Amide hydrogens which were relatively slow to exchange with solvent were identified in two experiments by dissolving protonated S4 peptide in deuterated solvent and recording spectra over a number of hours at 303 K.

The NOEs derived from the 2D NOESY spectra were classified according to relative strengths of the cross peaks. Using a manual contour counting method, NOEs were classified as weak, medium and strong and assigned to maximum distances between the interacting protons of 5, 3.5 and 2.5 Å, respectively, following established procedures [15]. We have previuosly found this generalization to hold reasonably well for peptides in organic solvents [14]. The computer program DISMAN [16] was used to generate 32 putative structures from the set of distance restraints starting from both random and semi-random conformations. A subset of these structures selected on the basis of minimum violation of the restraints was further refined by energy minimisation and restrained molecular dynamics (RMD) using the GROMOS package of programs [17] as described previously [14,18].

Table 1

Assignments of resonances to protons in S4 segment of sodium channel protein

Residue	Chemical shifts (ppm)			
	NH	αCH	βCH	Others
A1		4.12	1.67	
L2	8.28	4.34	1.76,1.72	γCH 1.72 δCH_3 1.00,0.94
R3	8.19	4.14	1.94,1.92	γCH_2 1.76,1.67 δCH_2 3.21 εiNH 7.17
T4	7.61	3.96	4.11	γCH_3 1.22
F5	7.69	4.37	3.26,3.20	2,6-H 7.24 3,5-H 7.31 4-H 7.26
R6	7.95	3.99	2.01	γCH_2 1.88,1.72 δCH_2 3.23 εNH 7.20
V7	7.80	3.72	2.21	γCH_3 1.07,0.98
L8	8.09	4.09	1.84,1.56	γCH 1.82 δCH_3 0.91,0.91
R9	8.06	3.94	1.88,1.82	γCH_2 1.68,1.58 δCH_2 3.11 εNH 7.09
A10	7.94	4.13	1.60	
L11	8.56	4.12	1.96,1.54	γCH 1.92 δCH_3 0.89,0.86
K12	8.42	4.06	1.98,1.86[a]	γCH_2 1.57[a] δCH_2 1.69 ϵCH_2 2.94 ϵNH_3^+ 7.48
T13	7.90	4.02	4.47	γCH_3 1.27
I14	8.13	4.02	2.02	γCH_2 1.66,1.34 γCH_3 0.98 δCH_3 0.88
S15	7.77	4.33		
V16	7.47	4.26	2.31	γCH_3 1.05,0.99
I17	7.54	4.35	1.99	γCH_2 1.72,1.27 γCH_3 0.99 δCH_3 0.91
P18		4.39	2.28,1.94	γCH_2 2.11,1.99 δCH_2 4.01,3.66
G19	7.82	4.07,3.84		
L20	7.62	4.38	1.67	γCH 1.69 δCH_3 0.96,0.90
K21	7.71[a]	3.71[a]	1.97[a],1.81[a]	γCH_2 1.47[a] δCH_2 1.70 ϵCH_2 3.02 ϵNH_3^+ 7.43

[a] Indicates some uncertainty in the value of chemical shift

3. RESULTS

Data from all the 2D NMR experiments contributed to the assignment of proton resonances, and values of chemical shifts are given in table 1 for an 9 mM solution of sodium channel S4 peptide in CF_3CD_2OH with approx. 11% water and 5.5 mM sodium chloride (pH* 2.74). It was not possible to be completely certain of the proton resonances of the two lysines nor to identify the β-proton resonances of the serine and Leu-20.

Changing sodium chloride concentration from 5.5 mM to 0.1 M and/or changing water content from

2.5% to 11.9% caused various changes, of the order of 0.05 ppm, in the chemical shifts of amide resonances but other resonances showed little movement.

From the NOESY spectra it was possible to trace connectivities between sequential NHs along the backbone of the peptide from Leu-2 to Ile-17 except between Leu-8 and Arg-9; a cross peak between these NH protons would be too near the diagonal to observe (fig.1). The NOEs observed between NH resonances of Leu-2 and Arg-3, Arg-3 and Thr-4 at the beginning of the peptide, and between Ser-15 and Val-16, Val-17 and Ile-17, situated just before Pro-18, were weak. Fig.2 summarises the interresidue NOEs observed in the S4 peptide. There are a considerable number of α_i-NH_{i+3} and α_i-β_{i+3} and a few α_i-NH_{i+4} NOEs, indicative of an α-helical structure [15].

Values of the $^3J_{NH\text{-}\alpha CH}$ coupling constants for residues Leu-2 to Ile-14 are given in table 2. They are all less than 6 Hz which is consistent with an α-helical structure [19]. Addition of water up to 24% caused an increase in the coupling constants for some amide resonances, particularly those of valine and threonine. The ϕ angles, calculated from the measured coupling constants using the Karplus equation as modified for proteins by Pardi et al. [19], are shown in table 2. In cases of degeneracy the members of the solutions set which are closest to α-helical values were chosen (for an α-helix, $\phi = -48°$). Table 2 also shows average values of ϕ for the structures shown in fig.3. The structural information gained from ^3J-coupling constant data was used to check the validity of the structures determined.

From the NOE data, 73 inter-residue restraints and 59 intra-residue restraints were used to calculate the structure. Twelve structures resulting from application of DISMAN and 10 ps of molecular dynamics treatment showed a well deefined α-helical region for the first 14 residues of the peptide (RMSD 1.6 Å for backbone atoms of these residues) followed by a poorly defined region from which very few NOE restraints were obtained. Fig.3 shows the 6 best-fitting structures after 10 ps RMD and energy minimisation. The side chains of Phe-5, Val-7, Leu-8 and Leu-11 are well defined.

It has been suggested [7] that the S4 peptide might take up a 3_{10} helical conformation. When the starting structure for the distance geometry calculations was a

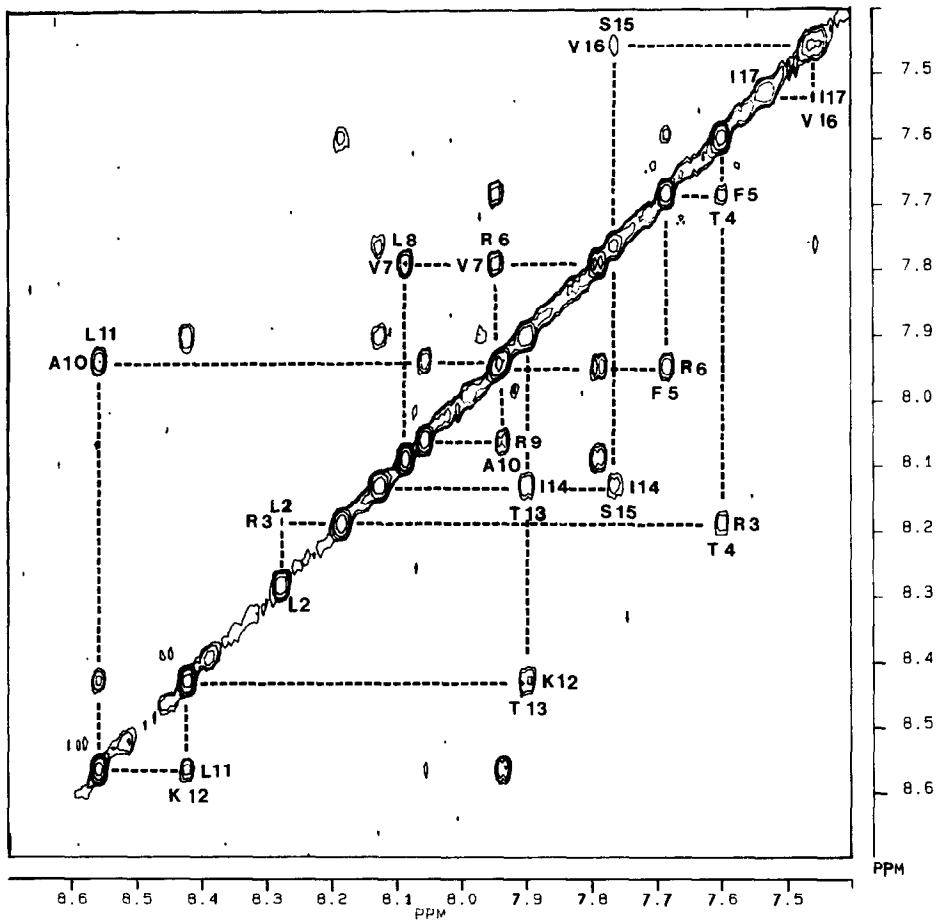

Fig.1. 500-MHz phase-sensitive NOESY spectrum of the S4 segment of the sodium channel protein (9 mM) in CF_3CD_2OH with 11% water (T = 303 K; mixing time = 400 ms; pH* = 2.74; NaCl = 5.5 mM). 4K (real and imaginary) points were collected in f_2 with 80 scans per t_1 increment; 504 t_1 values were acquired and the data were filtered by shifted sine bell multiplication in each dimension and zero filled to 2K in f_1. The NH-NH region is shown with connectivities traced between cross peaks.

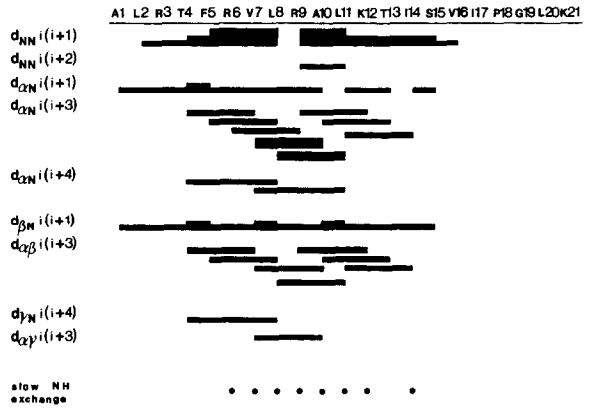

Fig.2. Summary of inter-residue NOEs observed for the S4 segment of the sodium channel protein and used in calculation of the structure. The bar height is an indication of the approximate intensity of the NOE. The amide hydrogens that remained protonated after 12 h in perdeuterated solvent are also indicated.

3_{10} helix, application of the DISMAN and GROMOS programs to the NMR data produced an α-helical structure. This demonstrates that a 3_{10} helix is not a conformation favoured by the experimentally derived distance restraints.

The occurrence of specific $C=O_i$ to $NH_{(i+4)}$ hydrogen bonds was estimated from restrained molecular dynamics calculations as the percentage of time for which a particular bond is present in the last 5 ps of the RMD trajectory (5–10 ps) [18]. Hydrogen bond occurrence was found to be over 50% for residues between Arg-6 and Ile-14. Confirmation of the presence of hydrogen bonds was provided by NMR data on the exchange rates of deuterium for the amide protons. The exchange rates were low over the same region of the peptide (fig.2); indeed no rate at all could be measured for the amide protons of Leu-8, Arg-9, Ala-10 and Leu-11 over a period of 18 h.

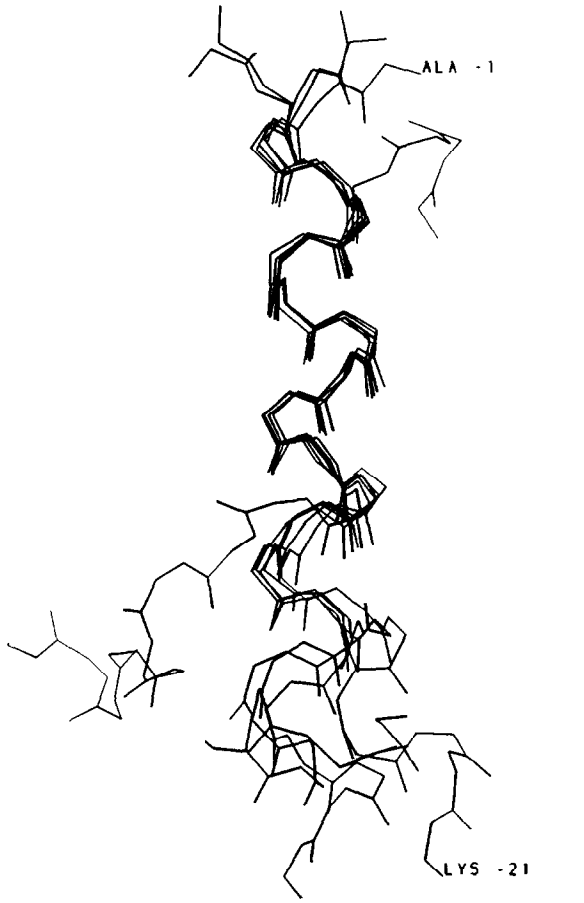

Fig.3. The backbone atoms (N, C, Cα, O) of the 6 best-fitting structures produced by energy minimisation of the DISMAN/RMD data. The structures are overlaid to minimise the RMSD of residues Leu-2 to Ile-14.

Table 2

Comparison of ϕ angles calculated from $^3J_{NH\text{-}\alpha CH}$ coupling constants with those from averaging over the last 5 ps of RMD for the structures shown in fig.3

Residue	Coupling constant $^3J_{NH\text{-}\alpha CH}$ (Hz)	Calculated ϕ angle (degrees)	Mean ϕ angle after DISMAN & RMD with standard deviation (degrees)
A1	–	–	–
L2	4.7	−64.0	–
R3	3.8	−56.7	−52.9 ± 5.9
T4	4.8	−64.6	−56.6 ± 4.4
F5	4.5	−62.7	−66.2 ± 3.5
R6	4.7	−64.2	−65.4 ± 2.5
V7	5.7	−72.2	−68.4 ± 1.7
L8	3.6	−54.6	−56.9 ± 2.9
R9	4.0	−58.6	−66.9 ± 2.8
A10	5.3	−68.7	−60.3 ± 4.8
L11	3.6	−54.5	−70.6 ± 10.5
K12	4.6	−63.2	−75.6 ± 8.4
T13	5.6	−71.2	−72.1 ± 10.5
I14	4.4	−61.5	−72.9 ± 7.0

4. DISCUSSION

The results indicate that the synthetic peptide, corresponding to the S4 sequence proposed for the internal repeat of the rat brain sodium channel by Noda et al. [1] forms a predominantly α-helical structure in TFE/water (9:1); there is no evidence for a 3_{10} helix as has been suggested [7]. Since the basic residues are evenly disposed about the helix face, the structural data are consistent with the 'sliding helix' model of channel activation [9,11], but the closely related ion-pair disruption model of Greenblatt et al. [8] is still a possibility.

The structures calculated from the NMR data indicate that the α-helical region of the peptide only extends from Leu-2 to Ile-14. There is no evidence from this study that the C-terminal region has a well defined conformation. The model of Greenblatt and coworkers [8], in which the S4 segment ends at Ile-17, is consistent with the NMR result. However, the S4 segment proposed in the model of Guy and Seetharamulu

[9], which ends at Ile-17 but starts four residues earlier than in the Noda and Greenblatt models, cannot be discounted on the basis of the present results. It is interesting to note that Stühmer et al. [13] showed by site-directed mutagenesis experiments on the S4 sequence of the first internal repeat of the rat sodium channel II that decreasing the number of positive charges caused a decrease in Z_m, the gating charge for activation; neutralisation of more than three positive charges produced no functional sodium channels. However, Z_m did not increase when positively charged residues replaced the amino acids corresponding to our Ser-15 and Pro-18. These results were interpreted as suggesting, that either the side chains at these positions 'are not located within the membrane and are thus unable to sense the transmembrane field, or that they do not move along the field with gating'. The unstructured nature of the synthetic peptide in the 15–21 region may be related to their observations.

The methodology presented here, which involves synthesis of a putative membrane-spanning helix and the determination of its structure in an organic solvent by high resolution NMR and computer based methods, promises to be a powerful way of gaining new information about membrane proteins consisting of helical bundles. It has been pointed out that the usual structure prediction methods may not be an ideal way of producing models of these proteins [20]. We are currently extending this approach to investigate the S2 peptide from the sodium channel and other S4 peptides of different lengths.

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