Eur Biophys J (1998) 27: 99–104

ARTICLE

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Structure of the inactivating gate from the *Shaker* voltage gated K^+ channel analyzed by NMR spectroscopy

Received: 25 August 1997 / Accepted: 7 November 1997

Abstract Rapid inactivation of voltage-gated K^+ (K_V) channels is mediated by an N-terminal domain (inactivating ball domain) which blocks the open channel from the cytoplasmic side. Inactivating ball domains of various K_V channels are also biologically active when synthesized separately and added as a peptide to the solution. Synthetic inactivating ball domains from different K_V channels with hardly any sequence homology mediate quite similar effects even on unrelated K_V channel subtypes whose inactivation domain has been deleted. The solution structure of the inactivating ball peptide from *Shaker* (Sh-P22) was analyzed with NMR spectroscopy. The NMR data indicate a non-random structure in an aqueous environment. However, while other inactivating ball peptides showed well-defined three-dimensional structures under these conditions, Sh-P22 does not have a unique, compactly folded structure in solution.

Key words *Shaker* · Potassium-channel · NMR · Structure

Introduction

The molecular mechanism of rapid inactivation of the so-called "A-type" K^+ channels was first studied in *Shaker* channels (Timpe et al. 1988; Pongs et al. 1988) from *Drosophila melanogaster*. Different *Shaker* K^+ channel subtypes are products of the same gene that is alternatively spliced to generate various N- and C-terminal variants which show functional differences. Rapidly inactivating *Shaker* channels have a particular N-terminal domain missing in the slow inactivating variants (Timpe et al. 1988; Iverson et al. 1988). It has been shown that treatment of inside-out patches with trypsin, N-terminal deletions and point mutations in this domain may destroy rapid inactivation (Hoshi et al. 1990), and that rapid inactivation can be transferred to a non-inactivating mammalian potassium channel by exchanging its N-terminus with that of a rapidly inactivating *Shaker* channel (Stocker et al. 1991). In a more detailed structure-function analysis it was possible to identify two functional domains in the N-terminus of *Shaker* channels (Hoshi et al. 1990). First, a very N-terminal domain of 21 amino acids in length which is assumed to bind to the open channel pore ("inactivating ball domain"). A deletion in this domain slows or destroys rapid inactivation. Second, a longer sequence domain which ties the inactivating ball domain (IB) to the first transmembrane segment and which was designated as "chain-domain". Deletions in the chain-domain speed-up inactivation, presumably by bringing the IB closer to the channel pore (Hoshi et al. 1990). When the *Shaker* IB was synthesized as a peptide and applied at micromolar concentrations to the cytoplasmic side of an inside-out patch carrying non-inactivating *Shaker* K^+ channels, it was able to restore inactivation (Zagotta et al. 1990). This indicated that the ball-domain represents an autonomous protein structure which may interact with the channel pore of voltage-gated K^+ (K_V) channels, independently of the chain domain. The sequence of the IB comprised a hydrophilic part, which carries several positive charges, and a hydrophobic part. Site-directed mutagenesis experiments have shown that the positive charges are important for electrostatic attraction of the IB while the hydrophobic part mediates the binding affinity of the IB to the mouth of the channel (Murrell-Lagnado and Aldrich 1993a, b).

Three types of rapidly inactivating mammalian K^+ channels have been cloned so far (Stühmer et al. 1989;

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Schröter et al. 1991; Pak et al. 1991) and named RCK4, Raw3 and mShal. Additionally, it was found that β-subunits of the K^+ channels mediate rapid inactivation when they are coexpressed with α-subunits of non-inactivating K_V channels (Rettig et al. 1994). By analogy with the *Shaker* K^+ channels, a mechanism of inactivation based on an IB and a chain-domain was also assumed for the fast inactivating mammalian A-type K^+ channels. For Raw3 the effect of a deletion of the N-terminal 28 residues was tested; this led to a phenotype which was indistinguishable from the non-inactivating Raw2 channels (Rettig et al. 1992). Moreover, the inactivating potency of peptides composed of the first 28 amino acids of the Raw3 sequence and of the 37 amino acids of the RCK4 sequence was tested and compared with the corresponding peptides from *Shaker* (Ruppersberg et al. 1991b). In these experiments the two peptides derived from mammalian K^+ channels were more potent than the peptide derived from *Shaker* channels. Although all three peptides showed functional similarity and appeared to be active at the same receptor site, they display no sequence homology except for a sequence motif responsible for glutathione modulation in RCK4 and Raw3 (Ruppersberg et al. 1991a).

We recently determined the three-dimensional structure of the IBs of RCK4 and Raw3 (Antz et al. 1997); it turned out that the IB of Raw3 shows a compactly folded "ball" structure and that the IB of RCK4 contains a well-defined helix-turn motif in addition to a N-terminal unstructured region.

The present study investigates whether the functional IB peptide of *Shaker* has a well-defined and unique structure in aqueous solution. Circular dichroism (CD) measurements of the *Shaker* IB showed that it is unfolded in aqueous solution but adopts some β-pleated structure in the presence of detergents (Fernandez-Ballester et al. 1995). In contrast, Aldrich et al. (1990) reported that NMR spectroscopy revealed that the peptide is unfolded but exhibits a propensity to form a helical conformation, an effect which is strengthened in the presence of trifluoroethanol. The aim of this paper is to present the data available from NMR spectroscopy in more detail, in order to obtain a more precise picture of the structural dynamics of the *Shaker* IB.

Materials and methods

Peptide synthesis

Peptide *Shaker*-P22 (MAAVA GLYGL GEDRQ HRKKQ QQ – $CONH_2$; molecular mass 2485 Da) was synthesized and purified by solid phase synthesis (Frank et al. 1988).

NMR measurements

The ^{1}H NMR spectra were recorded with a Bruker AMX-500 NMR spectrometer operating at 500 MHz. 2.5 mg of the *Shaker*-peptide was synthesized and dissolved in buffer A or B. Buffer A consists of 1 mM DTE, 0.5 mM EDTA, 0.8 mM NaN_3 in 450 ml H_2O, 50 ml D_2O, pH 3.9. Buffer B is a buffer devised to simulate physiological intracellular ionic conditions as far as possible (Freund and Kalbitzer 1995); it is composed of 47.0 mM K_3PO_4, 9.0 mM $KHCO_3$, 2.4 mM $MgHPO_4$, 0.3 mM K_2SO_4, 2.2 mM KCl, 4.5 mM Na_2HPO_4, 67.2 mM CD_3COOD, pH 7.2 in 450 ml H_2O, 50 ml D_2O. The pH values were measured with a combination glass electrode. The values obtained were not corrected for isotope effects. The spectra were recorded at 282 K. The water signal was suppressed by selective presaturation of 1.5 s duration. ^{1}H chemical shifts were referred to 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) used as internal standard. Spin-system identification and sequential assignments were achieved by two-dimensional homonuclear NOESY, ROESY, DQF-COSY and TOCSY experiments. NOESY spectra were recorded according to Jeener et al. (1979) with mixing times of 100 ms and 300 ms and z-filtered ROESY spectra according to Rance (1987) with mixing times of 150 ms and 200 ms. TOCSY spectra were recorded according to Braunschweiler and Ernst (1983) using a 60 ms MLEV-16 decoupling sequence (Levitt et al. 1982) for isotropic mixing. DQF-COSY spectra were obtained with the basic pulse sequence described by Rance et al. (1983). Phase sensitive detection in the ω_1-direction was obtained by the time proportional phase increments method (Marion et al. 1983). For echo suppression a pair of spin lock pulses of 5 ms and 2.5 ms duration with orthogonal phases were applied before the low-power water suppression pulse. Typical recording times for two-dimensional spectra were 12–48 h. H/D exchange rates were determined in a series of TOCSY-spectra (256×4096 time-domain data points, one scan per time-increment, total acquisition time per two-dimensional spectrum approximatly 9 min) recorded immediately after dissolving the protonated peptide in D_2O.

Spectral assignment

The spectra were processed with the program XWINNMR (Bruker). Data evaluation was performed with AURELIA (Neidig et al. 1995). Assignment of resonance lines was performed according to the standard procedure (Wüthrich 1986) using DQF-COSY and TOCSY spectra for the identification of the spin systems and NOESY spectra for the sequence-specific assignment. Amide-Hα coupling constants $^3J_{HN\text{-}H\alpha}$ were determined from a strongly resolution-enhanced DQF-COSY spectrum with high digital resolution (spectral width 5050.50 Hz, 1024 * 8192 time domain data points, sine filtering in t_1 dimension, Gaussian filtering in t_2 dimension, size of the frequency domain data 2048 * 16384 real data points, base line correction according to Saffrich et al. (1993)). The peak-to-peak separation was determined with the program AURELIA (Neidig et al. 1995), the J-coupling constants were obtained by fitting the corresponding DQF-COSY peaks to two antiphase Lorentzian functions. The digital resolution of the time-do-

Table 1 ^{1}H chemical shifts of *Shaker*-P22 peptide at pH 7.2 and 282 K [a]

Residue	H^N	H^α	H^β	H^γ	H^δ	Others
Met-1	–[b]	4.04	2.14	2.63		ε 2.11
Ala-2	8.54	4.36	1.42			
Ala-3	8.56	4.36	1.39			
Val-4	8.27	4.10	2.08	0.97		
Ala-5	8.55	4.35	1.41			
Gly-6	8.43	3.92				
Leu-7	8.10	4.27	1.36	1.46	0.80/0.87	
Tyr-8	8.33	4.61	2.93/3.15		$\delta_{1,2}$ 7.15	$\varepsilon_{1,2}$ 6.85
Gly-9	8.44	3.93				
Leu-10	8.28	4.37	1.35	1.46	0.89/0.96	
Gly-11	8.63	3.95/3.99				
Glu-12	8.41	4.23	1.95/2.05	2.26		
Asp-13	8.60	4.57	2.75/2.69			
Arg-14	8.37	4.22	1.78/1.86	1.60/1.75	3.19	ε 7.39
Gln-15	8.40	4.28	1.99/2.08	2.31		ε_2 6.99/7.67
His-16	8.32	4.60	3.22/3.12		δ_2 7.11	ε_1 8.06
Arg-17	8.41	4.29	1.83/1.87	1.63/1.76	3.19	ε 7.39
Lys-18	8.47	4.27	1.70/1.76	1.42	1.78/1.84	ε 2.99, ζ 7.69
Lys-19	8.50	4.30	1.71/1.79	1.45	1.81	ε 2.99, ζ 7.69
Gln-20	8.56	4.30	2.00/2.11	2.39		ε_2 6.98/7.65
Gln-21	8.58	4.34	2.03/2.15	2.41		ε_2 6.98/7.65
Gln-22	8.57	4.32	2.02/2.14	2.40		ε_2 6.98/7.65

[a] Shifts (ppm) were measured relative to DSS
[b] Resonance line not identified

main data used in the t_2 direction was 0.62 Hz/point and 0.31 Hz/point after Fourier transformation. Dihedral angles ϕ were calculated with the Karplus equation using the parameters from Pardi et al. (1984). The NOESY cross peaks were integrated by the automated segmentation procedure of the program AURELIA. Distances were calculated from NOESY spectra (with a mixing time of 100 ms) applying the initial slope approximation. A set of well-resolved methylene protons with the known separation of 0.176 nm was used as reference distance.

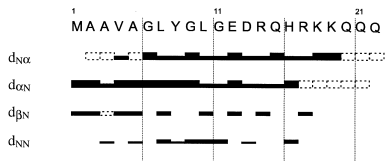

Fig. 1 Sequential NOEs and local structure of *Shaker*-P22. The strength of the intra residue H^α(i)–H^N(i) NOE $d_{N\alpha}$, the sequential H^α(i)–H^N(i + 1) NOE $d_{\alpha N}$, the sequential H^β(i)–H^N(i + 1) NOE $d_{\beta N}$ and the sequential H^N(i)–H^N(i + 1) or H^δ(i)–H^N(i + 1) (for prolyl peptide bonds) NOE d_{NN} is represented by the thickness of the bar. NOEs with NOESY-cross peaks in superposition free regions of the spectrum are depicted by filled bars, broken lines indicate NOEs which are present but could not be quantified precisely

Exchange rates

The experimental pseudo-first order H/D-exchange rates k_i were determined by fitting the NH-Hα cross peak volumes S_i of a series of TOCSY spectra recorded at different times t after dissolving the protonated peptide in D_2O to the equation $S_i(t) = S_i(t = 0)\exp(-k_i t)$. The theoretical exchange rates k_{theo} for random-coil peptides were calculated as described by Bai et al. (1993) with a program obtained from S. W. Englander; they are corrected for pH, temperature, ionic strength, and amino acid sequence effects.

Results and discussion

The NMR experiments on the IB-peptide were performed under various experimental conditions, in aqueous solution at low pH (buffer A) and in a "physiological" buffer at nearly neutral pH (buffer B). The spin systems were completely assigned under these conditions by one- and two-dimensional NMR methods (low pH is favourable for the observation of the exchangeable protons, neutral pH and a suitable ionic composition of the buffer is important to show the biological relevance of the possible structures). The chemical shifts obtained suggest that the conformation obtained in the "physiological" buffer is essentially retained at low pH. The missing concentration dependence of the line widths with an average value of 5.9 Hz at 282 K indicates that, in the millimolar concentration range used, the peptide is dissolved in a monomeric state and aggregation can be neglected. The ^{1}H NMR chemical shifts of the resonances of the *Shaker* peptide in the "physiological" buffer are given in Table 1. Figure 1 gives on overview of the most important NOEs obtained during the sequential assignment of the spin systems which simultaneously characterizes the local structure (secondary structure) of the *Shaker* IB. The pattern of sequential NOEs is dominated by $d_{\alpha N}$, the NOE between the α-proton of amino acid i and the amide proton of the following amino acids as is typical for more extended structures. Between Leu7 and Gly11 a stretch of additional amide-amide NOEs (d_{NN}) are observed, indicating a tendency for helical conformation in

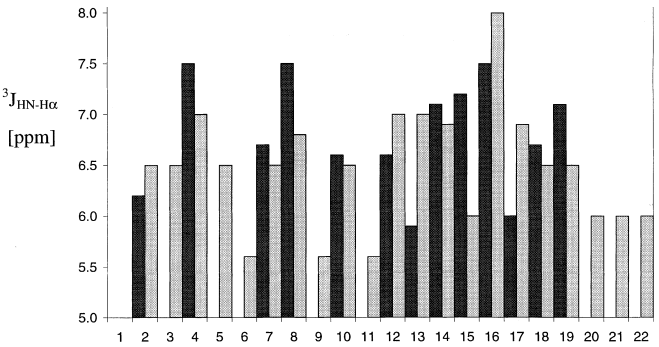

Fig. 2 Amide-H$^{\alpha}$ J-coupling constants $^3J_{HN-H\alpha}$ in the *Shaker* IB. The coupling constants $^3J_{HN-H\alpha}$ experimentally determined for the *Shaker* IB (black columns) are compared with the random-coil values (grey columns) taken from Bundi and Wüthrich (1979). The coupling constants were determined at 282 K and pH 7.2 in physiological buffer and pH 3.9. No pH-dependent changes were observed. An error of 0.5 Hz can be estimated from the limited digital resolution

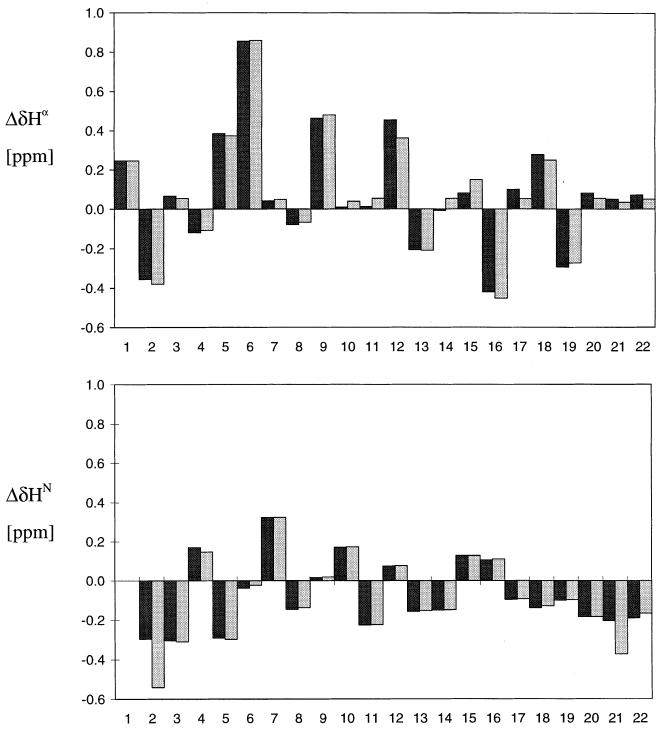

Fig. 3 Deviations of α-proton and amide proton chemical shifts from random-coil values. $\Delta\delta H^{\alpha}$ and $\Delta\delta H^{N}$ represent the differences of the experimentally determined H$^{\alpha}$- and amide chemical shifts to the standard random-coil values given by Wüthrich (1986) determined at 282 K, pH 7.2 (*black bars*) and pH 3.9 (*grey bars*), respectively

this region. No intermediate or long range NOEs (in the laboratory as well as in the rotating frame), expected for well-folded compact structures, could be observed. Most of the experimentally determined coupling constants between amide and α-proton, which indicate the average backbone ϕ-angle, correspond to the random-coil values reported by Bundi et al. (1979) (Fig. 2). However, there are a few exceptions which significantly deviate from the random-coil values, namely at positions 8, 13, 15 and 17, indicating that in time-average the backbone adopts a non-random conformation. As a further help in the qualitative structural analysis, the deviations $\Delta\delta H^{N}$ of the amide proton chemical shifts and $\Delta\delta H^{\alpha}$ of the H$^{\alpha}$-chemical shifts from the random-coil values (Wüthrich 1993) are depicted in Fig. 3. A number of amide-proton shifts and more pronounced of H$^{\alpha}$-shifts deviate significantly from the random-coil values; however no shift pattern typical for canonical secondary structure elements can be identified (Wishard et al. 1995).

Table 2 Exchange Rates for *Shaker*-P22 peptide at pH 3.3 and 283 K [a]

Residue	k_{exp} (10^{-3}/sec)	k_{theo} (10^{-3}/sec)	k_{theo}/k_{exp}
Met-1	– [b]	1320.00 (±330.00)	–
Ala-2	– [b]	41.60 (±10.82)	–
Ala-3	0.75 (±0.08)	0.93 (±0.24)	1.2
Val-4	0.25 (±0.02)	0.18 (±0.05)	0.7
Ala-5	0.75 (±0.08)	0.64 (±0.18)	0.9
Gly-6	0.86 (±0.09)	1.53 (±0.43)	1.8
Leu-7	0.52 (±0.05)	0.37 (±0.09)	0.7
Tyr-8	0.40 (±0.04)	0.30 (±0.08)	0.8
Gly-9	1.07 (±0.11)	1.65 (±0.46)	1.5
Leu-10	0.67 (±0.07)	0.37 (±0.09)	0.6
Gly-11	1.55 (±0.16)	0.95 (±0.28)	0.6
Glu-12	1.34 (±0.13)	1.96 (±0.51)	1.5
Asp-13	– [b]	7.88 (±2.13)	–
Arg-14	2.62 (±0.79)	3.28 (±0.89)	1.3
Gln-15	2.70 (±0.81)	1.50 (±0.46)	0.6
His-16	– [b]	7.75 (±2.52)	–
Arg-17	– [b]	6.26 (±1.82)	–
Lys-18	1.72 (±0.52)	1.19 (±0.34)	0.7
Lys-19	2.25 (±0.67)	0.95 (±0.28)	0.4
Gln-20	2.26 (±0.68)	1.20 (±0.34)	0.5
Gln-21	2.26 (±0.68)	1.44 (±0.42)	0.6
Gln-22	2.26 (±0.68)	1.44 (±0.42)	0.6

[a] Experimental exchange rates k_{theo} and theoretical (random-coil) exchange rates k_{theo} were determined from the data from Bai et al. (1993) as described in Materials and Methods. Note that the theoretical rates are corrected for pH, temperature, ionic strength and side-chain effects. Errors in k_{exp} are estimated from the exponential fit of the data; errors in k_{theo} were determined by assuming an error of ±1 K for the temperature and ±0.1 unit for the pH

[b] Exchange rates could not be determined with the method used since the amide protons were already exchanged in the first spectrum of the TOCSY time series

The H/D exchange rates of amide protons are usually assumed to be small when the corresponding hydrogen atom is involved in an internal hydrogen bond. For a few amino acids the ratio of the theoretically expected rate constant k_{theo} (Bai et al. 1993) to the experimentally determined rate constant k_{exp} is clearly greater than 1, indicating that these amide groups may be involved in transient internal hydrogen bonds. This is true for the amino acids Gly6, Gly9, and Glu12 (Table 2). Clearly, the values of k_{exp} found for Leu10, Gly11 and Lys19 are too high. An increase in the exchange rate can be induced by a negative charge in the close vicinity. The magnitude of the expected effect can be estimated from the random-coil values given by Bai et al. (1993): for example replacing Lys18

by an Asp residue would lead to an increase in k_{theo} from $1.4 \cdot 10^{-3}$ s^{-1} to $2.5\ 10^{-3}$ s^{-1}. This is just the order of magnitude observed in our peptide. Only two negatively charged groups are found in our peptide: the C-terminal carboxyl group and the carboxyl group of Glu12. A possible interpretation of the data would be that in the time average one (or both) of these groups come in the vicinity of Leu10, Gly11 and Lys19, stressing again the idea that the shaker peptide cannot be described by a random-coil.

The NOEs listed in Fig. 1 were determined at a pH of 7.2. However, the observed NOE pattern is not changed significantly at pH 3.9. The same is true for the chemical shifts (see Fig. 3 for H^N and H^{α}-shifts) and the J-coupling constant $^3J_{HNH\alpha}$. These data indicate that the conformational equilibrium ("the structure") is almost independent of pH, apart from trivial pH-dependent chemical shift changes caused by the protonation/deprotonation of functional groups in the side chains.

In summary, the results presented here suggest that the *Shaker* IB adopts no well-defined spatial structure including canonical secondary structure elements. However, it is clearly also not a random-coil structure, but is better described as a dynamic equilibrium of locally non-random structures. This is in line with the previously reported NMR and CD-data (Fernandez-Ballester et al. 1995) and the mutation experiments reported by Murrell-Lagnado and Aldrich (1993a, b). Many of these mutations do not significantly change the binding characteristics of the IB. It is difficult to imagine how a well-ordered three-dimensional structure could be insensitive to all these mutations. In contrast, the inactivation ball of Raw3 is compactly folded and that of RCK4 contains a well-defined helix-loop motif (Antz et al. 1997). Obviously, nature has solved the general problem of closing the inner pore of voltage-gated channels in different ways, ranging from the unordered structure of the *Shaker* peptide to the well-defined tertiary structure of the Raw3 peptide. On- and off-rates for the *Shaker* IB depend on different parts of the amino acid sequence: while the on-rate was mainly governed by the total electrostatic charge of the IB peptide, the off-rate could be increased by mutations of hydrophobic amino acids (Murrell-Lagnado and Aldrich 1993a, b). This led to the hypothesis that the IB finds the channel by electrostatic attraction involving the total positive charge and possibly the dipole moment of the IB, and binds to the channel by a site-to-receptor interaction involving coordination of a hydrophobic domain. We found that the isolated *Shaker* IB is not uniquely structured, indicating that a preformed, well-defined three-dimensional structure of the peptide is not mandatory for an effective inactivation of the channels. However, compared to completely random structures the existence of some local structure in the *Shaker* IB may have the advantage of facilitating the entry of the peptide into the inner pore. A well-defined structure may still be formed on contact with the receptor site of the protein. The absence or presence of a preformed three-dimensional structure probably influences the kinetic characteristics of the inactivation process; indeed, preliminary experiments indicate that the well-folded Raw3-IB is the most effective inactivator even for channels, such as the *Shaker* K_V channel, for which it is not designed.

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