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Pore mutations affecting tetrameric assembly and functioning of the potassium channel KcsA from *Streptomyces lividans*

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Abstract Designed mutations within the *Streptomyces lividans kcsA* gene resulted in a set of mutant proteins, which were characterized in respect to their assembly and channel activities. (i) The amino acid residue leucine 81 located at the external side of KcsA was found to be exchangeable by a cysteine residue without affecting the channel characteristics. (ii) Substitution of the first glycine (G77) residue within the GYG motif by an alanine or substitution of the tyrosine (Y) residue 78 by a phenylalanine (F) led to mutant proteins which form tetramers of reduced stability. In contrast to the AYG mutant protein, GFG functions as an active K^+ channel whose characteristics correspond to those of the wild-type KcsA channel. (iii) The investigated mutant proteins, which carry different mutations (T72A, T72C, V76A, V76E, G77E, Y78A, G79A, G79D, G79E) within the signature sequence of the pore region, do not at all or only to a very small degree assemble as tetramers and lack channel activity.

Key words: Tetramerization; Thermal stability; Electrophysiology; Mutant KcsA

1. Introduction

Within the Gram-positive soil bacterium *Streptomyces lividans*, we previously discovered a gene (*kcsA*) which encodes a protein of 17.6 kDa with two predicted transmembrane (TM) helices connected by a linker region. Twenty-four amino acids of this region shared a significant similarity with members of the growing class of eukaryotic K^+ channel proteins [1]. The *kcsA* gene could be linked to histidine codons and be successfully transcribed under the control of an inducible promoter within the Gram-negative bacterium *Escherichia coli*, resulting in the recovery of larger quantities of the KcsA fusion protein. Subsequent electrophysiological studies revealed that KcsA is the first identified functional, bacterial K^+ channel. It displays several subconductance states [1]. Occupancy of the subconductance levels depends on the direction of the electric field, leading to intrinsic rectification of currents [2]. Flux experiments had indicated that one side (suggested to be the external one) is pH-sensitive [3], electrophysiological studies, however, clearly showed that the internal side is the pH-sensitive one [2,4].

The sides of the KcsA channel are differently inhibited by quaternary ammonium ions. The most potent inhibitor for the external side is tetraethylammonium, tetrabutylammonium (TBA) blocks the internal side most efficiently. Ba^{2+} ions block K^+ currents from the internal side of the channel and cross a major part of the electrical distance to possibly interact with the external one of two K^+ binding sites within the selectivity filter. The smallest diameter of the pore of the functional KcsA channel was 5.8 ± 0.7 Å. Thus it could be concluded that each permeating K^+ ion is surrounded by $n \cong 6$ H_2O molecules [2]. Analysis of the KcsA crystal, which due to anisotropics could be resolved to various degrees in a range of 3.2–10 Å, allowed the prediction of a pore diameter within the selectivity filter of about 3 Å. Based on these calculations, it was moreover concluded that during permeation, K^+ ions have to shed all but two of the H_2O molecules in the hydration shell [5]. To account for the movement of TBA ($d_{min} \sim 9.5$ Å) from the internal side into the cavity, we assume that the internal entrance of the KcsA pore must widen to a larger extent [2] than assumed when analyzing the crystal structure [5]. This widening might be accompanied by rotations of the two TM helices, as deduced from site-directed spin labelling and electron paramagnetic resonance (EPR) analysis [6].

Comparisons of the pore regions (containing 24 amino acids) of several types of potassium channels showed that they share a signature sequence with the consensus TXTTXGYG. KcsA also has this signature [1]; notably, it is closely related to one of the Kv channels, namely to the *Shaker* channel. Previous mutational studies using various eukaryotic channel genes, as well as analysis of the channel activity, predominantly within oocytes of *Xenopus*, led to the conclusion that the GYG motif is necessary to determine K^+ ion selectivity [7–9].

In this report, we show that most mutations within the signature motif of the KcsA protein result in destabilization of the tetrameric assembly and concomitant lack of channel activity.

2. Materials and methods

2.1. Generation of mutants

The *Sph*I–*Sal*I fragment of the plasmid pKCS1 containing the *kcsA* gene [1] was cloned into the M13mp19 vector. Mutations were performed using the Sculpture in vitro Mutagenesis System (Amersham, UK) and corresponding deoxyoligonucleotides [10]. The mutation of

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Abbreviations: DDM, dodecyl β-D-maltoside; EPR, electron paramagnetic resonance; RT, room temperature; SDS, sodium dodecyl sulfate; TBA, tetrabutylammonium; TM, transmembrane; WT, wild-type

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the codon encoding cysteine in position 81 of KcsA was generated by PCR mutagenesis. The individually mutated genes were recloned into the vector pQE32 (Qiagen) in frame with six histidine codons and transformed into *E. coli* M15 (pREP4) (Qiagen). The correctness of the mutations was confirmed after sequencing.

2.2. Isolation of proteins

E. coli M15 (pREP4) transformants carrying the pQE32 construct with the wild-type (WT) or a mutated *kcsA* gene were grown to mid-logarithmic phase, then induced for 2 h in the presence of isopropyl β-D-thiogalactopyranoside (0.5 mM). The cells were washed and disrupted as previously described [1,2]. After removal of the supernatant by high-speed centrifugation (1 h, 100 000×*g*), the membrane proteins within the pellet were solubilized in 20 mM HEPES pH 7.5, 100 mM KCl, 10 mM imidazole, 0.75 mM pefabloc and 10 mM dodecyl β-D-maltoside (DDM) for 1 h at room temperature (RT). Having removed insoluble remains (1 h, 100 000×*g*), the solubilized His-tag protein was purified using affinity chromatography, as described [1].

2.3. Determination of melting points

A portion (10 μl) of the purified protein (~0.5 mg/ml) in a buffer containing 10 mM DDM, 100 mM KCl, 300 mM imidazole and 20 mM HEPES, pH 7.5, was incubated in 1.5 ml cups at the indicated temperature in a pre-heated water bath. After 20 min, the cups were placed on ice for 10 min and 3.5 μl of buffer (4% sodium dodecyl sulfate (SDS), 200 mM Tris, pH 6.8, 20% glycerine, 0.1% bromophenol blue) was added. Each sample was analyzed on an SDS (0.1%) polyacrylamide (12.5%) gel. Having been stained with Coomassie brilliant blue, the gels were scanned with the Cybertech program CAM 2.0, and the temperature-dependent tetrameric decrease was determined. The melting points (corresponding to the denaturation temperature at which 50% of the tetramer dissociated) were obtained by fitting the data using a modified Bolzmann equation (Microcal Origin 5.0): $[\text{tetramer}\%] = 100 \cdot \{1+\exp[(T-T_{50})/\mathrm{d}T]\}^{-1}$.

2.4. Electrophysiology

Using a dilution method, the protein was reconstituted into liposomes and proteoliposomes were fused with the planar lipid bilayer, as described [1,2,11]. If not indicated otherwise, the measurements were done in buffers containing 245 mM KCl and 5 mM KOH/citric acid pH 4.0. The Ag/AgCl electrode of the *trans* compartment was directly connected to the headstage of a current amplifier (EPC 7, List Medical). Currents were recorded and analyzed as reported previously [1,11]. Ion selectivities were calculated as described [2].

3. Results

3.1. Tetrameric assembly of KcsA and mutated proteins

In the range of 20–70°C, ~5% of the WT KcsA was found in the monomeric and ~95% in the tetrameric form. In contrast to the WT KcsA protein [1,2], the mutant proteins V76A or V76E (exchange of valine 76 by alanine or glutamic acid) did not form tetramers (Table 1). For the protein containing a substitution of glycine 77 by glutamic acid (G77E), no tetramerization was ascertainable, a mutation into the amino acid alanine (G77A), however, led to successful tetramerization. A substitution of the aromatic tyrosine residue Y78 by phenylalanine (F) allowed tetrameric assembly, a replacement of the same residue by alanine (A) prevented it. An exchange of glycine G79 by either alanine (A), glutamic acid (E) or aspartic acid (D) resulted only in the formation of monomers. A substitution of leucine L81 by cysteine (C) did not affect tetramerization. The mutant protein T72A (exchange of threonine 72 by alanine), in contrast, did not display a tetrameric

Table 1
Comparative characteristics of mutant channel proteins

		Signature sequence within the pore											Tetramer (%)	Melting point (°C)	Reference
			1	2	3	4	5	6	7	8					
Consensus 8 aa			**T**	**X**	**T**	**T**	**X**	**G**	**Y**	**G**					
			72	73	74	75	76	77	78	79	80	81				
KcsA			**T**	**A**	**T**	**T**	**V**	**G**	**Y**	**G**	D	L	A (K+S)	~95	84.8 ± 0.3	
	mutant								**F**				A (K+S)	~95	73.7 ± 0.3	
												C	A (K+S)	~95	75.7 ± 0.3	
								A					NC	~95	72.2 ± 0.3	
			C										NC	~5	nt	
			A				**A**		**A**	**A**			NC	0		
							E	**E**		E			NC	0		
										D			NC	0		
			439									448				
Shaker		H4	**T**	**M**	**T**	**T**	**V**	**G**	**Y**	**G**	D	M	A (K+S)			[7]
	mutant		S			A			**F**				A (K+S)			
			G				**A**	**A**		**A**			A (US)			
										S			A (US)			
									A				IIC			
			A				**E**	**E**					NC			
			V										NC			
			439									448				
		ShB	**T**	**M**	**T**	**T**	**V**	**G**	**Y**	**G**	D	M	A (K+S)			[12]
	mutant		**A**			A							NC			
			S										NC			
			370									379				
Kv 2.1			**T**	**M**	**T**	**T**	**V**	**G**	**Y**	**G**	D	I	A (K+S)			[13]
	mutant						C					**C**	A (nd)			
			C			C		C	C	C			NC			

The signature motif consensus sequence (amino acids 1–8) (bold font) and two amino acids (normal font) adjoining the pore region from the KcsA, *Shaker* (splice variants H4 and ShB) and Kv 2.1. Symbols: A = active, $K^+S = K^+$ selective, US = unselective, NC = no currents, IIC = immeasurable ionic currents, nd = selectivity not described, nt = not tested. Amino acids which have been exchanged in KcsA and other proteins are printed in bold font, substitutions by different amino acids are given in normal font.

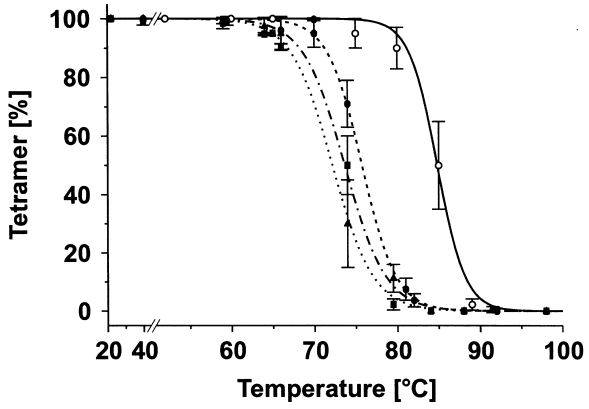

Fig. 1. Thermal stability of KcsA (○, solid line) and mutant proteins (L81C ●, dashed; Y78F ■, dotted and dashed; G77A ▲, dotted).

assembly, and only a small portion (~5%) of the T72C protein (exchange by cysteine) assembled as tetramer (Table 1).

3.2. Stability of the tetrameric proteins

To assess the stability of the WT or a mutated KcsA tetramer, the melting point was determined (corresponding to the denaturation temperature at which 50% of the tetramer dissociated; the amount of tetramer at RT was set to 100%). Significant reductions (Fig. 1) of the stability were ascertained for the proteins G77A, Y78F and L81C, as their melting points were 12.6, 11.1 and 9.1°C below the value of the WT protein (84.8 ± 0.5°C).

3.3. Electrophysiological characteristics of proteins

The mutant proteins were, like the WT KcsA protein (control), reconstituted into liposomes and electrophysiologically characterized in a bilayer system. All proteins which did not form tetramers (T72A, V76A, V76E, G77E, Y78A, G79A, G79D, G79E) or only a small portion thereof (T72C) showed no detectable K^+ channel activity; those proteins, however, which formed tetramers and whose melting point ranged between 84.8 and 73.7°C had channel activity. For the protein (G77A), which still assembles as tetramer, but has the lowest melting point (72.2°C), no measurable K^+ channel activity could be ascertained.

The properties of the mutant protein Y78F (Fig. 2), however, resemble those of the reconstituted WT KcsA channel: the channel activity is dependent on the internal pH and the relative permeabilities for monovalent cations hardly differ from those of the WT protein (Fig. 3). The general electrophysiological properties of the L81C mutant channel also corresponded to those of the WT channel protein.

4. Discussion

An exchange of threonine T72 located within the signature motif of KcsA by alanine (A) induced the formation of the mutant protein T72A, which did not assemble a stable tetramer in vitro and did not function as an active channel in a bilayer system (Table 1). This is in agreement with the lack of detectable currents within *Xenopus* oocytes after injection of mRNA derived from each of the mutant genes predicted to encode the corresponding mutant *Shaker* ShB protein T439A or T439S [12] or each of the *Shaker* H4 mutant proteins T439A or T439V [7]. The T439S mutant of *Shaker* H4, in contrast, has been reported to express a channel in *Xenopus* oocytes; the selectivity properties of this channel matched those of the corresponding WT protein.

Only a very small portion of the KcsA mutant protein T72C assembled as tetramer, its lack of functionality was thus not surprising. Also no currents were detected within *Xenopus* oocytes after injection of mRNA derived from a mutant gene predicted to encode the mutant T370C Kv 2.1 channel [13] (in a position corresponding to T72C in the mutant KcsA channel). As shown by EPR studies, the T72C-substituted KcsA displayed increased steric contacts while moving away from the symmetry axis [6]. Inspection of the eight-residue pore signature sequence (Table 1) of known deduced potassium channel proteins revealed that most of them comprise a threonine residue in the first position of the signature motif. A substitution by cysteine is only encountered within the deduced *Drosophila* EAG channel protein [14], the signa-

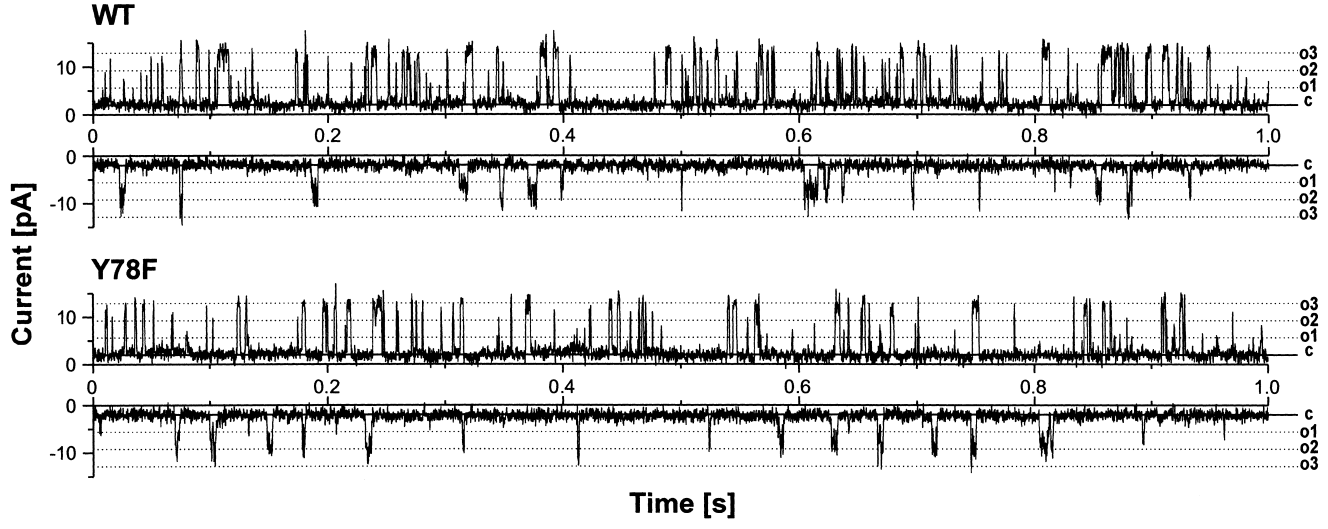

Fig. 2. Representative traces at ± 100 mV in symmetrical K^+ buffer (250 mM) for the WT KcsA channel (upper traces) and the Y78F mutant channel (lower traces). The closed state and the three subconductance levels open 1 (o1), open 2 (o2) and open 3 (o3) are indicated.

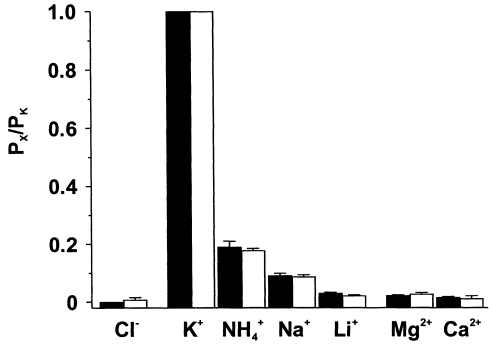

Fig. 3. Comparison of the permeability ratios of the WT KcsA (■) and the Y78F mutant (□) channels, calculated from the reversal potentials in 250 mM K^+/250 mM X^+ (or 125 mM X^{2+}). The potassium permeabilities were set as one.

ture motif of which however considerably differs from that of KcsA and all members of the Kv channels.

Replacement of the amino acid valine by either alanine (A) or glutamic acid (E) in position 76 of KcsA induces the formation of proteins which lack tetrameric assembly and functional channel properties in a bilayer. It had previously been shown that an exchange of the corresponding valine residue (V443) by alanine in *Shaker* H4 leads to a non-selective channel, whereas a substitution by E (glutamic acid) did not entail functional expression [7]. Whether this may also be due to lack of assembly or low stability of the mutant tetramer has however not been investigated. Within the crystal structure of KcsA [5], V76 is orientated toward the pore helix. Its exchange by glutamic acid may lead to a repulsion of the negatively charged glutamic acid in position 71, which has not yet been positioned in the crystal structure.

Most K^+ channels contain the conserved GYG motif, which is involved in ion selectivity [7–9]. Previous research [7,15] had indicated that neither G can be modified without seriously compromising selectivity. Our data reveal that the KcsA mutant proteins G77E, G79A, G79D and G79E neither assemble in vitro, nor form a functional channel. Replacement of the aromatic residue Y78 by alanine (A) stops tetrameric assembly, substitution by another aromatic residue (F), on the other hand, leads to the formation of tetramers with reduced stability. In contrast to the Y78A protein, Y78F still functions as K^+ channel whose electrophysiological properties correspond to those of the WT KcsA. On the basis of the crystal structure [5], the aromatic tyrosine 78 residue was shown to interact with the two tryptophan residues in positions 67 and 68. It is thus evident that a similar interaction is caused by a phenylalanine (F) residue, but not by an alanine (A) residue in position 78. In the position corresponding to Y78 in KcsA, a phenylalanine residue is present in a few deduced channel proteins, including EAG [14], HERG [16], Kir6.2 [17,18] and the deduced TREK-1 monomeric protein [19] with two predicted pore regions. The side chains of the mutated G79A subunits are assumed to be orientated towards the pore in the KcsA crystal structure. Considering the crystal structure of KcsA, it is not obvious why G79A does not form a stable tetramer. Since the protein G77A still assembles as a non-functional tetramer, this exchange may lead to a smaller diameter of the pore and thus hinder the permeation of K^+ ions. The exchanges G77E, G79E and G79D might be unstable, due to repulsion effects with other amino acids within the pore region. Mutations in corresponding positions (CYG and GYC) within the Kv 2.1 channel exhibited no currents, either [13]. All up to now known deduced channel proteins contain invariably glycines corresponding to G77 and G79 in KcsA. In view of these findings, it is surprising that previously functionality was reported for the mutants GYA and AYG from the *Shaker* channel [7].

Our data show that substitution of the amino acid residue L81 of KcsA by cysteine leads to the formation of a protein which still assembles as tetramer with a much lower melting point (75.7°C). In a previous attempt to define pH-dependent molecular movements near the selectivity filter [6], many amino acids had been replaced by cysteine residues. No significant change could be ascertained for exchanges of amino acid residues (including L81C) at the external side of the selectivity filter in probe mobility or spin–spin interaction. The corresponding mutant V378C from Kv 2.1 also formed an active channel [13].

In the crystal form used to elucidate the structure of the KcsA channel, the 4-fold symmetry cannot be seen within the X-ray diffraction patterns. Therefore, it cannot be concluded that the four subunits supply identical sets of atoms in a symmetrical manner to provide the pore lining [20]. However, it could be deduced that the TVGYG motif (amino acids 75–79) forms a stretch of 12 Å in length with the narrowest opening of the pore [5]. As the dimensions of the selectivity filter are determined by lining the groups, hydrogen bonds and van der Waals interactions with the remaining protein, it is conceivable that already subtle alterations of the amino acid composition in this region affect the stability of the tetrameric arrangement.

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