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Original paper

Monitoring the effects of strong cosolvent hexafluoroisopropanol in investigation of the tetrameric structure and stability of K^+-channel KcsA

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ABSTRACT

Adsorption of small chain alcohols into lipid membranes significantly changes the conformational states of intrinsic membrane proteins. In this study, the effects of membrane-active strong cosolvent hexafluoroisopropanol (HFIP) on the intrinsic tetrameric stability of potassium channel KcsA were investigated. Presence of acidic phosphatidylglycerol (PG) in non-bilayer phosphatidylethanolamine (PE) or bilayer phosphatidylcholine (PC) significantly increased the tetrameric stability compared to zwitterionic pure PC bilayers. The stabilizing effect of PG in both lipid bilayers was completely abolished upon deletion of the membrane-anchored N-terminus. Tryptophan fluorescence and circular dichroism experiments indicated that HFIP destabilizes the tetramer possibly via drastic changes in the lateral pressure profile close to the membrane–water interface. The data suggest that HFIP disturbs the ionic, H-bonding and hydrophobic interactions among KcsA subunits where N-terminus presumably plays a crucial role in determining the channel proper folding and tetrameric structure via ionic/H-bond interactions between the helix dipole and the membrane lipids.

Introduction

The oligomerization of protein macromolecules on cell surfaces is believed to play a fundamental role in the regulation of cellular function, including signal transduction and the immune response [1,2]. Oligomerization can bring several functionally important advantages [3,4]: (i) Oligomerization can give shape to active sites or even lead to their compartment-alization. It has been estimated that about one sixth of oligomeric enzymes have their active sites located at oligomeric interfaces; (ii) Oligomerization can allow cooperativity between subunits, enabling allosteric regulation as an additional level of control; (iii) Oligomerization can serve as a tool to create multivalency in active or interactive sites, increasing affinity of the complexes for substrates or binding partners; (iv) Oligomerization can enhance protein stability; (v) For those proteins that have different activities in their oligomeric and monomeric states, oligomerization can provide an additional level of regulation; and (vi) For hetero-oligomeric complexes, oligomerization may allow the formation of enzymatic and signaling cascades.

To remain soluble in the native state, oligomeric membrane proteins (MPs) need an amphiphilic environment, which can be provided by detergents or lipids. α-Helical MPs typically contain long consecutive sequences of hydrophobic amino acids in the transmembrane segments that allow the helices to dock against each other, stabilized through van der Waals interactions [5–7]. The environment of the MP stabilizing the native state can play a role in the protein's response to alcohols. Alcohols can also modulate the oligomerization of MPs in lipid bilayers. This can occur indirectly by redistributing lateral membrane pressure in a manner which correlates with alcohol hydrophobicity [8].

Among various alcohols, trifluoroethanol (TFE) is often used because of its high potential for stabilizing the α-helical structure [9,10]. The secondary structures stabilized by TFE are assumed to reflect conformations that prevail in the early stages of protein folding. However, hexafluoroisopropanol (HFIP), a compound with six F-atoms, is the most effective cosolvents for the structural stabilization of secondary structure forming peptides. In particular, it is one of the strongest helix-inducing and stabilizing cosolvents [11]. It has a pK_a of 9.3; hence, it is more acidic than its hydrocarbon analogue TFE (pK_a 12.4). The presence of two CF3 groups alters its properties to a great extent. It is a better H-bond donor and poorer H-bond acceptor than TFE. Hence, HFIP is potentially more powerful than TFE in terms of perturbing the ionic, H-bonding, and hydrophobic interactions in proteins. HFIP has been used to unfold aggregates of the Alzheimer's amyloid peptide [12] or prion protein peptides [13].

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The potassium channel KcsA is an oligomeric MP from *Streptomyces lividans*, which is a convenient model protein to study MP oligomerization [14]. The tetrameric structure of KcsA is highly stable in a wide range of detergents, even in SDS [15]. This high stability is caused not only by interactions between protein subunits but also by interactions between the protein and the surrounding lipid bilayer [16,17]. Previous studies on KcsA have shown that the stability of the tetramer in lipids is reduced by molar concentrations of alcohols, like TFE, in an indirect manner which can be related to their ability to alter the lateral membrane pressure and permeabilize the membrane [18,19]. More recently, TFE has been proven to be an authentic tool in investigation of channel assembly and stability of the tetrameric structure of KcsA [20]. The present study addresses the question that how does this tetrameric channel respond to HFIP-induced changes in membrane lateral pressure profile and how does any alteration in membrane profile affect the intrinsic stability, structure and conformation of KcsA in a lipid bilayer.

Experimental procedures

Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-glycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phospho-ethanolamine (DOPE) were purchased from Avanti Polar Lipids Inc. *n*-Dodecyl-*β*-D-maltoside (DDM)[1] was from Anatrace Inc. 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was obtained from Merck.

Protein expression, purification and reconstitution

Wild type (WT) and mutant-lacking the first 18 amino acids, named "(ΔN-KcsA)"-were expressed and purified according to the method described previously [17,20]. The purity of proteins was assessed by SDS–PAGE. Proteins were reconstituted in different lipid mixtures with a 1:1000 protein:lipid molar ratio, and the resultant proteoliposomes were obtained as described previously [17].

Stability assay of tetramer dissociation by HFIP

Small aliquots of proteoliposomes were incubated with variable concentrations of HFIP for 1 h at room temperature. Samples were mixed with an electrophoresis sample buffer (50 mM Tris–HCl, pH 6.8, 50% glycerol, 0.01% bromophenol blue and 10% SDS) and run on 15% acrylamide gel in the presence of 0.1% SDS. Gels were stained by silver nitrate, scanned by a densitometer (Bio-Rad Laboratories) and quantified with the program Quantity One. The amount of tetramer (%) was plotted against HFIP (vol.%) for the stability assay, as described previously [17].

Tryptophan fluorescence spectroscopy

All fluorescence experiments were performed in vesicle buffer at room temperature using a QuantaMaster QM-1/2005 spectrofluorometer (Photon Technology International, NJ) in a quartz cuvette. The samples were excited at 280 nm and emission spectra were collected between 300 and 400 nm. The bandwidths for both excitation and emission monochromators were 5 nm and the data were corrected as described previously [17]. All fluorescence experiments were performed at 3 μM protein concentration present in proteoliposomes.

Circular dichroism analysis

Far-UV CD spectra were recorded between 200 and 250 nm (350 μl sample volume) on a Jasco J-810 spectropolarimeter (equipped with a temperature-controlled incubator) at 20 °C using 1 mm optical path length quartz cells. The step size was 0.5 nm with a 1.0-nm-bandwidth at a scan speed of 10 nm/min. All measurements were performed under nitrogen flow. As a blank, the CD spectra of lipid mixture in the absence and presence of HFIP (vol.%) were measured. Averages of 10 scans were obtained for blank and protein spectrum and all spectra were corrected for the buffer and vesicle scattering effects. Predicted percentages for different kinds of secondary structures were calculated using the K2d computer modeling program as described previously [21], and the results were expressed as mean residue ellipticity in units of degrees/cm^2/dmol. The spectra were recorded at 0.1 mg/ml protein concentration in 50 mM Tris–HCl (pH 7.5).

Results

HFIP-induced tetramer dissociation in WT-KcsA

Fig. 1A (upper panel) illustrates silver-stained gels of WT-KcsA dissociation in PC:PG (7:3 mol.%) and PE:PG (7:3 mol.%) lipid bilayers as a function of HFIP (vol.%). Dissociation of WT tetramer (T), which runs at ~68 kDa, required ~6 vol.% HFIP to completely dissociate the tetramer into its monomeric (M) subunits, which run at ~18 kDa, as reported previously [17,20]. The amount of monomer in PC:PG bilayer almost remained the same throughout the whole range of HFIP; however, in PE:PG the monomer population was disappeared upon increasing HFIP concentration lipid bilayer, which reappeared upon complete tetramer dissociation. This effect can be explained by aggregation of KcsA in the presence of HFIP which could not be seen on SDS-gel. It was, however, interesting to note that the intensity of monomeric KcsA in PE:PG bilayer was relatively increased at higher HFIP concentration compared to PC:PG system. It suggests that non-bilayer lipid PE might be required for proper folding of monomeric KcsA as also reported previously [16].

Fig. 1B summarizes the data from the experiments illustrated in Fig. 1A. In addition, the summarized data also demonstrate KcsA tetrameric stability either in detergent micelles or in a pure PC bilayer (SDS-gels not shown) for comparison. DDM-solubilized WT-KcsA tetramer (0.1 mg/ml) was dissociated at 2 vol.% HFIP. In pure PC, the stability of KcsA was increased such that 4 vol.% HFIP was required for complete tetramer dissociation. In PC:PG or PE:PG, the tetrameric stability was significantly increased as discussed above. However, the quantification of gels clearly indicated that tetrameric stability was unaffected in the range of ~1–3 vol.% HFIP in PE:PG lipid bilayer compared to PC:PG system. These results agree well with the previous observation that non-bilayer lipid PE maximally stabilizes the tetramer [18,19].

HFIP-induced tetramer dissociation in ΔN-KcsA

ΔN-KcsA, which forms a stable tetramer, required significantly less amounts of HFIP to dissociate the tetramer such that 4 vol.% HFIP was able to completely dissociate the tetramer in both lipid bilayers (Fig. 1A lower panel). In addition, no monomeric ΔN-KcsA could be detected either in the absence or presence of HFIP. However, at 4 vol.% HFIP a monomeric band appeared upon tetramer dissociation indicating that HFIP solubilized the monomeric KcsA

[1] *Abbreviations used:* DDM, *n*-dodecyl-*β*-D-maltoside; HFIP, hexafluoroisopropanol; MP, membrane protein; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; TFE, trifluoroethanol; Trp, tryptophan; WT, wild type.