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Transmembrane and extramembrane contributions to membrane protein thermal stability: Studies with the NaChBac sodium channel

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ABSTRACT

The thermal stabilities of the extramembranous and transmembranous regions of the bacterial voltage-gated sodium channel NaChBac have been characterised using thermal-melt synchrotron radiation circular dichroism (SRCD) spectroscopy. A series of constructs, ranging from the full-length protein containing both the C-terminal cytoplasmic and the transmembranous domains, to proteins with decreasing amounts of the cytoplasmic domain, were examined in order to separately define the roles of these two types of domains in the stability and processes of unfolding of a membrane protein. The sensitivity of the SRCD measurements over a wide range of wavelengths and temperatures has meant that subtle but reproducible conformational changes could be detected with accuracy. The residues in the C-terminal extramembranous domain were highly susceptible to thermal denaturation, but for the most part the transmembrane residues were not thermally-labile and retained their helical character even at very elevated temperatures. The process of thermal unfolding involved an initial irreversible unfolding of the highly labile distal extramembranous C-terminal helical region, which was accompanied by a reversible unfolding of a small number of helical residues in the transmembrane domain. This was then followed by the irreversible unfolding of a limited number of additional transmembrane helical residues at greatly elevated temperatures. Hence this study has been able to determine the different contributions and roles of the transmembrane and extramembrane residues in the processes of thermal denaturation of this multipass integral membrane protein.

1. Introduction

Soluble proteins have been well characterised with respect to their unfolding/folding processes, thermal stabilities, and folding intermediates [1,2]. However, by comparison, relatively few studies have characterised the processes of unfolding and folding in membrane proteins. This is, in part, due to the challenge of obtaining accurate measurements on these scarce molecules with limited solubilities, the necessity of using detergent or lipid molecules to maintain their solubility, and also because of the difficulty in interpreting the convoluted effects present in multi-domain proteins (i.e. ones with both transmembrane (TM) and extramembrane (EM) regions).

As with studies on soluble proteins, membrane proteins can be denatured by both physical and chemical methods in order to gain insight into the mechanisms and thermodynamics of unfolding, as well as to provide information on the structure of the denatured protein [3]. Physical methods for unfolding include heat treatment [4,5], pressure [6], and dynamic force microscopy [7], whilst chemical denaturants [8] include guanidine hydrochloride, urea, the ionic detergent sodium dodecyl sulfate (SDS), and organic solvents [9]. Each denaturation method used to probe protein stability has its own set of advantages and limitations. Physical denaturation by heat treatment is advantageous in that it generally does not alter other properties of the solution such as ionic strength and does not require the presence of any denaturing chemical that itself can interfere with spectroscopic measurements. However, as with other techniques, it can result in irreversibly aggregated samples, and hence, may only permit qualitative interpretations rather than determination of thermodynamic parameters. Pressure denaturation can be difficult to control and may result from indirect effects on the lipid components [10]. Dynamic force measurements mostly provide information on the process of membrane protein interactions with bilayers rather than the unfolding process itself [11]. Chemical denaturations using guanidine hydrochloride or urea tend to only be effective at very high concentrations (near their solubility limits) and often do not significantly denature transmembrane α-helices as they tend to localise and act primarily in the EM region [12]. Organic solvents have converse limitations as they tend to partition preferentially

Abbreviations: CD, circular dichroism; Cymal-5, 5-cyclohexyl-1-pentyl-β-D maltoside; EM, extramembrane; FL, full-length (native) NaChBac; NaChBac, voltage-gated sodium channel from *B. halodurans*; SDS, sodium dodecyl sulfate; SRCD, synchrotron radiation circular dichroism; TM, transmembrane; VGSC, voltage-gated sodium channel

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into the membrane region [13], can be volatile and thus lost during the unfolding process, and some such as trifluoroethanol tend to induce helical structures rather than unfold them [9]. SDS has the advantage of being effective at millimolar concentrations and able to form mixed micelles with other detergents, but this denaturant often results in only partial unfolding of membrane proteins, and because it tends to induce helical structures, can result in "denatured" proteins with non-native helical structure [14]; indeed even β-pleated sheets can be transformed into α-helical structures in the presence of "denaturing" concentrations of SDS [15].

Overall, membrane proteins appear to be much more resistant to both chemical and thermal denaturation than are soluble proteins [16–18], with significant amounts of their secondary structures remaining even after extensive thermal or chemical treatment [8]. It has been suggested that the EM regions of the protein may be more susceptible to unfolding whilst the TM domains retain much of their native structure under various denaturing conditions [19], but there have been few studies defining the relative contributions of the extramembranous and transmembranous domains and the roles of different types of secondary structures in each of these regions in maintaining protein stability.

A very useful system for investigating the nature of unfolding in a multidomain membrane protein is the voltage-gated sodium channel (VGSC) from *Bacillus halodurans* (NaChBac). Bacterial sodium channels are tetrameric and hydropathy plots predict that all bacterial sodium channel monomers possess the same structural motif of six TM segments in addition to an extended cytoplasmically-exposed C-terminal domain (CTD) [20] (Fig. 1). In recent studies it was shown that the CTD of NaChBac is not required for tetramer formation [21], but may be important for initial assembly in membranes [22]. The structure of the C-terminal domain of NaChBac has been shown experimentally [22] to consist of a proximal unstructured region, which is followed by a distal helical structure.

In this study, we have produced both full length (FL) NaChBac and a series of C-terminally truncated constructs in order to separately dissect the thermal stabilities of the TM and EM regions, and to identify the different contributions of helical and unstructured residues in the EM domain to the thermal stability and unfolding processes. The highly sensitive method of synchrotron radiation circular dichroism (SRCD) spectroscopy was used to monitor and quantify relatively subtle changes in the secondary structure of FL NaChBac and the truncated constructs as a function of temperature, and to identify the pathways involved in its unfolding and refolding.

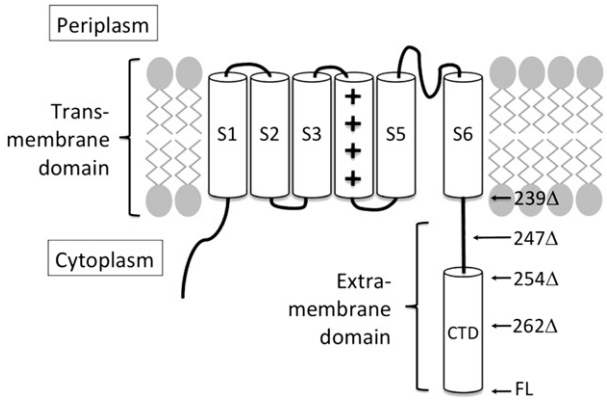

Fig. 1. Topology model of NaChBac, with the helical six transmembrane segments (S1 to S6) and the helical C-terminal domain (CTD) shown as cylinders. The sites of truncation of the various constructs (designated by the number of the first residue removed, with FL meaning full-length) are indicated by black arrows.

2. Materials and methods

2.1. Materials

The cDNA for NaChBac was provided by Prof. David Clapham of Harvard Medical School. N-dodecyl-β-D maltopyranoside and 5-cyclohexyl-1-pentyl-β-D maltoside (Cymal-5) were obtained from Anatrace. All other reagents were purchased from Sigma, unless otherwise stated.

2.2. Expression and purification

C-terminal truncated NaChBac constructs were generated using the QuikChange protocol from Stratagene to introduce stop codons into the full-length *NaChBac* cDNA as described in Powl et al. (2010) [22]. Protein samples were purified from *E. coli* C41(DE3) cells as described previously [22]. Their purity was assessed by solubilising the samples in NuPAGE lithium dodecyl sulfate sample buffer, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE 4–12% gradient Bis-Tris gel with MOPS). BenchMark molecular mass standards (Invitrogen) were used to calibrate protein molecular weights.

2.3. Synchotron radiation circular dichroism spectroscopy

Protein concentrations were determined immediately prior to measurement of the SRCD spectra using an extinction coefficient at 280 nm calculated from the amino acid sequence using Expasy ProtParam [23] and the A_{280} measured on a Nanodrop 1000 UV spectrophotometer.

SRCD measurements were undertaken on beamline CD1 at the ISA Synchrotron located at the University of Aarhus, Denmark, on beamline 4B8 at the Beijing Synchrotron Radiation Facility (BSRF) in China, and on the DISCO beamline at the Soleil Synchrotron, France. The beamlines were calibrated with camphorsulfonic acid at the beginning of each data collection run. Samples at concentrations of ~4 mg/ml in 50 mM NaCl, 20 mM sodium phosphate, pH 7.8, 0.3% Cymal-5 containing 10% v/v glycerol were loaded into a quartz Suprasil demountable cell (Hellma UK Ltd) with a pathlength of 0.0015 cm. On beamline CD1, SRCD spectra were measured over the wavelength range from 260 nm to 180 nm with a step size of 1 nm and a dwell time of 2 s. Three replicate scans were measured at each temperature between 20 °C and 85 °C. The temperature was raised in 5 °C increments allowing 3 min for the temperature to equilibrate prior to data collection. The actual temperature (as opposed to the set temperature) was determined from calibration curve measured using a thermistor probe inside a sample cell containing water. The first and third scans were compared to ensure that the sample had reached equilibrium before the measurements were made. Conditions and parameters on beamline 4B8 were identical except that the equilibration time was 5 min. Replicate scans were averaged and an averaged baseline (obtained using detergent-containing buffer without protein present) was subtracted. The spectra were smoothed with a Savitsky–Golay filter, and scaled to delta epsilon values with the CDTool software [24] using mean residue weight values of 114.4, 114.5, 114.4, 115.0, and 114.9 for the full-length and 262Δ, 254Δ, 247Δ and 239Δ mutants, respectively. Thermal denaturation curves were obtained from the delta epsilon values for each of the three peaks (222 nm, 209 nm and 192 nm) at each temperature; they were normalised by setting the peak values at 20 °C to 1.0. The data points were fitted with Boltzman functions in Origin (version 8.0).

2.4. Secondary structure analyses

Secondary structure analyses based on SRCD data were carried out using the DichroWeb analysis server [25]. Values reported are the