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Purification and reconstitution of the connexin43 carboxyl terminus attached to the 4th transmembrane domain in detergent micelles ☆

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ABSTRACT

In recent years, reports have identified that many eukaryotic proteins contain disordered regions spanning greater than 30 consecutive residues in length. In particular, a number of these intrinsically disordered regions occur in the cytoplasmic segments of plasma membrane proteins. These intrinsically disordered regions play important roles in cell signaling events, as they are sites for protein–protein interactions and phosphorylation. Unfortunately, in many crystallographic studies of membrane proteins, these domains are removed because they hinder the crystallization process. Therefore, a purification procedure was developed to enable the biophysical and structural characterization of these intrinsically disordered regions while still associated with the lipid environment. The carboxyl terminal domain from the gap junction protein connexin43 attached to the 4th transmembrane domain (TM4-Cx43CT) was used as a model system (residues G178-I382). The purification was optimized for structural analysis by nuclear magnetic resonance (NMR) because this method is well suited for small membrane proteins and proteins that lack a well-structured three-dimensional fold. The TM4-Cx43CT was purified to homogeneity with a yield of ~6 mg/L from C41(DE3) bacterial cells, reconstituted in the anionic detergent 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)], and analyzed by circular dichroism and NMR to demonstrate that the TM4-Cx43CT was properly folded into a functional conformation by its ability to form α-helical structure and associate with a known binding partner, the c-Src SH3 domain, respectively.

Gap junctions are integral membrane proteins that serve to directly interconnect the cytoplasm of neighboring cells, allowing the passage of ions, metabolites, and signaling molecules. They provide a pathway for the propagation and/or amplification of signal transduction cascades triggered by cytokines, growth factors, and other cell signaling molecules involved in growth regulation and development. Mammalian gap junction channels are formed by as many as 21 different connexin proteins [1]. Of these, connexin43 (Cx43)[2] is the most abundant connexin and best characterized isoform in terms of channel gating properties [2–4], phosphorylation sites [5–7], mechanisms of pH sensitivity [8–11], and overall molecular structure [12]. Cx43 is essential for normal cell growth [13], cardiac embryogenesis [14], and glial intercellular communication [15]. The functional importance of Cx43 has been illustrated through the identification of mutations that are associated with the human disease oculodentodigital dysplasia [16].

Cx43 is a tetraspan membrane protein with intracellular N- and C-termini. The Cx43 gap junction structure was initially determined by electron crystallography at 18 Å resolution by Unwin and Zampighi [17] and later at 7.5 Å resolution by Unger et al. [18]. These studies helped provide the first molecular view towards understanding the architecture of the channel. While the protein used in the Unger et al. [18] study was able to form functional channels [19,20], most of the carboxyl terminal domain (CT) was removed (residues 263–382) to improve the diffraction quality of the two-dimensional crystals [21]. Using a soluble version of the CT domain from Cx43 (Cx43CT; residues 255–382), we identified by nuclear magnetic resonance (NMR) that the Cx43CT is highly flexible and predominately disordered in structure [11,22,23]. The Cx43CT structure exemplifies many previous observations that highly flexible or completely unfolded fragments dramatically interfere with the crystallization process [24,25]. Based on the estimation that 41% of human membrane proteins have intrinsically disordered regions with more than 30 consecutive residues and these residues are preferentially localized at the cytoplasmic side [26], as well as, intrinsically disordered domains have been identified as playing an important role in cell signaling events [27], novel protein purification strategies need to be

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[1] These authors contributed equally to this work.
[2] *Abbreviations used:* NMR, nuclear magnetic resonance; Cx43, connexin43; CD, circular dichroism.

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developed not only to be able to characterize the structure-function correlates of these intrinsically disordered domains, but to characterize them when attached to the membrane.

Studies of the soluble Cx43CT indicate that the microenvironment of a soluble polypeptide versus that of the same sequence in the context of the native protein embedded in a lipid bilayer may not be the same [23,28]. For example, NMR studies indicate that the N-terminus of the soluble Cx43CT is highly flexible in comparison to the C-terminal domain and this maybe affecting the binding affinity of molecular partners interactions, whereas its association with the 4th transmembrane domain would provide the N-terminus with a more rigid and stable conformation. Also, region G261-N300, which is essential for normal pH gating [29], contains a region rich in proline residues. Proline-rich sequences commonly form left-handed type II polyproline helices [30,31], which was not observe in the NMR structure. These differences can be attributed to the constraints afforded by attachment to the membrane. Therefore, a purification and reconstitution protocol was developed to enable the biophysical characterization and structural determination by NMR methods of the CT when attached to the 4th transmembrane domain of Cx43 (TM4-Cx43CT) in detergent micelles. NMR is an ideal spectroscopic tool to characterize the structure and dynamics of intrinsically disordered proteins [32,33], unfortunately, size limitations do not support the feasibility of working with large molecular weight membrane proteins, such as the full-length Cx43. In general, this methodology will also be useful for the purification and reconstitution of other membrane-associated intrinsically disordered domains.

Materials and methods

Plasmid construction

DNA encoding the TM4-Cx43CT (G178-I382) was cloned by PCR from a G2A plasmid containing the *Rattus norvegicus* Cx43 gene and ligated into the *Escherichia coli* pET-14b expression vector (N-terminal 6× His-tag and thrombin cleavage site) (Novagen) using the restriction enzymes Nde1 and Xho1. All constructs were verified by the University of Nebraska Medical Center's DNA Sequencing Core Facility.

Protein expression and purification

The *E. coli* strains BL21(DE3) (Novagen), Rosetta-2(DE3) (Novagen), C41(DE3) (Lucigen, [34]), and C43(DE3) (Lucigen, [34]) were transformed with the TM4-Cx43CT expression plasmid (see above) and then inoculated into 1 L of Luria Bertani medium (LB), enriched minimal media [35], or ISOGRO media (Sigma–Aldrich). Cultures were incubated at 37 °C with continuous agitation. At an optical density of 0.6 at 600 nm, 1.0 mM isopropyl β-D-thiogalactopyranoside was added, and growth was allowed to proceed for 4 h (typical optical density ~ 1.6). The cells were harvested by centrifugation (1000*g* for 45 min), washed with PBS buffer, and stored at −20 °C.

Cells were suspended in 1× PBS buffer with a bacterial protease inhibitor cocktail (250 μl/5 g cells; Sigma–Aldrich) and disrupted with three passages through either an Emulsiflex or a French pressure cell at 15,000 psi. Cell debris was removed by centrifugation (1000*g* for 30 min) and a pellet containing the inclusion bodies was collected by a high-speed centrifugation step (25,000*g* for 45 min). The pellet was resuspended in 8 M urea, 1× PBS (pH 8.0), 1% Triton X-100, and 20 mM imidazole and placed on a rocker at 4 °C for ~2 h. The suspension was centrifuged again (25,000*g* for 45 min) and the supernatant was loaded onto a HisTrap HP affinity chromatography column using an ÄKTA FPLC (GE Healthcare). The TM4-Cx43CT polypeptides were eluted at 300 mM imidazole using a step gradient of 20, 40, 80, 100, 300, and 500 mM imidazole. Prior to the 300 mM imidazole elution step, four column volumes of a buffer containing 8 M urea, 1× PBS (pH 8.0), 1% Triton X-100, and 10% ethanol was used to wash the bound 6× His-tag TM4-Cx43CT. Fractions containing the TM4-Cx43CT were identified by SDS–PAGE. An estimate of the amount of TM4-Cx43CT after the HisTrap HP column when compared to BSA on a SDS–PAGE was ~6.0 mg/L

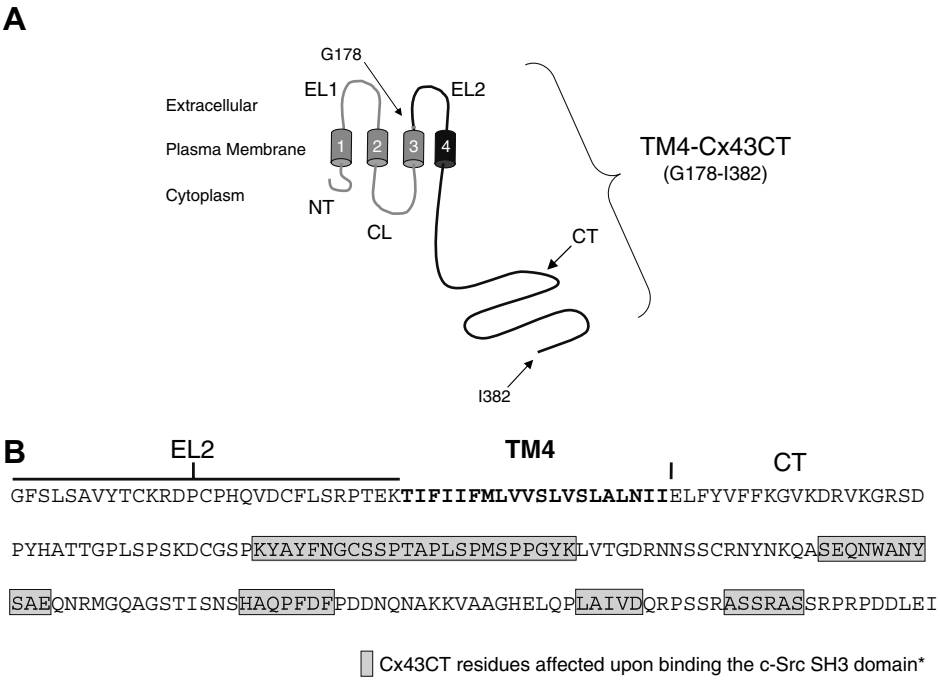

Fig. 1. Model of the TM4-Cx43CT construct. (A) Schematic diagram of full length Cx43. The black coloring represents the TM4-Cx43CT portion. The abbreviations are as follows: NT, N-terminus; CL, cytoplasmic loop; CT, C-terminus; EL1 and EL2, extracellular loops 1 and 2; 1–4, transmembrane segments 1–4. (B) Amino acid sequence of the TM4-Cx43CT construct. The EL2 (line), TM4 (bold), and the CT (arrow) domains have been labeled. The asterisk denotes that the study used to determine the Cx43CT residues affected by the c-Src SH3 domain used a soluble version of the Cx43CT without the TM4 domain [28].