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Crystal Structure of the Extracellular Domain of a Bacterial Ligand-Gated Ion Channel

Hugues Nury[1,2]†, Nicolas Bocquet[2]†, Chantal Le Poupon[2], Bertrand Raynal[3], Ahmed Haouz[4], Pierre-Jean Corringer[2] and Marc Delarue[1]*

[1]*Institut Pasteur, Unité de Dynamique Structurale des Macromolécules, CNRS URA 2185, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France*

[2]*Institut Pasteur, Channel-Receptors G5 Group, CNRS URA 2182, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France*

[3]*Institut Pasteur, Plate-forme de Biophysique des Macromolecules et de leurs Interactions, CNRS URA 2185, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France*

[4]*Institut Pasteur, Plate-forme de Cristallogenese et de Cristallographie, CNRS URA 2185, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France*

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The crystal structure of the extracellular domain (ECD) of the pentameric ligand-gated ion-channel from *Gloeobacter violaceus* (GLIC) was solved at neutral pH at 2.3 Å resolution in two crystal forms, showing a surprising hexameric quaternary structure with a 6-fold axis replacing the expected 5-fold axis. While each subunit retains the usual β-sandwich immunoglobulin-like fold, small deviations from the whole GLIC structure indicate zones of differential flexibility. The changes in interface between two adjacent subunits in the pentamer and the hexamer can be described in a downward translation by one inter-strand distance and a global rotation of the second subunit, using the first one for superposition. While global characteristics of the interface, such as the buried accessible surface area, do not change very much, most of the atom–atom interactions are rearranged. It thus appears that the transmembrane domain is necessary for the proper oligomeric assembly of GLIC and that there is an intrinsic plasticity or polymorphism in possible subunit–subunit interfaces at the ECD level, the latter behaving as a monomer in solution. Possible functional implications of these novel structural data are discussed in the context of the allosteric transition of this family of proteins. In addition, we propose a novel way to quantify elastic energy stored in the interface between subunits, which indicates a tenser interface for the open form than for the closed form (rest state). The hexameric or pentameric forms of the ECD have a similar negative curvature in their subunit–subunit interface, while acetylcholine binding proteins have a smaller and positive curvature that increases from the apo to the holo form.

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*Corresponding author. Department of Structural Biology and Chemistry, 25 rue du Dr Roux, 75015 Paris, France. E-mail address: marc.delarue@pasteur.fr.

† H.N. and N.B. contributed equally to this work.

Abbreviations used: pLGIC, pentameric ligand-gated ion channel; nAChR, nicotinic acetylcholine receptor; ECD, extracellular domain; TMD, transmembrane domain; AChBP, acetylcholine binding protein; GLIC, *Gloeobacter violaceus* ion channel; $GLIC_{ECD}$, extracellular domain of GLIC; ASA, accessible surface area; SeMet, selenomethionine; PDB, Protein Data Bank; SV, sedimentation velocity.

Introduction

Pentameric ligand-gated ion channels (pLGICs, also called Cys-loop receptors) constitute a major class of receptors within the nervous system and include nicotinic acetylcholine receptors (nAChRs), glycine receptors, and $GABA_A$ and $5HT_3$ receptors.[1] pLGICs are integral membrane proteins made up of five identical or homologous subunits symmetrically arranged around an axis perpendicular to the plane of the membrane. Neurotransmitters bind to pLGICs at the extracellular domain (ECD), governing the open-

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ing of the ion channel inside the transmembrane domain (TMD), made of a bundle of 20 α-helices.[2]

Because of the difficulty to express and determine the structure of entire pLGICs, extensive work has been devoted to the expression of isolated soluble ECDs, since early work on chimeric constructs suggested that ECDs constitute autonomous folding units.[3] The first ECD structure followed from the discovery of the acetylcholine binding protein (AChBP),[4] which is homologous to the ECD of nAChRs. It was expressed as a soluble homopentamer and crystallized, allowing its structural characterization by X-ray crystallography[5] and offering the first and best template until very recently of the ECD. AChBPs assemble with pseudo-5-fold symmetry; each subunit is folded as a β-sandwich with an immunoglobulin-like topology. X-ray structures obtained by co-crystallizations showed that agonists and competitive antagonists bind at the interface between subunits[6–11] in full agreement with a wealth of biochemical data accumulated on nAChRs.[1]

Attempts to produce native and soluble ECDs of eukaryotic pLGICs have met with limited success so far, both for nAChRs[12–14] and for the glycine receptor,[15] even after extensive mutagenesis designed to increase protein solubility. The best result was achieved with the α1-nAChR subunit, which does not form homopentamers *in vivo* but assembles into α1-γ-α1-δ-β heteropentamers. The initial expression of the α1-ECD in a monomeric state[16] was improved by the selection of a triple mutant with increased solubility, whose structure could be solved in complex with α-bungarotoxin.[17]

The discovery of bacterial members in the pLGIC family,[18] followed by the functional characterization of one of them,[19] recently changed this picture dramatically, leading to the crystallization and resolution of the X-ray structures of entire proteins of this family. Two conformations have been obtained: a closed-channel conformation for the ELIC homologue from *Erwinia chrysanthemi*[20] and an apparently open-channel conformation for the GLIC homologue from *Gloeobacter violaceus*.[21,22] These homologues are structurally similar to the X-ray structures of AChBPs and α1-ECD and to the 4-Å electron microscopy structure of the *Torpedo marmorata* nAChR[2] and are thus good structural prototypes of the pLGIC family.

In this article, we present the structure determination of the isolated ECD of GLIC; we study the flexibility and quaternary interactions of this autonomous folding unit that is normally assembled as a homopentamer in the entire protein.

Results

Soluble and monomeric expression of the ECD of GLIC in *Escherichia coli*

A construct coding for the mature ECD of GLIC, fused with a polyhistidine tag, was designed and expressed. In order to increase the protein solubility, we introduced four mutations within the β6–β7 loop: four hydrophobic residues (**F**RR**YPF**) in the β6–β7 loop were substituted by the corresponding hydrophilic residues (**G**RR**TES**) present in the AChBP (Fig. 1a). The GLIC structure[21] indeed shows that the bottom portion of the β6–β7 loop is engaged in hydrophobic interactions with the TMD. A non-modified ECD construct would expose this hydrophobic stretch to the solvent. In the whole article, we call this β6–β7 mutated construct $GLIC_{ECD}$ (extracellular domain of GLIC).

The mutated and the wild-type constructs express robustly as soluble proteins in *E. coli* C43 cells. After purification, we recovered 10 and 20 mg of proteins per liter of culture, respectively. The size-exclusion chromatography profiles of both proteins are identical and compatible with a monomer (Fig. 1b). Analytical ultracentrifugation experiments performed on the mutated protein confirm that it is in the monomeric form, even at the concentration used for crystallization ($s = 1.9 \pm 0.2$ S at both 4 and 12 mg/ml protein concentrations, Fig. 1c). Furthermore, circular dichroism spectra of both proteins are nearly identical, comparable with the spectrum of α1-ECD,[16] and point to a high content in β-sheet (data not shown). Taken together, this indicates that the quadruple mutation—that confers increased hydrophilicity—does not alter either tertiary or quaternary structures and only increases the production of soluble protein. It has also to be noted that the mutated stretch of residues does not contribute to packing interactions (see below).

Structure determination and refinement

The protein crystallizes in the $P2_12_12$ space group with six $GLIC_{ECD}$ molecules in the asymmetric unit. Data from a mercury derivative crystal diffracting at 2.3 Å resolution were phased experimentally using SIRAS method. Systematic Phased Translation function[23] using these experimental phases and scanning all possible rotations (grid search of 10° for the eulerian angles α and γ, 5° for β) allowed to orient and place six different copies of one AChBP in the asymmetric unit with correct packing. Initial phases from this preliminary model were used to calculate an anomalous map at 4.0 Å resolution with the selenomethionine (SeMet) derivative and showed peaks at the expected positions of the SeMet. After refinement, the final model encompasses residues from 3 to 193 with two disordered regions that were not completely modeled at the level of the β6–β7 (the Cys loop bearing the mutations introduced in $GLIC_{ECD}$) and β9–β10 (C loop) loops. Data collection and refinement statistics are presented in Table 1. $GLIC_{ECD}$ assembles as hexamers (Fig. 2a), an unexpected feature for the N-terminal domain of an otherwise pentameric protein. Modeling of the hydrodynamic properties of monomeric $GLIC_{ECD}$ using the crystal coordinates gives a theoretical sedimentation coefficient of 2.0 S, in good agreement with the experimental value, indicating little tertiary

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