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Probing the interaction mechanisms between transmembrane peptides and the chaperonin GroEL with fluorescence anisotropy

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ABSTRACT

Proper translocation, membrane insertion and folding are crucial biophysical steps in the biogenesis of functional transmembrane peptides/proteins (TMPs). ATP-dependent chaperonins are able to regulate each of these processes, but the underlying mechanisms remain unclear. In this work, interaction between the bacterial chaperonin GroEL and a synthetic fluorescent transmembrane peptide was investigated by fluorescence anisotropy. Binding of the peptide with GroEL resulted in increased fluorescence anisotropy and intensity. The dissociation constant and binding stoichiometry, as assessed by titration of the peptide with GroEL, were estimated to be 0.6 ± 0.2 μM and 2.96 ± 0.35, respectively. Complementary study with the single-ring version of GroEL confirmed the high-affinity peptide binding, and indicates that the two GroEL rings may function alternatively in binding the peptides. The co-chaperonin GroES was found to be effective at releasing the peptides initially bound to GroEL with the help of ATP. Moreover, our observation with the single-ring GroEL mutant demonstrated that during the encapsulation of GroEL by GroES, the bound peptides may either be confined in the cage thus formed, or escape outside. Competitive binding experiments indicated that the peptides studied interact with GroEL through the paired helices H and I on its apical domain. Our spectroscopic studies revealed some basic mechanisms of interaction between transmembrane peptides and GroEL, which would be instrumental for deciphering the chaperonin-mediated TMP biogenesis.

1. Introduction

Transmembrane peptides/proteins (TMPs) are encoded by about one-third of the genes in most genomes, and perform numerous biological processes from signaling to transport [1–3]. After being synthesized at ribosomes, the nascent chains of TMPs need to be translocated and inserted into biological membranes, where they fold into defined three-dimensional structures. Chaperonins are ubiquitous ATP-dependent protein complexes that can assist translocation, membrane insertion and protein folding [4–9]. For example, the bacterial chaperonin GroEL has been shown to interact with SecA, a central component of the translocation machinery, and promote post-translational membrane insertion of the membrane protein lactose permease, or solubilize the functional bacteriorhodopsin (bR) and deliver it to membranes [10,11]. It has also been demonstrated that incorrect orientation of TMPs in membranes can be prevented by the chaperonins in chloroplasts [12]. Moreover, GroEL is able to help the folding of newly translated membrane proteins in detergent micelles, apart from its well-known function in soluble protein folding [13,14]. In spite of the significance of interaction between TMPs and chaperonins *in vivo* and *in vitro*, the underlying mechanisms remain unclear. On the other hand, efficient translocation and proper membrane insertion and folding are crucial for TMPs to fulfill their normal biological functions. How such processes are accomplished with the help of chaperonins is also a problem of medical relevance [15,16]. For example, expression of physiologically important GPCR membrane proteins in eukaryotic cells has been shown to be regulated through an interaction with the chaperonin TRiC/CCT [17].

As one of the simplest models for TMPs, an individual transmembrane peptide is attractive for easily exploring the interactions between TMPs and chaperonins. One general structural feature common to all transmembrane peptides is that they are greatly enriched with aliphatic hydrophobic residues. It is hydrophobic interactions that play a decisive role in binding nonnative substrate polypeptides to chaperonins [18–21]. In addition, transmembrane peptides are usually only 20 amino acids in length [22], which can avoid a too stable fold and thus favor to the easy binding of chaperonins. In addition, the peptide approach would circumvent the complication of kinetic competition between intramolecular interaction and intermolecular binding to the chaperonin [23–25].

In this work, fluorescence anisotropy was employed to probe the interaction of GroEL with a transmembrane peptide from the prototypical

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integral membrane protein of bR. GroEL consists of two back-to-back stacked heptameric rings with a total molecular weight of ~800 kDa [26–29]. The initial molecular recognition and binding of the bR model peptides by GroEL were firstly examined, followed by the investigation of release of the bound peptides, induced by ATP-regulated binding of the co-chaperonin GroES or the mobile loop of GroES. In addition to wild-type GroEL, the single-ring mutant of GroEL was also used to further check the binding or release mode of bR peptides. This study aims at providing some basic thermodynamic and kinetic information on transmembrane-peptide-GroEL interaction at the molecular level. Moreover, as evidenced herein, the anisotropy measurement of fluorescent transmembrane peptides would offer great potentials for comprehensively exploring the chaperonin-mediated TMP biogenesis with highly dynamic feature.

2. Materials and Methods

2.1. Materials

The peptide we studied corresponds to the second transmembrane α-helix of bacteriorhodopsin (bR), a prototypical member of helical membrane proteins. There is a wealth of genetic, structural and biochemical information on bR, making it ideal for the development of methodologies [30]. Herein, the bR peptide labeled with 5(6)-carboxytetramethylrhodamine (TAMRA) at the N-terminus, TAMRA-KKAITTLVPAIAFTMYLSMLLKK, was synthesized and purified (>95% purity) by Nanjing Impact BioScience Ltd. according to standard Fmoc based solid phase approach. The lysine residues (K) underlined were added to the termini to increase the solubility. To prepare aqueous solutions of the TAMRA-peptide, the peptide was firstly dissolved in a small amount of hexafluoroisopropanol (HFIP), and then diluted by 150-fold into HEPES buffer (25 mM HEPES-NaOH, 120 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, pH 7.6). SBP (SWMTTPWGFLHP) and the mobile loop peptide from GroES (NH_2-KRKEVETKSAGGIVLTGSAA-COOH) used in this work were also synthesized and purified (>95% purity) by Nanjing Impact BioScience Ltd. For all the custom peptides, the certificates of analyses by high performance liquid chromatography (HPLC) and mass spectroscopy (MS) are provided as Supplementary data (see SI_Files 1–6).

GroEL was prepared to ~95% purity using *E. coli* strain BL21 harboring the plasmid pTric 99. As described [31], GroEL was first overexpressed in BL21 from the pTric 99 plasmid carrying the GroEL gene. The cells were subsequently induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside at 37 °C when OD600 reached 0.4–0.6. After 4 h, cells were harvested and then broken by passing cell suspension twice through a French pressure cell. The lysate was clarified by centrifugation at 10000*g* for 20 min at 4 °C, and the supernatant was collected into a fresh tube. Saturated ammonium sulfate was then added to a final concentration of 50% and incubated on ice for 1 h with gentle agitation. The sample was subsequently centrifuged (10,000 g) for 30 min at 4 °C. The pellet was collected and resuspended gently. This sample was then loaded onto a HiTrap Q Sepharose FF column (GE Healthcare), and the column was eluted with a linear gradient of 0–1 M NaCl. The GroEL-containing fraction was concentrated in Amicon Ultra-15 Centrifugal Filter Units (Millipore). The pTric 99 plasmids carrying the gene of GroEL, its single-ring mutant SR1 or GroES were a kind gift from Professor Arthur L. Horwich. The concentrations of purified proteins were estimated by the traditional Bradford method. The morphology of the purified GroEL was characterized by transmission electron microscopy (TEM) with a JEOL JEM 1400Plus electron microscope operated at 120 kV.

2.2. Fluorescence Anisotropy Measurements

The measurements were performed using a FluoroMax-4 instrument (HORIBA). The excitation and emission wavelengths for TAMRA were 560 nm and 582 nm, respectively.

2.2.1. Binding Isotherms

TAMRA-tagged bR peptide was diluted in HEPES buffer to a final concentration of 0.5 μM. Appropriate amounts of GroEL (or SR1) stock solution were added to multiple aliquots of peptide solution, with the final concentrations varied from 0 to 0.5 μM. The samples were pre-equilibrated at 20 °C for 30 min prior to measurements. Each data point represents an average of three readings of the same mixture.

2.2.2. Data Analysis

We adopted the following multi-site independent binding model to describe bR peptides binding to GroEL:

$$A + B \underset{K_D}{\rightleftarrows} AB \overset{+B}{\underset{K_D}{\rightleftarrows}} AB_2 \ldots\ldots \overset{+B}{\underset{K_D}{\rightleftarrows}} AB_n$$

A, *B*, AB_n, K_D and *n* ($n = 1, 2, 3\ldots\ldots$) represent GroEL, target peptide, their complex, equilibrium dissociation constant and binding stoichiometry, respectively. This greatly simplified approach to modeling the experimental data makes it easier to estimate K_D and *n* to describe the thermodynamic aspect of the binding of GroEL with the bR peptide. The K_D and fluorescence anisotropy (*r*) were defined as

$$K_D = \frac{[A][B]}{[AB]} = \frac{[AB][B]}{[AB_2]} \ldots\ldots = \frac{[AB_{n-1}][B]}{[AB_n]} \quad (1)$$

$$r = \frac{[B]}{[B_0]} r_B + \frac{n[AB_n]}{[B_0]} r_{AB_n} \quad (2)$$

$$[A] = [A_0] - [AB_n] \quad (3)$$

$$[B] = [B_0] - n[AB_n] \quad (4)$$

[*A*], [*B*] and [AB_n] represent the real-time concentrations of GroEL, target peptide and their complex; [A_0] and [B_0] represent the initial concentrations of GroEL and target peptide. r_B and r_{AB_n} denote the fluorescence anisotropy of target peptide and the corresponding GroEL-peptide complex. The following formula can be derived from Eqs. (1)–(4):

$$(K_D)^n \frac{(r - r_B)[B_0]}{n(r_{AB_n} - r_B)} = \left([A_0] - \frac{(r - r_B)[B_0]}{n(r_{AB_n} - r_B)}\right) * \left([B_0] - \frac{(r - r_B)[B_0]}{r_{AB_n} - r_B}\right)^n \quad (5)$$

The equilibrium dissociation constant K_D and binding stoichiometry *n* were obtained by fitting the experimental data using Eq. (5).

2.2.3. Kinetics Experiments

2.2.3.1. The Effect of GroEL, ATP or/and GroES on the TAMRA-peptide in Fluorescence Anisotropy. The anisotropy was recorded immediately after mixing GroEL (or its mutant SR1) with the bR peptide, and after the subsequent addition of ATP and GroES (or its mobile loop), either sequentially or simultaneously. The final concentrations of GroEL, SR1, ATP, GroES and its mobile loop were 0.5 μM, 1.0 μM, 5 mM, 1.0 μM and 7.0 μM, respectively.

2.2.3.2. The Competitive Binding of TAMRA-peptide and SBP to GroEL Measured by Anisotropy. The anisotropy of the bR peptide was monitored after it was added into a premix solution of GroEL (0.5 μM) and SBP (20.0 μM). Alternatively, the TAMRA-tagged bR peptide was mixed with GroEL firstly; after the anisotropy reached a steady state, SBP was added into the mixture and the anisotropy change was recorded.

2.3. Fluorescence Spectrum

The fluorescence spectra were measured using a FluoroMax-4 instrument (HORIBA) at 20 °C. The excitation wavelength was set to be