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Membrane protein crystallization in micelles conjugated by nucleoside base-pairing: A different concept

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ABSTRACT

The dearth of high quality, three dimensional crystals of membrane proteins, suitable for X-ray diffraction analysis, constitutes a serious barrier to progress in structural biology. To address this challenge, we have developed a new crystallization medium that relies on the conjugation of surfactant micelles *via* base-pairing of complementary hydrophobic nucleosides. Base-pairs formed at the interface between micelles bring them into proximity with each other; and when the conjugated micelles contain a membrane protein, crystal nucleation centers can be stabilized, thereby promoting crystal growth. Accordingly, two hydrophobic nucleoside derivatives – deoxyguanosine (G) and deoxycytidine (C), each covalently bonded to a 10 carbon chain were synthesized and added to an aqueous solution containing octyl β-D-thioglucopyranoside micelles. These hydrophobic nucleosides induced the formation of oil-rich globules after 2 days incubation at 19 °C or after a few hours in the presence of ammonium sulfate; however, phase separation was inhibited by 100 mM GMP. The presence of the membrane protein bacteriorhodopsin in the conjugated – micellar dispersion resulted in the growth within the colorless globules of a variety of purple crystals, the color indicating a functional protein. On this basis, we suggest that conjugation of micelles via base-pair complementarity may provide significant assistance to the structural determination of integral membrane proteins.

1. Introduction

Detergent micelles form spontaneously in aqueous media when the detergent concentration exceeds a broad threshold termed the critical micelle concentration (cmc) (Garavito and Ferguson-Miller, 2001; Wennerström and Lindman, 1979; Tanford, 1980; Rosen, 1989). In ideal dilute micellar suspensions, micelles do not interact with one another (Mitchell et al., 1983), but in response to global physical (*e.g.* temperature) or chemical (*e.g.* ionic strength, precipitants) modifications in the micellar environment, micelles can cluster and form aggregates (Garavito and Ferguson-Miller, 2001; Mitchell et al., 1983; Neugebauer, 1990). The initially transparent micellar solutions become turbid and enter a transient state termed the "*cloud-point*" representing micro-clusters of micelles (Loll et al., 2001). Further aggregation results in the separation of two distinct phases: a detergent rich phase and a detergent poor phase (Garavito and Ferguson-Miller, 2001; Mitchell et al., 1983; Nagarajan, 2002). Common micellar phase separation processes: (i) are non-specific; (ii) require high concentrations of salts, polymers (*e.g.* polyethylene glycols) or detergents well above the cmc; (iii) are difficult to fine-tune and control. Clearly, a mild, specific mechanism capable of controlling aggregation would be of significant value for applications which make use of micellar dispersions, such as: purification (Arnold and Linke, 2007; Dutta et al., 2015) and crystallization (Loll, 2003) of membrane proteins. This is particularly true if the mechanism is independent of the detergent chemical structure.

Membrane-associated proteins, comprising one third of the transcription products of the human genome (Wallin and von Heijne, 1998; Caffrey, 2003) are associated with diverse human disease states and currently represent more than 60% of the targets for drug development (Davey, 2004; Terstappen and Reggiani, 2001). Despite their obvious importance, obtaining high quality, three dimensional crystals of membrane proteins for structure

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determination remains a major barrier in structural biology. A significant degree of success has been achieved by embedding the proteins in detergent micelles (*i.e.* protein:detergent complexes, PDC) and then concentrating the PDC (Loll, 2003). Control over PDC aggregation is a central challenge of membrane protein crystallization. To this end, we have developed and reported on a mild, specific mechanism for conjugating *engineered-micelles,* composed of a non-ionic detergent and small amounts of a hydrophobic chelator (Patchornik et al., 2012a). The latter partition spontaneously into the micelle and position themselves at the micelle\water interface. Micellar conjugation is rapidly induced when metal ions, capable of binding two (or more) chelators simultaneously, are added. This mechanism was found to apply to diverse *engineered-micelles* comprised of a wide variety of non-ionic detergents, metals and hydrophobic chelators (Dutta et al., 2015; Patchornik et al., 2012a) as well as to natural lipid bilayers (Patchornik et al., 2012b). Imaging of the resulting conjugated *engineered-micelles* with cryo-transmission electron microscopy (cryo-TEM) (Patchornik et al., 2014) demonstrated that they undergo major structural transformations upon conjugation (*e.g.* initially spherical micelles are transformed into entangled thread-like micelles, membranes or multilamellar vesicles), presumably due to the strong binding affinity between the metal (Fe^{2+}) and hydrophobic metal chelator used (bathophenanthroline) (Smith and Martell, 1990).

We therefore sought to develop an alternative micellar conjugation mechanism that would rely on weaker binding affinities between adjacent micelles so as to suppress major structural transformations upon conjugation. This, in turn, might better preserve the native state of an embedded membrane protein and support crystal growth. As a first step towards this goal, we have studied micellar conjugation *via* hydrogen bonding of complementary base-pairs on hydrophobic nucleosides synthesized for this purpose (Fig. 1).

2. Materials and methods

2.1. Materials

Octyl β-D-thioglucopyranoside (OTG), ammonium sulfate (AS), tetracycline, *N,N*-dimethylformamide dimethyl acetal, 4,4′-dimethoxytrityl chloride and decanoyl chloride were obtained from Sigma-Aldrich (St. Louis, MO). 2′-Deoxycytidine and 2′-deoxyguanosine were purchased from Richem International Enterprise Co., Ltd., Shanghai, China.

2.2. General protocol for preparation of conjugated OTG-engineered-micelles containing hydrophobic nucleoside derivatives

Conjugated *OTG-engineered-micelles* were prepared according to the following protocol. 2.5 μL of the hydrophobic C derivative (25 mM in MeOH) were added slowly to 7.5 μL of 100 mM OTG in double distilled water (DDW) with continuous, vigorous vortexing (5–10 s). This was followed by further vortexing with the addition of 2.5 μL of the hydrophobic G derivative (25 mM in DMF) and 27.5 μL of DDW to a final volume of 40 μL. Aliquots (4 μL) of the resulting OTG\nucleoside solution were immediately placed on siliconized cover slides (Hampton Research, Aliso Viejo, CA) and mixed with an equal volume of an aqueous solution containing 1.5 M ammonium sulfate (AS). The combined drops were incubated at 19 °C over a reservoir containing 1.5 M AS in VDX™ crystallization plates (Hampton Research). Colorless, oil-rich globules appeared after 4–6 h of incubation.

2.3. Characterization of the synthesized oligonucleosides

The melting points recorded on a Bruker melting point apparatus are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR spectrometer. NMR spectra (^{1}H, ^{1}H decoupled ^{13}C and ^{1}H-^{1}H COSY) were recorded on a Bruker 400 or 500 MHz spectrometer with TMS as the internal standard. The coupling constants (*J* values) are given in Hz. High resolution mass spectra were recorded on a Bruker spectrometer under ESI Q-TOF conditions.

2.4. General protocol for the synthesis of 3′-O-(decanoyl)-2′-deoxycytidine (**5a**) and 3′-O-(decanoyl)-2′-deoxyguanosine (**5b**)

To a suspension of 2′-deoxycytidine/guanosine **1** (4.0 mmol, 1.0 equiv) in dry methanol (10 mL) was added N,N-dimethylformamide dimethyl acetal (8.26 mL, 7.38 g, 62.0 mmol, 15.5 equiv, excess) dropwise with vigorous stirring. The mixture was stirred at room temperature under N_2 atmosphere for about 28 h (monitored by TLC). The solid product **2** was isolated by filtration, washed with cold methanol (30 mL) and dried.

N^2-((Dimethylamino)methylene)-2′-deoxycytidine (**2a**): White solid; Yield 1.11 g, 99%; mp 196–198 °C; IR (KBr cm^{-1}) 3387 (br vs), 3113 (m), 2927 (s), 2871 (w), 1644 (vs), 1634 (vs), 1602 (vs), 1514 (s), 1429 (s), 1403 (s), 1383 (m), 1337 (s), 1286 (w), 1099 (vs), 1082 (vs), 1067 (vs), 998 (s) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.60 (s, 1H), 8.00 (d, *J* = 7.2 Hz, 1H), 6.15 (t, *J* = 6.6 Hz, 1H), 5.90 (d, *J* = 7.2 Hz, 1H), 5.23 (br unresolved t, 1H), 5.00 (br unresolved, 1H), 4.18–4.20 (m, 1H), 3.75–3.77 (m, 1H), 3.52–3.63 (m, 2H), 3.15 (s, 3H), 3.02 (s, 3H), 2.16–2.21 (m, 1H), 1.89–2.07 (m, 1H), Confirmed by ^{1}H-^{1}H COSY experiment; ^{13}C NMR (125 MHz, $CDCl_3$) δ 34.8, 40.7, 40.8, 61.2, 70.3, 85.4, 87.5, 101.6, 142.1, 155.1, 157.8, 171.1; HRMS (ES+, Ar) calcd for $C_{12}H_{18}N_4O_4$ (MH^+, 100) 283.1401, found 283.1382. Only ^{1}H NMR and CHN data are reported in (McBride et al., 1986).

N^2-((Dimethylamino)methylene)-2′-deoxyguanosine (**2b**): White solid, Yield 1.05 g, 97%; mp 230–232 °C; IR (KBr cm^{-1}) 3334 (s), 3107 (s), 2923 (m), 2865 (m), 2767 (w), 2688 (w), 1693 (s), 1683 (s), 1634 (m), 1543 (vs), 1488 (m), 1430 (m), 1349 (vs), 1323 (s), 1268 (m), 1251 (s), 1168 (m), 1159 (m), 1116 (s), 1098 (s), 1070 (s), 1035 (m), 1006 (m), 995 (m), 942 (m), 860 (m) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.94 (s, 1H), 6.14 (t, *J* = 6.3 Hz, 11H), 5.22 (d, *J* = 6.6 Hz, 1H), 4.87 (br t, *J* = 5.6 Hz, 1H), 4.25-4.32 (m, 1H), 3.72-3.78 (br m, 1H), 3.39-3.51 (m, 2H), 2.93 (s, 3H), 3.06 (s, 3H), 2.50 (ddd, *J* = 13.2, 7.4, 6.3 Hz, 1H), 2.11–2.16 (dddd, *J* = 13.2, 8.6, 6.3, 2.6 Hz, 1H), Confirmed by ^{1}H-^{1}H COSY experiment; ^{13}C NMR (100 MHz, $CDCl_3$) δ 34.7, 40.1, 40.6, 61.8, 71.0, 82.8, 87.7, 119.7, 136.7, 149.7, 157.3, 157.6, 158.0; HRMS (ES+, Ar) calcd for $C_{13}H_{19}N_6O_4$ (MH^+, 100) 323.1462, found 323.1438. Only ^{1}H NMR and CHN data are reported in (McBride et al., 1986).

A solution of **2** (0.4 mmol, 1.0 equiv), and DMTrt-Cl (162 mg, 0.48 mmol, 1.2 equiv) in dry pyridine (4 mL) was stirred under N_2 atmosphere at room temperature. After 3 h, complete consumption of the starting material was observed and a less polar spot was also observed on TLC (solvent system: CH_2Cl_2/MeOH 95:5 v/v) (Hasan et al., 1997). To the above reaction mixture was then added decanoyl chloride (0.17 mL, 152 mg, 0.8 mmol, 2 equiv) dropwise with vigorous stirring at 0 °C. After completion of the reaction as monitored through TLC, the reaction mixture was concentrated *in vacuo* and the residue was diluted with DCM (15 mL). The organic layer was washed with 5% $NaHCO_3$ solution (6 mL), water (2 × 10 mL), brine (5 mL) and dried over anhydrous Na_2SO_4. Then the organic layer was concentrated *in vacuo* and the crude product thus obtained was subjected to column chromatography using neutral alumina by eluting with CH_2Cl_2:MeOH (97:3 v/v) to afford