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Systematic optimization of cell-free synthesized human endothelin B receptor folding

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ABSTRACT

Cell-free production of G-protein coupled receptors is becoming attractive for biochemical characterization, ligand screening or even structural purposes. However, despite high production levels within the range of mg/mL, the fraction of functionally folded receptor is frequently below 1%. In synthetic cell-free reactions, numerous factors that affect the efficient folding and stability of translated membrane proteins can be addressed by the appropriate design of the synthetic expression environment. We demonstrate the systematic quality optimization of the cell-free synthesized human endothelin B receptor by a combined approach of lipid screening, redox optimization, and molecular engineering. Key parameters for receptor folding are the implementation of nanodiscs, the selection of suitable lipid environments for co-translational solubilization, as well as providing an optimized redox system for essential disulfide bridge formation. In addition, enrichment with chaperones as well as receptor engineering by thermostabilization further supported the folding into ligand binding conformation. In summary, we provide evidence that the initial co-translational folding process rather than long-term stability of the receptor is limiting. The folding efficiency could be improved by more than 10^3-fold and under optimized conditions, up to 1.6 nmol or ~100 μg of ligand binding competent receptor could be produced per mL of reaction mixture in a timescale of less than 24 h. The identified parameters affect rather common characteristics of G-protein receptors and are thus likely to improve the folding of similar targets as well. The optimized process provides full-length receptors embedded in defined membrane environments and in quantities and quality sufficient for throughput screening applications.

1. Introduction

G-protein coupled receptors (GPCRs) are one of the most important membrane protein families as they are involved in a multitude of physiological processes and play key roles in prevalent human diseases and disorders [1]. Consequently, they represent a major target fraction for modern pharmaceutical drugs with 475 drugs targeting 108 unique GPCRs, corresponding to ~34% of all drugs approved by the US Food and Drug Administration (FDA) by mid of 2017 [2]. Designing, screening and evaluation of new drugs requests fast and efficient platforms providing GPCRs in functionally folded and stable condition. Compared to classical detergent based approaches, lipid bilayers are more closely mimicking the native environment of membrane proteins and are therefore often superior for their characterization *in vitro* [3–5]. Most GPCRs are of low abundance in cells and their recombinant production is thus an almost mandatory prerequisite for their molecular analysis. Elaborated expression systems using insect or mammalian host cells are the current standard for GPCR production and they have considerably supported the progress in their structural evaluation [6]. With an optimized insect cell expression system, several mg of GPCR can be produced per L of cell culture. On the other hand, cell handling is relatively labor intense and the need to produce baculovirus for cell transfection is time consuming [7]. Further bottlenecks still exist in the efficient production and analysis of

Abbreviations: RT, room temperature; SEC, size exclusion chromatography; IMAC, immobilized metal-ion affinity chromatography; *CV*, column volume; CE, continuous exchange; CF, cell-free; RM, reaction mixture; FM, feeding mixture; DTT, dithiothreitol; TCEP, tris-2-carboxyethyl-phosphine; GSH, glutathione (reduced); GSSG, glutathione (oxidized); MSP, membrane scaffold protein; LP, liposome; ND, nanodisc; DPC, dodecylphosphocholine; DEPG, 1,2-dielaidoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serin; SOPG, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); ETB, endothelin receptor type B; BSA, bovine serum albumin; ET-1, endothelin-1.

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the vast majority of GPCRs as conventional cell-based expression systems are frequently faced with low synthesis rates, the need of potentially harmful membrane disruption procedures and altered expression efficiencies after genetical modification of the target protein.

Cell-free protein synthesis offers an alternative approach to get access to a GPCR target in a defined environment. It allows straight-forward optimization procedures that are exclusively focused on the production of a single protein without the need of compromises to cell viability and has therefore emerged as an alternative platform for the production and functional or structural characterization of membrane proteins [8]. Structural studies by crystallization and NMR are possible with samples isolated from few mL of CF reaction. These include the lipid metabolizing kinase DgkA [9], the K^+ channel KcsA [10] and the light-driven proton pump proteorhodopsin [11]. However, some types of membrane proteins such as the GPCRs are more difficult to address as their functional folding requires intensive screening of a variety of CF reaction conditions such as hydrophobic environments or redox conditions [12,13]. Despite quantities of several nmol of the human endothelin B (ETB) receptor could be synthesized per mL CF reaction, only a minor fraction of few pmol/mL and representing less than 0.1% of the synthesized protein appeared to be functionally folded [12]. A similar low quality was obtained from ETB samples isolated from CF reactions based either on *E. coli* lysates or on *Sf*9 insect cell lysates [14]. We therefore intended to identify and to optimize parameters in the CF reaction still being unfavorable or limiting for ETB folding.

We systematically analyzed reaction conditions with putative impact on ETB folding such as lipid environment, protein synthesis kinetics, disulfide bridge formation or presence of chaperones. Furthermore, the effect of thermostabilizing mutations on the quality of synthesized ETB samples was determined. We could identify limiting bottlenecks in ETB folding and the sample quality based on its competence to bind the cognate peptide agonist ET-1 could finally be improved from a less than pmol binding sites up to nmol quantities per mL CF reaction. High yields and fast production rates for ETB receptor as well as the general scalability and adaptability of the CF system are well suited for pharmacological profiling and optimized yields of 1.6 nmol corresponding to ~100 μg correctly folded receptor per mL of CF reaction mixture appear to be promising even for structural approaches. Furthermore, the exemplified strategy could provide a general basic guideline for the quality optimization of other CF synthesized GPCRs.

2. Materials and methods

2.1. Cell-free protein synthesis

Continuous Exchange Cell-free (CECF) protein synthesis was done in analytical scale reactions in mini-CECF reactions in a volume of 55 μL reaction mixture (RM) and 800–950 μL feeding mixture (FM) as previously described [13,15]. If not mentioned elsewhere, reactions were supplemented with 2 mM DTT in the RM and FM and 60 μM MSP1E3D1-nanodiscs or 4 mg/mL liposomes in the RM. Standard reaction time was 17 h at 30 °C and continuous shaking, if not stated elsewhere. For sample collection, RM was routinely centrifuged for 10 min at 18,000*g*, supernatant was separated from potential precipitates and used for further analyses.

2.2. Preparation of cell-free lysates

2.2.1. S30 lysate preparation

S30 lysate preparation was done as described previously. A 10 L fermentation of A19 in 2× YT medium yields approximately 60 mL of S30 lysate [16]. Alternatively, S30 lysate supplemented with T7RNAP can be obtained from commercial sources (Cube Biotech). In brief, the fermenter was inoculated 1:100 with a fresh preculture and operated at 37 °C with continuous stirring (500–700 rpm) and vigorous aeration until OD_{600} of 3.5–4.5 was reached. The broth was rapidly cooled to 18 °C and the cells were harvested by centrifugation at 6800*g* for 15 min. Pellets were washed three times with S30-A buffer (10 mM Tris-acetate (pH 8.2), 14 mM Mg $(OAc)_2$, 60 mM KCl, 6 mM β-mercaptoethanol) and centrifuged at 8000*g* for 10 min. The washed pellet was then suspended in 110% S30-B buffer (10 mM Tris-acetate (pH 8.2), 14 mM Mg $(OAc)_2$, 60 mM KCl, 1 mM DTT and 1 mM phenylmethanesulfonyl fluoride) and cells were disrupted by French Press. The lysate was centrifuged twice at 30,000*g* for 30 min. The supernatant was then transferred into a fresh tube and adjusted to a final concentration of 400 mM NaCl. The lysate was then incubated at 42 °C for 45 min and subsequently dialyzed overnight against 2 L S30-C buffer (10 mM Tris-acetate (pH 8.2), 14 mM $Mg(OAc)_2$, 60 mM KOAc, 0.5 mM DTT) with two buffer exchanges and by using a 12–14 kDa cutoff membrane. The lysate was then centrifuged twice at 30,000*g* for 30 min. The supernatant was finally aliquoted and adjusted with an appropriate volume of purified T7 RNA polymerase. The lysate can be shock-frozen in liquid nitrogen and stored at −80 °C for many months.

2.2.2. S30-H lysate preparation

S30-H lysate was prepared as published [17]. Preparation is identical to that of S30 lysate, with the modification that 300 mL ethanol were added at OD_{600} of 4.0 and the fermentation temperature was increased to 42 °C for 45 min before chilling the broth to 20–18 °C.

2.2.3. T7 RNA polymerase (T7RNAP) preparation

T7RNAP is necessary for the efficient transcription of the T7 promoter based DNA templates in CF synthesis reactions. One liter culture yields 20,000–40,000 units T7RNAP [16]. The enzyme was produced from the *E. coli* strain BL21 (DE3) Star x pAR1219 [18] by conventional cultivation in Erlenmeyer flasks. One liter LB medium was inoculated 1:100 with a fresh overnight culture and incubated at 37 °C on a shaker until OD_{600} = 0.6–0.8. The culture was induced with 1 mM IPTG and further incubated at 37 °C for 5 h. Cells were harvested by centrifugation at 4500*g* for 15 min and the pellets suspended in 30 mL resuspension buffer (30 mM Tris–HCl (pH 8.0), 10 mM EDTA, 50 mM NaCl, 5% glycerol *(v/v)*, and 10 mM β-mercaptoethanol). Cells were disrupted by French press and centrifuged at 20,000*g* for 30 min. Nucleic acids in the supernatant were precipitated with 4% streptomycin sulfate for 5 min on ice. The suspension was centrifuged at 20,000*g* for 30 min and the supernatant loaded onto a 40 mL Q-Sepharose column equilibrated with 2 column volumes of equilibration buffer. The column was washed with equilibration buffer (30 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol) at a flow rate of 4 mL/min and proteins eluted with a gradient from 50 to 500 mM NaCl at a flow rate of approximately 3 mL/min. Fractions with T7RNAP were pooled and dialyzed against dialysis buffer (10 mM K_2HPO_4–KH_2PO_4 (pH 8.0), 10 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 5% glycerol). The enzyme was concentrated to approx. 4 mg/mL by ultrafiltration, adjusted to 50% *(v/v)* glycerol and stored at −80 °C.

2.3. Preparation of nanodiscs and liposomes

2.3.1. Preparation and purification of MSP1E3D1 protein

MSP1E3D1 expression and purification followed the protocol previously described [15]. Briefly, expression was performed in *E. coli* strain BL21 (DE3) Star in LB medium supplemented with