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Energetics of OCP1–OCP2 complex formation

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Abstract

OCP1 and OCP2, the most abundant proteins in the cochlea, are putative subunits of an SCF E3 ubiquitin ligase. Previous work has demonstrated that they form a heterodimeric complex. The thermodynamic details of that interaction are herein examined by isothermal titration calorimetry. At 25 °C, addition of OCP1 to OCP2 yields an apparent association constant of 4.0×10^7 M^{-1}. Enthalpically-driven ($\Delta H=-35.9$ kcal/mol) and entropically unfavorable ($-T\Delta S=25.5$ kcal/mol), the reaction is evidently unaccompanied by protonation/deprotonation events. ΔH is strongly dependent on temperature, with $\Delta C_p=-1.31$ kcal mol^{-1} K^{-1}. Addition of OCP2 to OCP1 produces a slightly less favorable ΔH, presumably due to the requirement for dissociation of the OCP2 homodimer prior to OCP1 binding. The thermodynamic signature for OCP1/OCP2 complex formation is inconsistent with a rigid-body association and suggests that the reaction is accompanied by a substantial degree of folding.

Keywords: Ubiquitin ligase; SCF complex; F-box protein; Organ of Corti; Cochlea; ITC

1. Introduction

The organ of Corti (OC), the mammalian auditory organ, contains sensory and non-sensory cells. The sensory cell population includes the *inner*- and *outer hair cells*, responsible for acoustic signal transduction and amplification, respectively. The non-sensory cell population, also known as the supporting cell population or epithelial support complex (ESC), includes 12 morphologically distinct cell types [1]. The ESC sub-populations share two characteristics — an extensive gap-junction network and high-level expression of OCP1 and OCP2.

OCP1 and OCP2 comprise approximately 10% of the total cell protein in the OC [2]. Although their physiological role is presently unknown, sequence analysis suggests that they are components of an SCF E3 ubiquitin ligase. SCF complexes direct ubiquitination of specific target proteins. Ubiquitination is widely regarded as the signal for destruction by the 26S proteasome [3]. However, ubiquitin is known to have additional signaling functions. Recognition by the proteasome requires polyubiquitination ($N\geq4$). Mono-, di-, and tri-ubiquitinated species are not degraded. Protein translation, activation of protein kinases and transcription factors, and DNA repair are examples of ubiquitin-dependent phenomena not involving proteolysis [4].

Since their discovery in yeast [5,6], SCF ligases have been found in all eukaryotic species [7]. SCF is an acronym for *S*kp1, *c*ullin, and *F*-box protein. Cullin – named for the Cul1 gene product in yeast – serves as the scaffold for complex assembly [8–10]. Skp1 and the ubiquitin-conjugating enzyme (the E2 ligase) bind to cullin. The F-box protein, which dictates target protein specificity [11], binds to Skp1 through a characteristic F-box motif, present in the N-terminal half of the sequence. Interactions with the C-terminal domain of the F-box protein evidently position the target protein for ubiquitination by the E2 conjugating enzyme. The protein known as Rbx1 or Roc1 is also a subunit of the SCF complex [12].

The 163-residue sequence of OCP2 is identical to that of Skp1, although OCP2 is reportedly transcribed from a distinct

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gene [13]. The 299-residue sequence of OCP1 [14] harbors an F-box motif and exhibits 81% identity to Fbs1, or neural F-box protein [15]. OCP1 and OCP2 form a high-affinity heterodimeric complex [16]. Although several Skp1/F-box protein complexes have been characterized structurally, there is a paucity of thermodynamic data for the interaction. In this paper, we examine the energetics of OCP1–OCP2 complex formation by isothermal titration calorimetry (ITC).

2. Experimental

2.1. Reagents and chemicals

NaCl, $CaCl_2 \cdot H_2O$, $MgCl_2 \cdot 2H_2O$, trishydroxymethylaminomethane (Tris), monobasic anhydrous sodium phosphate (NaP_i), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), lysozyme, and Spectrapor 2 dialysis tubing (MWCO 12,000–14,000) were purchased from Fisher Scientific Co. Luria-Bertani (LB) broth (Miller), LB agar (Miller), ampicillin, kanamycin, and chloramphenicol were obtained from Research Products International. Isopropylthio-β-D-galactoside (IPTG) and dithiothreitol (DTT) were purchased from Gold Biotechnology. Diethylaminoethyl-Sepharose (DEAE-Sepharose), Sephadex G-75, Sephacryl S-100 HR, and trishydroxypropyl phosphine (THP) were obtained from Sigma-Aldrich Co. Complete® protease inhibitor tablets were obtained from Roche Applied Science. $^{15}NH_4Cl$ was purchased from Cambridge Isotope Laboratories.

2.2. Protein expression and purification

2.2.1. OCP2

The OCP2 coding sequence was cloned into pTriEx-1.1 (Novagen), between the NcoI and BamHI sites. That construction was used to transform *E. coli* BL21(DE3) Rosetta 2 cells (Novagen). Bacteria harboring the OCP2-pTRIex1.1 plasmid were cultured at 37 °C in LB broth containing ampicillin (100 μg/mL) and choramphenicol (30 μg/mL). When the turbidity of the culture, measured at 600 nm, reached 0.6, expression was induced with IPTG (0.25 mM). After an additional 3 h, the bacteria were harvested by centrifugation. The cell paste was resuspended in 20 mM Hepes, pH 7.4, containing protease inhibitors, and lysed by treatment with lysozyme and extrusion from a French pressure cell. The resulting suspension was heat-treated for 5 min at 60 °C, then centrifuged at 4 °C for 30 min at 27,000 ×g. OCP2 was isolated from the clarified lysate by NaCl-gradient (0–0.6 M, in 20 mM Hepes, pH 7.4) elution from DEAE-Sepharose, followed by gel-filtration through Sephadex G-75 in PBS (0.15 M NaCl, 0.010 M NaP_i, pH 7.4) containing 0.001 M DTT. A 1-L culture yields 20–30 mg with purity exceeding 95%.

The identical protocol was used to isolate ^{15}N-labeled OCP2, except that the bacteria were cultured on minimal medium (M9 salts plus glucose) containing 1.25 g $^{15}NH_4Cl$ per liter.

2.2.2. OCP1

The OCP1 coding sequence was cloned into pET28a (Novagen), between the Nde I and BamH I sites, downstream from the hexa-histidine tag and thrombin-cleavage site. Expression – in Rosetta 2 cells, in LB broth containing kanamycin (50 μg/mL) and chloramphenicol – and lysis were performed as described above. OCP1 was isolated from the clarified lysate by binding/elution from Ni-NTA His-Bind resin (Novagen). After overnight dialysis at 4 °C against PBS, the protein was treated, for 4 h on ice, with thrombin (Novagen, 1 U/mg protein). After confirming cleavage, the thrombin was inactivated with PMSF (1 mM) and DTT (10 mM). The preparation was then subjected to gel-filtration through Sephacryl S-100 HR, in PBS and 1 mM DTT. A 1-L culture yields 6–8 mg of OCP1 with purity exceeding 95%.

2.3. Quantitation

OCP1 and OCP2 concentrations were estimated spectrophotometrically. Extinction coefficients at 280 nm – 81,000 and 19,400 M^{-1} cm^{-1}, respectively – were obtained by parallel absorbance and interference measurements in a Beckman XL-I analytical ultracentrifuge.

2.4. Isothermal titration calorimetry

ITC was performed in a MicroCal VP-ITC. Prior to analysis, OCP1 and OCP2 were dialyzed extensively against a common buffer. THP (2.0 mM) was included in the buffer reservoir to maintain the cysteine sulfhydryl groups in the reduced state. Aliquots of the dialysis buffer were used to make dilutions.

Raw ITC data were integrated using the software supplied with the instrument. Single datasets were analyzed using the single-site model. An Origin script was written to simultaneously fit multiple datasets. All titrations included a 2.0 μL pre-injection. The heat associated with that addition, invariably low due to diffusion of titrant from the buret during thermal equilibration, was neglected during the fitting process. The integrated signals from the final three additions in each experiment were averaged and used to estimate the combined heat of mixing/dilution, which was subtracted from the data prior to least-squares minimization.

2.5. NMR spectroscopy

The ^1H,^{15}N-HSQC spectrum of OCP2 was obtained at 30 °C on a Varian Inova 600 MHz spectrometer, employing a triple-resonance cryoprobe equipped with pulsed-field *z* gradient. The 2.0 mM sample contained 0.15 M NaCl, 0.01 M NaP_i, pH 6.0, 0.002 M THP, 5% D_2O. Data were acquired with the BioPack N15-HSQC pulse sequence, processed with NMRPipe [17], and visualized with Sparky [18]. ^1H chemical shifts were referenced relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS); the ^{15}N shifts were referenced indirectly.

2.6. Accessible surface area calculations

The surface area buried in the Skp1/Fsb1 interface was analyzed with CNS [19]. Hydrogen atoms were added to the coordinates of the Skp1–Fsb1 complex (PDB code 2E31) using the ALLHDG topology and parameter files. Water molecules