Title: Cooperative subunit refolding of a light-harvesting protein through a self-chaperone mechanism

Authors: Alistair J Laos; Jacob C Dean; Zi S. D. Toa; Krystyna E Wilk; Gregory D Scholes; Paul M. G. Curmi; Pall Thordarson, PhD

To be cited as: 10.1002/anie.201607921

Link to VoR: https://doi.org/10.1002/anie.201607921

Cooperative subunit refolding of a light-harvesting protein through a self-chaperone mechanism

Alistair J. Laos, Jacob C. Dean, Zi S. D. Toa, Krystyna E. Wilk, Gregory D. Scholes, Paul M. G. Curmi* and Pall Thordarson*

Abstract: The fold of a protein is encoded by its amino acid sequence, but how complex multimeric proteins fold and assemble into functional quaternary structures remains unclear. Here we show that two structurally different phycobiliproteins refold and reassemble in a cooperative manner from their unfolded polypeptide subunits, without biological chaperones. Refolding was confirmed by ultrafast broadband transient absorption and two-dimensional electronic spectroscopy to probe internal chromophores as a marker of quaternary structure. Our results demonstrate a cooperative, self-chaperone refolding mechanism, whereby the β-subunits independently refold, templating the folding of the α-subunits which then chaperone the assembly of the native complex quantitatively returning all coherences. Our results indicate subunit self-chaperoning is a robust mechanism for heteromeric protein folding and assembly that could also be applied in self-assembled synthetic hierarchical systems.

Folding of proteins was shown to be amino acid encoded in 1960s.[1] Cooperativity[2-4] and the realization that multiple folding pathways are possible[5] are the keys to resolving Levinthal's famous paradox[6] which states that if a protein were to explore all possible conformations, it would take longer than the age of the universe to fold—yet most proteins fold within seconds. Many proteins in nature are found as multimeric or quaternary complexes[7] with database searches suggesting more than 80% of proteins are multimeric, with between 15-50% hetero-oligomeric.[8,9] The prevailing view on how these complexes form starts with the assumption that the individual subunits are first folded or sometimes, disordered (fuzzy), prior to the oligomerization step.[8,10] The folded monomer of the homo-oligmeric GroEL chaperonin catalyzis its own oligomerization[11] in a process known as self-chaperoning. Such behavior has also been observed in the folding of the bI5 RNA intron with its CBP2 protein cofactor.[12] How, or if, quaternary structures can be unfolded and then refolded in vitro, and whether the subunits need each other to fold and form a quaternary structure, remains an open question.

There are two different quaternary types of phycobiliproteins in the cryptophytes, the closed form and the open form protein. Both quaternary structures share the same types of subunits, that of two identical ~ 20 kDa β-subunits, and two identical or non-identical ~ 7-9 kDa α-subunits, which combine to make the ~ 60 kDa αβαβ heterodimer (Figure S1). The *Rhodomonas* sp. phycobiliprotein, phycoerythrin 545 (PE545[13]), is a dimer of two non-identical αβ monomers ($\alpha_1\beta\alpha_2\beta$, Figure 1a, PDB: 1XF6[14]). Each globular β-subunit covalently binds three linear tetrapyrroles (bilins), and the two non-identical α-subunits (α_1 and α_2) are short extended polypeptides, each with a single covalently bound bilin chromophore. The dimer adopts the closed form quaternary structure that brings two chromophores from each monomer into van der Waals contact. This feature is believed to allow electronic coherence in the closed form phycobiliproteins.[15,16] The phycocyanin 577 (PC577) phycobiliprotein from the *Hemiselmis pacifica* has identical αβ-monomers in contrast to PE545 (αβαβ, Figure S1,[17]) with chemically distinct chromophores. Along with other cryptophyte phycobiliproteins of the *Hemiselmis* clade, PC577 adopts a distinct, open form quaternary structure with a water-filled channel separating the central chromophores.[15]

Little is known about phycobiliprotein folding although interestingly, the α- and β-subunits are synthesized in two different compartments of the cryptophyte cell,[18] The question we address here is whether the subunits in these phycobiliproteins fold independently prior quaternary structure formation, or whether folding and assembly are cooperative.

Using pH titrations from pH 7, to pH 2, and then back to pH 7, we demonstrate the ability of PE545 and PC577 to reassemble and refold, from unfolded polypeptide states of the α/β-subunits without chaperones (Figure 1 – left side pathway: B→C→D→G→I). Denaturation and renaturation of PE545 was performed in 25 mM potassium phosphate buffer via simple titrations with hydrochloric acid to pH 2 and a subsequent titration back to pH 7 with sodium hydroxide after a time period of 10 minutes resulting in ca 80% yield for the recombined, refolded protein (Figure 1G and Figure S2). Leaving the proteins for longer than 10 min. at low pH reduced the yield of the refolded proteins while other refolding methods such as cooling after heat denaturation proved irreversible. At pH 2, the β-subunits remain relatively water soluble and stable for short time periods (< 60 min), while the water soluble α-subunits remain stable. Both subunits are positively charged at pH 2 (β subunit: +12; α_1: +7.5; and α_2: +8, including the tetrapyrrole chromophores).

* A. J. Laos, Prof. P. Thordarson
School of Chemistry, the Australian Centre for NanoMedicine and the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, The University of New South Wales, Sydney, 2052, NSW (Australia)
E-mail: p.thordarson@unsw.edu.au

A. J. Laos, Dr. K. E. Wilk, Prof. P. M. G. Curmi
School of Physics, The University of New South Wales, Sydney, 2052, NSW (Australia)
E-mail: p.curmi@unsw.edu.au

Dr. J. C. Dean, Z. S. D. Toa, Prof. G. D. Scholes
Department of Chemistry, Princeton University, Princeton, New Jersey 08544 (U.S.A.)

Supporting information for this article is given via a link at the end of the document.

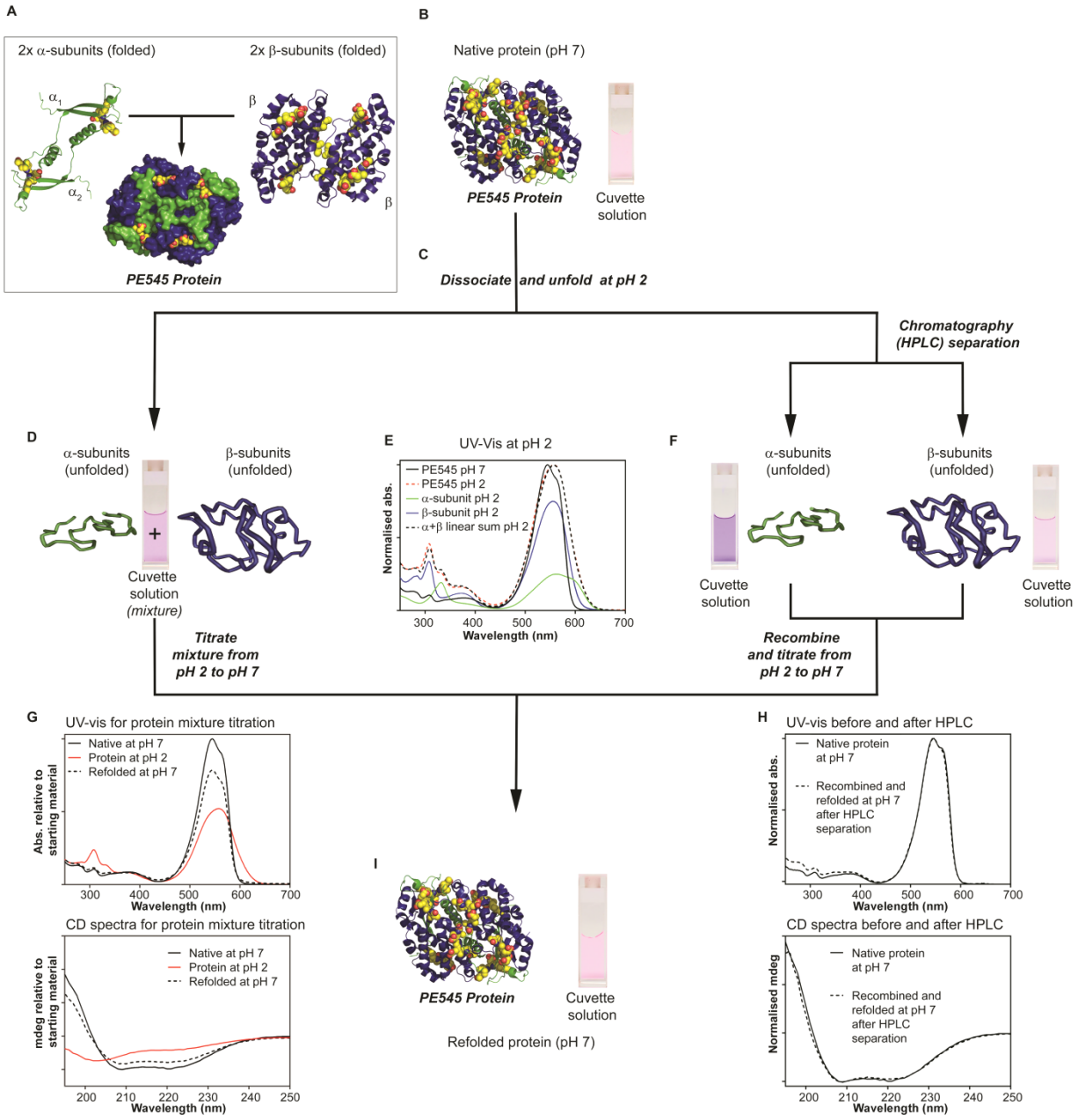


Figure 1. Unfolding and refolding of light-harvesting phycoerythrin 545 (PE545). A) The α- and β-subunits from the crystal structure of PE545 and the corresponding solvent accessible surface of the whole protein (pdb: 1XF6[14]). The two β-subunits are identical but α_1 and α_2 are not. B) A solution of native PE545 at pH 7 and the crystal structure of PE545. C) Lowering the pH to 2 leads to dissociation and unfolding of the PE545 protein. D) The solution of the PE545 after unfolding at pH 2. E) The normalized UV-Vis spectra at pH 2 of the unfolded protein in aqueous solution (red dotted line) represented schematically in D), as well as F, the reverse phase HPLC separated α-subunits (green line) and the β-subunits (blue line) from F in 4:1 (v/v) CH_3CN in 0.1 M $HCl_{(aq)}$, and the linear sum (dotted black line) of the α- and β-subunits spectra from F. G) The UV-Vis (upper panel) and CD (lower panel) spectra of the native protein (black line) from B, the unfolded protein (red line) from D, and refolded protein mixture at pH 7 (dotted black line). H) The UV-Vis (upper panel) and CD spectra of the native protein (solid line) from A, and recombined pure α- and β-subunits from F, after titrating it back to pH 7 (dotted line). I) A solution of the refolded protein from H, at pH 7 (the solution from G, is identical – not shown).

We performed a wide range of steady state spectroscopy to characterize the native, unfolded, and refolded proteins, (Figure 1G) along with their purified subunits from reverse phase HPLC using 1:2:12 (v/v) CH_3CN/isopropanol/0.1 M $HCl_{(aq)}$ as the running buffer (Figure 1F and Figure S3). Their purified α/β-subunits allowed deconvolution of the relative spectral contributions of the subunits in both the unfolded and native state, which further aided in elucidating the refolding events. Refolding experiments of the subunits show that the purified, isolated β-subunits refold (see below) whereas the α-subunits do not. When the two reverse phase HPLC separated subunits α and β were isolated at pH 2 and recombined at pH 7 (Figure 1, right side pathway: B→C→F→H→I) they refolded correctly to the native PE545 structure (Figure 1H and Figure S4). We also performed the same pH titrations and reverse phase HPLC separation experiments on the open form PC577 phycobiliprotein. Whether PC577 was titrated from pH 7 to pH 2, with or without reverse phase HPLC separation of subunits, and then titrated back to pH 7, the protein recovered and appeared spectroscopically identical to native PC577 (Figures S2-S5).

To provide an internal characterization of the refolded states of the phycobiliproteins we compared coherent oscillations recorded in the time-domain (and their frequency content in the spectral domain) for the native and refolded proteins using coherent electronic spectroscopy. The coherences serve as fingerprints of each chromophore as their frequencies and amplitudes are inherently sensitive to the chromophore conformation and its local protein environment.[19,20] In this way, coherent electronic spectroscopy can be utilized for its inherent selectivity of the eight structurally-distributed tetrapyrroles, exploiting them as local probes of the protein scaffold itself (Figure 2). Specifically, we implemented broadband transient absorption (BBTA) spectroscopy, where the coherent oscillations along the pump-probe time delay are recorded as a function of probe frequency (ν_3) and two-dimensional electronic spectroscopy (2DES),[21] which further disperses the BBTA signal into two dimensions by resolving the excitation axis (ν_1) in addition to the detection axis (ν_3). The combination of both methods allowed us to decisively compare coherences for the proteins from ultrafast (fs) to picosecond timescales.

As an example, the total 2D (magnitude) spectra at pump-probe time delay t_2 = 100 fs (Figure 2A), when the oscillatory amplitude at 500 cm^{-1} (Figure 2B) in the 2D oscillation maps are compared, the data recorded for native and refolded PE545 protein are nearly identical. More importantly, the 1D traces (Figure 2C) and Fourier (FT) power spectra (Figure 2D) of the BBTA spectra show that the fundamental coherences in the native and refolded are almost identical out to 2 ps. This is notable considering that coherences in and between protein chromophores are highly dependent on their conformational/torsional states. The measurements show that the proteins refold with high fidelity as demonstrated by comparing the PE545 power spectrum to that of the α-subunit (see Supporting Information, Figure S6). Similar broadband femtosecond coherent spectroscopy experiments on the open form PC577 protein also show excellent agreement between the native and refolded protein (Figure S7).

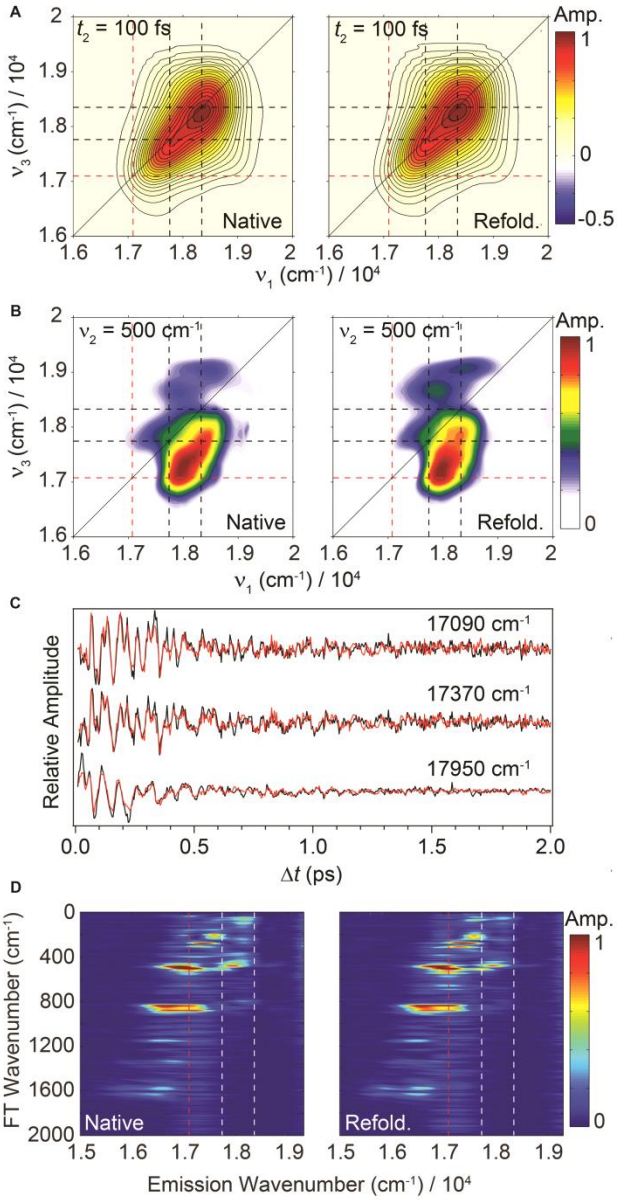


Figure 2. Coherent spectroscopy of the light-harvesting PE545 protein. A) Normalized 2D spectra at t_2 = 100 fs. B) 2D coherence maps at 500 cm^{-1} for native (left) and refolded (right) proteins. C) Broadband transient absorption (BBTA) traces of native (black) and refolded (red) proteins at various spectral positions (n=5). D) The power spectra for native (left) and refolded (right) proteins generated from coherent BBTA. Dashed lines indicate steady-state absorption (white) and fluorescence (red) maxima.

The high spectral yield (Figure 1G) and equivalence among coherences of the refolded and native proteins (Figure 2) indicates that refolding and subunit assembly proceeds via a well-defined thermodynamic and kinetic funnel.[22] This route is likely driven by the autonomous refolding of the β-subunit, evident in the CD spectra (Figure S8A and Figure S9A-B). At pH 7, the β-subunit secondary structure reforms, and importantly, the visible CD band re-appears as the β-subunit backbone refolds to its native chiral environment.

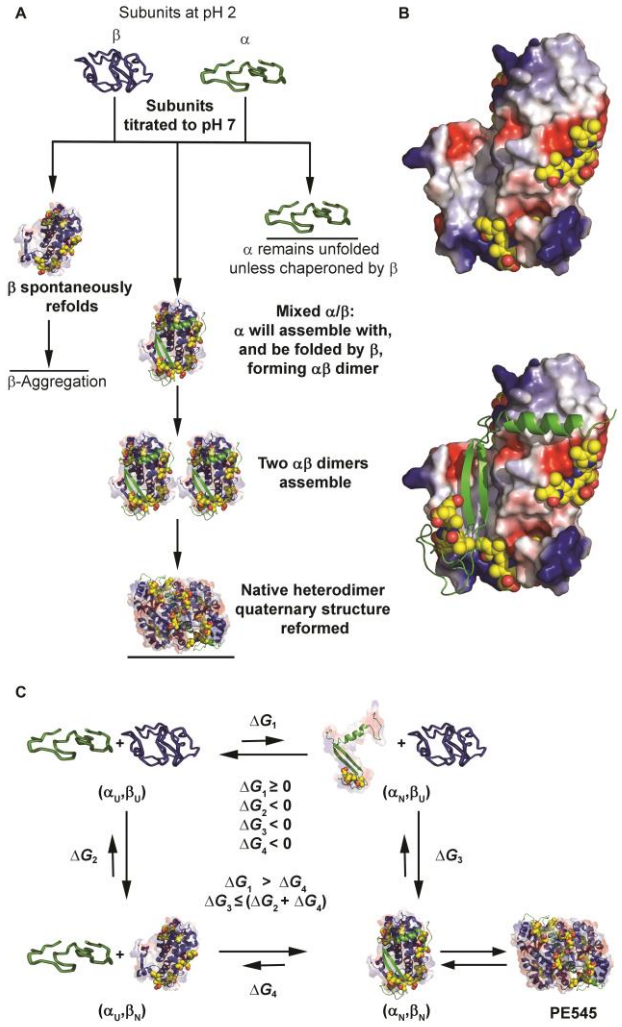


Figure 3. Proposed mechanism for the refolding of the PE545 protein. A) Diagram of the proposed PE545 subunit self-chaperone refolding mechanism. B) Top: Surface electrostatics (blue – positive, white – neutral/hydrophobic, red – negative) of PE545 β-subunit from crystal structure 1XF6[15] (with α-subunit hidden) showing the α-binding pocket. Bottom: Surface electrostatics of PE545 β-subunit shown with a cartoon representation of the α-subunit. C) The thermodynamic cycle for subunit folding leading to the formation of the folded αβ monomers starting from the unfolded α- and β-subunits (α_U,β_U).

The UV-Vis and fluorescence spectra (Figure S10) provide additional evidence for refolding. The α-subunits show unfolded (random coil) spectral structure at both pH 2 and pH 7 (Figure S8B and Figure S9C-D), with no resemblance to the native protein in the visible region. When titrated to pH 7, the α-subunits remain unfolded, and the refolding of the β-subunit leads to β-subunit aggregation. Therefore refolding and assembly of the protein complexes requires positive cooperativity[15] between α and β during refolding (Figure S8C and Figure 3).

We propose that upon titration to pH 7, native charge of the subunits is restored, leading initially to the spontaneous refolding of the β-subunit. This refolding promotes binding of the α-subunits to the hydrophobic pocket of the β-subunit, acting as a template for the folding of the water soluble α-subunit (Figure 3A). In binding to the β-subunit, the α-subunit acts as a chaperone preventing β-subunit aggregation. Finally, the αβ monomers bind one another to form the native $\alpha_1\beta\alpha_2\beta$ heterodimer quaternary structure. The native protein titrations (Figure 1G) and the exceptional agreement among the coherence fingerprints (Figure 2) together confirm that the final product is the $\alpha_1\beta\alpha_2\beta$ heterodimer and not a mixture of the heterodimer and the $(\alpha_1\beta)_2$ and $(\alpha_2\beta)_2$ homodimers. Noting that the two αβ monomers in PC577 are identical and therefore ruling out the possible formation of heterodimers. The data obtained for PC577 (Figures S7, S9 and S10) suggests PC577 unfolds and refolds by the same mechanism as PE545.

Cooperativity is the key to the high precision in re-folding and re-assembly of the cryptophyte phycobiliproteins. A clear sigmodal behavior is apparent when the characteristic absorptions of the α- and β-subunits at 602 nm and 306 nm, respectively, are plotted as a function of pH (Figure S8C). This is a clear sign of cooperativity[2] in the folding of these subunits. Moreover, the subunit absorbance changes are coupled; fitting the data to a simple sigmoid curve (four parameter logistic equation) gives an apparent pK_a = 3.5±0.3 and 3.1±0.3 (95% confidence interval) for the α- and β-subunits, respectively. In other words, the α- and β-subunits (un)fold simultaneously, which further confirms this highly cooperative process for both subunits.[2]

Cooperativity is further evident if one considers the thermodynamic cycle for the folding of the αβ monomer from the unfolded subunits (Figure 3C). The β-subunit folds autonomously, indicating the free energy is negative ($\Delta G_2 < 0$), whereas the α-subunit does not refold, implying $\Delta G_1 \geq 0$. Rather, the α-subunit folds spontaneously in the presence of a folded β-subunit ($\Delta G_4 < 0$). It follows that $\Delta G_1 > \Delta G_4$, i.e. positive cooperativity for the folding of the αβ monomers.[4] The hydrophobic binding pocket in β includes a negative electrostatic region and that the α-subunits are overall more positively charged (calculated charges of 0 and +2 at pH 7 for α_1 and α_2, respectively, including chromophores) than β (calculated charge of -2 at pH 7 including chromophores) suggesting that the cooperativity observed may be explained by initial electrostatic interactions between the positively charged α and negatively charged regions on β.

To confirm the proposed mechanism in Figure 3, kinetic experiments (Figure S12-S17) following protein fluorescence were performed. The kinetic data fitted well to a two-step model that assumes a fast first order process (k_{1app} = 1.4±0.6 s^{-1}), followed by a slower second order process (k_{2app} = 0.14±0.02 μM^{-1} s^{-1}). The β-subunit on its own also folds with a first order k = 2.1±0.6 s^{-1}. The protein refolding yield increases with concentration to a maximum at 80% around 5-10 μM concentration of the protein before decreasing again (Figure S18). This, and the second order slower step apparent in the kinetic experiments suggests that binding of folded β to unfolded α (self-chaperoning) or possibly the final monomer-to-dimer formation step is the rate-determining step. The decrease in protein yield at high concentration is readily explained by β-subunit aggregation competing with the folding pathway in Figure 3.

These results provide an insight into a possible in vivo mechanism of folding, and assembly of quaternary hetero-oligomers including PE545 and PC577. In the cryptophytes, the β-subunit is expressed in the plastid stroma[18] and folds, possibly with the aid of an unknown chaperone partner which prevents aggregation. This β-subunit is then on standby until the unfolded freshly synthesized nuclear encoded α-subunits are translocated across five membranes[18] to bind to the β-subunit in the stroma, triggering the folding of the α-subunit and the subsequent formation of the quaternary structure. This scenario bears some resemblance to the assembly of Ribulose-1,5-bisphosphate carboxylate/oxygenase (Rubisco) whereby chaperones fold and then keep the plastid encoded Rubisco large subunits (RbcL) from aggregating until the Rubisco small subunit (RbcS) becomes available.[23-25]

The work here has potential to inspire improvements in biotechnology methods for making quaternary protein complexes in vitro, and additionally in understanding ultrafast light-harvesting and protein folding in the quantum and chemical biology fields, respectively. Based on the mechanism proposed here, it should be possible to design and synthesize novel hybrid quaternary light harvesting protein complexes via complementary (cooperative) interactions between otherwise unrelated subunits. The lessons learned here could be extended to synthetic (macro)molecular systems in terms of how complex multimeric (quaternary) foldamers[26] could be constructed. Overall, our results suggest that subunit self-chaperoning[10,11] is important for the folding of the cryptophyte light harvesting complexes, and may be a widespread paradigm for the folding of heteromeric protein complexes.

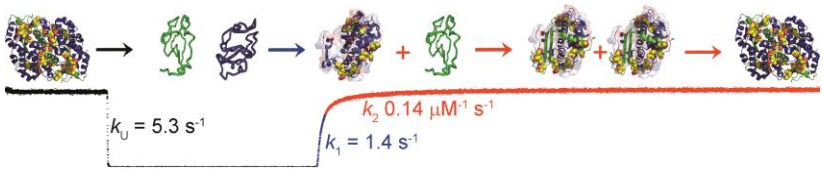
Acknowledgements

We thank the Australian Research Council (ARC) for an ARC Future Fellowship (FT120100101) and ARC Centre of Excellence Grant funding (CE140100036) to PT and an ARC Discovery Project Grant (DP150102580) to P.M.G.C. and G.D.S. We also acknowledge support by the U.S. Air Force Defence Research Scientific Program through the Asian Office of Aerospace Research and Development (FA2386-15-1-4078) to P.M.G.C and funding to G.D.S. from the U.S. Department of Energy, Office of Science, Basic Energy Sciences for the Photosynthetic Antenna Research Center (PARC), an Energy Frontier Research Center (#DE-SC0001035).

Keywords: Self-assembly • protein folding • protein complexes • cooperative effects • ultrafast spectroscopy

[1] C. B. Anfinsen, *Science* **1973**, *181*, 223-230.
[2] H. S. Chan, S. Bromberg, K. A. Dill, *Phil. Trans. R. Soc. Lond. B* **1995**, *348*, 61-70.
[3] A. Whitty, *Nat. Chem. Biol.* **2008**, *4*, 435-439.
[4] J. R. Williamson, *Nat. Chem. Biol.* **2008**, *4*, 458-465.
[5] M. Karplus, *Folding & Design* **1997**, *2*, S69-S75.
[6] C. Levinthal, in *Mossbauer Spectroscopy in Biological Systems* (Eds.: P. Debrunner, J. C. M. Tsibris, E. Münck) Univ. Illinois Press, Urbana, 1969, pp. 22-24.
[7] S. E. Ahnert, J. A. Marsh, H. Hernandez, C. V. Robinson, S. A. Teichmann, *Science* **2015**, *350*, aaa2245.
[8] J. A. Marsh, S. A. Teichmann, *Annu. Rev. Biochem.* **2015**, *84*, 551-575.
[9] B. J. G. E. Pieters, M. B. van Eldijk, R. J. M. Nolte, J. Mecinović, *Chem. Soc. Rev.* **2016**, *45*, 24-39.
[10] M. J. Ragusa, B. Dancheck, D. A. Critton, A. C. Nairn, R. Page, W. Peti, *Nat. Struct. Mol. Biol.* **2010**, *17*, 459-464.
[11] N. M. Lissin, S. Yu Venyaminov, A. S. Girshovish, *Nature* 1**990**, *348*, 339-342.
[12] I. Garcia, K. M. Weeks, *Biochemistry* **2004**, *43*, 15179-15186.
[13] K. E. Wilk, S. J. Harrop, L. Jankova, D. Edler, G. Keenan, F. Sharples, R. G. Hiller, P. M. Curmi, *Proc. Natl. Acad. Sci., USA* **1999**, *96*, 8901-8906.
[14] A. B. Doust, C. N. J. Marai, S. J. Harrop, K. E. Wilk, P. M. G. Curmi, G. D. Scholes, *J. Mol. Biol.* **2004**, *344*, 135-153.
[15] S. J. Harrop, K. E. Wilk, R. Dinshaw, E. Collini, T. Mirkovic, C. Teng, D. G. Oblinsky, B. R. Green, K. Hoef-Emden, R. G. Hiller, G. D. Scholes, P. M. G. Curmi, *Proc. Natl. Acad. Sci., USA* **2014**, *111*, E2666-E2675.
[16] E. Collini, C. Y. Wong, K. E. Wilk, P. M. G. Curmi, P. Brumer, G. D. Scholes, *Nature* **2010**, *463*, 644-647.
[17] S.D. McClure, D. B. Turner, P. C. Arpin, T. Mirkovic, G. D. Scholes, *J. Phys. Chem. B* **2014**, *118,* 1296–1308.
[18] S. B. Gould, E.; Fan, F. Hempel, U.-G. Maier, R. B. J. Klösgen, *J. Biol. Chem.* **2007**, *282*, 30295-30302.
[19] C. Y. Wong, R. M. Alvey, D. B. Turner, K. E. Wilk, D. A. Bryant, P. M. G. Curmi, R. J. Silbey, G. D. Scholes, *Nat. Chem.* **2012**, *4*, 396-404.
[20] G. D. Scholes, G. R. Fleming, A. Olaya-Castro, R. van Grondelle, *Nat. Chem.* **2011**, *3*, 763-774.
[21] J. C. Dean, S. Rafiq, D. G. Oblinsky, E. Cassette, C. C. Jumper, G. D. Scholes, *J. Phys. Chem. A* **2015**, *119*, 9098-9108.
[22] K. A. Dill, H. S. Chan, *Nat. Struct. Biol.* **1997**, *4*, 10-19.
[23] C. Liu, A. L. Young, A. Starling-Windhof, A. Bracher, S. Saschenbrecker, B. V. Rao, K. V. Rao, O. Berninghausen, T. Mielke, F. U. Hartl, R. Beckmann, M. Hayer-Hartl, *Nature* **2010**, *463*, 197-202.
[24] P. Durão, H. Aigner, P. Nagy, O. Mueller-Cajar, F. U. Hartl, M. Hayer-Hartl, *Nat. Chem. Biol.* **2015**, *11*, 148-155.
[25] J. Joshi, O. Mueller-Cajar, Y. C. C. Tsai, F. U. Hartl, M. Hayer-Hartl, *J. Biol. Chem.* **2015**, *290*, 1066-1074.
[26] G. W. Collie, K. Pulka-Ziach, C. M. Lombardo, J. Fremaux, F. Rosu, M. Decossas, L. Mauran, O. Lambert, V. Gabelica, C. D. Mackereth, G. Guichard, *Nat. Chem.* **2015**, *7*, 871-878.

Entry for the Table of Contents



Alistair J. Laos, Dr. Jacob C. Dean, Zi S. D. Toa, Dr. Krystyna E. Wilk, Prof. Gregory D. Scholes, Prof. Paul M. G. Curmi and Prof. Pall Thordarson**

Page No. – Page No.

Cooperative subunit refolding of a light-harvesting protein through a self-chaperone mechanism

Text for Table of Contents

Assisting each other: At least 50% of proteins are quaternary, yet how these proteins fold and form is poorly understood. Here, the subunits in a hetero-oligomeric light-harvesting proteis assist one another in folding; the large subunits (beta) fold the small subunits (alpha), and the small subunits chaperone and prevent aggregation of the large – a striking demonstration of the power of cooperativity in nature.