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METHOD ARTICLE

Making dimers of oligomeric membrane proteins using copper-free click chemistry [version 1; referees: 4 approved with reservations]

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Abstract

Here we describe the development of a protocol to make small oligomers, dimers and trimers, from highly oligomeric membrane proteins. The proteins that we used are the light harvesting 2 proteins and core complexes from photosynthetic bacteria, which contain respectively 16 and 56 individual polypeptides. Creating specific dimers between such multimeric protein poses several problems. We propose a protocol based on asymmetric lysine localization, thanks to the positive inside rule, and copper-free click chemistry. With this method we are able to produce specific dimeric complexes in detergent solution of possible biological relevance.

Open Peer Review

Referee Status: ? ? ? ?

	Invited Referees			
	1	**2**	**3**	**4**
version 1 published 01 Jun 2016	? report	? report	? report	? report

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Competing interests: The authors declare that they have no competing influences.

Introduction

The light harvesting apparatus of photosynthetic bacteria, and indeed of all photosynthetic organisms, is organized in a large array of complex structure. This array is designed to absorb light energy and efficiently funnel it to available reaction centers[1]. In most purple bacteria the light collection array is comprised of two proteins: the reaction center containing core complex (CC), and the peripheral light harvesting complex (LH2). In Figure 1A an AFM image of a fragment of photosynthetic membrane from *Rhodospirillum photometricum* is shown, immediately apparent is the extensive array of proteins. The visible proteins are of two types: smaller rings composed of LH2, a molecular model of which is shown in panel B, and larger rings composed of CC, a molecular model of which is shown in panel C. Analysis of the organization of these proteins[2] has shown that this organization can be described as a mixture of hexagonal packed arrays of the nonameric LH2, and a more randomly organized LH2 CC mixture.

The individual protein rings shown in Figure 1B & 1C are each oligomeric. The LH2 ring (Figure 1B) is made of a circular array, of usually 9, subunits each composed of two polypeptides α and β, shown in green and red respectively, which bind four pigment molecules, 3 bacteriochlorophyll *(BChl) a* and one carotenoid molecule[3,4]. In the case of *Phaeospirillum (Phsp.) molischianum* the ring is composed of 8 subunits[5]. The CC is slightly more complex,

and also variable, in architecture. In the center is a reaction center component, typically made of the subunits H, M and L with a bound tetraheme cytochrome subunit C, these are shown in Figure 1C colored cyan, yellow, orange and magenta respectively. This is surrounded by the light harvesting complex oligomer of 16 α and β subunits. These subunits are similar to those of LH2 but only bind 2 BChl a molecules[6]. There is considerable inter-species variability in the precise organization of the CC, with variants having less than 16 subunits, and additional subunits that do not bind pigment or complexes forming S-shaped dimers[7].

In order to better understand energy flow within the light harvesting array[8] it would be useful to study isolated parts of the array, larger than a single complex but smaller than the whole array, and with defined architecture.

This seemingly simple task has proved surprisingly complex. The main difficulties are the large size of the array *in situ*, the oligomeric structure of the pigment-protein complexes and the need to use detergents as membrane proteins are insoluble in standard biochemical buffers. Initial experiments[9,10] with chemical cross-linking in membranes gave very complex mixtures of intra-molecularly linked proteins, inter-molecularly linked homodimers and heterodimers with altered purification properties rendering the objective of reasonably homogeneous samples of defined architecture unattainable.
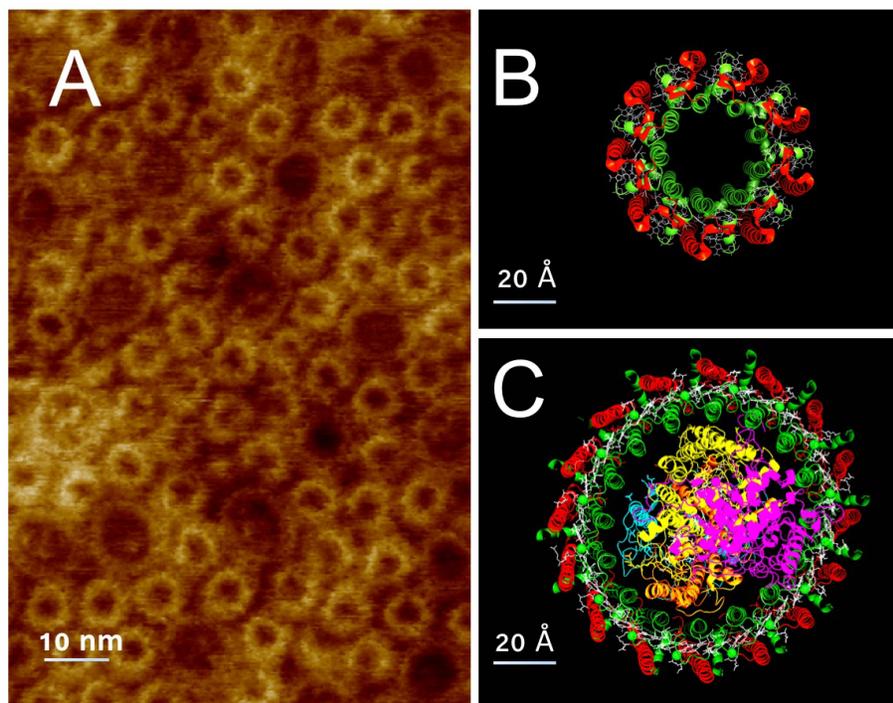
Figure 1. Organization of purple bacterial photosynthetic apparatus. A, AFM image of a fragment of photosynthetic membrane from *Rhodospirillum photometricum* showing the organization of LH2 (small rings) and CC (larger rings) in the membrane. **B**, Molecular model of LH2 viewed perpendicular to the membrane surface, the α polypeptides in green and β in red. **C**, Similar view of a molecular model of the CC, again the α subunits are in green and the β in red, the reaction center C,L,M and H subunits are in magenta, orange, yellow and cyan respectively.

Here we develop a protocol that allows us to form small relatively homogeneous oligomers (dimers and trimers) in a controlled manner. We show that the yields for the different steps are reasonable and that the end product is as expected.

Methods

Protein purification

The various pigment protein complexes were purified by standard methods as described previously[11]. Briefly photosynthetic membranes were solubilized with dodecyl-maltoside and pigment protein complexes isolated by sucrose density gradient centrifugation followed by anion exchange chromatography on a resource-Q column and gel filtration on a superose-6 column. The purity of the various complexes was evaluated by absorption spectroscopy (Shimadzu UV1800 spectrophotometer), measuring the ratio of the absorption peaks at 280 nm and 370 nm. This ratio varies somewhat depending on the source, but absorption ratios of 0.3 and 0.65 are typical for purified LH2 and CC respectively. Purified LH2 and CC proteins from *Phsp. molischianum*, *Rhodobacter (Rb.) sphaeroides* and *Roseobacter (Rsb.) denitrificans* were prepared in 20 mM sodium phosphate buffer (pH 7.2) containing 0.05% dodecyl maltoside. For each purified complex extinction coefficients were calculated from a knowledge of the stoichiometry, thanks to the known structures of the complexes, and BChl a extraction from purified complexes as described previously[12]. Briefly, the UV-visible absorption spectrum was measured, the BChla concentration of the sample was determined from the absorption of an acetone methanol (7:2) extract using the extinction coefficient of ϵ_{770nm} 76 $mM^{-1}cm^{-1}$[13].

The stoichiometry of the complex was calculated as 4 BChl per reaction center plus 2 BChl per LH1 type $\alpha\beta$ pair for core complexes and 3 BChl per LH2 type $\alpha\beta$ pair for LH2 complexes. The complex extinction coefficient was then calculated as $\epsilon(cm^{-1}mM^{-1})$ = *Absorbance* $*$ *stoichiometry/*$(1cm * [BChl])$, see Dataset 1.

Dataset 1. Extinction coefficients for purified complexes

http://dx.doi.org/10.5256/f1000research.8676.d121349

This table includes the extinction coefficients for the different complexes at the peak wavelengths in the near infra-red (IR). Column 1, species; column 2 protein; column 3, near-IR Absorption maximum in nm; column 4 extinction coefficient mM^{-1} cm^{-1} for the complete protein complex.

Protein chemistry

Succinimidyl esters, (5/6-carboxyfluorescein succinimidyl ester and succinimidyl-2-(biotinamido)ethyl-1,3- dithiopropionate), were purchased from Thermo Fisher Scientific (Waltham, USA) and dissolved in dry DMSO from Acros (Geel, Belgium) prior to use. Primary amine labeling was carried out at $4°C$ for 1 hour in 20 mM Na Phosphate buffer pH 7.2 containing 0.05% dodecyl maltoside.

Maleimides, dibenzylcyclooctyne-PEG4-maleimide and azido-PEG3-maleimide, were purchased from Jena Bioscience and dissolved in dry DMSO prior use. Sulfhydryl labeling was carried out at $25°C$ for 2 hours in 20 mM Na Phosphate buffer pH 7.2 containing 0.05% dodecyl maltoside. Coupling by copper-free click chemistry was performed in the same buffer for 10 hours at $4°C$.

After reaction with 5/6-carboxyfluresceine succinimidyl ester and the maleimides the labeled protein was separated from unreacted label using spin columns (Micro Biospin TM6 columns, Bio-Rad (Hercules,USA)), according to the manufacturer's instructions.

Reaction products after coupling were analyzed by HPLC. 20–40 μl samples were injected and separated on a Agilent technologies (Santa Clara, CA) 1260 infinity chromatography system equiped with a Biosep-SEC-s4000 analytical column ($300mmx4.60mm$) eluted with 20 mM Na Phosphate buffer pH 7.2 containing 0.05% dodecyl maltoside at a flow rate of 0.5 ml/min and followed by absorption at 280 nm. Absorption spectra of peaks were obtained from the integrated spectral detector (Agilent technologies G1315D diode array detector).

Modeling

Molecular models of proteins were prepared based on homology to proteins of known structure. In the case of LH2 the structures of the proteins from *Rhodopseudomonas (Rps.) acidophila*[14], and *Phsp. molischianum*[5] were used as templates. For core complexes the recent structure of the protein from *Allochromatium (Ac.) tepidum*[15] was used as a template.

Model structures were visualized and diagrams prepared with Pymol molecular graphics program, version 1.8[16].

Results

The general strategy

The general approach that we used is shown in Figure 2. In the first step the most reactive lysines in the complex are reacted with a succinimidyl-ester at a very low degree of labeling to ensure on average less than 1 reacted lysine per complex. This low degree of labeling is essential to ensure that during cross-linking the number of higher order oligomers formed is minimal. The choice of an
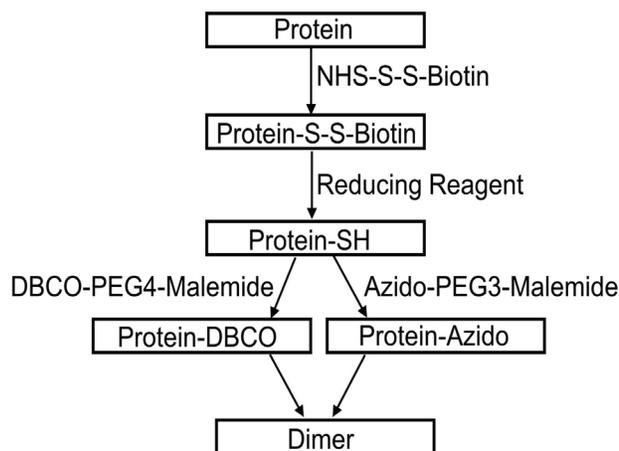
Figure 2. Flow diagram showing the general protocol used to prepare dimeric complexes of proteins from the purple bacterial photosynthetic apparatus. Complexes appropriate for copper-free click chemistry, labeled with DBCO- or azido- groups, were prepared in a two step procedure that allows affinity purification of a biotinylated intermediate.

amine directed reagent was governed by the known structure of the complexes where, thanks to the positive inside rule[17], a number of lysines predicted to be reactive are routinely found in the exposed cytoplasmic parts of the light harvesting ring. Indeed examination of the structures of LH2 and CC show that there is a ring of exposed lysines close to the cytoplasmic surface of the membrane.

Singly labeled proteins prepared by partial reaction of lysines were then purified, thanks to the use of a biotin containing reagent, by affinity on streptavidin beads, and eluted by reduction of the dithiol linkage in the reagent. The resulting proteins contain a unique reactive thiol, the proteins used do not have exposed cysteines. So in the next step of the protocol the thiols are reacted with maleimides appropriate for click chemistry. Allowing the formation of self-non reactive singly labeled proteins that can react with each other, an azido-labeled protein with an alkyne-labeled one.
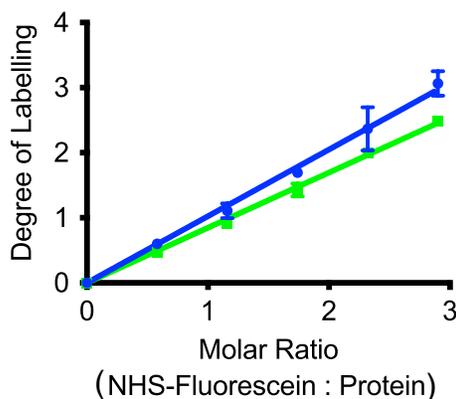
Controlling degree of labeling

> **Dataset 2. Degree of labeling for different complexes**
>
> **http://dx.doi.org/10.5256/f1000research.8676.d121350**
>
> This table includes the data used to generate Figure 3 and some additional data. Column 1, NHS-fluoresceine protein molar ratio; columns 2 to 6 the degree of labeling for, in order: Phsp. molischianum CC; Phsp. molischianum LH2 absorption maximum 850; Phsp. molischianum LH2 absorption maximum 820; Rb. sphaeroides LH2; Rsb. denitrificans LH2;

Critical for the general protocol outlined above (Figure 2) is the possibility of obtaining protein with a controlled low level of labeling on lysine residues. To assess this and verify that even under these somewhat non standard conditions we could obtain reproducible labeling we used carboxyfluoresceine succinimidyl ester to follow the degree of labeling. In Figure 3 we show the degree of labeling observed by absorption spectroscopy using the most

Figure 3. Curves showing how the degree of labeling, determined from carboxyfluorescine and bacterichlorophyll *a* absorption depends on the molar ratio of protein complex and carboxyfluoresceine succinimidyl ester. Curves are shown for two different purified complexes: LH2 of *Phsp. molischianum* (green), and CC of *Phsp. molischianum* (blue) (Dataset 2).

red Bchl a absorption band, with the extinction coefficients calculated as described above, and the fluorescine absorption at 494 nm, and the published extinction coefficient of 70 m$M^{-1}cm^{-1}$[18]. This graph shows that the degree of labeling was linear in the amount of succinimidyl-ester added without any noticeable threshold for labeling. The slopes of the relationship varied between proteins, presumably reflecting differences in the reactivity of the targeted lysines. In Figure 3 we show curves for CC (blue) and LH2 (green) of *Phsp. molischianum*, the slopes of the labeling relationship for all the different complexes we examined vary over a relatively narrow range of 0.98 to 0.81.

Purifying low yield labeled proteins

As the first step was designed to give a very low level of labeling, limited by the amount of succinimidyl ester, it was necessary to separate the labeled protein from unlabeled protein. To achieve this efficiently we chose to use succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate, this reagent allows us to strongly and specifically bind labeled protein to streptavidin beads, and elute the protein either with biotin or by reducing the disulfide bond.

Tests showed that, as expected, the binding was specific and typical yields with unlabeled protein, containing no bound biotin groups, was less than about 0.1% (the estimated detection limit). In contrast estimated yields of biotin containing proteins were greater than 95%.

We chose to use disulfide reduction to elute the proteins from the streptavidin beads, this approach gave better yields for the reacted proteins. Two different reducing agents were tested: tris(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT). Both proved able to release bound reacted proteins. However some proteins, notably several core complexes, proved rather sensitive to TCEP reduction losing their colored cofactors. We therefore decided to use DTT as the reductant, adding DTT to a final concentration of 50mM and allowing reduction to proceed for 4 hours at 25°C. The eluted protein was separated from excess reductant by gel filtration on a spincolumn.

The selectivity and specificity of this purification method ensures that the majority of the eluted protein, even at very low labeling levels will have one or more reacted lysines. For example, if a degree of labeling of 10% is targeted for a nonameric protein the probability of labeling on of the 9 equivalent most reactive lysines is 1.1% from this, assuming independent labeling of the different groups, we expect the reaction mixture to contain 90.4% unlabeled protein, 9.1% singly labeled protein and 0.5% multiply labeled protein. After purification, given the selectivity and specificity, the mixture is expected to contain less than 1% unlabeled protein and less than 5% doubly labeled protein and 94.6% singly labeled protein.

Click chemistry

Initial attempts to cross-link complexes using copper catalyzed click chemistry indicated that the colored cofactors were rather sensitive to monovalent Cu and lost their color under typical reaction conditions. Again this was particularly noticeable for CC and the B800 absorption band of LH2 complexes. In view of this we decided to use a copper-free click chemistry protocol for cross linking based on the strained cyclo-octyne ring[19]. Aliquots of the

proteins to cross-link were reacted with a 50 fold excess of dibenzylcyclooctyne-PEG4-maleimide or azido-PEG3-maleimide at room temperature for 1.5 hours. Unreacted reagent was removed by passage of the reaction mix over a spin column.

Immediately after preparation of the azide and cyclooctane derivatives they were mixed in a 1:1 molar ratio and allowed to react at 4°C over night. After the reaction was complete the sample was analyzed by HPLC.

Product characterization

> **Dataset 3. HPLC trace after cross-linking**
>
> **http://dx.doi.org/10.5256/f1000research.8676.d121351**
>
> This table includes the data used to generate Figure 4A. Column 1, retention time; column 2 absorbance at 280 nm in OD.

> **Dataset 4. Spectra of peaks in HPLC profile**
>
> **http://dx.doi.org/10.5256/f1000research.8676.d121352**
>
> This table includes the data used to generate Figure 4B. Column 1, wavelength (in nm); column 2 to 4 absorbance at in order 5.84, 6.13 and 6.60 min.

Reaction products were analyzed by HPLC on a size exclusion column. Typically, as shown in Figure 4A, several peaks could be observed, the first peak eluting at 5.84 min is specific to samples in which the alkyne and azide were present and corresponds to the cross-linked product. This is followed by peaks, at 6.13 and 6.6 minutes, corresponding to the unreacted CC and LH2 proteins respectively. Several smaller peaks can be observed in the later part of the chromatogram.

The identity of the different peaks was confirmed by absorption spectroscopy as can be seen in Figure 4B. The second and third peaks show the expected absorption of CC and LH2 respectively, while the first peak shows an absorption peak typical of a mixture of LH2 and CC. The absorption spectra of the first peak, when analyzed using the extinction coefficients determined for the individual pigment protein complexes gives a CC:LH2 ratio of 1.5, this would suggest either some contamination due to poor separation from CC and/or some higher order oligomers containing more than one CC attached to an LH2.

The presence of relatively large amounts of monomeric proteins and several peaks of low molecular components with UV absorption was neither expected nor desired. The most likely explanation of this is poor separation of labeled protein from unreacted or click chemistry reagents coupled with poor yield in the reaction between the maleimides and the thiol-containing protein. Unfortunately we have not as yet been successful in addressing this yield issue. Nevertheless, the protocol we have developed allows to form and separate heterodimers of several different proteins in a controlled manner.
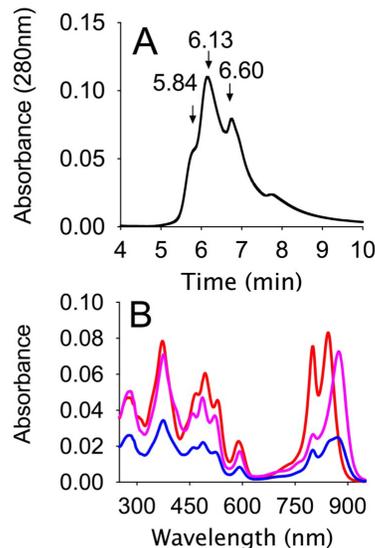
Figure 4. Characterization of reaction products after reaction of azide labeled LH2, with cyclo-octyne labelled CC. A, HPLC trace showing absorption at 280 nm of the reaction mixture separated on an analytical Biosep-SEC-s4000, in 20 mM Na phosphate buffer pH 7.2, containing 0.05% Dodecyl maltoside, at a flow rate of 0.5ml/min (Dataset 3). **B**, Absorption spectra of the major peaks in the HPLC trace: 5.84 min (blue), 6.13 min (magenta), 6.60 min (red) (Dataset 4).

While the polypeptides of certain LH2 complexes, for example those of *Rb. sphaeroides* or *Rsb. denitrificans* only contain lysine residues in the N-terminal, cytoplasmic, part of the sequence others such as those from *Rps. acidophila* or *Phsp. molischianum* have lysines in both the N and C terminal portions. Equally in core complexes there are lysines in the N terminal regions of the core antennae but also, depending on the species, various other potentially reactive lysines in the reaction center subunits and occasionally in the C-terminal region. To better understand the structure of the dimers formed, and assess their biological relevance, it would be useful to determine which lysines are linked together by cross-linking. Unfortunately our attempts to locate the labeling positions the labeling positions in the LH2 and core complexes from *Phsp. molischianum*, by MALDI-MS following digestion with various proteases (trypsin, chymotrypsin of V8 protease) were unsuccessful. Mass spectometry measurements were made on a Bruker Microflex II in positive reflectron mode with automatic sampling and peak identification followed by manual verification. The inability to determine the site of labeling in core complexes at low degrees of labeling, suggests perhaps that labeling is on the more numerous light harvesting polypeptides, since no difference was observed in the reaction center polypeptides detected before and after reaction even with degrees of labeling above 1. This however remains circumstantial and highly tentative.

Discussion

The general protocol we propose is able to produce purified dimeric, and possibly trimeric, complexes from the proteins of the

purple bacterial photosynthetic apparatus. The approach based on reaction at a very low degree of labeling to ensure essentially mono-derivatized oligomers, despite the large number of equivalent reactive groups, followed by high selectivity and specificity purification of the activated proteins and copper-free click chemistry is able to overcome several of the problems we have previously encountered in trying to obtain such complexes. Notably the formation of highly cross-linked products is not observed, such multimeric products would be expected to elute earlier from the gel filtration column (4 min). The destruction of pigments inherent to copper (I) based cyclo-addition was also avoided.

Nevertheless the approach is not without problems and two are particularly flagrant. First the yield in the final step is much less than expected, and appears to be rather variable. Second we have also been unsuccessful in determining the site of labeling.

The low yield in the final step could derive from several different causes. First, the presence of contaminant DBCO and Azide after purification of the labeled protein, could lead to side reactions inactivating the labeled proteins. Second, the reactivity of the Cu free reagents could lead to deactivation/reaction before the mixing of the two proteins. Third, the maleimide labeling could be less efficient than expected. We suspect at least the first two possibilities are partly responsible, but have been unable to resolve the issue.

The difficulty in determining the site of labeling is the consequence of 2 different factors. On the one hand membrane proteins are often rather hard to study by mass spectrometry, and this results in low yields and coverage with proteomic approaches and difficulties in obtaining total mass due to the presence of detergents.

The site of labeling of the different proteins does however need to be confirmed to build models of such complexes and understand their behavior. This absence of knowledge is less important for certain complexes, for example as mentioned above the LH2 of *Rsb. denitrificans* has only two lysines in the N-terminal portion of the protein, α_5 and β_7[20], thus we can be fairly certain labeling is close to the cytoplasmic side of the protein. Unfortunately for the majority of complexes such simple deduction is not possible. However for the core complex we have some circumstantial indications that labeling is of the more abundant light harvesting polypeptides as we do not observe changes in the mass spectrum of the reaction center components at a degree of labeling of 1.0.

Conclusions

We have developed a novel protocol based on copper-free click chemistry that allows the formation and purification of specific dimers between highly oligomeric proteins. This protocol can be used to prepare biologically relevant dimers of certain LH2s with very asymmetrical lysine distribution, for example the LH2 of *Roseobacter denitrificans* or *Rhodobacter sphaeroides*. Such dimers will be of considerable interest for studying energy migration in light-harvesting arrays.

Data availability

F1000Research: Dataset 1. Extinction coefficients for purified complexes, 10.5256/f1000research.8676.d121349[21]

F1000Research: Dataset 2. Degree of labeling for different complexes, 10.5256/f1000research.8676.d121350[22]

F1000Research: Dataset 3. HPLC trace after cross-linking, 10.5256/f1000research.8676.d121351[23]

F1000Research: Dataset 4. Spectra of peaks in HPLC profile, 10.5256/f1000research.8676.d121352[24]

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Data Source

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Data Source

24. Sturgis J, Dong W: **Dataset 4 in: Making dimers of oligomeric membrane proteins using copper free click-chemistry.** *F1000Research*. 2016.
Data Source

Open Peer Review

Current Referee Status: ❓ ❓ ❓ ❓

Version 1

Referee Report 20 June 2016

❓ **Takehisa Dewa**

Department of Frontier Materials, Nagoya Institute of Technology, Nagoya, Japan

The authors described an approach to hetero-coupling of bacterial photosynthetic apparatuses, LH2 and core complex. I think their approach using copper-free Huisgen cyclization is reasonable. Although reaction yields are unfortunately very low, careful and patient chemistry could overcome these problems that they described. I had tried similar hetero-coupling reactions but it could not work well. I appreciate their work because of the descriptions about some trials, e.g., using TCEP/DTT.

I like to suggest some points, for which I guess the authors have done experiments described below.

1. SDS-PAGEs may clearly indicate which polypeptides reacted with

   .

2. Reacted polypeptides (not only hetero-coupling products, but also NHS-SS-biotin adducts, DBCO- and Azide-bearing ones) may provide better MS after RP-HPLC purification.

3. To make sure the attachment and reactivity of DBCO and azide moieties, small molecules bearing azide and DBCO as their reaction counterparts should be useful. It may make clear whether DBCO and azide conjugates remain active.

4. Characterization using fluorescence spectroscopy is expected to see energy transfer from LH2 to cc.

5. It would be very helpful to understand the position of Lys if amino acid sequences of polypeptides of LH2 and cc used are listed.

Other comments:

1. In Abstract: "56 individual polypeptides"

   Where does this number come from?

   The title is "making dimers", but in Abstract description "making small oligomer, dimer and trimmers" is inconsistent. Major products seems dimer (heterodimer), so the description should be better to be just "dimer" to remove ambiguity.

2. page 4, 5th paragraph (right column): Explanations for reaction levels are hard to understand. Descriptions "90.4% unlabeled protein" and "94.6% singly labeled protein" are inconsistent.

3. Figure 4: This is one successful result showing the formation of LH2-cc hetero dimer. However, it is unclear what LH2 and cc were used. The authors used carious LH2 and cc from different photosynthetic bacteria. Origin of these complexes should be denoted.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 15 June 2016

Robert A Niederman
Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ, USA

These authors have attempted to develop a method for transforming the highly oligomeric light harvesting 2 (LH2) and LH1-reaction center (RC) core complexes into dimers (and trimers) of the *alpha/beta* hetrodimer, as a means of obtaining an improved understanding of energy transfer within isolated portions of the full arrays.

In ¶4 of the Introduction, the authors need to cite and elaborate on the work by Westerhuis *et al.* (2002), stating that a method was previously developed for the isolation of a series of light-harvesting 1 (LH1) oligomers from the fully arrayed LH1-RC core complex. In this procedure, a *Rhodobacter sphaeroides* mutant lacking LH2 was subjected to lithium dodecyl sulfate polyacrylamide gel electrophoresis, which gave rise to a ladder of LH1 bands representing a series oligomers of the *alpha/beta* heterodimeric unit, varying in size from $(alpha/beta)_{2-3}$ to $(alpha/beta)_{10-11}$. Moreover, these oligomers exhibited oligomeric-state dependent optical properties, characterized by red shifts in near-IR absorption and emission maxima of ~6 nm at 77 K, as the aggregate sizes increased from 3 to 7-8 *alpha/beta*-heterodimers, accompanied by shifts in highly polarized fluorescence from the blue to the red side of the absorption band. This has been explained by the oligomerization of heterodimers to form a curvilinear array of excitonically-coupled chromophores, with an anisotropic long-wavelength component corresponding to low energy excitonic transitions arising from interactions within inhomogeneous BChl clusters.

In light of these findings, the authors can further justify why their method has been developed for the isolation of dimers and trimers of defined architecture in a controlled manner. But will much useful information come out of dimers and trimers when the full LH1 red shift required going up to an octameric state? The type of proposed energy migration studies planned for the isolated small arrays should be mentioned?

Regarding Fig. 4, I don't believe the source of these complexes has been designated. Fig.4A, is a second purification done to further purify these peaks? Fig. 4B, all three spectra need to be normalized at 590 nm to better show the composition of the cross-linked entities. The complex ratios can then be more precisely determined.

Other errors found in text:
P. 2, cloumn1, ¶2, line 6: *Phaeospirillum (Phs.) molischianum*
P. 3, column 1, ¶2, line 14: *Phs. molischianum, Rhodobacter (Rba.)*

P. 3, column 1, ¶2, line 1: of the complexes
P. 3, column 1, 6 lines up dibenzylcyclooctyne (DBCO)
P. 3, column 2, ¶2, line 4: (300 *mm* x 4.60 *mm*)
P. 3, column 2, ¶3. line 3: *Rhodoblastus (Rbl.) acidophilus, Phs. molischianum*—2 lines down: *Allochromatium (Ach.)*
P. 4, column 2, ¶4, 3 lines from bottom: 50 *mM*
P. 5, column1, ¶1, line 1: 50-fold
P. 5, column1, ¶2: *Phs. molischianum omit "Phaeospirillum"*
P. 5, column 2, ¶1, line 2: *Rba. sphaeroides,* 2 lines down: *Rbl. acidophilus, Phs. molischianum* (and 12 lines up)

P. 6, column1, ¶4: This ¶ needs to be corrected (2 different factors??) line 4 after: with proteomic approaches, add: and on the other hand, difficulties occur in obtaining ………

It should be noted that difficulties, making integral membrane proteins hard to study by mass spectroscopy arise by virtue of their hydrophobicity leading to a bias toward soluble hydrophilic peptides. The latter are more easily recovered during sample processing and separation, and ionize and dissociate better during mass spectroscopy. Moreover, some membrane proteins are insoluble under enzyme digestion conditions and can also precipitate during the subsequent analysis steps.

References

1. Westerhuis W, Sturgis J, Ratcliffe E, Hunter C, Niederman R: Isolation, Size Estimates, and Spectral Heterogeneity of an Oligomeric Series of Light-Harvesting 1 Complexes fromRhodobacter sphaeroides. *Biochemistry*. 2002; **41** (27): 8698-8707 Publisher Full Text

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 06 June 2016

Kamil Woronowicz
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This paper lays a very important ground work and proof of principle for crosslinking photosynthetic complexes using click chemistry. It has already solved several major hurdles such as Cu-related loss of pigment color and similar effects due to reducing agent. It is using a very clever approach for low level modification and purification of these modified complexes using streptavidin beads, yet choosing to elute using DTT. It is somewhat surprising that TCEP, being a milder reducing agent, appears to be harsher on these pigmented complexes than DTT. It would also be great to see a comparison to betamercapthoethanol (BME), another well-known reducing agent, as well as an array of less known alternatives.

Nevertheless, the discussion identifies weaknesses of current work appropriately. These weaknesses could be addressed in a follow up work either by the Sturgis lab or anyone else following the precedent

set by this work. Some additional comments might include poor resolution of size exclusion HPLC (Fig. 4A), though absorption spectra (Fig. 4B) show acceptable spectral purity, especially in context of the discussion presented by the author, exhibiting keen understanding of the nature of some of the impurities.

In order to address my reservations, I would like to see the following:

1. The paper mentions low yield of the last step, but doesn't provide quantitative data. Judging by the HPLC chromatogram in Fig. 4B it could be as much as 33%, but most likely it is much lower. If possible, either a Clear Native Gel or ultracentrifugation (especially analytical ultracentrifugation) might show relative amounts of complexes. Protein assay or amino acid analysis might be helpful in this regard as well.

2. Showing normalized spectra shown in Fig. 4B (perhaps based on a ~600nm peak or one of the carotenoid peaks) could show the spectral shift and incorporation of both CCs and LH2s in the click-chemistry linked complex.

3. Providing insight into yield using Cu. Even though the pigment is lost, but is the final step yield different (especially if significantly higher) as judged by HPLC? This could provide evidence that Cu-free system is not as efficient and might need additional improvements.

4. Building on my previous remark, how optimized are current conditions? Have they been selected after a panel of unsuccessful trials or these represent the first or second trial? Specifically, are the temperatures and times of incubation at each step optimal? Click chemistry is typically thought of as almost quantitative, but it this very complex system there are several aspects to be considered that may decrease the yield.

5. I think it would be great to see the HPLC of these protein complexes before and after to show appearance of a new peak corresponding to the linked complexes. It is unlikely that LH2 absorption would be observed in first peak since it shows up last as the peak representing the smallest complex. Unless, of course, it is present in such oligomeric form even before the reaction.

6. Lastly, I would like to see several editing corrections to be made:

   a) page 4, in the paragraph that starts with "Singly labeled proteins..." second and third sentences should read " The resulting proteins contain a unique reactive thiol, SINCE THE NATIVE proteins used do not have exposed cysteines. In the next step..." new suggestion is shown in all CAPS, but does not need to be capitalized in the final text. Also, the word "So" has been removed from the beginning of the third sentence in this paragraph.

   b) continuing a few lines, instead of "each other" perhaps "a complimentarily modified counterpart" might be more suitable.

   c) Controlling degree of labeling paragraph, remove "this" in the second sentence.

   d) Same paragraph as part b) refer to Figure 1 when referring to "extinction coefficients calculated as described above, and then check spelling of fluorescein immediately following.

   e) same paragraph, when talking about threshold, could you mention how many lysines do you expect to have available? It could be at least a measure of expected threshold.

f) The second and third sentences under "Purifying low yield labeled proteins" I would paraphrase into "We chose to use succinimidyl-2-(biotinamido)ethyl-1,3,dithiopropionate, allowing strong and specific binding of labeled protein to streptaidin beads. Pure protein was eluted either with biotin or by reducing the disulfide bond"

g) Next sentence I would take out "as expected", and add "non-specific binding" when referring to contamination of unlabeled protein without biotin.

h) It would be great to have a mention of what the 5% impurities are in the biotin-containing proteins (or sizes, or at least how that was established)

i) Next paragraph, I would like to add "since" after the comma in the first sentence. Also, could you provide % purity or yields in support of your decision to use reducing agent?

j) Same paragraph (third under Purifying low yield labeled proteins, middle right hand side page 4 on PDF, roughly in the middle of the page) very end: How did this gel filtration step on a spin column affect the impurities (see h above)? Would another SEC be needed to show that?

k) Next paragraph, second sentence refer to nanomeric protein COMPLEX.

l) Same paragraph, impressive statistical analysis of the distribution of modifications. Could you provide that as a supplementary material or show it somewhere?

m) Last paragraph before discussion, about halfway: "the labeling positions" seems to be typed twice in a row.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

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Tatas Brotosudarmo

Ma Chung Research Center for Photosynthetic Pigments (MRCPP), Ma Chung University, Malang, Indonesia

The report of using click chemistry to label the protein of LH2 and core complex is interesting, however I have not been convinced by the data from spectroscopy nor biochemistry that can show the existence or the formation of a dimeric form of the LH2 or core complex polypeptides. The absorption spectra in Figure 4 resemble very much with the spectra of the native LH2 or core complex polypeptides. The B820 dimeric form was not there. CD spectrum might be a useful method to know.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.