

Site-specific incorporation of perylene into an N-terminally modified light-harvesting complex II†‡

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Employing the utility of the native chemical ligation, site-specific attachment of an ultrastable perylene dye to a derivative of the major light-harvesting complex (LHCII) was demonstrated. Biochemical analysis of the conjugate indicated that the structure and function of LHCII remain largely unaffected by the N-terminal modification.

Visualizing proteins using fluorescence and confocal laser scanning microscopy has enabled 3-dimensional imaging of protein dynamics and cellular events.¹ The broad utilization of fluorescent probes has led to better understanding of protein function and structure. Fluorescent molecules have been introduced into proteins by chemical modification of the reactive side chains of amino acids such as lysine and cysteine.² Selective chemical modification of proteins, however, can be complicated by the presence of several reactive side chains in the protein. Labeling of the proteins at defined positions has offered the ability to monitor conformational dynamics and protein folding at ensemble and single-molecule level using fluorescence resonance energy transfer (FRET) between a single donor and a complementary acceptor.³ For this purpose the incorporation of the label in a controlled and site-specific way is crucial.

Numerous chemical methods have been introduced that can be used for site-selective modification of proteins. Native chemical ligation (NCL) is one of the most versatile coupling techniques developed to prepare larger protein structures, to selectively introduce a biophysical probe or to synthesize proteins that are difficult to express.⁴ It involves the chemoselective coupling of unprotected peptide segments, one containing a C-terminal thioester and the other containing an N-terminal cysteine residue. Small molecule fluorescent probes that specifically react with N-terminal cysteine-containing proteins have been reported previously.⁵

Herein, we introduce a new member of the rylene family—an ultrastable, highly fluorescent, water-soluble perylenebis(dicarboximide) that possesses thioester functional group for site-selective labeling of proteins. The perylenebis(dicarboximide)thioester (PDI-thioester) underwent reaction under

standard “native chemical ligation” conditions with a derivative of the major light-harvesting complex (LHCII) protein containing an N-terminal cysteine.

Different classes of chromophores have been synthesized and utilized for site-specific labeling of proteins, cells and other biomolecules.⁶ For an increasing number of applications the photostability of the label is essential. The perylenebis(dicarboximide)s have proven to be an exceptional class of chromophores possessing excellent thermal and photochemical stabilities. Their unique sets of photophysical properties have provoked many studies in the area of single molecule spectroscopy. They have been used efficiently as label in single enzyme tracking using real time wide field microscopy of single phospholipases. The nitrilo-triacetic acid-functionalized perylenebis(dicarboximide) has shown an unexpected behavior in the presence of nickel ions since no change in the quantum yield was observed.⁷

However, so far no derivative of the rylenebis(dicarboximide) series is known to exhibit an appropriate functionality for covalently labeling either the N- or C terminus of proteins selectively. Therefore, we decided to synthesize a water-soluble perylenebis(dicarboximide) functionalized as a thioester to achieve covalent attachment to an N-terminal cysteine of proteins. Water-soluble perylene diimide containing a primary amine group was prepared as described in the literature. Monofunctionalized perylene diimide (**1**) (Scheme 1) was reacted with thiolane-2,5-dione, and subsequent addition of benzyl bromide at slightly acidic conditions resulted in the formation of thioester **2** in 50% yield. Applying the procedure described by Pannel *et al.* for conversion of succinimidyl ester of the chromophore *via* activation of benzyl mercaptan with trimethylaluminium did not lead to the formation of the target compound **2**.

Previously reported chromophore thioesters suffer from poor solubility, and their extinction coefficients or fluorescence quantum yields have not been reported.⁵ The PDI-thioester **2** exhibits excellent solubility in water, an absorption maximum at 568 nm ($\epsilon = 23\,400\text{ M}^{-1}\text{ cm}^{-1}$) and an emission maximum at 622 nm. Moreover, it displays a high fluorescence quantum yield (Φ_f) of 54% in aqueous medium measured at room temperature using Cresyl Violet as a reference. Normalized absorbance and fluorescence spectra of the chromophore are shown in Fig. 1.

To prove the suitability of PDI-thioester for protein labeling, the dye was attached to a derivative of LHCII containing an N-terminal cysteine. LHCII is the major light-harvesting complex in the photosynthetic apparatus of higher plants, non-covalently binding eight chlorophyll (Chl) *a*, six Chl *b*, and four carotenoid molecules (lutein, neoxanthin, violaxanthin). It is one of the few recombinant membrane proteins that assemble *in vitro* from the denatured, bacterially expressed apoprotein and plant pigments,

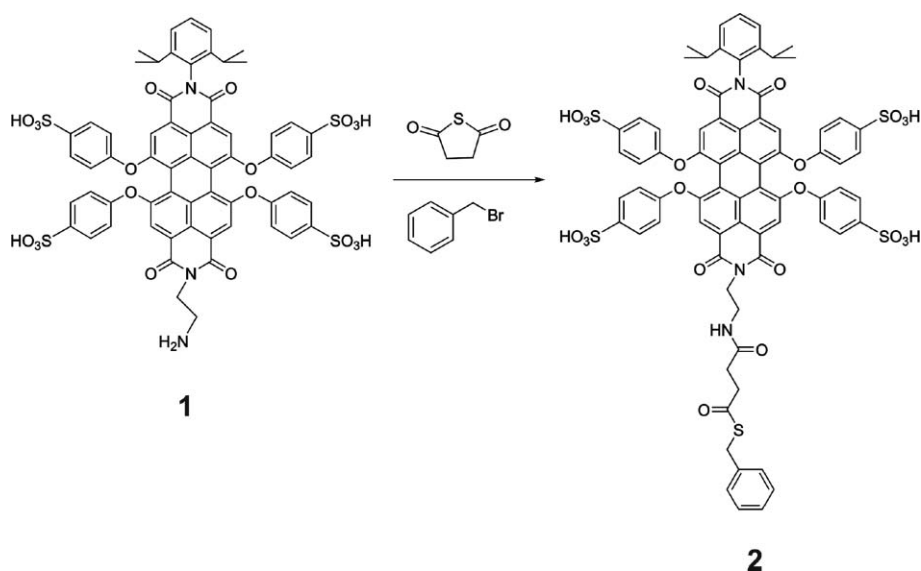
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Scheme 1 Preparation of PDI-thioester **2** via reaction with thiolane-2,5-dione and subsequent addition of benzyl bromide.

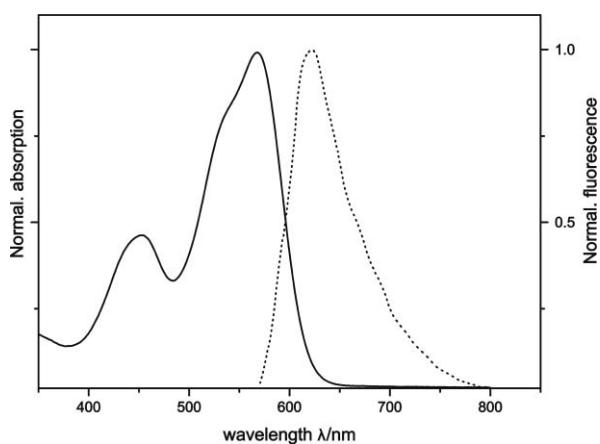


Fig. 1 Normalized absorption (solid line) and emission (dotted line) spectra of **2** in aqueous medium.

and the functionality of the refolded complex can easily be tested by spectroscopic measurements.⁸ Therefore possible distortions of the protein structure due to the PDI-thioester can be investigated.

For bacterial expression of a recombinant protein with an N-terminal cysteine, several problems have to be overcome. Bacterial proteins are always synthesized with a leading methionine, which in some cases is removed by endogenous enzymes; therefore, conditions had to be found in which methionine removal was as complete as possible. To avoid N-terminal modifications of the protein either at the amino or the sulfhydryl group, which both would impair the reaction with the thioester dye, the growth medium contained cysteine and methyl methanethiosulfonate according to Gentle *et al.*⁹ Analysis of the N-terminal amino acids by Edman degradation indicated that an estimated 25% of the protein still contained methionine as its first amino acid.

Labeling of this protein sample with PDI-thioester was performed according to the native chemical ligation procedure.¹⁰ Under these conditions transesterification of PDI-thioester with the Cys thiol of the protein occurs, followed by an S–N acyl shift to generate the conjugate that exhibits a stable amide

bond. To confirm successful labeling, samples were analyzed by SDS PAGE. N-terminally labeled PDI-LHCII was seen on the unstained electrophoresis gel as a fluorescent band (Fig. 2, lower panel lane 2). Upon Coomassie staining of the protein, two bands became visible (Fig. 2, upper panel lane 2). Superimposition of the stained and unstained gel (not shown) revealed that the upper band in the double band corresponds to the fluorescent one; the other one representing non-labeled LHCII apoprotein. By comparing the amount of Coomassie stain in the two bands, the yield of PDI labeling was estimated to be 40%. Labeled LHCII was separated from unlabeled material by preparative gel electrophoresis taking advantage of the reduced mobility of the PDI-labeled protein in a denaturing SDS gel. In the purified material, about 80% of the protein was labeled (Fig. 2A, lane 3).

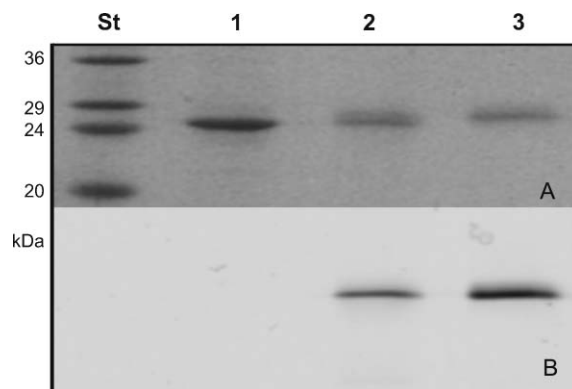


Fig. 2 Labeling efficiency can be calculated by gel electrophoresis. Equal amounts of unlabeled (**1**) and labeled LHCII prior to (**2**) and after (**3**) further purification. Protein signals were visualized by Coomassie staining (**A**) and fluorescence upon excitation by UV-light (**B**). St – protein standard.

The PDI-labeled protein could be folded and assembled with pigments by following the standard reconstitution procedure. The best functional test for recombinant LHCII is the intramolecular energy transfer from Chl *b* to Chl *a*.⁸ The fluorescence of

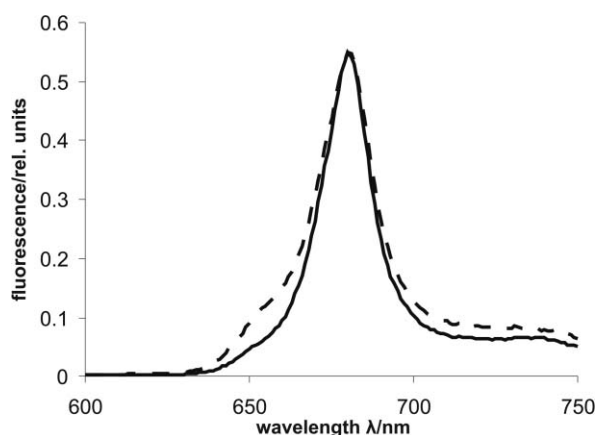


Fig. 3 Folded PDI-LHCII is functional. Emission spectra ($\lambda_{\text{ex}} = 470$ nm) of LHCII with (dashed line) and without PDI-thioester (solid line). Concentration of LHCII was always 37 nM.

PDI-labeled LHCII is shown (Fig. 3) upon excitation at 470 nm, near the absorption maximum of Chl *b*. Fluorescence emission is seen mostly around 680 nm, the emission wavelength of Chl *a*, and only very little around 660 nm where Chl *b* emits. This shows that in PDI-labeled LHCII, most of the excitation energy is transferred from Chl *b* to Chl *a*, demonstrating that these complexes are functional. The small contribution of Chl *b* fluorescence is probably due to unbound Chl *b* generated by dissociating LHCII. Monomeric LHCII slowly dissociates in aqueous detergent solution, particularly at the low concentrations used here for fluorescence measurements, whereas the trimeric form of LHCII is intrinsically more stable. We anticipate that trimeric PDI-LHCII (which has not been studied here) exhibits complete energy transfer from Chl *b* to Chl *a*. Proper folding and assembly of labeled LHCII was further verified by fluorescence excitation and CD spectra (ESI \ddagger). Furthermore SDS PAGE analysis after treating the pigment-protein complexes with the serine protease trypsin demonstrated that the PDI dye is specifically coupled to the N terminus of the LHCII protein, rather than being non-specifically attached to the pigment-protein complexes (ESI \ddagger).

Since the PDI-labeled LHCII apoprotein contained a small amount of unlabeled protein, it needed to be shown that it was in fact the labeled material that successfully folded into functional LHCII. This was proven by a significant drop in PDI fluorescence upon pigment binding, possibly due to possible energy transfer between the dye and LHCII. As the emission spectrum of PDI-thioester and the absorption spectrum of LHCII chlorophylls overlap, the chromophores can potentially engage in FRET. The critical Förster distance between PDI-thioester and Chl *a* or Chl *b* is 5.2 nm and 5.0 nm, respectively. With the maximum radius of LHCII being approximately 6 nm,¹¹ PDI and Chls are expected to be close enough in the labeled LHCII for efficient FRET to take place. To test this, emission spectra with excitation near the PDI absorption maximum (550 nm) were measured of trypsin-digested and non-digested PDI-LHCII. Undigested samples did not show any significant PDI fluorescence but did show LHCII fluorescence at 680 nm. On the other hand, in digested samples both LHCII and PDI fluoresced (Fig. 4).

A possible explanation is that in PDI-labeled LHCII, the dye transfers virtually all of its excitation energy to the LHCII Chls.

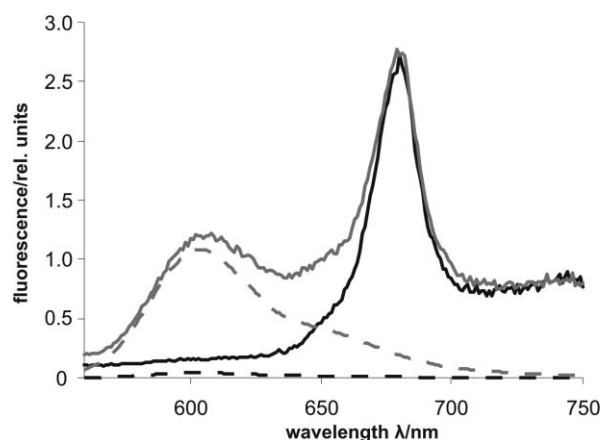


Fig. 4 Energy transfer measurements. Emission spectra ($\lambda_{\text{ex}} = 550$ nm) of PDI-LHCII with (solid gray line) and without (solid black line) trypsin digestion. Concentration of LHCII was always 37 nM. PDI fluorescence before (dashed black line) and after (dashed gray line) trypsin digestion were fitted by deconvolution.

As soon as the N-terminal amino acids are cleaved off, the dye becomes uncoupled from the acceptor Chls and resumes emission at around 600 nm. The Chls still emit because of their co-excitation at 550 nm. Assuming that neither the extinction coefficient nor the fluorescence quantum yield of the donor dye are affected by trypsin cleavage, the amount of donor quenching indicates an energy transfer efficiency of at least 96%. However, no commensurate sensitized acceptor (chlorophyll) emission is observed prior to trypsin cleavage. Therefore, other mechanisms leading to PDI quenching in the pigmented protein cannot be excluded. It should be noted, however, that PDI fluorescence quenching is dependent on pigment binding and, therefore, independently of the quenching mechanism proves that the dye-labeled protein is in fact assembled with chlorophylls and carotenoids.

There is only a limited number of native proteins possessing a starting Cys, however, bacterially expressed recombinant proteins as shown here in the case of LHCII can easily be engineered such that they contain Cys in position 2 by removal of the leading methionine in the expressing bacteria according to Gentle *et al.*⁹ Additional Cys residues elsewhere in the protein do not disturb the procedure because only N-terminal cysteine is susceptible to native chemical labeling. This reaction therefore provides an excellent possibility to attach PDI, or other dyes containing a thioester function, highly specifically to the N terminus of recombinant proteins. Where preparative gel electrophoresis is not suitable for isolating the labeled protein, an alternative possibility would be affinity chromatography using a sulfhydryl-binding matrix, provided the N-terminal cysteine is the only one in the protein. In this case; only the non-reacted protein would bind to the affinity column leaving the labeled material in the run-through. Thus, the labeling procedure described in this paper can be extended to a large number of very different proteins.

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