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Absorption spectra of chlorophyll *a* and *b* in Lhcb protein environment

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Abstract

The spectral forms of the two chlorophyll species in higher plant Photosystem II antenna proteins have been experimentally determined within their protein environment. Recombinant CP29 and LHC II antenna proteins missing individual chromophores were obtained by over-expression in bacteria without any changing of the primary protein sequence and *in vitro* reconstitution. Difference absorption spectroscopy with respect to the corresponding proteins binding the complete pigment complement yielded the spectral shape and extinction of single chlorophyll *a* and *b*. A functional relation of their absorption was given by Gaussian subband decomposition covering the entire Q_x and Q_y optical region together with the absolute value of the molar extinction coefficient. With respect to analogous determinations reported in the literature for organic solvents, this information is valuable for further understanding the in-protein chlorophyll excited states and excited state dynamics: in particular, for the calculation of Förster transfer rates by means of chlorophyll–chlorophyll overlap integral employing the Stepanov relation for emission and single chromophore transition energies according to the results of mutational analysis of chlorophyll binding sites [Bassi et al. (1999) Proc Natl Acad Sci USA 96: 10056–10061; Remelli et al. (1999) J Biol Chem 274: 33510–33521].

Abbreviations: Abs – absorption; Car – carotenoid; CD – circular dicroism; Chl – chlorophyll; CP29 – complex photosynthetic 29; DEPC – diethylpirocarbonate; LD – linear dicroism; Lhcb – light-harvesting complex of Photosystem II; LHCII – light-harvesting complex II; PSII – Photosystem II from higher plants; RT – room temperature; WT – wild type

Introduction

In higher plant chloroplasts, many pigment-binding proteins are inserted into the thylakoid membrane and organised into multisubunit complexes called photosystems. Photosystems (PS) catalyse the light absorption, the use of the excitation energy in transmembrane electron transport and, finally, the ATP and NADPH synthesis. PS I and II are both composed of a chlorophyll *a* binding core complex surrounded by a chlorophyll *a/b* binding antenna. Until recently, a detailed understanding of energy transfer processes in antenna and reaction centres has been prevented by insufficient knowledge of the major parameters controlling Förster coupling, namely: the distances between chromophores, the mutual orientation between dipole transitions, the absorption and fluorescence energy levels and their distribution within the photosystems. In the case of PS II light harvesting proteins, considerable insight has been obtained from elucidation of the major antenna complex (LHC II) structure to a resolution of 3.4 Å (Kühlbrandt et al. 1994) showing that nearest-neighbour chlorophylls (Chl) are spaced by 9÷13 Å (centre–centre distance). Absorption (Abs) spectra of Lhc proteins display a markedly heterogeneous broadening in the 630÷685 nm range, or so-called Q_y region, due to the presence of about 8–11 spectral forms identified so far with reasonable

certainty (Hemelijk et al. 1992) and which can be attributed to chlorophylls in different proteic sites (Bassi et al. 1999). Mutation analysis of chlorophyll binding residues allowed construction of mutant proteins lacking individual chromophores which were used for determination of the Abs energy levels for each Chl in Lhcb1 (LHC II) and Lhcb4 (CP29) proteins (Bassi et al. 1999; Remelli et al. 1999) and for the experimental analysis of the orientation of the transition moments of individual Chl molecules by Linear Dicroism (LD) (Simonetto et al. 1999). Thus, all the major parameters involved in the excitation energy transfer are being elucidated at least in the case of CP29. Nevertheless, Förster energy transfer rates strongly depend on the overlapping integral between the fluorescence emission spectra of the donor chromophores and the absorption spectra of the acceptors (Förster 1948, 1965). While fluorescence emission spectra of individual chromophores can be drawn from absorption spectra using the mirror image rule, and formally calculated through the Stepanov relation (Stepanov 1957), direct determination of the absorption spectrum of individual chromophores within a pigment–protein complex, containing many (8÷12) of them in a narrow spectral range, appears exceedingly difficult by direct spectroscopic methods. Present knowledge relies on few examples accessible to hole-burning studies (Gillie et al. 1989; Reddy et al. 1994). An alternative approach was recently attempted based on differential spectroscopy of mutant proteins missing individual Chls, which led to the proper identification of absorption peak wavelengths (Bassi et al. 1999; Remelli et al. 1999; Croce et al. 1999) of the individual chromophores and, furthermore, to the spectral reconstruction of LD and Abs spectra both at 100 K (Simonetto et al. 1999) and room temperature (Cinque et al. 2000). Nevertheless, the resolution of minor features of the absorption spectra which might, however, affect calculation of the Förster overlapping integral is hampered, possibly due to the side effects of amino acid substitutions. In this study, recombinant proteins missing single Chl *a* or Chl *b* chromophores have been produced in CP29 and LHC II, respectively, without modifying the primary sequence of the protein. Differential absorption spectroscopy with respect to the corresponding proteins carrying the complete chromophore complement allowed to obtain separately Chl *a* and Chl *b* absorption with enhanced resolution: these spectral forms are suitable for further studies on energy transfer in antenna, specifically for calculation of the energy overlap between donor and acceptor chromophores in the Förster mechanism.

Materials and methods

Sample preparation

Native CP29 protein was purified from maize PS II membranes as already described in Croce et al. (1996). Recombinant CP29 was obtained by *in vitro* reconstitution of the apoprotein overexpressed in *Escherichia coli* with purified pigments (Giuffra et al. 1996). Recombinant CP29 and Lhcb1 proteins carrying mutation in individual chlorophyll binding residues were produced according to Bassi et al. (1999) and Remelli et al. (1999). Wild type (WT) CP29 missing a Chl *a* chromophore was prepared by chemical modification of Lhcb4 protein during refolding. CP29 apoprotein from *E. coli* inclusion bodies was refolded *in vitro* as above reported (Giuffra et al. 1996) with the following modifications: following freeze-thawing of the pigment–protein mixture to induce folding and substitution of the SDS with OGP, glutaraldehyde was added to 1% and the mixture was incubated at 20 °C for 20 min in the dark. The reaction was blocked by adding 0.025 vol. of 2 M Na BH_4 in O.1 M NaOH. After 20 min incubation in ice, the mixture was dialysed against 0.5% OGP, 10 mM Hepes pH 7.6 for 1 h. Purification of the pigment–protein was then performed as in Giuffra et al. (1996).

WT LHC II missing a single Chl *b* chrompohore was prepared by setting the protein–pigment ratio to 0.9 (w/w) (0.57 in the control sample) during reconstitution as previously reported (Remelli et al. 1999).

For stoichiometric (pigments/protein ratio) determination, the protein concentration was measured by the ninhidrine method (Hirs 1967). Chlorophyll concentration was determined by the method of Porra et al. (1989). HPLC analysis was performed according to Gilmore and Yamamoto (1991).

Steady-state spectroscopy

Absorption spectra were performed by using an SLM-Aminco DW-2000 Spectrophotometer: the buffer was 10 mM Hepes (pH 7.6), 0.06% DM and 20% glycerol. Samples were measured at room temperature in a 1 cm pathlength cuvette. The total chlorophyll concentration was in the order of 10 μg/ml (about 1 OD at maximum Q_y absorption).