Pigment conformation and pigment–protein interactions in the reconstituted Lhcb4 antenna protein

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Abstract **Resonance Raman spectra of the native Lhcb4 antenna protein are compared with those of a recombinant protein prepared by in vitro refolding of its polypeptide, over-expressed in *Escherichia coli*, with added pigments [Giuffra et al. (1996) Eur. J. Biochem. 238, 112–120]. The results indicate that the native pigment conformation is reproduced almost perfectly in the reconstituted protein, with only small differences which are attributed to a slight shift in the Soret absorption peak of two or more chlorophylls. This procedure therefore represents a model system for the investigation of site-directed mutant LHC proteins, which are otherwise very difficult to obtain. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Light-harvesting protein; LHC gene family; Chlorophyll binding; Pigment–protein interaction; Photosystem II; Resonance Raman spectroscopy

1. Introduction

The harvesting of light energy in green plants is carried out by an extremely complex system of proteins. Among these, the members of the light-harvesting complex (LHC) multigene family, a set of homologous pigment-binding proteins, constitute the outer antenna system, and are organized around both photosystems I and II. An atomic model of the most abundant LHC protein, the bulk photosystem II LHC antenna or LHCIIb, presents the overall protein fold of the protein along with the position of most of the bound cofactors (chlorophylls (chl) *a* and *b* and xanthophylls; [1]). In this structure, however, important information is lacking, such as the precise position of most amino acid sidechains, the formal chemical identification of the cofactor pigments, pigment–protein interactions, and the detailed conformation of the different cofactors. In the absence of a more detailed structure, a less direct approach to define structural properties of these pigment cofactors has been used – that of reconstitution of wild type and mutated polypeptides in which pigment binding has been perturbed [2]. This system has been used to address, among other issues, the chemical identification of the bound cofactor molecules seen in the structural model (such as the assignment of chls *a* and *b*). These studies are particularly well advanced for

one of the minor LHCII proteins, Lhcb4, in which reconstituted mutant pigment–protein complexes have been produced lacking each seven of the eight bound chl molecules [3]. However, assessing precisely to what extent the complexes reconstituted from wild type apoproteins are similar to purified ones at a molecular level is particularly complex. First, the factors influencing the electronic absorption properties of the chl molecules bound to these complexes are little understood. Moreover, as the different chl molecules bound to these complexes share rather similar electronic properties, the fact that the electronic properties of reconstituted complexes are similar to that of purified ones does not constitute a formal demonstration that the reconstituted complexes are indeed identical, at a molecular level, to purified ones.

Resonance Raman spectroscopy is an ideal tool for studying these reconstituted proteins in terms of the molecular conformation of their bound pigments. It has been used extensively in the characterization of physico-chemical mechanisms underlying the absorption properties of bacteriochlorophyll cofactors in bacterial light-harvesting proteins [4] and to evaluate the quality of such proteins reconstituted with extrinsic pigments [5,6]. In particular, this technique allowed a quantification of the role of the different types of interactions in tuning the electronic properties of these molecules. Up to now, it has seen only limited use for chl-containing light-harvesting proteins, particularly because of the difficulty of identifying, in the resonance Raman spectra, the individual contributions of each of the bound chls *a* or *b* in the different LHCs. However, this method can yield the same specific information for chl molecules as for bacteriochlorophylls, i.e. on the conformation of, and intermolecular interactions assumed by, these pigments. Such information is of primary importance in evaluating the correctness of the refolding of the reconstituted LHC complexes. In the same way, as the resonance Raman technique is ideally suited to the study of the precise conformation of protein-bound carotenoids [7], it can also give information on how the latter cofactors are affected by the reconstitution procedure.

Recently we presented resonance Raman spectra for two of the photosystem II LHC proteins, LHCIIb and Lhcb4 [8]. While these data allowed us to define binding site properties for populations of bound chl molecules, it was not possible to identify these populations with the supposed structural positions of each chl in the atomic model [1]. The availability of mutant Lhcb4 proteins in which individual chl molecules are missing, produced by reconstitution of recombinant genes expressed in *Escherichia coli* [3], permits us to continue this work by carrying out such an identification. It is first neces-

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sary, however, to assess the quality of the wild type recombinant protein as compared to the native complex. The resonance Raman investigation of Lhcb4 reconstituted with the wild type apoprotein presented in this work, yielding precise information on pigment structure and interactions, is thus an important step to understanding the impact of the reconstitution protocol on the function of the wild type protein.

2. Materials and methods

Native Lhcb4 was isolated from maize PS II membranes as described previously [9,10]; reconstitution of the Lhcb4 protein has also been described elsewhere [2,11]. All samples were present in 0.06% β-dodecyl maltoside (DDM), 10 mM HEPES pH 7.6; 60% glycerol was added for low temperature absorption measurements.

4.2 K absorption spectra were recorded in a helium bath cryostat (Utreks), using a Varian Cary E5 double-beam scanning spectrophotometer. Resonance Raman spectra were collected at 77 K as described previously [8,12], on samples concentrated to an optical density in their Q_y absorption maxima of 20–50 cm^{-1} ($\sim$0.5 mg chl ml^{-1}).

3. Results

3.1. Absorption spectra

Fig. 1 compares the 4.2 K absorption spectra of native and reconstituted Lhcb4 complexes. The absorption spectrum of the recombinant protein is virtually identical to that of the protein isolated from maize thylakoids. There are, however, some differences observable in the spectra. In the Q_y region, the main chl a peak around 674 nm is slightly broader on the blue side. In the Soret region there are larger changes which can in the main be attributed to a slightly higher carotenoid stoichiometry for the recombinant protein (2.18 xanthophylls per 8 chls, as opposed to 2.05 for the native protein; data not shown). These additional xanthophylls may also contribute to much smaller differences in overall bandshape in the envelopes
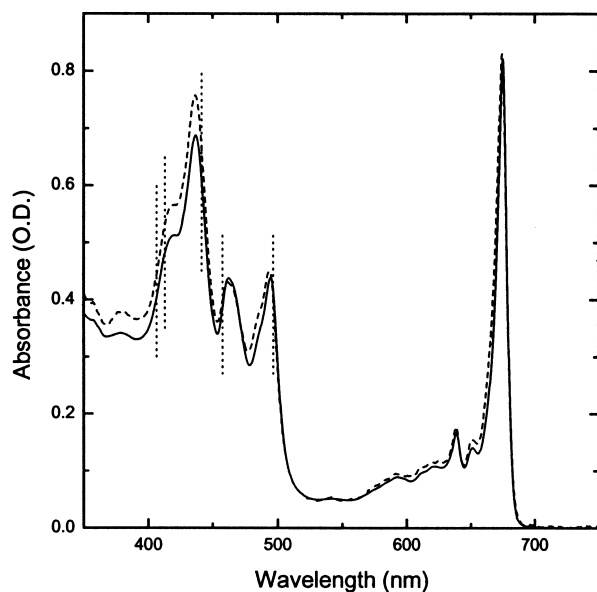

Fig. 1. 4.2 K absorption spectra of native (full line) and recombinant (dashed line) Lhcb4. Positions of the excitation wavelengths used for resonance Raman spectra, at 496.5 nm (carotenoids), 457.9 and 441.6 nm (chl b), and 413.1 and 406.7 nm (chl a), are indicated by dotted lines.
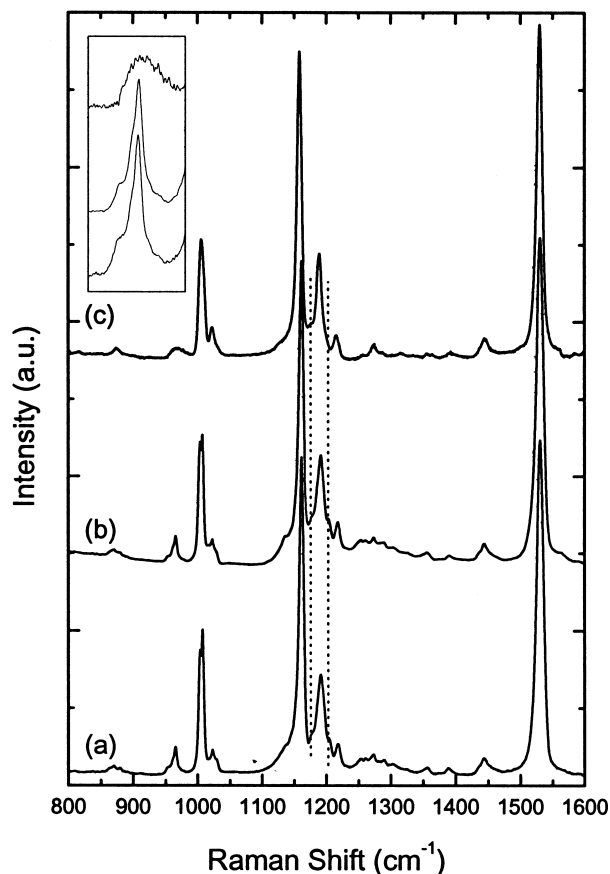

Fig. 2. Carotenoid resonance Raman spectra of native (a) and reconstituted (b) Lhcb4, excited at 496.5 nm. Also shown is the spectrum of isolated lutein in pyridine, excited at the same wavelength (c). Dotted lines at 1176 and 1203 cm^{-1} indicate modes referred to in the text. The ν_4 region (935–995 cm^{-1}) is shown enlarged in the inset.

of bands around 460–490 nm, although slight alterations in the absorption properties of one of the two chl b molecules may also contribute to these changes. The bandshape of the cluster at 420–440 nm shows no apparent differences, indicating that the chl a molecules bound by the reconstituted protein have largely unaltered absorption properties, although if only one of the six chls a present had its absorption peak shifted by a very small amount (less than 0.5 nm) it may not be observable.

3.2. Carotenoid molecules

Fig. 2 presents resonance Raman spectra of the two proteins in conditions favoring excitation of their bound xanthophyll molecules (496.5 nm). As can be seen from Fig. 2, the spectra are virtually identical and indicate that the carotenoids in the recombinant complex attain a configuration as near to the native conformation as can be measured by Raman spectroscopy. The same conclusions could be drawn for other excitations in resonance with carotenoids, from 476.5 to 514.5 nm (data not shown). In particular, the reconstituted protein shows all the features of protein-bound carotenoids rather than those free in solution. Around 1200 cm^{-1} in the ν_2 region, the minor bands at 1176 and 1203 cm^{-1} (indicated by dotted lines in Fig. 2) are present, whereas these are very small
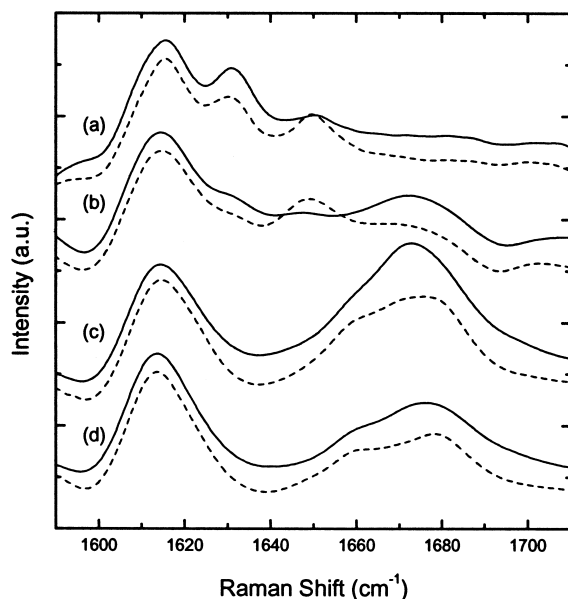
Fig. 3. Resonance Raman spectra of native (full lines) and reconstituted (dashed lines) Lhcb4, excited at 457.9, 441.6, 413.1 and 406.7 nm (a–d respectively).

or absent for the isolated carotenoids (Fig. 3c). The shape of the ν_3 region around 1000 cm^{-1} is more structured than for xanthophylls in vitro. However, a slight reduction in the structure of the latter feature is probably associated with the presence of a very small amount of non-specifically bound carotenoid, evidenced by the absorption spectra and pigment analysis (see above). The ν_4 region, around 960 cm^{-1} (see inset to Fig. 2), is particularly sensitive to slight changes in carotenoid configuration [13]. The fact that the bands are nearly identical in this region indicates that the xanthophyll molecules bound to the reconstituted complex adopt exactly the same molecular configuration as those bound to the native protein. Note that the same region for isolated lutein is smaller and unstructured (upper trace). The spectra are also identical to that already published for spinach Lhcb4 prepared by affinity chromatography [8].

3.3. Chl b molecules

Presented in Fig. 3a,b are resonance Raman spectra in conditions favoring chl b excitation (i.e. 457.9 and 441.6 nm respectively). The native protein measured here exhibits identical chl b spectra to those of spinach Lhcb4 [8]. In these spectra, bands are seen at 1630 and 1649 cm^{-1} which arise from the formyl carbonyl groups of the two apparent populations of chl b present in the protein. As discussed in [8], presence of these bands reveals that these groups are not in the same interaction state, that of one population being H-bonded with a neighboring amino acid sidechain, while that of the other is either weakly interacting or free from interaction in a rather polar environment. Presence of these two bands in the reconstituted protein indicates that it contains two populations of chl b whose formyl groups are involved in the same intermolecular interactions, and in similar immediate dielectric environments, as those of the native complex. The intensity of the 1649 cm^{-1} band is, however, observed to be higher in the recombinant protein. This cannot be explained in terms of an increase in the number of chl molecules contributing to

this mode, as pigment analysis of the sample clearly shows that the protein binds an equal number of chls a and b as the native complex [2]. Such an effect could easily be explained by a small shift of the Soret transition of one population of chl b, which would in turn modify the resonance conditions for this pigment. This would be consistent with the observed small changes in the Soret region of the absorption spectra, discussed above (see Fig. 1). We cannot formally rule out the possibility that, upon reconstitution, some chls b enter protein binding sites usually occupied by chl a, and which are unable to provide an H-bonding partner to its formyl carbonyl. However, this should result in a broadening of this band, rather than just an increase of its intensity, as these sites would not be expected to exhibit exactly the same dielectric properties.

3.4. Chl a molecules

Also shown in Fig. 3 are Raman spectra in resonance with the chl a molecules present (excited at 413.1 and 406.7 nm; Fig. 3c,d, respectively). The spectra for the native complex are again identical to Lhcb4 obtained from spinach thylakoids by affinity chromatography [8]. A comparison of the spectra in Fig. 3c and d indicates that the interactions assumed by the different bound chl a molecules are similarly conserved in the reconstituted protein. The position and width of methine bridge modes at 1613 cm^{-1} for the recombinant protein indicate that, as for the native complex, all chls a are five-coordinated, and that their macrocycle conformation is not significantly distorted when compared to the latter. The envelope of keto carbonyl modes, in the 1650–1700 cm^{-1} range, exhibits a similar distribution of carbonyl vibrators in the two samples, so that at least the large majority of the bound chls a in the recombinant protein find their native protein binding site, and bind to it with the proper geometry. There is, however, some difference in the shape of the envelope for the two complexes. It is difficult to judge whether this reflects small differences in the binding-site environment of a small number of the bound chl a molecules, or alterations in the resonance conditions with respect to the absorption transitions of some of the pigments concerned, as is the case for chl b excitations (see above).

4. Discussion

The use of resonance Raman spectroscopy has allowed us to investigate the result of in vitro reconstitution on detailed structural properties of the Lhcb4 antenna protein. The Raman bandshapes of the three types of pigment present (chls a and b and carotenoids) provide a measure of the effect of the reconstitution procedure on the overall protein folding pattern. No detectable deviations in xanthophyll conformation and configuration were observed. The conformation of, and the interactions assumed by, most of the chl molecules was also identical in the refolded complex and in the native one. There were, however, some small changes to the absorption properties of a few chl molecules bound to the protein, resulting in small differences in absorption spectra. Due to the resonance phenomenon, in which the contribution of absorbing pigments to Raman spectra is largely determined by their relative extinction coefficient at the excitation wavelength used, these small changes probably result in larger changes in the bandshape of the measured chl Raman spectra. The spectra nevertheless indicate that the reconstituted protein re-

folds to an excellent approximation of the wild type assembly. Reconstituted complexes thus represent a model system of choice for the precise, spectroscopic study of Lhcb4 proteins. Characterization of the different mutants of this protein obtained by reconstitution, particularly those lacking one chl *a* or *b*, is likely to yield useful information on the molecular mechanisms involved in tuning the properties of these molecules, and thus the function of their protein host.

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