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ORIGINAL PAPER

Coupling of laser excitation and inelastic neutron scattering: attempt to probe the dynamics of light-induced C-phycocyanin dynamics

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Abstract Excitation energy transfer (EET) in light-harvesting antennae is a highly efficient key event in photosynthesis, where light-induced dynamics of the antenna pigment-protein complexes may play a functional role. So far, however, the relationship between EET and protein dynamics remains unknown. C-phycocyanin (C-PC) is the main pigment/protein complex present in the cyanobacterial antenna, called "phycobilisome". The aim of the present study was to investigate light-induced C-PC internal thermal motions (ps timescale) measured by inelastic neutron scattering. To synchronize the beginning of the laser flash (6 ns duration) with that of the neutron test pulse ($\sim$87 μs duration), we developed a novel type of "time-resolved" experimental setup on MIBEMOL time-of-flight neutron spectrometer (LLB, France). Data acquisition has been modified to get quasi-simultaneously "light" and "dark" measurements (with and without laser, respectively) and eliminate many spurious effects that could occur on the sample during the experiment. The study was carried out on concentrated C-PC ($\sim$135 g/L protein in D_2O phosphate buffer), contained in an aluminium/sapphire sample holder (almost "transparent" for neutrons) and homogeneously illuminated inside an "integrating sphere". We observed very similar incoherent dynamical structure factors of C-PC with or without light. The vibrational density of states showed two very slightly increased vibrational modes with light, at $\sim$30 and $\sim$50 meV ($\sim$240 and $\sim$400 cm^{-1}, respectively). These effects have to be verified by further experiments before probing any temporal evolution, by introducing a time delay between the laser flash and the neutron test pulse.

Keywords Excitation energy transfer · C-phycocyanin · Photosynthesis · Inelastic neutron scattering · Time-resolved dynamics · Vibrational density of states

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Introduction

Light-harvesting antennae are pigment–protein complexes playing a key-role in the initial steps of photosynthesis. They are involved in light-absorption and excitation energy transfer (EET) to the so-called "reaction center" complexes, where the photochemical processes of photosynthesis take place. C-phycocyanin (C-PC) is one component of the phycobilisome, the light-harvesting system of cyanobacteria (Glazer 1989). Its structure has been determined by X-ray crystallography at a resolution of 1.45 Å (Nield et al. 2003). C-PC is composed of $\alpha\beta$ heterodimers, each heterodimer containing three linear tetrapyrrole chromophores, referred to as phycocyanobilins. Three $\alpha\beta$ units oligomerize as disc-shaped trimers $(\alpha\beta)_3$, which in turn form hexamers $(\alpha\beta)_6$. These hexamers stack one above the other forming the rod-like structure of the phycobilisome (Glazer 1989).

Phycobilisomes are very effective energy transducers, transferring the absorbed light energy to the photosystem II with more than 95% efficiency (Gantt et al. 1990; Porter et al. 1978), through different proposed pigment–pigment pathways (Contreras-Martel et al. 2007; Debreczeny et al. 1995; Glazer 1989; Padyana and Ramakumar 2006; Ren et al. 2006; Stec et al. 1999; Zhang et al. 1999). Short distances between chromophores of adjacent hexamers within rods provide the framework for efficient EET (Debreczeny et al. 1995; Gillbro et al. 1993; Glazer et al. 1985; Xia et al. 1991; Zhang et al. 1999).

However, a more detailed understanding of the physical processes leading to efficient EET would be of great interest for our knowledge of EET mechanism in photosynthesis. This generally requires determination of the energy level structure of the excited electronic states, which is governed by pigment–pigment and pigment–protein interactions. Electronic transitions in pigments have been shown to be coupled to low-frequency protein vibrations and higher-frequency localized vibrational modes of the chromophores, which serve as acceptor modes of excess energy during EET (Kühn et al. 1997; Pieper et al. 2004). In contrast to bacteriorhodopsin, a light-activated proton pump where the protein undergoes a sequence of structural changes during its photocycle, EET in C-PC does not require large-scale conformational changes of the protein-chromophore system. However, a change in protein dynamics of C-PC upon light-excitation may be expected via two possible mechanisms: (1) excess energy may be dissipated from distinct vibrational acceptor modes of the chromophores into a "heat bath" of protein motions (Kühn et al. 1997; Pieper et al. 2004), and (2) conformational protein dynamics may be directly induced by light excitation of a pigment, as the potential surface of the surrounding protein is generally disturbed in the pigment's excited state (Hayes et al. 1989; Tietz et al. 2001). The lifetime of such light-induced changes in the protein dynamics may not necessarily be coupled to the generally short time constants of EET (ps) or fluorescence lifetimes (ns) of C-PC.

Moreover, the validity of the employed models to study electronic interactions between pigment molecules in photosynthetic antennae is often restricted to low temperature (Novoderezhkin et al. 2005; Renger et al. 2001). One reason for this shortcoming might be the consideration of only harmonic protein motions. Recent quasielastic neutron scattering (QENS) experiments on photosystem II membrane fragments reported the onset of diffusive protein motions at $\sim$120 K in correlation with a drastic change in the spectroscopic properties of the embedded antenna complexes (Pieper et al. 2007). That means that protein dynamics may affect EET at physiological temperature.

Thus, the aim of the present study was to investigate whether dissipation of excess energy during light absorption and EET in the isolated pigment/C-PC protein system involves an activation of fast and localized protein motions at room temperature. The technique of inelastic neutron scattering is well suited to measure protein motions, such as vibrational modes, on an atomic length scale and a pico to nanosecond timescale (Smith 1991; Zaccai 2000). Structural and dynamical studies on stable light-induced intermediate conformational states have already been reported by neutron spectroscopy for bacteriorhodopsin (Dencher et al. 1989, 1990; Fitter et al. 1999; Weik et al. 1998). Very recently, "time-resolved" neutron spectroscopy revealed a transient protein softening correlated with the structural change in the M-intermediate of this protein (Pieper et al. 2008).

We used the time-of-flight spectrometer MIBEMOL at the Laboratoire Léon-Brillouin (LLB, France) to access motions of H atoms on timescales in the range 0.2–20 ps. To detect thermal protein motions related to light dissipation in C-PC, as well as to investigate the timescale of such dynamical changes, we developed a new experimental setup on this spectrometer. This new "time-resolved" method was technically challenging, since we had to synchronize pulsed inelastic neutron scattering measurements with repetitive light excitations.

"Laser/neutron" coupling

Inelastic neutron scattering

Neutrons are scattered by atomic nuclei. Since the incoherent neutron scattering cross section of hydrogen nucleus exceeds, by at least one order of magnitude, the scattering cross sections (coherent and incoherent) of all other atoms present in biological samples (mainly C, O, N, P, and S), as well as of deuterium atom (D), total scattering is dominated by incoherent scattering of individual H atoms. As hydrogen atoms are abundant and uniformly distributed in proteins, the scattering represents a mean global view of protein dynamics. The incoherent scattering from C-PC H atoms, i.e., from its non-exchangeable H atoms (70% of the total C-PC H atoms), contributes to 87.5% of protein total scattering signal (coherent and incoherent).

Experimental setup

The principle of the experimental setup is shown in Fig. 1. The laser was fixed outside the sample well of MIBEMOL spectrometer, perpendicularly to the neutron beam and in front of the detectors (Fig. 1a). The photon beam was directly aimed on the sample through the 10 mm-diameter

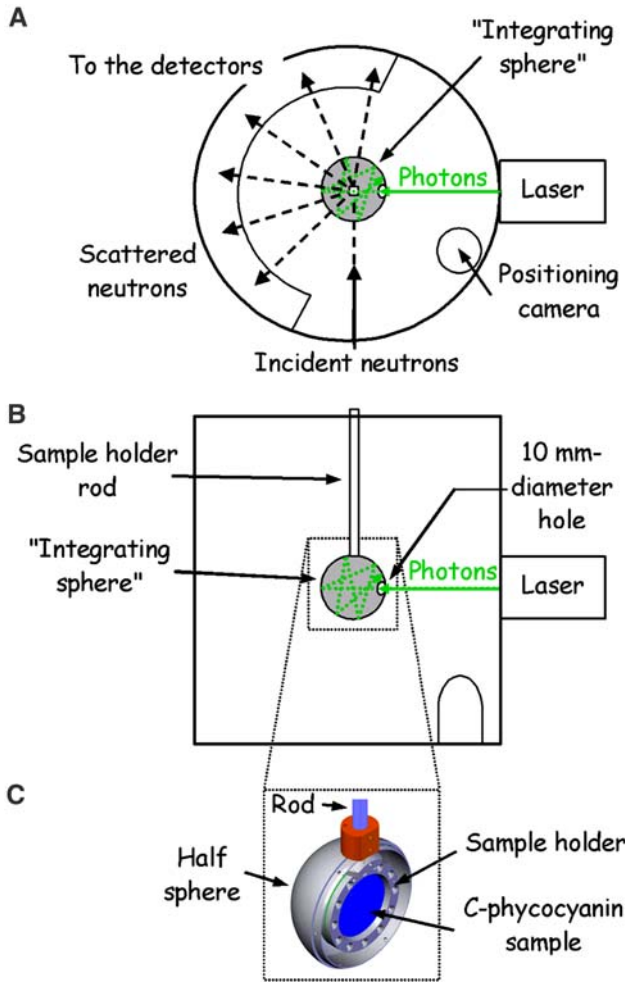

Fig. 1 Schematic experimental setup of the "laser/neutron" coupling on the time-of-flight MIBEMOL neutron spectrometer (LLB, France): top (**a**) and side (**b**) views. The neutron and photon beams were perpendicularly oriented. Homogeneous illumination of the protein was provided by an "integrating sphere" (a spherical and hollow aluminium chamber) containing the sample holder (**c**). Photons were aimed on the sample through a 10 mm-diameter hole in the sphere. Height and orientation positioning of the sphere inside the sample well was checked using a camera

hole of an "integrating sphere" (Fig. 1b, c), without any additional optical device (see "Sample environment" below). We used this spherical and hollow aluminium chamber, containing a highly reflective and diffusing interior coating, to illuminate the sample inside uniformly (Fig. 1c). The "integrating sphere" was positioned in front of the laser head using a camera located inside the sample well. First, we positioned and aligned the experimental setup, without the "integrating sphere" but using optic fibres illuminating both sides of the sample container (Fig. 2). In that configuration, we were able to check that the sample was entirely illuminated, since fluorescence was homogenously emitted from the surface of the sample (Fig. 2).

Data acquisition

MIBEMOL data acquisition system has been modified to synchronize the laser excitation flashes (6 ns duration) with the neutron pulses ($\sim$87 μs duration) at the sample position, in order to perform "time-resolved" measurements. Successive neutron scattering measurements (few hours duration), with or without the laser, gave access to the "light" and "dark" spectra, respectively. "Light" and "dark" spectra were separated by 50 ms in the case of a 10 Hz laser frequency (see Fig. 3). The quasi-simultaneous process of such "light" and "dark" relative measurements and their evolution as a function of time could be compared to measurements performed in a "double beam" spectrometer (Fig. 3a). The flash lamp pumping and the laser triggering have been controlled by a special device and checked all along the experiment by an oscilloscope (TDS 2024, Tektronix) (Fig. 3b). This procedure of alternating "light" and "dark" measurements was novel and eliminated many spurious effects that could occur in the sample during the experiment (e.g., positioning of the sample holder or protein denaturation). However, such device does not eliminate putative laser-induced heating dissipated before the "dark" spectra measurement. For the present study, only a "zero-time" experiment has been performed, i.e., no time delay has been introduced between the beginning of the laser flash and the beginning of the neutron pulse. However, for future measurements, a time delay between the laser flash and the neutron pulse can be introduced.

Sample environment

Measurements have been performed at constant room temperature (298 K) and pressure. The light source was a 6 ns pulsed Nd:YAG laser (Excel Technology, France), at $\lambda = 532$ nm, with maximum frequency and energy of 14 Hz and $\sim$25 mJ, respectively. Photons entered the "integrating sphere" through a 10 mm-diameter hole (the laser beam width has been evaluated to be $\sim$4.5 mm at the entrance of the sphere). Before protein sample measurements, we tested two different sample holders: a quartz Suprasil 300 (Hellma, France) rectangular one ($35 \times 40 \times 0.5$ mm^3) and a circular aluminium one surrounding 50 mm diameter sapphire glasses (Roditi, UK) with a 0.5 mm sample thickness. Such dimensions optimized the intersection with the neutron beam, whose size is approximately 25×50 mm^2. Inelastic neutron scattering by the sample containers was compared to the background measured in the empty sample well of MIBEMOL spectrometer. We observed that the elastic intensity level from the aluminium/sapphire (Al_2O_3) sample holder was much weaker than that of the quartz (SiO_2) one (Fig. 4). Indeed,

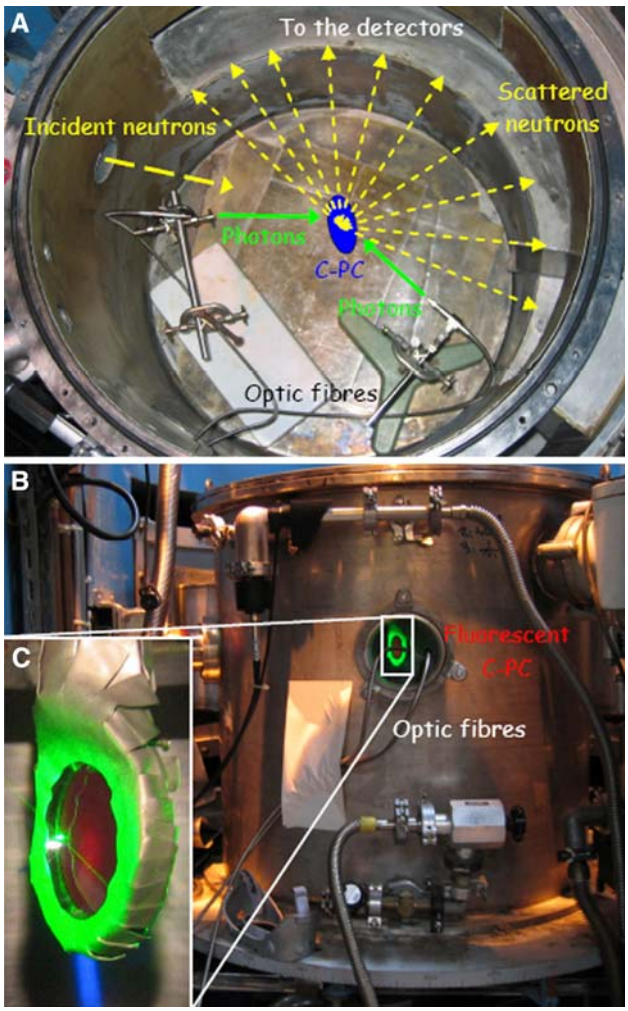

Fig. 2 Top (**a**) and side (**b**) views of the sample environment on MIBEMOL neutron spectrometer during illumination experiments on C-PC. To check that the protein solution was entirely illuminated, we made direct observation of the sample, without the sphere, by using optic fibres. Inset (**c**): fluorescence of the C-PC (*red colour*) in the sample holder with the green laser beam reflected on the cadmium mask

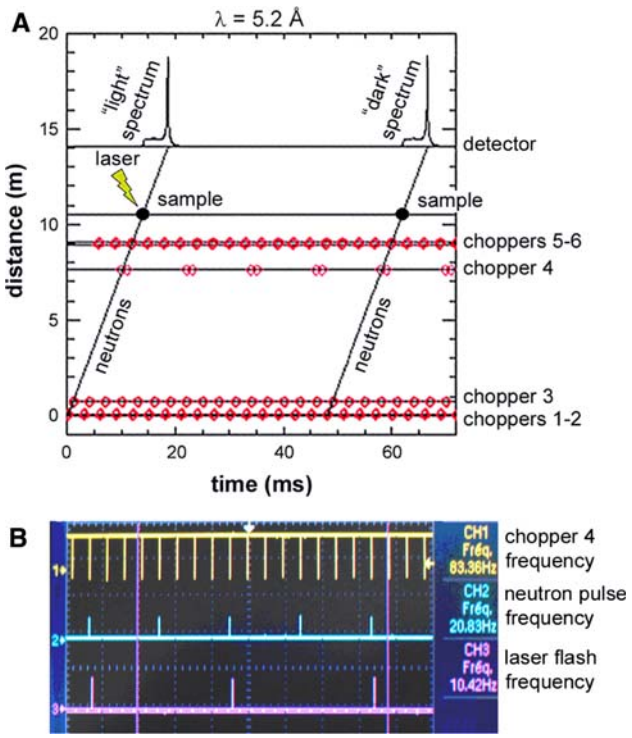

Fig. 3 **a** Diagram of the chopper velocities on MIBEMOL neutron spectrometer for the "laser/neutron" setup, at $\lambda = 5.2$ Å configuration ($\sim$140 μeV resolution). The incident and scattered neutrons are represented by a *solid line* and typical time-of-flight spectra have been drawn at the detector. The "light" spectrum was obtained when the beginning of the neutron test pulse ($\sim$87 μs duration) was synchronized with the laser flash, symbolized by a *green lightning symbol* ($\sim$25 mJ, 10 Hz, $\lambda = 532$ nm, 6 ns duration). The "dark" spectrum, resulting from each neutron test pulse between two laser flashes, was considered as the reference without laser. **b** Photograph of the oscilloscope screen representing the recording of the chopper #4 frequency (80 Hz, channel #1), the neutron pulse frequency on the sample (20 Hz, channel #2), and the laser flash frequency (10 Hz, channel #3)

quartz sample has a Bragg peak at 1.5 $Å^{-1}$, whereas, in the *Q*-window of our measurements, no Bragg peak was observed for sapphire glass, nor aluminium. The aluminium/sapphire container was therefore chosen for the protein dynamics measurements. The aluminium "integrating sphere" surrounding the sample holder was also almost "transparent" for neutrons (data not shown).

Materials and methods

C-PC purification

Protonated C-PC was prepared from the thermophilic cyanobacterium *Synechococcus elongatus*, grown as previously described (Boussac et al. 2004). Briefly, the cells were grown (1,500 mL DTN culture medium) in a CO_2 enriched atmosphere, in a rotary shaker, at 45°C, under continuous illumination from white lamps, until they reached an optical density close to 1 at 800 nm. After harvesting by centrifugation, the cells were washed with buffer (40 mM MES, pH 6.5, 15 mM $MgCl_2$, 15 mM $CaCl_2$, 10% glycerol, 1.2 M betaine) and resuspended in the same buffer, with 0.2% (*w*/*v*) bovine serum albumin, 1 mM benzamidine, 1 mM aminocaproic acid, and 50 μg ml^{-1} DNase I added to a chlorophyll concentration of $\sim$1.5 mg ml^{-1}. The cells were ruptured with a French press and unbroken cells were removed by centrifugation (1,000×*g*, 5 min). Thylakoids were eliminated by centrifugation (180,000×*g* for 35 min at 4°C) and the supernatant containing the phycobiliproteins was precipitated with 50% ammonium sulphate. C-PC was purified by hydrophobic interaction chromatography on HiLoad 16/10

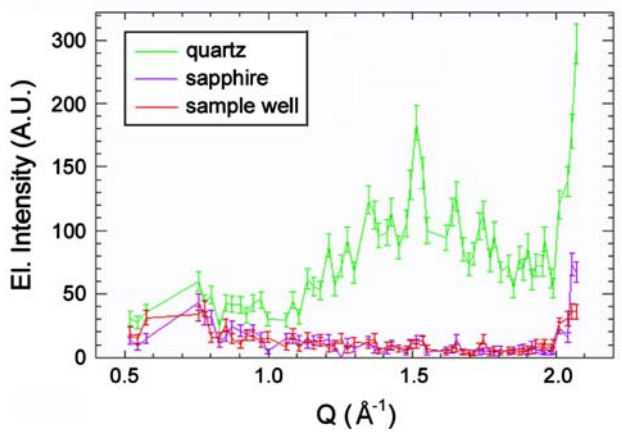

Fig. 4 Elastic neutron scattering levels for the sample holders we tested: a rectangular quartz one and a circular aluminium/sapphire glass one (see "Laser/neutron coupling" part for details), compared to the background of the empty sample well of MIBEMOL spectrometer. Measurements have been performed at $\lambda = 5.2$ Å and room temperature and pressure

Phenyl Sepharose High Performance resin (Amersham Biosciences) using an ÄKTA FPLC purification system (Amersham Biosciences). The column was first equilibrated with buffer A (50 mM sodium phosphate, 0.8 M ammonium sulphate, 10% ethanol, pH 7) and the C-PC protein was eluted with a linear gradient from 100% buffer A to 100% buffer B (50 mM sodium phosphate, 10% ethanol, pH 7) over six column volumes (120 mL). The column effluent was monitored at 280 nm. Purity and quality of the C-PC protein samples have been checked by SDS-PAGE electrophoresis (Bio-Rad) and silver coloration (Pierce), as well as by UV-visible spectrophotometry (Cary 100, Varian).

Sample preparation for neutron experiments

We used deuterated water environment to minimize the strong scattering contribution from the solvent. All labile protons have been exchanged against deuterons, by repeated mixing with heavy water (12 h, 4°C) and water evaporation by centrifugation (Concentrator 5301, Eppendorf). Samples were then dialysed three times in cassettes (10,000 MWCO, Pierce), at 4°C, against 20 mM sodium phosphate buffer prepared in D_2O (pH 6.6). Finally, protein concentration was adjusted to $\sim$135 g/L by centrifugal filter devices (10,000 MWCO Centricon, Millipore) at 6,500 r.p.m. and 4°C. Optical density of C-PC sample at $\lambda = 532$ nm is about 1/6 of that at the spectrum maximum, at $\lambda \approx 620$ nm. Since the absorption coefficient of C-PC at $\lambda \approx 620$ nm has been estimated to be $\sim 2.8\ 10^5\ M^{-1}\ cm^{-1}$ (Glazer et al. 1973; Svedberg and Katsurai 1929), the absorption coefficient at $\lambda = 532$ nm could be evaluated to be $\sim 0.5\ 10^5\ M^{-1}\ cm^{-1}$. With a protein concentration of $\sim$135 g/L, the optical density of the sample was close to $\sim$0.2, at $\lambda = 532$ nm, for an optical path of 0.5 mm. This introduced less than $\sim$10% of difference between the minimum and the maximum of light intensity inside the protein solution. The exact amount of C-PC solution in the sample holder and the proper sealing were determined by weighing on a high precision balance. Survival of the protein (non-bleaching) has been checked at the end of the experiment by UV-visible spectrophotometry.

Neutron scattering experiments

Inelastic neutron scattering experiments were performed on the time-of-flight spectrometer MIBEMOL (LLB, France) modified as described above. We obtained a set of energy-transfer spectra measured at different scattering angles (Q-range from 0.5 to 2.1 Å^{-1}). We used an incident wavelength of 5.2 Å to have the maximum of neutron flux with a reasonable resolution ($\sim$140 μeV). Such a resolution gives access to motions on 0.2–20 ps timescale. Data collection times were about 10–20 h to obtain sufficient statistics.

Inelastic neutron scattering data treatment

In an incoherent neutron scattering experiment, waves scattered by different nuclei do not interfere (Bée 1988). The scattering intensity measured is the sum of intensities scattered by individual nuclei, mainly H nuclei. This scattering intensity is proportional to the incoherent dynamical structure factor $S_{\mathrm{inc}}(Q,\omega)$, which provides the probability for an incident neutron to be scattered by the sample with a momentum transfer $\hbar Q$ and an energy transfer $\hbar\omega$, where $\hbar$ is the reduced Planck constant. Therefore, the individual dynamics of a single particle can be completely described by its incoherent dynamical structure factor $S_{\mathrm{inc}}(Q,\omega)$:

$$S_{\mathrm{inc}}(Q,\omega) = \exp(-Q^2\langle u^2\rangle/3) \left[\mathrm{EISF}(Q)\delta(\omega) + (1-\mathrm{EISF}(Q))L(Q,\omega)\right] + S_{\mathrm{inelastic}}(Q,\omega)$$

The exponential term is the Debye-Waller factor, which accounts for the spatial extension of the vibrational modes of the system, where $\langle u^2\rangle$ is the mean square displacements of overall atoms, mainly H ones. The vibrational modes contribute, at high energy transfer ω, to the inelastic part to the spectrum through $S_{\mathrm{inelastic}}(Q,\omega)$. δ is the Dirac delta function and EISF stands for the Elastic Incoherent Structure Factor, which modulates the elastic intensity and gives access to the geometry of the motions. EISF is defined as $\mathrm{EISF}(Q) = \frac{I_{\mathrm{el}}(Q)}{I_{\mathrm{el}}(Q)+I_{\mathrm{qe}}(Q)}$, where I_{el} and I_{qe}

represent the elastic and quasielastic scattering intensity, respectively. $L(Q,\omega)$ is a Lorentzian function, which appears as a broadening of the elastic peak and, so, is called the "quasielastic" signal. $L(Q,\omega)$ is related to the relaxational dynamics, attributed to nonvibrational, over-damped, or diffusive motions, or jumps between conformational substates.

Data have been analysed using standard LLB software. Correction and smoothing procedures were the same for both "light" and "dark" spectra.

Results and discussion

As shown in Fig. 5, C-PC in solution has a significantly higher elastic intensity level all along the Q-range, as compared to the buffer scattering and background due to the cell container and the sample well. As only small differences were expected, an accurate buffer subtraction would have been essential to avoid introduction of artefacts into the measured data. Therefore, we preferred to compare raw intensity measurements of the sample, illuminated or not by the laser, in a relative way. Moreover, such "time-resolved" experiments have poor statistics because only one neutron pulse within a 100 ms period (in the case of 10 Hz-frequency laser flashes) is counted for each "light" and "dark" spectrum (see Fig. 3a).

Incoherent neutron scattering intensity

Since the time-scale of a possible light-induced effect on the protein dynamics is unknown, the laser flashes were first synchronized with the neutron pulses (time delay = 0)

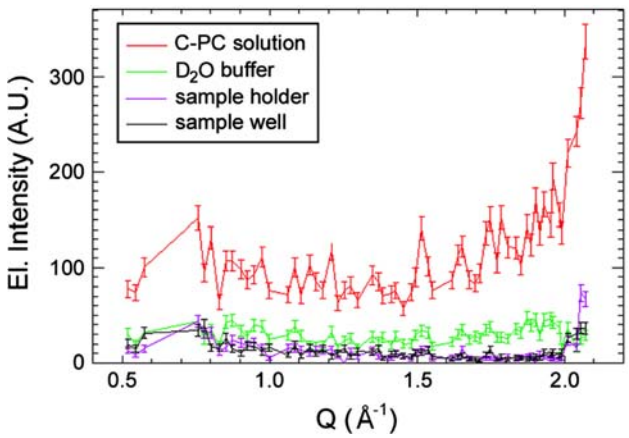

Fig. 5 Elastic neutron scattering intensity of the C-PC solution sample (~135 g/L protein concentration), in 20 mM sodium phosphate buffer in D_2O, compared to the intensity from the buffer alone, the aluminium/sapphire sample holder, and the empty sample well. Measurements have been performed on MIBEMOL spectrometer at $\lambda = 5.2$ Å and room temperature and pressure

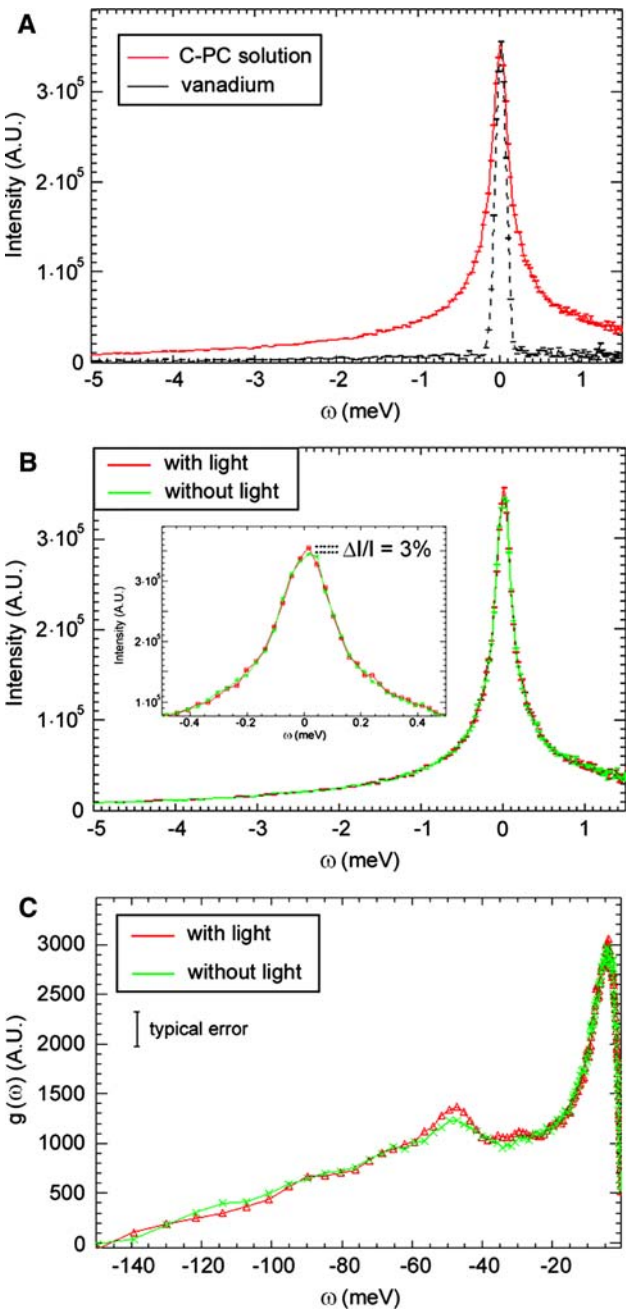

Fig. 6 **a** Typical incoherent inelastic scattering spectrum, as a function of energy transfer ω, of the C-PC solution sample (~135 g/L), compared to the vanadium (resolution) spectrum. **b** Incoherent inelastic scattering spectrum of the C-PC solution sample, illuminated or not by the laser (~25 mJ, 14 Hz flashes, $\lambda = 532$ nm). Inset: zoom-in of the elastic lines for ω from −0.5 to +0.5 meV, showing a tiny, non significant, 3% relative difference between the intensities of the sample illuminated or not. **c** Vibrational density of states of the C-PC solution sample, illuminated or not by the laser. A typical error bar is shown. A very slight increase of the vibrational mode was observed with light at ~50 and ~30 meV (~400 and ~240 cm^{-1}, respectively). The latter one corresponds to the methyl group librations. Measurements have been performed on MIBEMOL spectrometer at $\lambda = 5.2$ Å configuration (~140 μeV resolution) and room temperature and pressure

in order to achieve the highest possible temporal overlap between excitation laser flash and neutron test pulse. Figure 6a shows a typical QENS spectrum ($\lambda = 5.2$ Å) of C-PC sample, compared to the vanadium spectrum (resolution) essentially containing elastic scattering within the accessible energy transfer region. In Fig. 6b, we compare the C-PC QENS spectra $S(\omega)$ obtained with or without light. Except for the slight non significant $\sim 3\%$ relative difference at the maximum intensity of the elastic peaks (see inset, Fig. 6b), both spectra were perfectly superimposed all along ω-range. The spectra obtained at another configuration ($\lambda = 9$ Å) gave similar results (data not shown). We checked, by UV-visible spectrophotometry on the C-PC sample before and after the experiment, that the protein was not denaturated by the laser flashes (data not shown).

Density of states of the vibrational excitations

The incoherent inelastic neutron scattering is directly related to the density of states of the vibrational excitations of the H atoms present in the system, according to the relation: $G_{\mathrm{inc}}(Q,\omega) = \frac{\omega^2}{Q^2} S_{\mathrm{inc}}(Q,\omega)$. The amplitude-weighted density of states $g(\omega)$ can be obtained taking the limit $Q \to 0$ of the function above: $\lim_{Q \to 0} G_{\mathrm{inc}}(Q,\omega) \propto g(\omega)$.

Figure 6c shows the vibrational density of states $g(\omega)$ of the C-PC, illuminated or not by the laser. The data have been averaged over all available Q-values to improve data statistics. A slight light-induced increase of two vibrational modes at ~ 30 and ~ 50 meV (~ 240 and ~ 400 cm^{-1}, respectively) has been observed. The vibrational mode at ~ 30 meV may be related to the librations of the protein methyl groups (Drexel and Peticolas 1975). Moreover, such C-PC dynamics might be related to the internal molecular reorganization observed recently in light-irradiated phycobilisome (Stoitchkova et al. 2007). However, the magnitude of these effects is similar to the experimental uncertainty (see Fig. 6c). Thus, the statistical significance of the observed effects has to be verified before truly "time-resolved" experiments are performed, i.e., before the time delay between laser excitation and neutron test pulse can be varied to probe any temporal evolution.

Provided that further evidence for the observed effect can be obtained, a light-induced increase of inelastic intensity might be related to dissipation of excess energy during EET. In this case, the "overpopulated" vibrational modes observed in the present study would correspond to the energetic difference between electronic energy levels of the C-PC pigment molecules involved in EET. This means that the ~ 30 and ~ 50 meV-vibrations would serve as "acceptor modes" for excess energy before further relaxation, into a "heat bath" of protein vibrations. In the case of coincidence of laser excitation and neutron test pulse, as employed here, such an overpopulation of vibrational modes might appear, regardless of the fact that EET and vibrational relaxation might occur on much faster timescales (fs- and ps-range) in a truly time-resolved experiment. Such effects might be superimposed on others observed at longer timescales, since the minimum time resolution in QENS experiments is given by the width of the neutron pulse (μs-range).

In conclusion, we described a new experimental setup coupling light irradiation and inelastic neutron scattering spectroscopy in order to probe light-induced dynamics of the photoactive protein C-PC. The "light/dark" procedure employed has the advantage to eliminate many spurious effects that could occur in the sample during the experiment by relative measurements. We found that, in the case of C-PC, the incoherent dynamical structure factor was not significantly altered by light. Two modes within the vibrational density of states were slightly increased in C-PC upon illumination. These tiny effects observed on C-PC have to be verified by further experiments with improved data statistics. In particular, characterization, by time-resolved optical spectroscopy, of the excitation timescale and the fraction of excited sample, which has not yet been achieved in the present study, should be improved. A perspective would be to apply this coupling setup to more characterized and appropriate light-excitable systems.

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