Photochemically induced dynamic nuclear polarisation in entire bacterial photosynthetic units observed by $^{13}$C magic-angle spinning NMR*

5.1 Abstract

Photochemically induced dynamic nuclear polarisation has been observed from entire photosynthetic units (PSU) bound to chromatophore membrane (membrane-bound PSU) of the purple bacteria *Rhodobacter sphaeroides*, which have been selectively $^{13}$C-isotope enriched at all BChl and BPheo cofactors. These 1.5 MDa membrane-bound protein complexes comprise reaction centers as well as the antenna systems called light harvesting complexes I and II. Due to light-induced enhancement of nuclear polarisation, the $^{13}$C magic-angle spinning (MAS) NMR spectrum shows absorptive lines originating from the cofactors involved into the photochemical machinery and allowing the determination of the electronic ground state structure at atomic resolution. Addition of detergent released intact PSU from the chromatophore membrane (so called detergent-solubilized PSU) and caused significant changes in the sign and intensity pattern of the light-induced MAS NMR spectrum. In contrast, detergent-solubilised PSU and detergent-solubilised bacterial reaction centers with the same isotope label pattern exhibit essentially the same chemical shifts with only minor differences in the intensity pattern. The pronounced differences between intact membrane-bound and detergent-solubilised photosynthetic units are tentatively explained by the loss of self-orientation of the membrane-bound samples by solubilisation. This interpretation suggests that the theoretically predicted anisotropy of the light-induced nuclear polarisation has been observed for the first time.

5.2 Introduction

Due to the unsurpassed electron pumping efficiency of photosynthetic reaction centers (RCs), photon flux is limiting for photosynthesis (for a review on the photophysics of RCs of purple bacteria, see (Hoff and Deisenhofer, 1997). Therefore, increasing the number of RCs would not increase the photosynthetic activity. Instead, the RCs are embedded in arrays of antenna pigments. In purple bacteria, the entire photosynthetic apparatus containing RCs and light-harvesting (LH) antenna complexes is called the photosynthetic unit (PSU) (Papiz et al., 1996; Hu et al., 2002). In the PSU, an RC is directly surrounded by the core LH I (B875). Around such RC-LH I complexes, other antenna systems called LH II (B800-850) are located.

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Kinetics and optical properties of RCs in PSUs are slightly different from isolated and detergent-solubilised RCs. In *Rhodobacter (Rb.) sphaeroides*, isolation from membranes by detergents causes a small blue shift of the monomeric BCHls (from 802 to 801 nm) and a small red shift of the BPheos from 754 to 755 nm (Beekman et al., 1995). Absorbance-detected magnetic resonance (ADMR) experiments suggest interaction between exciton states of antenna and RCs (Owen et al., 1997). It has also been shown that the charge separation is slower in PSU ($\tau = 4.5$ ps compared to 3.3 ps), due to an increase of the slower of the two exponential components (Schmidt et al., 1993). It has been speculated that a slight increase of the redox midpoint potential $E_m$ for the $P/P^+$ couple may be the reason, since similar phenomena were observed in mutants of *Rb. capsulatus* (Jia et al., 1993). In *Rhodopseudomonas (Rps.) viridis*, the lifetime of the primary radical pair ($P^+H^-$) with pre-reduced secondary acceptor QA has been found to be 2.4 to 3 ns in intact membranes and about 5 ns in isolated RCs (Gibasiewicz et al., 1999). The differences in the recombination kinetics may be either due to efficient exciton back-transfer opening an additional decay path, or caused by an influence of the LH I antenna complex on the energetics of the primary charge separation (Visschers et al., 1999; Bernhardt and Trissl, 2000). Another difference between PSU membranes and detergent-solubilised RCs arises from the fluid mechanics of membranes: PSU samples have the tendency to orient under appropriate conditions, as under centrifugal forces or in thin layers (Alegria and Dutton, 1991; Hara et al., 1993).

In a first attempt to probe the differences between RCs embedded in intact PSUs and in solution conditions, we applied magic-angle spinning (MAS) solid-state NMR, which is a powerful method for studying structure and dynamics of membrane proteins (de Groot, 2000). In principle, NMR chemical shift information can allow for the exploration of spatial, protonic and electronic structure at atomic resolution in the electronic ground state, and NMR analysis can provide detailed insight into functional mechanisms of proteins. In case of several photosynthetic RCs of bacteria and plants, it has been shown that photochemically induced dynamic nuclear polarisation (photo-CIDNP) can overcome the intrinsic insensitivity of NMR spectroscopy by photochemical induction of non-Boltzmann nuclear spin states. Photo-CIDNP has been observed initially in quinone-blocked bacterial RCs from *Rb. sphaeroides* R26 (Chapter 3) (Zysmilich and McDermott, 1994, 1996b, 1996a; Matysik et al., 2000b). As a second system, the RC of *Rb. sphaeroides* wildtype (WT) has shown $^{13}$C photo-CIDNP of similar intensity as R26 (Chapter 2) (Matysik et al., 2001a). The third system, in which photo-CIDNP has been observed, is the D1D2 complex of the RC of photosystem II of plants, resulting in a hypothesis of the remarkable strength of the redox potential of the primary electron donor P680 (Matysik et al., 2000a; Diller et al., 2005). The fourth system in which photo-CIDNP has been observed is photosystem I of spinach leading to a complete set of assignments of the aromatic ring carbons to the P2 cofactor of the primary electron donor P700 (Alia et al., 2004b). Spatial selectivity and sensitivity of photo-CIDNP experiments can...
be further improved by application of selectively isotope labelled samples. From selectively
\(^{13}\text{C}\)-isotope labelled RCs of \textit{Rb. sphaeroides} WT, two-dimensional photo-CIDNP MAS NMR
spectra have been obtained, which clearly demonstrate that the electron density of the two
BChl molecules of the special pair is already different in the electronic ground state (for
details, see Chapter 4).

In addition to the information on the electronic ground state provided by the NMR
chemical shifts, photo-CIDNP solid-state NMR intensities are linked to the local electron spin
densities occurring in the radical-pair state. The exact link between the local electron-spin
densities and the photo-CIDNP intensities, however, remains the object of further studies
since the mechanism producing photo-CIDNP in solids is currently under discussion.
Originally, the net polarization in frozen RC under continuous illumination was assumed to be
due to significant relaxation of the nuclear spins in the special pair triplet \(^3\text{P}\), while nuclear
spins in the singlet ground state \(^1\text{P}\) with a much longer longitudinal relaxation time would
retain their polarization (McDermott et al., 1998). However, this differential relaxation
mechanism cannot explain photo-CIDNP effects for nuclear spins in the BPhe acceptor,
which also have been observed (Zysmilich and McDermott, 1996b; Jeschke, 1998; Polenova
and McDermott, 1999; Schulten et al., 2002). Two further mechanisms generating photo-
CIDNP have been proposed. In the three-spin mixing mechanism, the presence of both an
anisotropic hyperfine interaction and a coupling between the two electron spins, creates net
nuclear polarization in the spin-correlated radical pair (Jeschke, 1997, 1998). In the
differential decay mechanism, net nuclear polarization is accumulated due to the preferential
decay of triplet radical pairs \(^3(P^*\Phi^*)\) to special pair triplets \(^3\text{P}\), which is faster than that of
singlet radical pairs to the ground state (Polenova and McDermott, 1999). The singlet pairs
exist long enough for a fraction of the electron spin polarisation to be transferred to nuclear
polarization by evolution of the spin system under the anisotropic hyperfine interaction. Very
recently, both mechanisms have been reassessed and the effects have been simulated (Chapter
2). According to both mechanisms, the photo-CIDNP effect is predicted to be highly
anisotropic. The effect due to both mechanisms vanishes at the canonical orientations of the
hyperfine tensor where the pseudosecular component \(B\) to the hyperfine coupling, which
causes the electron-nuclear spin mixing, approaches zero. The contribution by the three-spin
mixing mechanism vanishes for zero coupling between the electron spins, which would be
expected if the dipole-dipole coupling compensates the \(J\) coupling at some orientations.
Judging from the known distance between the radical ions and values for the \(J\) coupling found
in the literature, this is unlikely to occur, however, some orientation dependence of the photo-
CIDNP effect is still induced by the orientation dependence of the dipole-dipole coupling
(Tang et al., 1996; Hulsebosch et al., 1999, 2001). Perhaps more significantly, the magnitude
of the effect for both mechanisms depends strongly on the secular hyperfine coupling \(A\); for
the differential decay mechanism even the sign of the effect depends on the sign of \(A\). This
may cause a strong orientation dependence of the magnitude of the effect and even sign changes, as for many \(^{13}\)C nuclei, \(A\) is close to zero for orientations within the plane of the macrocycle. While these qualitative predictions are well founded in the theory of the mechanisms, quantitative predictions are difficult to make because of uncertainties in too many parameters. In this situation a better understanding of the relation between photo-CIDNP effects and the electronic structure of the radical ion pair is hampered by a lack of data on oriented systems. The tendency of PSUs to self-orient may provide such an oriented system (Jeschke, 1997; Jeschke and Matysik, 2003).

Photo-CIDNP data from RC bound in PSUs may be interesting in two aspects. Comparison with data collected from detergent-solubilised RCs may provide (i) information on the mode of interaction between LH I and the RCs as well as (ii) a clue on the mechanism of photo-CIDNP in solids, especially on its anisotropy. In this paper, we report for the first time photo-CIDNP in PSUs observed by \(^{13}\)C MAS NMR, and discuss spectral differences with isolated RCs.

5.3 Materials and methods

5.3.1 Preparation of \(^{13}\)C-labeled PSUs

\(\delta\)-Aminolevulinic acid (ALA) is a precursor of naturally occurring tetrapyrroles, including BChl and BPhe (Jordan, 1991). In biosynthesis, two molecules of ALA are asymmetrically condensed to form the pyrrole porphobilinogen (Figure 1). Four molecules of porphobilinogen tetramerize and prior to macrocycle ring closure, the pyrrole ring IV is inverted via a spiro-intermediate. This sequence causes the asymmetry of the macrocycle backbones of BChl and BPhe. On the biosynthetic pathway, mono-\(^{13}\)C enriched ALA forms doubly \(^{13}\)C-enriched porphobilinogen and eightfold \(^{13}\)C-enriched BChl and BPhe macrocycles. Incorporation of (4-\(^{13}\)C)-ALA, as reported in this paper, produces BChl and BPhe macrocycles, labeled at the C-1, C-3, C-6, C-8, C-11, C-13, C-17 and C-19 (see Figure 5.1 for nomenclature). Cultures of \(Rb.\ sphaeroides\) WT (480 mL) were grown anaerobically in the presence of 1.0 mM (4-\(^{13}\)C)-\(\delta\)-aminolevulinic acid·HCl (COOH \(\text{CH}_2\text{CH}_2\text{COCH}_2\text{NH}_2\cdot\text{HCl}, 99\%\) \(^{13}\)C-enriched), which was purchased from Cambridge Isotope Laboratories (Andover, USA). The cultures were allowed to grow for 7 days in light. Prior to harvesting the cells for the preparation of chromatophore and RCs, a 4 mL aliquot was taken from the culture and the extent of \(^{13}\)C incorporation of (4-\(^{13}\)C)-ALA into BChl has been determined as described in detail earlier (Schulten et al., 2002). The total \(^{13}\)C-label incorporation in BChl/BPhe \((^{13}\text{C}_{0-8})\) was about 60±5\%. The chromatophore-membrane containing entire PSU (membrane-bound PSU) were prepared essentially as described in (Kondo et al., 2002).
Figure 5.1. Schematic representation of the biosynthesis of \((^{13}\text{C}_0-8\)-labelled BChl a starting from \((4-^{13}\text{C})-\delta\)-aminolevulinic acid (ALA). The positions of the \(^{13}\text{C}\)-labels are indicated with filled circles (●). BPhe a is a derivative of BChl a, in which the magnesium is replaced by two hydrogen atoms. The numbering of BChl a is according to the IUPAC nomenclature.

For the preparation of a detergent-solubilized PSU sample, chromatophore-membrane containing entire PSUs (A\(_{865}\) of 200) were treated with 0.5% detergent (LDAO, Fluka Chemie GmbH, Buchs, Switzerland) for 1.5 h at 4°C. As a result, membranes were partially solubilised and the intact PSU was released from the membrane. This has been confirmed by linear sucrose gradient ultracentrifugation which resulted in the clear separation of the band of detergent-solubilized PSU (at ~30% sucrose) from membrane-bound PSU (at ~40% sucrose). The RCs were purified as described by (Shochat et al., 1994).

5.3.2 MAS NMR Measurements

MAS NMR experiments were performed with a DMX-400 NMR spectrometer (Bruker, Karlsruhe, Germany) that was equipped with a double-resonance MAS probe operating at 396.5 MHz for \(^1\text{H}\) and 99.7 MHz for \(^{13}\text{C}\). The illumination setup has been described in detail in Chapter 1. A sample containing 30 mg wet weight of the PSU, which contains about 0.1 mg RC, was loaded into an optically transparent 7-mm sapphire rotor and \(^{13}\text{C}\) MAS NMR spectra were recorded at a temperature of 223 K with \(\omega_t/2\pi = 3.6\) kHz. Several minutes before the start of the experiment, 10 mM sodium ascorbate and 0.5 mM terbutyn were added to photo-reduce the acceptor site \(Q_A\) in situ. The sample was frozen in the dark under slow spinning (\(\omega_t/2\pi = 400\) Hz). During the course of the experiment, the sample was continuously illuminated with white light. Dark and photo-CIDNP spectra were measured by simple Bloch decay followed by a Hahn echo in order to delay the response. The FID was collected with TPPM proton decoupling (Bennett et al., 1995). A recycle delay of 12 s was used. Spectra of PSU were measured in 48 hours. The spectrum of solubilised RCs has been obtained in 30 min at a spinning frequency \(\omega_t/2\pi = 5\) kHz. For details about the experiment on solubilised RC, see (Schulten et al., 2002). In order to distinguish absorptive and emissive signals, a
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spectrum of solid \((u^{13}\text{C})\)-tyrosine\(\cdot\)HCl was recorded prior to the experiments on PSU. The phase correction for this reference was essentially conserved in the spectra of the PSU. The spectra were recorded in 2k data points with a sweep width of 50 kHz and an exponential line broadening of 70 Hz was used. All MAS NMR spectra were referenced to the \(^{13}\text{COOH}\) response of solid tyrosine\(\cdot\)HCl at 172.1 ppm.

5.4 Results

5.4.1 Apoprotein and lipids

The \(^{13}\text{C}\)-MAS NMR spectrum of the membrane-bound PSU in the dark (Fig. 5.2A) show the features of the apoprotein, which also occur in solubilised RC samples, as well as two signals from the lipid molecules at 57.5 and 17.7 ppm (Zysmilich and McDermott, 1996a; Matysik et al., 2000b; Matysik et al., 2001a). Detected after a Hahn echo, these signals appear to be out of phase, indicating the mobility of the lipid phase at 223 K. This assignment is backed by \(^1\text{H}\rightarrow^{13}\text{C}\) cross-polarisation experiments, in which the lipid signals appear with low relative intensity. The signals of the \(^{13}\text{C}\)-labelled cofactors are not observable under these experimental conditions.

5.4.2 The signs of the spectra

Upon illumination (Figure 5.2B), several weak absorptive (positive) features occur. Most prominent among the light-induced signals are the absorptive signals at 165 and 131 ppm. Their chemical shift anisotropy, which can be estimated from the side-band pattern at the low-frequency site, is in the range for aromatic carbons. Weaker signals occur at 155 and 150 ppm. No light-induced emissive (negative) signals are observed. Also in the aliphatic region, a light-induced absorptive signal occurs at about 50 ppm. Addition of detergent to the PSU sample changes the intensity pattern dramatically (Figure 5.2C). All signals appear to be emissive. In addition, several signals gain intensity and can be identified. Center bands appear at 168, 165, 160, 155, 150, 145, 139, 131 and 128 ppm in the aromatic region, and 56 and 50 ppm in the aliphatic region. These signals are in sign, intensity ratio, chemical shift and chemical shift anisotropy similar as those from detergent solubilised RCs (Figure 5.2D).

This spectrum has been reported recently by us, however, a careful re-investigation of the sign of this spectrum revealed that all signals appear to be emissive and not absorptive. This means that all signals in the photo-CIDNP spectrum of membrane-bound PSUs are absorptive (Figure 5.2B) whereas all signals from the detergent solubilised PSUs (Figure 5.2C) and isolated and detergent solubilised RCs are emissive (Figure 5.2D) (Schulten et al., 2002).
5.5 Discussion

5.5.1 Effects of spin diffusion

Light-induced signals in the aliphatic region have not been observed in continuous illumination experiments on samples of unlabelled RCs, but have been observed for the RCs with selectively labelled BChl and BPheo cofactors (Figure 5.2D) (Zysmilich and McDermott, 1996a; Matysik et al., 2000b; Matysik et al., 2001a). Also in spectrum 5.2B, a small light-induced signal can be observed at 50 ppm. The aliphatic chlorophyll carbons do not gain the photo-CIDNP from the primary mechanism (Matysik et al., 2001b).

Since the weak NMR signals of the apoprotein at about 15 and 30 ppm in Figure 5.2D appear emissive, the polarisation of these carbons of the protein pocket is most likely induced by spin diffusion from the cofactors of the photochemical machinery. This observation
demonstrates the possibility to explore the protein pocket of the photochemically active cofactors by photo-CIDNP. Spin diffusion complicates the correlation of measured photo-CIDNP intensities to local electron spin densities. On the other hand, steady-state photo-CIDNP intensities may allow for a rough assignment of signals to different cofactors. In the isolated selectively labelled RCs (Figure 5.2D), as shown by Schulten et al., the signal of the special pair carbons is about three-times stronger than that for the BPheo response (Schulten et al., 2002). All signals observed in Figure 5.2B have been assigned to special pair carbons. The absence of BPheo carbon signals is probably due to the generally lower intensity in this spectrum rather than due to a change in the intensity ratio between signals from the special pair and from the BPheo.

### 5.5.2 Comparison of membrane-bound and detergent-solubilised PSUs

In membrane-bound PSUs, five absorptive lines at 165, 155, 150, 131 and 50 ppm can be identified (Figure 5.2B). Upon addition of detergent to the sample, emissive lines at 168, 165, 160, 155, 150, 145, 139, 131, 128, 56 and 50 ppm occur (Figure 5.2C). Differences in the chemical shifts induced by the detergent cannot be detected unequivocally. The striking difference is the change of sign and increase for most of the signals. These findings suggest that the changes of the electronic structure and sample state induced by the detergent involve the radical-pair state and not the electronic ground state of the photochemically active region of the RCs. Rather, the strong spectral effect of the detergent can either be due to changes of the electronic structure of the RC by its embedding in the LH I antenna, or can be caused by the destruction of the membrane structure and its orientation. If the latter were true, it would suggest that the sample measured in spectrum 5.2B has been self-oriented by the sample spinning.

### 5.5.3 Comparison of detergent-solubilised PSUs and RCs

The comparison of detergent-solubilised PSU and purified RC samples may provide a route to probe the effect of the LH I on the RC. As indicated by the similarity between the spectra of PSU after addition of detergent (Figure 5.2C) and isolated and detergent-solubilised RCs (Figure 5.2D), the electronic structures of the photochemically active regions as well as the sample states of the RCs are similar. Most of the differences between the spectra 5.2C and 5.2D can be explained by (i) different spinning frequency, (ii) occurrence of lipid signals in spectrum 5.2C, and (iii) different concentration of the RCs. The signal at 139.1 ppm has been assigned to BPheo (Schulten et al., 2002). Its intensity relative to signals assigned to the special pair is similar in both spectra. This observation suggests that the efficiencies of the different mechanisms producing photo-CIDNP are similar in both samples. Figure 5.3 allows for comparison of both systems in more detail. The spectrum of detergent-solubilised PSUs (Figure 5.3A) is identical to spectrum 5.2C.
Figure 5.3. Expansion of the aromatic region of $^{13}$C Photo-CIDNP MAS NMR spectra of detergent-solubilised (BChl/BPhe)-labelled PSU (A) and purified (BChl/BPhe)-labelled RCs (B). Both spectra were recorded at 223 K with a spinning frequency of 3.6 kHz (A) and 5 kHz (B). The arrow points to the difference in the relative intensity of signal at 131.5 ppm in detergent-solubilized PSU and RCs.

The spectrum of purified RCs (Figure 5.3B) has been obtained with a MAS rotational frequency of 5 kHz, allowing an unequivocal separation of center and spinning side bands. The dotted lines in Figure 5.3 refer to the center bands resolved by band fitting in spectrum 3B. No change of a chemical shift is observed. Hence, the electronic ground states of PSU-bound and purified RCs are essentially the same. This again demonstrates the insensitivity of the electronic ground state to sample solubilisation. The only difference between the center bands of spectra 3A and B is related to the intensity of the signal at 131.5 ppm (Arrow in Figure 5.3). In spectrum 5.3B, this signal has an intensity double of most other signals assigned to special-pair carbons and has been assigned to the two carbons 13 localised on rings III (Jeschke, 1998). Spectrum 5.3A shows this signal with less relative intensity. The loss in intensity may be explained to some degree by the overlap of absorptive signals, as observed in spectrum 5.2B at this position, indicating some amount of membrane-bound sample. On the other hand, the signal at 164.9 ppm, for which also a strong emissive signal appears in spectrum 5.2B, is not so dramatically reduced in spectrum 5.3A. Probably, this
difference in intensity is related to a variation of the electron-spin density distribution. Since the two carbon atoms are localised at the ends of the special pair dimer, their involvement into the spin-diffusion driven polarization equilibration may be limited. The origin of the proposed difference in electron-spin density distribution in the radical-pair state may be related to the longer radical lifetime in membrane-bound RCs (Gibasiewicz et al., 1999).

5.5.4 Origin of the sign change

Photo-CIDNP $^{13}$C MAS NMR spectra of membrane-bound PSUs show a completely different intensity pattern compared to data collected from detergent-solubilised PSUs and RCs, while the differences between solubilised PSUs and RCs are minor. In principle, two explanations are possible: Either the remarkable differences between membrane-bound and detergent-solubilised PSUs are caused by orientation of intact PSU membranes, and demonstrate the anisotropy of photo-CIDNP, or they are induced by the mode of interaction of the antenna with the RC.

Since ground state electronic structure changes have been shown to be limited, explanation of the sign change by interaction of LH I antenna to the electronic structure of RCs in the radical-pair state is difficult. On the other hand, due to the anisotropy of g and hyperfine tensors as well as the dipole-dipole coupling between the two electron spins, theory predicts a strong anisotropy of the photo-CIDNP enhancement and strong effects on the photo-CIDNP intensity pattern upon orientation as discussed above. Therefore, we assign tentatively the differences between membrane-bound and detergent-solubilised PSUs (Figure 5.2B and C) to self-orientation of PSU membranes upon sample spinning and to the detergent induced solubilisation of RCs. Photo-CIDNP MAS experiments on purified and oriented bacterial RC samples are on the way in our laboratory and can provide a definite explanation of this phenomenon.

5.5.5 The effect of light intensity

The PSU sample contains about 0.1 mg of RCs. In experiments on detergent solubilised RCs, sample preparations of ~5 mg were used. At such high concentration, the question arises whether the photo-CIDNP intensities are limited by the number of photons penetrating into the highly absorbing sample. The effective signal-to-noise ratio is comparable while the sample concentration is different for the spectra shown in Figure 5.2C and D. This suggests that the strength of the photo-CIDNP effect in RCs is not limited by the light intensity.

In previous work, we estimated that under continuous illumination with white light about 50 photons are absorbed per RC and per second on average (Matysik et al., 2001a). In optically dense samples, the number of photons available inside the sample depends strongly on the distance from the surface. The penetration depth of light into absorbing and scattering material has been treated in 1931 theoretically by Kubelka and Munk in order to investigate...
the necessary thickness of coats of paint (Kubelka and Munk, 1931). Schrader and Bergmann extended in 1967 this concept in an attempt to optimise Raman scattering signals from crystal powders (Schrader and Bergmann, 1967). A quantitative theoretical treatment of photo-CIDNP in optically dense samples requires an analogous approach. One parameter, however, the build-up kinetics of photo-CIDNP, remains to be experimentally determined.

5.6 Conclusions

The occurrence of photo-CIDNP within membrane-bound PSUs opens up a new route to probe native photosynthetic proteins at atomic resolution and to compare their electronic structure to isolated and detergent-solubilised samples. For detergent-solubilised PSUs and RCs, the ground states are very similar, while there are clear indications for differences in the radical-pair states of both species. The pronounced variations of the photo-CIDNP intensity pattern between intact and partially solubilised PSUs can be explained by a loss of orientation of membrane-bound intact PSU in the rotating sample upon addition of detergent. This interpretation would imply that an anisotropy of the photo-CIDNP effect has been observed for the first time.