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Biosynthetic site-specific ^{13}C labeling of the light-harvesting 2 protein complex: A model for solid state NMR structure determination of transmembrane proteins

A.J. van Gammeren, F.B. Hulsbergen, J.G. Hollander & H.J.M. de Groot*
Leiden Institute of Chemistry, Gorlaeus Laboratoria, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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Abstract

Partly biosynthetic site-directed isotopically ^{13}C enriched photosynthetic light-harvesting 2(LH2) complexes have been prepared from *Rhodopseudomonas acidophila* strain 10050 by using chemically labeled [1,2,3,4-^{13}C], [1,4-^{13}C] and [2,3-^{13}C] succinic acid as a precursor in the growth medium. Two-dimensional proton driven spin diffusion (PDSD) solid state NMR correlation spectroscopy has been used to trace each individual ^{13}C isotope from the labeled succinic acid precursor to its destination into the protein and into the embedded major light-absorbing bacteriochlorophyll cofactors. For both the residues of the protein and for the cofactors distinct labeling patterns have been deduced, for protein complexes prepared from [1,4-^{13}C]-succinic acid or [2,3-^{13}C]-succinic labeled media. All residues, except isoleucine and leucine, have been labeled almost homogeneously by the succinic acid precursor. Carbonyl carbons in the protein backbone were labeled by [1,4-^{13}C]-succinic acid, while the Cα and Cβ carbons of the residues were labeled by [2,3 ^{13}C]-succinic acid. Leucine and isoleucine residues were labeled using a uniformly labeled amino acid mixture in the medium. The pattern labeling yields an increase of the resolution and less spectral crowding. The partial labeling technique in combination with conventional solid state NMR methods at ultra high magnetic fields provides an attractive route to resolve chemical shifts for α-helical transmembrane protein structures.

Abbreviations: LH2 – light-harvesting 2; MAS NMR – magic angle spinning NMR, PDSD – proton driven spin diffusion.

Introduction

In the past decade, Magic Angle Spinning (MAS) NMR has developed rapidly to resolve structures of microcrystalline protein samples at atomic resolution (Straus et al., 1998; Hong, 1999; Detken et al., 2001; Pauli et al., 2001; Castellani et al., 2002; Böckmann et al., 2003). At an early stage, the light-harvesting 2 (LH2) transmembrane protein complex from the anaerobic *Rhodopseudomonas acidophila* strain 10050 purple bacterium has been used to explore the range and resolution of the MAS NMR in the study of transmembrane proteins (Egorova-Zachernyuk et al., 2001). The LH2 complex, for which a detailed structure has been described elsewhere, is an intrinsic

*To whom correspondence should be addressed. E-mail: masnmr@chem.leidenuniv.nl

α: MNQGKIWTVVNPAIGIPALLGSVTVIAILVHLAIL SHT TWFPAYWQGGVKKAA

β: ATLTAEQSEELHKYVIDGTRVFLGLALVAHFLAFS ATPWLH

Figure 1. The primary structure for the α- and β-protein subunits of the LH2 complex from *R. acidophila* 10050. Residues in a α-helical structure are underlined.

transmembrane protein complex, built from nine identical units containing three bacteriochlorophyll cofactors each (McDermott et al., 1995; Prince et al., 1997; Papiz et al., 2003). The primary structure of one unit of the LH2 complex is represented in Figure 1. Here the α-helical part is underlined.

MAS NMR applied to biomolecules requires suitable [^{13}C, ^{15}N]-labeled samples of high structural homogeneity for optimal resolution, to resolve the large number of cross signals from the backbone nuclei of the protein in dipolar correlation spectra. Transmembrane proteins generally contain α-helices and there is little dispersion for the ^{13}C and ^{15}N resonances of backbone atoms in the α-helical structure, which makes the sequence specific chemical shift assignment difficult. A reduction of labeled carbon positions is a prerequisite to resolve the correlation responses in 2D spectra.

Our study aims to produce pattern labeled LH2 samples in an attempt to alleviate the problem of spectral crowding for chemical shift assignments and to reduce the broadening due to J-couplings between neighboring carbons for an increased spectral resolution. The use of site-directed reduced biosynthetic labeling in MAS NMR has been demonstrated before with *E. coli* and has appeared to be useful for the structure determination of the crystalline α-spectrin SH3 domain (LeMaster and Kushlan, 1996; Castellani et al., 2002). The proteins of this system were pattern labeled by using specifically labeled [1,3-^{13}C] or [2-^{13}C] glycerol as a carbon source (LeMaster and Kushlan, 1996). The anaerobic *R. acidophila* purple bacterium does not adapt to the medium with glycerol as a single carbon source and a different experimental labeling method is needed. For biosynthetic incorporation of the ^{13}C labels into the LH2 complex, chemically synthesized site-specific labeled [1,4-^{13}C] and [2,3-^{13}C]-succinic acid have been used, yielding the 1,4-LH2 and 2,3-LH2 sample, respectively. Due to the symmetry of the succinic acid precursor, using a singly labeled or [1,3-^{13}C]-succinic acid yields a 50% dilution of the labeled positions in the biomolecules. The labeling patterns of the 1,4-LH2 and 2,3-LH2 and the embedded BChl *a* cofactors have been analyzed using 2D PDSD dipolar correlation NMR spectra.

Materials and methods

Sample preparations

All [^{13}C, ^{15}N] isotopically enriched LH2 complexes were obtained by growing the photosynthetic purple bacteria *R. acidophila* strain 10050 anaerobically in light at 30 °C on a well-defined medium (Egorova-Zachernyuk et al., 2001). For an optimal cell growth, *R. acidophila* strain 10050 requires an elaborate medium containing two essential nutrient sources, consisting of succinic acid and a mixture of amino acids (Cambridge Isotopes Laboratories, Andover, MA, U.S.A). ^{13}C-labeled succinic acid was prepared by a multi-step synthesis, starting from ^{13}C-labeled acetic acid (Heinen, 1996). In the final step, fumaric acid was converted into succinic acid using a reduction procedure with H_2 and Pd-C as catalyst.

The label incorporation from the two different nutrient sources was investigated by labeling of one or both carbon nutrient sources. Three labeled LH2 samples were prepared: the uniformly ^{13}C-labeled LH2 sample (U-LH2), the 1,2,3,4-LH2 sample that was prepared from a uniformly ^{13}C labeled [1,2,3,4-^{13}C]-succinic acid and the AA-LH2 sample that was prepared from a [^{13}C,^{15}N]-labeled amino acid mixture. For uniformly labeled samples the metabolic pathway is irrelevant. The concentrations of the [^{13}C, ^{15}N]-isotope labeled amino acid mixture and [1,2,3,4-^{13}C]-succinic acid in the medium were optimized to 1.5 g of amino acid mixture and 2.0 g succinic acid per liter to minimize the label cost.

In contrast, for the preparation of specific biosynthetic labeled samples, the selection of one specific pathway to minimize the dilution or scrambling of the label pattern in the protein by synthesis of the same intermediates by different competitive non-equivalent metabolic pathways is

required. For the preparation of the 1,2,3,4-LH2 sample, the concentration of the unlabeled amino acid mixture was reduced to 1.0 g/l to enhance the biosynthesis from the labeled succinic acid source and to reduce the incorporation of unlabeled amino acids. For the AA-LH2 sample, both the concentrations of the unlabeled succinic acid and the labeled amino acid mixture were adjusted to 1.5 g/l to enhance the label incorporation from the amino acid source. An attempt to prepare an AA-LH2 sample from the hydrolysate of waste proteins from the *E. coli* BL21 that was used for the expression of the SH3 protein was not successful (Castellani et al., 2002). The uptake of residues from this hydrolysate was insufficient to obtain an extensive labeled AA-LH2 sample.

Proton driven spin diffusion (PDSD) spectra were recorded from the three test samples to gain insight into how nutrient sources are involved in the biosynthesis of residues in the LH2 complex. It was found that succinic acid is required for the synthesis of most residues. Subsequently, the pattern labeled 1,4-LH2 and 2,3-LH2 samples were prepared from a medium containing 2.0 g/l [1,4-^{13}C]-succinic acid and [2,3-^{13}C]-succinic acid, respectively, to introduce a labeling pattern. The amount of unlabeled amino acid mixture in the media was reduced to 1.0 g/l to enhance the biosynthesis from the labeled succinic acid source and to reduce the incorporation of unlabeled amino acids. All media, including the three test samples, contained also $^{15}NH_4OH$. The concentration was twice the molar succinic acid concentration for labeling all ^{15}N atoms in the protein complex.

The cell growth was followed with UV spectroscopy by measuring the optical density at $\lambda = 860$ (OD_{860}). A full-grown culture will enable alternative biosynthetic pathways to maintain its steady state, which may lead to dilution or scrambling of the labels. To prevent deterioration of the labeling pattern, cells obtained from media containing site-specific labeled succinic acid were harvested at $OD_{865} = 3.6\ cm^{-1}$, which is just before the cell culture reaches the steady state ($OD_{865} \geq 3.8$). The LH2 complex isolation was performed according to Hawthornthwaite-Lawless and Cogdell (Hawthornthwaite-Lawless and Cogdell, 1991). About 30 mg of LH2-protein was isolated, starting from 0.8 l medium.

MAS NMR experiments

1D and 2D ^{13}C-^{13}C homonuclear correlation spectra of labeled LH2 samples were recorded with a Bruker AV-750 spectrometer equipped with a double channel CP-MAS probe head and with a ^{13}C radio frequency of 188 MHz. The proton $\pi/2$ pulse was set to 3.1 μs, corresponding with a nutation frequency of 80.6 kHz. ^{13}C B_1 field strengths of 50 kHz corresponding with a cross polarization time of 2.0 ms were used during a 100–50% ramped CP sequence (Metz et al., 1994). In the PDSD experiment, two-pulse phase modulation (TPPM) decoupling was applied during the t_1 and t_2 periods (Bennett et al., 1998). A mixing time of 50 ms was used to transfer the magnetization into the side chains. All samples were cooled to 253 K, and the MAS spin frequency $\omega_R/2\pi$ was 8.5 kHz. The ^{13}CO resonance of U-[^{13}C,^{15}N]-Tyrosine·HCl at 172.1 ppm was used as an external reference for the determination of the isotropic ^{13}C chemical shifts.

Results and discussion

Five 1D ^{13}C PDSD spectra were recorded from U-LH2, 1,2,3,4-LH2, AA-LH2, 1,4-LH2 and 2,3-LH2 complexes (Figures 2a–e). Resonances between 0 and 75 ppm are mainly from the labeled Cα, Cβ, Cγ and Cδ carbons in the amino acids. A minor fraction is from aliphatic carbons of the BChl *a* cofactors. Between 100 and 170 ppm the signals from aromatic amino acids and the bacteriochlorin rings of the BChl *a* cofactors are detected. The carbonyl responses from the protein backbone and some carbonyl responses from the BChl *a* cofactors are observed between 170 and 190 ppm.

The U-LH2, 1,2,3,4-LH2 and AA-LH2 complexes in Figures 2a–c resonate in all three spectral regions. This shows that the succinic acid is essential for labeling the protein. The spectrum of the AA-LH2 in Figure 2c shows signals in the carbonyl area and in the aliphatic area, which indicates that a fraction of the labeled amino acids can be incorporated from the labeled amino acid mixture nutrient source. Resonances of the 1,4-LH2 and the 2,3-LH2 complexes are represented in green and red in Figures 2d and e, respectively. In the 1,4-LH2 spectrum in

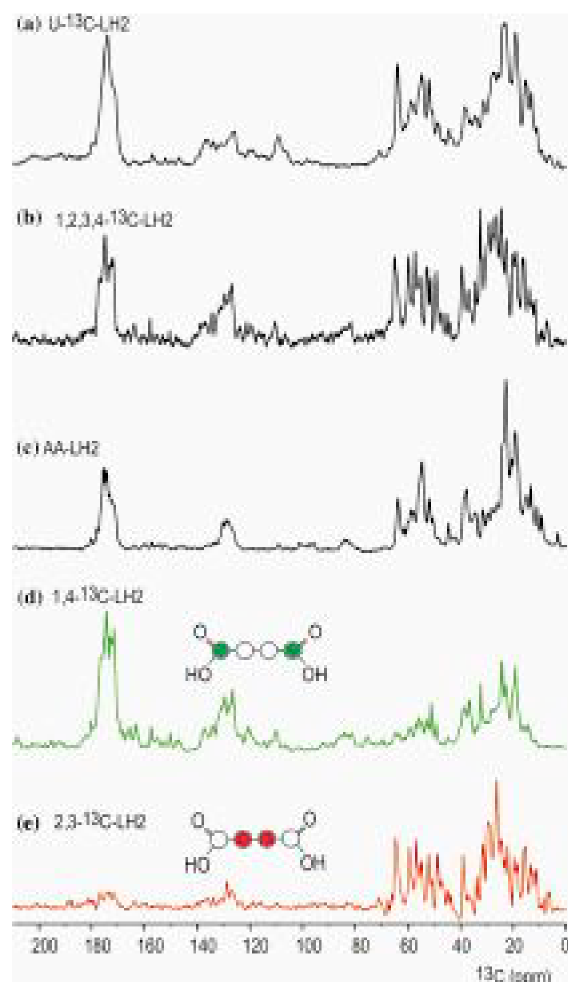

Figure 2. 1D PDSD spectra of U-LH2 (a), 1,2,3,4-LH2 (b), AA-LH2, 1,4-LH2 (d) and 2,3-LH2 (e) recorded with 32 scans. The green and red colors refer to the labeling by using [1,4-^{13}C]-succinic acid and [2,3-^{13}C]-succinic acid, respectively.

Figure 2d, intense resonances are observed in the carbonyl and aromatic regions, while relatively weak responses are observed in these regions for the 2,3-LH2 sample in Figure 2e. In contrast, many responses are observed in the aliphatic part of the 2,3-LH2 dataset, while for the 1,4-LH2 a less crowded spectrum is observed in this area.

The 2D PDSD correlation spectra reveal the labeled pairs of nuclei that are in the molecular structure of each sample. From this information it was deduced which residues are synthesized from [1,2,3,4-^{13}C]-succinic acid and which residues are taken up from the amino acid mixture, and how the ^{13}C labels of [1,2,3,4-^{13}C]-succinic acid are incorporated in the BChl *a* cofactors. The aliphatic part of the 2D PDSD spectrum between 0 and 75 ppm shows the cross signals between carbons of the amino acid side chains. A complete and specific set of correlations for almost every residue except Ile and Leu is detected. Ile and Leu residues comprise ~20% of the protein sequence and are responsible for a significant reduction of the correlations in the 2D spectra of the samples prepared by succinic acid. In the protein there is one carboxyl-methionine at the N-terminus of the α-subunit, which can not be identified in the spectrum. The data show that almost all amino acids, except Ile and Leu, are synthesized from the succinic acid carbon source. This is corroborated by a 2D spectrum of the AA-LH2 sample, which shows the correlation signals for Ile and Leu. The same spectrum also shows resonance sets for Val, Gly and Ala, which can be taken up from the amino acid mixture or synthesized from the succinic acid source. By using separately both the AA-LH2 and 2,3-LH2 samples, it is possible to observe all type of amino acids, which will be of use for an assignment.

The aliphatic parts of the 2D PDSD spectra of 1,4-LH2 and 2,3-LH2 in Figure 3 show a reduced number of cross signals compared to data collected from the 1,2,3,4-LH2. From the 1D PDSD spectra in Figure 2D and E, it was inferred that most of the labels from [1,4-^{13}C] succinic acid are introduced at the carbonyl positions, while the labels from [2,3-^{13}C] succinic acid are mainly introduced at the Cα, Cβ, Cγ and Cδ positions. This is confirmed by the aliphatic correlation area in the 2D PDSD spectra of the 2,3-LH2 and 1,4-LH2 samples, represented by the red and green color in Figure 3, respectively. The Cα–Cβ correlation area in spectrum of the 2,3-LH2 complex is more crowded than for the 1,4-LH2 complex. This demonstrates that the [2,3-^{13}C]-succinic acid labels are mainly introduced into the aliphatic Cα, Cβ, Cγ and Cδ positions.

The reverse is observed for the CO–Cα–Cβ correlation area in the spectra of the 1,4-LH2 and 2,3-LH2 in Figure 4, where the carbonyl response area for the 1,4-LH2 is more crowded than for the 2,3-LH2 sample. This confirms that the [1,4-^{13}C]-succinic acid labels are mainly incorporated in the carbonyls. The relatively weak cross resonances of the 1,4-LH2 complex indicated in green in the CO–Cα–Cβ correlation area

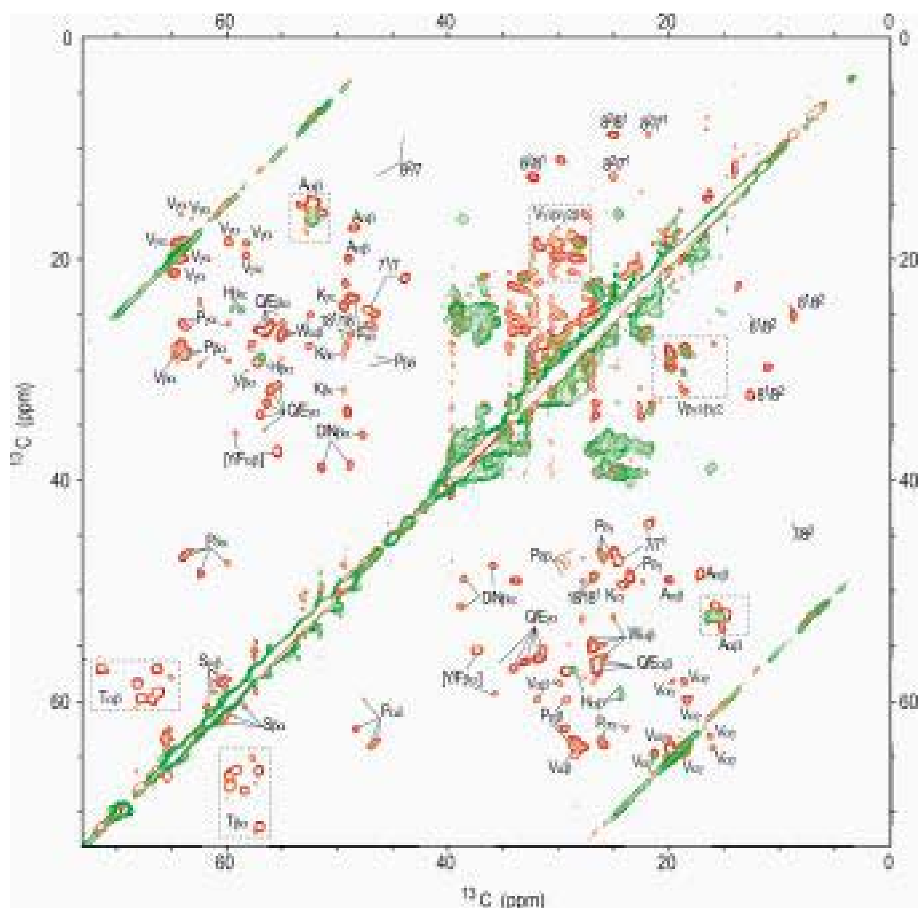

Figure 3. Superposition of the aliphatic parts of the PDSD 2D spectra collected from the 1,4-LH2 (green) and the 2,3-LH2 sample (red). Correlation signals are indicated by their single letter code for the corresponding amino acid residue. The resonances of the BChl *a* chromophore are indicated with a number corresponding to the IUPAC numbering of BChl *a* as represented in Figure 6.

between 170 and 180 ppm in Figure 4, represented the His, Thr, Ser, Val, Ala and Pro residues in which Cα carbons are partly enriched by label scrambling. The Cα carbons that correlate with the carbonyls are labeled from [1,4-^{13}C]-succinic acid. The Cα–CO responses are weak, indicating fractional labeling to multiple metabolic pathways. The carbonyls of the histidines are anomalous in the sense that the major fraction is labeled from [2,3-^{13}C]-succinic acid. This can be concluded from the relatively strong CO–Cα–Cβ responses in the spectrum of the 2,3-LH2 complex. Weaker CO–Cα cross correlation signals are detected with the same shifts in the spectrum of the 1,4-LH2. This indicates that a fraction of the CO and Cα carbons of the histidines can be enriched from [1,4-^{13}C]-succinic acid. The Cα–Cβ correlation signals of the histidine residues are only observed in the spectrum of the 1,4-LH2. The few remaining cross correlations in the spectrum of the 2,3-LH2 in Figure 4 are attributed to the labeled CO, the Cα, the Cβ and the Cγ carbons of the Gln and Glu residues.

Finally, by comparing the PDSD spectra of the 2,3-LH2 and 1,4-LH2 with the corresponding spectra of the U-LH2, the AA-LH2 and the 1,2,3,4-LH2, the isotope incorporation for each amino acid can be determined. The label incorporation is summarized in Figure 5, which represents the label transfer from succinic acid to the ^{13}C positions for each amino acid type by red and green circles. The small sections in the circles indicate minor fractions of ^{13}C-label rearrangement due to the biosynthetic pathways that are

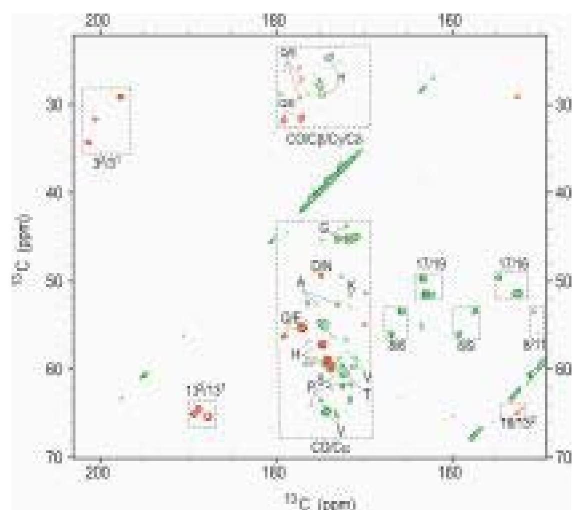

Figure 4. The superposition of the CO–Cα–Cβ–(Cγ) correlation areas in the data obtained from 1,4-LH2 (green) and 2,3-LH2 (red). Signals from the BChl *a* chromophores are numbered corresponding to the IUPAC carbon numbering of BChl *a* as represented in Figure 6.

different from the main pathway. As indicated by the relative site-specific biosynthetic incorporation in the Figure the succinic acid digestion follows mainly a single metabolic pathway. Evidently, the succinic acid concentration of 2.0 g/l medium to prepare the 1,2,3,4-LH2, the 1,4-LH2 and the 2,3-LH2 sample has appeared to be sufficiently high to suppress the alternative metabolic pathways.

It has been shown for a pattern labeled β-sheet protein that the decreased number of correlation signals in the pattern labeled sample in combination with MAS NMR techniques offers a simplified route to a sequence specific assignment of these proteins by MAS NMR (Castellani et al., 2002). The pattern labeling in the 1,4-LH2 and 2,3-LH2 complexes is also promising for a sequence specific assignment of the transmembrane LH2 complex.

BChl a cofactor isotope labeling.

From Figure 2D and E, it transpires that most aromatic carbons of the BChl *a* cofactors are labeled from the [1,4-^{13}C]-succinic acid. This is confirmed by the aromatic cross correlations in the 2D spectra that are colored green in Figure 4. The numbering and color coding for the BChl *a*

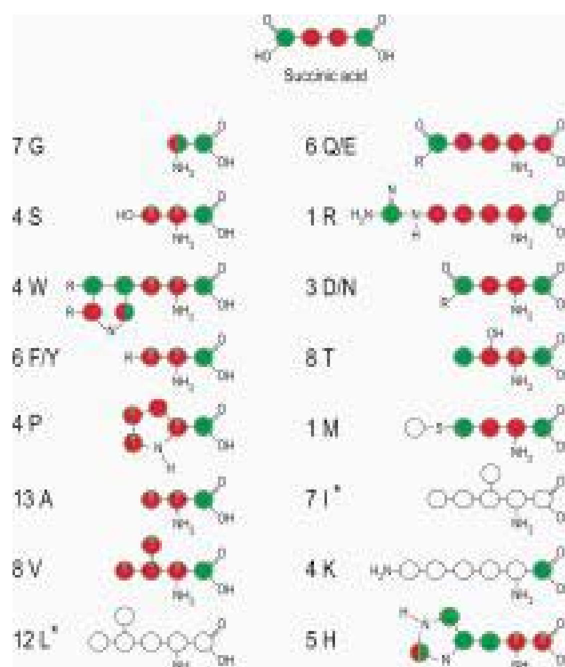

Figure 5. Schematic representation of the effective ^{13}C-isotope enrichment of the residues, obtained by protein expression in *R. acidophila* strain 10050. The green color corresponds to the ^{13}C labeling pattern that is obtained by growing on [1,4-^{13}C]-succinic acid, while the labeling pattern obtained by growing on [2,3-^{13}C]-succinic acid is represented in red. The small section indicates a small fraction of label scrambling. Ile and Leu are labeled due to the uptake from the amino acid nutrient source.

follows the scheme of Figure 6. Strong C2-C2^1 and C12-C12^1 are detected in the dataset collected from the 2,3-LH2 outside the response areas represented in Figures 3 and 4. The carbon positions in Figure 6 that are not colored are introduced by glycine in the biosynthesis of BChl *a*. They are slightly enriched by a small fraction of glycine synthesized from the labeled succinic acid, which is in competition with the main unlabeled glycine pool from the amino acid mixture. Correlations for C9 and C16 are weakly observed in the spectra.

The labeling pattern of the BChl *a* cofactors in the 1,4-LH2 and 2,3-LH2 complexes as represented in Figure 6 is well in line with the biosynthetic condensation pathway of succinyl-CoA and glycine to δ-aminoleuvalinic (δ-ALA) acid and from δ-ALA to BChl *a* (Schulten et al., 2002). A retro-synthesis from BChl *a* to succinic acid can reduce the labeled carbons to either the

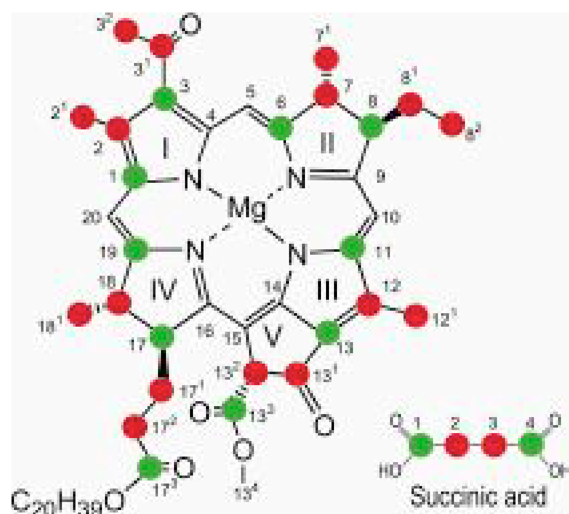

Figure 6. Schematic representation of the ^{13}C-isotope enrichment in the BChl *a* cofactors. The green and red colors indicate the labeling patterns obtained when [1,4-^{13}C] and [2,3-^{13}C]-succinic acid are used.

[1,4-^{13}C]-succinic acid or the [2,3-^{13}C]-succinic acid compound.

Conclusions

The 2D PDSD spectra provide pronounced evidence for the preparation of biosynthetic site-specific pattern labeled LH2-samples. A relatively homogeneous label incorporation from the [1,4-^{13}C] and the [2,3-^{13}C] succinic acid precursor into the protein and BChl *a* cofactor of the LH2 complexes is demonstrated. Carbonyl carbons from the membrane protein are mainly introduced from [1,4-^{13}C]-succinic acid and the $C\alpha$ and $C\beta$ are mainly labeled from [2,3-^{13}C]-succinic acid with only a small fraction of label scrambling. In both the 1,4-LH2 and the 2,3-LH2 complexes the J-couplings are reduced, because in general the labeled $C\alpha$ carbons in the 2,3-LH2 are adjacent to an unlabeled CO carbon and the labeled CO carbons in the 1,4-LH2 are adjacent to an unlabeled $C\alpha$ carbon. The line width in the 2D spectra is $\sim$1 ppm, which is larger than the effect of the J-coupling. The broadening can be attributed to moderate disorder in the LH2 sample. The labeled $C\beta$ carbons only have an adjacent labeled $C\gamma$ carbon in Pro, Val, Glu, Gln and Lys. Approximately each labeled $C\alpha$ has a labeled adjacent $C\beta$ which still form a pair of carbons with strongly coupled nuclear spins. The reduced labeling of the LH2 membrane protein conveniently reduces the spectral crowding and increases the resolution. This facilitates the identification of individual residues in comparison to uniformly labeled proteins. The specifically labeled 2,3-LH2 and 1,4-LH2 complexes provide a strong basis for a sequence specific assignment of the LH2 complex in a next step. These experiments are in progress.

Acknowledgements

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