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Photosynthesis Research **55:** 241–245, 1998.

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Received 11 September 1997; accepted in revised form 8 December 1997

Key words: ^{13}C MAS NMR, EPR, FTIR, spheroidene, stable isotope labelling, ubiquinone

Abstract

In this minireview the information at the atomic level that has been obtained by studying photosynthetic reaction centers with site-directed isotope labelling is discussed. The required isotopically labelled RCs are prepared using isotopically labelled amino acids and cofactors that have been prepared via organic total synthetic chemistry. In some cases the results of isotopically labelled RCs that are prepared via other methods are also discussed.

Introduction

Understanding photosynthesis at the atomic level is a challenge in structural and functional biology today. The primary event of photosynthesis is a light-induced trans membrane redox process, occurring in a pigment protein complex termed the reaction center (RC). The ground state structures of RCs of two purple bacteria *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* have been resolved by X-ray crystallography with nearly atomic resolution (Deisenhofer et al. 1984; Allen et al. 1988).

Even though this represents a fundamental understanding of the function of RCs, to achieve a complete picture their dynamic behaviour has to be determined at the atomic level by time resolved methods. Upon light excitation an electron is transferred from the primary donor P (bacteriochlorophyll *a* dimer) at the periplasmic side, via monomeric bacteriochlorophyll *a* and a bacteriopheophytin *a* molecule to the primary quinone Q_A, and finally to the secondary quinone Q_B at the cytoplasmic side of the membranes.

Although Q_A and Q_B are both ubiquinone-10 molecules in the RC of *Rhodobacter sphaeroides*, their functions are different. Q_A is a one electron gate only, whereas Q_B accepts two electrons and two protons and is finally released from the RC as QH_2.

These structural and functional differences of the cofactor ubiquinone-10 in the Q_A *versus* the Q_B side are the result of as yet unknown differences in the interaction of the quinone with the protein in the respective binding sites. It dramatically shows that detailed information on the structure and electronics of the cofactors and protein part is needed. The strategy of choice to obtain this information is site-directed isotope labelling of the functionally intact active protein, studied with non-invasive isotope sensitive techniques.

With other groups we have initiated collaborative research based on these strategies. It involves:

1. The total organic syntheses of amino acids and cofactors highly isotope enriched with stable isotopes in any position and combinations of positions starting from simple highly enriched commercially available starting materials.
2. Preparation of RCs with isotope label in the peptide chains by growing the photosynthetic bacteria on media containing the isotopically labelled amino acids in question and subsequent isolation in the RCs. In order to prevent isotope dillution the condition should be such that *de novo* biosynthesis is completely prevented. For the incorporation of cofactors the depleted protein is reconstituted with the isotopically labelled cofactor.
3. Study of the isotopically labelled RC by isotope sensitive techniques such as high resolution MAS solid state ^{13}C NMR spectroscopy, ESR spectroscopy, FTIR difference spectroscopy and laser Resonance Raman spectroscopy.

The great advantage of this strategy is that resolution at the atomic level is achieved with essentially intact and selective functionally active proteins. No

change in sterical and electronic properties are introduced by isotopic labelling; the natural RC also contains heavy isotopes at each position at the natural abundance level (e.g. 1.1% for ^{13}C).

Synthesis

Only few of the required isotopically labelled amino acids are commercially available. None of the required ubiquinone-10 and other cofactors are commercially available. We therefore started a synthetic programme to prepare these site-directed isotopically labelled molecules, with 99% incorporation at the required position, using commercially available highly enriched starting materials. In the meantime the synthesis of resp. L tryptophan (Berg et al. 1990), L tyrosin (Winkel et al. 1989), L lysine (Raap et al. 1995), L glutamic acid (Cappon et al. 1991), L proline (Cappon et al. 1992), L histidine (Cappon et al. 1994), L glutamine (Ogrel et al. 1994), L serine (Karstens et al. 1995), L threonine (Karstens et al. 1995) have been published.

The synthesis of ubiquinone-10 isotopomers with a ^{13}C label on resp. 1,2,3,4 and $3CH_3$ have been reported (Liemt van et al. 1994). Similarly, the synthesis of ubiquinone-3 labelled with ^{13}C at the 5 and 6 positions in the quinone nucleus has been published (Boullais et al. 1994). The synthesis of isotopically labelled spheroidenes have been published (Gebhard et al. 1991a, 1991b; Jansen et al. 1996).

In the meantime the efficient incorporation of isotopically labelled tyrosins, tryptophans and histidines in the reaction centers of *Rhodobacter sphaeroides*, R26, have been reported (Raap et al. 1990; Carnecki et al. 1997). Also the bacterio chlorophyll cofactors in the reaction centers have been labelled with ^{26}Mg by growing the bacteria on media containing ^{26}Mg (Carnecki et al. 1997).

Results

Tyrosine residues

The reaction centers of *Rhodobacter sphaeroides* R26 selectively enriched with [4′–^{13}C] tyrosine and the *Rhodobacter sphaeroides* 2.4.1 (M)Y210W mutant selectively enriched with [4′–^{13}C] tyrosin have been investigated with ^{13}C MAS NMR spectroscopy (Fischer et al. 1992; Shochat et al. 1995). The R26 RCs comprise a total of 28 tyrosines distributed over the complex. (M)Y210 is strategically located in the active branch near the special pair, bacteriochlorophyll and bacteriopheophytin. The signal of [4′–^{13}C] tyrosine R26 consists of at least seven narrow lines superimposed on a broad doublet. The two narrowest resonances corresponding to signals from individual tyrosins are 28 ± 5 Hz wide, compatable to what is observed for quaternary carbons in linearly elastic organic solids. The spectrum of the 2.4.1. (M)Y210 M mutant is very similar to the spectrum of the R26 system except that the most upfield narrow signal (at 152.2 ppm, in the region where non-hydrogen bonded tyrosins are expected), is lacking. This allows the assigment of the M210 tyrosin signal. The spectra also show that during the expression of the protein no back mutation has taken place.

The line width as well as the chemical shift of these signals is essentially independent of temperature. This provides strong evidence for an unusually ordered, well-shielded and structurally, electrostatically and thermodynamically stable interior of the protein complexes without structural heterogeneties in the time and length scales of the NMR. Also, the influences of the (M) Y210 M mutation on the global electrostatic properties and structures of the protein, as probed by the tyrosine labels, is minimal. When the R26 sample is frozen while subject to intense illumination, a substantial part of the protein is brought into the charge separated state $P^{\bullet+}Q_A^{\bullet-}$. At least three sharp resonances, including the narrowed lines at 152.2 ppm, are substantially reduced in intensity, resulting from the effect caused by the electronic spin density associated with the oxidized primary donor $P^{\bullet+}$. This suggests that part of the environment of the special pair probed by the tyrosine is extremely rigid and questions the role of protein conformational distortions on the time scale of the primary photoprocess.

The results of the mutant argues against an explanation of slow and non-experimental transfer kinetics in the (M)Y210W RC in terms of loss of structural integrity upon mutation. The NMR results strongly support current opinions that (M)Y210 contributes to the fine tuning of the energy levels of the prosthetic group involved in electron transfer.

Histidine

The central magnesium ion of each of the four BChl cofactors in the RCs is ligated to a histidine residue, which serves as the only covalent link to the protein.

Systematic spectroscopic studies aimed at elucidation of the characteristics and functional importance of the magnesium histidine linkage in RC have recently started. Via a laser Resonance Raman study on RC with isotopically labelled histidine, Charnecki et al. have recently identified the magnesium histidine stretching vibrations of the bacterio chlorophyll cofactors (Charnecki et al. 1997). Their implications for the photoexcitation dynamics in the RC have been discussed.

Photochemically induced nucleic spin polarization signals have been observed that were attributed to δ and ϵ nitrogens of histidine; presumably the ligand of P865 (Zysmilich et al. 1996).

Ubiquinone

The ^{13}C MAS NMR spectra of *Rhodobacter sphaeroides* R26 RC reconstituted with ubiquinone ^{13}C enriched on positions 1,2,3,4 and 3Me in the Q_A site have been obtained (Liemt van et al. 1995). They show a slight heterogenity reflected in the line width between 180 and 300 Hz. Electrostatic charge differences in Q_A induced by polarization from the protein are less than 0.02 electronic equivalent for any of the labelled positions, this includes the 4 ^{13}C carbonyl position. For the 4 ^{13}C carbonyl position a response can be observed at 183 ppm, but only with sample temperature below ~255 K. Although the asymmetry of the anisotropy of the 4 ^{13}C signal from Q_A is only moderately different from the anistropy of this position in crystallin ubiquinone-10, it is concluded that this result is compatible with a decrease of the 4 C–0 bond order upon binding to the protein. The temperature dependent asymmetry between the signal strengths of the two carbonyls in Q_A indicates that the putative strong interaction with the protein at position 4 involves dynamic character, which may be of importance to the specific Q_A redox chemistry (Groot et al. 1995).

These isotopically labelled systems have been investigated in the $Q_A^{\bullet -}$ state with 35 GHz EPR spectroscopy. The direct observation of the ^{13}C hyperfine splitting of the g_z component of UQ $10_A^{\bullet -}$ in the RC shows that the electronic spin distribution is asymmetric in the RC. The carbonyl oxygen of the semiquinone anion nearest to the S = 2 Fe^{27} and Q_B is shown to acquire the highest (negative) charge density in the reduced state (Brink et al. 1994).

Reaction centers reconstituted in the Q_A site with resp. ^{13}C labelled ubiquinone-10 and ^{13}C labelled ubiquinone 3 have been studied with FTIR difference spectroscopy in the dark adapted state versus the $Q_A^{\bullet -}$ state (Brudler et al. 1994; Breton et al. 1994). The mode dominated by the 4 C–0 stretch vibration is drastically down-shifted in the Q_A site compared to its frequency in free ubiquinone-10. In contrast the mode dominated by the 1 C=0 contribution absorbs at similar frequencies in the free and the bound forms.

In the charge-separated state the mode dominated by the semiquinone 4 C⎓0 vibration is characteristic of a strong hydrogen binding to the micro environment, whereas the mode dominated by the 1 C⎓0 vibration indicates a weaker interaction.

Similar to the reaction centers of *Rhodobacter sphaeroides*, R26, *Rhodopseudomonas viridis*, reconstituted with ^{13}C labelled ubiquinone in the Q_B site, have been studied with FTIR difference spectroscopy (Brudler et al. 1995; Breton et al. 1995, 1996) showing a symmetric environment to the cofactor in this site in contrast to the Q_A site. Comparison between the quinone in the Q_A of *Rb. sphaeroides* versus the *Rp. virides* shows an almost identical interaction in both cases. Indications have been obtained that in *Rb sphaeroides* the ubiquinone-10 in the Q_B site is bound in two different fractions in the ratio of 1:3.

Spheroidene

In the RC of *Rhodobacter sphaeroides* 2.4.1. spheroidene is present. Probably the most important role of the spheroidene is to act as a protective device against irreversible photodestruction of the RC. The spheroidene is the only cofactor that does not adhere to the approximate twofold rotation symmetry of the RCs. It is located near the monomeric accesory bacteriochlorophyll in the photosynthetically inactive branch. In order to resolve structural ambiquities of the bound spheroidene, ^{13}C MAS NMR of *Rhodobacter sphaeroides* R26 reaction centers reconstituted with 14,14′ and 15,15′ spheroidene were carried out, showing chemical shift values in agreement with a bound 15,15′Z structure (Groot et al. 1992).

Resonance Raman spectroscopy on RCs reconstituted with specific ^{2}H labelling in the central part of the spheroidene revealed evidence for out of plane distortions of the bound 15,15′Z spheroidene in the central C_{14} to $C_{14'}$ region of the system (Kok et al. 1997).

Pheophytin

RC of *Rhodobacter sphaeroides* R26 were reconstituted with uniformly ^{13}C labelled (plant) Pheo *a* in the two pheophytin binding sites. New solid state ^{13}C NMR techniques have been applied to extract the chemical shift values of each of the ^{13}C atoms and their modifications due to binding in the protein (Egorova-Zachernyuk et al. 1997). No evidence for electrostatic differences between the two Pheo *a*s were found. The first results indicate that the symmetry breaking between the A and B branch is not due to differences in electronic ground states of the pheophytin cofactors, and a thorough investigation to put these conclusions on a firm ground is currently being performed.

Photochemically induced nuclear spin polarization on ^{15}N enriched RCs have been observed that can be attributed to the bacteriopheophytin acceptor tetrapyrrole nitrogens in addition to the signals of the tetrapyrrole nitrogens of the special pair (Zysmilich et al. 1996).

Conclusion

The precise information at the atomic level described by investigation of photosynthetic reaction centers specifically labelled with stable isotopes with non-invasive isotope sensitive techniques has been obtained in the last five years.

New developments in isotope sensitive spectroscopic techniques, such as the recent method to determine the value of torsional angles and precise distance measurements in membrane proteins by solid state NMR (Feng et al. 1997; Verdegem et al. 1997), coupled with development in the synthetic and genetic area indicate that in the near future a wealth of new essential information about the structure and function in photosyntheses will become explored via site directed isotopically labelled systems.

Acknowledgment

Thanks are due to all the persons who contributed to the success of the study of isotopically labelled reaction centers. Their names are listed in the references. The work was sponsored by the Netherlands Foundation for Chemical Research (SON) with financial support from the Netherlands Organization for Scientific Research (NWO). The financial support by the European Commission Biotechnology grant PL92-0417 and by the HFSPO, grant R6469/94M is gratefully acknowledged.

References

Allen JP, Feher G, Yeates TO, Komiya H and Rees DC (1988) Structure of the Reaction Center from *Rhodobacter sphaeroides* R26. The cofactors. Proc Natl Acad Sci USA 84: 5730–5734

Berg EMM van den, Jansen FJHM, de Goede ATWJ, Baldew AU and Lugtenburg J (1990)Chemo-enzymatic synthesis and characterization of L-tryptophans selectively ^{13}C-enriched or hydroxylated in the six-membered ring using transformed *Escherichia coli* cells. Recl Trav Chim Pays-Bas 109: 287–297

Boullais C, Breton J, Nabedryk E and Mioskowski C (1994) Synthesis of ubiquinones-3 specifically labelled with ^{13}C at C (5)- and C (6)-positions. Tetrahedron 53: 2505–2515

Breton J, Boullais C, Burie JR, Nabedryk E and Mioskowski C (1994) Binding sites of quinones in photosynthetic bacterial reaction centers investigated by light-induced FTIR difference spectroscopy: Assigment of the interactions of each carbonyl of Q_A in *Rhodobacter sphaeroides* using site-specific ^{13}C labelled ubiquinone, Biochemistry 33: 14378–14386

Breton J, Boullais C, Berger G, Mioskowski C and Nabedryk E (1995) Binding sites of quinones in photosynthetic bacterial reaction centers investigated by light-induced FTIR difference spectroscopy: Symmetry of the carbonyl interactions and close equivalence of the Q_B vibrations in *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* probed by isotope labelling. Biochemistry 34: 11606–11616

Brink JS van, Spoyalov AP, Gast P, Liemt WBS van, Raap J, Lugtenburg J and Hoff AJ (1994) Asymmetric binding of the primary acceptor quinone in reaction centers of the photosynthetic bacterium *Rhodobacter sphaeroides* R26 probed with Q band (35 GHz) EPR spectroscopy, FEBS Lett 353: 273–276

Brudler R, Groot HJM de, Liemt WBS van, Steggerda WF, Esmeijer R, Gast P, Hoff AJ, Lugtenburg J and Gerwert K (1994) Asymmetric binding of the 1- and 4-C=O groups of Q_A in *Rhodobacter sphaeroides* R26 reaction centres monitored by fourier transform infra-red spectroscopy using site-specific isotopically labelled ubiquinone-10. The EMBO J 13 23: 5523–5530

Brudler R, Groot HJM de, Liemt WBS van, Gast P, Hoff AJ, Lugtenburg J and Gerwert K (1995) FTIR Spectroscopy shows weak symmetric hydrogen banding of the Q_B carbonyl groups in *Rhodobacter sphaeroides* R26 reaction centres, FEBS Lett 370: 88–92

Brudler R, Groot HJM de, Liemt WBS van, Gast P, Hoff AJ, Lugtenburg J and Gerwert K (1996) in: ME Michel-Beyerle (ed) Reaction Centers of Photosynthetic Bacteria; Structures and Dynamics, pp 395–404. Springer-Verlag, Berlin 1996

Cappon JJ, Baart J, van der Walle GAM, Raap J and Lugtenburg J (1991) Chemo-enzymatic synthesis of specifically stable-isotope labelled L-glutamic acid and 2-oxoglutaric. Acid Recl Trav Chim Pays-Bas 110: 158–166

Cappon JJ, Walle GAM van der, Verdegem PJE, Raap J and Lugtenburg J (1992) Synthesis of specifically stable-isotope labelled L-proline via L-glutamic. Acid Recl Trav Chim. Pays-Bas 111: 517

Cappon JJ, Witters KD, Baart J, Verdegem PJE, Hoek AC, Luiten RJH, Raap J and Lugtenburg J (1994) Synthesis of L-histidine

specifically labelled with stable isotopes. Recl Trav Chim Pays-Bas 113: 318–328

Czarnecki K, Chynwat V, Erickson JP, Frank HA and Bocian DF (1997) Identification of the magnesium-histidine stretching vibration of the bacteriochlorophyll cofactors in the photosynthetic reaction centers via ^{15}N labelling of the Histidines. J Am Chem Soc 119: 2594–2595

Deisenhofer J, Epp O, Miki K, Huber R and Michel H (1984) X-Ray structure analysis of a membrane protein complex J Mol Biol 180: 385–398

Egorova-Zachernyuk T, Rossum BJ van, Boender GJ, Franken E, Ashurst J, Raap J, Gast P, Hoff AJ, Oshkinat H and Groot HJM de (1997) Characterization of pheophytin ground states in *Rhodobacter sphaeroides* R26 photosynthetic reaction centers from multispin pheophytin enrichment and 2-D ^{13}C MAS NMR dipolar correlation. Biochemistry 36: 7513–7519

Feng X, Verdegem PJE, Lee YK, Sandström D, Edén M, Bovee-Geurts P, Grip WJ de, Lugtenburg J, Groot HJM de and Levitt MH (1997) Direct determination of a molecular torsional angle in the membrane protein rhodopsin by solid-state NMR. J Am Chem Soc 119: 6853–6857

Fischer MR, Groot HJM de, Raap J, Winkel C, Hoff AJ, and Lugtenburg J (1992) ^{13}C Magic angle spinning NMR Study of the light-induced and temperature dependent changes in *Rhodobacter sphaeroides* R26 reaction centers enriched in [4'-^{13}C] tyrosines. Biochemistry 31: 11038–11049

Gebhard R, Hoef K van der, Lefeber AWM, Erkelens C and Lugtenburg J (1991) Synthesis and Spectroscopy of (14′-^{13}C)- and (15′-^{13}C) spheroidene. Recl Trav Chim Pays-Bas 109: 378–387

Gebhard R, Dijk JTM van, Boza MVTJ, Hoef K van der and Lugtenburg J (1991) Synthesis and spectroscopic properties of 14-, 15-, 15′- and 14′-monodeutero and 15,15′-dideuterospheroidenes. Recl Trav Chim Pays-Bas 110: 332–341

Groot HJM de, Gebhard R, Hoef K van der, Hoff AJ, and Lugtenburg J (1992) ^{13}C magic angle spinning NMR evidence for a 15,15′-cis Configuration of the spheroidene in the *Rhodobacter sphaeroides* reaction center. Biochemistry 31: 12446–12450

Groot HJM de (1995) Asymmetric primary quinone hydrogen bonding in *Rhodobacter sphaeroides* reaction centers In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol. 1, pp 401–406. Kluwer Academic Publishers, Dordrecht, the Netherlands

Jansen FJHM and Lugtenburg J (1996) Part VI: Labelled Carotenoids, of Chapter 3: Carotenoid Synthesis, in: Carotenoids, Vol 2: Synthesis, pp 233–258. 1996 Birkhäuser, Verlag, Basel

Karstens WFJ, Berger HJFF, Haren ER van, Lugtenburg J and Raap J (1995) Enantioselective synthesis of isotopically labelled L-α-amino acids. Preparation of ^{13}C-, ^{18}O- and ^{2}H-labelled L-serines and L-threonines. J Labelled Compounds and Radiopharmaceuticals XXXVI 11: 1077–1096

Kok P, Kohler J, Groenen EJJ, Gebhard R, Hoef K van der, Lugtenburg J, Farhoosh R and Frank HA (1997) Resonance Raman spectroscopy of ^{2}H-labelled spheroidenes in petroleum ether and in the *Rhodobacter Sphaeroides* reaction centre. Spectrochim Acta, Part A, 53: 381–392

Liemt WBS van, Steggerda WF, Esmeijer R and Lugtenburg J (1994) Synthesis and spectroscopic characterisation of ^{13}C-labelled ubiquinone-0 and ubiquinone-10. Recl Trav Chim Pays-Bas 113: 153–161

Liemt WBS van, Boender GJ, Gast P, Hoff AJ, Lugtenburg J and Groot HJM de (1995) ^{13}C Magic angle spinning NMR characterization of the functionally asymmetric Q_A binding in *Rhodobacter sphaeroides* R_{26} photosynthetic reaction centers using site-specific ^{13}C-labeled ubiquinone-10. Biochemistry 34: 10229–10236

Ogrel A, Vasilenko IA, Lugtenburg J and Raap J (1994) Enzymatic synthesis of specifically ^{2}H -labelled L-glutamic acids and ^{2}H-, ^{15}N-, ^{13}C-labelled L-glutamines on a preparative scale. Recl Trav Chim Pays-Bas 113: 369–375

Raap J, Winkel C, Wit AHM de, Houten AHH van, Hoff AJ and Lugtenburg J (1990) Mass spectrometric determination of isotopically labeled tyrosines and tryptophanes in photosynthetic reaction centers of *Rhodobacter sphaeroides* R26. Anal Biochem 191: 9–15

Raap J, Wolthuis WNE, Hehenkamp JJJ and Lugtenburg J (1995) Enantioselective syntheses of isotopically labelled α-amino acids, preparation of specifically ^{13}C-labelled L-lysines. Amino Acids 8: 171–186

Shochat S, Gast P, Hoff AJ, Boender GJ, Leeuwen S van, Liemt WBS van, Vijgenboom E, Raap J, Lugtenburg J and Groot HJM de (1995) ^{13}C MAS NMR evidence for a homogeneously ordered environment of tyrosine M210 in reaction centres of *Rhodobacter sphaeroides*. Spectrochimica Acta 51A: 135–144

Verdegem PJE, Helmle M, Lugtenburg J and Groot HJM de (1997) Internuclear distance measurements up to 0.44 nm for retinals in the solid state with 1-D rotational resonance ^{13}C MAS NMR Spectroscopy. J Am Chem Soc 119: 169–174

Winkel C, Aarts MWMM, Heide FR van der, Buitenhuis EG, and Lugtenburg J (1989) Synthesis and NMR-spectroscopy of stable isotope-labelled phenols and L-tyrosines. Recl Trav Chim Pays-Bas 108: 139–146

Zysmilich MG and McDermott A (1996) Photochemically induced nuclear spin polarisation in bacterial photosynthesis reaction centers: Assigments of the ^{15}N SSNMR Spectra. J Am Chem Soc 118: 5867–5873