^{13}C Magic angle spinning NMR evidence for a 15,15'-Z configuration of the spheroidene chromophore in the *Rhodobacter sphaeroides* reaction center; synthesis of ^{13}C - and ^2H -labelled spheroidenes

R. Gebhard¹, K. van der Hoef¹, C.A. Violette², H.J.M. de Groot¹, H.A. Frank²
and J. Lugtenburg¹

¹ Department of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands

² Department of Chemistry, University of Connecticut, Storrs, Connecticut, 06269-3060, U.S.A.

Abstract - The synthesis of the isotope-labelled spectroscopic probes [$14'^{13}\text{C}$]-, [$15'^{13}\text{C}$]-, [$14'^2\text{H}$], [$15'^2\text{H}$]-, [$15'^2\text{H}$]- and [$14'^2\text{H}$]-spheroidene, all with more than 98% enrichment, is described. In addition, the structure of the carotenoid in the photosynthetic reaction center of *Rhodobacter sphaeroides* has been studied with solid state magic angle spinning (MAS) ^{13}C NMR spectroscopy on R-26 reaction centers reconstituted with [$14'^{13}\text{C}$]-spheroidene. The isotope-label is found to resonate at 125.3 ppm, which is 6.6 ppm upfield from the resonance in all-*E* spheroidene. This indicates the presence of a γ -effect at $\text{C}_{14'}$ and provides evidence for a 15,15'-Z configuration of the chromophore in the active site.

INTRODUCTION

Life on earth depends on photosynthesis. In green plants, algae and phototrophic bacteria the light energy is collected by the light-harvesting protein-pigment complexes, and is transported to the photosynthetic reaction center (RC) for energy transduction and storage (for recent reviews see e.g. refs. 1-4). The RC contains one tightly-bound carotenoid in the active site (ref. 5). The main function of this carotenoid is to quench triplets, thereby preventing the formation of singlet oxygen, which is a powerful oxidizing agent of chlorophyll (ref. 6). Photosynthetic bacterial mutants that lack carotenoids, and higher plants that are treated with herbicides which prevent carotenoid biosynthesis, are rapidly killed by exposure to light (refs. 7 & 8).

The RCs of the photosynthetic bacteria *Rhodospseudomonas viridis* (ref. 9) and *Rhodobacter sphaeroides* R-26 and 2.4.1 (ref. 10) have recently been crystallized and their structures have been elucidated. To date, these are the largest molecules that have been analyzed by X-ray crystallography and they are the only membrane proteins of which the tertiary and quaternary structures have become known. Spheroidene (Fig. 1) is the carotenoid that is bound to the RC of *Rhodobacter sphaeroides* 2.4.1. According to the X-ray data, the protein-bound spheroidene has a curved shape (ref. 10). However, the resolution and quality of the electron density map is insufficient to establish the structure of the carotenoid unambiguously. The X-ray data indicate a 15,15'-*E* double bond for the protein-bound spheroidene. On the other hand, resonance Raman spectroscopy and chemical extraction experiments provide strong evidence that spheroidene occurs in the 15,15'-Z configuration (refs. 11-13). Since these spectroscopic investigations do not address the structure of the spheroidene chromophore *in situ*, a different approach, preferably spectroscopy on the intact and functionally active system, is necessary to solve this discrepancy.

Fig. 1: Structure and numbering of all-*E* spheroidene

In collaboration with others we have worked out a three-step strategy that has made it possible to establish the detailed structure of the retinal chromophore in the active site of the visual pigment bovine rhodopsin and the light-driven proton pump bacteriorhodopsin (ref. 14). First, isotopically labelled retinals with a high level of enrichment were synthesized (ref. 15). Second, active biomolecules were prepared with the isotope-labelled retinal chromophore in the active site. In the third step, the selectively enriched systems were studied with isotope-sensitive non-invasive spectroscopic techniques, in particular magic angle spinning (MAS) NMR (refs. 16-18) and resonance Raman spectroscopy (refs. 19 & 20). The introduction of an isotope as a specific spectroscopic probe does not introduce any changes in steric or electronic properties of the chromophore, since the native protein-pigment complex also contains the same isotopes at the natural abundance level.

The purpose of the present paper is to show that the same strategy can be used to obtain detailed structural and functional information about the spheroidene in the active site of the RC. Specifically labelled spheroidenes with high enrichment were obtained in high yield by means of total synthesis. We have demonstrated that MAS ^{13}C NMR spectroscopy of specifically ^{13}C -labelled biomolecules can be used to analyze details of the structure of a 100 kDa protein at the atomic level. We have investigated the 15,15'-double bond configuration in the RC by high resolution solid state ^{13}C NMR on RCs of the carotenoid-less mutant R-26, reconstituted with specifically ^{13}C -labelled spheroidene. This pigment is functionally active and has the same properties as the wild-type *Rhodobacter sphaeroides* RC (ref. 21).

No specifically labelled spheroidenes have been described before. We first present a novel reaction scheme for the synthesis of the two NMR spectroscopic probes, $[14'\text{-}^{13}\text{C}]$ - and $[15'\text{-}^{13}\text{C}]$ -spheroidene, with >98% ^{13}C incorporation (ref. 22). By slight modification of the synthetic scheme used for the synthesis of these ^{13}C -labelled spheroidenes, we have also prepared the Raman probes $[14'\text{-}^2\text{H}]$ -, $[15'\text{-}^2\text{H}]$ -, $[15'\text{-}^2\text{H}]$ - and $[14'\text{-}^2\text{H}]$ -spheroidene, all with more than 98% deuterium incorporation. In addition we report about the solid-state ^{13}C NMR spectroscopy of RCs reconstituted with $[14'\text{-}^{13}\text{C}]$ -spheroidene. These data are compared with results obtained on model compounds. They provide an independent *in situ* analysis of the configuration of the 15,15'-bond and strongly support the 15,15'-Z structure for the protein-bound spheroidene.

SYNTHESIS OF $[14'\text{-}^{13}\text{C}]$ - AND $[15'\text{-}^{13}\text{C}]$ -SPHEROIDENE

For the synthesis of the C_{40} -carotenoid spheroidene **1** and its $[14'\text{-}^{13}\text{C}]$ - and $[15'\text{-}^{13}\text{C}]$ -isotopomers **1a** and **1b**, we needed to develop the $\text{C}_{18} + \text{C}_2 + \text{C}_5 + \text{C}_5 + \text{C}_{10}$ approach depicted in scheme 1. The previously reported synthetic scheme for the preparation of spheroidene (ref. 23) whereby a symmetric C_{10} -dialdehyde was used as the building block for the center of the molecule, was deemed inappropriate for selective introduction of ^{13}C into the spheroidene molecule. In our scheme, the C_2 -unit is derived from acetonitrile whose $[1\text{-}^{13}\text{C}]$ - and $[2\text{-}^{13}\text{C}]$ -isotopomers are commercially available with 99% ^{13}C incorporation. After optimizing the reactions depicted in scheme 1 for the synthesis of unlabelled spheroidene **1**, $[2\text{-}^{13}\text{C}]$ - and $[1\text{-}^{13}\text{C}]$ -acetonitrile were used for the synthesis of respectively $[14'\text{-}^{13}\text{C}]$ -spheroidene **1a** and $[15'\text{-}^{13}\text{C}]$ -spheroidene **1b**.

The first step in the synthesis of spheroidene **1** was the conversion of C_{18} -ketone **2** into C_{20} -nitrile **3**. For this reaction 250 μl (1 eq.) acetonitrile was first deprotonated at -70°C in THF with one equivalent of lithium diisopropylamide (LDA) in the presence of a second equivalent of LDA. In the subsequent $\text{S}_{\text{N}}2$ -reaction of the lithioacetonitrile with one equivalent of diethylchlorophosphate the ensuing diethyl cyanomethylphosphonate is immediately deprotonated by the second equivalent of LDA. Thus, acetonitrile is efficiently converted *in situ* into the anion of diethyl cyanomethylphosphonate (ref. 24). A Horner-Wadsworth-Emmons (HWE) reaction between this phosphonate-anion and C_{18} -ketone **2** [prepared from the commercially available geranylacetone in two steps; (ref. 22)] gave, after purification, the C_{20} -nitrile **3** in 71% yield based on acetonitrile. Reduction with diisobutylaluminium hydride (Dibal) of the nitrile function of **3** in petroleum ether afforded the corresponding

Scheme 1

Synthesis of spheroidene **1** and its $[14'\text{-}^{13}\text{C}]$ - and $[15'\text{-}^{13}\text{C}]$ -isotopomers **1a** and **1b**, respectively.

C_{20} -aldehyde **4** in 76% yield after purification. For efficient conversion of C_{20} -aldehyde **4** into C_{25} -aldehyde **7** a new C_5 -phosphonate synthon 4-(diethylphosphono)-2-methyl-2-butenenitrile **5** was developed (scheme 2; *vide infra*). HWE reaction in THF of C_{20} -aldehyde **4** with the anion of **5**, prepared with $n\text{-BuLi}$ at -70°C , gave the C_{25} -nitrile in 95% yield after flash chromatography. Dibal reduction of the nitrile function, and purification by column chromatography gave the corresponding aldehyde **7** in 83% yield. Aldehyde **7** reacted in a HWE reaction with the anion of phosphonate **5** (prepared with $n\text{-BuLi}$ at -70°C) to give C_{30} -nitrile in 94% yield. This rendered, after reduction with Dibal and purification, the corresponding C_{30} -aldehyde **8** in 80% yield. A Wittig reaction in THF between aldehyde **8** and phosphonium salt **9** (scheme 3), using $n\text{-BuLi}$ as a base, gave spheroidene **1** in 100% yield. The product is a mixture of *E/Z* isomers from which the all-*E* isomer is separated with difficulty. The overall yield of the isomer mixture of spheroidene, based on acetonitrile, is 32%.

In the HWE reactions depicted in scheme 1, the newly formed double bond predominantly has the *E* configuration. However, an appreciable amount (~20%) of the *Z* isomer is also formed. Following reduction with Dibal, the isomers were separated and the pure all-*E* isomer was used in the next step to prepare **1**. The *Z* isomers can be converted efficiently into the all-*E* isomer by irradiation with visible light in the presence of a catalytic amount of iodine. This procedure was repeated after each HWE/Dibal reduction sequence. In this way, the final product consisted predominantly (> 85%) of all-*E* spheroidene **1** and only minor quantities of two *Z* isomers. The pure all-*E* isomer was now easily obtained by crystallization from ether/petroleum ether. Starting from 250 μl $[2\text{-}^{13}\text{C}]$ -acetonitrile, $[14\text{-}^{13}\text{C}]$ -spheroidene **1a** was obtained in six steps in 38% yield based on the labelled acetonitrile. Similarly, 250 μl $[1\text{-}^{13}\text{C}]$ -acetonitrile afforded $[15\text{-}^{13}\text{C}]$ -spheroidene **1b** in an overall yield of 40%.

In scheme 2 the gram-scale synthesis of the C_5 -phosphonate 4-(diethylphosphono)-2-methyl-2-butenenitrile **5** is depicted. First, propionitrile was treated with two equivalents of LDA in THF at -70°C . After deprotonation of propionitrile, one equivalent of diethyl chlorophosphate was added. An $\text{S}_{\text{N}}2$ -reaction of lithiopropionitrile with diethyl chlorophosphate gave the corresponding phosphonate with simultaneous deprotonation by the second equivalent of LDA. Reaction of this ylide with chloroacetaldehyde **10** (obtained in pure form by ether extraction of the commercially available 50% solution in water), dissolved in THF, gave 4-chloro-2-methyl-2-butenenitrile **11** in 63% yield after column chromatography. The Arbuzov reaction between **11** and triethyl phosphite gave in 95% yield, after distillation *in vacuo*, the C_5 -phosphonate **5**.

Scheme 2

Synthesis of C_5 -phosphonate **5**.

In scheme 3 we present a novel and improved synthesis of phosphonium salt **9** (refs. 22 and 25). In the first step methylcrotonaldehyde **12** was coupled to give the poly-unsaturated ester **14** in 91% yield in a HWE reaction with C_5 -phosphonate **13** using NaH as a base. Treatment of **14** with *m*-chloroperbenzoic acid cleanly leads to epoxidation of the most electron-rich 6,7 double bond and pure epoxide **15** was obtained in 84% yield. Lithium aluminiumhydride reduction of **15** gave in 82% yield the corresponding 1,7-diol (no traces of the 1,6-diol were found) which was subsequently treated with three equivalents of sodium hydride and ten equivalents of methyl iodide in THF. Under these conditions the primary hydroxyfunction is methylated within a few minutes. For the complete methylation of the sterically hindered tertiary hydroxyfunction it proved necessary to reflux the mixture for several hours. After purification the dimethoxy-compound **16** was obtained in 75% yield. Reaction of **16** with triphenylphosphine hydrobromide gave, after crystallization from diethyl ether/ethyl acetate the desired phosphonium salt **9** in 51% yield.

Scheme 3

Synthesis of C_{10} -phosphonium salt **9**.

SYNTHESIS OF [14'-²H]-, [15'-²H]-, [15'-²H]- AND [14'-²H]-SPHEROIDENE

For the specific introduction of the deuterium atoms in the central part of spheroidene, we used the reactions depicted in scheme 1 with a few minor modifications. For the conversion of C₂₀-aldehyde **4** into C₂₅-aldehyde **7**, we changed the C₅-synthon **5** into a C₂ + C₃ synthon, as is depicted in scheme 4. Since reduction with Dibal-²H is the method of choice for the conversion of nitriles into aldehydes, we decided to use Dibal-²H for the preparation of deuterated aldehydes. Dibal-²H was prepared from Li²H and diisobutylaluminium chloride (ref. 26). Dibal-²H reduction of the C₂₀- and C₂₂-nitriles in scheme 4 gave the corresponding deuterated aldehydes **4n** (m=1 and n=2) and **6r** (m=n=q=1 and r=2) in high yield with the same level of enrichment as in the starting Li²H (>98% ²H). The conversion of aldehydes **4n** and **6r** into the corresponding [15'-²H]- and [14'-²H]-spheroidene **1n** and **1r** was performed according to the reactions in schemes 1 and 4. For the introduction of a deuterium atom at the 14' position of spheroidene with >98% enrichment, we used a novel one-pot procedure. First, the anion of deuterated acetonitrile was prepared with *n*-butyllithium at -70 °C and was reacted with C₁₈-ketone **2**. The resulting tertiary alkoxy-compound was subsequently treated with rigorously dried methanesulfonyl chloride and triethylamine, which led to the introduction of the desired α,β-unsaturation. No scrambling between the α protons of C₁₈-ketone **2** and deuterium atoms or loss of label was observed. After Dibal reduction deuterated aldehyde **4m** (n=q=r=1 and m=2) was obtained. The conversion of **4m** into [14'-²H]-spheroidene is most easily performed according to scheme 1. For the synthesis of [15'-²H]-spheroidene **1q**, we used a similar procedure: coupling of the anion of deuterated acetonitrile with C₂₀-aldehyde **4**, followed by dehydration with mesyl chloride and triethylamine, afforded the C₂-elongated nitrile. After Dibal reduction, mono-deuterated aldehyde **6q** (m=n=r=1 and q=2) was obtained with >98% ²H incorporation. The conversion of **6q** into [15'-²H]-spheroidene **1q** was performed according to schemes 1 and 4. Combinations of the reactions depicted in scheme 4 can be used to prepare all possible ²H combinations in the central part of spheroidene.

Scheme 4

Synthesis of specifically deuterated aldehydes **7**, intermediates in the synthesis of [14'-²H]-, [15'-²H]-, [15'-²H]- and [14'-²H]-spheroidene **1m**, **1n**, **1q** and **1r** respectively.

SPECTROSCOPIC IDENTIFICATION

Mass spectrometry

The double-focus mass spectrum (e.i.: 70 eV) of spheroidene **1** shows the parent peak at *m/z* 568.4646 (calculated for C₄₁H₆₀O: 568.4644). Other strong peaks in the mass spectrum are at *m/z* 476 (M⁺ - C₇H₈), 462 (M⁺ - C₈H₁₀), 91 (C₇H₇⁺) and 73 (C₄H₉O⁺). The fragments at *m/z* 476 and 462 correspond to the loss of toluene and *m*-xylene, well known fragments in the mass spectra of carotenoids (ref. 27 & 28). The peak at *m/z* 91 (tropylium ion) is also a common fragment in the mass spectra of other carotenoids. The (C₄H₉O⁺)-ion is due to the tertiary ether function. The fragment is the result of cleavage of the 1,2-bond, a common fragmentation in aliphatic ethers, and is found in the mass spectra of all carotenoids possessing this type of end-group (ref. 28).

The double-focus mass spectra of [14'-¹³C]-spheroidene **1a** and [15'-¹³C]-spheroidene **1b** showed the parent peak at respectively *m/z* 569.4658 and *m/z* 569.4679 (calculated for ¹³C₁¹²C₄₀H₆₀O: 569.4678). The isotope incorporations were 99% ¹³C for **1a** and 98% ¹³C for **1b**. The ¹³C incorporations were the same as in the starting acetonitriles, indicating that during the reactions no loss of label has occurred. The mass spectra of **1a** and **1b** both show strong peaks at *m/z* 477 (M⁺ - C₇H₈), 476 (M⁺ - ¹³C₁¹²C₆H₈), 463 (M⁺ - C₈H₁₀), 91 and 73. Both in the spectrum of **1a** and **1b**, the intensity ratio between the peaks at *m/z* 477 and *m/z* 476 is 5 : 95 respectively, after correction for the natural-abundance amount of ¹³C. This means that the atoms C14' and C15' are involved in 95% of the toluene eliminations and not in the loss of xylene (ref. 22).

At the time this paper was written, a detailed analysis of the mass spectra of the deuterated spheroidenes **1m**, **1n**, **1q** and **1r** was not available.

^1H and ^{13}C NMR spectroscopy

From the 300 MHz ^1H NMR and the 75 MHz ^{13}C NMR spectra of the ^{13}C -labelled spheroidenes **1a** and **1b** and the ^2H -labelled spheroidenes **1m**, **1n**, **1q** and **1r** the position of the label and the incorporation (>98%) are evident. This is illustrated for the ^1H NMR spectra of $[14'\text{-}^{13}\text{C}]$ - and $[15'\text{-}^{13}\text{C}]$ -spheroidene **1a** and **1b**. In figure 2A the resonances of the protons of the polyene chain of spheroidene **1** are given, together with their assignment (ref. 22). In figures 2B and 2C the low-field part of the spectra of, respectively, $[14'\text{-}^{13}\text{C}]$ -spheroidene **1a** and $[15'\text{-}^{13}\text{C}]$ -spheroidene **1b** are presented. In the spectrum of **1b** (fig. 2C), four signals near 6.90 ppm are present that are not observed in the spectrum of **1** and **1a**. They belong to the low-field part of the $\text{H}_{15'}$ resonance that has been split by the $15'\text{-}^{13}\text{C}$ atom. Around 6.35 ppm, the high-field part of the same signal is present ($J_{\text{CH}} = 151.4$ Hz). The protons 14, 15, $15'$ and $14'$ in **1b** are mutually interacting, and are coupled to the spin 1/2 of the ^{13}C nucleus of the label (ABCMX pattern of an ABCMX spin system, see e.g. ref. 29). This pattern has been analyzed with the PANIC programme from Bruker. Besides the precise chemical-shift values, the coupling constants are obtained. For the $^3J(\text{H}_{15}\text{-H}_{15'})$ the value of 14.0 Hz has been found, establishing that the configuration around the 15-15'-double bond is *E*. In the spectrum of **1a** (fig. 2B) the large splitting of the $\text{H}_{14'}$ signal at 6.20 ppm is observed (J_{CH} is 150.0 Hz). Due to the strong coupling between H_{15} and $\text{H}_{15'}$, this spectrum could not be analyzed completely. The signal of $\text{H}_{12'}$ shows an additional splitting of 5.8 Hz [$^3J(^{13}\text{C}_{14'}\text{-H}_{12'})$] and the $13'\text{-CH}_3$ group shows a 6.9 Hz coupling with $^{13}\text{C}_{14'}$. In the signals of H_{11} , a broadening due to the long-range coupling (J) with $^{13}\text{C}_{14'}$ is apparent.

Fig. 2: Low-field part of the 300 MHz ^1H NMR spectrum of all-*E* spheroidene **1** (A), all-*E* $[14'\text{-}^{13}\text{C}]$ -spheroidene **1a** (B) and all-*E* $[15'\text{-}^{13}\text{C}]$ -spheroidene **1b** (C).

SOLID STATE ^{13}C NMR SPECTROSCOPY

Cross-polarization Magic Angle Spinning (CP/MAS) NMR difference spectroscopy is an excellent method for observing the contribution of a label in the ^{13}C NMR spectra of large proteins (ref. 30 & 31). In Fig. 3 the ^{13}C CP/MAS NMR spectrum of reconstituted R-26 RCs is shown. A MAS-NMR spectrum (Fig. 3) is obtained on a frozen, and hence solid, sample that is rapidly rotating, in this case 6 kHz, around an axis at the magic angle of $54^\circ 44'$ with the magnetic field. Due to the low temperature (~ 200 K) the vesicles containing the photosynthetic RCs are immobilized and cross-polarization from the protons to the ^{13}C nuclei can be used to enhance the signal. In a previous note, it was demonstrated that it is possible to achieve atomic resolution with this technique in a bacterial photosynthetic reaction center selectively enriched with ^{13}C at the 4' position of the tyrosines (ref. 32). For the present work we reconstituted ~ 40 mg of RC of the carotenoid-less mutant *Rhodobacter sphaeroides* R-26 with the synthesized $[14'\text{-}^{13}\text{C}]$ -spheroidene. It was determined that $\sim 70\%$ of the RC had incorporated the carotenoid.

The NMR data were collected with a MSL-400 spectrometer and a probe from Bruker with 7 mm rotors. The ^{13}C frequency of the spectrometer is 100 MHz with a 90° pulselength of 5 μsec for both the ^{13}C and the ^1H . The chemical shift is referenced to the carboxyl signal of an external sample of solid glycine (176.04 ppm relative to TMS) and has an absolute accuracy of less than one ppm.

From the comparison of the spectrum in Fig. 3 with data for an unlabelled sample, it is possible to identify the contribution of the label to the spectrum. After subtraction of a natural abundance spectrum, followed by a cubic spline background correction, the difference spectrum shown in the insert in Fig. 3 is obtained. It is evident that a tiny peak at 125.3 ppm (as indicated by the asterisk in the spectrum of the reconstituted sample) represents the contribution from our label.

A comparison with model compound data provides an independent *in situ* analysis of the configuration around the 15,15' double bond of spheroidene in the photosynthetic RC. CP/MAS NMR spectra were collected on (all-*E*)- β -carotene, (15,15'-*Z*)- β -carotene and (all-*E*)- $[14'\text{-}^{13}\text{C}]$ -spheroidene in the solid state. It appears that the 14 (or 14') ^{13}C is shifted by more than 5 ppm upfield when the β -carotene undergoes a *trans* to *cis* isomerization around the central double bond. This is thought to occur mainly because of the steric hindrance between the hydrogens attached to C_{14} and $\text{C}_{14'}$, and is known as the γ -effect. It has been observed in proteins (ref. 34), polyenals and aromatic compounds in the solid state (refs. 34 & 35), and is also present in solution (ref. 36). It amounts to an upfield shift of 5-12 ppm. With MAS NMR the ^{13}C label in the solid (all-*E*)- $[14'\text{-}^{13}\text{C}]$ -spheroidene was found to resonate at a chemical shift of 133.9 ppm. We expect the $14'\text{-}^{13}\text{C}$ resonance of the 15,15'-*Z* isomer to be in between 129 and 122 ppm. The value of 125.3 ppm found in our protein experiments therefore supports earlier suggestions from resonance Raman studies (ref. 11-13) and favours a 15,15'-*Z* configuration, as opposed to the central *trans* structure suggested by the X-ray data.

Fig. 3: CP/MAS ^{13}C NMR spectrum of R26 reaction centers reconstituted with $[14'\text{-}^{13}\text{C}]$ -spheroidene 1a.

CONCLUDING REMARKS

It may be mentioned that information about the 15,15'-double bond configuration of the RC-bound spheroidene can also be obtained from CP/MAS ^{13}C NMR on RCs reconstituted with $[15'^{13}\text{C}]$ -spheroidene. From solution studies on carotenoids, it is known that the $15'^{13}\text{C}$ resonance shifts upfield by ~ 5 ppm upon *trans-cis* isomerization. Therefore the difference between a 15,15'-*E* and a 15,15'-*Z* configuration of the RC-bound chromophore should be reflected also in the CP/MAS NMR spectrum of RCs reconstituted with $[15'^{13}\text{C}]$ -spheroidene. In addition detailed information about the configuration and conformation of the protein-bound chromophore can be obtained with resonance Raman studies of RCs reconstituted with deuterium-labelled spheroidene. In the Raman spectrum of the RC-bound spheroidene, an intense hydrogen-out-of-plane vibration (HOOP) is observed at 958 cm^{-1} , which shows that the chromophore is twisted into a non-planar conformation (ref. 13). From the Raman work on bathorhodopsin, it is known that the HOOPs in a distorted polyene-chain can be assigned by specific deuteration (ref. 38). Similar experiments on RCs reconstituted with $[^2\text{H}]$ -spheroidenes are in progress. Finally, time-resolved Raman spectroscopy of RCs reconstituted with isotope-labelled spheroidenes may give information about the structure-function relation of the carotenoid in the protein.

At present a widely used technique for bio-molecular research of larger proteins is site-directed mutagenesis. A non-negligible problem with these studies is that only disturbed systems are available and that the mutations can result in a major perturbation of the system. In recent years the number of isotope-labelled compounds that can be used as a spectroscopic probe (e.g. ^{13}C , ^{15}N , ^{17}O , ^2H) has been increasing rapidly. Various labelled amino-acids are nowadays commercially available. As is also demonstrated by the present work, the custom-synthesis of specific spectroscopic probes is very well possible with today's chemical technology within a reasonable amount of time. Therefore we anticipate that labelling biomolecules followed by spectroscopy will evolve into an indispensable technique for biomolecular research in the very near future and we strongly advocate more research along this line.

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