# Magic-angle spinning 13C NMR with atomic resolution of a photosynthetic reaction center enriched in [4'-13]tyrosine

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# MAGIC-ANGLE-SPINNING $^{13}$C NMR WITH ATOMIC RESOLUTION
# OF A PHOTOSYNTHETIC REACTION CENTER ENRICHED IN [4'-$^{13}$C]TYROSINE

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Low-temperature magic-angle-spinning (MAS) $^{13}$C NMR was used to investigate reaction centers of *Rhodobacters sphaeroides* R26 specifically enriched in [4'-$^{13}$C]tyrosine, allowing the investigation of variations in chemical environment, in particular at the phenolic oxygen. The level of incorporation was determined with mass spectrometry and is ≈98%. Magic-angle-spinning $^{13}$C difference NMR spectra with atomic resolution were obtained. In the signal of the 28 tyrosines only five MAS patterns with different isotropic shifts and intensities are observed. Most of the spectral intensity is spread over two components (≈60% and ≈30%). This suggests that the protein provides mainly two different chemical environments, which is ascribed to the polar and non-polar protein regions. Two small MAS patterns contain ≈4% of the total integrated intensity, corresponding with the signal intensities of individual tyrosines, and have an unusually low isotropic shift. This may reflect interactions with the cofactors, for instance ring-currents in the macroaromatic cycles. The data provide compelling evidence that all tyrosines in the dark-adapted R26 photosynthetic reaction centers are normally protonated.

## 1. Introduction

One of the most important advancements in photosynthesis research of the last few years has been the X-ray structure determination of bacterial photosynthetic reaction centers (RCs) [1–3]. In order to relate these structures to their function, spectroscopic techniques with sufficient sensitivity to investigate in particular the amino-acid side chains with atomic resolution, need to be applied. Among the residues that are probably crucial for the photochemistry are the tyrosines. Several tyrosines are likely to be involved in the various mechanisms of the photosynthetic process in the R26 RCs. Two, M210 and L162, are possibly involved in charge transfer, contribute to the breaking of the nearly twofold symmetry in the RC, and are part of the aromatic environment of the cofactors [4]. Other tyrosines may play a role in proton transport to the quinone acceptors.

MAS difference NMR on specifically enriched samples has proven to be an excellent technique for the investigation of large proteins on a microscopic scale. We have performed low-temperature $^{13}$C MAS NMR spectroscopy on RCs of *Rhodobacter sphae-roides* R26 that are specifically enriched in [4'-$^{13}$C]tyrosine. The tyrosines are labeled at the 4' position, because this offers the best possibility to characterize the differences in chemical environment at the phenolic oxygens, an important step towards understanding the function of the tyrosines in the protein.

The purpose of this note is thus twofold: It is demonstrated that photosynthetic RCs are well within the scope of the MAS difference NMR method; and we shall discuss our first $^{13}$C NMR data obtained on R26 RCs enriched in [4'-$^{13}$C]tyrosine. The most striking result of these experiments is that the chemical environment is in fact very well defined, since the signal of most of the tyrosines, ≈25 from a total of 28, is divided over two resonances with slightly different isotropic chemical shifts. The three remaining residues are "special". Two of them are upfield, and one downfield shifted from the bulk signal.

## 2. Experimental

About 4 g L-[4'-$^{13}$C]tyrosine was synthesized fol-

lowing previously published procedures [5]. A synthetic medium for *Rhodobacter sphaeroides* R26 was developed and used to prepare $\approx 15$ mg labelled RCs. In addition a natural abundance sample ($\approx 50$ mg) was grown. The RCs consist of the L, M, and H subunits, with a total molecular weight of $\approx 101000$ [6], and are embedded in LDAO vesicles in a buffer. The $^{13}C$ incorporation was determined with mass spectrometry as $\approx 98\%$.

NMR data were collected with an MSL-400 spectrometer and 8 mm probe from Bruker. Spectra were collected with cross-polarization-magic-angle-spinning (CPMAS) NMR, where the sample is rapidly rotating, in this work $\omega_r/2\pi = 5$ kHz, around an axis at an angle of $54°44'$ with the magnetic field. This is the method of choice to observe NMR of specific labels in large membrane proteins [7]. The pulse lengths ($^1H$ and $^{13}C$) were 4.5 µs.

The experiments were performed on very dense (OD > 100) frozen solutions of RC vesicles. The low temperature, $\approx 200$ K, was necessary to immobilize the vesicles. For each spectrum $\approx 40000$ transients were accumulated in 512 channels with a recycle delay of 2 s and a sweep width of 50 kHz. Use was made of MAS difference spectroscopy, which permits suppression of the natural abundance background, and therefore yields high-resolution spectra of only the labeled positions [8]. For the analysis of these difference spectra we used the sideband intensities of Herzfeld and Berger [9], to generate simulations of MAS spectra that were fitted to the experimental data, using the CERN (Geneva) MINUIT fitting package. These calculations were done on a VAX-750 computer.

## 3. Results and discussion

A MAS NMR spectrum is a set of resonances centered around the isotropic shift and equally spaced by multiples of the spinning speed. This is demonstrated by the difference spectrum from the tyrosine labels in fig. 1c. The increase in sensitivity by isotopic labelling is evident: the signals of the tyrosines are dramatically enhanced in the spectrum of the enriched species (fig. 1a), with respect to the natural abundance background (fig. 1b). This allows the detailed analysis of the lineshape and the chemical-shift
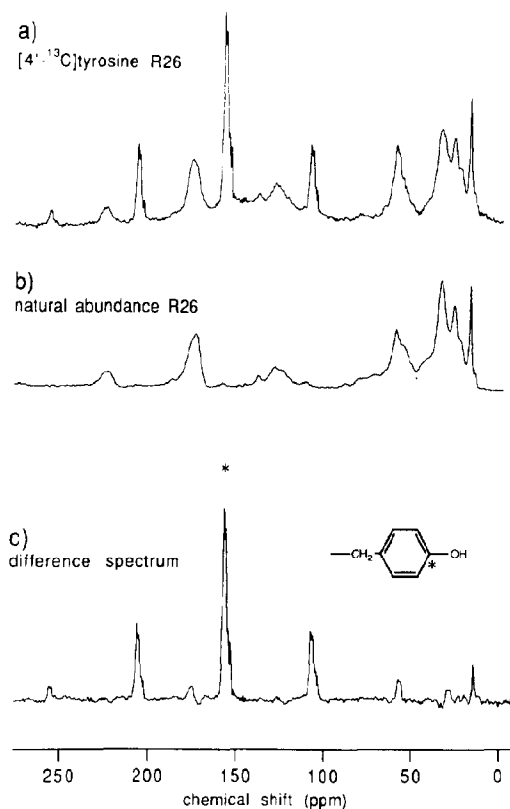


Fig. 1. Solid-state $^{13}C$ CP/MAS NMR of *Rhodobacter sphaeroides* R26 RCs at a temperature of 200 K and a spinning speed of 5 kHz. (a) [4'-$^{13}C$]tyr R26, (b) natural abundance R26, and (c) difference (a) minus (b), with a cubic spline background correction. The position of the $^{13}C$ enrichment in the tyrosine sidechain and the centerband of the resonances from the label are indicated with asterisks in (c). Chemical shifts are relative to external TMS.

anisotropy. The principal components of the chemical-shift tensors are calculated from the peak intensities as $\sigma_{11} = 60(3)$ ppm, $\sigma_{22} = 160(4)$ ppm, and $\sigma_{33} = 245(3)$ ppm.

Fig. 2 shows that in fact four different centerbands are observed, at 156.0, 154.8, 153.6 and 152.5 ppm. Errors are $\approx 0.2$ ppm. In addition there is a shoulder at $\approx 157.1$ ppm. The intensities of the 154.8 and 156.0 ppm resonances may be associated with approximately 8 and 17 tyrosines per RC, respectively. It is possible to achieve atomic resolution in this large protein since the two small upfield resonances contain $\approx 4\%$ of the total integrated intensity each and
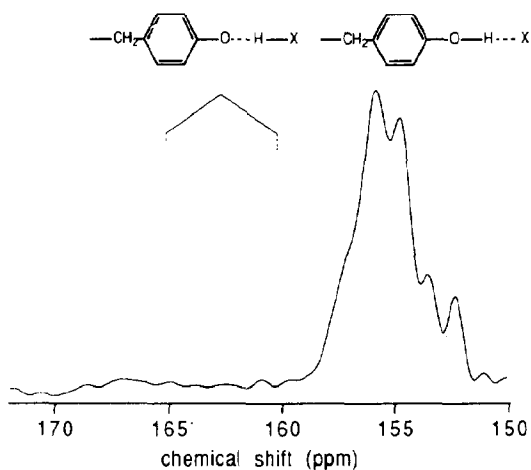
Fig. 2. Exploded view of the centerband region of the difference spectrum in fig. 1c, showing the details of the lineshape from the neutral tyrosine side chains (drawn at right in the figure). There are no tyrosinates (drawn at left in the figure) in the protein.

are both probably due to one tyrosine per protein molecule. The isotropic shifts of the two small signals are unusually low. This may reflect the influence of the cofactors. For example, the accessory chlorophyll $B_A$ is expected to cause an upfield ring-current shift of $\approx 3$–4 ppm for the label in M210, which is situated $\approx 4.5$ Å below the aromatic macrocycle [4,10]. The downfield shoulder accounts for the remaining tyrosine, making a total of 28.

It was totally unexpected that most of the signal ($\approx 25$ residues) is divided over only two MAS patterns. This suggests that the protein provides mainly two different well-defined chemical environments and that the small but significant shift difference between these two is caused by only one predominant mechanism. The X-ray structure shows a striking difference between the trans-membrane region, which is non-polar, and the exposed part of the RC that contains the charged residues [11]. Since the $4'$-$^{13}C$ is close to the phenolic oxygen the chemical shift is sensitive to variations in hydrogen bonding associated with the variation in protein environment. This is probably the origin of the small difference between the 156.0 (polar) and 154.8 (non-polar) signals, an explanation supported by studies on tyrosine, soluble oligopeptides and small soluble proteins [12]. Furthermore, the MAS spectrum of $[4'$-$^{13}C]$tyrosine-

labelled bacteriorhodopsin ($MW \approx 27000$), where also the trans-membrane region is thought to be polar, shows only one main peak at 156 ppm, with no additional resonances upfield [13].

The details of the hydrogen bonding in the RC have attracted considerable attention lately. NMR is an excellent method to determine protonation states in situ. Our data provide compelling evidence that all tyrosines in the dark-adapted R26 RCs are neutral (protonated). The $[4'$-$^{13}C]$ resonance moves 9 ppm downfield upon deprotonation [12], and a tyrosinate in the RCs would have caused a centerband signal in the 161 to 166 ppm region, with an intensity and linewidth comparable to the 152.5 ppm resonance. This should have been easily discernable in the exploded view in fig. 2.

It is concluded that MAS difference NMR on specifically enriched samples is an excellent technique to investigate photosynthetic RCs. Amino-acid residues and prosthetic groups may be studied in situ, as well as protein–cofactor interactions. Also changes induced by the photochemistry in the RC are accessible, provided that these can be fixed, for instance by freezing or with chemical methods. The information provided by these experiments cannot be obtained in any other way at present. It is also important to realize that the strategy of isotope labelling followed by spectroscopy, is in many respects complementary to studies involving site-directed mutagenesis, an approach frequently undertaken to investigate structure and function of RCs and many other proteins. The method used here has the advantages that an unperturbed system is investigated, that standard procedures are used for protein purification and sample preparation, and that chemical shifts of different residues of one type are compared in only one experiment. In combination with the recently developed techniques for site-specific incorporation the strategy will be even more powerful [14].

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