Incorporation of ^{19}F-substituted aromatic amino acids into membrane proteins from chromatophores of *Rhodospirillum rubrum* G9^{+}

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Chromatophore membranes were isolated from cells of the carotenoidless mutant *Rhodospirillum rubrum* G9^{+} grown in the presence of several fluorinated aromatic amino acids, solubilized using SDS and the extent of incorporation analyzed using high-resolution ^{19}F-NMR spectroscopy. 3- and 4-^{19}F-phenylalanine, 6-^{19}F-tryptophan and 3-^{19}F-tyrosine were biosynthetically incorporated into membrane proteins whereas 5-^{19}F-tryptophan and 2-^{19}F-phenylalanine were inhibitors of cell growth. The polypeptide chains of the major chromatophore membrane protein the light-harvesting complex, were isolated and shown by high-resolution ^{19}F-NMR to contain 3-^{19}F-phenylalanine, which is known to be situated principally within the membrane hydrocarbon layer. Broad-band ^{19}F-NMR spectra of 3-^{19}F-phenylalanine-labelled chromatophores showed the phenyl ring to be immobilized within the membrane.

^{19}F-NMR *Membrane protein* *Membrane dynamics* *Chromatophore membrane* *Light-harvesting complex* *Protein modification*

1. INTRODUCTION

Although ^{19}F-NMR has been used extensively to study water-soluble proteins [1–3], to date only a single example exists of similar studies applied to integral membrane proteins [4]. Indeed it is generally thought that the high electrical dipole moment of fluorine would preclude its insertion into the low dielectric hydrocarbon layer of a phospholipid bilayer without perturbation of the native structure in some way. Such perturbations, albeit small, have in fact been shown experimentally for ^{9}F-substituted bilayers of dipalmitoylphosphatidylcholine [5,6].

On the other hand, fluorine has several advantages as a possible nucleus for NMR studies of molecular structure: due to the high gyromagnetic moment the sensitivity of detection is very high. This allows studies of samples available in only small quantities or conversely, it raises the possibility of performing 2-D NMR studies without the prohibitively long instrument times necessary for many other dipolar nuclei of biological interest (e.g. ^{13}C, ^{31}P, ^{15}N).

With these possibilities in mind we attempted to incorporate ^{19}F-labelled aromatic amino acids into the proteins of the chromatophore membrane of the photosynthetic bacterium *Rhodospirillum rubrum* strain G9^{+}, using biosynthetic methods [7]. From these membranes we isolated the extremely hydrophobic light-harvesting polypeptides of the membrane to demonstrate that the fluorinated amino acids were indeed incorporated into the protein structure.

2. MATERIALS AND METHODS

Fluorinated amino acids of high purity were ob-

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tained from EGA Chemie and Fluka and added individually to the growth medium (final concentration 2 mM). Cells of *R. rubrum* were grown in the medium of Ormerod as described before and chromatophores were prepared by conventional methods [8]. For NMR measurements the washed membranes were solubilized in 1% SDS at 4°C, then transferred to an NMR tube and measured immediately.

Light-harvesting polypeptides were prepared from intact chromatophores as follows; a chromatophore suspension was lyophilized onto the side of a round-bottomed flask and then extracted with 10% formic acid. The solubilized fraction was then made up to 50% methanol and applied to a column (50 cm × 2.5 cm) of Sephadex LH60 equilibrated in the same solvent. Peaks from the column were then pooled and tested for protein using the method of Peterson [9], for phospholipids using TLC and for bacteriochlorophyll using the extinction coefficient of free bacteriochlorophyll at 772 nm [10]. The pools containing protein were dried overnight under high vacuum and then dissolved in buffer containing 1% SDS, 10 mM mercaptoethanol and applied to an SDS-polyacrylamide gel according to the method of Laemmli [11]. Staining was performed using Coomassie blue R250.

^{19}F-high resolution NMR spectra were obtained using a Bruker AM-300 NMR instrument at a measuring frequency of 282.4 MHz. All spectra were obtained using 8K of memory, a spectral width of 30 kHz and an acquisition time of 0.129 ms.

Broad-band ^{19}F-NMR spectra were obtained using a Bruker CXP-300 NMR instrument with a special probehead which contained no Teflon or other ^{19}F-containing compounds. 2K spectra were taken at 282.5 MHz using an offset of −20.5 kHz and a spectral width of 1 MHz. Data acquisition was performed using a spin-echo sequence $[90_x\text{-}\tau_1\text{-}180_x\text{-}\tau_1\text{-AQ-}\tau_2]_n$ with a 90° pulse of 3 μs, acquisition time (AQ) or 1 ms, echo refocussing time (τ_1) of 3 μs, and a recovery time (τ_2) of 20 s. A PAPS phase alternation sequence to minimize phase errors was also used.

3. RESULTS AND DISCUSSION

Fluorine-labelled amino acids were incorporated into the chromatophore membrane by providing external amino acids in the medium. In some cases (4-^{19}F-Phe, 3-^{19}F-Tyr, 6-^{19}F-Trp) the usual growth rate was slowed down partially or completely by the added amino acids at a concentration greater than 2 mM, although the near IR (660 nm–940 nm) spectra of the isolated chromatophores was identical to those obtained from bacteria grown under the normal culture conditions (i.e. in the absence of ^{19}F-labelled amino acids). The near IR absorption spectrum is usually the best criterion that the protein-pigment interactions which are critical for the phototransducing function of the chromatophore membranes are unperturbed.

Fig.1 shows the ^{19}F-NMR spectra of SDS-solubilized chromatophores from *R. rubrum* G9$^+$ after biosynthetic incorporation of various fluorinated amino acids. For 3-^{19}F-Phe, 4-^{19}F-Phe, 6-^{19}F-Trp, and 3-^{19}F-Tyr cell yield was smaller but in all cases several distinct resonance lines were observed. Little or no growth was observed using the amino acids 5-^{19}F-Trp and 2-^{19}F-Phe.

To determine whether the fluorinated amino acids were truly incorporated into the polypeptide of the light-harvesting pigment proteins [14] we have purified the ^{19}F-Phe substituted chromatophore proteins using Sephadex LH60 chromatography (fig.2a). Three separate fractions were obtained containing proteins of large molecular mass (fraction I), peptides of the light-harvesting complex (fraction II) which comprises about 50% of the total membrane protein [12], and a third fraction containing the free bacteriochlorophyll and phospholipids, respectively. For both fractions I and II the single resonance observed in the ^{19}F-NMR spectrum obtained in HCOOH/MeOH corresponded to that of the free amino acid dissolved in water (fig.2b,c). This result shows that 3-^{19}F-Phe was indeed incorporated into the component proteins of the chromatophore proteins and the single resonance observed sugggests that the amino acid was not modified by cell metabolism, since large chemical shifts are observed for amino acids with different chemical structures. (We note in passing that these results represent only the second report (see [3]) of the biosynthetic replacement of amino acids by their fluorinated analogues for an organism other than *E. coli* [7].)

Examination of the sequence of the 2 light-harvesting polypeptides (fig.2d) [13,14] shows that

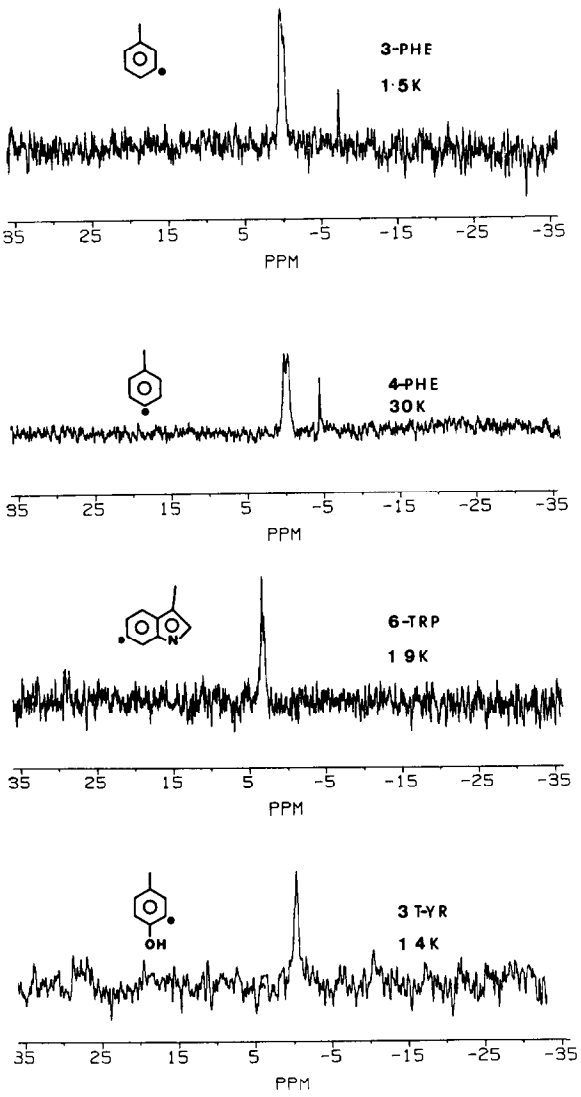

Fig.1. ^{19}F-NMR spectra of SDS-solubilized chromatophores from *R. rubrum* G9^{+} labelled with various ^{19}F-amino acids. (●) The position of the fluorine label. The spectral width used was 30 kHz and the number of scans used to obtain each spectrum is shown on the figure. For each spectrum the reference refers to the resonance position of the free amino acid dissolved in water. All spectra were obtained without decoupling and a line-broadening factor of 20 Hz was used for exponential multiplication prior to Fourier transformation.

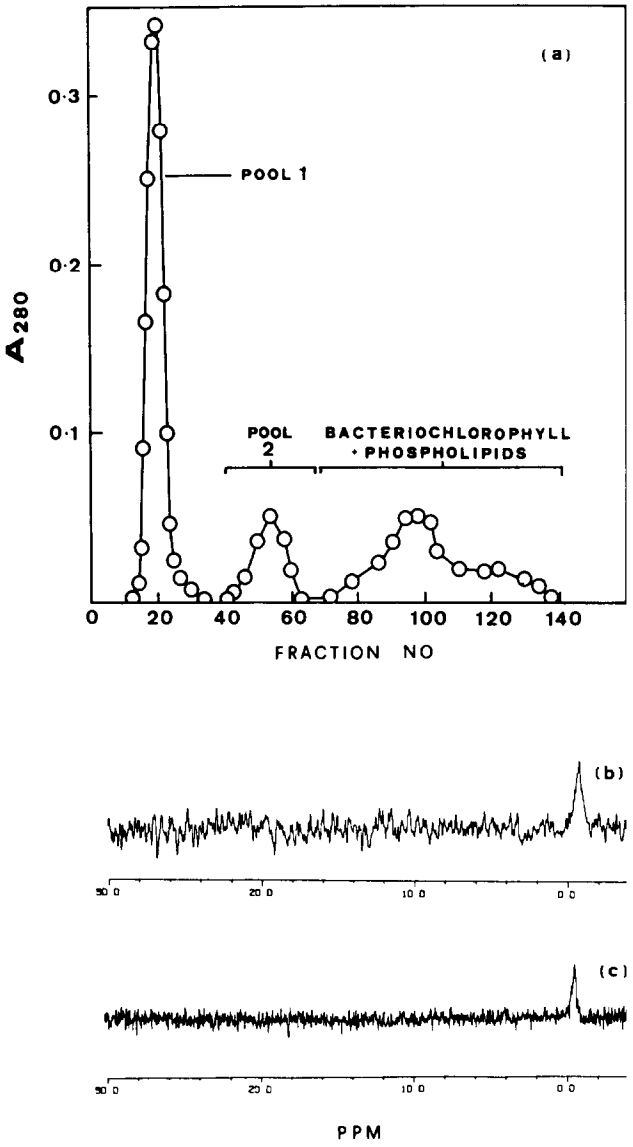

Fig.2. (a) Elution profile of chromatophores extracted with formic acid and then chromatographed on Sephadex LH60, using formic acid/methanol [1:2.8) (v/v)] as eluting solvent. Pools 1 and 2 contained lipid-free protein. ^{19}F-NMR spectra were obtained from pools 1(b) and 2(c) using a line-broadening factor of 1 Hz, a spectral width of 30 kHz and without proton decoupling. Pool 2 was found to contain a mixture of only light-harvesting polypeptides. The sequences of these polypeptides are shown in (d) (15). The sequences shown between the 2 dotted lines define the region of the polypeptide chains thought to exist within the hydrocarbon layer of the membrane (see text).

a total of 11 phenylalanine residues are present. Assigning the membrane embedded fragments [14,15] shows that at least 9 of these residues lie within the hydrocarbon layer and labelling studies

show that the remaining residues are very close to the polar/hydrocarbon interface [15]. The observation of fluorine resonances from the isolated protein are therefore by no means trivial since the electric dipole moment of fluorine is extremely large compared to that of hydrogen and it has been occasionally suggested that this might prevent the incorporation of fluorine into intrinsic membrane proteins. Indeed, to date only a single fluorine substituted membrane protein has been reported, the coat protein for the M13 virus [4] and in this case the protein is only transiently membrane-bound during the viral cycle. The present study shows that in the case of the light-harvesting complex of *R. rubrum* $G9^+$, fluorine-substituted amino acids may be incorporated into these very hydrophobic polypeptides without perturbing their functional state in vivo.

Finally, in a preliminary experiment, the feasibility of obtaining ^{19}F-NMR spectra from proteins embedded in pellets of liposome membranes was determined. Fig.3 shows the broad-band ^{19}F-NMR spectrum of pelleted native chromatophore membranes labelled biosynthetically with 3-^{19}F-Phe. Although this spectrum is the sum of contributions of various chromatophore membrane proteins (of which the light-harvesting complex constitutes 50%), the most striking fact is that the spectral form is very similar to that obtained from a dry powder of the pure amino acid. These spectra were taken using a rather long recovery time between acquisition so that a narrow, slowly relaxing component should be clearly visible if present. This is not the case, suggesting that in almost all the constituent membrane proteins 3-^{19}F-Phe residues are immobilized, probably within the membrane interior. Unfortunately, it was possible only to obtain spectra in the absence of proton decoupling. Thus the dipole-dipole interactions cause the broad resonance to have a near-Lorentzian shape and the true spectral form of the chemical shielding anisotropy averaging is not observable. This means that we cannot say unequivocally whether the 'immobilized' ^{19}F-labelled phenylalanine residues are really fixed in a single state, or whether they are undergoing some sort of 'flipping' motion. However, the immobilized, as opposed to freely rotating, interpretation remains unequivocal and is indeed in accord with recent ^{2}H-NMR studies of ^{2}H-labelled phenylalanine residues in bacteriorhodopsin [16]. We are presently using ^{19}F-NMR to study the structure of fluorine-labelled light-harvesting complexes reconstituted into an artifical membrane.

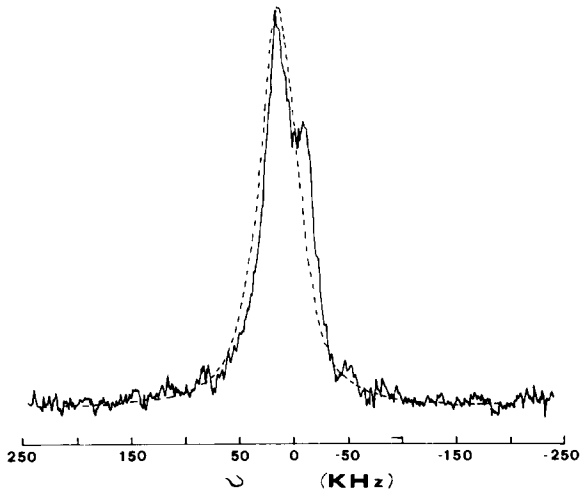

Fig.3. Broad-band ^{19}F-NMR spectra of native chromatophore membranes from *R. rubrum* labelled biosynthetically with 3-^{19}F-phenylalanine (——), and a dry powder (– – –). The spectra were taken using the spin-echo sequence described in the text. 210 scans were used to acquire the spectrum for the native membrane, and both spectra shown were transformed using an exponential line-broadening factor of 5 kHz prior to Fourier transformation.

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