INVESTIGATION OF THE LOWEST TRIPLET STATE OF THE PYROCHLOROPHYLLIDE a–APOMYOGLOBIN COMPLEX BY ZERO-FIELD OPTICALLY DETECTED MAGNETIC RESONANCE SPECTROSCOPY

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The zero-field fluorescence-detected triplet state magnetic resonance spectra have been obtained for the pyrochlorophyllide a–apomyoglobin complex at 2 K. The triplet state zero-field splittings and spin sublevel dynamics were detected on the resolved features of the structured low-temperature fluorescence. Structured fluorescence is not observed for pyrochlorophyllide a in an organic matrix under identical conditions. These data are interpreted in terms of the local binding site of the pyrochlorophyllide a chromophore in the protein and the low-temperature conformation of the protein matrix

1. Introduction

The interaction of chlorophyll pigment molecules with a protein environment plays a fundamental role in the processes of photosynthesis. Proteins provide the principal structural elements in both antenna and reaction center complexes, and the positioning of in vivo chlorophylls for efficient energy and electron transfer may be determined by such interactions. Furthermore, it is likely that chemical properties of chlorophyll in vivo, such as reduction potentials and the stabilities of radical intermediates, are modulated by the protein environment.

Perhaps the most detailed information obtained to date on chlorophyll–protein complexes in green plant systems comes from spectroscopic studies of the antenna chlorophylls associated with the light harvesting chlorophyll complex (LHCP) [1,2]. This complex can be isolated from chloroplasts [2,3], and its electronic spectra have been investigated [1,4,5]. But the most revealing spectroscopic data, obtained by resonance Raman spectroscopy demonstrates that the antenna chlorophyll pigment exists in the LHCP as *monomeric* units, bound to the protein through their magnesium central atoms and through hydrogen bonding of their carbonyl groups with various amino acids [3,6].

The essentially monomeric nature of the antenna chlorophyll in its protein environment has prompted our examination, by triplet state spectroscopic techniques, of a model monomeric chlorophyll–protein complex, the pyrochlorophyllide a–apomyoglobin system, in which pyrochlorophyllide a is substituted for heme in the extensively characterised protein myoglobin (Mb). This 1 : 1 complex has been studied in detail by optical spectroscopy in solution [7,8], in single crystals [9] and by NMR [7,8]. Our interest in the triplet state properties, as revealed through zero-field optically detected magnetic resonance (ODMR), arises because the zero-field splittings and sublevel decay rates are well known to be highly sensitive to the nature of the surrounding environment for monomeric chlorophyll system [10].

In this paper we present results of low-temperature ODMR spectroscopy which provide an indication of the nature of the local binding site for the pyrochloro-

phyllide a molecule unit within the apomyoglobin protein environment. These data are discussed with regard to the presence of two conformational states of the protein, evidence for which has been noted in th NMR spectrum at room temperature in previous work [8].

2. Experimental techniques

The preparation of the pyrochlorophyllide a–apomyoglobin complex has been extensively described in previous publications [7,8]. Samples of the complex were prepared for ODMR experiments by dispersing the complex in a 1 : 1 water . glycerol solution. The samples were then slowly cooled to ≈ 77 K in the nitrogen-purged inner compartment of a liquid-helium dewar. After this slow cooling procedure, liquid helium was introduced directly into the sample chamber.

The arrangement for fluorescence-detected ODMR experients, run at 2 K, were the same as utilized in previous chlorophyll ODMR experiments [11]. The samples were excited by direct irradiation with either the 514.5 nm or the 457.9 nm line of an argon-ion laser (Spectra-Physics model 164) operated at 0.5 W output power for the measurement of the ODMR transition frequencies and over the range 0.5–0.05 W for the triplet spin sublevel dynamics experiments [11].

3. Results

The laser-excited (457.9 nm) low-temperature (2 K) fluorescence of the pyrochlorophyllide a–apomyoglobin complex in solution is shown in fig. 1. Changing the excitation wavelength changes the relative contribution to the fluorescence intensity of the two peaks observed at 671 nm and 685 nm; for example, using laser excitation at 514.5 nm, the spectrum consists of virtually only the lower-wavelength feature, with the 685 nm band a slight shoulder on the high-wavelength side. A similar doubling could be observed when zinc was substituted for magnesium in the complex, but in this case the peaks were not as clearly resolved as in fig. 1.

Zero-field fluorescence-detected ODMR spectra

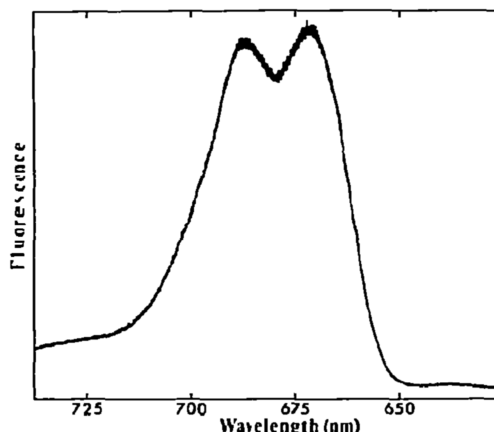

Fig. 1. The pyrochlorophyllide a–apomyoglobin complex fluorescence spectrum in a frozen solution of water and glycerol (1 : 1) at 2 K. The fluorescence was excited by the 457 9 nm line of an ar on-ion laser.

could be observed on the two fluorescence peaks, giving two different sets of triplet state zero-field transitions whose frequencies are listed in table 1. The ODMR spectra obtained were in both cases strong and reasonably sharp, with linewidths on the order of 30 MHz. The ODMR transitions in all cases corresponded to a microwave-induced decrease in the fluorescence intensity at 671 and 685 nm for the magnesium complex.

For comparison with a simple organic solution environment, ODMR results for the pyrochlorophyllide a molecule in frozen toluene matrix are also presented in table 1. No attempt was made to rigorously dry the toluene used in these experiments. The linewidths for the ODMR zero-field transitions in toluene are comparable to those observed in the apomyoglobin complex.

4. Discussion

The appearance of two peaks in the low-temperature laser-excited fluorescence spectrum of the magnesium pyrochlorophyllide a–apomyoglobin complex is in contrast to the fluorescence observed for magnesium pyrochlorophyllide a in hydrocarbon solution, which is identical in features to chlorophyll a in solution at low temperature, viz., a single broad

Table 1
Pyrochlorophyllide a–apomyoglobin complex ODMR data (2 K)

λ_{ex} (nm)	$\lambda_f(max)$ (nm)	$\lvert D-E \rvert$ (MHz)	$\lvert D+E \rvert$ (MHz)	k_x (s^{-1})	k_y (s^{-1})	k_z (s^{-1})	k_T (s^{-1})
457.9	671	780	1002	814(70)	714(10)	31(5)	520
	685	752	949	804(70)	832(25)	69(8)	568
514.5	668	784	1012	–	–	–	–
pyrochlorophyllide a in toluene							
457.9	680	724	943	–	–	–	720

band in the region 670–680 nm, the exact position of which depends on the wavelength of excitation [10]. That the two fluorescence peaks observed in the complex correspond to two distinct pyrochlorophyllide emitting units is corroborated by the two sets of ODMR frequencies detected at each fluorescence maximum. Both sets of ODMR frequencies are in the general region expected for the pyrochlorophyllide a molecule, as seen in table 1.

A most revealing result concerning the nature of the two pyrochlorophyllide a emitting sites in apomyoglobin is found in the triplet state intersystem crossing rates. It is expected from previous ODMR results on chlorophylls that the intersystem crossing rates will be most sensitive to the pyrochlorophyllide a local site environment, particularly with regard to ligand attachment at the magnesium center [10,12]. Such an effect has been shown for chlorophyll a when different ligand states associated with different numbers of water molecules complexed to the metal center of the molecule may be selected by laser excitation, and the triplet lifetime changes appreciably with the change in laser excitation wavelength [10]. As described in previous work [10], the chlorophyll a triplet lifetime decreases by an approximate factor of two as one goes from monoligated to biligated monomer. A similar situation applies to the values for pyrochlorophyllide a in toluene (not rigorously treated for removal of water). The results in table 1 for laser excitation of pyrochlorophyllide a at 457.9 nm parallel exactly the results observed at that wavelength for chlorophyll a (the zero-field frequencies and triplet lifetime are, within experimental error, the same as for chlorophyll a, detected at 680 nm), and the triplet species reported in table 1 is, by comparison with the chlorophyll a triplet results, assigned as pyrochlorophyllide a·$2H_2O$ [10]. Yet, in apomyoglobin the overall triplet lifetime *and* the individual rates of intersystem crossing give, within experimental error, the same results when detecting on either the 671 or 685 nm fluorescence bands, with the overall triplet lifetime in each case approximately equal to the average of the two values reported for monoligated and biligated chlorophyll a in solution [10].

The differences in zero-field splittings observed at the two fluorescence detection wavelengths and the simultaneous agreement of intersystem crossing rates suggest a straightforward explanation of the pyrochlorophyllide emitting sites in the protein environment. If the protein is capable of dynamical interconversion between two conformations at room temperature, as suggested by earlier NMR work [8], at the cryogenic temperatures of the ODMR measurements, the protein complex will freeze into a statistical distribution of *both* conformations. Each conformation will result in a slightly different electrostatic protein environment about the pyrochlorophyllide ring, resulting in two sets of triplet states with different zero-field splitting parameters. However, since changes in the metal coordination would produce substantial changes in the triplet state intersystem crossing rates [10], we conclude that the *local* binding of the chromophore is identical in the species giving rise to the different fluorescence peaks. It is interesting to note that the triplet lifetime measured by ODMR in the pyrochlorophyllide–apomyoglobin complex lies between the values associated with the monoligated and biligated forms of chlorophyll a [10]. Such a value suggest binding of a single external ligand in strong coordination to the metal center, as might be provided by an amino acid residue such as proximal histidine-F8, which strongly coordinates iron in native Mb. It is possible,

however that the triplet lifetime is also decreased over that expected for a monoligated chlorophyll ring in hydrocarbon solution due to a second, more distant interaction with water or another amino acid side chain (such as distal His-E7) which is weakly associated with the sixth coordination site of the metal center. Another possibility is that the distinguishable fluorescence peaks in fig. 1 and the corresponding triplet state features arise from the two possible five-coordinate chromophores distinguished by inversion about the chromophore α–γ axis in the heme pocket and ligation on either side by His-F8. NMR data at room temperature in aqueous solution suggest that the NMR peak doubling does not arise from such a mixture of complexes in which the macrocycle is inverted in the heme pocket [8], though this possibility cannot be ruled out completely. Such a mechanism has been suggested to explain similar NMR observations on re-on reconstituted heme proteins [13]. A fuller description of the details of the metal coordination will have to await the complete X-ray analysis currently in progress on single crystals of the complex [9]. Finally, we note that there is evidence for multiple, spectroscopically and kinetically distinguishable states of native Mb [14,15], and the ODMR measurements described in this note on heme analogous with diamagnetic ground states may relate to those observations.

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