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Protein and chlorophyll in photosystem II probed by infrared spectroscopy

Camille Chapados [a], Sylvie Lemieux [b] and Robert Carpentier [b]

[a] *Département de chimie-biologie and* [b] *Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières, Trois-Rivières, Québec G9A 5H7, Canada*

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The infrared spectra of photosystem II (PS II) enriched submembrane fractions isolated from spinach are obtained in water and in heavy water suspensions. Other spectra are obtained after a photooxidation reaction was performed on PS II to bleach the pigments. The water bands are removed by computer subtraction and the amide bands (A, B, I, II, and III) of the protein are identified. Computer enhancement techniques are used to narrow the bandwidth of the bands so that the weak chlorophyll bands, buried in the much stronger protein bands, can be observed. Comparing the spectra of native and photooxidized PS II preparations in water and in heavy water, we determine that three polypeptide domains are present in the native material. The first domain, which contains 22% of the polypeptides, is situated in the peripheral region of the PS II system. The polypeptides in this region are unfolded and devoid of chlorophyll. The second domain contains 41% of the polypeptides, is more organized, and contains the chlorophylls. The third domain has an α-helix configuration, does not contain chlorophyll, and is not affected by the photooxidation reaction or by the proton/deuteron exchange. Three different types of chlorophyll organisation are identified: two have their ketone carbonyl groups non-bonded, differing from one another only in their hydrophobic milieux; the third is weakly bonded to another unidentified group. Other forms of chlorophyll organisation are present but could not be observed because their absorption is buried in the protein amide I band.

1. Introduction

In the chloroplast of higher plants the major photosynthetic pigments are the chlorophylls and the carotenoids. These pigments which harvest light energy are associated with specific polypeptides to form pigment-protein complexes. These are organized in core or peripheral antenna complexes which are assigned to PS I or II. The reaction centre of PS I, P-700, is buried between two or four large polypeptides (about 70 kDa each) which also contains the core antenna Chl *a* [1,2]. To this core complex are associated two peripheral antenna complexes which contain Chl *a* and Chl *b*. One of these, LHC Ia, emits fluorescence at 680 nm, the other, LHC Ib, emits at 730 nm [3].

The PS II light-harvesting system has a different organization concerning the core antenna which is not located on the same polypeptides that contain the reaction centre P-680. The latter is imbedded within two polypeptides of 32 kDa each named D1 and D2 [4]. Two core antenna complexes, CP47 and CP43, are linked to this reaction centre complex and the peripheral antenna is composed of the major Chl-protein complex LHC II complemented by at least two minor light-harvesting units, CP29 and CP24 [5].

Correspondence address: C. Chapados, Département de chimie-biologie, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Québec G9A 5H7, Canada.

Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; FWHH, full-width at half-height; IR, infrared; LHC, light-harvesting complex; Mes, 2-(*N*-morpholino)ethanesulphonic acid; PS II, photosystem II; P-700, reaction centre of PS I.

The organization of the pigment molecules within their apoproteins and the relation between the complexes are not completely understood. These matters are the object of close examinations. Summarizing the status on this question, Eisenberg [6] gives the following resumé: the polypeptides in the membrane form α-helices whose hydrophobic portions cross the lipid bilayer while the more hydrophilic portions of the amino acid chains reside in the aqueous phase. For LHC II, three potential membrane-spanning α-helices have been identified from the analysis of the amino acid sequence of the apoprotein [7,8]. Although the exact nature of the Chl-protein interaction is still unknown, Hinz and Welinder [9] and Butler and Kuhlbrandt [10] have suggested that 13–15 Chl molecules lie within the α-helices of the LHC protein monomers.

In LHC II, the electronic Q_y dipoles of the Chls are preferentially oriented parallel to the membrane plane and therefore mostly parallel to the loops of the α-helices within the apoprotein [11,12]. The porphyrin ring of the Chl molecules could be imbedded between the α-helices where they would interact with the side groups of the protein amino acids.

From studies of the aggregation of chlorophylls, using model systems, it is known that the central Mg^{2+} and the carbonyl groups in the porphyrin ring are the groups that can be involved in intermolecular bonding with available donor groups [13–17]. Of the three carbonyls in Chl *a*, the ketone carbonyl on C_{28} (notation in ref. 18) is the group that makes the stronger intermolecular bond which makes it the favoured group for such a bond [19].

It was suggested that charged amino acid residues [20] or hydrophilic moieties on the protein [19] could be responsible for the bathochromic shift of the red band in the absorption spectrum of chlorophyll. Therefore, the position of the red peak could depend on the interaction of the Chl molecules with various amino acids. Because each Chl molecule is probably located in slightly different surroundings, it is conceivable, that after complete spectral separation, one could find a different absorption spectrum for each Chl molecule of each Chl-protein complex.

The above presentation certainly underlines the difficulties encountered when studying Chl-protein organizations. To obtain new information on the type of interactions involved and on the general structure of the Chl-protein organization, we undertook an infrared (IR) spectroscopic study of some photochemically active chloroplast preparations. The advantage of the IR technique is that it allows examination of the vibrational bands as they are modified by their interacting milieux. Infrared spectroscopy has already been used to characterize a light-minus-dark infrared spectrum of photochemical reaction centres [21,22].

To simplify the problem, we have used a PS II submembrane fraction instead of the whole thylakoid membrane. Although the analysis of the IR spectra of chloroplast involves several species: water, different proteins, Chl *a*, Chl *b*, lipids, carotenoids, etc., we show in this IR study that, besides the strong water and protein bands, some Chl bands can be identified.

Because all the IR bands of pure Chl are not assigned [23,24] and of those that are properly assigned, many are buried in much stronger protein bands, it is impossible at the present time to have a complete picture of the interaction of the Chl molecules with the surrounding proteins. However, by carefully analyzing the behaviour of the major protein and Chl IR bands before and after deuteron/proton exchange and upon degradation of the pigments and alteration of the membrane by a photooxidation reaction, we obtained some information on the structure of the membrane proteins and on the Chl molecules which interact with them.

2. Materials and methods

2.1. *Preparation of PS II submembrane fractions*

PS II submembrane fractions depleted of PS I are isolated by the method of Ghanotakis et al. [25]. The resulting preparation is resuspended in 20 mM Mes-NaOH (pH 6.5), 1 mM NaCl, and 0.5 mM $MgCl_2$ to give a Chl concentration of 2 mg/ml. The suspension is stored in liquid nitrogen until needed. The Chl concentration is determined by the method of Arnon [26].

2.2. Photooxidation experiments

Photooxidation consists in oxidizing the PS II preparations with light [27]. A 2 mg Chl/ml sample is diluted to 10 μg Chl/ml in 20 mM Mes-NaOH (pH 6.2), 1 mM NaCl, and 0.5 mM $MgCl_2$. The suspension is illuminated with a 150 W quartz-halogen lamp until the sample is completely bleached (~ 30 min) [28]. To minimise heating, a glass container (2 cm thick) filled with water is placed between the lamp and the sample. The bleached material is recovered by centrifugation at $35000 \times g$ for 30 min and resuspended in the buffer.

2.3. Preparation of the samples for IR spectroscopy

For the native and photooxidized PS II, the samples are centrifuged for 5 min in an Eppendorf microcentrifuge. The supernatant is aspirated with a Pasteur pipette and the pellet is suspended in ordinary water or heavy water as required. After washing with H_2O or D_2O, the pellet is pressed between two BaF_2 windows and their IR spectra taken.

PS II proteins are extracted by mixing 250 μl of native or photobleached preparations with 5 ml of pure acetone. After washing several times with acetone, the proteins are dried for 24 h in a desiccator. The dried samples are mixed with KBr powder and pressed into pellets. As a comparison, the spectrum of BSA (97–98% pure from Sigma) in a KBr disk is obtained.

2.4. Spectrum recording and computer treatment

The IR spectra are recorded at 33°C on a model 180 Perkin-Elmer spectrophotometer with a resolution near 2 cm^{-1}. Dry air is circulated in the spectrometer to remove atmospheric water and CO_2. The calibration is made with the water vapour bands; the precision of the PS II bands is better than 1 cm^{-1} except for broad bands. For each sample, three spectra are recorded on floppy discs using the 3600 Data Station of Perkin-Elmer. The spectral data are transferred to a Cyber 830 of Control Data for treatment. For each spectrum, the average of the original spectra is taken, calibrated and subtracted from the background.

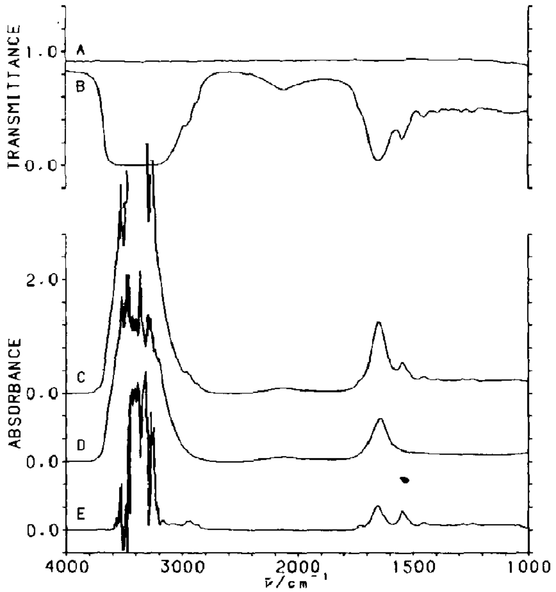

Fig. 1. Treatment of the IR spectra of PS II preparations. (A) Background in transmittance; (B) PS II preparation; (C) same as B but in absorbance with the background subtracted; (D) liquid water; (E) same as C with water subtracted.

For native or photooxidized PS II preparations, a fraction of the spectrum of a water film kept between BaF_2 windows, is subtracted as illustrated in fig. 1. The water band near 1640 cm^{-1}, that near 2200 cm^{-1}, and the absorption far from resonance of the 3400 cm^{-1} band are used to determine the fraction of the water spectrum to be subtracted. The procedure is correctly performed when the absorption of water falls to zero with no negative lobes. This is verified in fig. 1E where we see that the result is very good.

The absorption spectra are obtained on a Perkin-Elmer model 553 UV-Vis spectrophotometer using 10-mm quartz cells. The resolution and the precision of the bands are 1 nm. The spectral data are stored on floppy discs and are treated as for the IR data. The experimental details for obtaining the electronic and IR spectra of pure Chl *a* are given elsewhere [19].

Since different chloroplast extracts were used for the spectra of each species given here it is necessary, after removal of the water bands, to normalize the intensity of the spectra in order to

Table 1

Position in (cm^{-1}) of the infrared bands of Chl and proteins

s, strong; m, medium; w, weak; v, very; sh, shoulder; sym, symmetric; asy, asymmetric; def, deformation.

Chl *a*			BSA in KBr (fig. 3)	PS II protein in KBr		PS II/H_2O		PS II/D_2O		Assignment
Amorphous (ref. 19 and fig. 4)	Solvated	In KBr [23]		Native (fig. 3)	Oxidized	Native (fig. 2)	Oxidized	Native (fig. 2)	Oxidized	
								3417		
			3308	3296	3340			3302	3285	amide A
				3180	3180	3182				
			3060	3073	3062	3063	3070	3067	3065	amide B
								3014		
			2960	2960	2958	2957	2957	2956	2957	CH_3 asy. stretch
			2940	2935	2927	2927	2930	2927	2929	CH_2 asy. stretch
			2872	2874	2872	2875	2875	2871	2872	CH_3 sym. stretch
						2856	2857	2856	2856	CH_2 sym. stretch
1735	1735	1737 vs			~1735	1737	~1730 sh	1736	~1733 sh	CO ester
1690	1696	1690 vs				~1680				Chl free CO keto
			1660	1658	1665	1653	1651	1653	1650	amide I
1651	1659	1657 vs								Chl assoc. CO keto
1608	1609	1607 vs	1607			~1610				Chl C = C, C = N
								1575sh	1582	
1550	1552	1550 vs								Chl C = C, C = N
1532	1533	1533 vs								Chl C = C, C = N
			1532	1531	1530	1547	1545	1548	1550	amide II
						1530	1528	1526	1525	
			1519	1520	1517					
1489	1489	1491 m								
		1462 sh								
				1468						
1447		1451 m	1452	1450	1452	1454	1453	1453	1451	def. CH asy.
			1443							
		1433 m					1416			
1376	1377	1378 w	1387	1390	1385	1397		1400		
								1365	1366	
1343	1344	1344 m				1346		1343	1350	Chl
		1326 w								
		1303 vw								
			1303	1325	1307	1303				amide III
1283	1285	1284 s				1287	1278 sh	1286		Chl
		1239 vw						1249		
			1237	1234	1237	1243	1244	1240	1249	
								1227		
1186	1184	1185 s				1186		1193	1193	Chl
			1170	1169	1168	1169	1165	1165	1168	
	1159	1160 m								
		1148 m								
1129	1129	1130 m				1125		1127		
			1121							
		1102 vw	1105	1110	1112			1107		
			1096							
1057		1068 w		1076	1073	1080	1082	1070	1083	skeletal mode
	1053									
1041		1042 m		1037	1036	1047		1048		Chl
	1032									

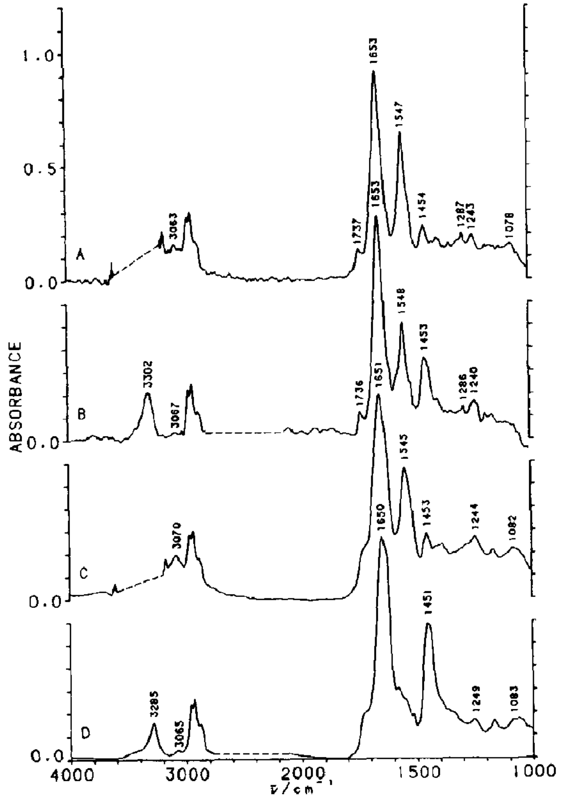

Fig. 2. (A) Native PS II preparation in H_2O; (B) in D_2O; (C) photooxidized PS II preparation in H_2O; (D) in D_2O. Water (H or D) is subtracted from the spectra.

evaluate correctly the spectral modifications following the different perturbations that the preparations endure. For this purpose, we have used the CH_2 asymmetric stretch band situated near 2930 cm^{-1} (table 1), since this group is not affected by the oxidation reaction. Also, the hydrogens in this group do not exchange with the deuteriums of D_2O when the extract is washed with heavy water. For the samples in ordinary water, the slanting background is subtracted. To verify whether the normalisation procedure had proceeded correctly we examined the intensity of the amide I band at 1653 cm^{-1} of the native sample in water and in heavy water (fig. 2A and B), since this band contains mainly CO stretch vibrations which are unaffected by the type of water surrounding this functional group. Also, the conformation of the polypeptides is not affected by the type of water used. For the same reasons, we have compared the intensity of the amide I band of the oxidized samples in water and in heavy water (fig. 2C and D). The results are satisfactory.

To enhance the spectra so as to reveal concealed features in the bands, two computer techniques are used: the second derivative and Fourier deconvolution methods. The separation of the components in the amide A and amide II bands is carried out by computer band fitting using Cauchy-Gauss product functions. Details of the computer methods used in the enhancement and the band fitting procedures are described in refs 29 and 19.

3. Results

The different steps involved to obtain useful IR spectra from the PS II preparation are illustrated in fig. 1. The background spectrum A and the spectrum B of the native PS II preparation both in transmittance are transformed in absorbance and subtracted one from the other to give spectrum C. From the latter, a fraction of the water spectrum D is subtracted to give the final spectrum E. The above procedure allowed us to analyze the IR spectrum of PS II preparations between 4000 and 1000 cm^{-1} except in the OH stretch region (3700–3200 cm^{-1}) where water absorbs strongly. By carefully performing these operations, it was possible to obtain the small amide B band at 3063 cm^{-1} (figs 1E and 2A) buried in the strong OH absorption (fig. 1C). These results give us confidence that the intensity of the amide I band observed near 1653 cm^{-1} and that of the amide II at 1547 cm^{-1} are reliable (fig. 2A). Accordingly, the amide I band is free of the absorption of water near 1650 cm^{-1}.

To observe the bands in the 3700–3200 cm^{-1} region, the samples are resuspended in heavy water. In this case the OD stretch blackout is situated between 2780 and 2000 cm^{-1} (fig. 2B). After subtracting the heavy water spectrum, a small amount of H_2O was detected in the remaining spectrum and subtracted as before. This procedure enables the observation of amide A and B bands situated at 3302 and 3067 cm^{-1}, respectively.

3.1. Identification of the protein bands in PS II

The first goal in this study is to identify the origin of the bands obtained in the complex PS II spectra presented in fig. 2A and B. To do this, we first identify the protein bands by isolating them from acetone extracts and preparing KBr pellets as decribed in section 2.3. In fig. 3, we compare the spectrum of native PS II proteins (trace B) and oxidized PS II proteins (trace C) with that of BSA (trace A). In these spectra, we observe some diffusion which gives slanted spectra; the strong broad absorption band near 3300 cm^{-1} is due in most part to residual water. However, all the major protein bands observed in fig. 3 are identified in table 1: amide A (near 3300 cm^{-1}), amide B (near 3065 cm^{-1}), amide I (near 1660 cm^{-1}), amide II (near 1531 cm^{-1}), and amide III (near 1300 cm^{-1}). The bands identified in the pure protein extracts are used to assign the protein bands in the native PS II preparations (table 1).

The amide A band of native PS II is masked by the strong OH band of water (fig. 2A). This band is observed in the spectrum taken in D_2O (fig. 2B). In the latter, the relative intensity of the amide II band compared to the amide I band is smaller than that obtained with H_2O. This event coincides with a stronger band appearing at 1450 cm^{-1} which is assigned to the amide II band involving deuterium instead of hydrogen as a result of proton/deuteron exchanges.

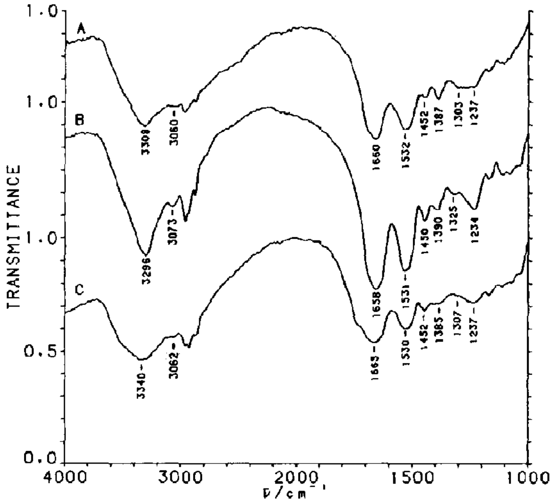

Fig. 3. Dry proteins in KBr disks. (A) Bovine serum albumin (BSA); (B) PS II protein; (C) photooxidized PS II protein.

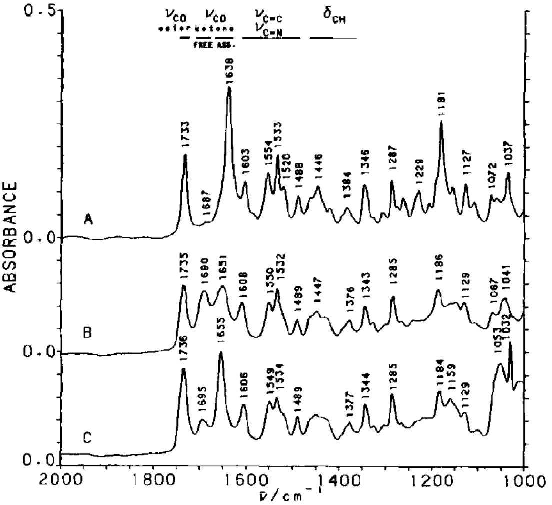

Fig. 4. IR spectra of: (A) crystallised hydrated Chl *a*; (B) amorphous Chl *a*; (C) propanol solvated Chl *a*.

3.2. Identification of the chlorophyll bands

The protein bands dominate the IR spectra of PS II in fig. 2. Some of the weak bands that cannot be identified as protein bands are assigned to Chl. This is done by comparing the spectra of PS II with those of pure Chl *a* in various aggregation states given in fig. 4 where some spectral regions are identified. We note that on the spectra of the crystallized (A), amorphous (B), or alcohol solvated (C) Chl *a*, the bandwidth and the band shape of the bands are similar.

Excluding the ketone carbonyl stretch bands in the 1720–1620 cm^{-1} region where aggregation manifests itself, the positions of the bands of Chl *a* in different aggregation states do not change greatly (fig. 4). In chloroplasts, one out of three Chl molecules is a Chl *b* molecule. The spectrum of the latter is similar to that of Chl *a* save for the absorption of the formyl carbonyl groups (1650–1600 cm^{-1}) [16]. Therefore, except in the ketone carbonyl region, the position of the IR Chl bands in PS II should not be much different from that of amorphous Chl *a*.

The ester carbonyl band is located at 1735 cm^{-1} for amorphous Chl *a* (fig. 4B), at 1736 cm^{-1} for alcohol solvated Chl *a* (fig. 4C), and at 1733 cm^{-1} for crystallized Chl *a* (fig. 4A). The position of this band is less sensitive to aggregation than the ketone carbonyl bands. The band observed at 1737 cm^{-1} in PS II (fig. 2) is therefore partly assigned to both the ester carbonyl of Chl and to the lipid esters which also absorb in this region.

In the ketone carbonyl region, the Chl bands are overlapped by the strong amide I band at 1653 cm^{-1}. Hence, it is difficult to evaluate the situation of this group. The same occurs for the C = C, C = N bands situated around 1600 cm^{-1} which are overlapped by the strong amide II band. We have used computer band enhancement techniques to retrieve the overlapped bands (see section 3.5).

The small bands observed at 1346, 1287, and 1186 cm^{-1} for PS II in H_2O (fig. 2A) are recognized as Chl bands because similar bands are observed in the spectra of pure Chl *a* (fig. 4) and because they are modified by the oxidation procedure (fig. 2C) which destroys the chlorophylls (see section 3.5). Similar bands are observed for PS II in D_2O (fig. 2B and D).

The CH stretch bands of the protein, lipid, and chlorophyll molecules appear in the 2900 cm^{-1} region. These bands are not modified by the deuteration and oxidation procedures. Therefore, this region is not useful for identification purposes but may be used for normalisation of the spectra.

From all these observations we see that the readily resolved bands in the spectrum of fig. 2A are assigned to protein, chlorophylls or lipids (table 1). This assignment is verified by the study of photooxidized PS II. Upon illumination, excited Chl molecules trigger a photoreaction which produces some highly reactive oxygen radicals and singlet oxygens [30,31]. During photooxidation, the Chl absorption is lost which is an indication that the chlorophylls are destroyed. The result of this situation is the opening of the porphyrin ring, the breaking of most of the C = C, C = N double bonds, and the destruction of the Chl-protein complex. As a consequence of these modifications, smaller molecules than chlorophyll or porphyrin are formed. The hydrophilic molecules formed are eliminated during the washing-centrifugation cycles.

One exception to this pattern is the ester groups. As can be seen from fig. 2, the intensity of this band is not greatly decreased after photooxidation although its shape and position are modified compared to that of the native samples. The shoulder near 1730 cm^{-1} in the spectra of the oxidized samples (fig. 2C and D) is an indication that the environment is modified by the photooxidation process compared to the native samples where a neat band appears at 1737 and 1736 cm^{-1} (fig. 2A and B) for the sample in water and heavy water, respectively. The ester groups are end products of an oxidation process and are more difficult to destroy photochemically than the other double bonds. Therefore, the ester groups are not decomposed and form insoluble molecules containing aliphatic chains.

3.3. Electronic spectrum of PS II

The electronic absorption spectra of PS II were recorded before and after photobleaching. The spectra (not shown) of the photobleached samples showed only a continuous absorption which increased as the wavelength decreased. This constitutes evidence that the pigments have been decomposed. The continuous absorption is attributed to diffusion from the membrane aggregates. Since the sizes of the aggregates are approximately the same in native and bleached samples, we have used the spectrum of the oxidized PS II as the background for the spectrum of native PS II to correct for the diffusion. The resultant spectrum presented in fig. 5A shows a band at 681 nm ascribed to Chl *a*, a shoulder at around 654 nm due to Chl *b*, a band at 483 nm ascribed to carotenoids and Chl *b*, and a band at 439 nm assigned to Chl *a*.

As a comparison we have included in fig. 5B, the spectrum of amorphous Chl *a* obtained by the electrodeposition technique [19]. There is a general agreement between this spectrum and that of na-

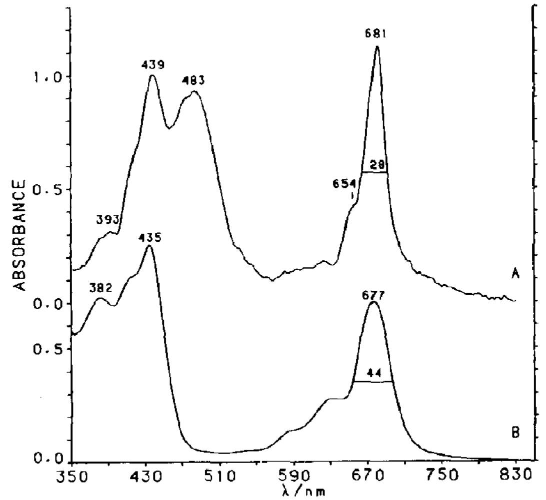

Fig. 5. Absorption spectra of: (A) PS II minus photooxidized PS II; (B) amorphous Chl *a*.

tive PS II if we do not consider the bands situated at 483 and 654 nm. However, several differences between the two spectra are worth appraising. In particular: (i) the position of the red band maximum is located at 681 nm for PS II and at 677 nm for amorphous Chl *a*; (ii) the FWHH of PS II is 28 nm and that of amorphous Chl *a* 44 nm. The latter is larger than the former which also contains a small amount of Chl *b*.

A red band situated at long wavelengths indicates that the molecular arrangement of Chl *a* in a system is more organised than one whose red band appears at lower wavelength [16]. As for the bandwidth, work performed in this laboratory showed that the FWHH of monolayers is smaller than that of multilayers. The Chl molecules of the former are more ordered than those in the latter [17,29]. When Chl *a* is solvated in the walls of vesicles made with depigmented chloroplast lipids, the FWHH of the red band is 28 nm [32]. The lipids provide similar environments for the chromophores and explain the narrow bandwidth.

The position at a relatively high wavelength and the narrow FWHH of the red absorption band of native PS II are indications that the Chl molecules are well organized in this system, much more than in amorphous Chl *a*.

3.4. Photooxidized PS II

The IR spectra of photooxidized PS II preparations obtained in the presence of H_2O or D_2O are illustrated in fig. 2B and 2D, respectively. The IR spectrum from proteins isolated from this photooxidized material is also shown in fig. 3C. The most apparent feature observed on these spectra is a general broadening of the amide bands compared to those of native PS II. Furthermore, in the PS II spectrum taken with D_2O, the amide II band at around 1548 cm^{-1} has decreased substantially and the band at 1451 cm^{-1} has increased correspondingly (fig. 2D). These modifications coincide with a simultaneous decrease in the intensity of the amide A band near 3300 cm^{-1} (fig. 2B and D).

Changes in the IR bands of Chl *a* are more difficult to observe owing to the strong intensity of the amide bands and to the low intensity of the Chl bands. However, from the electronic spectra we know that the chromophores are decomposed by fragmentation. The soluble molecules are washed away in the aqueous solutions. For instance, the bands located at 1287 cm^{-1} in the spectrum of native PS II in water (fig. 2A) and at 1286 cm^{-1} in heavy water (fig. 2B) have completely vanished from the spectra of the photobleached materials (fig. 2C and D). The 1287 cm^{-1} band which is assigned to Chl as well as the other bands identified as Chl bands is discussed below.

3.5. Enhanced IR spectra

To establish more clearly the differences between the spectrum of the native sample (fig. 6B) and that of the photooxidized one (fig. 6C), we used two enhancement procedures which were employed previously in the study of pure Chl *a* [29,19]: the Fourier deconvolution technique (spectra indicated by prime (′)) and the second derivative technique (spectra indicated by double prime (″)). We see that all the small features barely visible in the regular spectra become obvious in the enhanced spectra.

Comparing the Fourier deconvoluted spectra (fig. 6B′ and C′) with the second derivative spec-

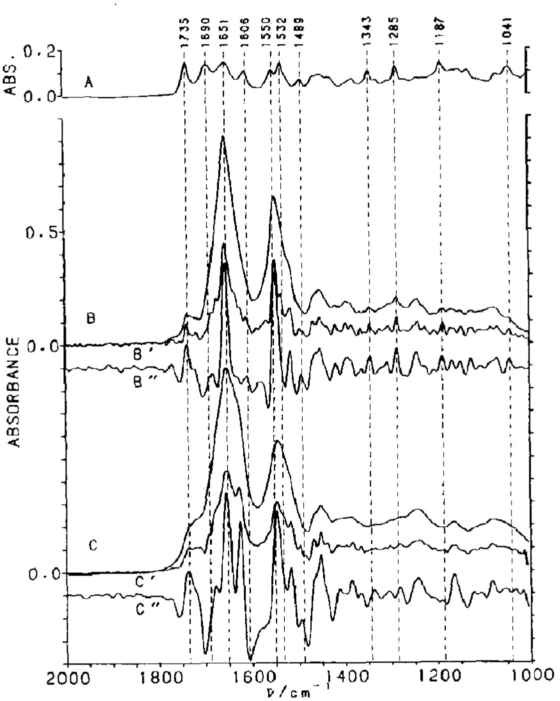

Fig. 6. IR spectra of: (A) amorphous Chl *a*; (B) native PS II preparation in H_2O; (B′) Fourier deconvoluted; (B″) second derivative; (C) photooxidized PS II in H_2O; (C′) Fourier deconvoluted; (C″) second derivative.

tra (fig. 6B″ and C″), we note that the same features are apparent in both enhanced spectra. Because negative lobes appear in the second derivative spectra, some of the features are less obvious in these enhanced spectra than in the Fourier deconvoluted ones. We used the second derivative spectra essentially as a check to ascertain that the features obtained in the Fourier deconvoluted spectra are real. In the following analysis we will use mainly the Fourier deconvoluted spectra.

The Fourier deconvoluted spectrum of PS II in water (fig. 6B′) shows some features that disappear almost completely in the spectrum of the oxidized sample (fig. 6C′). These features, indicated by dashed lines, coincide with the bands of amorphous Chl *a* (fig. 6A) and confirm the presence of Chl bands in the native PS II spectrum. The spectrum of the oxidized sample (fig. 6C) shows fewer small features than the native sample (fig. 6B) because the oxidizing process destroys all the chromophores by breaking these molecules into smaller ones. The process also disrupts the membrane organization. The water-soluble molecules are eliminated by the washing-centrifugation cycles. Very little is left of the original chromophores; only the solid insoluble fragments remain in suspension and these do not absorb at the position of the chromophores except for the ester groups.

The bands near 1735 cm^{-1} are assigned to ester groups of chlorophylls and lipids. Following photooxidation, the bands are modified although their integrated intensities are not appreciably decreased compared to those of the bands before oxidation. This is due to the fact that the ester groups are not decomposed by the oxidation reaction. However, the bands are displaced because the environment of these groups is modified.

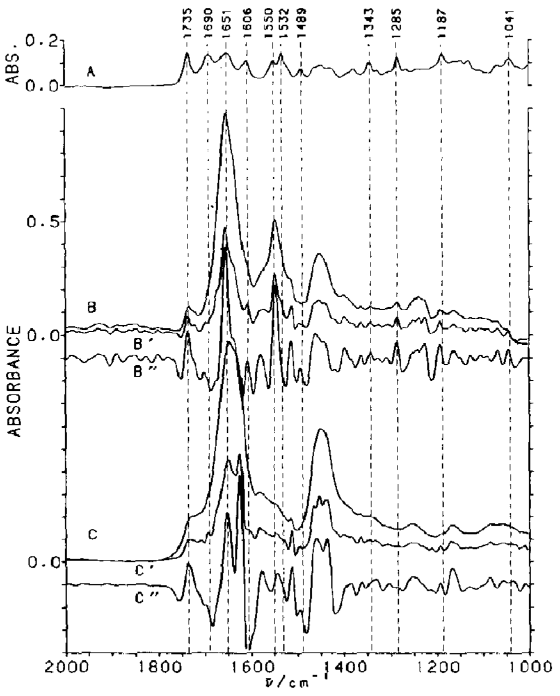

Fig. 7. IR spectra of (A) amorphous Chl *a*; (B) native PS II preparation in D_2O; (B′) Fourier deconvoluted; (B″) second derivative; (C) photooxidized PS II in D_2O; (C′) Fourier deconvoluted; (C″) second derivative.

The amide I band is modified by the oxidation process. The strong band situated at 1654 cm^{-1} in native PS II (fig. 6B) has broadened and a new feature situated at 1624 cm^{-1} has appeared. These modifications indicate that some of the α-helical structures of the native sample have unfolded.

On the high frequency side of the amide I band of native PS II (1654 cm^{-1}, fig. 6B′), we observe some low intensity bands situated around 1690 cm^{-1}. In this region no conformational organization of polypeptides absorbs but the free ketones of Chl *a* do (fig. 6A). On the low-frequency side of the amide I band (1654–1600 cm^{-1}) we observe some low-intensity bands. The associated ketone band and one C = C, C = N band of Chl *a* are situated in this region (fig. 6A and ref. 19). Also, some conformers of polypeptides absorb in this region. PS II in heavy water (fig. 7) shows the same features as PS II in water and following photooxidation demonstrates the same decrease in intensity of these features. The presence of these small features in native PS II in water and in heavy water followed by their decrease upon photooxidation are indications that many features can be assigned to the chromophores of which Chl *a* is the principal one.

3.6. Chlorophyll in PS II

Comparing the enhanced spectra of PS II in H_2O (fig. 6) with those of PS II in D_2O (fig. 7) we observe that the enhanced features ascribed to Chl (dashed lines) are situated at the same positions while the features associated with the polypeptides are different depending whether the samples are in native or oxidized forms and whether they are in water or heavy water.

To gain more knowledge of the situation of the chromophores in the native chloroplast extract, we subtracted the spectrum of the oxidized sample in water (fig. 6C or C′) from that of the native sample (fig. 6B or B′). Between 1500 and 1000 cm^{-1} the results showed predominantly the bands of the chromophores. Between 1750 and 1500 cm^{-1}, the situation is less clear because the protein bands show major modifications. Similar results are obtained with the samples in heavy water.

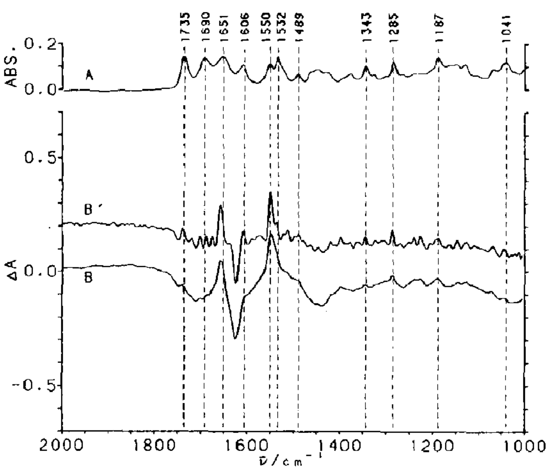

Fig. 8. IR spectra of: (A) amorphous Chl *a*; (B) mean of native PS II preparation in H_2O and in D_2O subtracted from the mean of the photooxidized PS II in H_2O and in D_2O; (B′) Fourier deconvoluted.

The spectra of the amide bands in the native PS II preparations are different in water and in heavy water. They are also different in the photooxidized samples. The noise in these spectra is also different. In this section, we are interested only in obtaining the IR bands of the chromophores in PS II preparations. Thus, by taking the average of the spectra of the oxidized samples in water and heavy water and subtracting it from the average of the native samples in water and heavy water, we decrease the noise level while spreading the conformal modification of the PS II polypeptides bands and intensifying the chromophores bands. The result is presented in fig. 8B and the Fourier deconvoluted spectrum in fig. 8B′.

By this procedure, it was not possible to eliminate all the conformational differences of the polypeptides. The positive features observed at 1657 and 1549 cm^{-1} and the negative one at 1626 cm^{-1} are the most evident features remaining. However, without performing the averaging procedure just described, these features are stronger and mask the chromophores components (specially in the 1715–1670 cm^{-1} region) which were unravelled by carrying out the data treatment.

Comparing the enhanced spectrum (fig. 8B′) with that of Chl *a* (fig. 8A) we see that, here again, there is coincidence of the bands, but in a way that is more obvious than previously. Of special interest is the group of bands situated between 1715 and 1670 cm^{-1}. The positions of these two bands along with the other carbonyl bands are compared in table 2 with the separated carbonyl components of amorphous Chl *a* [19] and the Raman spectra of spinach and *B. alpina* [33].

In native PS II, the bands at 1701 and 1687 cm^{-1} are assigned to free ketone carbonyls and that at 1675 cm^{-1} to bonded ketone carbonyl. The high-frequency position of the latter band compared to a mean position of 1651 cm^{-1} for the associated ketone indicates that the intermolecular bond associated with this band is weak. Below 1670 cm^{-1}, the conformational modifications of the proteins perturb the difference spectrum which render the retrieving of the chlorophyll bands difficult.

To determine the possibility of having other Chl carbonyl bands situated between 1670 and 1620 cm^{-1}, we have compared the integrated intensity of the PS II Chl carbonyl bands with those of amorphous Chl *a*. In the latter, the ratio of the bands situated between 1710 and 1620 cm^{-1} to the intensity of the 1285 cm^{-1} band is 3.4. The 1285 cm^{-1} band is a Chl band which is not strongly perturbed by the aggregation state (fig. 4). For Chl in PS II, the ratio of the bands situated between 1710 and 1670 cm^{-1} and the 1285 cm^{-1} band is 1.7 (fig. 8B′). Because of the presence of Chl *b* in the PS II preparation, the integrated intensity of the carbonyl bands in this system should be stronger than in pure Chl *a*. Since the ratio of the intensity of the observed bands is less in the PS II preparation than in Chl *a*, we conclude that some unobserved associated carbonyl bands are present in PS II and are masked by the amide I band.

Table 2

Position (in cm^{-1}) of the Chl carbonyl bands

Symbols as in table 1.

IR amorphous Chl *a* [19]	Raman *B. alpina* Chl *a* [33]	Raman spinach Chl [33]	IR PS II Chl (fig. 8)	Assignment
1761 vvw				free ester CO
1745 vw				free ester CO
1738 vs			1738 s	free ester CO
1731 s				free ester CO
1721 w			1719 w	ass. ester CO
1714 w				ass. ester CO and free keto CO
1705 m	1704 sh			ass. ester CO and free keto CO
1698 s		1700 sh	1701 s	free ketone CO
1692 s	1691 sh	1689 w	1687 s	free ketone CO
1685 s	1683 m			free ketone CO
1676 s		1680 w	1675 s	ass. ketone CO
1667 s	1670 sh	1671 w		ass. ketone CO
1660 s	1660 m	1660 sh	(1657 vs) [a]	ass. ketone CO
1653 s	1651 sh			ass. ketone CO
1647 s				ass. ketone CO
1642 s	1638 vvw	1640 m		ass. ketone CO and formyl CO
1635 m		1631 sh	(1636 sh) [a]	ass. ketone CO and formyl CO
1628 m		1625 sh		ass. ketone CO
1618 m	1614 m	1618 m		ass. ketone CO
1609 s				C = C, C = N stretch
1605 m			1608 m	C = C, C = N stretch

[a] Uncertain.

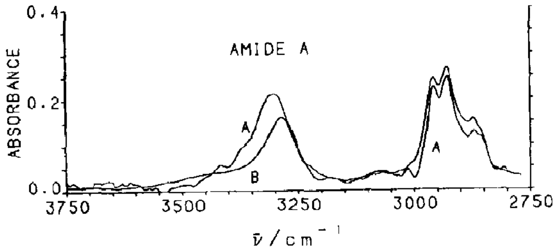

Fig. 9. Comparison of the IR spectra of amide A of PS II in D_2O: (A) native; (B) photooxidized.

3.7. *Intensity of the amide bands*

In fig. 9, the spectra of the amide A band of native and photooxidized PS II in heavy water are compared. By performing computer fitting [19,29], these bands were simulated with two components: 3325 and 3285 cm^{-1} (table 3). The high-frequency component disappears after photooxidation which indicates that during photooxidation a portion of the polypeptides is disorganized while the others remain unaffected. The disorganization produces labile hydrogens available for deuterium exchange. The resulting band appears in the 2500 cm^{-1} region which is, however, masked by the heavy water absorption. This decrease is also observed in the amide II band at 1547 cm^{-1}.

To gain further understanding of the conformational modifications occurring in the PS II preparation, we also examined the bands situated between 1600 and 1400 cm^{-1} where the amide II and the CH_2 groups absorb (table 3). In heavy water, the amide II band, situated at 1547 cm^{-1}, decreases by 0.13 in relative intensity while the band at 1450 cm^{-1} increases proportionally. This decrease represents $0.13/0.60 = 22\%$ of the intensity of the band and therefore 22% of the polypeptides. Since the amide II band contains NH deformation vibrations, the decrease is due to the exchange of NH for ND groups. These modifications indicate the presence of available NH groups in the peripheral regions of the PS II polypeptides. This situation precludes that this portion of the polypeptides is well organized. Therefore, in these regions, the polypeptides are not in an α-helical or β-pleated sheet conformation where deuteron/hydrogen exchange is less feasible. Since the chlorophyll bands are not modified by the NH/ND exchange, the chlorophyll molecules should not be located within this portion of the polypeptides.

During the photooxidation reaction, the decomposition of chlorophyll is followed by polypeptide reorganization which provides new exchangeable hydrogens. The 3325 cm^{-1} component of the amide A band is decreased during photo-

Table 3

Relative intensities of some PS II protein bands

Position	Native PS II		Oxidized PS II		Native PS II minus oxidized PS II in D_2O
	In H_2O	In D_2O	In H_2O	In D_2O	
Amide A					
3325		0.12		0.02	0.10
3285		0.14		0.15	−0.01
Amide II					
1547	0.60	0.47	0.46	0.22	0.25
1517			0.20		
~1452	0.22 [a]	0.36 [b]	0.26 [a]	0.35 [b]	0.01
1438				0.30	−0.30
$\sum$Amide II [c]	0.82	0.83	0.92	0.87	−0.04

[a] CH_2 deformation; [b] amide II and CH_2 deformation; [c] $\sum$Amide II, sum of the relative intensities of the amide II bands.

oxidation. This band represents $0.12 \times 78/26 =$ 36% of the polypeptides available for exchangeable hydrogens. In native PS II, the chlorophylls should be located within this polypeptide domain. The band situated at 3285 cm^{-1} is not modified by the exchange with heavy water nor by photooxidation. This band represents $0.14 \times 78/26 =$ 42% of the polypeptides.

4. Discussion

The IR spectra of thylakoid membrane fractions are composed of the vibrational bands of several molecular species and therefore its analysis is not a simple task. Furthermore, water, which is a strong absorber in the IR, is present in large quantities in the material studied and is a prerequisite for the further investigation of the membrane in its native form. Fortunately, water present in the material is mostly liquid water. By subtracting a spectrum of liquid water or heavy water, as the case may be, from the spectrum of the condensed suspension of the submembrane fractions we obtained the spectrum of the membrane fraction between 4000 and 1000 cm^{-1}. It is possible to assign most of the bands to either protein or Chl groups (table 1). The very good subtraction of the water bands (fig. 1) permitted us to retrieve the amide B band near 3065 cm^{-1} not only in the heavy water solution but also in the light water solution (fig. 2).

4.1. Conformation of the polypeptides

The protein amide bands are identified in the spectra of PS II submembrane fractions (fig. 2 and table 1). When we compare the position of these bands with those in the literature [34] we find that a large proportion of the polypeptides is present in an α-helical configuration. These results are similar to those obtained from electronic circular dichroism that were used to determine that the polypeptides in isolated PS II reaction centre complexes and in isolated LHC form at least 50% α-helices and only 10% β-pleated sheets [35–39]. All these observations are in agreement with the general idea that the fraction of integral membrane proteins that crosses the membrane plane are mostly from α-helices [6,40].

4.2. Effect of deuterium exchange on the polypeptides

When the preparation is made in heavy water, available protons are expected to exchange with deuterons: the amide A (3302 cm^{-1}) and amide II (1548 cm^{-1}) bands which are largely produced by NH groups [34] are displaced to lower frequency; the ratio of the intensity of the 1548 cm^{-1} band to the 1653 cm^{-1} band (amide I) is significantly decreased (fig. 2B) in comparison to that obtained in water (fig. 2A). The decrease of the 1548 cm^{-1} band is compensated by the increase in the 1453 cm^{-1} band due to the ND group. Therefore, the amide II absorption of PS II preparation in D_2O is situated in two bands: 1548 cm^{-1} for the remaining NH vibrations and 1453 cm^{-1} for the ND vibrations.

The intensity of the amide A band of the spectrum obtained in water is impossible to determine because of the saturating absorption of water in that region. For the same reason, the amide A band due to the ND stretching mode cannot be seen in the spectrum obtained with D_2O because it is situated between 2800 and 2000 cm^{-1} where saturating absorption of D_2O occurs. The remaining amide A band due to NH stretch is observed at 3302 cm^{-1} (fig. 2B).

4.3. Effect of photooxidation on the polypeptides

The exchange between proton and deuteron is more pronounced after photooxidation than before (fig. 2D). The amide A band is much weaker in the spectrum taken with D_2O after photooxidation than before. Furthermore, the amide II band at 1548 cm^{-1} is replaced by a strong band at 1451 cm^{-1} (fig. 2D). The above results are explained by a more effective replacement of protons by deuterons in the photooxidized samples. This is an indication that some protein unfolding is induced as Chl is bleached during illumination. The unfolding is also demonstrated by a general broadening of the amide bands whether the sample is in water or heavy water (fig. 2C and D). The reason for this unfolding could be a direct effect of protein photooxidation. Another hypothesis would be that Chl/protein interaction is necessary to maintain the complete tertiary structure of the Chl-containing polypeptides. In that case, the degradation of Chl molecules would destroy the intermolecular bonds between the Chl molecules and the protein necessary to maintain the α-helix configuration. This idea relates to a previous demonstration that, in *chlorina f-2* mutant of barley, the LHC proteins present in the chloroplast stroma cannot accumulate in the thylakoid membranes in the absence of Chl *b* synthesis [41,42].

4.4. Polypeptide domains

From the results obtained, we conclude that, in native PS II, there are three polypeptide domains of various sensitivity. One domain, lacking chromophores, contains unfolded polypeptides which renders the hydrogen available for deuteration. This portion, which represents the extrinsic part of PS II, constitutes nearly 22% of the polypeptides of the PS II preparation.

Another domain contains some polypeptides in an α-helical configuration, is not modified by the photooxidation process and is not modified by the proton-deuteron exchange. This domain, which represents around 42% of the PS II polypeptides, does not contain the chlorophyll molecules.

Finally, a third domain of polypeptides, in an α-helix configuration, is situated between the two previous ones, is modified by the photooxidation process which liberates protons that can exchange with deuterons. This domain contains the chlorophyll molecules and represents nearly 36% of the PS II polypeptides.

4.5. Chlorophylls in PS II

The IR absorption bands of Chl in the PS II preparation are isolated in the native minus photooxidized spectrum by Fourier deconvolution (fig. 8). In this enhanced difference spectrum, several weak positive bands could be identified with bands in the spectrum of pure Chl *a*. The region of the spectrum assigned to ketone is very important because this group is implicated in the ligation of Chl molecules to various donors [13–19]. Between 1715 and 1670 cm^{-1}, we observe three ketone bands compared to five in amorphous Chl *a* (table 2). This is an indication that in the chloroplast, the free ketone carbonyl groups are exposed to less interactions than in amorphous Chl *a*. This can also be related to the high position of the red band at 682 nm with a relatively narrow FWHH observed in the spectrum of the chloroplast extract compared to that of amorphous Chl *a* (fig. 5).

The bands at 1701 and 1685 cm^{-1}, observed in the enhanced spectra of the chloroplast extract, indicate the presence of free ketone groups in two different milieux. The band observed at 1675 cm^{-1} is situated in the upper limit of the associated ketone region and is therefore assigned to weakly associated ketone carbonyl. At present, the type of association remains undetermined. At lower frequencies other associated ketone bands must exist but they were impossible to observe owing to the intensity variation caused by the protein unfolding.

Acknowledgements

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