Biophysical Chemistry, 47 (1993) 193–202
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01768

Chlorophyll *a* auto-aggregation in water rich region

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(Received 13 August 1992; accepted in revised form 15 March 1993)

Abstract

Visible absorption and fluorescence spectroscopy, togheter with circular dichroism were employed to investigate the equilibrating chlorophyll *a* species formed in solution at different water/acetonitrile content and different pigment concentrations. Changes in chlorophyll *a* auto-aggregation occurring in water containing acetonitrile in the range 0.1–33% (v/v) were observed and related to the bulk properties of the medium. A 713 nm absorbing species, occurring in solution for water concentrations exceeding 99% was characterized. The influence of the ionic strength of the solution was also subject of the present study, together with the time evolution of the different aggregates.

Keywords: Chlorophyll *a*; Auto-aggregation; Water rich region; Circular dichroism; Fluorescence

1. Introduction

Chlorophyll *a* (Chl *a*) plays a fundamental role in the photosynthetic processes of higher plants and algae, acting as energy collector and primary electron carrier. Its functional differences are closely related to the hydration and the aggregation of the pigment [1,2]. Molecular organization of Chl *a* has been studied in several laboratories [3–10] using a variety of techniques, such as absorption and fluorescence spectroscopy [7–10], fluorescence lifetime [7,8], photoinduced charge separation [9].

Study of Chl *a* aggregation in a variety of polar [7,11–13] and non-polar [4,6,14–16] solvents containing different amounts of water have been extensively reported in literature. The studies on water–polar solvents mixtures are of particular interest because they supply information on the influence of the hydrophobic effect and on the relative competition between the functional groups in determining Chl *a* aggregation. This could help to understand how the polar side chains of amino acids influence the physicochemical properties of Chl *a* in natural Chl *a*–protein complexes [1].

It is well known that in a wide range of polar solvents Chl *a* is present in solution as solvated monomer. Upon addition, water replaces the solvent ligands to the Mg atoms allowing the formation of aggregates. The number of hydration and the aggregation state depend on water amount, organic solvent nature and pigment concentration. In nearly all the studies in this field, the organic solvent is used as a majority component.

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The problem of Chl *a* aggregation in water containing trace amount of organic solvent has been scarcely investigated. An early study by Hochapfel et al. [11] reported the observation of long-wavelength absorbing Chl *a* in acetone containing up to 88% in volume of water. On the other side, in a more recent study of Khanova and Tarasevich [17] the monomeric Chl *a* adsorbed on electrode from acetone solution containing 99% (v/v) of water was detected by means of electrochemical and spectroscopic techniques. Finally, the formation of a 718 nm absorbing species was reported by our group [18] and ascribed to a likely micelle formation whose contact area with the electrode was three times that of the monomeric Chl *a*.

This paper reports the observation of the changes in Chl *a* aggregation occurring in solutions of water containing acetonitrile (ACN) in the range 0.1–33% (v/v). The spectroscopic techniques of absorption, circular dichroism (CD) and fluorescence were concurrently employed in order to investigate the equilibrating Chl *a* species formed in solution at different pigment concentration and at different water/ACN content. Particular attention was devoted to characterize a new species absorbing at 713 nm. The influence of the ionic strength of the solution was also studied, together with the time evolution of the different aggregates.

2. Materials and methods

Chl *a* was isolated from fresh spinach leaves as previously described [19,20] and stored in pentane at −30°C in the dark. Purity and concentration were routinely checked using the criteria described elsewhere [21,22].

An appropriate amount of the stock solution was evaporated under N_2 flow to dryness and the desired volume of ACN, usually 30 μl, was added to prepare the Chl *a* solutions. The ACN/water mixtures (0.1–1.0% v/v)) were prepared by addition of small quantities of concentrated ACN

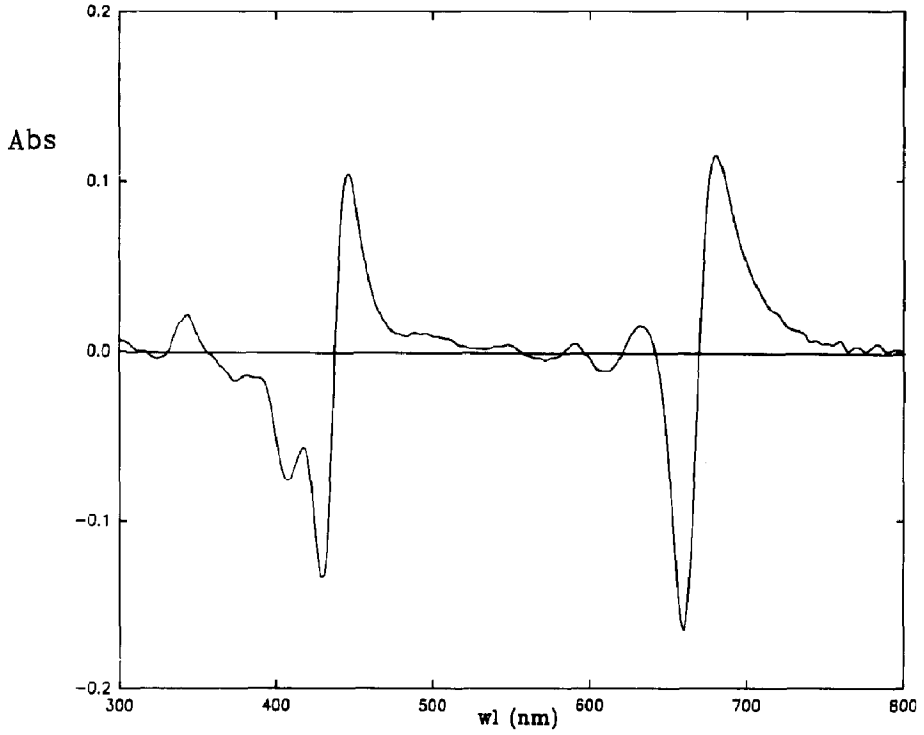

Fig. 1. Differential spectrum of 5×10^{-6} *M* Chl *a* in ACN/water 0.1% (v/v) against Chl *a* in pure ACN at the same concentration. The spectrum was recorded less than one minute after sample preparation.

solutions of Chl *a* to water or KCl aqueous solutions. For larger amount of ACN the Chl *a* was dissolved in the organic solvent and the necessary amount of water was added dropwise. The solutions were prepared directly in the measurement vessels. All the operations were performed under dim green light.

Visible absorption spectra were recorded in the 350–800 nm interval with a Cary 3 UV–Vis spectrophotometer (Varian s.p.a.). CD spectra were measured in the 300–700 nm interval, on a JASCO J600 Spectropolarimeter (Japan) under N_2 atmosphere, all CD data are reported as ellipticity.

For the fluorescence experiments an argon laser (Spectra Physics) equipped with a Ramanor HGLS monochromator and a RCA 31034A02 photomultiplier were used.

All the spectroscopic measurements were performed in 1 cm path length cells.

All chemicals were pure grade reagents from Carlo Erba (Italy). The water was purified by reverse osmosis with the Purite Lab Water (England) apparatus.

3. Results

The absorption spectrum of Chl *a* in pure ACN shows the characteristic behaviour of the monomeric species, with maximums at 662 and 432 nm [23].

When small amounts of the ACN concentrate Chl *a* solution are injected in water, the absorption spectrum shows dramatic changes. Immediately after the sample injection, the monomeric bands are shifted to 671 and 437 nm, respectively, and the Full Width at Half Height (FWHH) of the red band increases from ≈ 20 nm to ≈ 40 nm. The blue-to-red intensity ratio also increases from 1.24 to 1.46.

The changes in peak position and intensity are shown in the differential spectrum (Fig. 1) where the absorption of Chl *a* in water/ACN is

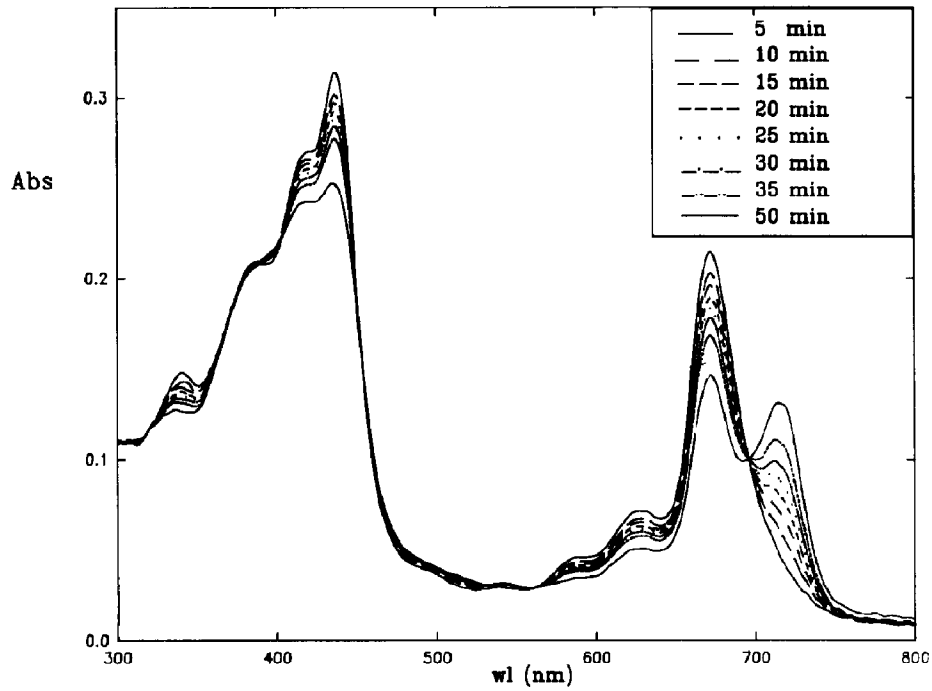

Fig. 2. Absorption spectra of a 5×10^{-6} *M* Chl *a* solution in ACN/water 0.1% (v/v). Spectra were recorded at different times after sample preparation.

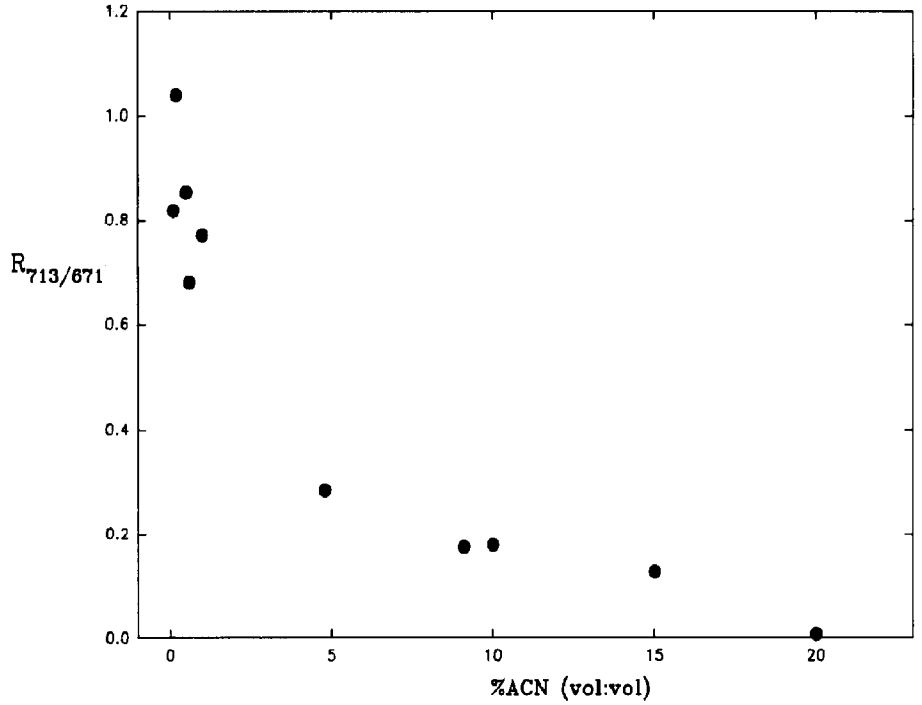

Fig. 3. Effect of variation of acetonitrile percentage on the ratio of $R_{713/671}$. The Chl *a* concentration was 5×10^{-6} *M*.

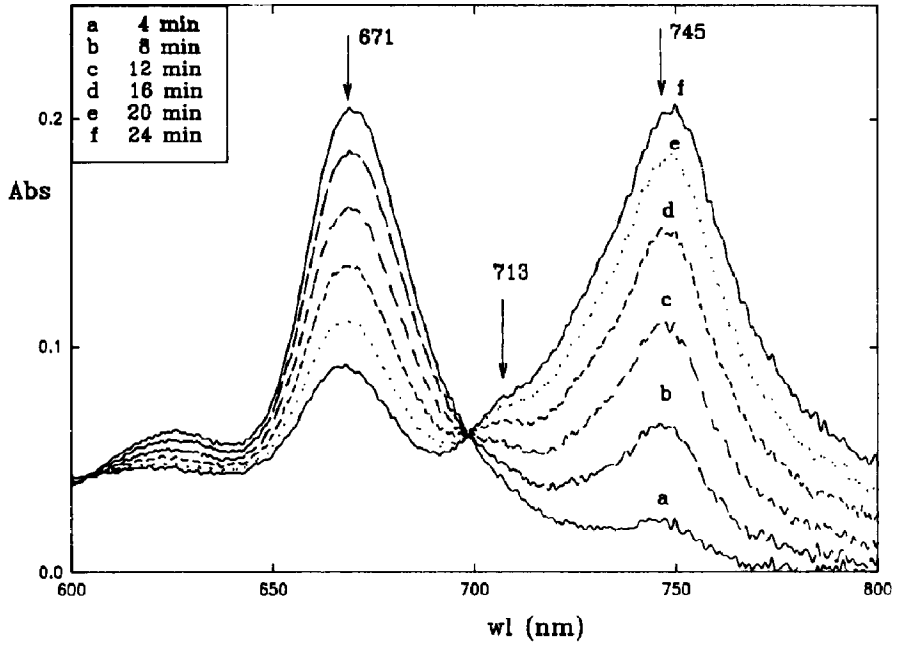

Fig. 4. Formation of long-wavelength absorbing species in a solution ACN/water 33.3% (v/v). Spectra were recorded at different times after sample preparation. The Chl *a* concentration was the same as in Fig. 2.

recorded against a solution containing the same amount of Chl *a* in ACN.

Figure 2 shows the time evolution of the absorption spectrum of the Chl *a* in water/ACN solution: 10 min after the sample preparation a peak at 713 nm appears. The intensity of this peak increases in time while the intensity of the 671 nm and 436 nm peaks decreases.

The peak at 713 nm is present in the absorption spectra of Chl *a* in the concentration range 10^{-6} *M* to 3×10^{-5} *M* and also in the range 0.1–4% in volume of ACN. The relative intensity of the bands at 713 nm and 671 nm ($R_{713/671}$) is largely influenced by the last parameter, as illustrated in Fig. 3. Up to 1% in ACN, the peaks have almost the same intensity and the ratio $R_{713/671}$ is close to unity. The ratio decreases with the increasing of the ACN percentage, reaching zero when the ACN content exceeds 20%, where the 713 nm peak is not present. Solutions containing more than 30% of ACN show the well known long wavelength band around 745 nm [3,24], characteristic of the oligomeric form of Chl *a*. The formation in time of these polymeric species (Fig. 4) shows unexpectedly the reappearance of the 713 nm band after 8 min from the water addition to the Chl *a* solution in ACN.

It is interesting to note that the polymer formation takes place at a much lower (1%) content of ACN if KCl is added to the water. The salt concentration is a critical parameter in the formation of the species in solution. For concentrations lower than 0.1 *M* the 713 nm peak is formed, while for greater concentrations the 745 nm peak appears and its relative intensity increases with the KCl concentration.

The CD spectrum of freshly prepared sample of Chl *a* in the water/ACN mixture does not evidence any appreciable increase of the intensity compared to the corresponding spectrum of a monomeric species. Ten minutes after the sample preparation a single non-conservative negative band at 450 nm appears, its intensity increases in time (Fig. 5) reaching a steady value after 50 min.

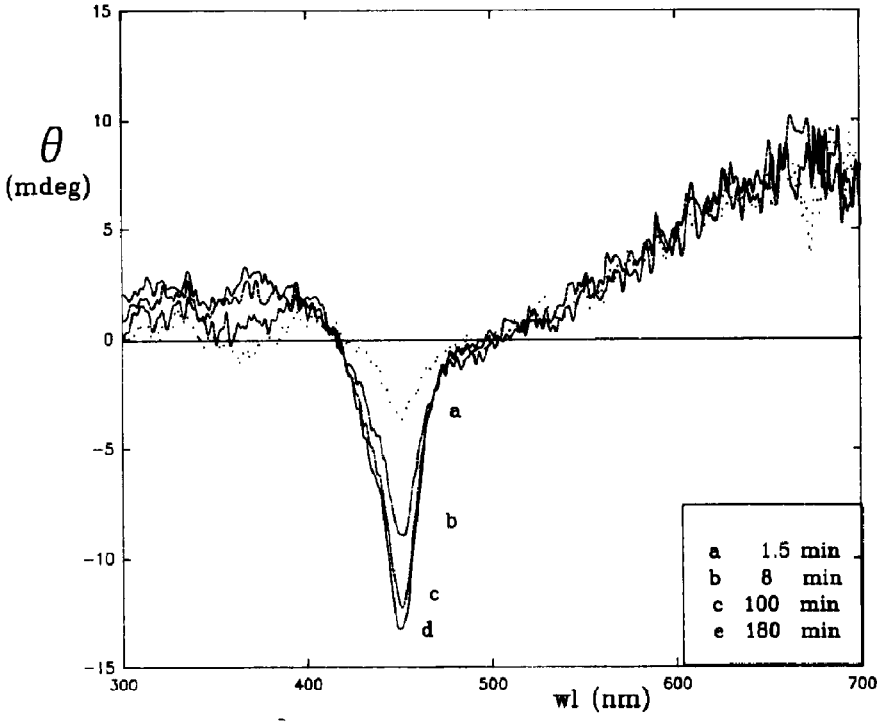

Fig. 5. Circular dichroism spectra of a 5×10^{-6} *M* Chl *a* solution in ACN/water 0.1% (v/v). The spectra were recorded at different times after sample preparation.

The kinetic behaviour of this band closely resembles the time course of the 713 nm peak formation in the optical absorption spectrum as illustrated in Fig. 8.

The same band also appears in the CD spectrum of the polymeric Chl *a* (curve f of Fig. 4). Here, however, the negative band at 450 nm is accompanied by a second negative band at 325 nm and by two well defined positive signals at 372 and 425 nm respectively (curve a of Fig. 6). The relevant aspect of the spectrum reported in Fig. 6 is the thousand-fold increase in the ellipticity with respect to the monomeric Chl *a* at the same concentration (curve b of Fig. 6).

The solutions of Chl *a* in ACN show the typical monomeric fluorescence spectrum (See inset of Fig. 7). The samples whose absorption spectrum shows the 713 nm band, also show a fluorescence band with intensity ten times lower than the one recorded in pure ACN (Fig. 7) that probably can be attributed to a loose aggregate of the Chl *a*. The fluorescence spectra recorded after excitation at different wavelength in the range 400–700 nm did not evidence any change in the shape and peak position compared to that of the monomer reported in the inset of the figure, as further evidenced by the excitation spectrum.

4. Discussion

In the water rich region of the water/ACN mixture, for an ACN content not exceeding 1%, the spectra of Chl *a* undergoes a number of modifications. The position of the porphyrin Qy-transition shifts ≈ 9 nm and FWHH increases ≈ 10 nm upon addition of ACN to water (see Fig. 1). Part of these modifications can be attributed to the variation in the dielectric constant and refractive index of the bulk solution. Moreover, an additional contribution can be ascribed to the replacement of ACN molecules by water in solvating the Mg atom of the pigment macrocyclic

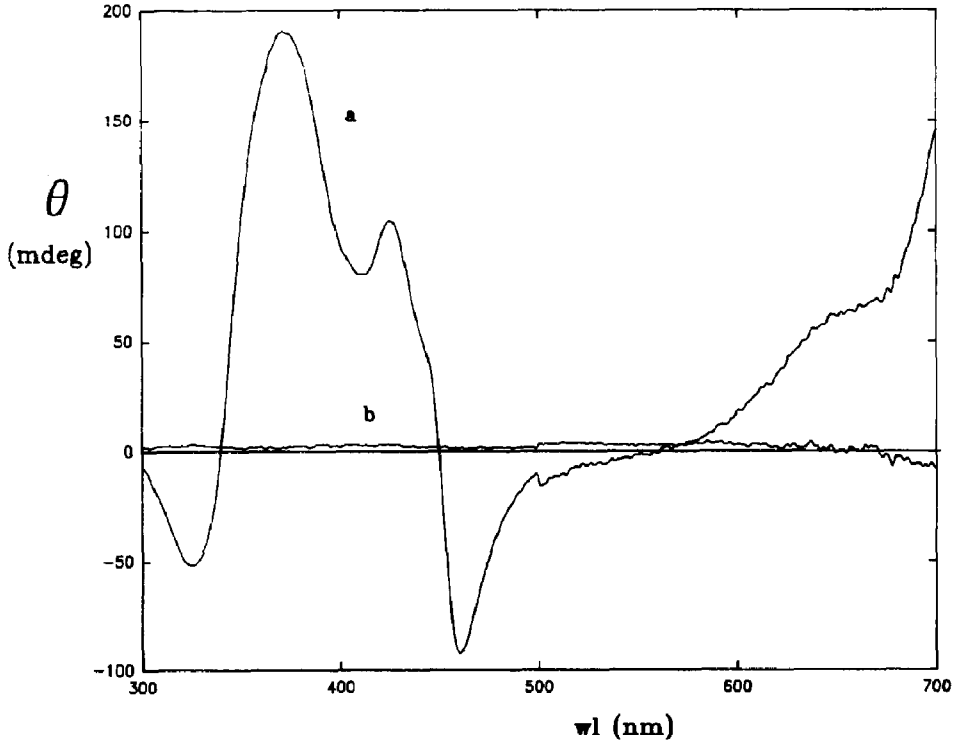

Fig. 6. Circular dichroism spectra of 5×10^{-6} *M* Chl *a* solution in: (a) ACN/water 33.3% (v/v) and (b) in pure ACN.

head. The species absorbing at 671 nm can be identified as the hydrated monomer of Chl *a*. The formation of a dimer can be excluded since no strong CD signal, characteristic of dimeric species [6,25], is present in freshly prepared solution (Fig. 5).

In time a new peak at 713 nm appears that we attribute to the formation of an aggregated species of Chl *a* based on a number of observations. In the first place this species is a product of an equilibrium conversion of the hydrated monomer, as indicated by the presence of the isosbestic point at 695 nm (Fig. 2). Secondly the peak position shift of ≈ 42 nm and the broadening of the FWHH of ≈ 23 nm are too large to be only a result of variations in the bulk properties of the solution, but can reasonably be attributed to the excitonic coupling of several Chl *a* molecules.

In literature peaks absorbing close to 713 nm have already been reported. A 717 nm absorbing species, revealed on cooling hydrocarbon solutions of Chl *a*, was reported by Fong et al. [26]. Fong and Alfano [27] observed a similar absorption at 715 nm in aged 4:1 water/acetone Chl *a* solutions in equilibrium with a 675 nm species. Agostiano et al. [18] reported a 718 nm absorbing species in a water solution of Chl *a* containing trace amount of acetone. Finally an absorption peak at 715 nm has been reported by Worcester et al. [24] in the organic solvent region of the water/(toluene–octane) mixture. This species was assigned to the formation of an oligomeric form of Chl *a* with an aggregation number larger than 20.

The CD spectrum associated with the 713 nm absorbing species (Fig. 5) shows a weak single

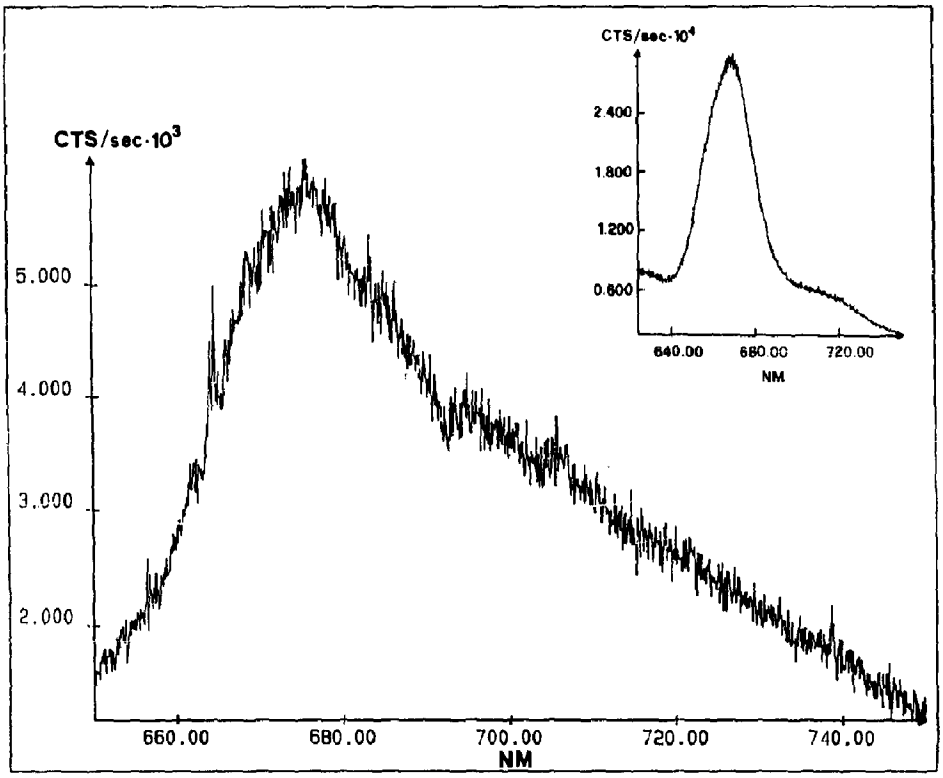

Fig. 7. Fluorescence spectra of 3×10^{-5} *M* Chl *a* solution in ACN/water 0.6% (v/v). Inset: fluorescence spectra of 5×10^{-6} *M* Chl *a* solution in pure ACN. All the spectra are excited by light of 488 nm.

band at 450 nm. This excludes the possibility of identifing it with the exo-dimer proposed by Fong et al. [26], whose CD spectrum is expected to have intense conservative signals. These signals, due to the coupling of the oscillators associated with corresponding electronic transitions in the different molecules, should be splitted into the two components of the electronic transition. On the other side we can also exclude that the 713 nm is a small sub-unit of the oligomeric form of Chl *a* absorbing at 745 nm based both on the CD and fluorescence data. The CD spectrum of the oligomeric Chl *a* (Fig. 6) clearly shows the characteristic strong coupling between the dipole transitions of different molecules of the aggregate [28–30], indicating a repetition in space of dimeric units, as well as the splitting in the bands. Both characteristic are absent in the 713 nm species CD spectrum. Moreover no fluorescence at 755 nm, typical of the oligomeric Chl *a* [30], has been detected in solutions containing 0.1% of ACN in water where only the typical monomeric fluorescence appears. Also should be noticed that the formation of the 713 nm and 745 nm species are parallel and not sequential processes as indicated by the presence of the isosbestic point in Fig. 8. The formation of one or the other species seems to be closely related to the bulk properties of the solution as already hypothesized [12]. It is indeed possible to obtain an oligomeric form of Chl *a* (745 nm) in ACN/water mixtures only for ACN exceeding 30% where the dielectric constant of the solution is ≈ 70 [31]. The oligomers can form in the water rich region (ACN < 1%) if the dielectric constant of the solution is lowered to ≈ 70 by adding a high salt concentration ([KCl] $\approx$ 1 *M*) [32]. Finally even after several hours of centrifugation no precipitation was obtained from the 713 nm species containing solutions, as opposed to the 745 nm containing solutions, where a micro-crystalline precipitate is formed after 5 min of centrifugation. The above data are consistent with a 713 nm aggregate in which the phytylic chains of the Chl *a* are surrounded by ACN, anchoring the pigments with the macrocyclic heads displayed toward the water bulk. A Chl *a* auto-aggregation is expected to minimize the strong hydrophobic effect due to the injection of

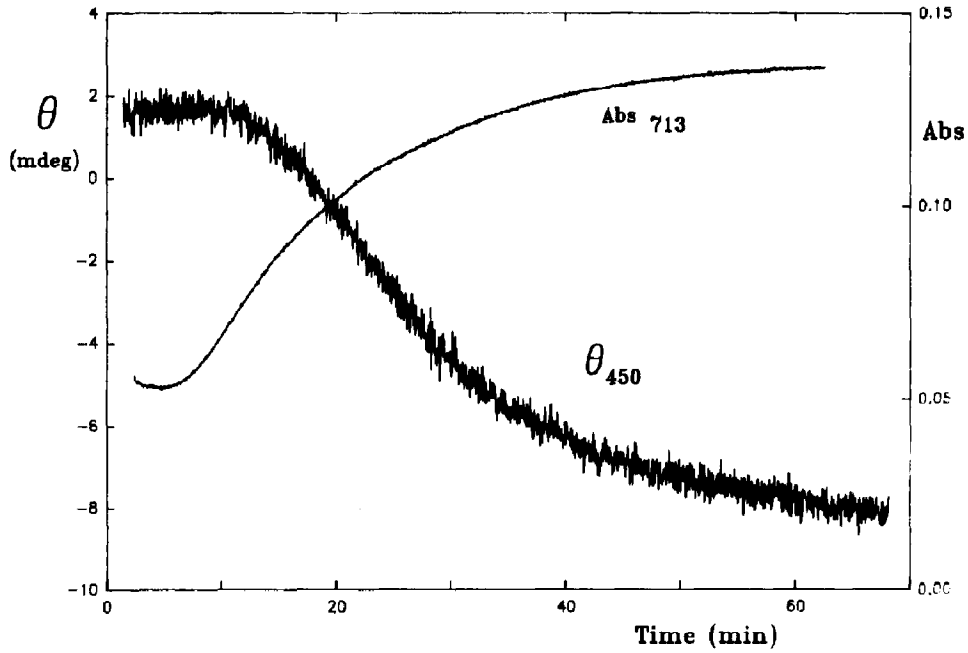

Fig. 8. Time evolution of 450 nm CD band (left scale) and of 713 nm absorption peak (right scale). The sample was the same as Fig. 5.

the Chl *a*, dissolved in a small amount of ACN, into water. In this structure the *y*-axes of neighbouring porphyrinic rings should be anti-parallel: a large splitting but a negligible oscillator strength can be predicted from such geometry. This can account for the red shift in the absorption band of ≈ 42 nm observed in Fig. 2 and for the absence of the second peak. The CD signal is also assumed to vanish as suggested by the excitonic theory [29,33,34]. Although our CD spectra do not cover wavelength longer than 700 nm, all the spectra referred to the 713 nm species do not show any conservative signal even at 693 nm (isosbestic point) where the transition under examination is surely present. The only observable CD signal that appears is due to the non-conservative term generated by the coupled interactions with non degenerate excited states of different Chl *a* molecules. A similar effect has been reported both for synthetic Chl *a* dimers and in presence of monomeric Chl *a* interacting with proteins [35]. In conclusion the spectroscopic data here presented seem to support our hypothesis that in almost pure water the Chl *a* autoaggregates to give a novel kind of structure. Further experiments are in progress in our laboratories to better characterize the structure of the aggregate and to identify the kinetic and thermodynamic of its formation together with study on its reactivity in photoinduced processes.

Acknowledgements

We thank prof. P.L. Luisi for the opportunity to use the CD apparatus and Dr. G. Colafemmina for helpful discussions. Thanks also to Dr. F. Avventurato for her patient help in reviewing the manuscript. This work has been funded by CNR – Progetto Finalizzato Chimica Fine.

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