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Preservation of long-wavelength fluorescence in the isolated thylakoids of two phytoplanktonic algae at 77 K

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Loss of long-wavelength fluorescence upon isolation of chloroplasts or thylakoids has long been a problem in studies of the chromophyte algae, that is, algae possessing chlorophyll *c* and related algae. We now report the preservation of long-wavelength fluorescence, using two such algae, *Phaeodactylum tricornutum* (Bacillariophyta) and *Vischeria helvetica* (Eusigmatophyta). Preservation was achieved using an isolation buffer of high osmotic and ionic strength. Anions high on the Hofmeister series were more efficient than anions low on the Hofmeister series. The most efficient buffer was one high in phosphate (0.5 M) and citrate (0.3 M) combined with 0.3 M sucrose. The results indicate that there are basic differences at the molecular level between the arrangement in the thylakoid membrane of chlorophyll-protein complexes of the chromophyte and related algae compared to green algae and higher plants.

Introduction

Fluorescence spectra are routinely used in the characterization of whole cells, chloroplasts and isolated pigment-protein complexes. Long-wavelength fluorescence (705–740 nm), has been observed in many photosynthetic systems at low temperature (77K) [1–4]. In higher plants, on which most studies have been carried out, it is now apparent that two components (a) a core complex of Photosystem I (PS I) fluorescing at 722 nm and (b) light-harvesting chlorophyll *a/b* binding protein complex of PS I (LHC I) fluorescing at 735 nm, give rise to the fluorescence at long wavelength [5–7]. Thus, under defined conditions, long-wavelength fluorescence may be taken as a reliable indicator of PS I and/or its light-harvesting complex.

Chromophyte algae, that is, algae possessing Chl *c*, and related algae that do not possess Chl *c* such as the Eustigmatophyta, generally have long-wavelength fluorescence in vivo but show a characteristic reduction or loss upon isolation of chloroplasts or thylakoid fragments in vitro [8,9]. The aim of the present work was the preservation of the long-wavelength fluorescence during thylakoid isolation from two chromophyte algae, *Phaeodactylum tricornutum* (Bacillariophyta) which possesses Chl *a* and *c*, and *Visheria helvetica* (Eustigmatophyta) which possesses only Chl *a*.

Previously, it has been found that high concentrations of salts containing anions high on the

Abbreviations: Chl, chlorophyll; CP I, chlorophyll-protein complex I; LHC I and II, light-harvesting chlorophyll *a/b* protein I and II; PMSF, phenylmethylsulphonylfluoride; PF, protoplasmic face; PS I and II, Photosystem I and II; RC, reaction centre; CD, circular dichroism.

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Hofmeister [10] or lyotropic series stabilised a number of protein systems. Ribonuclease can be protected against thermal denaturation by high concentrations of phosphate and sulphate [11,12]. Similar stabilizing effects of phosphate, sulphate, citrate and acetate have also been observed for collagen, gelatin and the mitochondrial complex II-succinate dehydrogenase multiezyme complex [13,14].

Previously, high concentrations of phosphate have been shown to stabilize phycobilisomes on the thylakoid membranes of blue-green and red algae and to preserve energy transfer between the phycobilisomes and PS II after thylakoid isolation [15,16]. Further, Stewart [17] showed that high rates of electron transport through PS II in thylakoid particles from spinach depended on high concentrations of salts containing anions high in the Hofmeister series.

Thus, there is widespread evidence that high concentrations of salts containing ions on the Hofmeister series stabilise a number of protein systems. Franks [18] proposed that the correlation between the effects of anions on proteins and their position on the Hofmeister series was due to the ability of these anions to order water molecules around amphipathic protein molecules thus increasing conformational stability. In the present study anions most effective in preserving long-wavelength fluorescence in both *Phaeodactylum tricornutum* and *Vischeria helvetica* are ranked in an order consistent with the Hofmeister series.

Materials and Methods

V. helvetica (CSIRO culture collection) was grown in Bold's Basal Medium [19] enriched with vitamins at 18°C under continuous cool white light (35 $\mu E \cdot m^{-1} \cdot s^{-2}$). *P. tricornutum* (CSIRO culture collection) was grown in Provasoli-enriched sea-water medium [20]. Both cultures were shaken.

Cells were harvested in the late log phase by centrifugation in disruption buffer (Table I) and resuspended in the same buffer and broken by two passages through a French pressure cell at 80000 kPa at 4°C. Cell debris and unbroken cells were removed by centrifugation at $3000 \times g$ for 10 min. Breakage was approx. 60% for *V. helvetica* and 45% for *P. tricornutum* calculated on chlorophyll released.

Fluorescence emission and excitation spectra were carried out at 77 K or 293 K in a Perkin-Elmer 44B fluorescence spectrophotometer with a low-temperature attachment. The instrument was used in the ratio mode, but the emission spectra were otherwise uncorrected. Samples contained 66% (v/v) glycerol.

Spinach chloroplasts were isolated by the method of Dunkley and Anderson [21]. Chlorophyll was assayed by the method of Arnon [22].

Circular dichroism studies were carried out using a Jasco spectropolarimeter at room temperature.

Results

The fluorescence emission spectra of the whole cells of *V. helvetica* (Fig. 1) showed a room-temperature maximum at 690 nm and a 77 K maximum at 715 nm with a significant shoulder at 695 nm. The fluorescence emission spectra of the whole cells of *P. tricornutum* (Fig. 2) showed a room temperature maximum at 680 nm and two peaks at 690 nm and 715 nm at 77 K.

When the cells of these two organisms were broken in the French pressure cell (Fig. 2) the fluorescence spectra changed considerably, both losing much long-wavelength fluorescence. This contrasted directly with spinach chloroplasts where the excitation spectrum was virtually unaffected by the French pressure cell treatment in a number of buffers (Fig. 1). The absorption spectra and P-700 activity of the thylakoid fragments have been detailed previously [23].

Table I gives the ratio of F_{715}/F_{695} for thylakoid fragments isolated in each of the disruption buffers tested. Tricine-KCl (pH 7.2) was used initially but was later replaced by Tris-acetate (pH 9.2) which has better penetration ability. Triethanolamine penetrates membranes even faster than Tris-acetate, however, the results were comparable to those obtained with Tris-acetate. Neither PMSF, previously used to reduce protease activity during pigment protein complex isolation [24], nor the cross-linking agents glutaraldehyde and suberimidate had any effect in preserving long-wavelength fluorescence.

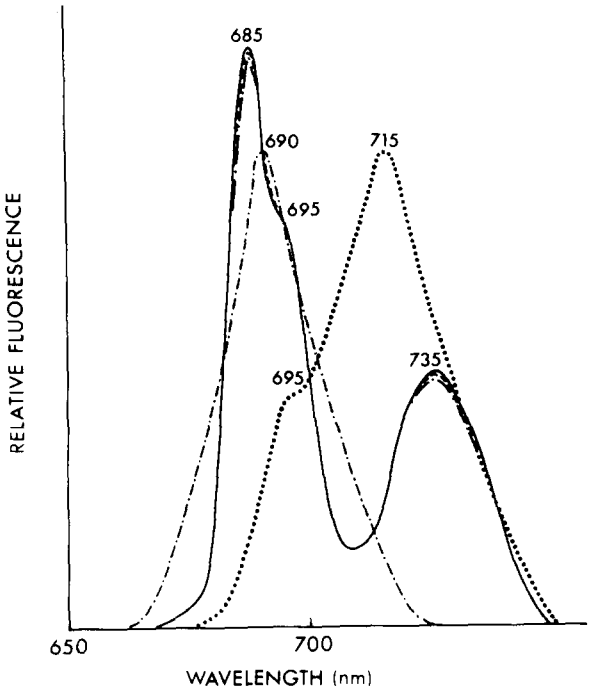

Fig. 1. Fluorescence emission spectra of *Vischeria helvetica* and spinach chloroplasts. *V. helvetica*: (a) whole cells at 77 K (·····); (b) whole cells at 293 K (-·-·-); Spinach: (c) chloroplasts at 77 K (———); (d) thylakoid fragments from chloroplasts broken in 0.3 M sucrose, 0.5 M KH_2PO_4, 0.3 M citrate at 77 K (------); (e) thylakoid fragments from chloroplasts broken in 0.1 M sorbitol, 0.1 M Tris acetate at 77 K (-·-·-). Samples were in 66% glycerol (v/v). Samples were excited at 436 nm. Chlorophyll concentration was 25 μg/ml. Solutions had a pH of 7.2.

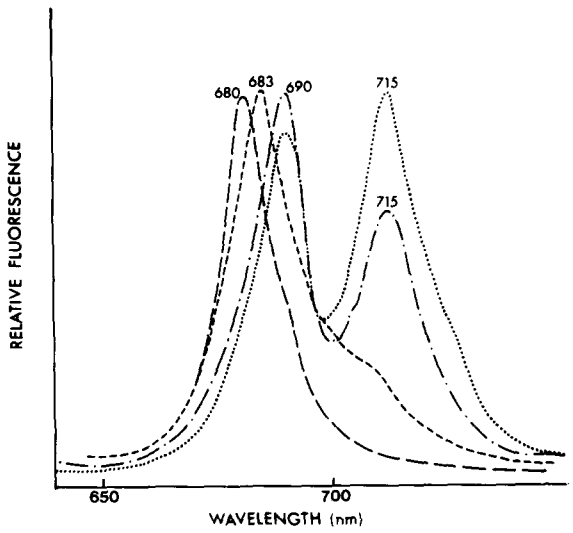

Fig. 2. Fluorescence emission spectra of *Phaeodactylum tricornutum*. (a) Whole cells at 77 K (·····); (b) whole cells at 293 K (------); (c) thylakoid fragments from cells broken in 0.3 M sucrose, 0.5 M KH_2PO_4, 0.3 M citrate (-·-·-); (d) thylakoid fragments from cells broken in 0.3 M sorbitol, 0.05 M sodium potassium phosphate, 0.01 M NaCl (------). Samples were in 66% glycerol (v/v). Samples were excited at 436 nm. Chlorophyll was at a concentration of 25 μg/ml. Solutions had a pH of 7.2.

Only the high ionic strength of the buffer in combination with appropriate ions high on the Hofmeister scale preserved long-wavelength fluorescence to a greater extent. Citrate was the most effective anion when used in combination with a high concentration of phopshate. As can be seen from Table I, phosphate alone was ineffective in preserving long-wavelength fluorescence at concentrations below 0.45 M. The anions when ranked in order of effectiveness resemble a Hofmeister series.

Circular dichroism spectra were measured to determine relationships between long-wavelength fluorescence changes and changes in the structuring of chlorophyll in the thylakoid membranes. CD spectra (Fig. 3) showed that whole cells were characterised by a positive peak at 672 nm. The

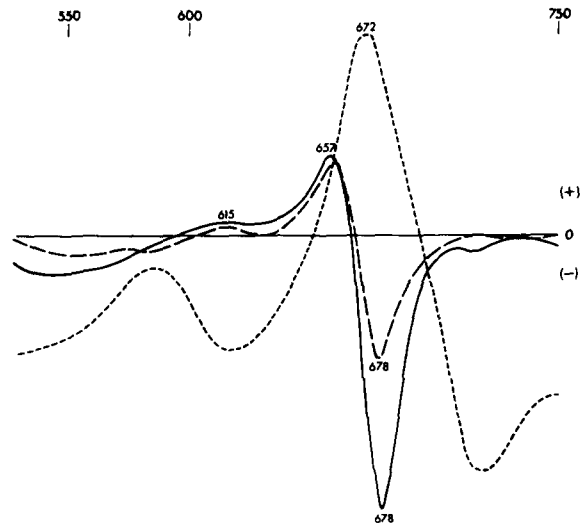

Fig. 3. Circular dichroism spectra of *Vischeria helvetica*. (a) Whole cells (------); (b) thylakoid fragments from cells broken in 0.3 M sucrose, 0.5 M KH_2PO_4, 0.5 M citrate (------); (c) thylakoid fragments of cells broken in 0.3 M sucrose, 0.1 M Tris acetate (———). Chlorophyll was at a concentration of 25 μg/ml. Spectra were measured at 293 K.

TABLE I

THE RATIO OF THE 715 nm PEAK TO THE 695 nm PEAK IN THE FLUORESCENCE EMISSION SPECTRA FROM WHOLE CELLS OF *VISCHERIA HELVETICA* OR THYLAKOID FRAGMENTS ISOLATED IN THE 17 BUFFERS SHOWN

Samples were excited at 436 nm and contained 66% glycerol (v/v). Chlorophyll concentration, 25 μg/ml. At pH 7–9 the error in pH due to the use of glass electrodes at high salt concentrations is negligible (0.05 pH unit in the presence of 3 M sodium [34]).

Buffer/sample	F_{715}/F_{695}	% Control
Whole cells (control)	2.00	100.0
1 0.2 M Tricine/0.1 M KCl (pH 7.5)/0.3 M sucrose	0.24	12.0
2 0.2 M Tris-acetate (pH 9.2)/0.3 M sucrose	0.16	8.0
3 0.05 M triethanolamine (pH 8.6)/0.3 M sucrose	0.16	8.0
4 0.10 M triethanolamine (pH 8.6)/0.3 M sucrose	0.17	8.5
5 0.2 M PMSF-Tris-acetate (pH 9.2)/0.3 M sucrose	0.17	8.5
6 0.05 M KH_2PO_4/0.3 M potassium citrate/0.3 M sucrose	0.56	23.0
7 0.2 M KH_2PO_4/0.3 M potassium citrate/0.3 M sucrose	0.59	29.5
8 0.35 M KH_2PO_4/0.3 M potassium citrate/0.3 M sucrose	0.63	31.5
9 0.45 M KH_2PO_4/0.3 M potassium citrate/0.3 M sucrose	0.90	45.0
10 0.50 M KH_2PO_4/0.3 M potassium citrate/0.3 M sucrose	1.25	62.5
11 0.5 M KH_2PO_4/0.3 M KCl/0.3 M sucrose	0.60	30.0
12 0.5 M KH_2PO_4/0.3 M KNO_3/0.3 M sucrose	0.53	26.5
13 0.5 M KH_2PO_4/0.3 M KI/0.3 M sucrose	0.48	24.0
14 0.5 M KH_2PO_4/0.3 M KBr/0.3 M sucrose	0.26	13.0
15 0.3% glutaraldehyde/0.1 M Tris-acetate/0.3 M sucrose	0.17	8.5
16 0.02 M suberimidate/0.1 M Tris-acetate/0.3 M sucrose	0.18	9.0
17 2 mM Tris-maleate/0.1 M sorbitol/0.5 mM e-amino caproic acid/1 mM PMSF	0.28	14.0

spectra of the two thylakoid preparations both showed a negative peak at 678 nm. The CD spectrum of the thylakoid fragments isolated in a high osmotic strength buffer containing anions high on the Hofmeister differed from the CD spectrum of the thylakoid fragments isolated in a medium osmotic strength buffer containing anions low on the Hofmeister preserved.

Discussion

In this study a high concentration of citrate in combination with a high level of phosphate was required for maximum preservation of long-wavelength fluorescence in in vitro preparations of thylakoid particles. Only anions high on the Hofmeister series were effective. Franks [18] proposed the explanation for the correlation of various effects of anions on proteins with the position of the anions on the Hofmeister series as an effect on conformational stability instigated by the water molecule orientation (see Introduction). The wavelength of chlorophyll fluorescence is determined by the orientation and conformation of the chlorophyll which influences the transition from first excited state to the ground state [1]. Since it is now widely accepted that all chlorophylls are specifically attached to binding proteins by hydrogen bonding and salt bridges [9,25,26], the particular binding conformation of the chlorophyll is directly involved in determining the fluorescence characteristics. As detailed in the Introduction, the core complex of PS I in higher plants is now widely regarded as being the source of the long-wavelength fluorescence component with a maximum emission close to 720 nm. The long-wavelength fluorescence in chromophyte algae at 715–722 nm is likely, therefore, to come from the PS I core complex and its loss upon physical breakage of the cells (accompanied by increased fluorescence at shorter wavelengths) indicates a conformational change in the protein(s) binding the pigments involved in the fluorescence emission. Preliminary evidence [27] suggests that these Chl *a* are bound to intramembrane helices of the polypeptides involved (i.e., the Chl *a* are held in a hydrophobic 'cage') and thus it is unlikely that direct reactions with the aqueous medium could

occur, even in the isolated chloroplasts.

Since spinach chloroplasts showed no change from the initial 77 K fluorescence emission after being exposed to the same treatment that caused the loss in the two phytoplankton species, it seems clear that a real difference exists between the structure of the membranes of these two groups, involving specifically PS I. It might be argued that the loss of PS I complex emission (at 715–722 nm) in spinach chloroplasts was masked by the unaffected LHC I emission (at 735 nm), but this is discounted by the ability to isolate a PS I core complex with long-wavelength (722 nm) fluorescence [28]. Further, it is possible to cause the loss of the long-wavelength fluorescence in this complex with SDS or Triton X-100 and to restore it by replacement of these detergents with dodecylmaltoside [28]. In the two phytoplankton species studied here it was not possible to restore the long-wavelength fluorescence after it had been lost following treatment in the French pressure cell by changing to a high concentration of phosphate/citrate (Chrystal, J. and Larkum, A.W.D., unpublished data).

The circular dichroism spectra support these conclusions although linear dichroism studies are needed for further confirmation [29]. The large change between whole cells and thylakoid fragments (Fig. 3) has been observed in previous studies where it has been assumed to result from relaxation of tightly packed bulk pigments upon cellular disruption (e.g., Ref. 31). The higher negative peak at 678 nm and the preservation of the 705 nm peak in high osmotic, high phosphate/citrate medium indicates greater preservation of the native conditions of binding chlorophyll *a* within RC I. The 705 nm peak may arise from a complex similar to CP0 of *Chlamydomonas reinhardtii* [30].

The foregoing leads us to question whether there are structural differences involving especially PS I between the thylakoid membranes of chromophyte algae, on the one hand, and the blue-green, red and green algae (and higher plants) on the other. Chromophyte algae are known to have their thylakoids stacked characteristically in groups of three [9]. Evidence from freeze-fracture studies indicate that lateral heterogeneity occurs with small PF (PS I) particles on the non-appressed membranes [32]. In chromophyte algae the thylakoids are long linear structures so that if lateral heterogeneity [33] does exist it would mean that PS I complexes would exist in a very different environment to either that in blue-green and red algae (PS I and PS II in close proximity) or green algae and higher plants (PS I predominantly in areas of high curvature at the perimeter of thylakoids).

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