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ANALYSIS OF ABSORPTION SPECTRA CHANGES INDUCED BY TEMPERATURE LOWERING ON PHYCOBILISOMES, THYLAKOIDS AND CHLOROPHYLL-PROTEIN COMPLEXES

J.C. LECLERC, J. HOARAU and R. REMY

Laboratoire de physiologie Cellulaire Végétale, associé au C.N.R.S. (L.A. 40), 91405 Orsay Cedex (France)

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Summary

Using fourth derivative analysis, differences between room and low temperature absorption spectra were studied. The positions of most absorption bands of the water-soluble, accessory pigment complex,the phycobilisome, remained unchanged after cooling. The stability of the wavelength positions of chlorophyll *a* forms in vivo as a function of temperature (Gulyaev, B.A. and Litvin, F.F. (1967) Biofizika 12, 845—854) was generally confirmed. The wavelength positions of all chlorophyll *a* forms in the *P*-700 chlorophyll *a* protein complex were unchanged when the preparations were cooled to —196°C. Likewise, with other chlorophyll-containing materials: the light-harvesting chlorophyll *a/b* protein complex and the thylakoids of higher plants, algae, and cyanobacteria, the wavelength positions of most chlorophyll *a* forms were stable upon cooling. An exception was a 680 nm chlorophyll *a* band which was generally split at low temperature into two bands with the materials investigated. An interpretation of the multiplicity of chlorophyll spectral forms and the spectral changes induced by cooling for these forms is given using exciton theory and the energy-coupling variation of chlorophyll *a* molecules.

Introduction

After several studies showing two or three forms of chlorophyll (Chl) *a* [1—3], Thomas [4] proposed the hypothesis that Chl *a* exists in vivo in more

Abbreviation: Chl, chlorophyll.

than three forms. This hypothesis has been widely discussed [5—11], and further details on this subject are available in some extensive reviews [12—15]. Actually, the existence of several Chl *a* forms at 20°C and the possibility of artifactual forms at −196°C is still a subject of discussion [16]. For example, Gulyaev and Litvin [11] consider that there is no fundamental difference in the multiplicity of spectral forms found in room and low temperature spectra. On the contrary, Cotton and coworkers [17] have questioned the importance of the multiplicity of spectral forms found at low temperature because cooling can induce the formation of artificial chlorophyll associations. Finally, Picaud [18] interprets the spectral changes induced by cooling to be due to a splitting of some spectral bands already present at room temperature.

The idea of the formation of new Chl *a* forms at low temperature is largely based on the observed formation of chlorophyll aggregates in vitro with cooled or concentrated organic solutions of chlorophyll [19,20]. Although highly concentrated in vivo, chlorophyll is organized in the photosynthetic membranes by specific associations with proteins. Consequently, chlorophyll interactions probably already exist at 20°C but these membrane-bound protein-chlorophyll complexes could prevent further associations at low temperature.

The purpose of this investigation was to make a precise comparison of room and low temperature absorption spectra using several materials: thylakoids of algae and higher plants, chlorophyll-protein complexes of higher plants, and phycobilisomes, the water-soluble antenna pigment complexes of cyanobacteria and red algae. Since the fourth derivative of an absorption spectrum is a very sensitive method for the determination of the number and wavelength position of close absorption bands [21], this technique was employed to see if changes occur in the number and positions of absorption bands upon cooling even when the spectrum does not appear markedly altered. A hypothesis is proposed to explain the stability of some bands and the changes of others observed by this technique.

Materials and Methods

For thylakoid studies the following materials were used:

(1) Higher plants: *Nicotiana tabacum* L. var. Wisconsin and *Spinacia oleracea* L. var. America.

(2) Cyanobacteria: *Fremyella diplosiphon* (strain No. 7601) and *Phormidium* sp. (strain No. 7409, Institut Pasteur, Paris); *Spirulina platensis* (Institut Français du Pétrole) and *Anacystis nidulans* (strain No. 625 from Bloomington, IN, U.S.A.).

(3) Green alga: *Chlorella pyrenoidosa* (strain 211/8 b, Algal collection of Göttingen University, F.R.G.

The chlorophyll-protein complexes (*P*-700 Chl *a* protein and light-harvesting Chl *a/b* protein), as well as free chlorophyll were electrophoretically isolated from SDS-solubilized tobacco thylakoids [22]. To minimize the influence of the 'sieve effect' and light scattering on absorption spectra, algae and higher plant plastids were mechanically disrupted (French press 20 000 lb/inch^2, twice). Phycobilisomes from *Phormidium* sp. were isolated according to Gantt and Lipschultz [23] with some modifications [24] and analyzed in 0.75 M

phosphate buffer after dialysis to remove sucrose.

Absorption spectra were recorded at 20°C and −196°C with a single beam spectrophotometer previously described [25,26]. The fourth derivatives were calculated according to Butler and Hopkins [21]. The fourth derivative of an absorption spectrum shows significant peaks corresponding to absorption bands but occasionally, some minor irreproducible peaks result from the background noise of the apparatus. Additionally, the fourth derivation of a single absorption band gives on each side of its main derivative peak two minor peaks (seven to nine times smaller) which are generally not apparent when the derivation is performed on a mixture of absorption bands except at the ends of the whole absorption envelope. Analytical methods using Gaussian models (fourth derivatives of single Gaussian curves having a diversity of bandwidths) and calculation of derivative spectra with two differentiation intervals allow the identification of such artifactual peaks. For this study, our aim was only to interpret the changes which affected the major peaks, two or three times above background noise, and always distinct of artifactual peaks after analysis.

In this report we have used either 8.5 or 17-nm differentiating intervals [26,28]. The 8.5 nm differentiating interval (D 8), with a resolution of 4 or 5 nm, allowed a satisfactory separation of the Chl *a* forms absorbing from 650 to 695 nm; this differentiating interval was generally used for this work. However; the D 8 was found to be inadequate for the study of some broad absorption bands of chlorophyll in the 700—730 nm region. Because of a low signal to noise ratio at 20°C, it was impossible to obtain a satisfactory comparative study of phycobilisome absorption bands at room and low temperature using the 8.5 nm differentiating interval. Consequently, a 17 nm differentiating interval (D 17), well adapted for absorption bands having a half-bandwidth of about 20 nm, was used for the phycobilisome study.

Results

Spectral forms of isolated phycobilisomes from Phormidium No. 7409

Fig. 1 shows that at 20°C and −196°C the absorption spectra of isolated phycobilisomes are quite similar except that the broad absorption bands are resolved at −196°C into several individual bands with no major shifting of the spectrum. Using D 17 it is possible to determine precisely the position of the absorption bands (Fig. 2). The bands between 540 and 575 nm belong to C-phycoerythrin [13] and peaks in the 620—640 nm region are attributed to two phycocyanins as occur in *Agmanellum* [38]. For all bands at the two temperatures investigated, calculations show the same peak locations within ±1 or 2 nm. After normalization of the absorption spectra in the phycoerythrin region at 565 nm (Fig. 2) we observe at 572—574 nm an amplitude of the derivative signal about three times higher at −196°C than at 20°C; the calculation of fourth derivatives of Gaussian curves shows that this corresponds to about a 30% decrease of the absorption bandwidth at this temperature.

Especially in the case of phycocyanin, the decrease in bandwidth upon cooling leads to a better partition of derivative peaks at −196°C. Moreover, the phycocyanin absorption band at 640 nm is probably greater at −196°C than at

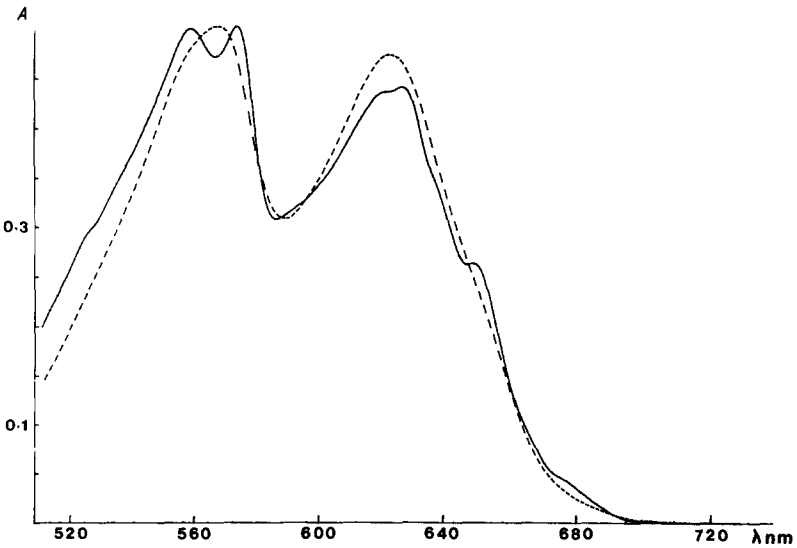

Fig. 1. Absorption spectra of phycobilisomes from *Phormidium*. ———, −196°C spectrum; - - - - - -, 20°C spectrum.

20°C. A small but significant absorption band is observed at 675–676 nm (20°C) and at 676–678 nm (−196°C). Such a band has been observed in phycobilisomes of other species and is probably due to allophycocyanin B [39].

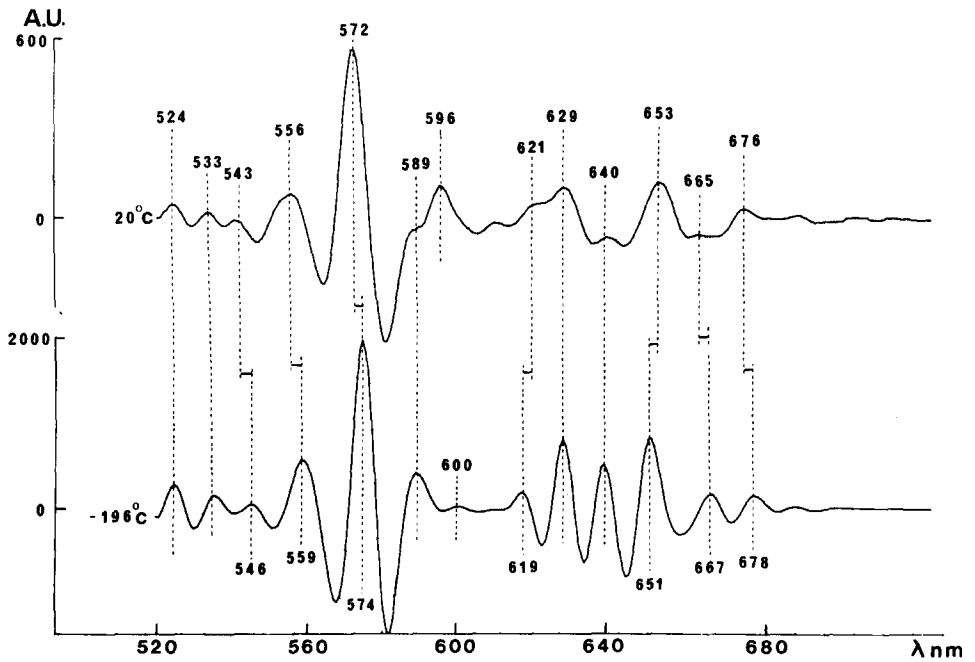

Fig. 2. Fourth derivative analysis of absorption spectra of phycobilisomes calculated with 17-nm differentiating intervals (D 17). The absorption bands having a common origin are connected by vertical dashed lines and little horizontal lines. A.U., arbitrary units.

Spectral forms of chlorophyll

Cyanobacteria. In the three species investigated, *Spirulina*, *Anacystis* (results not shown) and *Fremyella* (Fig. 3), the position of the long wavelength chlorophyll absorption maximum changes only slightly upon cooling, as previously observed by Cho and Govindjee for *Anacystis* [30]. However, some differences appear in the fourth derivative spectra in the number and position of Chl *a* forms absorbing around 680 nm.

For *Anacystis*, the significant fourth derivative peaks are reported in Table I. Using a derivation interval of 8.5 nm with *Anacystis* and *Spirulina* (Table I, lines E–G), the broad band at 680 nm and 20°C splits, at low temperature, into two bands absorbing at 677–678 nm and 683 nm. For *Fremyella*, two close bands at 678 and 682 nm at 20°C (Fig. 3 and Table I, lines E and F) give at low temperature two bands at 677 and 684 nm.

In those species studied, absorption bands at longer wavelengths (between 685 and 700 nm) do not change significantly either in position or in number as a function of temperature. Although the *Fremyella* long wavelength Chl *a* forms are more easily visible in the absorption spectrum at −196°C (Fig. 3 and Table I), these Chl *a* forms (694 and 703 nm) are already visible at 20°C on the D 8 derivative spectrum (Fig. 3).

Rhodophyta. With *Porphyridium*, the spectroscopic behavior of Chl *a* forms as a function of temperature between 658 and 696 nm is similar to that observed in cyanobacteria. In this region the absorption of the phycobilipro-

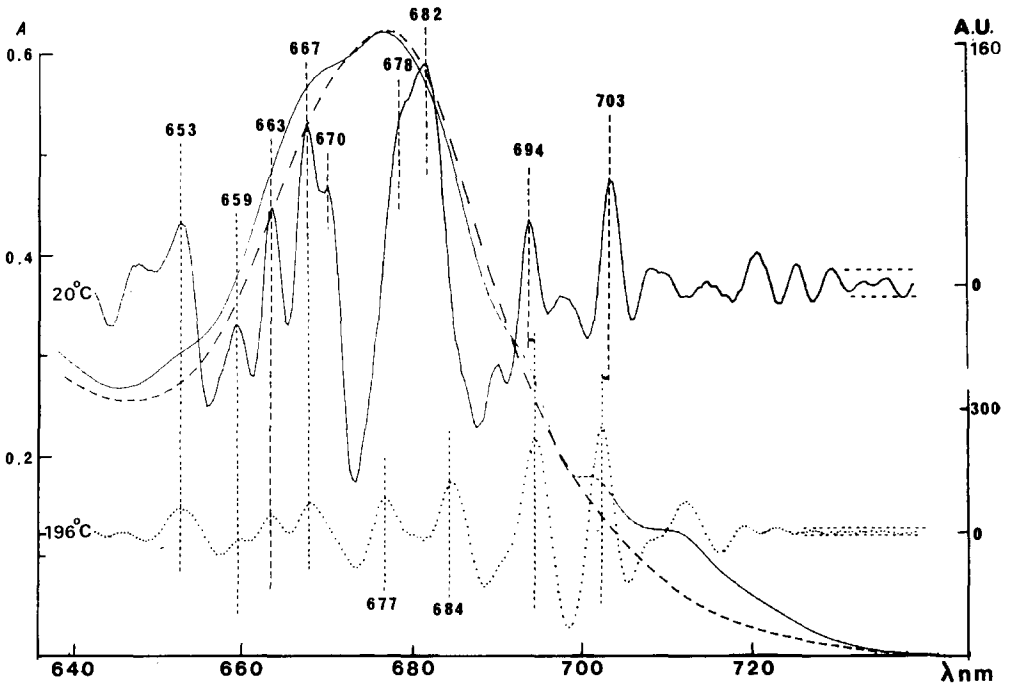

Fig. 3. Fourth derivatives of absorption spectra of *Fremyella* thylakoids at 20°C and −196°C. ------, absorption spectra at 20°C, and ———, at −196°C. The horizontal dashed lines show the level of background noise.

TABLE I

WAVELENGTH POSITIONS OF THE MAIN ABSORPTION BANDS BETWEEN 650 AND 700 nm FROM THYLAKOIDS OF SOME ORGANISMS POSSESSING PHYCOBILIPROTEINS

Absorption bands are revealed by fourth derivative spectra using a differentiating interval of 8.5 nm. A—L, classification of the derivative peaks in order of their decreasing wavelength. + —, signals slightly higher than background; +, 1.5—2 times higher than background; ++, 2—4 times higher than background; +++, 4—10 times higher than background; ++++, 10—30 times higher than background; +++++, more than 30 times higher than background; WB, wide band

	Cyanobacteria						Rhodophycae (*Porphyridium*)	
	Anacystis		*Spirulina*		*Fremyella*			
	−196°C	20°C	−196°C	20°C	−196°C	20°C	−196°C	20°C
A	703 ++	703 +	704 ++	704 ++	702 +++++	703 +++	702 +	
B	696 ++++	698 ++	696 ++++	697 +++			696 ++	697 +—
C		693 +	693 +—	693 ++	694 +++++	694 +++		692 +
D	688 +++++	688 +++	687 ++++	688 WB +++	689 +—	689 +	687 WB ++++	687 ++
E	683 ++++		683 +		684 ++++	682 ++++	683 +	682 ++
F		680 WB ++++		680 WB +++++		678 ++++		679 ++
G	677 +++++		678 +++++		677 ++++		677 +++++	673 +—
H	670 +—	669 WB ++++	671 +++++	671 ++++	670 ++++	670 ++++	669 +++	670 ++
I	667 +++++			668 ++++	667 +—	667 ++++	666 +++	667 ++
J	663 ++	663 +	663 ++	663 ++	663 ++	663 +++		662 ++
K	659 ++	659 +—	658 ++	657 ++	659 +	659 +	658 +—	657-8 +—
L	651 ++++	651WB ++	652 ++	653 ++	653 +++	653 +++	652 +++	653 ++

teins is weak except for a 665 nm band [31]. The broad 680 nm Chl *a* band which is more or less separated into two bands of 679 and 682 nm in the fourth derivative (D 8) spectrum at 20°C, is clearly split into two sharp bands at 677 and 683 nm at −196°C (Table I, lines E—G). For long wavelength forms the derivative peak observed at 692 nm at 20°C disappears at −196°C while the band at 696—697 nm quantitatively increases (Table I, lines B and C).

Chlorophyta. Results obtained with *Chlorella* are reported in Table II. As noted for the cyanobacteria and the red alga *Porphyridium*, the absorption bands from 662 to 668 nm are stable with temperature change. The absorption band at 681 nm at 20°C, however, gives a main band at 677 nm and a secondary band at 684 nm (−196°C) (Table II, lines D—F).

Thylakoids from higher plants. (1) The Chl *a* forms observed for the two

TABLE II

WAVELENGTH POSITIONS OF THE MAIN ABSORPTION BANDS BETWEEN 630 AND 700 nm OF THYLAKOIDS, OR CHLOROPHYLL-PROTEIN COMPLEXES, FROM ORGANISMS POSSESSING CHLOROPHYLL *b*

Symbols are the same as in Table I. LHP, light-harvesting Chl *a*/*b* protein.

	Higher plants (Tobacco)								Chlorophycae (*Chlorella*)	
	Thylakoids		LHP		Free Chl		*P*-700 Chl*a* protein		Thylakoids	
	−196°C	20°C	−196°C	20°C	−196°C	20°C	−196°C	20°C	−196°C	20°C
A	697 +++	697 +							696 ++	696 +
B		692 +		693 +			692 ++	693 +—		693 +
C	688 +++	687 ++	687 +	687 +			687 +++	688 +	687 ++	687 ++
D	683 ++++								684 +—	
E	679 +—	680 WB +++++	678 +—	680 WB +++++			681 ++++	681 ++		681 ++++
F	675 +++++		675 WB +++++		675 ++	675 ++	676 ++	676 +	677 WB +++++	
G	669 ++++	670 +++	669 WB ++++	670 +++	669 ++	669 ++	670 +++	670 +++		670 +++
H		667 +++		667 +++	667 ++		667 +++	667 ++	668 WB ++++	668 +++
I	662 +++	662 ++	660 WB ++++	662 +++			662 ++	662 ++	662 +	662 ++
J	655 ++	657 +—	655 ++	655-8 +—			657 ++	658 +	659 +	658 +
K							651 ++	651 +		
L	649 ++++	649 WB +++	648 +++++	648 +++++	649 +	649 +	648 ++	647 +	647 ++++	648 WB +++
M	638 +++	639 ++	638 +++	638 ++					637 ++	

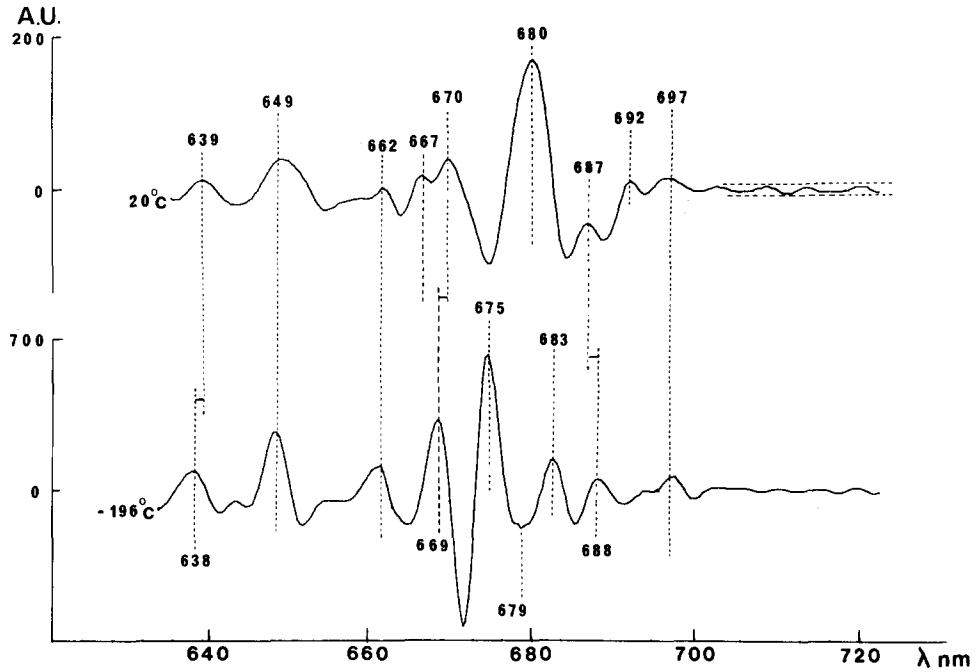

Fig. 4. Fourth derivatives (D 8) of the absorption spectra of tobacco thylakoids at 20° and —196°C. The background noise at —196°C is negligible in this figure.

plants studied, tobacco and spinach, were very similar. (2) At —196°C the red absorption maximum is shifted 2 or 3 nm towards shorter wavelengths, and a very broad absorption band is observed between 700 and 730 nm [12,32]. In the D 8 derivative at 20°C, (Table I and Fig. 4), the 680 nm peak is broad but does not clearly appear to be composed of two bands; while at low temperature, two principal bands are found at 675 and 683 nm plus a minor band at 678 nm. The position of the 670 nm absorption band does not change upon cooling, and the absorption band at 662 nm is present at 20°C and —196°C in both higher plant species.

The major absorption band of chlorophyll *b* occurring at 648—649 nm at 20°C (Table II, line L) remained unchanged at —196°C, and for this reason Chl *b* was used as a marker in the shift analysis of the other forms of chlorophyll. In the same way, the 638 nm peak which is clearly observed in derivative spectra of certain higher plants and chlorophytae [32] but which is not due to Chl *b* [33], does not significantly change in position or relative intensity upon cooling.

Chlorophyll-protein complexes from higher plants. (a) Light-harvesting Chl *a/b* protein. As previously demonstrated by curve analysis [34,35] and fourth derivative analysis at —196°C [22], light-harvesting Chl *a/b* protein is enriched in short wavelength Chl *a* forms. At 20°C the fourth derivative (D 8) contains a main band at 680 nm which shifts to 675 nm at low temperature (Table II, lines E and F). The 662 nm band shifts slightly (2 nm) toward shorter wavelengths at —196°C, while the position of the main Chl *b* absorption band at 648 nm (Table II, line L) remains unchanged.

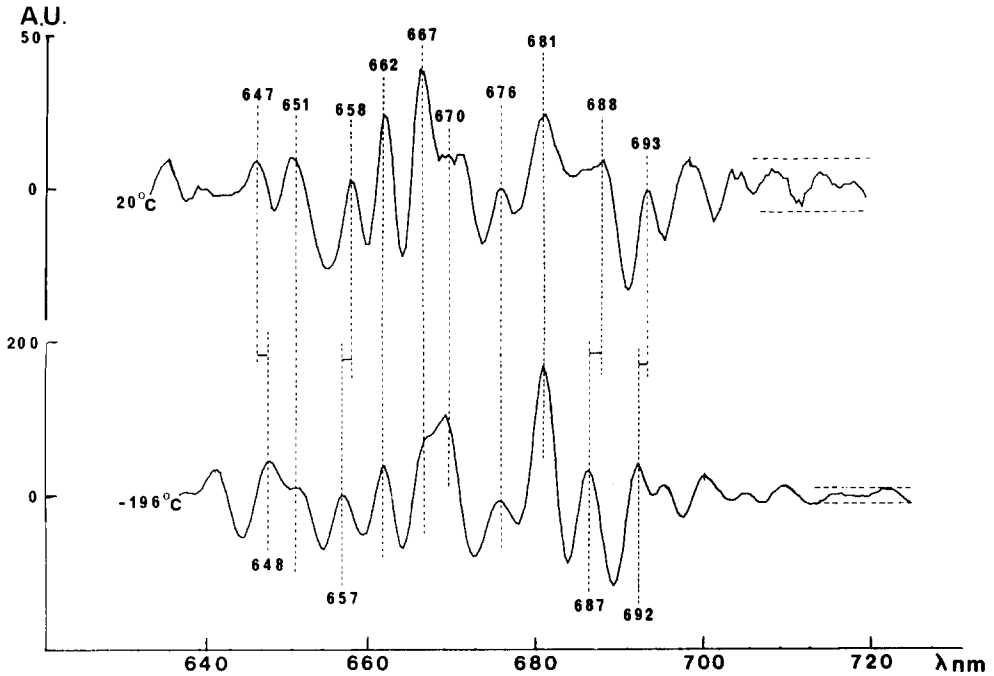

Fig. 5. Fourth derivative (D 8) of the absorption spectra of tobacco *P*-700 Chl *a* protein complex at 20°C and −196°C.

(b) *P*-700 Chl *a* protein. With this complex a remarkable stability (Fig. 5) is observed in the positions of the derivative signal with temperature. Only some relative amplitude variations are noted. Contrary to what is observed with light-harvesting Chl *a/b* protein, the 681 nm band does not change upon cooling (Table II, line E).

(c) Free chlorophyll. Contrary to what is observed with light-harvesting Chl *a/b* protein and thylakoids a derivative signal at 675 nm is already visible at 20°C (Table II, line F). The broad 669 nm band at 20°C, gives two bands at 667 and 669 nm at −196°C.

Discussion and Conclusions

When compared with their room temperature counterparts, the fourth derivative curves obtained from low temperature absorption spectra clearly reflect two types of changes in the absorption bands: (1) band narrowing, and (2) band shifting or band splitting. The derivative signals increase in amplitude and decrease in width because of the absorption bandwidth diminution which occurs at low temperatures. This phenomenon is due to the fact that molecular vibrations and rotations are lower at low temperature, as described in the recent work or Whitten et al. [36] on a bacteriochlorophyll-protein complex.

Excitonic theory can explain some of observed changes in absorption wavelength which occur upon cooling and can explain some aspects of the spectral

diversity of Chl *a* in vivo. According to the excitonic theory [37], a mutual perturbation of electrical origin between identical molecules which are more or less coupled introduces a variation in electronic excitation energy when compared to that of non-interacting molecules. The tightness of coupling between molecules is dependent on the distances between these molecules [38] as well as the orientations of their transition dipoles [37]. Simpson and Peterson [38] have described weak, strong, and intermediate coupling interactions. According to Kasha [37], these different couplings can lead to the formation of one or two new absorption bands from the principal absorption band of uncoupled molecules. The changes in the absolute energy levels for the principal electronic transitions, and thus the magnitude of the changes observed in absorption wavelength, are dependent on the degree of coupling. Moreover, the sign of the energy level change is dependent on the mutual molecular orientations of the interacting molecules; thus, band splitting or band shifting toward higher or lower wavelengths may be observed. Consequently, among a number of chemically identical chlorophyll molecules, the existence of excitonic coupling, of the types described above, can explain a part of the observed multiplicity of absorption bands of chlorophyll in vivo. Moreover, the spectral changes observed upon cooling may also be explained by modifications of some of these coupling interactions, either by changing intermolecular distances or by changes in mutual orientation.

In higher plant thylakoids, the 680 nm absorption band observed at 20°C develops at −196°C into a strong 675 nm band and a weaker band at 683 nm. This splitting (8 nm) is twice or three times greater than would be expected for a simple resolution of a two absorption band system due to narrowing of the absorption bandwidths and their corresponding derivative peaks. An exciton model in which occurs an increase of coupling at low temperature between two Chl molecules obliquely oriented towards one another could explain the observed splitting. This coupling of Chl *a* molecules is not strictly analogous to the dimer described in the Cotton et al. model [17] of the Chl *a* antenna, because in our model a chemical bond between the two molecules is not required but only a sufficiently short distance between the two molecules to allow electronic interaction. Weak exciton coupling is expected with Chl molecules having intermolecular distances up to about 20 Å [39].

At low temperature, the splitting of the 680 nm absorption band (or a further splitting of the 678—682 nm absorption band pair) is also observed with algal and cyanobacterial thylakoid preparations (Table I). This splitting can also be explained by a stronger coupling of Chl *a* molecules at low temperature rather than by the formation of artifactual aggregates or interactions. The argument that the 683 and 678-nm bands share a common origin is strengthened by previous observations concerning *Porphyridium* grown under different culture conditions. When the light quality or the medium composition where changed during cell growth, it was observed that the height ratios of the Gaussian components located at 683 and 678 nm were unchanged while the relative heights of other components showed reproducible variations [26].

Splitting of the 680 nm absorption band at low temperature is not universal. With light-harvesting Chl *a/b* protein, a simple shift from 680 to 675 nm is observed upon cooling. The absorption bands of the *P*-700 Chl *a* protein com-

plex show no variation in position with temperature (Fig. 5). In some Xanthophyceae (unpublished ovservations), the absorption bands in the 680 nm region are unaffected by cooling.

In all chlorophyll membranes investigated, no significant spectral shifts are observed between 650 and 670 nm. This fact correlates well with the low degree of organization of the transition dipoles of the monomeric chlorophyll absorbing in this region [40]. Beddard and Porter [41] recently reported the chemical stability of these Chl *a* fluorescent forms which must be separated from one another by strongly coordinating molecules. These molecules might also prevent removal and reorientation of chlorophyll molecules in the thylakoids upon cooling. In another study, stable hexacoordinated species absorbing mainly at 670 nm were recognized in vitro [42]. However, in vivo, up to now there is no evidence for the presence of such molecules [43]. At long wavelengths (around 690 nm), the absence of shifts could be due to the existence of stable dimers or polymers of Chl whose coupling interactions are little affected by temperature changes. Different data were obtained with chromophores of photosynthetic bacteria [44].

In summary, this spectroscopic study demonstrates that: (1) The use of low temperature spectral analyses does not introduce serious artifacts because most of the spectral bands seen early at −196° C are also detectable at 20° C. Only few modifications are seen at −196° C, principally with the 680 nm band.

Absorption band of thylakoid preparations, indicate only a slight change in the structure which is already present at 20°C. (2) The multiplicity of Chl spectral forms must be due to a high degree of pigment organization. This result is in agreement with the observed loss of spectroscopic resolutions when denaturing treatments (French press disruption in low ionic strength medium, detergents, and heating) are used on chlorophyll-containing materials.

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