The Calculated In Vitro and In Vivo Chlorophyll a Absorption Bandshape

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ABSTRACT The room temperature absorption bandshape for the Q transition region of chlorophyll a is calculated using the vibrational frequency modes and Franck–Condon (FC) factors obtained by line-narrowing spectroscopies of chlorophyll a in a glassy (Rebane and Avarmaa, *Chem. Phys.* 1982; 68:191–200) and in a native environment (Gillie et al., *J. Phys. Chem.* 1989; 93:1620–1627) at low temperatures. The calculated bandshapes are compared with the absorption spectra of chlorophyll a measured in two different solvents and with that obtained in vivo by a mutational analysis of a chlorophyll–protein complex. It is demonstrated that the measured distributions of FC factors can account for the absorption bandshape of chlorophyll a in a hexacoordinated state, whereas, when pentacoordinated, reduced FC coupling for vibrational frequencies in the range 540–850 cm^{-1} occurs. The FC factor distribution for pentacoordinated chlorophyll also describes the native chlorophyll a spectrum but, in this case, either a low-frequency mode ($\nu < 200$ cm^{-1}) must be added or else the 262-cm^{-1} mode must increase in coupling by about one order of magnitude to describe the skewness of the main absorption bandshape.

INTRODUCTION

In plants, the primary photosynthetic pigments are chlorophyll (chl) molecules that are bound to an ensemble of different proteins to form the structurally complex photosystems (Golbeck, 1992; van Grondelle et al., 1994; Green and Durnford, 1996; Jennings et al. 1996; Hankamer et al., 1997; Boekema et al., 2000). The individual chlorophyll–protein complexes that make up the photosystems have all been isolated, and considerable progress has been made in their biochemical and spectroscopic characterization (Gillie et al., 1989; Tang et al., 1990; Hemelrijk et al., 1992; Jennings et al., 1993; Zucchelli et al., 1994, 1996; Giuffra et al., 1997). Moreover, several of these complexes have been successfully reconstituted in vitro and a site-specific mutagenetic analysis is in progress (Bassi et al., 1999; Remelli et al., 1999; Rogl and Khülbrandt, 1999; Yang et al., 1999, He et al., 1999). It has become evident that the spectral characteristics of protein-bound chlorophyll molecules are modulated by interaction with their environment (Renge and Avarmaa, 1985; Gudowska-Nowak et al., 1990; Giuffra et al., 1997; Rogl and Kühlbrandt, 1999; van Amerongen and van Grondelle, 2001; Nishigaki et al., 2001), giving rise to spectroscopically different chl forms (French et al., 1972; Zucchelli et al., 1990, 1994; Hemelrijk et al., 1992; Krawczyk et al., 1993; Nussberger et al., 1994; Schubert et al., 1997; Nishigaki et al., 2001). The high level of spectral congestion, with the different chl spectral forms overlapping strongly, is responsible for the broad and featureless Q_y absorption band of pigment–protein complexes. This high spectral congestion renders it impossible, with conventional absorption and fluorescence spectroscopies, to directly access the spectral characteristic of the chlorophyll spectral forms. The absorption profile displays limited structure even on lowering the sample temperature, and a careful derivative analysis is required to indicate the presence of hidden features (e.g., Krawczyk et al., 1993; Zucchelli et al., 1996; Kleima et al. 1999; Kochubey and Samokhval, 2000).

Line-narrowing spectroscopies have been successfully used by a number of laboratories (Gillie et al., 1989; Tang et al., 1990; Reddy et al., 1992; Kwa et al., 1994; Peterman et al., 1997; Pieper et al., 1999a, 2000; Rätsep et al., 2000) and have provided a wealth of detailed information on the absorption properties of protein-bound chlorophylls. However, these techniques, which require the use of very low temperatures, are in themselves unable to describe the absorption characteristics at physiologically relevant temperatures and, therefore, must be augmented by other approaches. One such approach is the numerical decomposition of the absorption profile, in terms of a chosen absorption bandshape for the spectral forms.

Probably the most common bandshape choice is that of a substantially symmetrical Gaussian function to model the 0–0 absorption. As far as it goes, the choice is physically reasonable due to the presence of a statistical broadening of the 0–0 line (the inhomogeneous contribution), which gives rise to a Gaussian "dress" to the bandshape, even at low temperature (Schomacker and Champion, 1986; Hayes et al., 1988; Di Pace et al., 1992). This model bandshape can also describe the contribution of a low-frequency phonon to the 0–0 band-

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shape (Chan and Page, 1983; Di Pace et al., 1992; see also Methods). The principal drawback is that the intramolecular vibrational contributions are ignored and can, at best, be described by other Gaussian contributions in the high-energy region of the absorption spectrum (e.g., Shipman et al., 1976; Umetsu et al., 1999). It should be noted, however, that, although the Gaussian model is physically reasonable for the 0–0 bandshape, description of the vibrational part of the absorption spectra in terms of Gaussian bands has numerical value only, because groups of vibrational modes are arbitrarily correlated in a Gaussian envelope. To overcome these shortcomings, a number of attempts at using the chlorophyll solution absorption bandshape for spectral decomposition have been made (Trinkunas and Holtzwarth, 1994; Trinkunas et al., 1997). Although this chlorophyll bandshape approach is, at face value, sounder than the use of Gaussians, it lacks a theoretical basis of just how the protein matrix modifies the absorption properties. Very recently, Bassi and coworkers (Cinque et al., 2000) have obtained a "native" chlorophyll a (chla) room-temperature (RT) bandshape by the mutational deletion of a chla binding site in the antenna chl–protein complex, CP29. This mutational-deletion approach opens up the possibility of understanding just how the protein environment modifies the vibronic interactions that determine the absorption bandshape at RT, thus permitting the use of a correct 0–0 and vibrational transitions bandshape in spectral decomposition.

Information on the vibrational modes of the electronic ground state of chl molecules, both in solution and in a host matrix, has conventionally been obtained by resonance Raman spectroscopy (Lutz and Breton, 1973; Lutz, 1974, 1977; Fujiwara and Tasumi, 1986a,b; Krawczyk, 1989; Mattioli et al., 1993; Sato et al., 1995). This technique, however, is incapable of reliably detecting the presence of very low-frequency modes (e.g., Cupane et al., 1998) and the intensity of the vibronic contributions are not simply related to the coupling strengths with the electronic transition (Franck–Condon coupling factors). These Franck–Condon (FC) factors can be obtained, in principle, by using a variety of analyses of the resonance Raman data (Schomacher and Champion, 1986; Schweitzer-Stenner et al., 1997), but these approaches have never been used in the context of chla measurements. In recent years, line-narrowing spectroscopies, such as hole burning or fluorescence narrowing, have been extensively used to obtain detailed information on the chla vibrational modes coupled to the Q_y electronic transition (Rebane and Avarmaa, 1982; Gillie et al., 1989; Kwa et al., 1994; Peterman et al., 1997; Pieper et al., 1999a, 2000).

Of particular importance is that these line-narrowing spectroscopies permit determination of the FC coupling factors for each vibronic mode. This knowledge, in principle, allows theoretical modeling of the absorption bandshape of the Q_y transition, not only for the low temperatures at which measurements are performed ($T \leq 5$ K), but also at physiological temperatures (e.g., Jia et al., 1992). The first attempt to obtain a complete map of vibronic frequencies of chlorophyll with their FC factors was carried out by Rebane and Avarmaa (1982) and Avarmaa and Rebane (1985). In these studies, using both fluorescence line-narrowing and hole-burning spectroscopies, 37 vibrational modes in the energy range 263–1530 cm^{-1} were detected for chla dissolved in a glass of diethyl ether, but only relative FC factors were estimated. More recently, the same spectroscopic techniques have been used to analyze the vibronic distribution for antenna chla molecules in a native host protein matrix (Gillie et al., 1989; Peterman et al., 1997; Pieper et al., 1999a, 2000).

Using PSI200 particles, Small and coworkers (Gillie et al., 1989) obtained a map of the vibrational modes in the 262–1524-cm^{-1} wave number interval, the same energy region of the Rebane measurements, with the corresponding absolute FC coupling factors. These factors are weak for all modes, falling in the range 0.004–0.044. They also detected the presence of a bath of low-frequency phonons, due to the host protein, that could be described by a mean frequency mode of $\sim$22 cm^{-1} with a coupling factor of 0.8. Measurements on other chl–protein complexes indicate the presence of phonon frequencies in the 20–30-cm^{-1} range with coupling strengths between 0.3 and 0.8 (e.g., Jankowiak et al., 1993). These are relatively low values for electron phonon coupling and seem to be common to all antenna chls, with the exception of the very red shifted chls responsible for the long wavelength absorption tail of PSI, which have higher values (Croce et al., 1998; Cometta et al., 2000; Rätsep et al., 2000). An inhomogeneous contribution $\sigma_{inh} = 50$–55 cm^{-1} is associated with the chla bandshape in a protein environment (Reddy et al., 1994; Zucchelli et al., 1996) that, together with the phonon data, indicate a RT main bandwidth $\sigma \leq 98$ cm^{-1}, whereas, for chla in solution, a wider bandwidth, $\sigma \geq 160$ cm^{-1}, is measured (e.g., Shipman et al., 1976).

Another set of vibronic frequencies and FC factors, obtained using fluorescence line-narrowing spectroscopy, for chla in a native host protein environment, have been published by Peterman et al. (1997). Their measurements, which display a similar pattern of vibrational modes to those of Small and coworkers (Gillie et al., 1989; Pieper et al., 1999a,b), however, indicate FC factors that are vanishingly small and which yield the improbably high value of 0.98 for the Debye–Waller factor (DWF), the contribution of the 0–0 transition to the total normalized spectrum. A value for the DWF of 0.55 was obtained in the above-mentioned absorption hole-burning studies of Gillie et al. (1989) which is reasonably close to the value 0.64 for chla in solution (Shipman et al., 1976; see Methods). The origin of this discrepancy is not clear (for a discussion, see Pieper et al., 1999b), but the DWF = 0.98 of Peterman et al. implies the absence of a vibronic contribution to Q_y absorption. There are, however, similarities between the frequency set $\{\nu_r\}$

of vibronic modes obtained with these different measurements (Peterman et al., 1997; Pieper et al., 1999a,b), indicating a similarity in the vibronic pattern for the ground and the first excited state.

In the present study, which aims at obtaining a model bandshape for chla in a native protein environment at physiologically relevant temperatures, we have used the set $\{\nu_r, S_r\}$ of the vibrational frequencies ν_r and FC factors S_r to calculate the corresponding absorption bandshape in terms of the Fourier transform of a complex function containing the set $\{\nu_r, S_r\}$ and the temperature (Lax, 1952; Dexter, 1958; Markham, 1959; Pryce 1965; Page and Tonks, 1981). A Fast Fourier Transform (FFT) algorithm has been used to numerically estimate the chla bandshape, with the data sets $\{\nu_r, S_r\}$ obtained both by Rebane and Avarmaa (1982) in a glassy solution, and Gillie et al. (1989) in a native protein environment. Only chla is considered, because there are insufficient data available on chlb in a native protein environment (Gillie et al., 1989). The calculated absorption spectra are compared with the experimental RT absorption spectra of chla in a solution of diethyl ether (Shipman et al., 1976) and pyridine (Umetsu et al., 1999). The comparison is then extended to the RT chla spectrum in a native protein environment obtained by site-selective mutation by Cinque et al. (2000). It is demonstrated that the data sets $\{\nu_r, S_r\}$ of both Rebane and Avarmaa (1982) and Gillie et al. (1989) provide a good description of the absorption bandshape of an hexacoordinated rather than a pentacoordinated chla. In contrast, the RT absorption bandshape obtained by site-selective mutagenesis has a similar vibronic pattern to chla in the pentacoordinated state.

METHODS

When a single electronic excited state with the electronic transition linearly coupled to N harmonic FC active vibrational modes is considered in the context of the Born–Oppenheimer and Condon approximation, the normalized spectral line-shape function $B(\nu) \propto \varepsilon(\nu)/\nu$, where $\varepsilon(\nu)$ is the extinction coefficient spectrum, is given by the Fourier transform of a time-dependent function $f_N(t)$,

$$B(\nu) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp(-it(\nu - \nu_0) - \gamma|t|) f_N(t), \quad (1)$$

with

$$f_N(t) = \exp\left\{ -\sum_{r=1}^{N} S_r \left[\coth\left(\frac{h\nu_r}{2k_B T}\right) \times (1 - \cos \nu_r t) - i \sin \nu_r t \right] \right\}, \quad (2)$$

that contains the thermal average over the initial vibrational states; h is the Planck constant; k_B is the Boltzmann constant, and T the absolute temperature. In this expression, γ is the "natural" width, due to a finite lifetime of the state, and the presence of this damping term gives a finite width to each line of the line-shape function; ν_0 is the 0–0 transition frequency and S_r is the FC factor for the vibrational mode of frequency ν_r. All these parameters are temperature independent. This is a quite complex but standard expression (Lax, 1952; Dexter, 1958; Markham, 1959; Pryce 1965; Page and Tonks, 1981) that represents the overall absorption bandshape in terms of the "microscopic" parameters of a two-level system. The Q_y absorption band of chla molecules is due to an electric dipole-allowed $\pi \rightarrow \pi^*$ transition, which has a large extinction coefficient, thus allowing the interaction of the electronic transition with the set of vibrations to be described in the framework of the FC approximation, neglecting higher order effect. Thus, Eq. 1 can be used to describe the absorption (or fluorescence) bandshape for chla at different temperatures in terms of frequencies and FC coupling factor $\{\nu_r, S_r\}$ for each vibrational mode r.

Eq. 1 has been widely used as the starting point to obtain approximate expressions, in principle, more manageable (Pryce, 1965; Chan and Page, 1983). These approximate expressions have found application in the description of the site-selected absorption and fluorescence spectrum of chla in chl–protein complexes (e.g., Hayes et al., 1988; Peterman et al., 1997) and in the analysis of the spectroscopic properties of hemeproteins (Chan and Page, 1983; Di Pace et al., 1992). There are, however, drawbacks to their use and, in particular, that derived by Pryce (1965) neglects any broadening mechanisms ($\gamma = 0$) and uses the property of the Dirac δ function. This approach is mathematically well founded only for δ function and may not be used when the phonon profiles are used to calculate bandshapes. We have, therefore, preferred to use the more complete Eq. 1, which has been done by numerically calculating the Fourier transform with FFT algorithm, that is both reliable and fast. We have chosen to directly integrate Eq. 1 numerically because it contains the exact behavior of the bandshape at all temperatures, irrespective of the set of vibrational frequencies utilized. In this way, all approximations are eliminated and the microscopic parameters $\{\nu_r, S_r\}$ obtained by selective spectroscopies of chla at low temperature can be used to calculate a theoretical bandshape for all temperatures. The numerical integration process is, however, not free of troubles. The integral in Eq. 1 must be truncated at a finite time, and the choice of this time window is quite critical. The number of points used in the calculation is also critical.

To find the best choice of these parameters, it is important to have an exact result for comparison with the numerical solution. The possibility of integrating Eq. 1 in a closed form is without hope, but, in the presence of only one vibrational mode, it is possible to obtain a quite manageable expression for $B(\nu)$ that can be solved exactly and used to compare with the numerical results. This expression, derived in the Appendix, is used to calculate exact vibronic spectra with the vibrational frequencies characteristic of chla molecules and at different temperatures. These exact spectra are then used as a ruler to establish the time window and number of points to be used in the FFT calculations to avoid distortions. The FFT of Eq. 1 is a progression of Lorentzian peaks of width γ and height that depends on the coupling strength (FC factors S_r) and, in general, on the temperature used in the calculation. The inhomogeneous contribution, of statistical nature (Schomacker and Champion, 1986), is treated separately and modeled as a Gaussian distribution G_{inh} of width σ_{inh}, temperature independent, that is convolved with the bandshape $B(\nu)$,

$$A(\nu) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp(-it(\nu - \nu_0) - \gamma|t|) f_N(t) \otimes G_{inh}, \quad (3)$$

giving a Gaussian dress to each vibronic peak. The main input parameters are the set $\{\nu_r, S_r\}$, that contain also the low-frequency phonons modes, when present. In this way, a model bandshape for chla, comprising the vibrational region, can be obtained in terms of its microscopic, experimentally established, characteristics.

It is interesting to note that, when a number, L, of phonon modes, with $h\nu_r \ll k_BT$, is present, the set of N modes $\{\nu_r, S_r\}$ can be divided into two subsets: one containing the L phonon modes and the other containing all the remaining M vibrational modes. With this partition, Eq. 1 can be written as

$$B(\nu) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp(-it(\nu - \nu_0) - \gamma|t|) f_M(t) f_L(t). \quad (4)$$

We can regard $\exp(-\gamma|t|)f_M(t)$ as the Fourier transform of a function $H(u)$ and $f_L(t)$ as the Fourier transform of a function $L(u)$. Using the convolution theorem for Fourier transforms (e.g., Sneddon, 1972), Eq. 4 can be written as

$$B(\nu) = \int_{-\infty}^{+\infty} du\, L(u)H(\nu - \nu_0 - u)$$

$$\equiv L(\nu - \nu_0) \otimes H(\nu - \nu_0). \quad (5)$$

$B(\nu)$ is then the convolution ($\otimes$) of the two function,

$$H(\nu - \nu_0) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp(-it(\nu - \nu_0) - \gamma|t|) f_M(t), \quad (6)$$

equivalent to Eq. 1, but with $f_M(t)$ (see Eq. 2) written in terms of the subset of the M vibrational modes, whereas

$$L(\nu - \nu_0) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp[-it(\nu - \nu_0)] f_L(t)$$

$$\cong \frac{1}{\sqrt{2\pi}\sigma} \exp\left[- \frac{\left(\nu - \nu_0 - \sum_{r=1}^{L} S_r\nu_r\right)^2}{2\sigma^2}\right] \quad (7)$$

is a Gaussian with

$$\sigma^2 = \sum_{r=1}^{L} S_r \coth\left(\frac{h\nu_r}{2k_BT}\right)\nu_r^2,$$

a result obtained following Chan and Page (1983). The bandshape $A(\nu)$ can then be written as

$$A(\nu) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp(-it(\nu - \nu_0) - \gamma|t|) f_M(t)$$

$$\otimes L(\nu - \nu_0) \otimes G_{inh}, \quad (8)$$

where $L(\nu - \nu_0)$, the so called homogeneous contribution, gives a further term to the Gaussian dress, because the convolution of two Gaussians is itself a Gaussian, that now has the width $\sigma_{tot}^2 = \sigma^2 + \sigma_{inh}^2$. This shows that, also in the presence of an unknown phonon and inhomogeneous contribution, the calculated band shape is a correct representation of the absorption spectrum at high temperature, but the bandwidth of the Gaussian term cannot be interpreted as the pure inhomogeneous contribution. When the phonon contributions are known, their frequency and coupling factors are inserted directly in the set $\{\nu_r, S_r\}$ used to calculate $B(\nu)$.

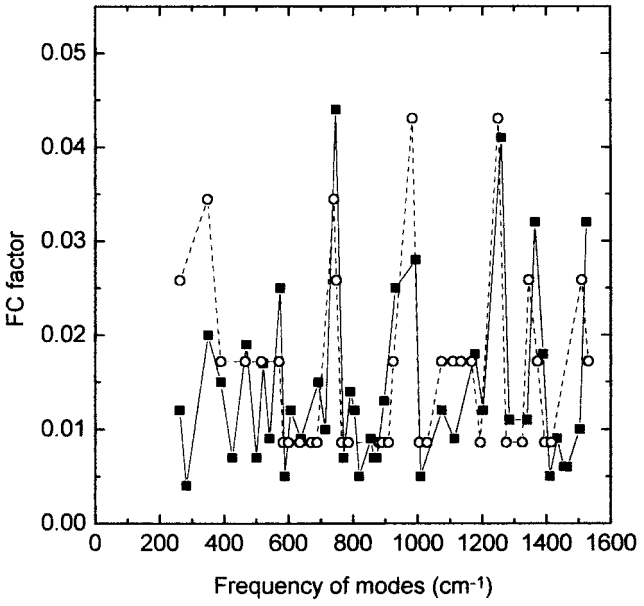

FIGURE 1 The vibrational modes and Franck–Condon (FC) factors for chlorophyll a. (—■—) chlorophyll a in native host protein environment at 1.6 K (Gillie et al., 1989); (- - ○ - -) chlorophyll a dissolved in a glass of diethyl ether at 5 K (Rebane and Avarmaa, 1982), the FC values are normalized to the value $\Sigma_{i=1}^{N} S_i = 0.594$ obtained by Gillie et al. (1989).

To check the results of this procedure, the theoretical bandshape should be compared with the measured absorption band shape for an absorber molecule of known parameters $\{\nu_r, S_r\}$. Chlorophyll a in a glassy solution is ideal for this purpose, but, unfortunately, there is no absorption spectrum available at 5 K, the temperature of the published data $\{\nu_r, S_r\}$ (Rebane and Avarmaa, 1982). To overcome this problem, we have taken advantage of the fact that Eq. 3 describes the bandshape as a function of temperature. We have therefore used the set $\{\nu_r, S_r\}$ measured at 5 K to describe the RT absorption spectra of chla measured in different solvent environments. The FFT of Eq. 3 is the model band shape used to fit an experimental spectrum in a fitting procedure that uses Minuit (CERN Program Library, Version 94.1) as minimizer. At a given temperature, a number of parameters can be independently selected as free-fit parameters; these include the area of the bandshape, the 0–0 frequency, the width of the Gaussian distribution σ_{tot}, all the vibrational frequencies ν_r and the corresponding FC coupling factors S_r. For each of these free parameters, an interval of variation can be defined, which allows the experimental errors to be taken into account. The fitting routine may also be used in an analytical way, searching for the values of selected parameters that describe the input bandshape.

We have used, as input spectra, the measured RT absorption spectra of chla in different environmental conditions: chla in dry diethyl ether (Shipman et al., 1976); chla in dry pyridine (Umetsu et al., 1999); chla in the chl–protein complex CP29 (Cinque et al., 2000). It is known that chla in dry diethyl ether has a pentacoordinated Mg, whereas chla in dry pyridine is hexacoordinated (Shipman et al., 1976; Fujiwara and Tasumi, 1986a,b; Fujiwara et al., 1988; Krawczyk, 1989). The measured absorption spectra of chla are described in terms of the vibrational frequencies and FC factors $\{\nu, S_r\}$ obtained both by Rebane and Avarmaa (1982) for chla in a glassy matrix, and by Gillie et al. (1989) using a PSI200 preparation. The two data sets $\{\nu_r, S_r\}$, compared in Fig. 1 after a normalization to $\Sigma_{r=1}^{41} S_r = 0.598$

(Gillie et al., 1989), are similar (see also Peterman et al., 1997; Pieper et al., 1999a,b).

RESULTS AND DISCUSSION

The set $\{\nu_r, S_r\}$ of vibrational frequencies and FC coupling factors obtained by Rebane and Avarmaa (1982) for chla in a glassy matrix of diethyl ether is the input parameter set used to describe the absorption spectrum of chla in a diethyl ether solution (RT). In these fits the 0–0 frequency and the Gaussian bandwidth σ_{tot} are free parameters, whereas the frequencies ν_r are taken as fixed values, and the FC factors S_r were allowed to vary within ±20%, twice the error associated by Pieper et al. (1999b) with their measurements. The parameter γ is fixed to 5 cm^{-1}, a value of the order of the inhomogeneous line width obtained by Avarmaa and Rebane (1985). It should be noted that this choice is not critical because a width of this order has no impact on the overall band width. The FC factor distribution must be correctly normalized before being used in the fit procedure and the DWF, which represents the contribution of the 0–0 line to the total spectrum, numerically equal to $\exp(-\sum_{r=1}^{N} S_r)$, is the suitable parameter to use. An estimation of the DWF for the Q_y absorption spectrum of chla in diethyl ether, using the band-shape Gaussian partitioning of Shipman et al. (1976), gives a value ~0.64, and this has been used as the normalizing factor. It should be noted that a DWF = 0.55 is obtained using the FC values of Gillie et al. (1989), possibly indicating a smaller contribution of the 0–0 transition to the absorption spectrum when chla is in a native protein environment. The results in Fig. 2 *A* show that, although, as expected, a good description of the main Q_y band is obtained, a marked discrepancy is present in the vibronic wave-length region between 610 and 645 nm. This discrepancy can be largely removed if all S_r values are allowed to vary much more than 20%, but this leads to a distribution of FC factors very different from the measured ones. A similar result is obtained when the set $\{\nu_r, S_r\}$ determined by Gillie et al. (1989) for chla in a protein environment is used (Fig. 2 *B*). This is not surprising, because the two data sets are rather similar (Fig. 1), indicating that the protein environment only slightly perturbs the distribution of the characteristic vibrational modes of chla. The source of the discrepancy in the wave-length region 610–645 nm is mainly due to the presence of strong vibrational modes around 750 cm^{-1} (see Fig. 1; Peterman et al., 1997; Pieper et al., 1999a,b), which are present in both membrane-bound and solvated chla at low temperature. Vibronic peaks around this frequency are also suggested by Raman spectra for chla in different environmental conditions (e.g., Fujiwara and Tasumi, 1986b; Mattioli et al., 1993; Sato et al., 1995).

It is clear from Fig. 2 that the vibronic pattern $\{\nu_r, S_r\}$ obtained for chla in a glassy environment (5 K) does not accurately describe the absorption spectrum of chla in a solution of the same solvent (dry diethyl ether) at RT. Chlorophyll a in a dry diethyl ether solution is known to be pentacoordinated (Shipman et al., 1976; Fujiwara and Tasumi, 1986a,b; Fujiwara et al., 1988; Krawczyk, 1989) at RT, as is usual in nonpolar solvents. In contrast, it has been shown that chla in dry diethyl ether (Krawczyk, 1989) changes from the pentacoordinated to the hexacoordinated

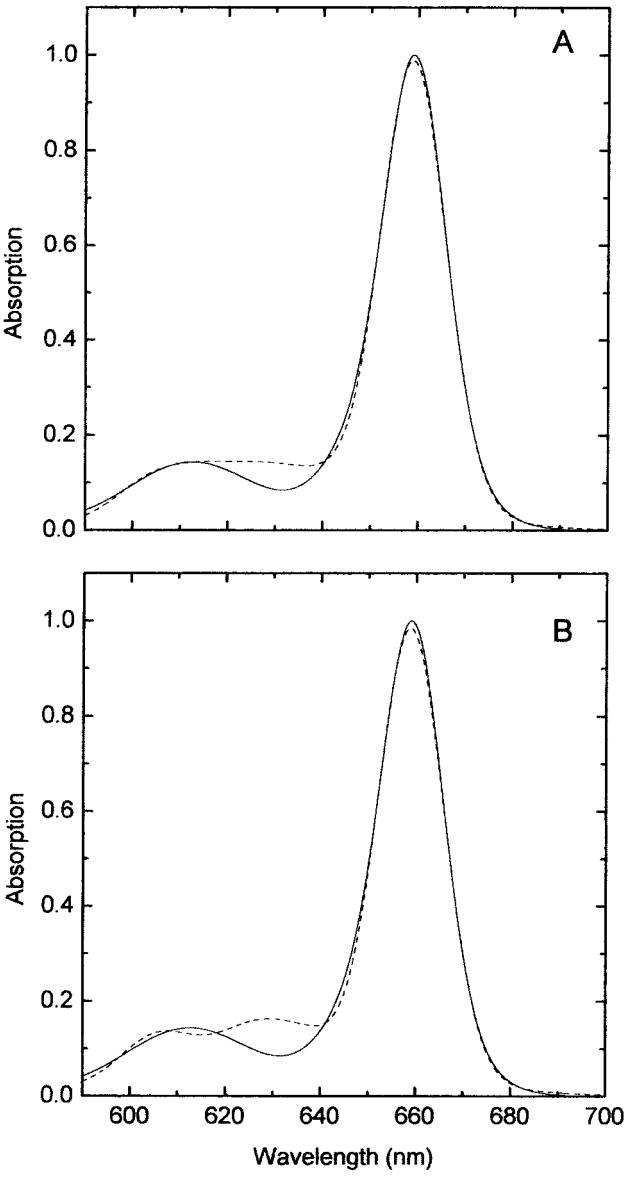

FIGURE 2 Absorption spectrum of chlorophyll a in diethyl ether at room temperature. The experimental spectrum is taken from Shipman et al. (1976). The dashed lines are fits to the measured spectrum using Eq. 3. (*A*) Fit obtained using, as input parameters, the set $\{\nu_r, S_r\}$ of the frequency modes ν_r and FC factors S_r obtained by Rebane and Avarmaa (1982) after normalization to a proper DWF (see text). In this fit, ν_r are taken as fixed values, whereas S_r were allowed to vary within ±20%. The parameter γ = 5 cm^{-1} and a value σ_{tot} = 159 cm^{-1} is obtained. (*B*) As in (*A*), but using the set $\{\nu_r, S_r\}$ obtained by Gillie et al. (1989). The parameter γ = 5 cm^{-1} and a value σ_{tot} = 161 cm^{-1} is obtained.

state on passing from RT to 77 K. Because line-narrowing spectroscopies are performed at very low temperatures, it is possible that Rebane and Avarmaa (1982), measuring chla in diethyl ether at 5 K instead of a pentacoordinated chla, in fact measured the hexacoordinated molecule, with all the changes in the vibronic pattern associated with the different configurational state of the chla macrocycle induced by this configuration (Fujiwara and Tasumi, 1986a). To test this hypothesis, we have attempted to describe the absorption spectrum of a hexacoordinated chla using, as input spectrum, that measured by Umetsu et al. (1999) in dry pyridine at RT. Both sets of data $\{\nu_r, S_r\}$ obtained by Rebane and Avarmaa and by Gillie et al. have been used. As in the previous fits, the 0–0 frequency and the bandwidth contribution, σ_{tot}, are free-fit parameters, but the frequencies ν_r are taken as fixed values and the FC factors S_r are allowed to vary by up to ±25%. In this case, the DWF = 0.55 (Gillie et al., 1989) was used to normalize the S_r distribution of Rebane and Avarmaa, due to the smaller 0–0 contribution apparent in the absorption spectrum (see also Umetsu et al., 1999). A good description of the RT chla–pyridine absorption spectrum is obtained (Figs. 3 and 4) with a distribution of S_r substantially similar to that measured for both chla in a glassy and in a native protein environment. Thus, the presence of the vibrational modes around 750 cm^{-1} with relatively high FC factors accounts for the description of the hexacoordinated chla bandshape (pyridine solvent). At the same time, it is the main source of discrepancy in the description of the pentacoordinated chla (diethyl ether). The vibrational region around and below 750 cm^{-1} is considered as characteristic of overall deformations of the tetrapyrrole macrocycle (Ogoshi et al., 1972; Lutz, 1974). Such a deformation could follow changes in the Mg coordination state due to the displacement of the Mg atom of ~0.4 Å above the nitrogen plane (when pentacoordinated) to the in-plane position (when hexacoordinated).

We have then checked whether both sets $\{\nu_r, S_r\}$ obtained by low-temperature line-narrowing spectroscopies can describe the pentacoordinated chla band shape when major changes in the FC parameter values are permitted. To this end, the absorption spectrum of chla-diethyl ether, measured by Shipman et al. (1976), was taken as the input spectrum and the FC factors S_r of the vibrational modes $\nu_r <$ 900 cm^{-1} were only upper bounded. The other fit parameters were subject to the previous fit conditions. The results are shown in Figs. 5 and 6. A good overall description is obtained with distributions of FC factors similar to those measured, with the exception of a number of frequency modes around 750 cm^{-1} that change considerably (S_r very low).

Taken together, these results indicate that Eq. 3, using the sets of vibrational data $\{\nu_r, S_r\}$ obtained by selective spectroscopies of both solvated and protein-bound chla, provides a good description of the RT absorption spectrum of chla in a hexacoordinated state, whereas, in a pentacoordinated state, changes in the coupling factors for vibrational modes in the range 540–850 cm^{-1} are needed. In other words, the distribution $\{\nu_r, S_r\}$ obtained using a PSI200 preparation (Gillie et al., 1989) seems to have the FC characteristic for chla in a hexacoordinated state, whereas it is generally thought that chla in a membrane–protein envi-

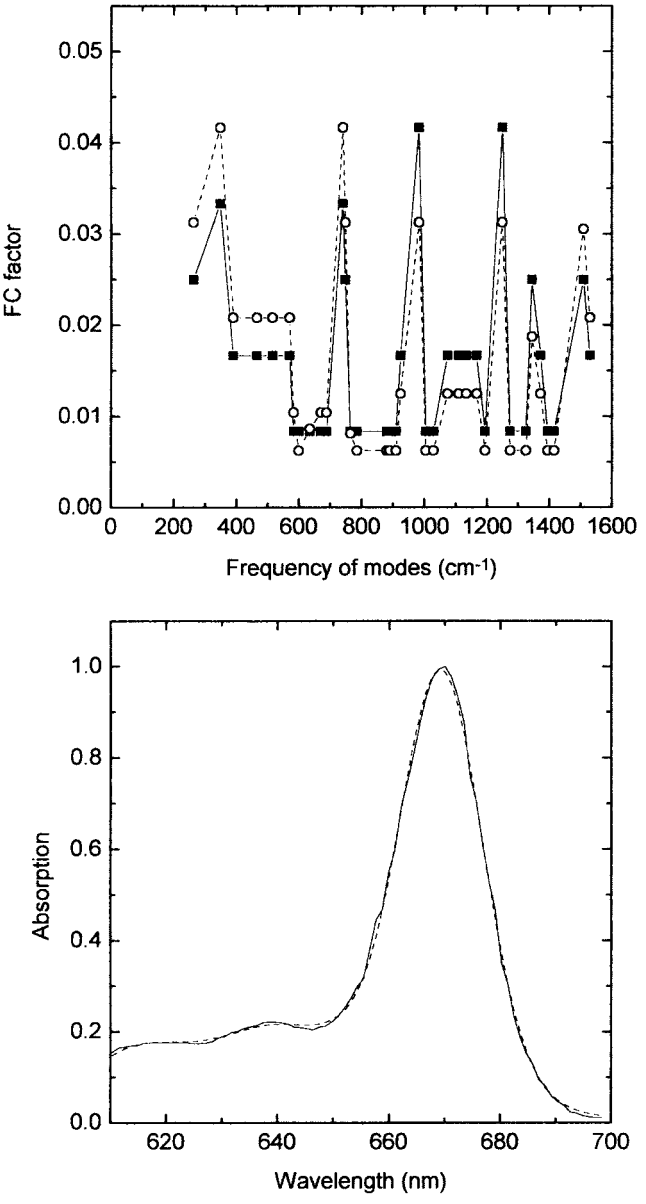

FIGURE 3 Fit of the absorption spectrum of chlorophyll a measured in pyridine at room temperature. The experimental spectrum is taken from Umetsu et al. (1999). Fit obtained using, as input parameters, the set $\{\nu_r, S_r\}$ of Rebane and Avarmaa (1982) after normalization to a proper DWF (see text). In this fit, ν_r are taken as fixed values, whereas S_r were allowed to vary within ±25%. The parameter γ = 5 cm^{-1} and a value σ_{tot} = 166 cm^{-1} is obtained. *Upper panel*: the comparison between the experimental FC factor distribution (—■—) and that obtained by the fit procedure (- - ○ - -). *Lower panel*: the comparison between the measured (——) and the calculated spectrum (- - - -).

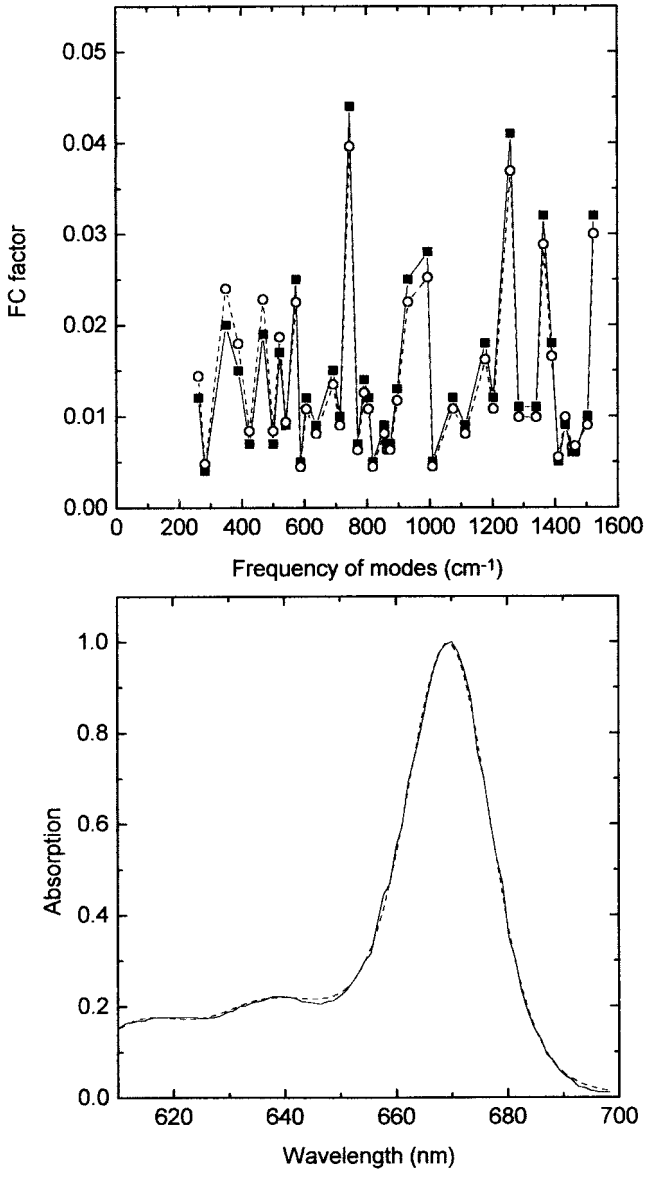

FIGURE 4 Fit of the absorption spectrum of chlorophyll a measured in pyridine at room temperature. The experimental spectrum is taken from Umetsu et al. (1999). Fit obtained using, as input parameters, the set $\{\nu_r, S_r\}$ of Gillie et al. (1989). In this fit, ν_r are taken as fixed values, whereas S_r were allowed to vary within ±25%. The parameter $\gamma = 5\ cm^{-1}$ and a value $\sigma_{tot} = 170\ cm^{-1}$ is obtained. *Upper panel*: the comparison between the experimental FC factor distribution (—■—) and that obtained by the fit procedure (- - ○ - -). *Lower panel*: the comparison between the measured (——) and the calculated spectrum (- - - -).

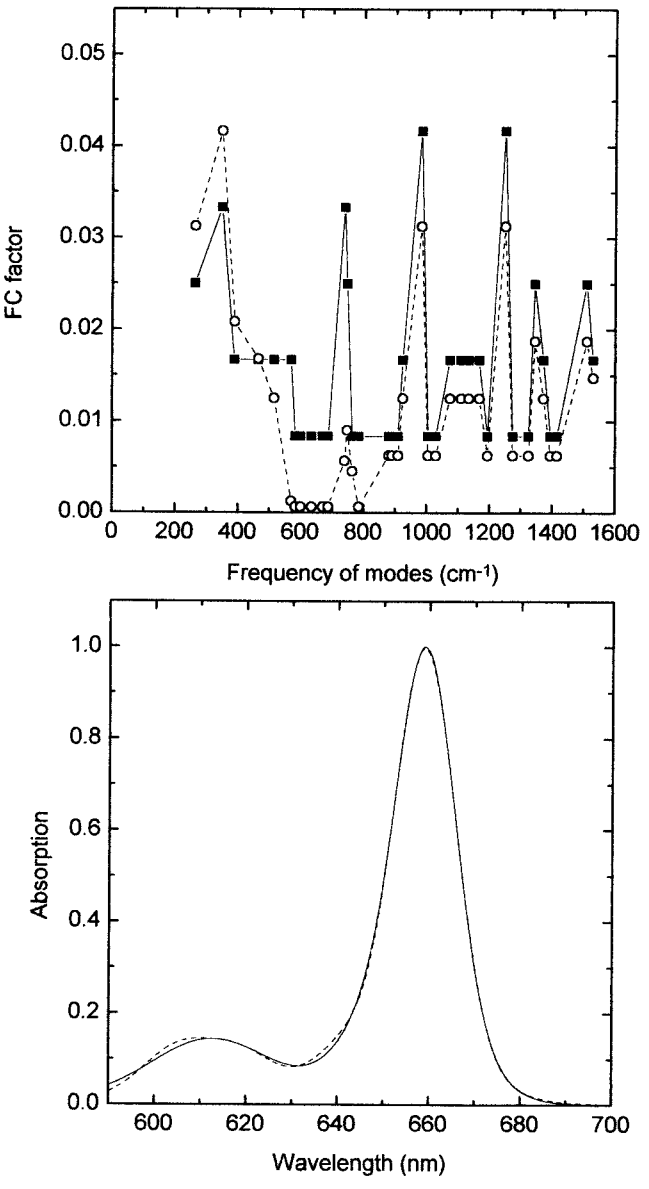

FIGURE 5 Fit of the absorption spectrum of chlorophyll a measured in diethyl ether at room temperature. The experimental spectrum is taken from Shipman et al. (1976). Fit obtained using, as input parameters, the set $\{\nu_r, S_r\}$ of Rebane and Avarmaa (1982) after a normalization to a proper DWF (see text). In this fit ν_r are taken as fixed values, whereas S_r were allowed to vary within ±25% except for the FC factors of the frequency modes $\nu_r < 900\ cm^{-1}$, which were allowed to vary between 0 and +25%. The parameter $\gamma = 5\ cm^{-1}$ and a value $\sigma_{tot} = 155\ cm^{-1}$ is obtained. *Upper panel*: the comparison between the experimental FC factors distribution (—■—) and that obtained by the fit procedure (- - ○ - -). *Lower panel*: the comparison between the measured (——) and the calculated spectrum (- - - -).

ronment is pentacoordinated (Matthews et al., 1979; Peterman et al., 1997), a coordination state recently confirmed by the three-dimensional structure of PSI (Jordan et al., 2001). This suggests that changes of the chla coordination state at cryogenic temperatures may occur in membranes, as is the case in some solvents (Krawczyk, 1989; Bellacchio and Sauer, 1999).

It is interesting to consider, in this context, the chla absorption band shape in a native protein environment recently obtained, at RT, by the mutational deletion of the chla binding site A2 in the chl–protein complex CP29 (Cinque et al., 2000). The chla spectrum is obtained by

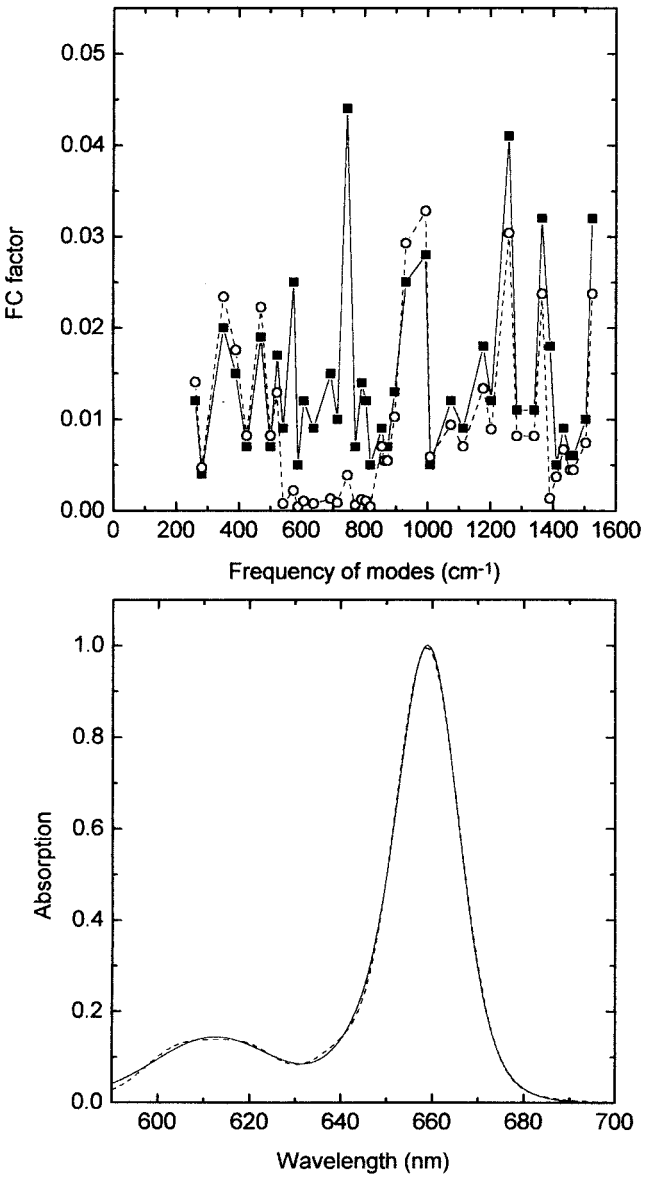

FIGURE 6 Fit of the absorption spectrum of chlorophyll a measured in diethyl ether at room temperature. The experimental spectrum is taken from Shipman et al. (1976). Fit obtained using, as input parameters, the set $\{\nu_r, S_r\}$ of Gillie et al. (1989). In this fit, ν_r are taken as fixed values, whereas S_r were allowed to vary within ±25% except for the FC factors of the frequency modes $\nu_r < 900\ cm^{-1}$, which were allowed to vary between 0 and +25%. The parameter $\gamma = 5\ cm^{-1}$ and a value $\sigma_{tot} = 159\ cm^{-1}$ is obtained. *Upper panel*: the comparison between the experimental FC factor distribution (—■—) and that obtained by the fit procedure (- - ○ - -). *Lower panel*: the comparison between the measured (——) and the calculated spectrum (- - - -).

the difference of the wild-type and mutant absorption spectra after appropriate normalization to the total chlorophyll. The chla spectrum thus obtained has its maximum at 680 nm and is believed to represent the electronic transition of lowest frequency in CP29 (see also Pieper et al., 2000). This approach can only yield the spectrum of a chl molecule bound to a particular protein site if its absorption band is not perturbed by interactions with other pigments. The mutated chl site, in this case, is known as A2. We have analyzed this difference spectrum in terms of the data set $\{\nu_r, S_r\}$ obtained by Gillie et al. (1989), plus a phonon mode (input data: $\nu_m = 20\ cm^{-1}$, range of allowed variation, 0–30 cm^{-1}; $S_m = 0.5$, range of allowed variation 0–1) that has been inserted to account for the hole-burning data and thermal band shape analysis (Zucchelli et al., 1996). The results are shown in Fig. 7. Due to the possible distortion of the experimental chla spectrum, we have not attempted to obtain a description closer than that reported in the figure. A distribution of FC factors substantially similar to that obtained for chla in the pentacoordinated state, with frequency modes in the range 390–855 cm^{-1} having very low FC factors, describes the chla absorption band shape in a native environment. However, to describe the skewness of the main spectral band, the FC factor of the vibrational mode at 262 cm^{-1}, the lowest active chla modes observed in the hole-burning spectra of PSI200 (Gillie et al., 1989), becomes unreasonably high; more than one order of magnitude higher than that obtained by hole burning. Alternatively, the skewness of the chla main absorption band requires, at least, one other vibrational mode with frequency $\nu_1 < 250\ cm^{-1}$. When this new active mode is included in the parameter set used to fit the absorption spectrum (Fig. 8), the distribution of FC factors, S_r, is similar to that obtained for chla in the pentacoordinated state (Fig. 6).

It should be noted that an exact analysis of the low frequency modes is beyond the possibility of this fit due to the interplay between the inhomogeneous distribution width and the contribution of these modes to the overall main band shape. It is worth mentioning that a vibrational mode at 140 cm^{-1} was observed in single-site absorption spectra of chla in an n-octane matrix (Platenkamp et al., 1980) and the presence of an allowed low-frequency vibrational mode for chla in chl–protein complexes comprising the external antenna of PSII has been proposed (Zucchelli et al., 1995, 1996; Schubert et al., 1997) to account for thermal changes in the optical properties of these complexes.

CONCLUSIONS

The spectral band shape of chla in the Q absorption region has been calculated by numerical integration of its Fourier transform representation in terms of the frequencies of the vibrational modes and their coupling factors giving a model band shape, as a function of temperature, which may be used in the analysis of the congested spectra of chla in chl–protein complexes. To check whether the result of these calculations, using the microscopic parameters experimentally determined at low tem-

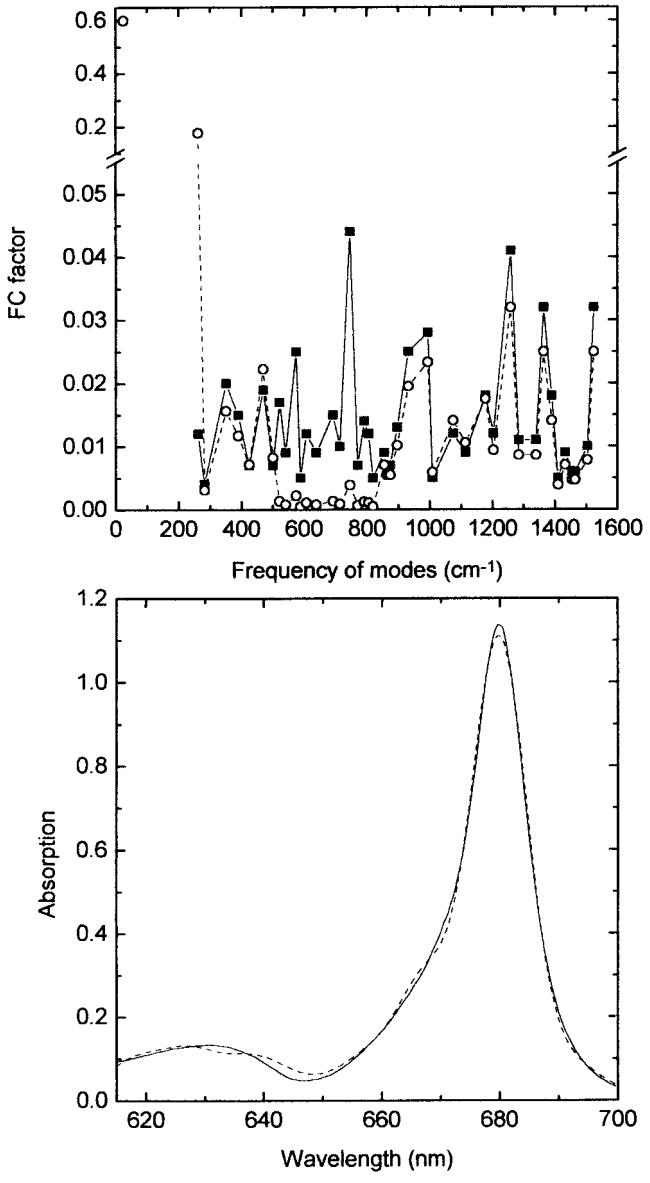

FIGURE 7 Fit of the absorption difference spectrum of chlorophyll a obtained by mutational analysis. The experimental spectrum is taken from Cinque et al. (2000). Fit obtained using, as input parameters, the set $\{\nu_r, S_r\}$ of Gillie et al. (1989) after a normalization to a proper DWF (see text). In this fit, ν_r are taken as fixed values, whereas S_r were allowed to vary within $\pm 25\%$ except for the FC factors of the frequency modes $\nu_k < 900\ cm^{-1}$, which were free. With respect to the previous fits, one phonon mode ($0 \leq \nu_1 \leq 30\ cm^{-1}$, $0 \leq S_1 \leq 1$) is added to the input set of parameters, according to the hole-burning data (Gillie et al., 1989). The parameter $\gamma = 5\ cm^{-1}$ and a value $\sigma_{inh} = 65\ cm^{-1}$ is obtained. *Upper panel*: the comparison between the experimental FC factors distribution (—■—) and that obtained by the fit procedure (- - ○ - -). *Lower panel*: the comparison between the measured (——) and the calculated spectrum (- - - -).

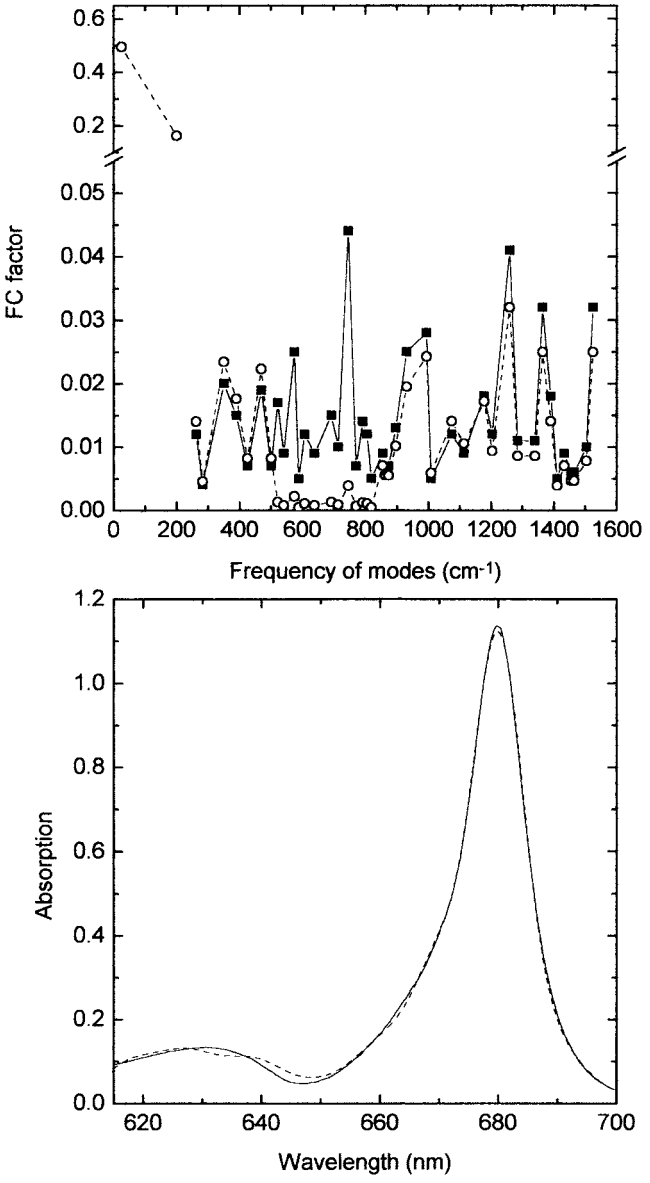

FIGURE 8 Fit of the absorption-difference spectrum of chlorophyll a obtained by mutational analysis. The data set is as in Fig. 7, but one vibrational mode ($\nu_2 \geq 80\ cm^{-1}$, $0 \leq S_2 \leq 1.5$) is added to the input set of parameters. The parameter $\gamma = 5\ cm^{-1}$ and a value $\sigma_{inh} = 56\ cm^{-1}$ is obtained. *Upper panel*: the comparison between the experimental FC factors distribution (—■—) and that obtained by the fit procedure (- - ○ - -). *Lower panel*: the comparison between the measured (——) and the calculated spectrum (- - - -).

perature, can describe chla absorption spectra in different environments, we have used the model band shapes to fit the following RT chla absorption spectra: chla in a solution of diethyl ether, a solvent in which chla has its Mg pentacoordinated; chla in a pyridine solution, where chla has its Mg hexacoordinated; chla in a protein environment, where the Mg is pentacoordinated. We conclude that:

1. The RT absorption spectrum of chla in diethyl ether cannot be described either by the distribution of frequencies and FC factors obtained for chla in a glassy host of the same solvent at low temperature or by the

distribution of the same parameters obtained in a host protein environment.

2. A good description of the RT pentacoordinated chla absorption spectrum (diethyl ether solution) is obtained when the vibrational modes in the range 540–850 cm^{-1}, characteristics of overall deformations of the tetrapyrrole macrocycle, became negligible.
3. Both the distributions of vibrational modes and FC factors obtained in a glass of diethyl ether host and in a protein environment can account for the RT absorption spectra of a hexacoordinated chla.
4. The RT absorption bandshape for chla in a native protein environment is described by a distribution of vibrational modes and FC factors similar to those used to describe a pentacoordinated chlorophyll, except for the presence of a strong asymmetry of the Q_y band which requires either that the lowest frequency mode becomes strongly allowed or the presence of at least one more low-frequency mode, $\nu < 250\ cm^{-1}$.

APPENDIX

The absorption bandshape $B(\nu)$, written in terms of the set $\{\nu_r, S_r\}$ of vibrational frequencies ν_r and FC factors S_r, is given by (see Methods)

$$B(\nu) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp[-it(\nu-\nu_0) - \gamma|t|] \times \exp\left\{-\sum_{r=1}^{N} S_r\left[\coth\left(\frac{h\nu_r}{2k_BT}\right)(1-\cos\nu_r t) - i\sin\nu_r t\right]\right\},$$

that can be rewritten, in term of the mean occupation number $\langle n_r\rangle \equiv (\exp(h\nu_r/k_BT) - 1)^{-1}$, as

$$B(\nu) = \frac{1}{2\pi}\exp\left[-\sum_{r=1}^{N} S_r(1+2\langle n_r\rangle)\right]\sum_{-\infty}^{+\infty} dt\, \exp[-it(\nu-\nu_0) - \gamma|t|]\exp\left\{+\sum_{r=1}^{N} S_r[(1+\langle n_r\rangle)e^{i\nu_r t} + \langle n_r\rangle e^{-i\nu_r t}]\right\}. \quad \text{(A1)}$$

In the presence of one vibrational mode, with frequency ω and coupling S, Eq. A1 becomes

$$\begin{aligned} B(\nu) &= \frac{1}{2\pi}\exp[-S(1+2\langle n\rangle)]\int_{-\infty}^{+\infty} dt\, \exp[-it(\nu-\nu_0) - \gamma|t|] \times \exp[S(1+\langle n\rangle)e^{i\omega t}]\exp(S\langle n\rangle e^{-i\omega t}) \\ &= \exp[-S(1+2\langle n\rangle)]\sum_{k=0}^{\infty}\sum_{m=0}^{\infty}\frac{S^{k+m}}{k!\,m!}(1+\langle n\rangle)^k\langle n\rangle^m \times \frac{1}{2\pi}\int_{-\infty}^{+\infty} dt\, \exp[-it(\nu-\nu_0-(k-m)\omega) - \gamma|t|] \\ &= \exp[-S(1+2\langle n\rangle)]\sum_{k=0}^{\infty}\sum_{m=0}^{\infty}\frac{S^{k+m}}{k!\,m!}(1+\langle n\rangle)^k\langle n\rangle^m \times \frac{\gamma}{(\nu-\nu_0-(k-m))^2+\gamma^2}, \end{aligned} \quad \text{(A2)}$$

a double sum of weighted Lorentzian having the width γ.

The double series $\sum_{k=0}^{\infty}\sum_{m=0}^{\infty} a_{k,m}$, considered as a sum over a bidimensional array $\{a_{k,m}\}$, can be written as

$$\sum_{k=0}^{\infty}\sum_{m=0}^{\infty} a_{k,m} \equiv \sum_{k=0}^{\infty} a_{k,k} + \sum_{m=1}^{\infty} a_{0,m} + \sum_{k=1}^{\infty} a_{k,0} + \cdots + \sum_{m=n}^{\infty} a_{n-1,m} + \sum_{k=n}^{\infty} a_{k,n-1} + \cdots.$$

In this way, $B(\nu)$ becomes

$$\begin{aligned} B(\nu) = \exp[-S(1+2\langle n\rangle)]\Bigg\{&\frac{\gamma}{(\nu-\nu_0)^2+\gamma^2} \times \sum_{k=0}^{\infty}\frac{S^{2k}}{k!\,k!}(1+\langle n\rangle)^k\langle n\rangle^k + \cdots + \frac{(1+\langle n\rangle)^{r-1}}{(r-1)!} \\ &\times \sum_{m=r}^{\infty}\frac{S^{m+r-1}}{m!}\langle n\rangle^m\frac{\gamma}{(\nu-\nu_0-(r-1-m)\omega)^2+\gamma^2} \\ &+ \frac{\langle n\rangle^{r-1}}{(r-1)!}\sum_{k=r}^{\infty}\frac{S^{k+r-1}}{k!}(1+\langle n\rangle)^k \times \frac{\gamma}{(\nu-\nu_0-(k-r+1)\omega)^2+\gamma^2} + \cdots \end{aligned}$$

$$r = 1, 2, 3 \ldots,$$

and, after a rearrangement of the series indexes m and k,

$$B(\nu) = \exp[-S(1 + 2\langle n\rangle)]\left\{\frac{\gamma}{(\nu - \nu_0)^2 + \gamma^2}\right.$$

$$\times \sum_{k=0}^{\infty} \frac{S^{2k}}{k!\,k!}(1 + \langle n\rangle)^k\langle n\rangle^k + \cdots$$

$$+ \sum_{m=1}^{\infty} S^m\langle n\rangle^m \frac{\gamma}{(\nu - \nu_0 + m\omega)^2 + \gamma^2}$$

$$\times \frac{S^{2(r-1)}(1 + \langle n\rangle)^{r-1}\langle n\rangle^{r-1}}{(m + r - 1)!\,(r - 1)!} + \sum_{k=1}^{\infty} S^k(1 + \langle n\rangle)^k$$

$$\times \frac{\gamma}{(\nu - \nu_0 - k\omega)^2 + \gamma^2}$$

$$\left.\times \frac{S^{2(r-1)}(1 + \langle n\rangle)^{r-1}\langle n\rangle^{r-1}}{(k + r - 1)!\,(r - 1)!} + \cdots\right\}$$

$$r = 1, 2, 3 \ldots,$$

which, finally, can be written as

$$B(\nu) = \exp[-S(1 + 2\langle n\rangle)]\left\{\frac{\gamma}{(\nu - \nu_0)^2 + \gamma^2}\right.$$

$$\times \sum_{k=0}^{\infty} \frac{S^{2k}}{k!\,k!}(1 + \langle n\rangle)^k\langle n\rangle^k + \sum_{m=1}^{\infty} S^m\langle n\rangle^m$$

$$\times \frac{\gamma}{(\nu - \nu_0 + m\omega)^2 + \gamma^2} \sum_{i=0}^{\infty} \frac{S^{2i}(1 + \langle n\rangle)^i\langle n\rangle^i}{(m + i)!\,i!}$$

$$+ \sum_{k=1}^{\infty} S^k(1 + \langle n\rangle)^k \frac{\gamma}{(\nu - \nu_0 - k\omega)^2 + \gamma^2}$$

$$\left.\times \sum_{j=0}^{\infty} \frac{S^{2j}(1 + \langle n\rangle)^j\langle n\rangle^j}{(k + j)!\,j!}\right\}. \quad \text{(A3)}$$

Using the identity

$$\sum_{k=0}^{\infty} \frac{z^k}{k!\,(k + s)!} = z^{-s/2}I_s(2z^{1/2}),$$

where s is an integer, and $I_s(x)$ is the modified Bessel function of integer order (Erdélyi et al., 1953), Eq. A3 can be written as

$$B(\nu) = \exp[-S(1 + 2\langle n\rangle)]\left\{I_0[2S((1 + \langle n\rangle)\langle n\rangle)^{1/2}]\right.$$

$$\times \frac{\gamma}{(\nu - \nu_0)^2 + \gamma^2} + \sum_{m=1}^{\infty} I_m[2S((1 + \langle n\rangle)\langle n\rangle)^{1/2}]$$

$$\times \left(\frac{\langle n\rangle}{1 + \langle n\rangle}\right)^{m/2} \frac{\gamma}{(\nu - \nu_0 + m\omega)^2 + \gamma^2}$$

$$+ \sum_{k=1}^{\infty} I_k[2S((1 + \langle n\rangle)\langle n\rangle)^{1/2}]$$

$$\left.\times \left(\frac{1 + \langle n\rangle}{\langle n\rangle}\right)^{k/2} \frac{\gamma}{(\nu - \nu_0 - k\omega)^2 + \gamma^2}\right\}, \quad \text{(A4)}$$

or, using again $\langle n\rangle \equiv (\exp(h\omega/k_BT) - 1)^{-1}$, in the form

$$B(\nu) = \exp\left[-S\coth\left(\frac{h\omega}{2k_BT}\right)\right]$$

$$\times \left\{I_0\left(S/\sinh\frac{h\omega}{2k_BT}\right)\frac{\gamma}{(\nu - \nu_0)^2 + \gamma^2}\right.$$

$$+ \sum_{m=1}^{\infty} I_m\left(S/\sinh\frac{h\omega}{2k_BT}\right)\exp\left(-m\frac{h\omega}{2k_BT}\right)$$

$$\left.\times \frac{\gamma}{(\nu - \nu_0 + m\omega)^2 + \gamma^2}\right.$$

$$\left. + \sum_{r=1}^{\infty} I_r\left(S/\sinh\frac{h\omega}{2k_BT}\right)\exp\left(r\,\frac{h\omega}{2k_BT}\right)\frac{\gamma}{(\nu - \nu_0 - r\omega)^2 + \gamma^2}\right. . \quad \text{(A5)}$$

The first term is the 0–0 contribution, whereas the other terms are all the vibronic bands due to the vibrational mode at frequency ω with coupling S, weighted by a temperature-dependent contribution. The Lorentzian contributions are located at multiple integers of the frequency ω both at higher and lower energy with respect to the 0–0 transition energy ν_0.

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