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DERIVATIVE ABSORPTION SPECTROSCOPY FROM 5—300 K OF BACTERIOCHLOROPHYLL *a*-PROTEIN FROM *PROSTHECOCHLORIS AESTUARII* *

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Summary

Absorption spectra of the bacteriochlorophyll *a*-protein from *Prosthecochloris aestuarii* were measured at temperatures from 2.9 to 300 K. Fourth and eight derivatives of the spectra were calculated from the digital data. From an analysis of 34 scans taken from 750 to 850 nm at 5 K, and 130 scans taken from 822 to 838 nm, we find evidence for nine peaks, six of which are probably 0-0 excitonic and three probably higher vibronic features. The major peaks are resolved in the derivative spectra to 300 K, and all shift with temperature by less than 1 nm compared to their 5 K positions, except for the 825 nm peak which shifts about 2 nm. The most prominent fourth derivative peak at 300 K shifts from 812.9 nm in the standard buffer solution to 814.1 nm in the cryogenic solution in which our low temperature measurements were made. We conclude that the conformation of the protein at 5 K is essentially the same as at 300 K.

Introduction

The Bchl *a*-protein from the green photosynthetic bacterium *Prosthecochloris aestuarii* [1] is apparently an intermediate in the transfer of excited electronic singlet state energy from the antenna chlorobium chlorophyll to the bacteriochlorophyll of the reaction centers [2]. This particular chlorophyll-protein is of special interest because the atomic coordinates of the bacteriochlorophylls contained within it have bee determined from X-ray crystallographic studies [3]. Thus it should be possible to calculate the exciton inter-

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actions among the Bchl molecules and their effects on the absorption spectrum [4–6].

The absorption spectrum of the Bchl *a*-protein at 77 K shows three prominent peaks as well as some unresolved shoulders [2,7,8]. The structure exhibited by the low temperature absorption has been interpreted in terms of exciton splitting that results from resonant interactions among the Bchl molecules within the protein [7]. The circular dichroism spectrum also shows considerable structure at 77 K [7,8] and even at 300 K [7], although the high temperature circular dichroism spectrum is weaker and less well resolved because of thermal broadening. The room temperature electric field oriented linear dichroism spectrum [9] displays a single narrow peak corresponding in wavelength to the principal peak of the 77 K absorption spectrum.

Preliminary attempts to calculate the absorption spectrum from the known structure have had only qualitative succes [4,6]. Such calculations would be greatly helped if the energy levels of all of the exciton states were precisely known. Because of the poor spectral resolution even at 77 K it is not possible to make these energy assignments unequivocally.

If the broadening of the spectral lines at 77 K is primarily of thermal origin, increased resolution can be obtained by reducing the sample temperature. In addition, Butler [10] has shown that if the signal to noise ratio of the experimental spectrum is sufficiently high, it is possible to obtain enhanced resolution of spectra of biological materials by numerically differentiating the spectrum versus wavelength; 4 *n*-th derivatives (*n* integral) are most useful. The primary limitation of the technique is noise. Random noise is amplified in the numerical differentiation, increasing relative to the signal as the effective resolution is increased. The noise problem can be reduced with numerical techniques that use the spectral data more effectively than a simple linear appoximation to the derivative (see Materials and Methods). Further improvement in the results, however, can only be obtained by increasing the signal to noise ratio in the original data, by signal averaging for example.

We present here the results of a study of the absorption spectrum of Bchl *a*-protein from *P. aestuarii* at temperatures as low as 2.9 K and at a number of higher temperatures to 300 K. At 5 and 300 K, a large number of spectral measurements were made and the results combined to give an enhanced signal to noise ratio. The spectral data are analyzed by numerical differentiation techniques. The results are interpreted in terms of the exciton structure for the Bchl *a*-protein.

Materials and Methods

The Bchl *a*-protein was extracted from cells of *P. aestuarii* strain 2K which had been cultured previously and frozen [9]. The method of purification was that described by Olson [1]. In an attempt to attain higher sample purity and possibly higher low temperature spectral resolution, the final step in purification, i.e. the DEAE chromatography, was repeated. At 300 K the purified protein had absorbance ratios $A_{809}/A_{371} = 2.1$, $A_{267}/A_{371} = 0.56$.

For most of the measurements a cryogenic solvent containing potassium glycerophosphate was employed. This solvent, used for this Bchl *a*-protein by

Phillipson and Sauer [7], consists of 4 parts by volume of 75% potassium glycerophosphate (K and K Chemical Co.), 2 parts protein in 10 mM Tris with 0.2 M NaCl (pH 8.0), and 1 part glycerol. Samples with this solvent formed glasses of good optical quality with only a few cracks when cooled in 1 cm square cuvettes.

The spectral measurements were performed on a Cary 14 spectrophotometer with digital encoders on the pen and wavelength drive mechanisms. To achieve higher spectral resolution and signal to noise ratio, the light which had passed through the sample and reference chamber was led via a fiber optic light pipe to an external RCA C31034 photomultiplier with a GaAs cathode. The anode of the photomultiplier was connected by a low capacitance cable to the anode pin of the photomultiplier socket within the spectrometer. This arrangement was checked for absorbance accuracy with neutral density filters. No noticeable improvement in signal to noise ratio was observed when the cathode voltage of the photomultiplier was decreased below 600 V. At this voltage, the slits on the spectrometer operated at 0.05 mm or less over the 860—750 nm range, so that the spectral resolution was always better than 0.2 nm. With the cathode voltage increased to 800 V the spectral resolution is limited by the inherent resolution of the monochromator, $\approx$0.1 nm, but the signal to noise ratio is reduced somewhat. Wavelength accuracy of the spectrometer was checked by replacing the visible source with a sodium lamp. Sodium lines at 819.48 and 818.33 nm [11] were detected and a wavelength correction obtained for the instrument.

Digital records of absorbance and wavelength were punched on paper tape every 0.1 nm while the spectrometer was scanning at 0.25 nm/s. For enhanced signal to noise ratio at 5 and 300 K a large number of scans of the spectra was made and the spectra were subsequently added together.

For the low temperature measurements the samples were cooled in a liquid He cryostat (Janis Varitemp) with Spectrasil windows. Temperature was controlled by a Lake Shore Cryotonics controller that heats cold He gas to the desired temperature. The samples were frozen at slightly less than ambient pressure in a stream of cold He gas while the cryostat was being filled with liquid He. In all measurements the samples were frozen within 1 h of mixing in the cryogenic solvent.

The 4th and 8th derivatives of the spectra were calculated numerically by fitting a number of points in the region of each wavelength to a least squares quadratic curve. For an odd number of points symmetric about the center wavelength, the derivative of the quadratic, $A(\lambda)$, evaluated at the center wavelength, λ_i, is

$$\frac{dA(\lambda_i)}{d\lambda} = \frac{\sum_{j \neq i} (\lambda_j - \lambda_i) A(\lambda_j)}{\sum_{j \neq i} (\lambda_j - \lambda_i)^2} .$$

Higher derivatives can be obtained by repeating the process. This numerical differentiation technique is different from Butler's method [10], in which the difference spectrum $A(\lambda + \Delta\lambda) - A(\lambda)$ is calculated and assigned to the wavelength $\lambda + \Delta\lambda/2$, i.e., a linear approximation to the derivative is used. Our

method, which is equivalent to Butler's if only three points are used in the fit region of each wavelength, in general reduces spurious noise generated by numerical differentiation.

The amount of resolution which can be obtained in the derivatives depends primarily on the random noise present in the original spectrum. As Butler has shown, the effects of random noise can be reduced somewhat by varying the wavelength intervals used in successive differentiations. For the results reported in this paper the derivatives at 5 K were taken with groups of five points spaced 0.4 and 0.5 nm. For analysis of temperature dependence, since such high resolution was not attainable at the higher temperature, derivatives were calculated with seven points, spaced 0.5 and 0.6 nm apart.

Results

The absorption spectra of the Bchl-protein at various temperatures in the potassium glycerophosphate buffer are shown in Fig. 1. The spectral resolution increases markedly as the temperature is lowered. The main features of the 5 K spectrum (Fig. 2) are the three peaks at 825, 814 and 805 nm with shoulders at 801 and 793 nm. In addition there are minor peaks at 760 and 768 nm and a small shoulder at 778 nm which appear consistently. Measurements were also made in two samples at 2.9 K; the spectra are indistinguishable from the 5 K spectra.

The spectra in Fig. 1 at 5 and 300 K were measured on different samples than those for the other temperatures so the absolute heights do not correspond to a single concentration. In Table I the peak absorbance vs. temperature relative to the 300 K value is tabulated for a single concentration. These data can be used with Fig. 1 to obtain corrected spectra at each temperature.

Before presenting results of numerical differentiation of the experimental spectra we demonstrate what happens when synthetic spectra are differentiated. In Fig. 3 a Gaussian peak along with its 4th and 8th derivatives are shown. The primary peak at the central wavelength is sharpened considerably with higher differentiation. It is this property that makes the higher derivatives useful for spectral interpretation. The height of this peak is proportional to that of the undifferentiated peak divided by the 4th or 8th power of the width. The derivatives are also characterized by a number of subsidiary maxima and minima. The amplitudes of these peaks depend on both the height and width of the original Gaussian while the wavelengths depend only on the width. In particular, the separations, $\Delta\lambda_4$ or $\Delta\lambda_8$, of the two undershoots in the 4th or 8th derivative are related to the full width at half maximum, Γ, of the Gaussian by

$$\Delta\lambda_4 = 1.15\Gamma$$

$$\Delta\lambda_8 = 0.87\Gamma$$

It should be noted in Fig. 3 that the positions of the subsidiary maxima and minima move towards the central peak as the order of the derivative is increased. This shift will be used later to discriminate between central and subsidiary peaks of adjacent spectral features.

When spectra of partially resolved multiple peaks are differentiated, inter-

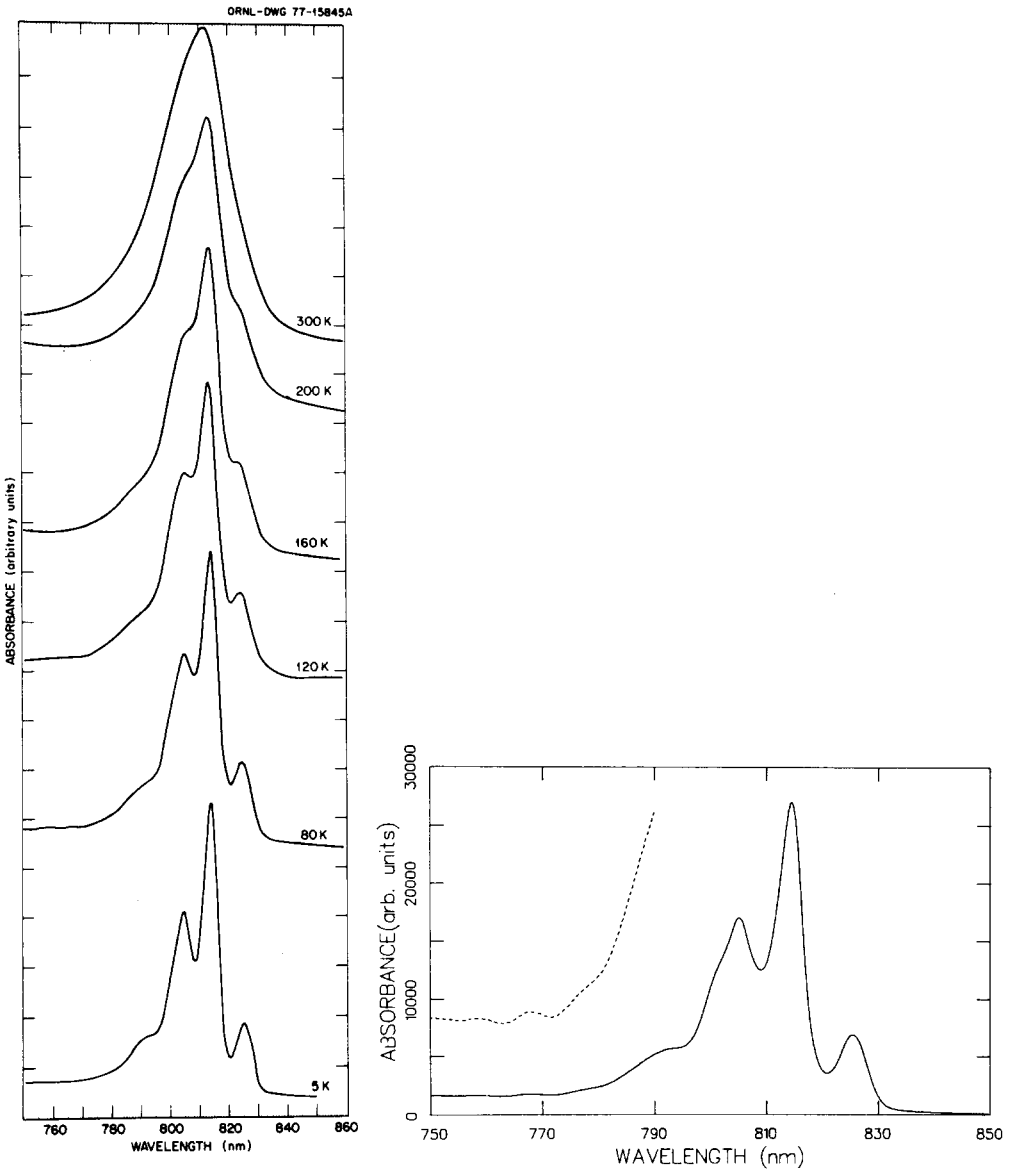


Fig. 1. Absorption spectra of the Bchl *a*-protein at 5, 80, 120, 160, 200, and 300 K. The spectra at 5 and 300 K are the averages of 34 and 16 scans, respectively. The other spectra are single scans.

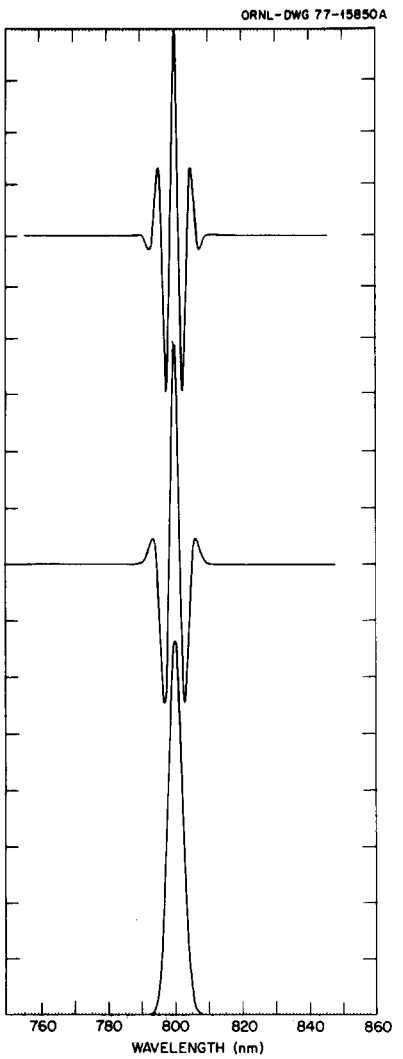
Fig. 2. Absorption spectrum of the Bchl *a*-protein at 5 K (34 scans) with 5X expanded scale from 750 to 790 nm (dashed curve).

ference may occur between the derivatives of the individual spectral peaks. An example of a spectrum more representative of real data is shown in Fig. 4. This spectrum, a sum of six skew-symmetric Gaussians, is the result of a numerical fit by Olson et al. [8] to the 77 K spectrum, minus the baseline correction, of the *P. aestuarii* protein in 50% glycerol. The wavelengths, heights, and widths of the six components are given in Table II. The maxima

TABLE I

PEAK ABSORBANCES OF BCHL *a*-PROTEIN AT SEVERAL TEMPERATURES

Temperature (K)	Relative maximum absorbance
5	2.01
80	1.79
100	1.71
120	1.59
140	1.52
160	1.46
180	1.38
200	1.37
270	1.12
300	1.00



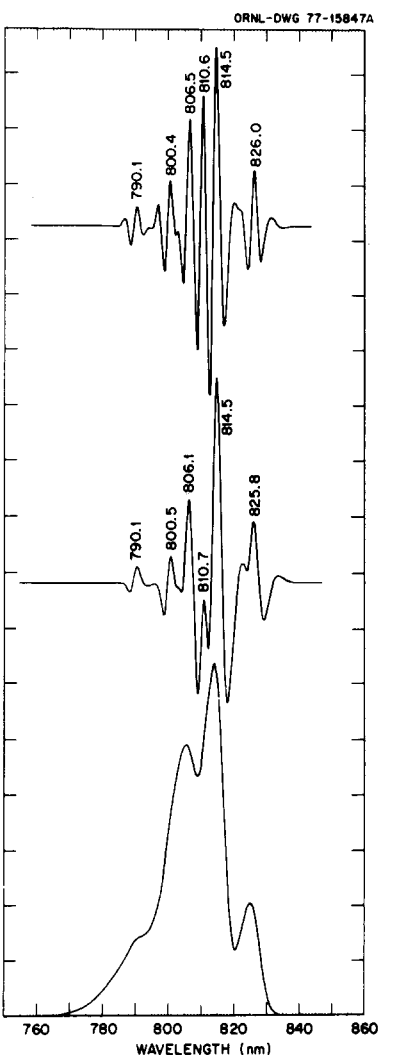


Fig. 3. Absorption spectra of a single Gaussian peak. The lower curve is the absorption spectrum for a Gaussian with full width at half maximum of 5 nm. The middle and upper curves show the 4th and 8th derivatives, respectively. The outermost subsidiary maxima of the 8th derivative spectrum are too small to be noticeable on this scale.

Fig. 4. Absorption and derivative spectra of synthetic data. The absorption spectrum (lower curve) is the sum of 6 skew-symmetric Gaussian peaks from Olson et al. [8], with maxima at 789.2, 799.5, 805.4, 810.1, 813.9, and 825.1 nm. The middle and upper curves show the 4th and 8th derivatives, respectively.

TABLE II

SKEW-SYMMETRIC GAUSSIAN PEAKS USED FOR SYNTHETIC SPECTRUM

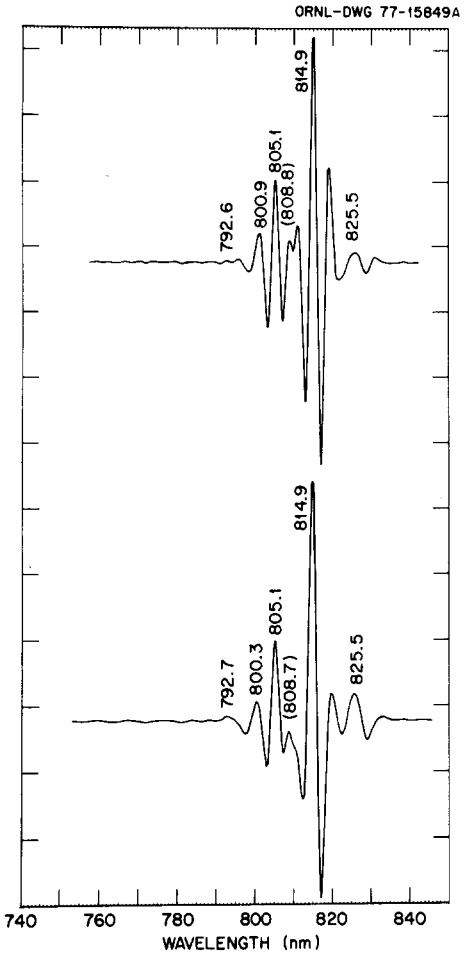
Width is twice the halfwidth at half maximum of the short-wavelength side of each peak. Skew is the ratio of the long-wavelength to short-wavelength halfwidths. Data from ref. 8.

λ (nm)	Heigth	Width (nm)	Skew
789.2	0.650	18.6	0.43
799.5	1.323	14.3	0.49
805.4	3.333	8.9	0.78
810.1	0.356	5.7	0.48
813.9	4.719	7.3	0.86
825.1	1.534	8.4	0.75

in the 4th and 8th derivatives which correspond to the original centers of the skewed Gaussians are labeled with their peak wavelengths. It can be seen that in addition to the six primary peaks, several additional maxima are present, particularly in the 8th derivative. In some cases, such as at 797 nm in the 8th derivative spectrum, the subsidiary peaks can be as pronounced as the primary peaks so that considerable care must be taken in identifying derivative peaks with spectral features. It should also be noted that, depending on the symmetry of the original peak and the amount of interference with neighbouring features, the wavelengths of the maxima in the derivatives can be shifted somewhat from the peaks in the undifferentiated spectrum.

The 4th and 8th derivatives of the 5 K spectrum are shown in Fig. 5. To reduce the effects of random noise which are magnified in the higher derivatives, a large number of spectra were averaged together. The results at 5 K in Figs. 2 and 5 represent the sum of 34 spectral runs at that temperature. The 4th derivative spectrum shows peaks corresponding to the five main features of the 5 K spectrum. There are two smaller peaks at 820 and 833 nm which are probably overshoots. There is a small peak at 809 nm and a shoulder at 810.5 nm, both with maxima less than zero. These two features will be discussed below. The shoulder at 795 nm is probably an overshoot from the 800 nm peak.

The 8th derivative was also taken to see if additional resolution could be obtained and to help distinguish between overshoots and peaks. It can be seen in Fig. 5 that the chief result is the sharpening of the peaks and shoulders which were visible in the 4th derivative. There are no new peaks apparent although the 825 nm peak now appears somewhat broadened. The two presumed long wavelength overshoots have shifted to 831 and 819 nm and both increased substantially relative to the central peak as would be expected for subsidiary maxima. The shoulder at 810.5 nm in the 4th derivative has grown in the 8th derivative and shifted to 811 nm. The peak at 809 nm did not shift appreciably from the 4th to the 8th derivative, nor did it increase as much as th 811 nm peak. Olson et al. [8] found that a small peak at 810.1 nm was necessary for their Gaussian fit of the 77 K spectrum. If there is a peak in this region of the spectrum it appears that the 809 nm derivative peak would be a better choice than the 811 nm peak which behaves more nearly like a subsidiary maximum. The shoulder at 795 nm in the 4th derivative also grows and



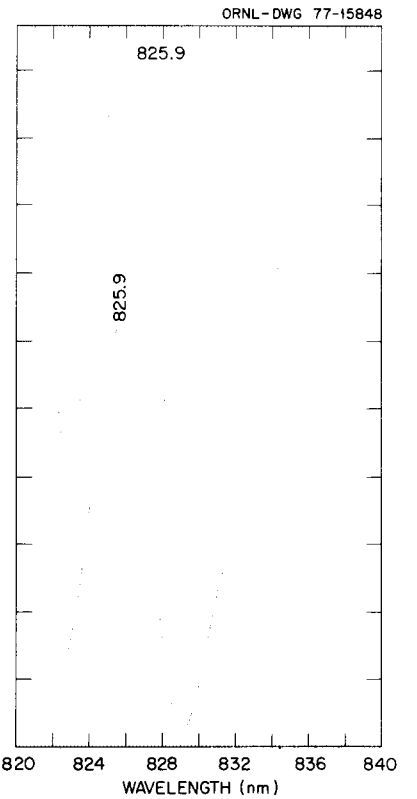


Fig. 5. The 4th derivative (lower curve) and 8th derivative (upper curve) of the 5 K spectrum of the Bchl *a*-protein.

Fig. 6. The 4th derivative (lower curve) and 8th derivative (upper curve) of the 5 K spectrum in the vicinity of the 825 nm absorption peak. The absorption spectrum is the average of 130 scans.

shifts to 796 nm in the 8th derivative, which reinforces the suggestion that it is an overshoot of the 800 nm peak.

The apparent flattening of the 825 nm peak in the 8th derivative suggests that this feature might consist of two narrower peaks. A series of measurements was made on a sample of four times higher concentration over the limited spectral region 820 to 840 nm with more scans to enhance the signal to noise ratio. In Fig. 6 are shown the 4th and 8th derivatives of the sum of 130 scans. With the higher signal to noise ratio only a single peak can be resolved which suggests that the flattening seen in the 8th derivative spectrum (Fig. 5) is not due to two partially resolved peaks.

With numerical differentiation techniques we can locate the wavelegths of the principal features as a function of temperature, even though those features

TABLE III

TEMPERATURE DEPENDENCE OF MAJOR PEAK POSITIONS IN 4th AND 8th DERIVATIVE SPECTRA

5 K derivatives taken with 5 points spaced 0.4 and 0.5 nm, others with 7 points spaced 0.5 and 0.6 nm.

Temperature (K)	4th derivative λ (nm)				8th derivative λ (nm)			
	1	2	3	4	1	2	3	4
5	800.3	805.1	814.9	825.5	800.9	805.1	814.9	825.5
80	799.8	805.0	814.4	825.0	799.5	805.8	814.4	825.3
100	799.9	805.0	814.1	824.7	799.2	805.9	814.1	825.1
120	800.0	805.0	814.1	824.6	799.3	806.2	814.1	824.7
140	800.5	804.9	814.1	824.5	799.5	806.2	814.1	824.8
160	800.0	804.9	814.1	824.5	799.0	806.2	814.1	824.6
180	800.0	804.9	814.0	824.4	799.4	806.2	814.0	824.3
200	800.0	804.5	814.0	825.5	799.5	806.2	814.0	824.0
270	803		814.0	824.7	800.5	805.8	814.2	823.0
300	802		814.1	825.2	800.3	805.0	814.2	823.1

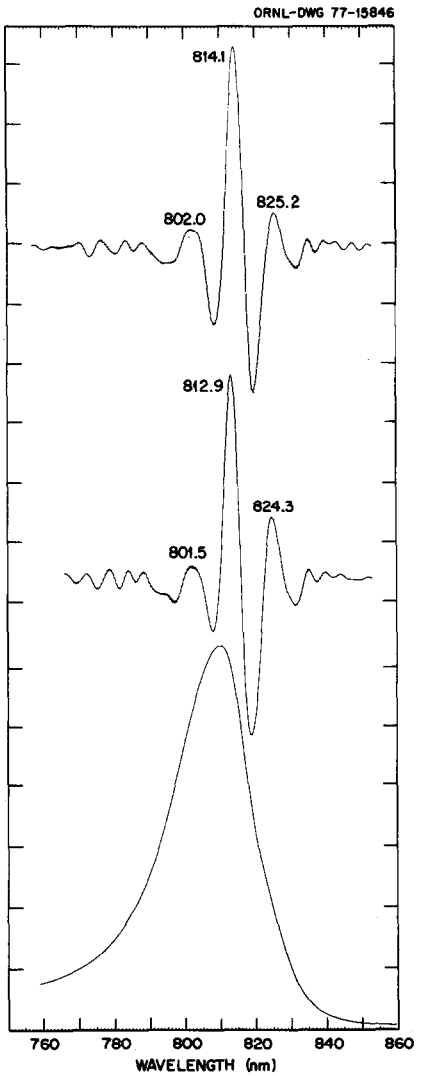


Fig. 7. Absorption and derivative spectra of the Bchl *a*-protein at 300 K. The lower and middle curves are the absorption and 4th derivative spectra of the Bchl *a*-protein in 10 mM Tris · HCl/0.2 M NaCl (pH 8.0) buffer (66 scans). The upper curve is the 4th derivative of the 300 K spectrum in Fig. 1 (cryogenic solvent, 16 scans).

may not be resolved in the absorption spectrum itself. In Table III the wavelengths of the four most prominent peaks from the 4th and 8th derivatives are listed for various temperatures. At the higher temperatures, interference between adjacent features can cause apparent shifts in peak positions. These shifts are much smaller in the 8th than in the 4th derivative, although the former has more pronounced noise peaks. By using the 4th derivative to identify real peaks, we were able to locate the corresponding 8th derivative features and thus obtain peak positions more accurately. Most noteworthy in Table III is that with the exception of the 825 nm peak, the wavelengths are essentially constant from 5 to 300 K. The 825 nm peak shows a slight blue shift with increasing temperature.

An example of the applicability of the numerical differentiation techniques at high temperatures is shown in Fig. 7. The 300 K absorption spectrum and 4th derivative spectrum of the protein in the buffer solution normally used for room temperature measurements [1], 10 mM Tris · HCl and 0.2 M NaCl (pH 8.0), as well as the fourth derivative spectrum in the cryogenic solvent, are presented. The three prominent peaks in the 4th derivative spectra correspond to the 825, 815, and merged 805 and 800 nm peaks of the low temperature spectra. We believe most of the other structure in these 4th derivative spectra is due to a periodic reinforcement of random noise that results from numerical differentiation [10]. It can be seen that the main derivative peak is red shifted by 1.2 nm in the glycerophosphate solvent, the others somewhat less. Our results at 300 K in the glycerophosphate solvent differ slightly from those reported previously [8].

Discussion

The spectrum of the Bchl *a*-protein at 5 K shows considerably more resolution than at approx. 80 K but the spectral peaks still cannot be unequivocally assigned as individual exciton transitions. The major differences between our results and earlier work at approx. 80 K are the appearance of a shoulder at 801 nm in the undifferentiated 5 K spectrum and the possible resolution of a small peak at 808.8 nm in the 8th derivative of the 5 K spectrum. Of the three minor features at 778, 768, and 760 nm, the former two are evident in the published spectra of Philipson and Sauer [7] and of Olson et al. [8] although these authors do not comment about them. Aside from these three minor peaks which are probably higher vibronic rather than 0-0 excitonic transitions, we discern using numerical differentiation of the absorption spectrum the same six principal features that Olson et al. [8] found upon simultaneously fitting skew-symmetric Gaussians to their absorption and circular dichroism data, with only slight differences in wavelengths.

The maximum number of features that can appear in the absorption spectrum as a result of exciton interactions among the Bchl molecules in the protein is a direct consequence of the protein's structure [7]. X-ray analysis has shows that the Bchl *a*-protein from *P. aestuarii* consists of three identical subunits each containing seven nonequivalent Bchl *a* molecules [3]. The three subunits are arranged about a 3-fold symmetric axis. If the subunits were completely isolated from each other, the lowest singlet excited state (Q_y) of the

Bchl *a* would be split into seven exciton levels. Fenna and Matthews [4] have pointed out, however, that some of the intersubunit interactions may not be negligible. Because of the threefold rotational symmetry, it can be shown that if these interactions are included each of the seven levels is split into three, one with transition moment parallel to the symmetry axis and two, which are degenerate, with moments perpendicular to the axis. Thus a maximum of 14 exciton levels should be observed for the Bchl *a*-protein trimer, corresponding to seven nondegenerate exciton states and seven pairs of degenerate states.

Clearly we have not resolved this theoretical maximum number of exciton levels. In fact, since it is more likely from their small size and short wavelengths that the three minor peaks are vibronic overtones rather than 0-0 exciton transitions, we have probably resolved no more than six exciton features. Unfortunately, from symmetry considerations alone, one cannot decide how the 14 theoretical levels are grouped to form absorption spectral features, and as we have already pointed out theoretical calculations based on the detailed structure of the protein's Bchl core have so far not been very succesful in accounting for such features. Nonetheless, the more resolved spectrum at 5 K provides additional detail that any further attempt to calculate the spectrum from the structure must take into account.

The sharpening of the lines with cooling, i.e., the 25% reduction in width from 80 to 5 K, is not as great as would be expected if thermal broadening were predominantly responsible for the line widths. Contributions to the low temperature ($\leq$5 K) linewidth could come from low-lying vibrational levels that might be expected in an aggregate of this size, and from slight variations in conformation from one protein to another. At higher temperatures, thermal broadening does become important (Figs. 1 and 7).

At 300 K, 4th derivative spectroscopy resolves the principal peaks seen in low temperature absorption spectra. The most prominent of these 300 K derivative peaks, at 812.9 nm, coincides in wavelength and has the same width as the single peak in the room temperature electric field oriented linear dichroism spectrum of the Bchl *a*-protein [9]. This result strongly supports the earlier conclusion that the linear dichroism spectrum is due to the same exciton state or states that give rise to the main peak in the 77 K absorption spectrum.

We have shown that the locations of the principal absorption peaks of the Bchl *a*-protein are essentially constant from 5 to 300 K. Differences in peak positions revealed by derivative spectroscopy at 300 K between Bchl *a*-protein in the cryogenic solvent and in the buffer solution are at least an order of magnitude smaller than the separations of adjacent peaks. Olson et al. [12] showed that at 300 K the absorption spectrum of the crystallized protein is essentially the same as that of the protein in buffer. We conclude, therefore, that as far as absorption is concerned the conformations of the protein at 300 K in the crystal and at 5 K in the cryogenic solvent are virtually identical. Thus, conformational change cannot be invoked to explain the difficulties in obtaining agreement between the spectrum calculated from the X-ray structural data and the observed low temperature spectra.

Acknowledgments

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