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Fluorescence Line Narrowed Spectra of Zn and Metal-Free Cytochrome c

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Fluorescence line narrowing (FLN) spectroscopy was used to study the role of the polypeptide chain in influencing the spectrum of Zn-substituted cytochrome c (Zn cyt c) and metal-free cyt c (porphyrin cyt c). For both derivatives the spectra show characteristics of relaxed fluorescence from an inhomogeneously broadened sample. Zero phonon lines and phonon wings can be clearly distinguished, and vibrational frequencies of the ground and excited states were identified. The inhomogeneous distribution width for porphyrin cyt c is slightly wider than that of Zn cyt c and a second population of molecules was apparent in the porphyrin cyt c. The phonon coupling was greater for Zn cyt c than for porphyrin cyt c, which may be due to the extra coupling to the polypeptide chain by metal ligation.

KEY WORDS: Cytochrome c; fluorescence; fluorescence line narrowing; inhomogeneous broadening; site selection; site distribution function; porphyrin.

INTRODUCTION

When a wide light source is used at temperatures above 70 K, electronic absorption and emission spectra of polyatomic organic molecules in solutions consist of broad bands characterized by widths of 100 to 1000 cm^{-1} [1]. Such spectra yield very little useful information and do not allow for any detailed studies of complex organic molecules such as proteins. Two types of broadening are responsible for the wide spectral bands: homogeneous and inhomogeneous. For homogeneous broadening every individual molecule has a spectrum consisting of a broad band. In inhomogeneous broadening every molecule has a well-resolved emission signal at a frequency determined by the specific solute–solvent interactions. For this case the concealed vibrational structure can be obtained using very narrow, highly selective laser excitation at cryogenic temperatures to ensure that molecules are maintained in the lowest vibrational state. This method used to measure fluorescent emission is called fluorescence site selection or fluorescence line narrowing (FLN)[5] spectroscopy.

Previously it had been shown that under site selection conditions, high-resolution spectra are obtained for porphyrin cyt c [2] and Zn cyt c [3,4]. These are derivatives of cyt c in which the iron is removed. Without iron the porphyrin becomes fluorescent and can be used as a probe to investigate porphyrin/protein interactions. Incorporation of Zn into the porphyrin produces a derivative in which the symmetry of the porphyrin is similar to that of the native iron protein but which, like porphyrin cyt c, also fluoresces. In the present work we compare the FLN spectral features of metal-free and Zn cyt c. We examine here in more detail the features of

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[5] Abbreviations used: cyt c, horse heart cytochrome c; porphyrin cyt c, metal-free cyt c; Zn cyt c, cytochrome c in which Fe has been replaced by Zn; FLN, fluorescence line narrowing.

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these high-resolution spectra and we compare the inhomogeneous broadening for the two derivatives of cyt c.

BACKGROUND TO FLN

The principles of FLN spectroscopy have been described in detail in reviews [1,4,5]. Apparently quite complex spectra are obtained, but these spectra become clear if one understands three basic features of FLN, described below.

The first feature is that by exciting with narrowband excitation at a low temperature, one subpopulation ("site") of the ensemble of molecules is selectively excited (Fig. 1, top). This arises because the broad absorption bands of many molecules observed using conventional spectroscopy are composed of individual sharp lines, determined by a solvent shift specific for the individual solute–solvent unit interaction potential. The subpopulation of molecules excited using narrowband excitation emits to the ground state, and vibrational features of the ground state are apparent in the emission spectra. When the excitation is changed, the entire emission spectrum shifts because a new subpopulation of molecules is selectively excited. It has been observed that for many molecules the vibrational features of the ground state are nearly the same irrespective of the excitation frequency within the inhomogeneous distribution. The intensity of the emission is a function of the number of molecules that absorb light, and by varying the excitation and observing one vibrational mode, we can obtain information on the distribution of the electronic transitions. (The "site distribution function" is defined below.)

The second feature apparent in FLN spectra is that the chromophore couples with low-frequency modes of the surrounding matrix, which in our case is the polypeptide chain. This coupling is called the phonon interaction, and it raises the energy of the levels, as illustrated in Fig. 1 (middle). Each transition consists of a sharp line, called the zero phonon line, with a typical width of ~0.05–5 cm^{-1}, and a broader band, the phonon wing, with a width of the order of ~100 cm^{-1}. The phonon wing appears to the high-energy side of the absorption line and to the low-energy side of the emission line. The existence of phonon interactions explains why FLN spectra are obtained only at low temperatures, because as the temperature is raised the phonon wings become populated as described by a Boltzmann distribution. Another observation of FLN spectroscopy—that the most highly resolved spectra are obtained using excitation at the low-energy side of an absorption band—is also explained by

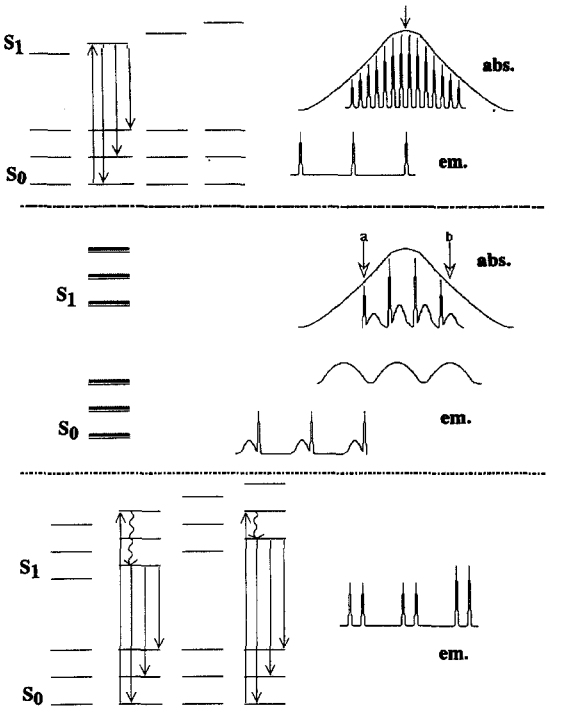

Fig. 1. Diagram to illustrate FLN. Left, energy levels for an inhomogeneous distribution of molecules; right, spectra. Top: Excitation into an absorption band (abs.) yields emission spectra (em.) that give information on the vibrational levels of the ground state. As one changes the excitation, another subpopulation of molecules are excited, and the emission spectrum will shift with the excitation. Middle: Each transition has a phonon wing associated with it and each transition consists of a sharp line (zero phonon line) and an unstructured band (phonon wing). The phonon wing raises the energy of the transition. Therefore, the phonon wing is on the high-energy side of the absorption and the low-energy side of the emission. Bottom: When the inhomogeneous distribution of the electronic transitions is shifted to a greater extent than the energy difference between vibrational levels, then it is possible that a given frequency of light can excite into multiple vibrational levels. Since emission occurs from the lowest vibrational level of the excited state, multiple emission spectra are obtained. These spectra are shifted relative to each other by the difference in energy of the excited-state vibrational levels. Energy increases from left to right in the spectra.

the phonon wing. As diagrammed in Fig. 1 (middle), since the phonon wing lies to the high-energy side of the zero phonon line when excitation is at the blue edge of the inhomogeneous absorption band, many populations of chromophores will be excited through their phonon wings and the resulting emission is unresolved.

The third feature of FLN occurs when the inhomogeneous broadening is greater than the vibrational levels of the molecule. In this case, excitation into a higher vibrational level will lead to multiple emission spectra,

as illustrated in Fig. 1 (bottom), because more than one subpopulation of molecules will be excited. Personov [1] pointed out that the emission spectra will be separated by the vibrational frequencies of the excited state and therefore FLN spectra uniquely contain information on the vibrational levels of both the excited- and the ground-state molecules.

MATERIALS AND METHODS

Porphyrin and Zn cyt c were prepared from type III horse heart cyt c from Sigma Chemical Co. (St. Louis, MO) as previously described [7,8].

Conventional fluorescence spectra were obtained on a Perkin Elmer 650 spectrophotometer, with temperature regulation of the sample achieved by a circulating water bath. High-resolution emission spectra were obtained using the instrument as described [2]. Excitation was achieved using a Coherent 599 dye laser with rhodamine 560 and rhodamine 575 (Exciton Co., Dayton, OH), pumped by a continuous wave Coherent INNOVA 70 argon ion laser (Palo Alto, CA). The power of the laser beam, measured with a Coherent 210 power meter, was attenuated with Newport FSQ neutral density filters. The intensities are given in the figure legends. The emission spectrum was measured at 90° from the excitation using a 1-m JY Ramanor HG2S double monochromator with holographic gratings. The resolution of the spectrophotometer was about 1 cm^{-1}. The detector was a cooled GaAs photomultiplier (RCA 3C1034-RF). The sample was cooled by a helium gas flow Dewar (APD Cryogenics, Alltentown, PA).

Calculation of Site Distribution Function. The site distribution function of the 0,0 transition energies (E) of the chromophore, $I(E)$, was calculated by measuring the intensities of the sharp 0,0 emission lines originating from the same vibronic level in FLN spectra as a function of emission frequencies. Without phonon interactions, these line intensities can be described by the following functional form:

$$I(E) = Kpn(E)f \qquad (1)$$

where I is the line intensity of a sharp 0,0 line of emission energy E, originating from the same vibronic level, p is the probability of a transition from ground state, S_0^0, to the chosen vibrational level m in the first excited state, S_1^m, f is the probability of an emission from S_1^0 to S_0^0, and K is a constant [9]. We define $n(E)$ to be the inhomogeneous distribution function of a chromophore embedded in a protein and $I(E)$, when considered as a function of 0,0 transition energies (E) for different excitation energies, to be the site distribution function of the chromophore. To determine $I(E)$ we measured the intensity of the 0,0 emission frequency in increments of 5 to 15 cm^{-1} of the excitation frequency.

To obtain the site distribution function of the Zn porphyrin in cyt c, we used Asystant Plus (Asyst Software Technologies, Inc.) to fit the data points to a Gaussian distribution function of the form $I(\nu) = I_{max} \times \exp\{-(\nu-\mu)^2/2\sigma^2\}$, where ν is a 0,0 transition wavenumber; μ is the mean 0,0 transition wavenumber, and σ is the standard deviation of the distribution. The width of the site distribution function was considered to be 2σ. A similar method was used to obtain the site distribution function of porphyrin cyt c, but the data were fitted to a double Gaussian distribution.

RESULTS

Total Emission Spectra of Porphyrin and Zn cyt c. The absorption spectra of porphyrin and Zn cyt c are shown in Fig. 2. These spectra have been published previously, but are included to indicate the excitation wavelengths used for FLN.

As previously reported, the emission spectra for these two derivatives are resolved under site selection conditions [2–4]. In Fig. 3, three emission spectra of por-

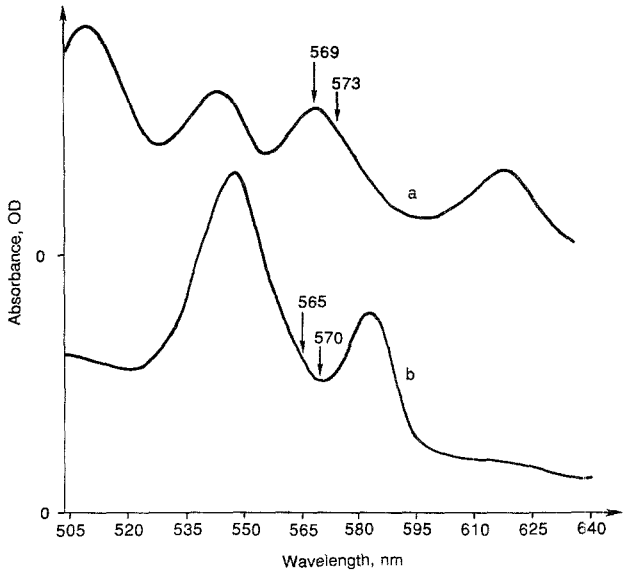

Fig. 2. Q-band absorption spectrum at room temperature of porphyrin cyt c in 50 m*M* ammonium acetate buffer, pH 6.6 (a), and of Zn cyt c in 10 m*M* KPO_4 buffer, pH 7 (b). The sample concentration was ~10–100 μ*M*. Arrows indicate the range used for excitation in Fig. 7.

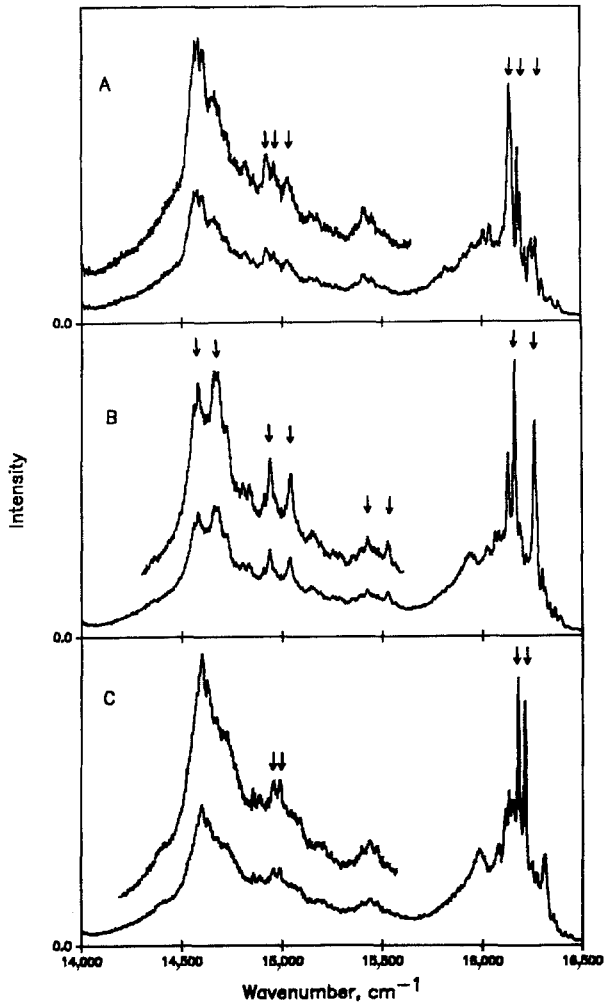

Fig. 3. Multiple emission spectra of ~10μM porphyrin cyt c in 50 mM ammonium acetate buffer, pH 6.6, and 50% glycerol. (A) Excitation at 17,350 cm^{-1}. (B) Excitation at 17,470 cm^{-1}. (C) Excitation at 17,520 cm^{-1}. Temperature: 4.2–5.0 K. Irradiation power: 3.5–5.0 mW. Inset: 2× magnification of the intensities.

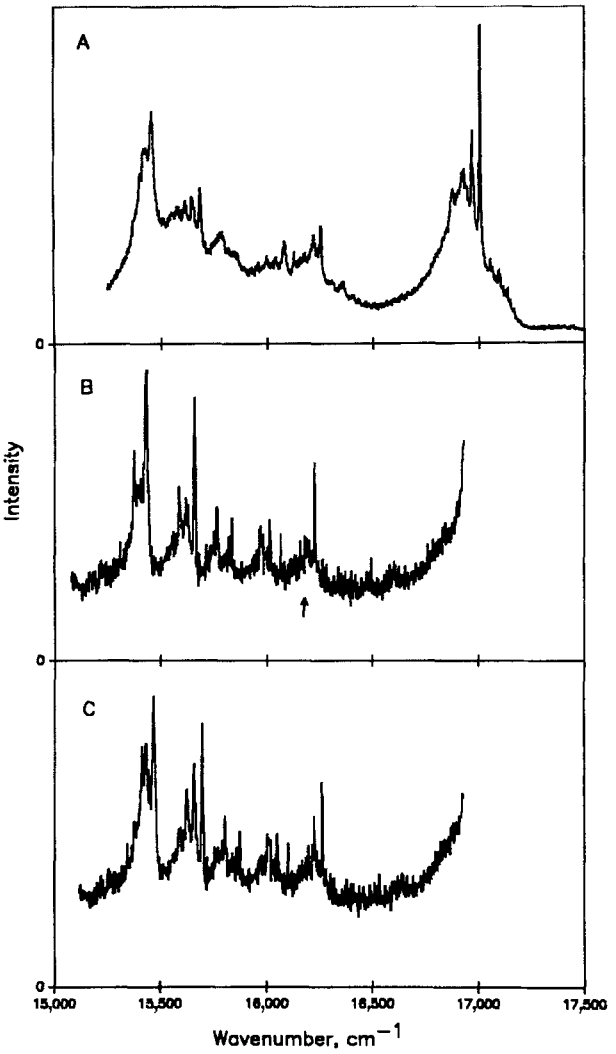

Fig. 4. Multiple emission spectra of ~30 μM Zn cyt c in 10 mM KPO_4 buffer, pH 7, and 50% glycerol. (A) Excitation at 17,600 cm^{-1}. Temperature: 4.2–4.5 K. Irradiation power: 10 mW. (B) Excitation at 17,000 cm^{-1}. Temperature: 7.1–11.8 K. Irradiation power: 9.2–10.0 mW. (C) Simulated spectrum based on the spectrum of B: two spectra of B, one shifted by 38 cm^{-1} and multiplied by a factor of 3, summed.

phyrin cyt c are shown. Features of site selection can be seen in these spectra. The spectra vary with excitation, and multiple 0,0 lines in the frequency range of 16,100–16,400 cm^{-1} are apparent. Vibrational details are also apparent in the 0,1 range, but the lines are broader. Nevertheless, the emission in this range is also a composite of more than one spectra. Lines shifted by the same amount as the prominent 0,0 bands are indicated by arrows in Fig. 3.

The emission spectra of Zn cyt c excited at the low-energy side of the beta band and the alpha band are compared in Figs. 4A and B. For both excitation frequencies the same lines are observed in the 0,1 region, indicating that relaxation to the lowest excited vibrational level has occurred. The emission lines of Zn cyt c are sharp, and in some cases they approach the resolution of the instrument, ~ 1 cm^{-1}. Each zero phonon line has an associated phonon wing, one of which is indicated by the arrow in Fig. 4B. The background relative to the zero phonon lines is higher for Zn cyt c than for the porphyrin case (compare Figs. 3 and 4A), and we attribute this to stronger phonon coupling in the Zn cyt c case. The spectrum of Zn cyt c obtained using 17,000 cm^{-1} as excitation is somewhat more resolved

than for excitation at 17,600 cm^{-1}, and two sharp emissions lines at 16,968 and 17,006 cm^{-1} indicate that two subpopulations of the porphyrin are excited for the latter case. An attempt was made to simulate the emission of Fig. 4A, by adding two vibrationally resolved spectra (Fig. 4B) which are shifted by 38 cm^{-1}, the difference of the two prominent 0,0 lines observed in Fig. 4A. The sum is given in Fig. 4C. The spectrum resembles the experimental spectrum (Fig. 4A), consistent with the view that multiple vibrationally resolved spectra are obtained in Fig. 4A, but the simulated spectrum is more resolved than the experimental spectrum. We attribute the lower resolution and higher background for the experimental spectrum excited at 17,600 cm^{-1} to be due to excitation into phonon bands.

Figures 3 and 4A indicate that for both metal-free cyt c and Zn cyt c, the most intense lines of the fluorescence spectra are in the region of ~ 1600 cm^{-1} from the 0,0 frequency. These lines are characteristic of most of the porphyrins and have been identified as corresponding to the vibrations of the carbon atoms of the pyrrole rings and methine bridges [10–13].

Vibrational Structure of the S_1 State and Site Distribution Function. When Zn cyt c and porphyrin cyt c are excited in the wavenumber range of 17,490–17,730 and 17,400–17,580 cm^{-1}, respectively, multiple 0,0 emission lines are seen for both derivatives. As described above, these multiple lines reflect the vibrational levels of the excited state. These spectral regions were examined in more detail here, and in Figs. 5 and 6 we show the emission of porphyrin and Zn cyt c at various excitation wavelengths. The spectra are particularly rich in structure, and the difference of the excitation frequency from the 0,0 emissions yields the vibrational frequencies of the excited states (Tables I and II). These values are given for reference only; at this time we do not interpret the vibrational frequencies in terms of the structure of the porphyrin. (The frequencies for porphyrin cyt c are shifted by ~ 5 to 10 cm^{-1} from the previously reported values [2]. This discrepancy is attributed to the poorer instrumentation used in the previous work.)

When we examine the transitions, we see that a shift in the excitation frequency results in an equal shift in the emission frequency. Focusing on the 0,0 transition at 1308 or at 595 cm^{-1} from the excitation frequency for porphyrin and Zn cyt c (marked by an asterisk in Figs. 5 and 6), the intensity of the chosen peak can be seen to increase continuously, reaching a maximum value and then decreasing as the excitation frequency is scanned. When the intensity of a peak at a given energy difference from the excitation wavelength is plotted as a function

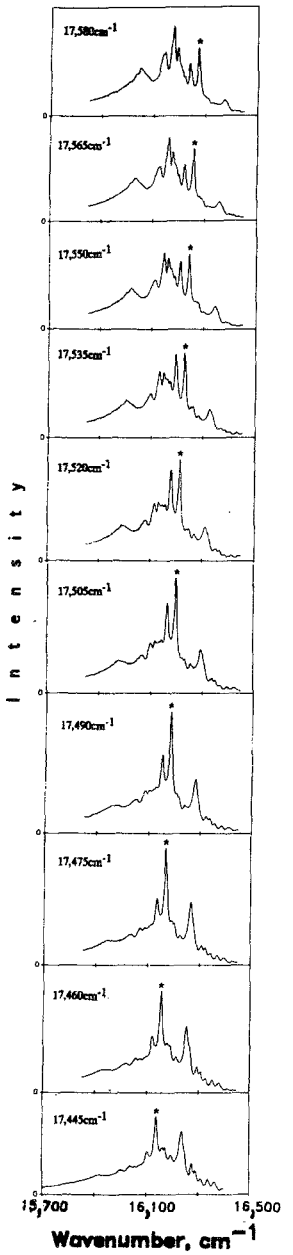

Fig. 5. The 0-0 emission lines at various excitation wavenumbers for porphyrin cyt c. Excitation wavenumbers are given. Asterisks indicate peaks at 1308 cm^{-1} from excitation. Temperature range: 4.5–8.5 K. Irradiation power: 5–8 mW. The intensities of the spectra are normalized for excitation power. Other conditions given in the legend to Fig. 3.

of the emission wavenumber, the site distribution function, as defined in Eq. [1], can be obtained. For porphyrin cyt c, the site distribution function was bimodal with peaks at 16,181 and 16,271 cm^{-1}. The Zn cyt c distribution is somewhat narrower (65 vs 76 cm^{-1} for

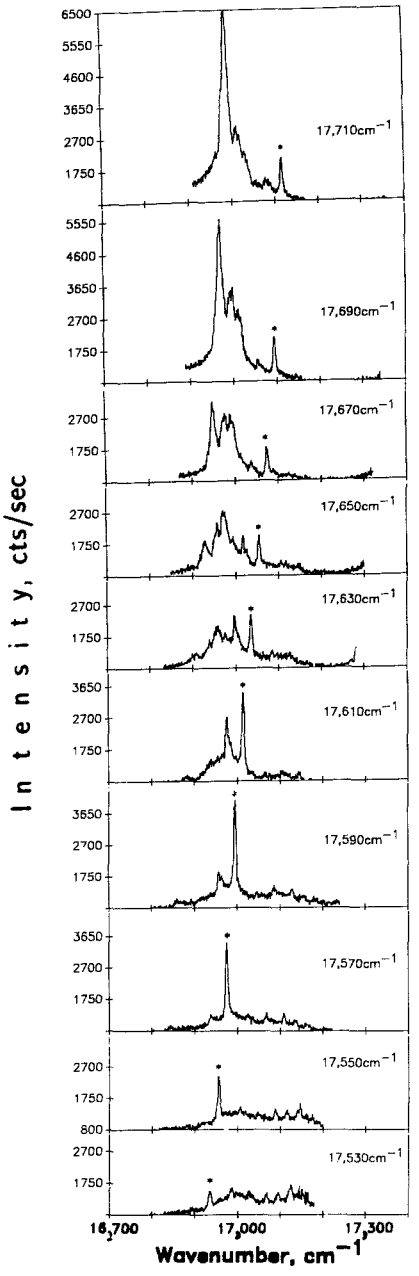

Fig. 6. Variation of the 0-0 emission lines as a function of excitation wavenumber for Zn cyt c. Excitation wavenumbers are given. Asterisks indicate peaks at 595 cm^{-1} from excitation. Temperature range: 5.0–10.0 K. Irradiation power: ~5 mW. The intensities of the spectra are normalized for excitation power. Other conditions given in the legend to Fig. 4.

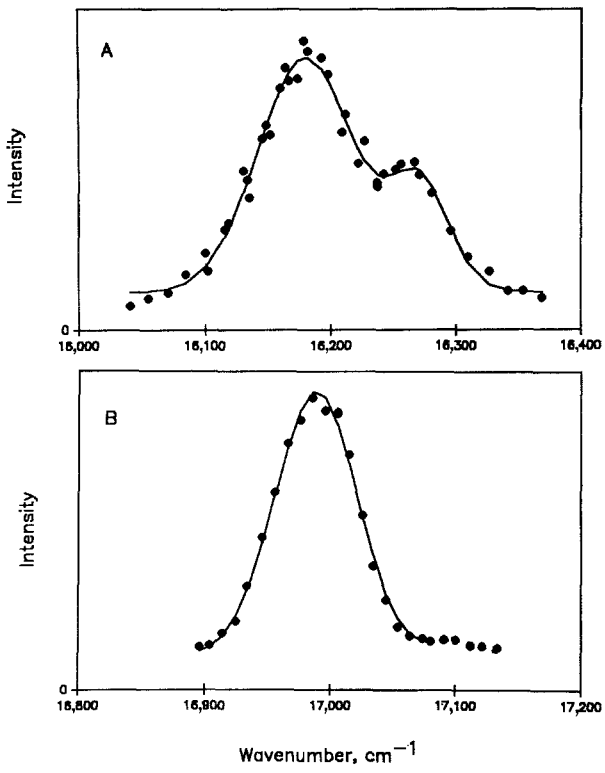

Fig. 7. Site distribution function of porphyrin (A) and Zn cyt c (B). Data taken from Figs. 5 and 6 and sample conditions given in the legends to Figs. 3 and 4. Excitation was into the beta band as shown in Fig. 2. The peaks at 1210, 1,308, 1341, and 1404 cm^{-1} for porphyrin and 595 cm^{-1} for the Zn cyt c from the excitation were used to calculate the site distribution function. Solid lines indicate the computer best fit. The widths of the fits are given in Table III. For porphyrin cyt c the ratio of the amplitude of the major to minor component was 2.2.

the major component of porphyrin cyt c) and showed one major modality. There is an indication of a small second component centered at ~ 17,100 cm^{-1}. When comparing the widths of the distributions for the two derivatives, one must make an allowance for the fact that the peaks used to calculate the site distribution function for the porphyrin cyt c are in a more vibrationally dense region than the peak used to calculate the site distribution function for Zn cyt c. The widths and peak positions are given in Table III.

DISCUSSION

Characterization of Porphyrin and Zn cyt c by Fluorescence Line Narrowing Spectroscopy. The emission spectra of both derivatives show the characteristics of FLN spectra of a chromophore in a glass. The 0,1 spectra showed the same vibrational pattern and energies irrespective of 0,0 or 1,0 excitation (Fig. 4). These results are consistent with relaxed fluorescence (and inconsis-

Table I. S_1 Vibrational Frequencies of Zn cyt c (cm^{-1})[a]

375
402
434
464
503
543
595
625
633
657
674
681
692
698
720

[a]Conditions given in the legend to Fig. 3.

Table II. S_1 Vibrational Frequencies in Porphyrin cyt c (cm^{-1})[a]

1048
1080
1135
1154
1169
1208
1250
1274
1282
1308
1341
1362
1376
1387
1404
1439
1529

[a]Conditions given in the legend to Fig. 4.

Table III. Characteristics of the Site Distribution Function

	0,0 energy, (cm^{-1})	2σ (cm^{-1})	Reference
MP HRP	16,000	52	[9]
	16,100	52	[9]
Zn MP HRP	17,034	35	[9]
Porphyrin cyt c	16,181	76	This work
	16,271	48	This work
Zn cyt c	16,989	65	This work

tent with a resonance Raman process). The spectra show a wealth of detail, including vibrational features of the ground state, of the excited state, and of phonon interactions. Although both derivatives showed resolved spectra, the spectra for Zn cyt c show more phonon coupling than for porphyrin cyt c, and resolution was somewhat less for this derivative. Since Zn is able to coordinate to the polypeptide, the extra coordination may give added phonon interactions.

Degree of Inhomogeneous Broadening. The site distribution functions for Zn cyt c and porphyrin cyt c are continuous (Figs. 5 and 6); continuous distributions have been observed for other proteins [14,15]. The continuous nature of the distribution is consistent with the model of a very large number of substates for proteins [16].

The shape and the width of the distribution are also of interest. It has been observed experimentally that a homogeneously broadened line is fitted well by a Lorentzian shape, whereas an inhomogeneously broadened line for a chromophore in a glass is almost a Gaussian [16]. Two-level spin glass models applied to conformational substates in proteins suggest that the density of substates is a Gaussian distribution [18]. In addition, Laird and Skinner [19] have developed a statistical, microscopic theory showing that the inhomogeneous line shape for dye molecules in polymer glasses is also a Gaussian function. The results of Laird and Skinner's theory indicate that the mean frequency and the width of the inhomogeneous distribution for S_0–S_1 transition frequencies increase with larger solute–solvent unit interaction potential. Although their theory has been developed for a much simpler system of dye molecules in a homogeneous polymer glass and only van der Waal's types of interactions between the solute and the solvent molecules have been taken into account, we consider their results to be relevant to our work. In a more recent work, Kador *et al.* [20] extended the statistical, microscopic approach to include the electrostatic interactions between the solute and the solvent. Their results agree to a large extent with the results of Laird and Skinner's theory. They suggest that the most significant contribution to the solvent shift of the solute absorption lines is made by van der Waals' (dispersion) interactions.

Although we cannot interpret the site distribution function in terms of atomic interactions, it does give clues of porphyrin/protein interaction. The site distribution function for Zn cyt c (65 cm^{-1}) is broader than for Zn mesoporphyrin in horseradish peroxidase (35 cm^{-1}; Table III), where Zn mesoporphyrin is embedded completely in the protein and has very little solvent exposure. In cyt c the porphyrin may already be significantly inhomogeneously broadened by a partial exposure to the solvent and interactions with water dipoles or some other

interactions. The site distribution function for porphyrin cyt c is definitely not a single Gaussian, whereas the Zn cyt c distribution shows non-Gaussian behavior at the low sides of distribution, indicating the possibility of a secondary component of small amplitude. At present we have not established the origin of the second component for porphyrin cyt c. It is known that free-base porphyrin systems undergo tautomerization reactions at cryogenic conditions under illumination, and this reaction has been studied for porphyrin derivatives of horseradish peroxidase [15]. The questions whether a tautomerization reaction also occurs in porphyrin cyt c and whether it contributes to the bimodal distribution require more experimentation. For both horseradish peroxidase and cyt c, the Zn porphyrin derivative showed a narrower distribution function than did the non-metal-containing porphyrin protein.

Finally, the site distribution function is the only correct way to estimate the inhomogeneous distribution of a chromophore. The inhomogeneous width is narrower than what would be estimated from the nonselected line width of absorption bands obtained with a monochromatic light source at cryogenic temperatures.

ACKNOWLEDGMENT

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