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Conformational substates of ferricytochrome *c* revealed by combined optical absorption and electronic circular dichroism spectroscopy at cryogenic temperature

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ABSTRACT

We have investigated the heterogeneity of the Fe(III)–Met80 linkage of horse heart ferricytochrome *c* by probing the 695 nm charge transfer band with absorption and electronic circular dichroism (ECD) spectroscopy. In order to verify the connection between conformational substates of the Fe(III)–Met80 linkage and the 695 nm band spectral heterogeneity, we have performed experiments as a function of pH (neutral and acidic) and temperature (room and 20 K). At room temperature, the ECD spectrum is blue shifted with respect to the absorption one; the shift is more pronounced at acidic pH and is compatible with the presence of sub-bands. ECD measurements at 20 K highlighted the heterogeneous nature of the 695 nm band and provided direct experimental evidence for the presence of sub-bands. Indeed, while the absorption spectra remained deceivingly unstructured, the ECD spectra showed well resolved peaks and shoulders. A consistent fit of the 20 K absorption and ECD spectra showed that five Gaussians (each centered at the same frequency in the absorption and ECD spectrum) are able to reproduce the observed lineshapes. A careful analysis of frequency shifts and intensity ratios of these sub-bands enabled us to identify at least three distinct sub-bands arising from taxonomic conformational substates of the Fe(III)–Met80 linkage. In view of the major influence of the Fe(III)–Met80 linkage on the redox potential of ferricytochrome *c*, we speculate that these spectrally distinguishable substates may have different functional roles.

1. Introduction

The three dimensional native structure of proteins is characterized by a highly complex energy landscape in which the global minimum (corresponding to the native conformation) is made up of a large number of quasi-isoenergetic minima (corresponding to the so-called conformational substates). A hierarchical organization of the landscape has been suggested [1,2], whereby a small number of substates (the taxonomic substates) is, in turn, structured in a large number of higher tier(s) substates (the statistical substates). From the spectroscopic point of view, the statistical substates contribute to the rather large spectral Gaussian widths of protein absorption bands [3–5], while taxonomic substates are responsible for their sub-bands structure, like e.g. in the case of the near infrared charge transfer bands of deoxyhemoglobin, deoxymyoglobin and superoxide dismutase [6,7], and of the infrared stretching band of CO bound to myoglobin [2,8,9]. Protein conformational substates have substantial functional relevance, since they are responsible for the heterogeneity observed in many dynamical processes like the binding of ligands to the active site; a role of conformational substates in protein folding/unfolding processes has also been suggested [10–12].

Cytochrome *c* is a small globular protein with a molecular weight of about 12.4 kDa. It contains a single heme group whose central iron atom is coordinated to a histidine residue (His18, proximal side) and a methionine residue (Met80, distal side), besides the four pyrrole nitrogens of the porphyrin. Its main physiological role is to mediate the electron transfer from cytochrome *c* reductase to cytochrome *c* oxidase. The absorption spectrum of cytochrome *c* in the ferric state is characterized by the well known B (Soret) and Q bands and by a weaker charge transfer (CT) band in the near infrared at about 695 nm [13]. This CT band had initially been assigned to an $A_{2u}(\text{porphyrin}) \rightarrow d_z^2(Fe^{3+})$ transition involving a molecular orbital of the porphyrin ring and an atomic orbital of the iron [14]. However, this band is absent in low-spin ferric forms of cytochrome *c* whenever the Met80 residue is replaced by a different ligand [15,16]. This observation indicated that the 695 nm band should be assigned to a $S(\text{Met80}) \rightarrow Fe^{3+}$ transition involving atomic orbitals of the methionine sulfur and the iron ion. Indeed, the dependence of this band on the axial iron–sulfur linkage has made the band a frequently used tool to monitor the presence of intermediates in the folding/unfolding transition of ferricytochrome *c* [17–23]. Structural heterogeneity of the heme pocket is expected to result in a spectral heterogeneity of the 695 nm band. Schweitzer-Stenner et al. have measured the absorption and electronic circular dichroism (ECD) spectra of ferricytochrome *c* in the 283–333 K temperature interval

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[22]. Their data show that the absorption band at 695 nm is shifted with respect to the corresponding ECD band, both bands being highly asymmetric. The authors interpreted this finding as an evidence of the presence of taxonomic conformational substates and proposed the existence of at least three sub-bands (S2, S3, and S4 in their terminology). A frequency shift between absorption and ECD can be attributed to the heterogeneous nature of the spectra; indeed, if the spectra are heterogeneous and if the underlying sub-bands have different rotational and oscillator strengths, the frequency shift can be easily rationalized. A similar argument has been already used by Schweitzer-Stenner et al. also in the case of the Soret band of several myoglobin derivatives [24], although in that case the origin of spectral heterogeneity is not the presence of conformational substates, but an *x*–*y* splitting due to symmetry lowering distortions. Although appealing, such evidences for sub-bands remain indirect since no clear peaks or shoulders could be resolved in the spectra close to room temperature, especially in the ECD ones. This consideration motivated the present study: in order to resolve the spectral heterogeneity of the 695 nm band in a clear-cut way, we have performed absorption and ECD experiments at 290 K and 20 K, exploiting the widely known fact that optical absorption bands of proteins become narrower on lowering the temperature [3,25–27].

2. Materials and methods

Horse heart cytochrome *c* was obtained from Sigma-Aldrich. To ensure full oxidation of the iron, a 4-fold molar excess of potassium ferricyanide was added and subsequently removed by prolonged dialysis. The protein solution was mixed with phosphate buffer and glycerol to obtain a sample of 1 mM ferricytochrome *c* in 65% (vol/vol) glycerol/water and 0.1 M phosphate buffer. The sample, contained in a 1 cm path length PMMA cuvette, remained homogeneous and transparent upon lowering the temperature down to 20 K. Measurements have been performed at two different pH values, 4 and 7, in order to modulate the spectral heterogeneity while preserving intact functional and spectral properties of the protein [17,28].

Absorption spectra were obtained with a Jasco V-570 spectrophotometer operated with a scan speed of 40 nm/min, 1 nm bandwidth, and 1 s response time. The experimental setup for the cryogenic temperature optical absorption measurements has been described in previous publications [27,29]. For ECD measurements at cryogenic temperatures a Jasco J-715 spectropolarimeter was equipped with a home-built apparatus comprising an Oxford Optistat cryostat and an Oxford ITC 503 temperature controller; spectral parameters were as follows: scan speed = 50 nm/min, bandwidth = 2 nm, response time = 1 s; 20 accumulations resulted in spectra having good signal-to-noise ratio. The frequency calibration of the spectrophotometer and spectropolarimeter were checked by measuring the absorption spectrum of a neodymium glass and the ECD spectrum of a camphor standard, respectively. We estimate that both instruments are calibrated within 20 cm^{-1}.

At each temperature, the absorption and ECD spectra of a 65% (vol/vol) glycerol/water mixture containing 0.1 M phosphate buffer were measured and subtracted from the corresponding spectra of the protein containing sample. Control experiments made at room temperature showed that, at both pH values, the presence of glycerol does not introduce any relevant spectral alteration in both the absorption and ECD spectral profiles.

3. Results and discussions

Fig. 1 reports the absorption and ECD bands at around 695 nm (~14,400 cm^{-1}) measured at room temperature and normalized with respect to their peaks. Since both bands lie on the tail of a much more intense band, a suitable baseline has been subtracted from each spectrum: while a cubic baseline has been subtracted from the absorption spectra, a linear baseline was enough in the case of the ECD ones (see panels c and f of Fig. 2 below). The peak of the ECD band is blue shifted with respect to the absorption one in the ferricytochrome *c* sample at pH 7 (Fig. 1a,b); this effect is even larger in an analogous sample at pH 4 (Fig. 1c,d). A frequency shift between an absorption band and its corresponding ECD one can be interpreted as an evidence of spectral heterogeneity as already mentioned in the Introduction. To further investigate such heterogeneity we have performed measurements at cryogenic temperatures. Fig. 2 shows the absorption spectra at 290 and 20 K of ferricytochrome *c* at pH 7 (panel a) and pH 4 (panel b); the baseline subtracted from the spectrum measured at 20 K (pH 4) is depicted in panel c. The low temperature absorption spectrum, although being, as expected, more intense and narrower, remains deceivingly

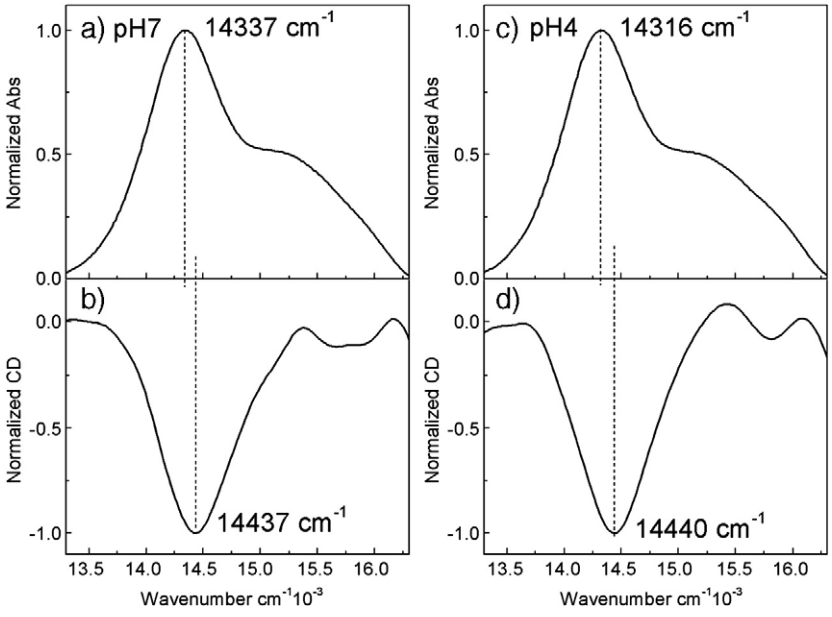

Fig. 1. Room temperature absorption and ECD spectra of horse heart ferricytochrome *c* at pH 7 (panel a and panel b) and pH 4 (panel c and panel d) in the 695 nm band region. Each spectrum has been normalized with respect to its maximum after subtraction of a suitable baseline (see text). The dashed lines mark the position of the respective main peaks.

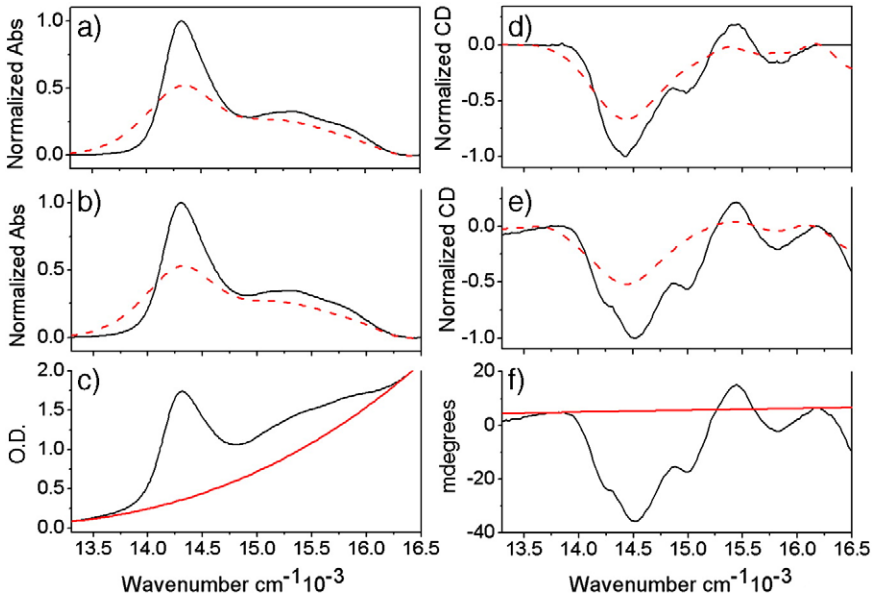

Fig. 2. Absorption (panels a–c) and ECD (panels d–f) spectra of ferricytochrome *c* at 20 K (black lines) and 290 K (red dashed lines) in the 695 nm band region. Panels a and d refer to a ferricytochrome *c* sample at pH 7, while panels b and e to a ferricytochrome *c* sample at pH 4. Spectra have been scaled with respect to the maximum at 20 K. The baseline has been taken into account with a cubic polynomial in the case of absorption spectra (panel c) and with a linear polynomial in the case of ECD ones (panel f).

unstructured and gives no direct evidence for the presence of sub-bands. We note that the clear asymmetry of the ~14,250 cm^{-1} absorption peak is not a proof of the presence of sub-bands since this could be related to the coupling of the underlying electronic transition with low frequency vibrational modes [26,29]. Following the idea of Schweitzer-Stenner et al. [22,23,28,30], we complemented the absorption data with ECD data on the very same samples. The ECD spectrum of ferricytochrome *c* at 20 K and pH 7 (Fig. 2d) shows an asymmetric peak at ~14,500 cm^{-1} and a clearly resolved peak at ~15,000 cm^{-1}; the high frequency wing of the spectrum is characterized by a positive peak a ~15,400 cm^{-1} and negative one at ~15,800 cm^{-1}. The residual marked asymmetry of the peak at 14,500 cm^{-1} seems to suggest the presence of further sub-bands. Indeed, the ECD spectrum of the sample at pH 4 (Fig. 2e) gives direct evidence of this hypothesis. This spectrum is very well resolved: it shows a shoulder at ~14,250 cm^{-1}, a peak at ~14,500 cm^{-1}, and a clearly resolved peak at ~15,000 cm^{-1}; as for the pH 7 sample, the high frequency wing is characterized by peaks at approximately 15,400 and 15,800 cm^{-1}. Note that the small band at about 13,650 cm^{-1} reported by Dragomir et al. [23] and named S1 by the authors, is never observed in our experiments (both in absorption and ECD) even at 20 K.

Based on direct evidence given by the 20 K ECD spectrum at pH 4, we conclude that the 695 nm absorption and ECD bands of ferricytochrome *c* are composed of several spectrally distinguishable sub-bands having different oscillator and rotational strengths. These spectroscopically distinct sub-bands likely correspond to different structures, i.e. to different conformational substates. Note that this spectral heterogeneity is already evident by inspection of the raw data, and it does not depend on the details of the baseline subtraction procedure.

To obtain quantitative information on the composite structure of the 695 nm band, and to check the consistency of our hypotheses, we analyzed simultaneously the absorption and ECD spectra measured at 20 K between 13,500 and 16,500 cm^{-1}; five different Gaussian components sharing the same peak frequencies in the absorption and ECD spectra have been used to reproduce the observed spectral profiles. Fig. 3 shows the result of the fitting procedure and demonstrates that the absorption and ECD spectra can be well fitted with a single set of five Gaussian sub-bands that are named A1 to A5 in the figure. Best fit parameters for the areas (I_k, $k=1 \ldots 5$), peak frequencies (ν_k, $k=1 \ldots 5$), and widths (σ_k, $k=1 \ldots 5$) of the five Gaussian components are reported in Table 1. The fractional areas of the sub-bands are reported in Fig. 4.

The data in Figs. 3 and 4 and in Table 1 show that:

- The area of the sub-bands depends sizably on pH, particularly in the case of the ECD spectrum: indeed, at pH 4, the ratio I_2/I_1 is ~1.37 (i.e. the rotational strength of A1 is smaller than that of A2), while at pH 7 it is ~0.83 (i.e. the rotational strength of A1 is larger than that of A2). This makes the ECD spectrum at pH 4 more resolved than the corresponding absorption spectrum.
- At a given pH, the areas of the sub-bands scale differently in the absorption and ECD spectra (Fig. 4): e.g., in the case of the sample at pH 7, I_2/I_1 is ~0.45 and ~0.83 for the absorption and ECD spectrum, respectively, while, at pH 4, I_2/I_1 is ~0.50 for absorption and ~1.37 for ECD.
- Frequency separations between sub-bands depend weakly on pH and are ~300 cm^{-1} ($\nu_2-\nu_1$), ~700 cm^{-1} ($\nu_3-\nu_1$), ~1100 cm^{-1} ($\nu_4-\nu_1$), and ~1530 cm^{-1} ($\nu_5-\nu_1$).
- Although they were left as free parameters in the fitting procedure, the widths of the sub-bands in the ECD spectrum are very similar to those of the absorption one (Table 1). A significant discrepancy is observed only in the case of the A4 sub-band (at ~15,400 cm^{-1}) whose width is, however, very sensitive to the details of the baseline subtraction procedure, especially for the ECD spectrum.

On the basis of our data we conclude that the spectrally distinguishable A1, A2, and A3 components are different sub-bands of the same $p(S) \rightarrow d_\pi(Fe^{3+})$ transition arising from different cytochrome *c* taxonomic conformational substates [16]. Supporting evidence for this conclusion comes from the following arguments:

1) Though it would seem possible to assign the different sub-bands to different $p(S) \rightarrow d_\pi(Fe^{3+})$ transitions, the observed sub-bands frequency differences are at variance with such conclusion. In fact, the magnetic circular dichroism (MCD) data reported by McKnight et al. [16] suggest a wavenumber difference of nearly 1000 cm^{-1} between, e.g., $p_y \rightarrow d_{yz}$ and $p_z \rightarrow d_{yz}$, while the $\nu_2-\nu_1$ and $\nu_3-\nu_1$ frequency differences are sizably smaller than 1000 cm^{-1}.
2) Our spectra rule out the hypothesis of a vibronic origin of these sub-bands since the I_2/I_1 and I_3/I_1 ratios scale differently in the

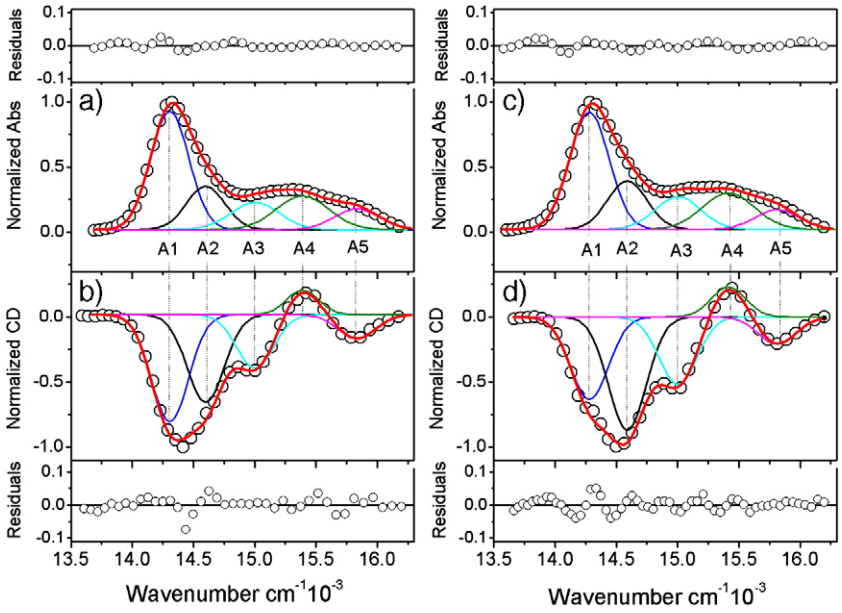

Fig. 3. Simultaneous fit of absorption and ECD spectra of ferricytochrome *c* at 20 K. Panels a and b refer to the sample at pH 7, while panels c and d to that at pH 4. The spectra have been decomposed in five Gaussian profiles (A1–A5): each component has the same peak frequency in ECD and absorption. Parameter values of the Gaussian components are reported in Table 1.

absorption and ECD spectra. As a matter of fact, it is possible to demonstrate that vibronic replicas in the ECD spectra are simply proportional to those in the absorption ones [31,32].

Coming to the origin of the high frequency wing centered at ~15,400 cm^1 (~650 nm) and corresponding to the A4 and A5 Gaussian components, our data do not allow us to make an unambiguous assignment since the arguments used above cannot be applied. Indeed, the $\nu_4 - \nu_1$ and $\nu_5 - \nu_1$ frequency separations are greater than 1000 cm^{-1}, and indeterminations in the baseline subtraction procedure (that has a stronger influence in this frequency region) do not allow us to draw definitive conclusions on the intensity scaling. The high frequency wing could, thus, well originate also from a different CT transition, or from porphyrin skeletal vibrations coupled to the main CT transition [14,16,33]. As far as the first hypothesis is concerned, the most reasonable assignment, following the arguments of McKnight et al. [16], is that of a $p_z(S) \rightarrow d_{yz}(Fe^{3+})$ transition as opposed to the $p_y(S) \rightarrow d_{yz}(Fe^{3+})$ transition at 695 nm. According to such assignment the low frequency transition is expected to be *z*-polarized, while the high frequency one should be *x,y*-polarized.

Table 1
Best fit parameters of the five Gaussian sub-bands (A1–A5) used to fit simultaneously the absorption and ECD spectra at 20 K. The typical uncertainty on the best fit values for the areas (I_k, $k=1 \ldots 5$) is ≤0.005, while that for frequencies (ν_k, $k=1 \ldots 5$) and widths (σ_k, $k=1 \ldots 5$) is ≤20 cm^{-1}.

	Abs pH 7	ECD pH 7	Abs pH 4	ECD pH 4
I_1 (a.u.)	0.410	−0.323	0.425	−0.244
I_2 (a.u.)	0.185	−0.268	0.211	−0.335
I_3 (a.u.)	0.159	−0.170	0.178	−0.200
I_4 (a.u.)	0.196	0.107	0.210	0.081
I_5 (a.u.)	0.129	−0.088	0.129	−0.081
ν_1 (cm^{-1})	14310		14280	
ν_2 (cm^{-1})	14600		14590	
ν_3 (cm^{-1})	15000		15000	
ν_4 (cm^{-1})	15390		15420	
ν_5 (cm^{-1})	15830		15810	
σ_1 (cm^{-1})	160	150	160	150
σ_2 (cm^{-1})	160	150	160	150
σ_3 (cm^{-1})	160	150	160	150
σ_4 (cm^{-1})	320	130	320	140
σ_5 (cm^{-1})	150	150	150	150

Accurate low temperature polarized absorption, resonance Raman with excitation in the near infrared, and spectral hole burning experiments on the 695 nm band would be needed in order to make an unambiguous assignment of the high frequency side-bands.

In the absence of direct structural evidence, we can only speculate about the structural origin of the A1, A2, and A3 sub-bands. In view of the charge transfer character of the associated transition, the iron–methionine linkage must obviously be involved. Different orientations of the S–Fe bond might be a plausible origin. However, the pH dependence of sub-bands intensity ratios suggests that they may be related to substates having different protonation states of residues pointing inside the heme pocket (close to the iron–methionine linkage). Finally, since the spectral heterogeneity is more pronounced for ECD than for absorption, a difference in the overall conformation and chirality of the heme pocket is also expected. In view of the major influence of the Fe^{3+}–Met80 linkage on the redox potential of the protein [34], we speculate that the above spectrally distinguishable substates may as well have different functional roles. Such hypothesis should be tested and taken into account also in future studies employing the 695 nm band as a conformational marker sensitive to the folding/unfolding of cytochrome *c*.

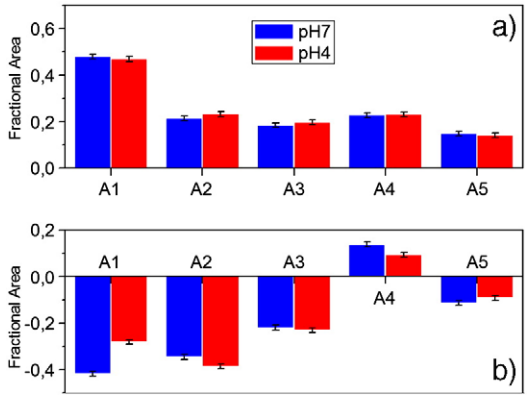

Fig. 4. Fractional areas of the Gaussian sub-bands for the absorption (panel a) and ECD (panel b) spectra with corresponding error bars. Fractional areas have been calculated as the ratio between the area of a single sub-band and the total area of the spectrum.

4. Conclusions

The combined use of low temperature optical absorption and ECD spectroscopies unambiguously shows that the ferricytochrome *c* spectrum in the 13,500–16,500 cm^{-1} (610–740 nm) region can be decomposed in five sub-bands that we label A1–A5. Detailed inspection of frequency shifts and intensity ratios enabled us to identify components A1, A2 and A3 as sub-bands of the same $p_y(S) \rightarrow d_{yz}(Fe^{3+})$ electronic transition, arising from different cytochrome *c* taxonomic substates. For A4 and A5 the assignment is more ambiguous and needs further experimental investigations with different spectroscopic techniques.

The present results highlight the potentialities of combining low temperature absorption and ECD for the resolution of highly heterogeneous protein spectra.

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