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Dynamic properties of the active site of azurin studied by the temperature dependence of the optical spectrum

Antonio Cupane, Maurizio Leone, Eugenio Vitrano, and Lorenzo Cordone

Istituto di Fisica dell'Università and GNSM-CISM, Via Archirafi 36, I-90123 Palermo, Italy

Summary. We report the optical absorption spectra of azurin (*Pseudomonas aeruginosa*) in the temperature range 290–20 K. The samples used are protein aqueous solutions containing 65% (by Vol.) glycerol as cryoprotectant. The measured spectra are deconvoluted in gaussian components and the temperature dependence of the zeroth, first and second moment of the observed bands is analyzed using the harmonic Franck-Condon approximation for the coupling between electronic transitions and nuclear vibrations. The analysis provides information on the stereodynamic properties of the active site of this protein. The possible functional relevance of these results is also suggested.

Key words: Azurin – Blue copper proteins – Optical spectroscopy at cryogenic temperatures – Protein dynamics – Charge transfer transitions

Introduction

The temperature dependence of the optical spectra of proteins is related to the interactions of optical electrons with the nuclear vibrations. The theory, developed long ago to study the properties of color centers in crystals (Markham 1959), has recently been applied to heme proteins (Cordone et al. 1986; Leone et al. 1987; Cordone et al. 1988; Cupane et al. 1988). It can be shown that, within the Franck-Condon approximation, the temperature dependence of the first (M_1) and second (M_2) moment of an absorption band can be expressed as:

$$M_1 = D + F\coth(h\langle\nu\rangle/2kT)$$
$$M_2 = A\coth(h\langle\nu\rangle/2kT) + C^2 \qquad (1)$$

where $\langle\nu\rangle$ is the 'mean effective' frequency of the nuclear motions coupled to the electronic transition and D, F, A and C are parameters linked to the Franck-Condon linear and quadratic coupling constants. Eqs. (1) show that information on the stereodynamic properties of a protein in the proximity of the chromophore can be obtained from the thermal behavior of the optical spectrum over a wide temperature range.

In this paper we use the above approach to analyze the temperature dependence of the optical absorption spectrum of azurin. Our aim is to obtain information on the stereodynamic properties of the active site of this protein since a deeper knowledge of these properties will also add to the understanding of the functional behavior.

Materials and methods

Azurin (from *Pseudomonas aeruginosa*) was purchased from Sigma and was used without further purification. Samples for spectrophotometric measurements contained 0.1 M phosphate pH = 7 (in water at room temperature) and 65% (by Vol.) glycerol/water; the protein concentration was 0.12 mM. Plastic cuvettes with an optical path of 1 cm were used; the samples remained homogeneous and transparent in the whole temperature range investigated. Glycerol (Carlo Erba) was RP grade and was used without further purification. Spectra (1100–350 nm) were measured with a Cary 2300 spectrophotometer controlled by an IBM PC; the spectral bandwidth was always less than 1.2 nm. The experimental setup and methods for spectral measurements have already been described (Cordone et al. 1986). The temperature dependence of the baseline was measured by performing measurements with a solvent sample. No baseline variations were observed in the region 800–350 nm over the whole temperature range; baseline variations with temperature were, however, observed in the region 1100–800 nm due to the absorption of the glycerol/water mixture. The baseline measured at the same temperature was subtracted from each spectrum before analysis.

The spectral deconvolution in terms of gaussian components $G(\nu) = A\cdot\exp[-(\nu-\nu_0)^2/2\sigma^2]$ was performed on an HP 1000 A 900 computer. The reduced-mean-square deviation was minimized using a non-linear least-squares algorithm. The zeroth, first and second moment of each band were calculated according to

$$M_0 = \int_0^\infty A(\nu)\,d\nu \qquad (2a)$$

$$M_1 = M_0^{-1}\int_0^\infty \nu A(\nu)\,d\nu \qquad (2b)$$

Offprint requests to: A. Cupane

$$M_2 = M_0^{-1} \int_0^{\infty} \nu^2 A(\nu)\, d\nu - M_1^2 \qquad (2c)$$

where $A(\nu)$ is the absorbance of the deconvoluted band at the optical frequency ν. Within the so-called narrow-band approximation (Dexter 1958), M_0 is proportional to the oscillator strength and Eqs. (2b) and (2c) are the first and second spectral moment which satisfy Eqs. (1).

Results and discussion

Fig. 1A shows the temperature dependence of the spectrum between 290–20 K; by lowering the temperature, an increase of the central band at ≈600 nm and a parallel decrease of the near-infrared band at ≈850 nm are evident. This is even more clearly shown in Fig. 1B where we report the temperature-induced difference spectra. A typical spectral deconvolution in terms of gaussian components is shown in Fig. 1C. The four

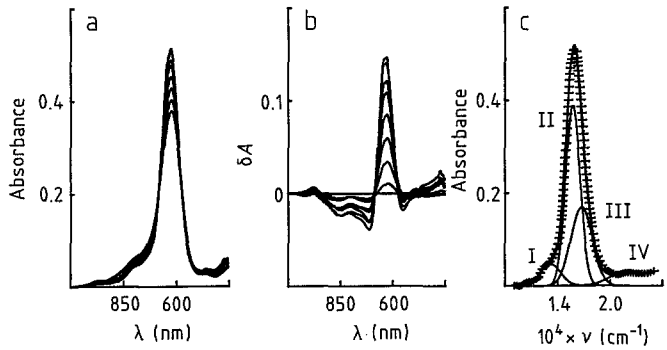

Fig. 1. (**A**) Spectra of azurin at various temperatures. (**B**) Temperature-induced difference spectra; δA is defined as $A(T) - A(290\ \mathrm{K})$. (**C**) Deconvolution of the 20 K spectrum in terms of gaussian components. Crosses are the experimental points; the continuous lines represent the gaussian components and the synthesized band profile

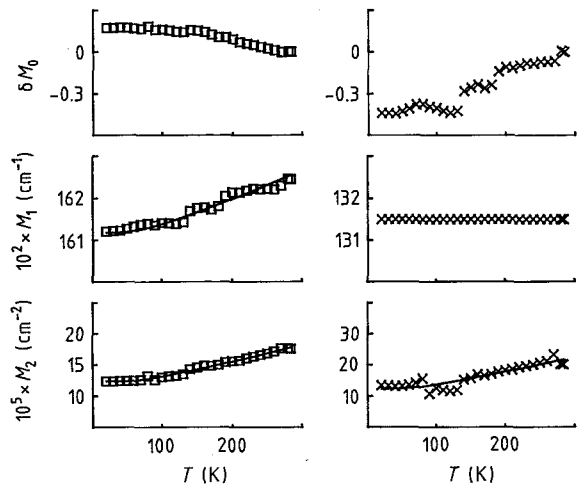

Fig. 2. δM_0, M_1 and M_2 as a function of temperature. **Left:** band II + III; **right:** band I. δM_0 is defined as $[M_0(T) - M_0(290\ \mathrm{K})]/M_0(290\ \mathrm{K})$. The continuous lines represent the best fit of Eqs. (1) to the experimental points; note that points at low temperatures are essential for a correct determination of parameters

Table 1. Values of the parameters obtained by fitting M_1 and M_2 in terms of Eqs. (1)

Band	$10^{-4} \times A$ (μm^{-2})	$\langle \nu \rangle$ (cm^{-1})	$10^{-2} \times C$ (μm^{-1})	D (μm^{-1})	$10^{-2} \times F$ (μm^{-1})
I	50±10	150±35	8.0±2.0	1.3069	0
II+III	30±5	150±20	9.5±0.3	1.6043±0.003	0.75±0.04

main bands, labeled I–IV in the figure, are attributed to ligand-metal charge-transfer transitions (Solomon et al. 1980; McMillin and Morris 1981) and in particular to the following orbital promotions: πS (Cys) → $d_{x^2-y^2}$ Cu (band I); σS (Cys) → $d_{x^2-y^2}$ Cu (band II); σS (Met) → $d_{x^2-y^2}$ Cu (band III); πN (His) → $d_{x^2-y^2}$ Cu (band IV). As can be seen from Fig. 1, band IV is too small to be able to make a meaningful analysis of its temperature dependence while bands II and III are not resolved separately, even at low temperature. For this reason, we considered a single band arising from the sum of the two gaussian bands II and III. Moreover, a temperature-independent M_1 value was imposed on band I to avoid artifacts arising from mixing with the central band.

In Fig. 2 we report the values of δM_0, M_1 and M_2 relative to band I and II + III as a function of temperature. As can be seen, lowering the temperature from 290 K to 20 K causes an integrated intensity decrease of about 50% for band I and an increase of about 20% for band II + III. The values of the parameters obtained by fitting $M_1(T)$ and $M_2(T)$ with Eqs. (1) are reported in Table 1.

Concerning the data reported in Table 1, we note that the value of the mean effective frequency of the vibrational modes coupled to the electronic transitions is about 150 cm^{-1} both for band I and band II + III. A previous report gave a $\langle \nu \rangle$ value of ≈350 cm^{-1} (Solomon et al. 1980). This value was obtained from the temperature dependence of optical spectra measured in thin films of dried protein; experimental data, however, were available at only four temperatures (i.e. 35, 60, 120 and 200 K).

In the resonance Raman spectra of azurin (Woodruff et al. 1984; Nestor et al. 1984; Ainscough et al. 1987; Mino et al. 1987), intense bands are observed near 400 cm^{-1} and are assigned to Cu–ligand stretching motions. Weaker bands are also evident in the range 120–170 cm^{-1} and are attributed to ligand–Cu–ligand deformations and/or to ligand torsions. Our data show that the ligand-to-metal charge transfer transitions responsible for the optical spectrum of azurin are substantially coupled with low-frequency modes; the deformation modes mentioned above are likely to be involved. We stress, however, that our data do not suggest a strong coupling with a single vibrational mode, but rather a weak coupling with several modes whose 'mean effective' frequency is about 150 cm^{-1}.

Our data clearly show that the integrated intensities of the observed bands (M_0) are temperature-dependent. This result implies that, as the temperature is varied, the overlap between the copper and ligand orbitals in-

volved in the charge-transfer transitions also varies, due to anharmonicity. Variations of the metal-ligand relative positions, i.e. deformations of the active site, therefore occur as the temperature is varied.

We wish now to speculate on the possible biological relevance of these results. Low-frequency ligand–metal–ligand deformation modes populated at room temperature ($kT \approx 215\ cm^{-1}$ at 300 K) could add to the conformational flexibility of the active site; moreover, the variation of the screening of the copper $d_{x^2-y^2}$ orbital, controlled by anharmonicity, could be relevant in regulating the electron transfer reaction to/from the orbitals of the redox partners (Nishida 1987).

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