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Vibrational coherence in Azurin with impulsive excitation of the LMCT absorption band

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Abstract

We report the results of femtosecond pump–probe measurements on Azurin, a blue copper protein. The decay of the pump-induced ground-state bleaching, following the excitation of the ligand-to-metal charge-transfer absorption band, is modulated by well visible oscillations and occurs exponentially with a time constant of about 270 fs. The Fourier transform of the oscillatory component, besides providing all the vibrational modes found by conventional resonance Raman, presents additional bands whose attribution is discussed also in connection with the protein dynamics.

1. Introduction

Azurin (Az) is a blue copper protein which acts as an electron transfer (ET) agent from cytochrome c_{551} to nitrite reductase in *Pseudomonas aeruginosa* bacteria [1]. Very recently, scanning tunneling microscopy (STM) studies of the ET behaviour of Az molecules immobilised onto gold electrodes, via their SS bridge, have drawn much attention also for the challenging perspectives of these systems in the frontier field of biosensors and biomolecular electronics [2–5].

The active site of Az contains a type I copper ion ligated to five amino acid side chains (two sulfurs from Cys112 and Met121, two nitrogens from His46 and His117 and an oxygen from Gly45) in a distorted trigonal bipyramidal geometry [6]. In the oxidised form (Cu^{++}), the optical absorption spectrum (see Fig. 1) shows an intense, broad band peaking at 625 nm ($\varepsilon \approx 5000\ M^{-1}\ cm^{-1}$) and some other weaker bands from 400 to 1000 nm [7,8]. The strong band centred at 625 nm has been formally attributed to S(Cys–π) → Cu charge-transfer transition, although actually only a small charge redistribution occurs in this transition [9].

It has been suggested that, generally in blue copper ET proteins, the Cu–S(Cys) bond plays a key role in the physiological ET reaction [10,11].

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Moreover, resonance Raman (RR) studies performed by exciting the strong ligand-to-metal charge-transfer (LMCT) band of blue copper proteins [12–14], including Az [14], have shown that the optical transition is coupled to well identified vibrational motions involving displacements along several ligand vibrational coordinates. Some of these vibrations appear to be coupled to protein atomic motions along proposed physiological ET paths [8,14]. Femtosecond spectroscopy enables to follow in real time, in molecules and solids, vibrational motions coupled to the electronic transition. If the system is excited by a pulse shorter than the vibrational period, vibrational coherence is induced both in the ground and the excited electronic state. Time-domain vibrational spectroscopy is complementary to frequency-domain RR spectroscopy [15], since it provides information also on the excited-state nuclear dynamics and on the coupling among the protein electronic levels. Such an information is important because low-level excited-states act as acceptor levels when the protein receives an electron from a redox partner [16]. In addition, it circumvents the experimental difficulty of RR in discriminating the low-frequency modes against the laser line. To date, there have been limited reports of observation of coherent vibrational dynamics in biomolecules, such as proteins [16–21]. Very recently, femtosecond pump–probe spectroscopy has been applied to study the LMCT band dynamics of two blue copper proteins, plastocyanin [16,20,22] and ceruloplasmin [16]. In this Letter we report on, to the best of our knowledge, the first femtosecond pump–probe experiments on Az; this study is motivated by our strong interest in the spectroscopic, MD and ET properties of this biomolecule [5,23–28].

Following excitation with $\approx$10 fs pulses resonant with the LMCT transition, we observed a fast ground-state recovery with a time constant of about 270 fs. Fourier Transform (FT) analysis of oscillatory pattern superimposed to the signal reveals almost all the vibrational frequencies observed in the conventional RR studies of this protein in addition to a new intense mode at about 500 cm^{-1} whose dynamics is consistent with the excited-state. Finally, three low-frequency modes are observed between 30 and 80 cm^{-1}.

2. Experimental

Azurin from *P. aeruginosa* was purchased from Sigma. The purity was assessed by a SDS PAGE which showed that no protein contaminants are present in our sample. The stock sample was prepared by dissolving 2 mg of the protein in 170 μl of sodium phosphate buffer solution (20 mM) at pH = 6 and stored at −20 °C. The purity ratio (A_{625}/A_{280}) was of 0.42. Optical absorption was measured by using a Jasco V-550 UV/visible spectrophotometer.

Steady-state fluorescence emission spectra were measured in a 500 μl Az sample at a concentration of 30 μM in a 1-cm-thick cuvette (600 μl) by using a Spex FluoroMax spectrofluorometer at room temperature. The buffer solution background was subtracted from all emission spectra.

For pump–probe experiments 50 μl of sample at a concentration of 0.8 mM were kept in a 0.5-mm-thick cuvette. The pump–probe experiments were performed using a non-collinear visible optical parametric amplifier, pumped by the second harmonic of a Ti:sapphire laser at 1 kHz. The system

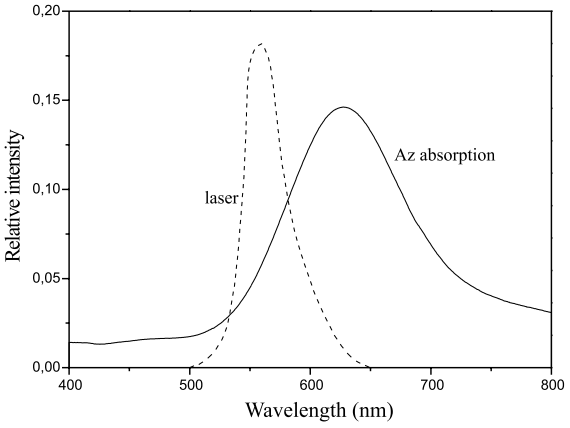

Fig. 1. Absorption spectrum of Az (solid line) in the visible region and the laser spectrum used in the pump–probe measurements (dashed line). The absorption band peaking at 625 nm correspond to the S(Cys–π) → Cu LMCT transition (see text).

generates pulses with ultrabroad bandwidth, extending from 500 to 650 nm, compressed to near transform-limited, sub-10-fs duration by multiple reflections onto chirped dielectric mirrors [29]. Since the main focus of this study is vibrational dynamics of Azurin, we chose to work with the highest possible temporal resolution. To this purpose we selected a pulse spectrum peaking at around 550 nm (see Fig. 1), that allows to achieve the shortest pulses [29].

Absorption spectra of Az were taken immediately before and immediately after the pump–probe measurements to check that no photodegradation has occurred.

3. Results and discussion

Fig. 2a shows the time evolution of the pump–probe signal (dots) resolved at a wavelength of 580 nm. The signals analysed at other wavelengths show an analogous behaviour, with all of them being characterised by a strong spike at zero time delay (for clarity it was removed in the figure). The inset in Fig. 2a, that shows the pump–probe signal from the buffer only, indicates that this spike is due to the buffer solution. Although the solvent contribution covers the protein signal until a delay time of about 100 fs, no oscillations are observed beyond this time delay.

The differential optical transmission shown in Fig. 2a decays exponentially with well visible superimposed oscillations. The decaying component is due to the recovery of the ground-state population, while the oscillatory component can be assigned to vibrational coherence, created by the very short pump pulses in the excited and ground states [16]. We have used several approaches to fit the pump–probe signal, but the best fit of the differential transmission dynamics was eventually obtained by a sum of a decaying exponential with a constant offset. The data fit is performed starting at the delay time for which the buffer response has ceased and is shown in Fig. 2a (solid line). All the fitting function parameters are reported in the legend of the figure. It turns out that the excited-state population created by the pump pulse returns to the ground-state with a time constant of about

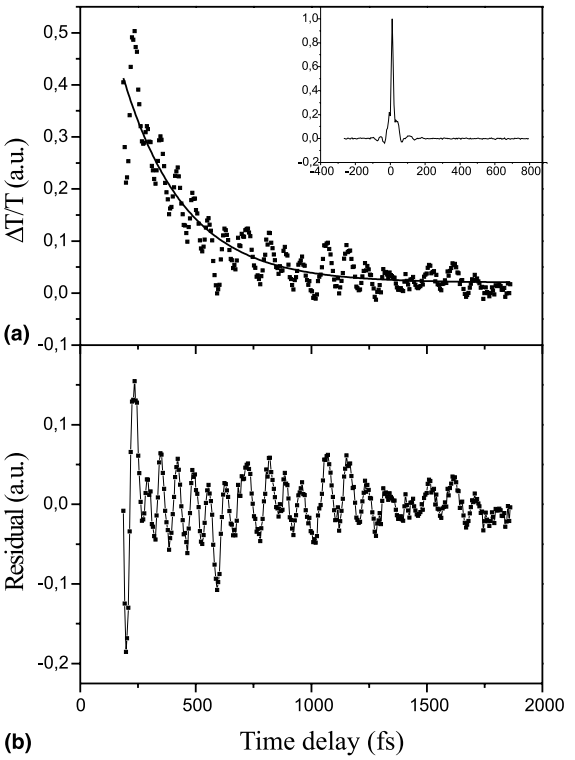

Fig. 2. (a) Differential optical transmission of Az, as a function of pump–probe delay, analysed at 580 nm. The dots represent the experimental data and the solid line is the best fit performed by using a single exponential decay with offset: $y(t) = A + Be^{-t/\tau}$. The best fit values are: $A = 0.0199 \pm 0.0035$; $B = 0.788 \pm 0.042$; $\tau = 268 \pm 13$. Inset: wavelength-resolved pump–probe signal for the buffer solution containing no protein. (b) Oscillatory components (residual) obtained after subtraction of the exponential fit from experimental data of Fig. 2a (the line is a guide to the eye).

270 fs by a non-radiative relaxation process accompanied by stimulated emission (see below). The decaying exponential accounts for about 97.5% of the initial fit amplitude, while the constant offset accounts for the remaining 2.5%. Due to the large similarity among the spectroscopic properties of blue copper proteins, it is interesting to compare our results with those obtained for plastocyanin and ceruloplasmin. Edington et al. [22] excited spinach plastocyanin into its LMCT band with 80-fs pulses. They inferred that the excited-state decays, by a hole transfer process, into an intermediate excited d state with a 125-fs lifetime, from which a return to the ground-state with

a time constant of 285 fs occurs. Book et al. [16] reported pump–probe measurements, for poplar and spinach plastocyanins and for human ceruloplasmin, by exciting with 16-fs pulses centred at 770 nm. They fitted the pump–probe signal evolution with a sum of a decaying exponential with an offset, obtaining a time constant of about 280 fs and an offset of about 2%. At variance with Edington et al. [22], these authors observed a single exponential decay but, since they excited the samples at 770 nm, corresponding to a weak absorption band due to a combination of three d–d transitions, they concluded that were probing the relaxation process corresponding to the second part of the two-step decay of the charge-transfer excitation observed by Edington et al.

We remark that in our case we irradiate just in the Az LMCT band, and we nevertheless observe a single exponential decay. This might be interpreted as an evidence of a single relaxation process ($d_{x^2-y^2} \rightarrow S(Cys–\pi)$) or of the final step of a multistage relaxation process, the initial stage of which is masked by the solvent contribution at early times. However, owing to the different excitation wavelengths used, we cannot affirm with certainty that the relaxation processes in Az and plastocyanin are completely different or are almost identical. The offset (2.5%) that appears stationary in the timescale of the measurement could be due to a small amount of population becoming trapped on the excited-state surface, preventing the reestablishment of equilibrium within this timescale [16].

To analyse the oscillatory component of the signal, the exponential fit of Fig. 2a was subtracted from the pump–probe signal and the residual shown in Fig. 2b was obtained. Successively, a careful Fourier transform [30] of the residual was performed (see Fig. 3). The Fourier spectrum is very similar to those obtained at other wavelengths (not shown) and strikes for the richness of the spectral content, especially if compared with those obtained in previous works for other blue proteins [16,20]. Actually, almost all the vibrational modes obtained by conventional RR spectroscopy [14,31] are retrieved. These are labelled with the value of their frequency in Fig. 3. In particular, the intense bands at 336, 364, 404 and 431 cm^{-1} could very likely correspond to the RR bands centred at 343,

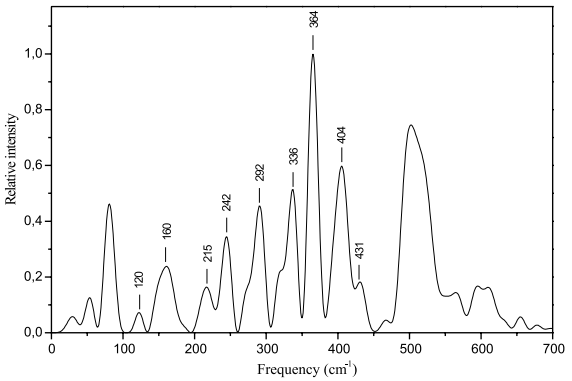

Fig. 3. Fourier spectrum of residual shown in Fig. 2b. The bands which are labelled with their corresponding frequency have been found in the Az RR spectrum. Unreproducible features are observed at frequencies higher than 700 cm^{-1}.

369, 404 and 424 cm^{-1}, respectively. They have been assigned to normal modes involving the Cu–S stretching mixed with other internal coordinates [14,31]. The bands at 215 and 242 cm^{-1}, that could correspond to the 218 and 260 cm^{-1} RR bands, have been assigned to modes involving the Cu–N(His) stretching [14,31].

Additional bands appear in the Fourier spectrum. Three low-frequency bands are clearly visible in the 30–80 cm^{-1} region. Some of these modes, have already been observed in Az by RR [31]. The mode at 30 cm^{-1} is strikingly similar to the vibrational feature observed at about 3.5 meV by inelastic neutron scattering spectroscopy in Az by some of the authors [25,26]. This vibrational bump, called 'boson peak', is a vibrational anomaly often appearing in disordered system such as proteins and glasses [25,26,32,33, and references therein] and might be related to collective modes [32]. Indeed Nakashima et al. [20], studying the charge-transfer dynamics in the plastocyanin by ultrafast pump–probe spectroscopy, focused the attention just on the strong and quite broad band observed at about 30 cm^{-1} attributing it to a delocalised mode involving the protein skeleton motion. Book et al. [16] found low-frequency modes in the 35–55 cm^{-1} region and assigned these vibrational features to protein 'phonon' modes coupled to the optical excitation.

The Fourier spectrum of Fig. 3, contains a new intense and quite broad vibrational mode at about

503 cm^{-1}. Such a band is not present in the Az RR spectrum. Furthermore it is not due to the solvent contribution since water does not have any peak in its Raman spectrum at this frequency and because a pump–probe experiment on the pure solvent did not reveal any oscillation. In order to better investigate the origin of this mode, we analysed the residual of Fig. 2b by linear-prediction singular-value decomposition (LPSVD) [34], using as initial parameters the frequencies extracted from the Fourier spectrum. For the 503-cm^{-1} mode we obtained a damping time of about 300 fs, close to the value of the excited-state lifetime, while for the other relevant vibrational modes the damping time was found in the range from 0.6 to 1.3 ps. All these considerations could indicate that the 503-cm^{-1} mode corresponds to the excited-state. On the other hand, Book et al. [16] also found a similar mode in spinach plastocyanin and human ceruloplasmin and attributed it to the Duschinsky rotation in the excited-state; with our finding confirming their hypothesis that the mode at $\approx$500 cm^{-1} is a general characteristic of blue copper proteins.

In order to better understand the contributions to the ground-state recovery, we analysed the wavelength dependence of the Az differential transmission at three probe delays following the excitation (see Fig. 4). The spectra are shown over the 560–680 nm region were an acceptable signal-to-noise ratio was obtained. The signals in Fig. 4 present three maxima at 580, 600 and 620 nm, whose relative intensity varies as the time delay increases. The peak at 620 nm, corresponding to the maximum of Az LMCT band, can be assigned to ground-state absorption bleaching. The attribution of the other two maxima is less straightforward; they could be due to stimulated emission from excited-state. To support this hypothesis, according to the literature [21,22], we attempted to measure steady-state fluorescence emission in the active-site spectral region. Actually, Loppnow and co-workers [13] who observed a fluorescence emission in plastocyanin when it is excited on the low-energy side of the LMCT band, did not found any fluorescence for Az [14].

Fluorescence emission is observed by us in Az when it is excited from 430 to 600 nm, and almost

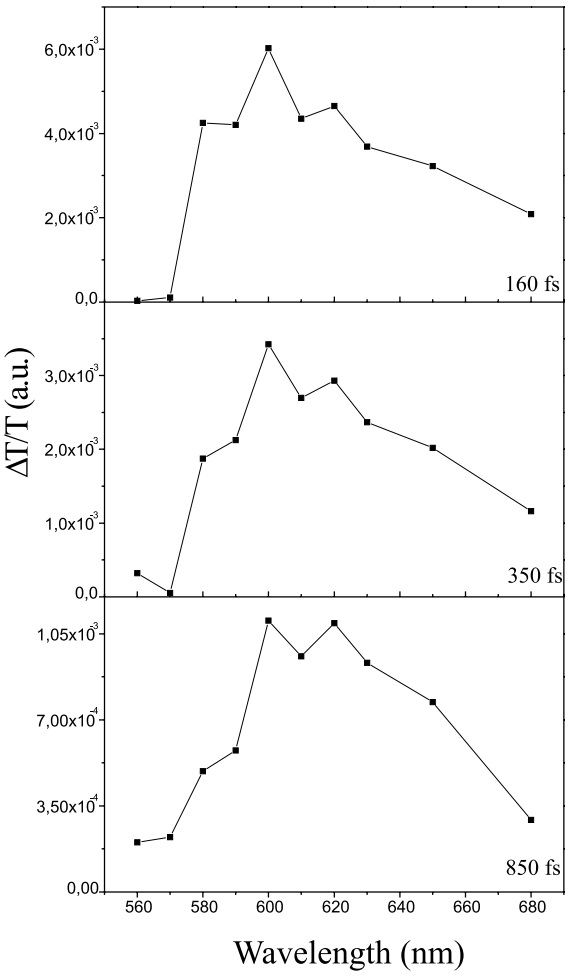

Fig. 4. Wavelength dependence of the Az differential transmission at three probe delay times (160, 350, 850 fs, from the top to the bottom). The dots represent the experimental data while the line is a guide to the eye.

all the emission spectra are characterised by two maxima at about 587 and 610 nm (see Fig. 5 dashed line), although the emission intensity varies. By exciting from 520 to 560 nm the fluorescence emission reaches the highest intensity. The steady-state fluorescence excitation spectrum at an emission wavelength of 610 nm is shown in the inset of Fig. 5. This spectrum indicates clearly that the fluorescent behaviour of Az is not due to the strong absorption band peaking at 625 nm, but to the weaker band at lower wavelength (see Fig. 1

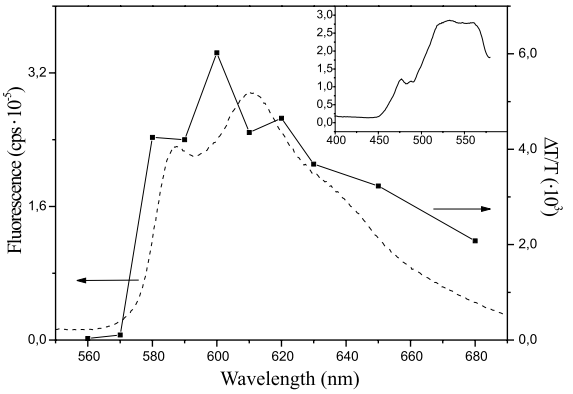

Fig. 5. Wavelength dependence of the Az differential transmission at the delay time of 160 fs (solid line) compared with steady-state fluorescence emission spectrum (dashed line) at the excitation wavelength of 520 nm. Inset: wavelength dependence of the fluorescence excitation spectrum obtained at an emission wavelength of 610 nm. The vertical scale reports the fluorescence intensity (cps) and the values have been multiplied by 10^{-5}.

and related legend). This band, which would partially underlie the strong LMCT band, corresponds to a combination of three charge-transfer transitions [8].

Furthermore, it can be observed in Fig. 5 that the fluorescence emission spectrum, obtained by exciting at 520 nm, matches quite perfectly with the two peaks in the first region of the differential transmission at 160 fs probe delay, although it is red shifted. This finding would suggest that the stimulated emission contributes to the signal at short probe delay. The shift could be explained by considering that the probe pulse stimulates the emission from the excited-state hot vibrational levels, while steady-state fluorescence emission takes place only when the relaxation to the lowest vibrational level of the electronic excited-state has occurred. Finally, as the probe delay increases, a gradual loss of stimulated emission character occurs and the differential transmission spectrum evolves toward the absorption spectrum shape (Fig. 4). This behaviour appears quite similar to that observed by Edington et al. [22] on plastocyanin, indicating that the excited-state dynamics of the two proteins displays some similarities.

4. Conclusions

The pump–probe measurements of Az, performed for the first time in this study, allow us to make some considerations concerning the LMCT state dynamics. Such state decays exponentially with a time constant of about 270 fs. Fluorescence measurements in the active-site spectral region put into evidence that stimulated emission could contribute to the early time differential transmission dynamics. The coherence created by the very short pump pulses in the ground and excited states, allows us to observe the vibrational features of both these electronic states. In fact, in addition to the vibrational bands typical of the RR spectrum of the protein, a new band at about 500 cm^{-1} is observed and attributed to an excited-state mode. Moreover, three low-frequency modes are found in the 30–80 cm^{-1} region; their attribution being still debated.

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