Luminescence emission from *Neurospora* copper metallothionein

Time-resolved studies

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The luminescence lifetime of Cu-metallothionein from the fungus *Neurospora crassa* has been studied by the frequency-domain emission technique. Lifetimes of 10.3 and 3.4 μ s have been found for the protein in the absence and in the presence of oxygen respectively. Binding of Hg(II) results in a quenching of the luminescence correlated to the shortening of lifetime to 0.3–0.4 μ s. No quenching by oxygen is found for the Hg(II)–Cu-metallothionein adduct. By analogy to model compounds, luminescence emission is attributed to a triplet excited state of a Cu(I)-to-sulphur charge-transfer complex. The comparison of bimolecular quenching constants for O₂ and acrylamide indicates a highly compact structure of the protein.

INTRODUCTION

Metallothioneins (MTs) are low-molecular-mass cysteine-rich proteins binding high amounts of metal ions such as Cd(II), Zn(II) and/or Cu(I) (Kägi & Nordberg, 1979). They are found in micro-organisms, plants, invertebrates and vertebrates, where they are believed to play an important role in metal metabolism and/or detoxification. The metal-binding mode has been investigated intensively in the case of mammalian MTs. Chemical, spectroscopic and X-ray-diffraction studies showed that seven group-IIB-metal ions are bound to 20 cysteine residues to form two clusters in which each metal ion is tetrahedrally co-ordinated by four thiolate ligands (Otvoš & Armitage, 1980; Boulanger *et al.*, 1982; Vašák & Kägi, 1981; Braun *et al.*, 1986; Furey *et al.*, 1986). In contrast with MTs of higher eukaryotic organisms, fungal MTs contain copper exclusively (Lerch, 1980, 1981; Weser *et al.*, 1977; Münger & Lerch, 1985). When the ascomycete *Neurospora crassa* is grown on a Cu-supplemented medium, a small Cu-MT can be isolated from the cytosolic extract. The protein consists of a single polypeptide chain of 25 amino acid residues (M_r 2600), seven of which are cysteine, and binds 6 g-atoms of copper/mol of protein (Lerch, 1980; Lerch & Beltramini, 1983). X-ray-absorption and e.x.a.f.s. analysis unambiguously showed the existence of a compact polynuclear cluster with thiolate ligation to Cu(I) ions (Smith *et al.*, 1986).

The spectroscopic properties of *Neurospora* Cu-MT arise predominantly from the Cu(I)-to-ligand complexation. This is borne out by the comparison of the absorption and c.d. spectra of native MT and its apoprotein. In agreement with the lack of aromatic

residues, the spectra of apo-MT are negligible above ~ 250 nm, the features in the far-u.v. being attributable to amide transitions of the peptide chain. Binding of Cu(I) intensifies the far-u.v. absorption spectrum, introduces a characteristic shoulder at 250 nm and the broad red-edge extending to 400 nm. At least five Cotton maxima are resolved in the c.d. spectrum of native Cu-MT over the range 220–400 nm (Beltramini & Lerch, 1983, 1986). The attribution of these spectral properties to the metal–ligand chromophore is also supported by the observation that remarkably different absorption and c.d. spectra are obtained upon substituting Cu(I) with group-IIB-metal ions or Co(II) and Ni(II) (Beltramini *et al.*, 1984; Beltramini & Lerch, 1986). In agreement with a d¹⁰ metal complex, *Neurospora* Cu-MT is e.p.r.-‘silent’ and does not display d–d absorption bands in the visible region. Upon excitation in the u.v. region, *Neurospora* Cu-MT emits luminescence characterized by a large Stokes shift with a maximum at 565 nm. The quantum yield is 0.013 at 10 °C in air and 0.033 in the absence of O₂ (Beltramini & Lerch, 1983). The luminescence emission is an inherent property of the Cu(I)–thiolate complex, as suggested by the lack of emission of both apo- and oxidized Cu-MT and by the close similarity of corrected excitation and absorption spectra. The emission is quenched by binding stoichiometric amounts of Hg(II) [the Hg(II)/MT ratio showing a linear dependence on the amount of Hg(II) added]. The effect is complete, and the dependence no longer linear, when the Hg(II)/Cu-MT ratio reaches 1.0, although copper is not displaced by Hg(II) from the thiolate ligands (Beltramini & Lerch, 1983).

To obtain more information on the peculiar emissive properties of this molecule we have studied the lifetimes

Abbreviations used: bpy, 2,2′-bipyridine; 6,6′-dmbp, 6,6′-dimethyl-2,2′-bipyridine; MT, metallothionein; mbp, 6-methyl-2,2′-bipyridine; phen, 1,10-phenanthroline; tmbp, 4,4′,6,6′-tetramethyl-2,2′-bipyridine; PPh₃, triphenylphosphine.

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of luminescence in deoxygenated buffer and in the presence of quenchers such as O₂, Hg(II) and acrylamide. Reference to model compounds, whose emissive properties have already been characterized, is also given.

This study will enable us to correlate luminescence emission with a possible peculiar electronic configuration of the metal-protein complex. The study of permeability of the protein to the quenchers will provide information on the shielding of the luminophore exerted by the protein structure and on the possible fluctuations of the protein matrix itself.

MATERIALS AND METHODS

Neurospora Cu-MT was purified under an N₂ atmosphere by a combination of gel-filtration and ion-exchange chromatography as described by Beltramini & Lerch (1983). The protein was stored as a freeze-dried powder in vials under argon atmosphere at -80 °C until used. Before lifetime measurements, samples were dissolved in 12 mM-potassium phosphate, pH 7.2. Spectroscopic measurements were carried out at 10 ± 0.1 °C. Lifetimes were determined by frequency-domain phase and modulation measurements using a GREG 200 (Industria Strumentazioni Scientifiche I.S.S., La Spezia, Italy) cross-correlated phase and modulation fluorimeter. The basic design of the apparatus, the theory and data analysis are as described by Lakowicz *et al.* (1984a,b, 1985). The light emitted by an ILC 150 W xenon lamp was phase- and amplitude-modulated with a PTS 250 frequency synthesizer. The synthesizer has limits of 0.3–200 MHz and was used in the 0.3–0.9 MHz range. Control experiments were carried out in a lower frequency range (5–10 kHz) by modulating directly the xenon lamp with a home-made synthesizer. Excitation at 310 nm was used throughout, monochromatization being obtained through a Jobin Yvon H 10 monochromator. Total emission was collected at wavelengths above 380 nm by using a cut-off filter. Data were obtained by averaging 12 measurements at each frequency, each measurement resulting from the average of approx. 300 counts. Data were stored on an IBM XT personal computer and analysed by a GREG 200 dedicated non-linear least-squares routine (ISSC 6-87) to optimize the goodness-of-fit corresponding to the minimization of χ^2 . This parameter, as defined by Lakowicz *et al.* (1984a,b), includes measurement errors. The data were analysed by using the frequency dependence of either phase and modulation or modulation only.

RESULTS AND DISCUSSION

The basis of phase and modulation measurements of fluorescence lifetimes is as follows (Lakowicz *et al.*, 1984b). The sample is excited with light whose intensity is modulated in a sinusoidal manner. Because of the time lag between absorption and emission, the emission is delayed in phase and demodulated relative to the incident light. The frequency-dependent phase delay (ϕ_ω) and demodulation factor (m_ω) can be used to calculate the fluorescence lifetime:

$$\tan \phi_\omega = \omega \cdot \tau_p \quad (1)$$

$$m_\omega = (1 + \omega^2 \cdot \tau_m^2)^{-\frac{1}{2}} \quad (2)$$

where τ_p and τ_m are the emission lifetimes calculated by phase delay and demodulation factor and ω is the circular frequency ($\omega = 2\pi \cdot \text{frequency in Hz}$).

A typical experiment consists in measuring ϕ_ω and m_ω as a function of ω . The experimental data are then compared with the set of phase delay and demodulation factor ($\phi_{\omega c}$, $m_{\omega c}$) values, calculated for a given lifetime whose value is chosen as to minimize the χ^2 coefficient defined as:

$$\chi^2 = \sum \frac{1}{\sigma_{\phi_\omega}^2} (\phi_\omega - \phi_{\omega c})^2 + \sum \frac{1}{\sigma_{m_\omega}^2} (m_\omega - m_{\omega c})^2 \quad (3)$$

where $\sigma_{\phi_\omega}^2$ and $\sigma_{m_\omega}^2$ are the frequency-dependent uncertainties of ϕ_ω and m_ω respectively (Lakowicz *et al.*, 1984b). The overall relaxation process of Cu-MT luminophore is treated in terms of a sum of exponentials:

$$F(t) = \sum A_i \exp(-t/\tau_i) \quad (4)$$

where $F(t)$ is the intensity of fluorescence signal at time t ; A_i and τ_i are empirical parameters selected to provide an estimation of the time-resolved decays. Within this approximation, the fraction of fluorescence decaying with the time constant τ_i is given by the product of the pre-exponential factor, A_i , and the lifetime, τ_i ($A_i \cdot \tau_i$).

Fig. 1. Effect of oxygen on the lifetime of *Neurospora* Cu-MT

(a) Frequency-dependence of modulation amplitude obtained for Cu-MT in N₂-saturated buffer. Curves 1, 2 and 3 refer to the expected dependence calculated for a single component with lifetimes of 5.0, 10.3 and 15.0 μs respectively. Data points refer to two Cu-MT preparations. (b) Frequency-dependence of modulation amplitude obtained for Cu-MT in air-equilibrated buffer; [O₂] = 0.28 mM. Curves: 1, 2.0 μs; 2, 3.3 μs; 3, 4.0 μs. For details, see the Materials and methods section.

Table 1. Luminescence lifetime analysis of *Neurospora* Cu-MT

Samples were measured in 12 mM-potassium phosphate buffer, pH 7.2, at 10 °C and under excitation at 310 nm. The symbols τ and A are respectively the lifetime in μs and the pre-exponential factor proportional to the relative concentration of the species emitting with time constant τ . χ^2 is defined in eqn. (3).

Conditions	τ_1	τ_2	A_1	A_2	χ^2
(1) N_2	10.3	—	1.0	—	2.2
(2) O_2^*	3.4	—	1.0	—	1.2
(3) $\text{N}_2 + \text{Hg(II)}$ (60.9)†	10.3	0.4	0.31	0.69	1.9
(4) $\text{O}_2 + \text{Hg(II)}$ (28.0)†	3.1	0.3	0.56	0.44	0.7
(5) $\text{O}_2 + \text{Hg(II)}$ (49.0)†	3.1	0.4	0.44	0.56	6.4

* Oxygen concentration 0.28 mM.

† In samples 3, 4 and 5, Hg(II) was added to a concentration suitable to quench respectively 60.9 % (sample 3), 28.0 % (sample 4) and 49 % (sample 5) of the luminescence emitted by the unquenched samples.

Obviously, the physical meaning of A_i is the relative concentration of the species emitting with time constant τ_i (Lakowicz *et al.*, 1984b).

In Fig. 1(a) are presented the data obtained with *Neurospora* Cu-MT in N_2 . It shows the fitting of modulation (m_w) versus log frequency. Curves 1, 2 and 3 represent the theoretical dependence expected for a single component with lifetime of 5.0, 10.3 or 15.0 μs respectively. Data points refer to two different protein preparations. The best fit gives a value of $10.3 \pm 0.35 \mu\text{s}$ with a χ^2 of 2.2 (Table 1). Analysis by a two-exponential model did not decrease χ^2 .

After equilibrating Cu-MT in air (final O_2 concentration in solution 0.28 mM), again a single component is found ($\chi^2 = 1.2$). The lifetime, however, decreases to $3.3 \pm 0.07 \mu\text{s}$ (Fig. 1b and Table 1). This effect of O_2 on lifetime parallels the 3-fold decrease of the quantum yield as previously reported by Beltramini & Lerch (1983). The calculated bimolecular rate constant for O_2 quenching is $6.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is about 30-fold lower than that expected for a diffusion-limited process.

In addition to O_2 , acrylamide was used as a luminescence quencher. In Fig. 2 the results of quenching experiments obtained with Cu-MT and acrylamide in the absence (a) and in the presence of O_2 ($[\text{O}_2] = 0.28 \text{ mM}$) (b) are reported. The data are plotted according to the Stern–Volmer equation:

$$F^0/F = 1 + K_Q[Q] \quad (5)$$

where F^0 and F are emission intensities in the absence and the presence of quencher, $[Q]$ is quencher concentration and K_Q is the Stern–Volmer constant. The plots become curved downward upon increasing acrylamide concentration, indicating shielding of the emitting species from the quencher. A modified Stern–Volmer plot allows one to calculate the effective Stern–Volmer constant attributable to a given fraction of luminescence:

$$\frac{F^0}{F^0 - F} = \frac{1}{[Q]} \cdot \frac{1}{\sum f_i K_{Q_i}} + \frac{\sum K_{Q_i}}{\sum f_i K_{Q_i}} \quad (6)$$

Fig. 2. Acrylamide quenching of the *Neurospora* Cu-MT luminescence

Stern–Volmer plots obtained according to eqn. (5) in the text are shown. (a) Cu-MT in N_2 -saturated buffer; (b) Cu-MT in air-saturated buffer; $[\text{O}_2] = 0.28 \text{ mM}$.

where $\sum K_{Q_i}$ (= intercept/slope) is the effective quenching constant, $(K_Q)_{\text{eff}}$, and $\sum f_i K_{Q_i} / \sum K_{Q_i}$ (= 1/intercept) is the effective maximum accessible fluorescence (f_a)_{eff}. (Lehrer, 1971). From the modified Stern–Volmer plots (Fig. 3) it can be calculated that (f_a)_{eff} is approx. 0.7, both in the absence and in the presence of O_2 . In the former case (Fig. 3a), $(K_Q)_{\text{eff}}$ is 38.9 M^{-1} . From this value, taking into account the relationship (Eftink & Ghiron, 1981):

$$K_Q = k_q \cdot \tau_0 \quad (7)$$

where k_q is the bimolecular rate of quenching and τ_0 is the lifetime of the emitting species in the absence of the quencher, k_q for acrylamide is found to be $3.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, two orders of magnitude lower than the k_q for O_2 . When acrylamide quenching is measured in the presence of O_2 (Fig. 3b), $(K_Q)_{\text{eff}}$ is 1.3 M^{-1} , corresponding to a k_q of $3.9 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The effect of Hg(II) was studied by adding substoichiometric concentrations of this metal ion to a solution of Cu-MT. In Fig. 4 the best fit is shown for the frequency dependence of modulation for a sample whose emission was quenched by Hg(II) to the extent of 60.9 %. In this case the lowest χ^2 obtained by a single exponential model is 11.7, with a lifetime of 4.6 μs . A much lower value of χ^2 (1.9) is, however, obtained for a two-component analysis. The resulting lifetime values were $\tau_1 = 10.3 \mu\text{s}$ and $\tau_2 = 0.4 \mu\text{s}$ with pre-exponential factors $A_1 = 0.31$ and $A_2 = 0.69$ respectively (Table 1). In the presence of both O_2 and Hg(II), minimization of χ^2 still results from a two-component fitting with lifetimes $\tau_1 = 3.1 \mu\text{s}$ and $\tau_2 = 0.3\text{--}0.4 \mu\text{s}$. However, the values of pre-exponential factors, namely the relative concentrations of species decaying with τ_1 and τ_2 , change as a

Fig. 3. Corrected acrylamide quenching of the *Neurospora* Cu-MT luminescence (using eqn. 2)

Modified Stern–Volmer plots obtained according to eqn. (6) in the text are shown. (a) Cu-MT in N_2 -saturated buffer; (b) Cu-MT in air-saturated buffer; $[O_2] = 0.28$ mM.

Fig. 4. Lifetime measurements of *Neurospora* Cu-MT in the presence of a heavy-metal quencher

Frequency-dependence of modulation amplitude obtained on Cu-MT in N_2 saturated buffer in the presence of Hg(II) to yield 60.9% quenching of native Cu-MT luminescence. Curve 1, best fit for a single component with $\tau = 4.6$ μ s. Curve 2, best fit for two components with parameters $\tau_1 = 10.3$ μ s, $A_1 = 30.9$, $\tau_2 = 0.4$ μ s, $A_2 = 60.1$. For details, see the Materials and methods section.

function of the amount of Hg(II) added (Table 1). Extrapolation of data reported in Table 1 indicates a lifetime of 0.3–0.4 μ s for the single emitting component at complete quenching.

Although our measurements necessarily are performed close to the lower limit of the modulation-frequency synthesizer, owing to the lifetime domain explored, both the reproducibility of the results using different Cu-MT preparations and the satisfactory fit of the data points allow us to conclude that the Cu(I)–thiolate complex of *Neurospora* Cu-MT behaves as a single emitting species with the lifetime reported. In the case of experiments in the absence and in the presence of O_2 , where the lifetimes are long enough, we have also confirmed the data at high frequency with measurements in the low-frequency range (5–10 kHz) by direct modulation of the light source.

The time-domain of lifetime and the ready quenching by O_2 reported in the present paper, together with the large Stokes shift of emission (Beltramini & Lerch, 1983), are rather strict indications of a triplet character of the emissive state.

Cu(I)–thiolate model compounds have not been thoroughly discussed in terms of their emissive properties; however, detailed studies are available for $[CuL(PPh_3)_2]^+$, where PPh_3 is triphenylphosphine and L is a nitrogen bidentate ligand such as 2,2'-bipyridine (bpy), 1,10-phenanthroline (phen) or dimethylphenanthroline (Reder *et al.*, 1981). Beside ligand emission, emission from a rather well characterized $^3d-\pi^*$ state was found in these charge-transfer complexes with lifetimes in the glassy state at room temperature ranging between 5 and 200 μ s depending on the particular ligand used. Completely analogous metal-to-sulphur charge-transfer transitions are conceivable in the Cu(I)–thiolate chromophore, the π^* aza-aromatic orbital being substituted by an appropriate d or π sulphur orbital.

The lifetimes of *Neurospora* Cu-MT in the presence of Hg(II) are quite easily interpreted. When Hg(II) quenching is incomplete, two forms of the protein are present: the uncomplexed one, with a lifetime of 10.3 μ s, quenched diffusively by O_2 , which decreases the lifetime to 3.1 μ s, and the Hg(II)-complexed species, with a lifetime more than one order of magnitude lower (0.3–0.4 μ s). The increase of relative concentration of species emitting with lifetime τ_2 parallels the amount of Hg(II) added, namely the luminescence quenching. It can be expected, therefore, that when all molecules are converted into an Hg(II)–Cu-MT adduct, a single lifetime of 0.3–0.4 μ s is exhibited. This lifetime is not affected by O_2 quenching at the concentration used (0.28 mM), the rate of quenching, $k_q \cdot [O_2]$ ($= 1.8 \times 10^5$ s $^{-1}$), being one order of magnitude lower than the rate of emission. These results further support the hypothesis that the quenching process is a consequence of Hg(II) binding, as already proposed by steady-state emission titrations (Beltramini & Lerch, 1983). Extrapolation of quenching plots (Beltramini & Lerch, 1983) predicted no luminescence emission at room temperature for the Hg(II)-complexed Cu-MT. This finding receives an explanation by the lifetime data obtained in the present investigation: upon Hg(II) binding, τ decreases by one order of magnitude, and the proportional decrease of the quantum yield brings the luminescence emission below the detection limit of the spectrofluorimeter. The heavy-atom effect is probably responsible for the strong quenching by Hg(II) and further supports the triplet character of the emissive state. It is noteworthy that we are dealing with more than one potential chromophore per molecule (up to six), and the quenching shows a stoichiometry of one Hg(II) ion per molecule. This strongly suggests the existence of a

metal cluster and would point toward an excitonic character of the emissive state.

A striking property of the Cu-MT triplet is its ability to emit in solution at room temperature. Luminescence spectra similar to that of Cu-MT were reported for the Cu(I)-mercaptoethanesulphonic acid complex, Cu(I)-reduced glutathione or Cu(I)-cysteine at liquid-nitrogen temperature (Beltramini & Lerch, 1983); their emission, however, was found to be completely quenched at room temperature. These findings can again be interpreted by comparing the emissive properties of non-thiolate Cu(I) model compounds. The triplet emission is observed in solution for the $[\text{Cu}(\text{dmp})(\text{PPh}_3)_2]^+$ (Blaskie & McMillin, 1980), whereas emission can be hardly detected from the phen analogue. This finding led to the conclusion that bulky ligands could play an important steric role by shielding the chromophore from the solvent environment, hence increasing the lifetime of the charge-transfer complex. In agreement, a further increase of quantum yield is observed for ligands in the order $\text{bpy} < \text{mbp} < 6,6'\text{-dmbp} < \text{tmbp}$ (McMillin *et al.*, 1983). It is also quite conceivable that, in a large molecule, the triplet-triplet annihilation process is drastically decreased by both diminished diffusion and more strict requirements for intermolecular chromophore interactions. Although *Neurospora* Cu-MT exhibits a rather simple primary structure lacking bulky and hydrophobic aromatic residues, shielding from solvent interaction could be elicited by a compact tertiary structure. A compact structure of the Cu(I)-thiolate complex is suggested by a recent e.x.a.f.s. analysis (Smith *et al.*, 1986), which showed an average copper environment of three or four S atoms at 0.22 nm and one or two Cu atoms at 0.271 nm. Our finding of a 30-fold reduced efficiency of quenching by oxygen is indicative of a steric constraint to oxygen diffusion through the protein moiety. Furthermore, the large decrease of K_q reported in the present paper when going from O_2 to a more bulky quencher like acrylamide [although both quenchers have comparable intrinsic efficiency (Eftink & Ghiron, 1981)], as well as the downward curvature of Stern-Volmer plots with acrylamide, also supports the view of a compact protein structure shielding the luminescent Cu(I)-S⁻ chromophore. Thus, in the case of a compact protein structure, the permeability to the quenchers is expected to be strictly modulated by the frequency of motions of amino acid residues and peptide backbone. Since such frequency is constant at a given temperature, saturation phenomena are expected with increasing quencher concentration.

Experiments were extended also to the Cu-MT isolated from the common mushroom, *Agaricus bisporus*. This metallothionein has the same metal-to-sulphur stoichiometry as *Neurospora* Cu-MT and shows comparable luminescence properties (Münger & Lerch, 1985). Lifetime analysis yields a single component with a lifetime of

1.8 μs in air, suggesting that the luminophore of this MT has a structure quite similar to that of *Neurospora* Cu-MT. The shorter lifetime in air, however, could result from different factors like a greater permeability of the protein to O_2 or greater rate constants of other non-radiative relaxation processes (interaction with the solvent or with neighbouring quenching groups).

This work was partly supported by a grant from the Swiss National Foundation (no. 3.285-082).

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