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A time-resolved fluorescence study of human copper-zinc superoxide dismutase

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The intrinsic fluorescence decay of human Cu,Zn superoxide dismutase was measured by frequency-domain techniques. The protein consists of two subunits, each containing one tryptophan and no tyrosine residues. Using a synchrotron radiation source, which allows facile selection of the excitation wavelength, the dependence of the emission decay upon excitation was studied. No significant excitation wavelength effects were found. The two tryptophans contained in the dimer, although fully equivalent and exposed to solvent, showed a fluorescence decay that cannot be described by a single lifetime. Either two lifetimes, or one Lorentzian-shaped continuous distribution of lifetimes, are needed to obtain a good fit. Under identical experimental conditions, control experiments showed that *N*-acetyltryptophanamide, an analogue of tryptophanyl residues in proteins, decays with a single lifetime. The heterogeneous decay of tryptophan fluorescence in superoxide dismutase is interpreted as due to the presence of static and/or dynamic conformers in the protein that decay with different lifetimes. The two models of discrete lifetimes and continuous distribution of lifetimes are discussed with reference to measurements on holo- and apo-human superoxide dismutase.

1. Introduction

Fluorescence is a suitable spectroscopic tool for the study of protein conformation and dynamics. Due to its high sensitivity, fluorescence can be used on very dilute samples; moreover, fluorescence explores a time-window (from pico- to nanoseconds) which is particularly useful for the study of protein motility. Protein intrinsic fluorescence arises from aromatic amino acid residues, among which tryptophan makes the most important contribution, not only in qualitative but also in quantitative terms. In fact, tryptophan

fluorescence emission may peak anywhere between 308 and 360 nm, depending on the physico-chemical properties of the environment. Therefore, tryptophan is an ideal intrinsic probe of protein structure. However, proteins often contain more than one tryptophan and, thus, the fluorescence signal is the sum of contributions from each emitting moiety.

Time-resolved measurements may provide further information on individual fluorescence species, since spectroscopically distinct moieties may decay with different time constants. However, even in the case of proteins containing only one tryptophan residue, the fluorescence decay often shows complex behavior. This finding has been generally ascribed to molecular or conformational heterogeneity [1].

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In a search for spectroscopically 'simple' proteins, we studied human Cu,Zn superoxide dismutase by time-resolved fluorescence. This enzyme appears to offer several advantages over other proteins. First, it contains only one tryptophan per subunit in a symmetric dimer [2,3]. Second, in both subunits this tryptophan is located at the protein surface, protruding into the solvent, so its steady-state fluorescence parameters are similar to those of *N*-acetyltryptophanamide (NATA) in aqueous solution [4,5]. Third, it does not contain tyrosine, thus avoiding the need of exciting at a wavelength where this residue does not absorb. Thus, it is possible to excite the fluorescence throughout the entire lowest energy absorption band of tryptophan.

2. Materials and methods

2.1. Sample preparation

Human Cu,Zn superoxide dismutase (HSOD) was purified from human erythrocytes according to the method of Bannister et al. [6]. A sample of recombinant HSOD was obtained from Bio-Technology General (Israel) through the courtesy of Professor G. Rotilio (University of Rome, Tor Vergata). Metal-free HSOD was prepared by dialyzing the protein against 1 mM EDTA in 0.05 M sodium acetate buffer (pH 3.5). The samples of HSOD used for the fluorescence experiments were dissolved in 0.1 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.0).

2.2. Fluorescence measurements

Fluorescence spectra were recorded on a Jobin-Yvon 3D spectrofluorometer. The multi-frequency phase-shift and demodulation measurements [7] were performed using the apparatus built at the synchrotron radiation facility of the ADONE storage ring, PULS Laboratory, Frascati, Italy. The experimental set-up has been previously described [8]. For data acquisition, commercially available electronics (I.S.S., La Spezia, Italy) were used. Excitation was at several wavelengths, ranging from 290 nm down to 260 nm, with a band-

width of 8 nm. The absorbance of the sample at 280 nm was about 0.2. Fluorescence was collected after passing through a 320 nm cut-off filter to remove scattered light. Fluorescence experiments were carried out at 25°C. Data obtained with the phase-shift technique were analyzed either in terms of a single- and a double-exponential decay [9] or with a model based on a continuous distribution of lifetimes having a Lorentzian shape [10]. In this latter case, the center (*C*) and the width (*W*) of the Lorentzian function were obtained by minimizing the χ^2 values with a routine based on the Simplex method, running on an IBM personal computer equipped with an 8087 mathematical coprocessor (software provided by I.S.S., Champaign, IL). Global analysis of the data was performed on the same computer with a program provided by the Laboratory for Fluorescence Dynamics (LFD), University of Illinois at Urbana-Champaign [11].

3. Results and discussion

The corrected fluorescence excitation and emission spectra of native and metal-depleted forms of HSOD are shown in fig. 1. The emission spectrum of both samples is broad (FWHM apo-HSOD = 80 nm and FWHM holo-HSOD = 60 nm), structureless, and centered at 344 nm. The excitation spectrum is independent of the emission wavelength

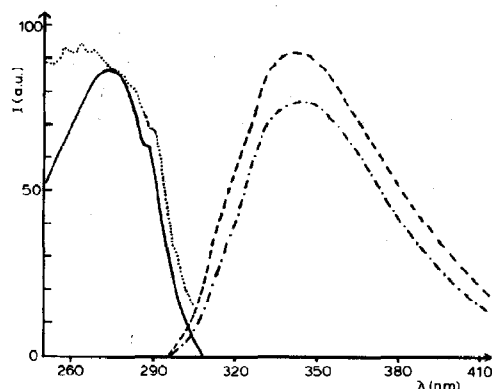


Fig. 1. Absorption (·····), corrected excitation (—) and emission spectra of holo (---) and apo (- - -) forms of HSOD.

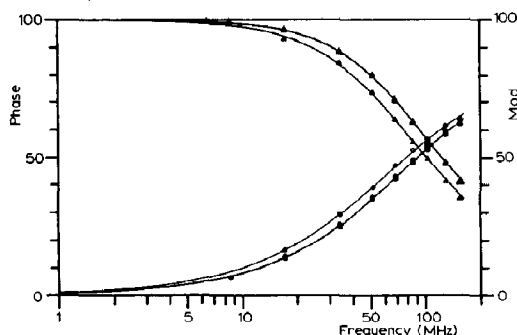


Fig. 2. Phase (\circ) and modulation (Δ) values as a function of light modulation frequency for holo (filled symbols) and apo (open symbols) HSOD. The excitation wavelengths (for holo-HSOD) spanned between 260 and 290 nm, giving almost superimposable phase and modulation values. Solid lines correspond to best fits obtained using one Lorentzian distribution of lifetimes.

and is similar to that of NATA. Also excitation and absorption spectra are significantly different (fig. 1), particularly in the region below 270 nm. The absence of fine structure in this region indicates that the phenylalanines present in HSOD do not transfer their energy to tryptophans. The quantum yields relative to NATA are 0.51 for holo-HSOD and 0.68 for apo-HSOD, indicating a weak quenching by the metal atoms.

Fig. 2 shows plots of phase and modulation values for holo-HSOD for excitation wavelengths between 260 and 290 nm. The values are almost identical at all modulation frequencies; however, the phase and modulation values for the apo-protein are well separated from those of the holo-protein. The decay has been fitted using one- and two-exponential functions or using a continuous Lorentzian-shaped distribution of lifetimes (table 1). The same fitting functions were used for NATA. In the case of single- or double-exponential functions, the values of the lifetime (τ) and the relative fraction (f) are given, while for the distribution of lifetimes, table 1 lists the values of the center (C) and width (W) of the Lorentzian distribution. The errors reported in table 1 were determined using the correlation error analysis procedure of the LFD software. This procedure moves one parameter at a time in the vicinity of the minimum until the value of the χ^2 increases

1.2-fold. During this exploration of the χ^2 surface, the other parameters are allowed to readjust to minimize the χ^2 . This method of error analysis corresponds to the worst-case scenario and the error obtained can be treated as an absolute confidence interval [12].

The best fit to the fluorescence decay of NATA is by a single exponential. However, in the case of holo- and apo-HSOD, the fit using two exponentials or a continuous distribution of lifetimes gave a significantly lower χ^2 than the single-exponential fit. No statistically significant reduction of χ^2 value was obtained using three exponentials or a bimodal continuous distribution of lifetimes.

The absence of tyrosines allowed us to establish, for the first time in a protein, whether any significant dependence occurs for fluorescence decay when the excitation is varied between 260 and 290 nm, i.e., throughout the L_a and L_b absorption bands of tryptophan (fig. 2 and table 1). On the basis of our excitation wavelength studies, it appears that: (1) the emission of tryptophanyl residues in HSOD arises from the same excited state in analogy with studies conducted with tryptophan in solution; (2) no photoselection among the population of protein molecules occurs; (3) the two tryptophans in the dimer are equivalent; and (4) the phenylalanines present in HSOD do not contribute in a significant way to the overall emission nor do they transfer their energy to tryptophan.

The analysis of the decay of the holo-protein shows different values of the lifetimes and fractional intensity contributions from those of the apo-protein (fig. 2 and table 1). In fact, while no significant change in the overall χ^2 values was obtained linking all the data of holo-HSOD taken at different excitation wavelengths of HSOD, the global analysis showed that the value of χ^2 increased dramatically when the data of apo-HSOD were also included (see table 1). This is evidence of a real difference between apo- and holo-HSOD decay data not found for the holo-HSOD at various excitation wavelengths. The decay for the apo- and holo-protein indicates that the protein matrix influences the decay even though the tryptophan residue is exposed to the solvent. Recombinant HSOD shows static and dynamic fluorescence

Table 1
Fluorescence decay parameters of HSOD and NATA
 τ , τ_1 , τ_2 , lifetime values (ns); f_2 , fluorescence fraction relative to τ_2 ; C , W , center, width of the Lorentzian distribution (ns); χ^2 , reduced chi-square; (1), global analysis of holo-HSOD decay data taken at different excitation wavelengths (260, 270, 280, 290 nm); (2), global analysis of holo-HSOD and apo-HSOD decay data.

| Sample | Excitation (nm) | One lifetime | | Two lifetimes | | | One Lorentzian distribution | | | |
|----------------------------|-----------------|--------------|----------|----------------|----------------|-----------------|-----------------------------|-----------------|----------|----------|
| | | τ | χ^2 | τ_1 | τ_2 | f_2 | C | W | χ^2 | χ^2 |
| NATA | 290 | 3.01 | 9 | — | — | — | 3.04 | 0.1 | — | 10 |
| Holo-HSOD | 290 | 2.17 | 33 | 0.8 \pm 0.4 | 2.4 \pm 0.1 | 0.91 \pm 0.04 | 2.22 \pm 0.03 | 0.42 \pm 0.08 | 2.1 | 3.0 |
| | 280 | 2.14 | 18 | 0.9 \pm 0.5 | 2.3 \pm 0.2 | 0.91 \pm 0.08 | 2.18 \pm 0.03 | 0.32 \pm 0.07 | 1.2 | 1.2 |
| | 270 | 2.16 | 25 | 1.2 \pm 0.9 | 2.9 \pm 0.6 | 0.80 \pm 0.50 | 2.20 \pm 0.03 | 0.35 \pm 0.07 | 4.9 | 3.7 |
| | 260 | 2.20 | 13 | 1.8 \pm 0.6 | 2.9 \pm 0.6 | 0.50 \pm 0.30 | 2.24 \pm 0.02 | 0.26 \pm 0.07 | 1.0 | 0.8 |
| Apo-HSOD | 290 | 2.53 | 73 | 0.93 \pm 0.6 | 2.96 \pm 0.2 | 0.9 \pm 0.1 | 2.65 \pm 0.04 | 0.73 \pm 0.07 | 6.0 | 3.3 |
| Global analysis | | | | | | | | | | |
| (1) Holo-HSOD | | 2.17 | 22 | 1.07 \pm 0.8 | 2.4 \pm 0.6 | 0.87 \pm 0.11 | 2.21 \pm 0.02 | 0.34 \pm 0.07 | 3.8 | 3.6 |
| (2) Holo-HSOD and apo-HSOD | | — | — | 1.07 | 2.55 | 0.86 | 2.29 | 0.41 | 45 | 45 |

parameters quite similar to those of native HSOD (data not shown).

The heterogeneous fluorescence decay of HSOD deserves some comments. The sum of two-exponential functions or one Lorentzian-shaped continuous distribution of lifetimes fit the fluorescence decay with similar statistical significance. It should be emphasized, however, that the fit obtained using the continuous distribution requires only two fitting parameters (C and W), while a two-exponential fit needs three parameters. In any case, either model requires the assumption of an intrinsic molecular (static or dynamic) heterogeneity. The presence of a contaminant is ruled out on the basis of analytical, as well as spectroscopic, evidence. In our opinion, it seems unreasonable to interpret the two lifetime values obtained for HSOD using the two-exponential model with the presence of two molecular species, one of which accounts for about 10% of the total. At variance with other cases reported and with the classic example of horse liver alcohol dehydrogenase [13,14], the two tryptophanyl residues of HSOD are equivalent. In addition, it is very common to find single-tryptophan proteins with double- or triple-exponential decay [1]. On the other hand, if one assumes that a continuous distribution of lifetimes is present, the width of this distribution may be indicative of a number of species, each emitting with a slightly different lifetime. This interpretation would be consistent with the model of proteins as dynamic structures fluctuating among a large number of slightly different conformations. In a previous publication, we suggested that a wider distribution of lifetimes indeed reflects a greater spectroscopic heterogeneity of apoferritin polymer with respect to the subunits [15]. Here, we report that the distribution of lifetimes is consistently wider in metal-free HSOD than in the metal-containing protein (fig. 3 and table 1). This finding may be consistent with a looser conformation of the apo-proteins, since the presence of metals is known to stabilize the three-dimensional arrangement of HSOD [16].

The tryptophanyl residues of HSOD belong to class III of Burstein et al. [17]; i.e., they are fully exposed to the solvent. Nevertheless, they show an emission maximum still 10 nm blue-shifted with

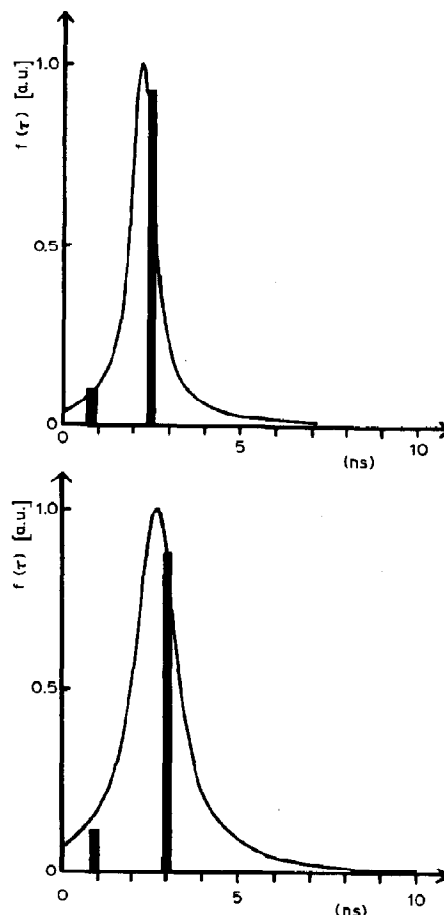


Fig. 3. Continuous distributions of lifetimes of holo (top) and apo (bottom) forms of HSOD. The vertical bars (heavy lines) correspond to the lifetimes and fractional intensity recovered using an analysis with two lifetimes.

respect to NATA, indicating the incomplete solvation of the indolyl moiety. This situation would allow many different interactions between the tryptophanyl side chain and the neighboring amino acid residues, which can be affected by the removal of metals.

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