Biol Metals (1990) 3:133–136

BIOLOGY OF METALS

Dynamic fluorescence in copper proteins

Selected examples

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Summary. The fluorescence properties of three copper proteins, namely human superoxide dismutase, *Pseudomonas aeruginosa* azurin and *Thiobacillus versutus* amicyanin have been studied. All these proteins show a non-exponential decay of fluorescence, though the tryptophanyl residues responsible for the emission are very differently located in the three proteins. All the three decays can be fitted by at least two lifetimes or better with one or two lorentzian-shaped, continuous distributions of lifetime. In each case the removal of copper affects the quantum yield of fluorescence without affecting the shape of the emission.

Key words: Dynamic fluorescence – Copper proteins – Human superoxide dismutase – *Ps. aeruginosa* azurin – *Thiobacillus versutus* amicyanin

Introduction

The fluorescence decay of tryptophanyl residues in proteins is related to the nature, the conformation and the dynamics of their microenvironment. The dependence of lifetime on protein conformation has been used to explain the heterogeneous decay of many single-tryptophan-containing proteins (Beechem and Brand 1985; Hutnick and Szabo 1989). It is generally recognised that proteins in solution are dynamic structures than can assume a large number of different configurations, possibly associated with different lifetime values. If the conformations and the lifetime are only slightly different from each other the fluorescence decay can be fitted with a continuous distribution of lifetimes characterized by two parameters, the center and the width (Alcala et al. 1987a, b, c). Here we report some results of the fluorescence decay of copper proteins. Two of them contain one tryptophan residue only, namely azurin from *Pseudomonas aeruginosa* and amicyanin from *Thiobacillus versutus*. The third one is human superoxide dismutase (HSOD), a dimer of two identical subunits, each containing a tryptophan residue. The lifetime dependence on temperature and denaturation has also been studied.

Human superoxide dismutase

The two equivalent tryptophan residues of HSOD are exposed to the solvent (Barra et al. 1980; Tainer et al. 1983). The emission spectrum of the protein, structureless and centered at 344 nm, is similar to that of *N*-acetyl-tryptophanamide ($AcTrpNH_2$) in water. The decay parameters depend on protein structure, presence of the metal, temperature and addition of denaturants.

The fluorescence decay of the various samples studied can be satisfactorily fitted with a continuous distri-

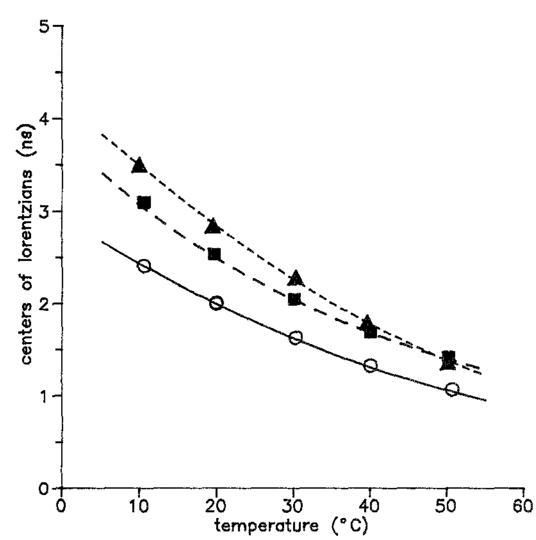

Fig. 1. Dependence of distribution centers on temperature for holo-HSOD (○), apo-HSOD (■) and $AcTrpNH_2$ (▲) in 0.05 M phosphate pH 7.2. The lines correspond to the best fit obtained with an Arrhenius-type function. Excitation wavelength 295 ± 1 nm; emission 320-nm cut-off filter; absorbance of protein at 295 nm 0.1

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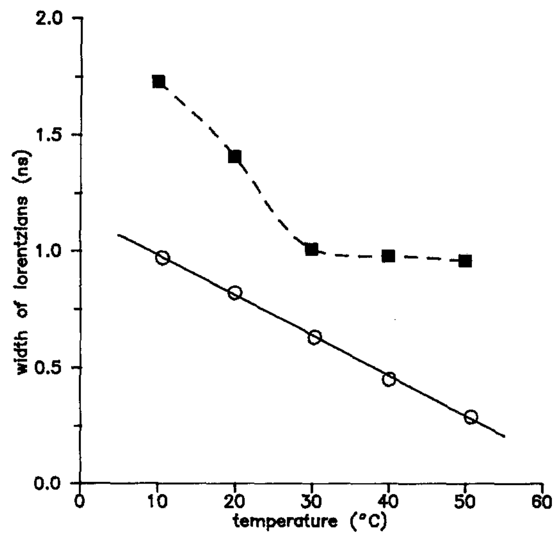

Fig. 2. Dependence of distribution widths on the temperature for holo-HSOD (○) and apo-HSOD (■). The line for the holo-protein corresponds to the best linear fit. Experimental conditions as in Fig. 1

bution of lifetimes, with a lorentzian shape (Rosato et al. 1990). The temperature dependence of the center of distributions for holo- and apo-protein and for Ac-TipNH_2 in buffer is shown in Fig. 1. The experimental points are adequately fitted by an Arrhenius-type law. The activation energy of holo-HSOD is greater than that of apo-HSOD, probably due to a greater rigidity of the native form. This difference vanishes upon denaturation of the protein, pointing to the protein structure as the origin for the different thermal quenching parameters.

The temperature dependence of the width of the lorentzian distributions of holo- and apo-HSOD is shown in Fig. 2. In all samples an increase in temperature is always accompanied by a decrease in the width. This effect can be explained by assuming that temperature increases the rate of structural fluctuations in the nanosecond range which renders the different conformational states spectroscopically identical.

The protein samples denaturated with guanidinium/chloride (GdnHCl) show wider distributions than the native samples, indicating the presence of a larger number of subconformations in the denaturated samples.

The dependence of the center and of the width of the lorentzian on the denaturant concentration is shown in Fig. 3 and 4, respectively. The denaturation of the apo-HSOD occurs at a lower concentration of denaturant. The dependence of the width on the GdhHCl concentration is worth mentioning. A maximum is reached at about half of the GdnHCl concentration needed for complete denaturation. This finding suggest a multi-path process during the unfolding of the protein giving the largest number of conformations at about the middle of the process.

Azurin

Azurin is a low-molecular-mass protein (14 kDa) containing a blue copper atom and acting as a redox partner in electron transfer reactions in the bacterium (Holwerda et al. 1976). *Ps. aeruginosa* azurin contains a single tryptophanyl residue with unique spectroscopic features. Its fluorescence spectrum is structured with a resolved vibronic structure at 295 nm and a peak at 308 nm. This is the bluest value obtained in proteins and it is indicative of highly non-polar and rigid environment of the tryptophan. In fact, the emission spectrum of 3-methylindole in methylcyclohexane is very similar to that of azurin (Szabo et al. 1983). The apo-azurin dis-

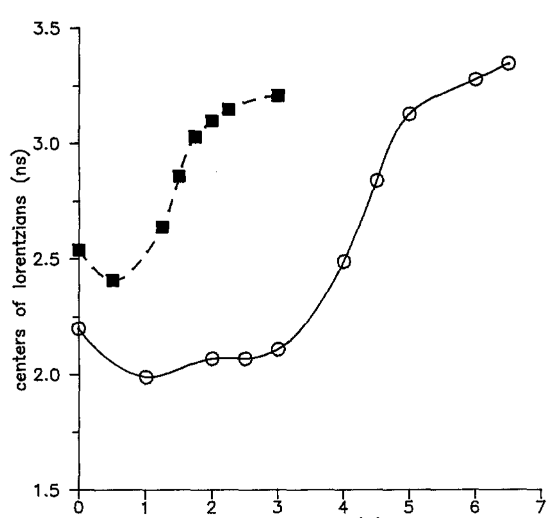

Fig. 3. Dependence of distribution centers on GdnHCl concentration for holo-HSOD (○) and apo-HSOD (■). Experimental conditions as in Fig. 1

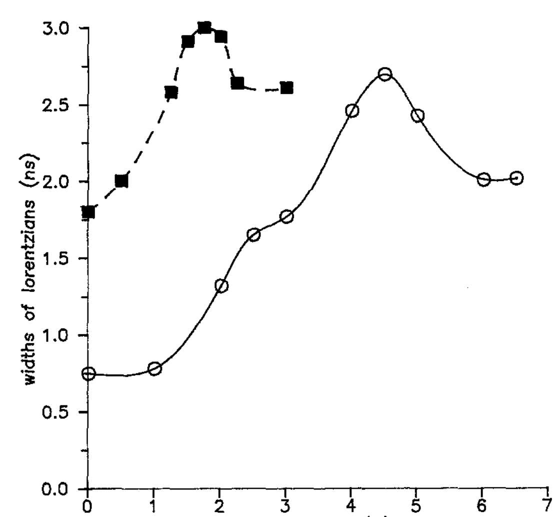

Fig. 4. Dependence of distribution widths on GdnHCl concentration for holo-HSOD (○) and apo-HSOD (■). Experimental conditions as in Fig. 1

plays a spectrum identical to that of holo-azurin but with a relative quantum yield about six times larger.

There is still some debate about the fluorescence decay of holo-azurin. In fact, two lifetimes are required to fit its fluorescence decay while the decay of the apo-azurin is monoexponential (Grinvald et al. 1979; Munro et al. 1979; Szabo et al. 1987). The larger lifetime of holo-azurin (about 5 ns) is similar to the lifetime of apo-azurin and relates to 5% of the molecules. These findings led Petrich et al. (1987) to suggest that this long decay time is due to an 'apo-like' contaminant. Recently, however, Hutnick and Szabo (1989) reported that on accurately purified samples of azurin and three lifetimes were necessary to fit the data the heterogeneity was present to holo-azurin. We have found that a satisfactory fit can be obtained using either two exponential components or two narrow distributions whose centers coincide with the two lifetimes found with the double-exponential analysis. The temperature dependence of the two lifetimes of the holo-azurin is reported in Fig. 5. The shorter lifetime (0.1 ns) of holo-azurin is hardly affected by temperature, like the relative pre-exponential factor that is about 95% at all temperatures. The centers and widths of the distribution of lifetimes required to fit the data of apo-azurin are reported in the same figure. Only at low temperature (< 20° C) or at 50° C is the width of the distribution

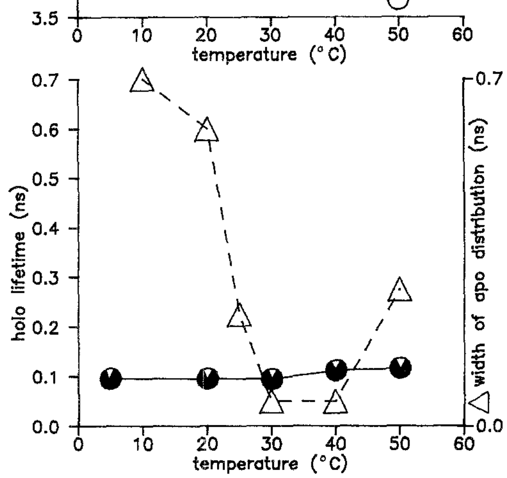

Fig. 5. *Upper part:* dependence of longer-lifetime of holo-azurin (○) and of center of distribution of apo-azurin (□) on temperature. *Lower part:* short lifetime of holo-azurin (○) and width of distribution of apo-azurin (△); the solid part in the symbols for the holo-protein are proportional to the pre-exponential factors relative to two lifetimes. Excitation wavelength 295 ± 1 nm; emission 305-nm cut-off filter. The proteins are in 0.01 M sodium acetate pH 5.2

significant. The specific mechanism of fluorescence quenching occurring in the azurin is still unexplained. Some authors (Petrich et al. 1987) suggest an electron transfer from the excited indole moiety to the copper atom. If this were the case, the Cu(I) and the Cu(II) azurin fluorescences would be quite different, contrary to experiment. At present, and with the available spectroscopic data, it seems more plausible that the quenching is due to conformational effects. Further studies on the dynamic polarization and on the phosphorescence of this protein will help in elucidating this question.

Amicyanin

Amicyanin is a mononuclear blue-copper protein involved in the electron transfer from the methylamine dehydrogenase to cytochrome *c* im methylotrophs (van Houwelingen et al. 1985). It contains a single residue of tryptophan with a structured emission spectrum similar to that of azurin. But, at variance with azurin (Finazzi Agrò et al. 1970), the spectrum is red-shifted by about 10 nm, suggesting that the tryptophanyl residue is not completely buried.

The emission decay of amicyanin is heterogeneous and two lifetimes are required to fit the data. This heterogeneity is due to the presence of metals. In fact, it is present also in the Zn derivative but disappears in the apo-amicyanin whose decay is monoexponential. At variance with azurin, the longer lifetime of the holo-protein (5.7 ns at 20° C) relative to 90% of the total molecules is nearly twice that of the apo-protein (3.3 ns). This finding rules out the hypothesis of an 'apo-like' form as responsible for the longer lifetime component for this protein.

Acknowledgements. The authors thank Dr G. W. Canters and co-workers for providing them with the amicyanin.

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