Fluorescence lifetime distributions in human superoxide dismutase

Effect of temperature and denaturation

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ABSTRACT The internal dynamics of human superoxide dismutase has been studied using time-resolved fluorescence. The fluorescence decay has been analyzed using continuous distribution of lifetime values. The effect of temperature and conformational state on the lifetime distribution has been investigated. The emission of the single tryptophan residue depends on the nature and dynamics of the protein matrix. Conformational changes have been induced by increased concentration of guanidinium hydrochloride. We found that both temperature and conformation strongly effect the width of the lifetime distribution.

INTRODUCTION

Steady-state fluorescence has become a popular tool to investigate conformation of proteins in solution. In particular, the denaturation of proteins has been studied by this technique because tryptophan, the main intrinsic probe, shows a large shift in emission on transfer from a completely or partly solvent inaccessible environment to a fully solvated environment. Fluorescence spectral shift can be interpreted in terms of solvent exposure. The correlation between emission maximum and solvent polarity has been established for some proteins and recently reviewed by Creed (1984). Less common, but similarly straightforward, is the application of fluorescence polarization to the study of protein denaturation. Generally, a significant fluorescence polarization decrease is observed upon denaturation. In this case, the polarization changes are usually interpreted in terms of the hydrodynamic volume changes of domains to which the tryptophan residue is attached (Weber, 1953). Both changes of local tryptophan mobility and shape of the protein due to conformational transition have been measured (Weber, 1953). Fluorescence intensity changes are less well characterized. Both increase and decrease of the fluorescence intensity have been observed upon denaturation (Shortle, 1986; Burstein et al., 1977). What is still lacking is a thorough understanding of the relationship between the fluorescence decay and the physico-chemical properties of the fluorophore microenvironment. Because average fluorescence intensity and fluorescence decay time are correlated, the investigation of the effect of solvent exposure on lifetime, and then on intensity, is better studied using time-resolved fluorescence (Beechem and Brand, 1985).

The improvement in technology and computational power makes the time mature for a more detailed investigation of the relationship between changes of fluorescence lifetime and protein conformation. The explanation of the effects observed must take into account the details of the microenvironment of tryptophan in the protein and of tryptophan at the interface between solvent and protein (Leibman and Prendergast, 1985). Furthermore, it is now accepted that proteins can exist in a large number of subconformations, all similar in energy and rapidly interconverting at room temperature (Austin et al., 1975). In this context, it became evident that a continuous distribution of lifetimes representative of the subconformations can be used instead of a discrete lifetime model to analyze the fluorescence decay in proteins (Alcala et al., 1987 *a–c*). Intrinsically, lifetime analysis contains more information than steady-state parameters, but is more difficult to analyze and understand. We believe that under proper treatment, the analysis of the intensity decay can provide detailed information on protein structure and protein dynamics. Analysis of the fluorescence decay using distribution of lifetime values gives a new parameter: the width of the lifetime distribution, which is related to the microheterogeneity of the tryptophan environment. The purpose of this investigation is to show that we can rationalize some of the features of the lifetime distribution, in particular, the effect on the distribution width of temperature and protein structure. To compare the effect of the tryptophan microenvironment on the native and denatured form, we have studied the intrinsic fluorescence

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[1] *Abbreviations used in this paper:* Gd Hcl, guanidinium hydrochloride; HSOD, human superoxide dismutase; NATA, *N*-acetyltryptophanamide.

decay of human Cu, Zn superoxide dismutase (HSOD),[1] in the apo and holo forms, at temperatures ranging from 10° to 50°C and in presence of different concentrations of guanidinium hydrochloride (Gd HCl). The HSOD is a dimeric protein, the two monomers are symmetrical and contain one tryptophan residue. In both subunits, this residue is at the solvent interface as demonstrated by x-ray structure and fluorescence spectra (Barra et al., 1980; Tainer et al., 1983).

The fluorescence decay of apo and holo forms of the protein has been analyzed using continuous distributions of lifetimes. In a previous work, the fluorescence decay of HSOD has been fitted with a Lorentzian-shaped lifetime distribution (Rosato et al., 1990). The width of the Lorentzian distribution of apo HSOD is about twice that of the holo HSOD. This difference has been attributed to the greater flexibility of the protein in the apo form and is related to a larger number of conformations when the metal is removed.

MATERIALS AND METHODS

Sample preparation

HSOD was purified from human erythrocytes according to Bannister et al. (1972). Metal-free HSOD was prepared by dialyzing the protein against 1 mM EDTA in 0.05 M Na acetate buffer, pH 3.5. The presence of metals were tested by atomic absorption. Gel electrophoresis was used to test that the apo protein was in the dimeric form. The samples used for the fluorescence experiments were dissolved in 0.01 M K phosphate buffer, pH 7.6. *N*-acetyltryptophanamide (NATA) (Serva Biochemicals, Heidelberg, West Germany) was used without further purification. Denatured protein samples were prepared by diluting 0.5 ml of protein stock solution in 2 ml of Gd HCl buffer. Fluorescence measurements were run after 20 h incubation in the presence of Gd HCl at 4°C. The optical density of samples at 295 nm was between 0.1 and 0.2. Molar extinction coefficient used was $\epsilon = 2{,}000\ M^{-1}\ cm^{-1}$ (Bannister et al., 1972).

Fluorescence measurements

Fluorescence decay measurements were performed using the frequency domain technique in a range of modulation frequencies between 20 and 300 MHz (Gratton et al., 1984). The apparatus was composed of a Neodymium-YAG mode-locked laser, an optical module, and the electronics for data acquisition and frequency modulation (Gratton and Limkeman, 1983). Excitation was at 295 nm and the fluorescence was collected using a 325-nm cut-off filter to remove scattered light. Experiments were performed at five different temperatures (10°, 20°, 30°, 40°, and 50°C). Sample temperature was measured with a thermocouple in a cuvette containing water placed in the sample holder.

Protein denaturation was checked by measuring the steady-state polarization of the intrinsic fluorescence, at various concentrations of Gd HCl at 20°C, with a photon-counting spectrofluorometer (model GREG PC; ISS, Inc., Urbana, IL). The denaturation was complete at ~3 M Gd HCl for the apo and at ~6 M Gd HCl for the holo protein. At 4 M Gd HCl the steady-state polarization of the holo protein decreased by ~50% of the total decrease.

The analysis of phase and modulation data was performed with the Globals Unlimited® software provided by the Laboratory for Fluorescence Dynamics (Beechem and Gratton, 1988). Several different models were used to fit the data, namely one and two discrete lifetimes, one and two Lorentzian continuous distributions of lifetimes, and a combination of Lorentzian distributions of lifetimes and discrete lifetimes. In each case the best fit parameters were obtained by minimization of the reduced χ^2 value. Parameter uncertainties were evaluated using the rigorous error analysis procedure of the Globals Unlimited® software (Beechem and Gratton, 1988). The relationship between the centers of the fluorescence lifetime distributions (roughly corresponding to the mean lifetime) and temperature was analyzed in terms of an Arrhenius model (Eq. 1) or following the model proposed by Bushueva et al. (1978) (Eq. 2)

$$1/C(T) = K_1 + K_0 \exp(-E/RT) \quad (1)$$

$$C(T/\eta) = a + b(T/\eta), \quad (2)$$

where C is the center of the Lorentzian distribution; K_1 is the radiative decay rate; K_0 is the nonradiative thermal rate constant at infinite temperature; E is the activation energy for the thermal quenching; R is the gas constant; T is the absolute temperature; and η is the solvent viscosity. The fit to Eqs. 1 and 2 was performed with a nonlinear least squares procedure, based on the Marquardt algorithm, written in Fortran and running on a PDP-11/23 Digital computer. Eq. 2 was derived from the model of Bushueva et al. (1978) to take into account the mobility of protein structure surrounding the fluorophore.

RESULTS

Phase shift and demodulation data for HSOD fluorescence decay obtained at different modulation frequencies was well fitted with a single, continuous, Lorentzian-shaped distribution of lifetimes (Fig. 1) (Rosato et al., 1990). The single distribution fit previously reported (Rosato et al., 1989) has been confirmed and extended to several derivatives of HSOD (holo HSOD in buffer, or in 4 M and 7.2 M Gd HCl, apo HSOD in buffer and in 4 M Gd HCl) each at 5 temperatures (10°, 20°, 30°, 40°, and 50°C). The is not a trivial result, because the fit requires only two floating parameters (the center and width of the Lorentzian distribution) as compared, for instance, with the more familiar two exponential fit, which requires three parameters. NATA in buffer and in 4 M Gd HCl can be fit by the same function, but the width of the distribution was very narrow (< 0.05 ns) at all temperatures investigated. We have systematically attempted to improve the quality of the fit using other models, like two and three exponentials, one Lorentzian and one exponential and two Lorentzians, without improving the χ^2 value (Rosato et al., 1990). In particular the fit for holo HSOD and apo HSOD in buffer showed a lower χ^2 value for a Lorentzian distribution than for two discrete lifetimes

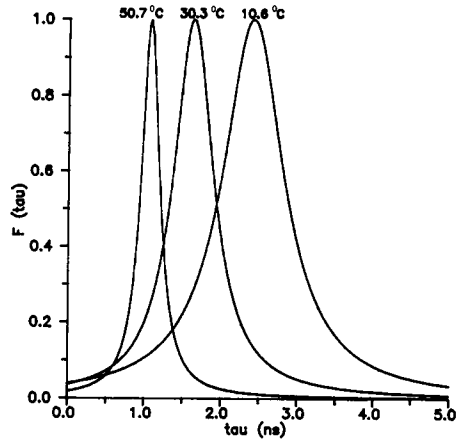

FIGURE 1 Continuous distributions of fluorescence lifetimes for holo HSOD at three different temperatures. The three lorentzian distributions (normalized at the maximum) correspond to the best fit of phase and modulation data vs. the modulation frequency at three different temperatures.

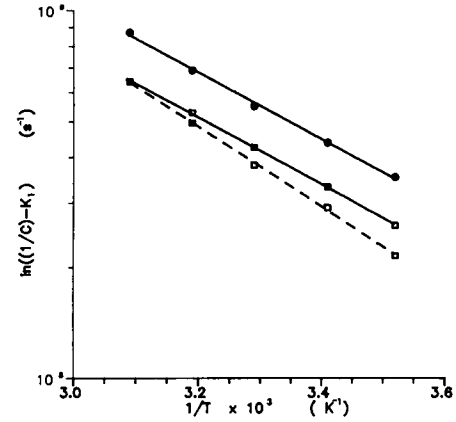

FIGURE 3 Dependence of distribution centers (according to Eq. 1) on temperature for apo HSOD (——□——), apo HSOD in 4 M Gd HCl (— —□— —), and holo HSOD (——·——) (reported again for comparison). The lines correspond to the best fit obtained with Eq. 1 (cf. Materials and Methods). The value of K_1 was assumed to be 1/16 ns^{-1}.

(Rosato et al., 1990). The primary aim of this study was to find a possible correlation of centers and widths of the Lorentzian distributions on protein structure and on temperature. The data in Fig. 1, the normalized lifetime distributions for holo HSOD at three different temperatures, provide evidence for this assumption. As the temperature increases, the centers shifted to lower lifetimes whereas the widths are significantly narrower. Figs. 2 and 3 show the temperature dependence of the centers of the lifetime distribution for native and Gd HCl-denatured

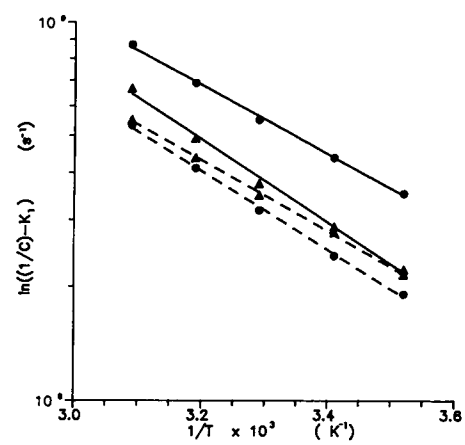

FIGURE 2 Dependence of distribution centers (according to Eq. 1) on temperature for holo HSOD (——·——), holo HSOD in 7.2 M Gd HCl (— —·— —), NATA (——▲——), and NATA in 7.2 M Gd HCl (— —▲— —). The lines correspond to the best fit obtained with Eq. 1 (cf. Materials and Methods). The value of K_1 was assumed to be 1/16 ns − 1.

holo and apo HSOD, following Eq. 1. These figures also show data for NATA in buffer and in buffer containing 7.2 M Gd HCl. All the experimental points, except those obtained at intermediate Gd HCl concentrations, are well fitted by Eq. 1 (*solid and dashed lines*). The relative errors are smaller than the height of the symbols used in the figures whereas the residues (i.e., the differences between the calculated and the experimental values) are always randomly distributed around zero.

A thermal denaturation process seems to take place in the case of holo HSOD in 4 M Gd HCl at higher temperature (data not shown). A partial thermal denaturation is probably occurring also in the apo HSOD, as suggested by the hysteresis observed upon return to lower temperature (data not shown).

Table 1 reports the thermal rate constants derived from the best fit according to Eq. 1. In all cases satisfactory χ^2 values were obtained. Fitting the data according to Eq. 2 was satisfactory for holo HSOD, but it did not give good fits for the apo protein; therefore, the empirical model used by Bushueva et al. (1978) is not applicable to our

TABLE 1 Thermal quenching parameters using the Arrhenius equation

Sample	K_0	E	χ^2
	$(1/s) \times 10^{-11}$	(*Kcal*)	
NATA	15.9	5.5	1.5
NATA + 7.2 M GdHCl	4.4	4.7	0.3
holo HSOD	5.6	4.5	0.9
holo HSOD + 7.2 M GdHCl	8.5	5.2	1.0
apo HSOD	4.4	4.6	1.1
apo HSOD + 4 M GdHCl	15.3	5.5	0.3

protein. We attempted to apply Eq. 1 to the two discrete lifetimes decay model. In this model only the longer lifetime seems to be affected by temperature whereas the short lifetime is temperature independent (Fig. 4). The amplitude and the lifetime of the two components varies in a complex fashion not interpretable using a two-state model. Using two discrete exponential components, the temperature behavior cannot be well fit using Eq. 1.

We have also studied the dependence of the width of Lorentzian distributions on temperature. The pertinent data are reported in Fig. 5. The experimental error of the width value is ~0.1 ns as estimated using the rigorous errors analysis of the Globals Unlimited® program. A linear dependence of widths on temperature was observed for the native holo protein, the distribution being narrower at higher temperatures. Significant exceptions are apo HSOD and holo HSOD in 4 M Gd HCl. In those cases, a more complex behavior is observed due to denaturation occurring at higher temperature values, as evidenced by nonreversibility after returning to lower temperature (data not shown). In all cases, the width of the distribution for the denatured protein is larger than for the corresponding native proteins.

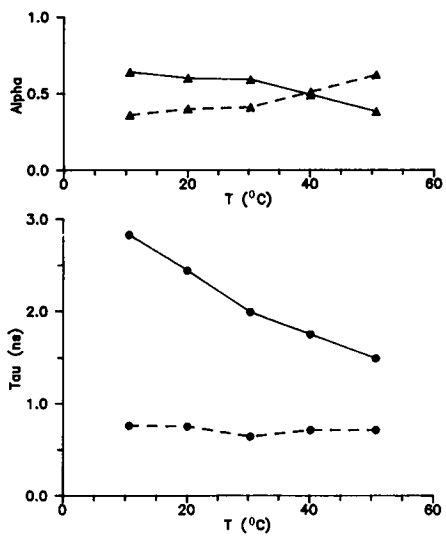

FIGURE 4 Dependence of the two lifetimes (*lower frame*) and their relative contribution (*upper frame*) on temperature. The two lifetimes and the relative contributions were calculated by fitting the intensity decay according to the equation

$$f(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2); \quad \alpha_1 + \alpha_2 = 1.$$

Longer lifetime (——·——) and its relative contribution (——▲——). Shorter lifetime (— —·— —) and its relative contribution (— —▲— —).

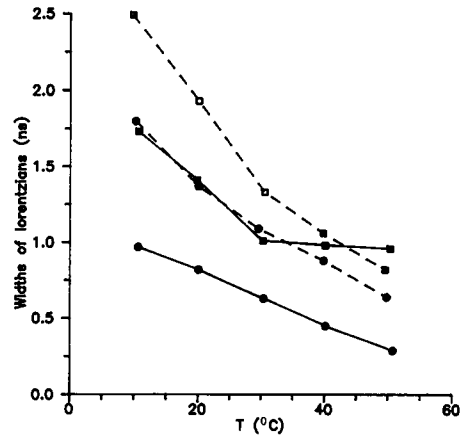

FIGURE 5 Dependence of distribution widths on temperature for holo HSOD in 7.2 of Gd HCl (— —·— —), holo HSOD (——·——); apo HSOD (——□——); and apo HSOD in 4 M GdHcl (— —□— —).

DISCUSSION

The study of proteins containing one tryptophan, or few equivalent tryptophans, often shows complex fluorescence decay that can be fitted using a multi-exponential decay (Beechem and Brand, 1985). Generally, this complex decay has been attributed to some sort of heterogeneity of the protein samples studied. However, no final evidence for a correlation between the number of decay components and conformations of the macro-molecule under study have been reported. We realized that complex decays of fluorescence in proteins frequently can be better described by a continuous distribution of lifetimes. In the distribution model, the centers roughly correspond to the average fluorescence lifetimes obtained with the "discrete lifetime model" whereas the distribution widths are novel parameters related to the quasi-continuous distributions of conformations and/or to the dynamic interconversion among them (Alcala et al., 1987 *b* and *c*).

In this paper we have studied how these parameters are affected by temperature and conformational state of the protein, in search of a simple correlation. Figs. 2 and 3 show that the centers of the Lorentzian distribution of lifetimes for several HSOD derivatives follow an Arrhenius behavior. According to this model it was possible to calculate various thermodynamic parameters related to the average value of the lifetime or center of the distribution (Table 1). Instead, the model with two discrete lifetimes should correspond to two protein conformations and the pre-exponential factors to their relative population. A temperature study should provide the relative change of population of the two conformations. Of course, we can also have a reduction of the average lifetime of the

two protein conformations, due to thermal quenching processes. The two-lifetime model, analyzed as a function of temperature, did not allow a straight-forward interpretation. In fact, whereas the longer lifetime was affected by temperature, more or less in an Arrhenius-like fashion, the shorter one was almost constant and its contribution increased gradually in a different way compared with that expected from a simple two-state equilibrium (Fig. 4).

The thermal deactivation rates and energies obtained from the Arrhenius analysis of the distribution center deserve comment (Table 1). The activation energy for thermal quenching, E, calculated for the native proteins is significantly lower than that for NATA. This result is compatible with a partial shielding of the solvent due to the protein. The frequency factor, K_0, is also lower in the native proteins. The differences between thermodynamic parameters related to the behavior of the average lifetime for the holo and apo protein are very small, both for the native and the denatured proteins.

Another interesting result concerns the width of distributions in the various samples and at different temperatures. (*a*) The width for all protein samples is remarkably larger than that of NATA. (*b*) The distribution of the holo protein is always narrower (~50%) than that of the apo protein. This is in line with a greater rigidity induced by the metals which then reduces the number of protein conformations (Roe et al., 1988). A biphasic behavior of the width vs. temperature was observed with apo HSOD and with the holo HSOD in 4 M Gd HCl. This behavior can be explained by a thermal denaturation occurring at ~40°C. Another possible explanation is the initial disruption of the dimer followed by denaturation of the monomers. (*c*) Denaturation broadens the distribution. This statement seems to contradict the concept of absence of structure in denatured proteins. Denatured proteins in which the tryptophan is supposedly fully exposed to the solvent were expected to have a width similar to that of NATA. Also, the emission spectrum and average lifetime are also similar. Instead, data from the lifetime distribution width for denatured HSOD suggest the presence of a greater number of conformations than in native protein. (*d*) An increase in temperature decreases the width of the distribution. This observation was already reported by Alcala et al. (1987*b*). A likely explanation is that rapid interconversion, in times shorter than nanoseconds, among energetically close structures occurs at higher temperature. This motional narrowing at high temperature makes the interconverting conformations indistinguishable. Because the interconversion "melts" more states as the temperature increases, the lifetime heterogeneity reflects structural heterogeneity which is larger at lower temperatures.

The description of fluorescence decay of HSOD by a continuous distribution of lifetimes gives two parameters, the center and the width, both affected by the protein structure and by temperature in a way readily interpretable by simple physico-chemical principles. This was not true for other fitting models, such as a two-conformational model.

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