Time-resolved fluorescence studies on site-directed mutants of human serum albumin

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Abstract Human serum albumin (HSA) contains a single tryptophan residue at position 214. The emission properties of tryptophan 214 from recombinant albumins, namely, normal HSA, FDH-HSA and a methionine 218 HSA were examined. In all cases, the excited state lifetimes were best described by a two component model consisting mainly of a Lorentzian distribution. The centers of these distributions were 5.60 ns for HSA, 4.23 ns for FDH-HSA, and 6.08 ns for Met-218 HSA. The global rotational correlation times of the three HSAs were near 41 ns while the amplitude and rate of the local motion varied. These changes in the lifetimes and mobilities suggest perturbation in the local protein environment near tryptophan 214 as a consequence of the amino acid substitutions.

Key words: HSA; FDH-HSA; Tryptophan; Site-directed mutagenesis; Time-resolved fluorescence

1. Introduction

Human serum albumin (HSA) contains 585 amino acid residues arranged into three distinct domains (I–III) [1,2]. Its single tryptophan residue is located at position 214 in subdomain IIA. More than 40 single-site point mutations in HSA have been identified [see 2 for a compendium]. Many of these genetic variants have little or no effect on the physiological function of the protein. However, the conversion of arginine to histidine at position 218 results in higher affinity of albumin for thyroxine (T4), and hence the condition familial dysalbuminemic hyperthyroxinemia (FDH) [3], which is characterized by elevated levels of total serum thyroxine.

The excited state properties of Trp-214, including both lifetime and rotational properties, have been studied for normal HSA [4–14]. The average lifetime of Trp-214 in FDH-HSA was reported to be less than in normal HSA [15], but was difficult to quantify since the data were obtained on proteins isolated from persons heterozygous for FDH-HSA, i.e. the sample contained both normal and FDH-HSA. The difficulty inherent in separating FDH-HSA from normal HSA, due to their very similar isophoretic mobilities, prevented an examination of pure FDH-HSA until recently when the protein was cloned and expressed [3]. Molecular genetic techniques also permitted the construction of other recombinant HSA variants with mutations at or near the 218 position. The effects of these mutations on the thyroxine binding properties of HSA have been reported [16]. Here we describe time-resolved fluorescence studies on recombinant FDH-HSA as well as recombinant normal HSA and an Arg-218 to Met-218 HSA. Specifically, multifrequency phase and modulation fluorometry was used to measure the excited state lifetime of the tryptophan residues and the dynamic polarization method (decay of anisotropy) was used to study the motional properties of these residues.

2. Materials and methods

2.1. Preparation of HSA

FDH-HSA and Met-218 HSA were prepared using site-directed mutagenesis and a yeast protein expression system as previously described [3,16]. Recombinant normal HSA was also prepared using this expression system. In all cases the absence of protein dimers, known to be present frequently in commercial or lyophilized preparations, was verified by native gel electrophoresis. All measurements were carried out at 20°C using 40 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, and 0.3 mM EDTA. The protein concentration in all experiments was approximately 0.1 mg/mL.

2.2. Fluorescence spectroscopy

The time-resolved fluorescence experiments were performed at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois, Urbana-Champaign using a multifrequency phase and modulation fluorometer. In the instrument utilized, the frequency modulation of the excitation source is realized using the harmonic content approach [17,18]. The exciting light was from a Coherent Nd:YAG mode-locked laser pumping a rhodamine dye laser. The dye laser was tuned to 600 nm, which was then frequency doubled to 300 nm. Emission was observed through a Schott WG-320 filter to isolate the emission of tryptophan and block scattered light. Phase and modulation values for both lifetime and dynamic polarization data were obtained as previously described [19–23]. The theoretical expressions describing the dynamic polarization data, which is the frequency domain equivalent of anisotropy decay, are more easily apprehended when written in the time domain. Specifically, the time-resolved anisotropy of a tryptophan residue contains contributions from the overall rotational diffusion of the protein (global motion) as well as from segmental motions of the tryptophan with respect to the rest of the protein (local motion). Assuming that the segmental motions occur independent of the overall protein rotation the anisotropy decay, $r(t)$, is the product of separate processes [12,23], and in the simplest, limiting case:

$$r(t) = r_0[f_1 \exp(-t/\phi_1) + f_2 \exp(-t/\phi_2)]$$

where r_0 is the time zero anisotropy (the limiting anisotropy; fixed in our analysis to 0.31 [12]), t is time after excitation, τ is the fluorescent lifetime, ϕ_1 and ϕ_2 are rotational correlation times (equal to the Debye rotational relaxation time divided by 3) associated with the 'global' and 'local' rotations, respectively, and f_1 and f_2 are the fractional changes in anisotropy associated with ϕ_1 and ϕ_2, respectively.

2.3. Data analysis

The data were analyzed using Globals Unlimited software, which allows for simultaneous fitting of multiple data sets, with or without parameters in common, i.e. with 'linked' or 'unlinked' parameters [24,25]. Models containing discrete components and/or continuous distributions [26–28] were compared. The goodness of fit of a particular model was judged by the value of the reduced chi-square (χ^2) [22,23]. Correlated error analyses (i.e. one parameter is varied near the minimum while the other parameters are all free) were performed on

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the lifetimes and rotational correlation times, and the rigorous 67% confidence limits are reported for each parameter.

3. Results

The results of the lifetime analysis, using Lorentzian distributions, for the three protein samples are shown in Table 1. Fig. 1 shows the Lorentzian distribution functions corresponding to these analyses for FDH-HSA (A), Met-218 HSA (B) and normal HSA (C). As indicated in the table, the major component of the FDH-HSA was the shortest having a center lifetime value of 4.23 ns while the normal HSA and Met-218 HSA demonstrated center lifetime values of 5.60 ns and 6.08 ns, respectively. The normal HSA had the largest width associated with its distribution, namely, 1.57 ns while the widths associated with the distributions for FDH-HSA and Met-218 HSA were both less and comparable, namely 0.94 ns and 0.82 ns, respectively. The dynamic polarization results are shown in Fig. 2 and the best fits corresponding to these data (the solid lines) are given in Table 2. In all cases the data fit best to two rotational components, namely a slower component attributed to 'global' protein rotation and a faster component attributed to 'local' mobility of the tryptophanyl residue. In all cases the majority of the depolarizing motion was attributed to the 'global' motion (fractional contributions ranging from 0.75 to 0.84). In the case of the unlinked analysis the 'global' rotational correlation times ranged from 37.4 ns (Met-218 HSA) to 45.0 ns (normal HSA). The rate associated with the 'local' motion in both the FDH-HSA and Met-218 HSA cases were comparable (0.30 ns and 0.31 ns) and faster than that found for normal HSA (0.58). In the case of the 'linked' analysis, wherein all 'global' rates are obliged to be equal, the 'global' rotational correlation time recovered was 40.6 ns and the 'local' rotational correlation times recovered were comparable to those in the 'unlinked' analysis.

4. Discussion

Previous investigators have found that Trp-214 in normal HSA was characterized by a heterogeneous lifetime. In most cases this heterogeneity was analyzed in terms of discrete exponential decays. DeLauder and Wahl [5], for example, found two components (in charcoal treated HSA at pH 5.5) of 3.3 ns (66%) and 7.8 ns (34%) and Wahl and Auchet [6] later analyzed the decay as a function of the emission wavelength and, upon 295 nm excitation, found three components, namely, 1.5 ns, 6.17 ns and 12.08 ns, with fractional contributions dependent upon the emission wavelength but with 6.17 ns being by far the major component. Hazan et al. [7] resolved the decay of HSA, upon 296 nm excitation and at pH 7.4, into two exponentials having lifetimes of 6.1 ns (45%) and 1.5 ns (55%) while Kasai et al. [8] found two components of 6.89 ns (46.5%) and 3.22 ns (53.5%) using 295 nm excitation at pH 7.4. Using multifrequency phase and modulation fluorometry, Lakowicz and Gryczynski [13] found for HSA, upon excitation at 298 nm at 20°C and pH 8, two decay components of 6.06 ns (95.7%) and 1.42 ns (4.3%). More recently, Davies et

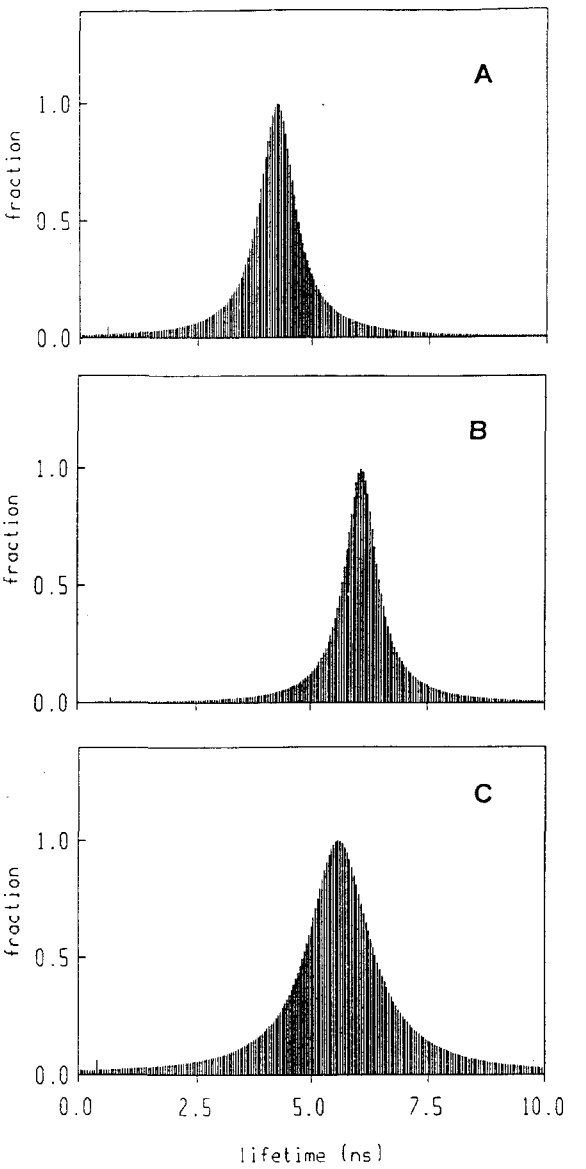

Fig. 1. Lorentzian distribution curves corresponding to the fit to the multifrequency phase and modulation lifetime data for (A) FDH-HSA, (B) Met-218 HSA, and (C) normal HSA. The recovered parameters are given in Table 1.

Table 1
Lifetime analysis for albumin samples

Sample	τ_1 (ns) [fraction]	Width (ns)	τ_2 (ns) [fraction]	χ^2
FDH-HSA	4.23 (+0.08, −0.09) [0.97]	0.94 (+0.22, −0.20)	0.59 (+0.14, −0.21) [0.03]	1.2
Met-218 HSA	6.08 (+0.04, −0.06) [0.98]	0.82 (+0.18, −0.17)	0.67 (+0.13, −0.15) [0.02]	0.57
Normal HSA	5.60 (+0.10, −0.09) [0.96]	1.57 (+0.33, −0.39)	0.40 (+0.09, −0.09) [0.04]	2.7

Errors given in parentheses represent the correlated 67% confidence limits of the reduced χ^2.

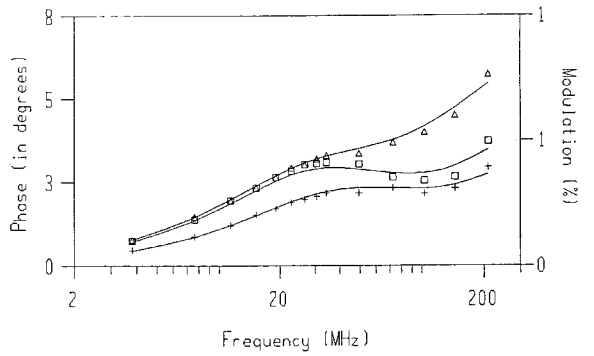

Fig. 2. Dynamic polarization data and fits for FDH-HSA (+), Met-218 HSA (□) and normal HSA (△). The solid lines represent the fits to two rotational correlation times with values and relative amplitudes given in Table 2A.

al. [14] found two components of 3.72±0.45 (27.8%) and 7.70±0.09 (72.2%) at pH 7.1 and 295 nm excitation while Vos et al. [11], using 300 nm excitation, analyzed their data in terms of three exponentials of 0.11 ns (41%), 3.7 ns (29%) and 7.3 ns (30%), giving an average lifetime of 6.0 ns. Using a Gaussian distributional fit for the lifetime of HSA, at pH 7.0, Marzola and Gratton [12] recovered a center value of 5.99 ns with a width of 3.8 ns, using pH 7.0 and 300 nm excitation. Considering that all of these lifetime measurements were carried out under slightly different conditions (pH, ionic composition, excitation and emission wavelength, temperature) the general agreement between the various laboratories, and our own results, for normal HSA is fairly good – namely a heterogeneous lifetime with an average value near 6 ns. In our case we found that the data could not be well fit to two discrete exponentials and that three discrete exponentials were required to give fits with χ^2 values comparable to distribution models. Moreover, we found that the Lorentzian distribution gave a slightly improved fit over the Gaussian function. It is interesting to note that the width of the recovered distribution was significantly less for both mutant HSAs compared to the normal HSA (Fig. 1). Gratton and coworkers [26–30] have demonstrated that the widths of the lifetime distributions recovered in the cases of single tryptophan proteins can be related to the extent of motion of the fluorophore, namely, decreasing as the rate of motion increases. Our observations show an increased rate of local mobility of Trp-214 in FDH-HSA and Met-218 HSA over that of Trp-214 in normal HSA and, hence, correlate well with the decreased widths of the recovered lifetime distributions for FDH-HSA and Met-218 HSA.

Our dynamic polarization results, summarized in Table 2, demonstrated good fits for all HSA samples using a two component model. One notes that the extent of 'local' mobility of Trp-214 varies markedly depending on the amino acid residue at position 218. In particular, replacement of the normal Arg-218 by either histidine (FDH-HSA) or methionine (Met-218 HSA) results in a significant enhancement in the rate of 'local' motion of the tryptophan residue. This result suggests that replacement of the Arg-218 with either histidine or methionine removes some steric barrier to movement from the vicinity of the tryptophan residue.

In the case of 'unlinked' data one notes that the 'global' rotational correlation times recovered varied from 37.4 ns to 45.0 ns. When these 'global' rates are 'linked' across the three data sets the rotational correlation time recovered is 40.6 ns with comparable χ^2 values. The question of which analysis, 'linked' or 'unlinked', is more appropriate is debatable. One may consider that the 'global' rotational correlation time should be the same in all cases since the overall rotation of the protein should not change significantly simply upon substitution of a single amino acid residue. However, in the case of asymmetric proteins it is well known that the alignment of the fluorophore's excitation and emission dipoles with respect to the rotational axes of the protein will affect the observed rotational correlation times [31,32] and, hence, one could envisage that the difference in the recovered 'global' rates reflect slightly altered alignments of the tryptophan residues in the three HSA variants, *presuming that the HSA molecule is non-spherical.* It may also be, however, that the overall solution conformation of HSA is particularly sensitive to small perturbations (vide infra) and hence the differences in the recovered 'global' rates may reflect significant differences in the HSA conformation. These 'global' values may be compared to the rotational correlation time expected for a spherical protein of

Table 2
Dynamic polarization analysis of albumin samples

(A) The global reduced χ^2 was 1.3, with Φ_1 not linked across data sets			
Sample	Φ_1 (ns) [fraction]	Φ_2 (ns) [fraction]	χ^2
FDH-HSA	41.7 (+5.8, −4.6) [0.84]	0.30 (+0.06, −0.06) [0.16]	0.92
Met-218 HSA	37.4 (+4.0, −3.4) [0.82]	0.31 (+0.06, −0.06) [0.18]	1.4
Normal HSA	45.0 (+9.9, −6.7) [0.75]	0.58 (+0.11, −0.09) [0.25]	2.9
(B) The global reduced χ^2 was 1.6, with Φ_1 linked across data sets			
Sample	Φ_1 (ns) [fraction]	Φ_2 (ns) [fraction]	χ^2
FDH-HSA	40.6 (+4.3, −3.5) [0.84]	0.29 (+0.10, −0.08) [0.16]	0.94
Met-218 HSA	40.6 (+4.3, −3.5) [0.81]	0.32 (+0.08, −0.07) [0.19]	1.6
Normal HSA	40.6 (+4.3, −3.5) [0.76]	0.55 (+0.10, −0.08) [0.24]	2.2

The limiting anisotropy was fixed at 0.31 for all analyses. Errors given in parentheses represent the correlated 67% confidence limits of the reduced χ^2.

HSA's molecular mass at 20°C, namely 26.5 ns (assuming a partial specific volume of 0.735 cm^3/g and 0.2 cm^3/g hydration [11]). In all cases then, 'linked' or 'unlinked', our data indicates that HSA, under our solution conditions, is non-spherical and consistent with the asymmetric (generally prolate ellipsoid) models proposed by a number of groups utilizing different experimental approaches [2, 33,35 and references therein].

As pointed out by Carter and Ho [2], however, other groups have reported data from electron microscopy and energy transfer which seemed to support the more globular model consistent with their X-ray structure. Some recent time-resolved fluorescence results using Trp-214 also address this question of HSA's overall shape. Marzola and Gratton [12], for example, fit their dynamic polarization data, obtained at 25°C, to two components, namely, rotational correlation times of 34 ± 3 ns and $0.14 \pm .05$ ns with fractional contributions of 0.76 and 0.24, respectively. Munro et al. [9] reported time-decay anisotropy results at 8°C and 41°C and concluded that at the higher temperature the data fit best to two rotational correlation times, namely, 14 ns and 0.14 ns while at the lower temperature only one correlation time was evident, namely 31.4 ns. They concluded that the local mobility of Trp-214 was not significant at lower temperatures but, upon raising the temperature, the internal flexibility of the protein greatly increased. Vos et al. [11] found, at 20°C, a single rotational correlation time of 26 ± 0.6 ns for HSA. Lakowicz and Gryczynski [13], on the other hand, found that their dynamic polarization data, taken at 20°C, fit well to two rotational correlation times, namely, 38.6 ns and 0.17 ns. Clearly there is a noticeable lack of agreement about the overall solution hydrodynamics for HSA and one must consider the possibility that the protein can facilely adopt a variety of conformations ranging from a more compact form (consistent with the latest X-ray based model [2]) to a more extended form (consistent with our results and others [33,35], including an earlier X-ray based model [34]) and that the details of the solvent conditions (pH, ionic strength, temperature et cetera) will determine the relative occupancy of these conformational forms.

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