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5-Fluorotryptophan as dual probe for ground-state heterogeneity and excited-state dynamics in apoflavodoxin

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ABSTRACT

The apoflavodoxin protein from *Azotobacter vinelandii* harboring three tryptophan (Trp) residues, was biosynthetically labeled with 5-fluorotryptophan (5-FTrp). 5-FTrp has the advantage that chemical differences in its microenvironment can be sensitively visualized via ^{19}F NMR. Moreover, it shows simpler fluorescence decay kinetics. The occurrence of FRET was earlier observed via the fluorescence anisotropy decay of WT apoflavodoxin and the anisotropy decay parameters are in excellent agreement with distances between and relative orientations of all Trp residues. The anisotropy decay in 5-FTrp apoflavodoxin demonstrates that the distances and orientations are identical for this protein. This work demonstrates the added value of replacing Trp by 5-FTrp to study structural features of proteins via ^{19}F NMR and fluorescence spectroscopy.

1. Introduction

The recent discovery of more homogenous fluorescence decay of single 5-fluorotryptophan-containing proteins as compared to single tryptophan-containing ones has considerably simplified interpretation of excited-state kinetics of tryptophan [1]. Experiment and theory show that photo-induced electron transfer to a nearby amide group in the peptide bond is responsible for lifetime heterogeneity. Photo-induced electron transfer is largely suppressed by fluoro-substitution at the 5th position of the indole of tryptophan [2]. This decrease in electron transfer rate is due to a higher ionization potential of the fluoro-indole residue compared to the one of the indole of tryptophan. Consequently, compared to tryptophan, the fluorescence lifetime of 5-fluorotryptophan is longer and the corresponding fluorescence decay kinetics is much more homogenous.

Since the fluorine nucleus has a magnetic moment, ^{19}F NMR spectroscopy can be used to obtain information about the chemical environment of 5-fluorotryptophan (5-FTrp) in proteins. By using both NMR and time-resolved fluorescence techniques one should in principle be able to obtain detailed information on ground- and excited-state properties of tryptophan residues in multi-tryptophan proteins.

In this study, we follow the latter approach to characterize apoflavodoxin (i.e., flavodoxin without cofactor FMN) from *Azotobacter vinelandii*, which is used as a prototype to investigate denaturant-induced protein (un)folding [3–7]. The three-dimensional structure of the 179-residue protein is characterized by a five-stranded parallel β-sheet surrounded by α-helices. This α–β parallel topology is one of the most common protein folds [8]. Fluorescence of apoflavodoxin arises mainly from its three tryptophans (Trp74, Trp128 and Trp167). Trp74 is located in α-helix 3, Trp128 is close to β-strand 5a and Trp167 is in helix 5 of the protein.

Using time-resolved fluorescence anisotropy of wild-type apoflavodoxin and of mutant proteins lacking one or two tryptophans, it has been recently demonstrated that photo-excited tryptophan residues of apoflavodoxin exchange energy through a Förster-type of dipolar coupling mechanism [9]. Of the tryptophans, Trp128 is located in the most polar environment and consequently its fluorescence spectrum is most red-shifted, which causes unidirectional radiationless energy migration from Trp74 and Trp167 to Trp128. Energy transfer from Trp167 to Trp128, residues that are 6.8 Å apart, is revealed by experiment because it leads to a rapid decay of the anisotropy signal with a 50-ps transfer correlation time. Although all three tryptophans of apoflavodoxin are excited, excitation energy

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rapidly migrates to Trp128, which acts as energy sink and is as a result the main emitting fluorophore of apoflavodoxin.

In this paper we compare the spectroscopic properties of native apoflavodoxin with those of apoflavodoxin, in which fluorinated tryptophan residues are incorporated.

2. Materials and methods

2.1. Replacement of tryptophan residues by fluorinated analogs in apoflavodoxin

Replacement of the tryptophans of apoflavodoxin by 5-FTrp analogs is done using an *Escherichia coli* tryptophan auxotroph system, as previously described [10]. The cells produce flavodoxin in which 5-FTrp analogs instead of tryptophan are incorporated. Flavodoxin is isolated and purified and subsequently apoflavodoxin is obtained through removal of the non-covalently bound FMN. The gene for *Azotobacter vinelandii* flavodoxin II was excised from the Lac-promoter containing pUC18 vector, using restriction enzymes KpnI and SphI, and ligated into the KpnI/SphI digested pMa plasmid [11]. This vector contains the heat-inducible lambda Pr promoter, allowing strict expression control of flavodoxin. Mass spectrometry showed that the 5-FTrp incorporation efficiency was at least 99%.

2.1.1. ^{19}F NMR measurements

The ^{19}F NMR spectrum has been recorded at 376.5 MHz on a Bruker DPX 400 MHz NMR machine, which is equipped with a 10 mm ^{19}F probe. For chemical shift referencing, trifluoroacetic acid and 4-fluoro-phenylalanine are added to the flavodoxin sample. The NMR sample contains 100 mM potassium pyrophosphate and 10% D_2O, pH 6.0.

2.2. Fluorescence measurements

Steady-state fluorescence spectra were obtained with a Horiba Jobin Yvon Fluorolog 3.2.2 spectrofluorometer. All spectra were corrected for wavelength-dependent instrumental response characteristics. Time-resolved polarized fluorescence measurements and associated analysis of total fluorescence intensity decay and fluorescence anisotropy decay are described in detail in [9]. 5-FTrp shows a low intrinsic anisotropy ($r_0 = 0.13$) when excited at 300 nm [2] and therefore an excitation wavelength of 308 nm ($r_0 = 0.26$) was chosen.

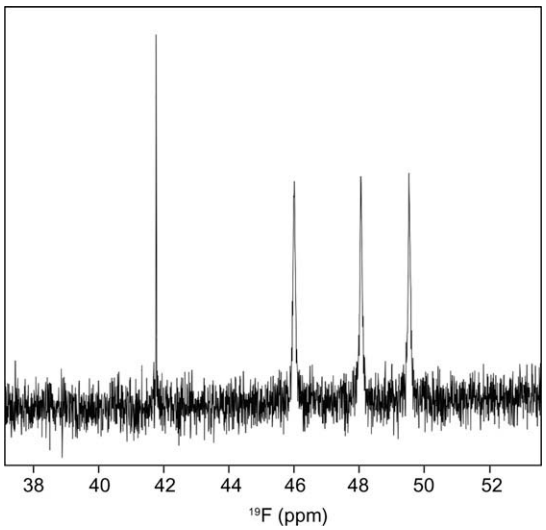

Fig. 1. ^{19}F NMR spectrum of 5-FTrp flavodoxin in 100 mM potassium pyrophosphate, pH 6.0, at 25 °C. The resonance at about 42 ppm is due to the chemical shift reference 4-fluoro-phenylalanine.

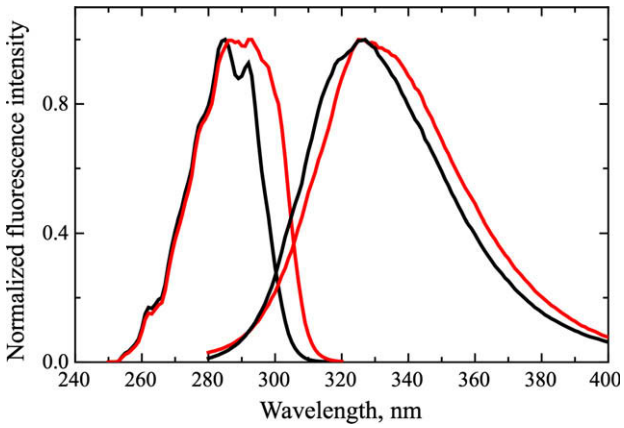

Fig. 2. Normalized steady-state fluorescence excitation and emission spectra of native WT apoflavodoxin (black) and native 5-FTrp apoflavodoxin (red). Apoflavodoxin concentration is 4 μM in 100 mM potassium pyrophosphate, pH 6.0, 25 °C. The excitation wavelength is 280 nm for emission spectra. The emission wavelength is 330 nm for excitation spectra. The excitation and emission slit widths are 2 nm.

3. Results and discussion

To verify whether uniform ^{19}F-labelling of the tryptophans of flavodoxin is achieved, a ^{19}F NMR spectrum of flavodoxin has been acquired (Fig. 1). The ^{19}F NMR spectrum clearly shows that indeed all three tryptophans of flavodoxin have been replaced by 5-FTrp in 5-FTrp flavodoxin, as three distinct ^{19}F NMR resonances of equal amplitude are observed. These three distinct NMR resonances also show that the chemical environments of the three 5-FTrp residues differ in flavodoxin.

The fluorescence excitation and emission spectra of 5-FTrp apoflavodoxin are red-shifted as compared to the ones of native apoflavodoxin (Fig. 2). A similar observation is made for the absorption and fluorescence spectra of N-acetyl-L-tryptophanamide and N-acetyl-DL-5-fluorotryptophanamide in different solvents, particularly dioxane [2]. The more red-shifted excitation (absorption) spectrum of 5-FTrp compared to the one of tryptophan allows excitation of 5-FTrp at wavelengths between 305 and 310 nm, where tryptophan hardly absorbs light.

Time-resolved fluorescence properties of 5-FTrp apoflavodoxin are determined in this study using excitation at 308 nm and detection at 363 nm. Total fluorescence decay of native 5-FTrp apoflavodoxin turns out to be mono-exponential, with a fluorescence lifetime of 4.14 ns (Fig. 3A). In contrast, in case of non-fluorinated native apoflavodoxin the measured fluorescence decay is slightly heterogeneous and consists of a short lifetime component of 1.34 ns (7% amplitude) and a predominant long lifetime component of 4.18 ns (93%), leading to an average fluorescence lifetime of 4.0 ns (see Supporting information). Time-resolved fluorescence anisotropy of native 5-FTrp apoflavodoxin shows rapid decay on the picosecond time scale followed by a much slower decay process on the nanosecond time scale (as well as depolarization due to the rotational correlation time of the protein of 10.4 ns), similar to as detected for native apoflavodoxin [9] (Fig. 3B). The 60-ps correlation time observed is due to resonance energy transfer from 5-FTrp167 to 5-FTrp128 and matches, within the 30-ps error, the 50-ps correlation time detected for native apoflavodoxin [9]. The 3.3-ns transfer correlation time observed is due to resonance energy

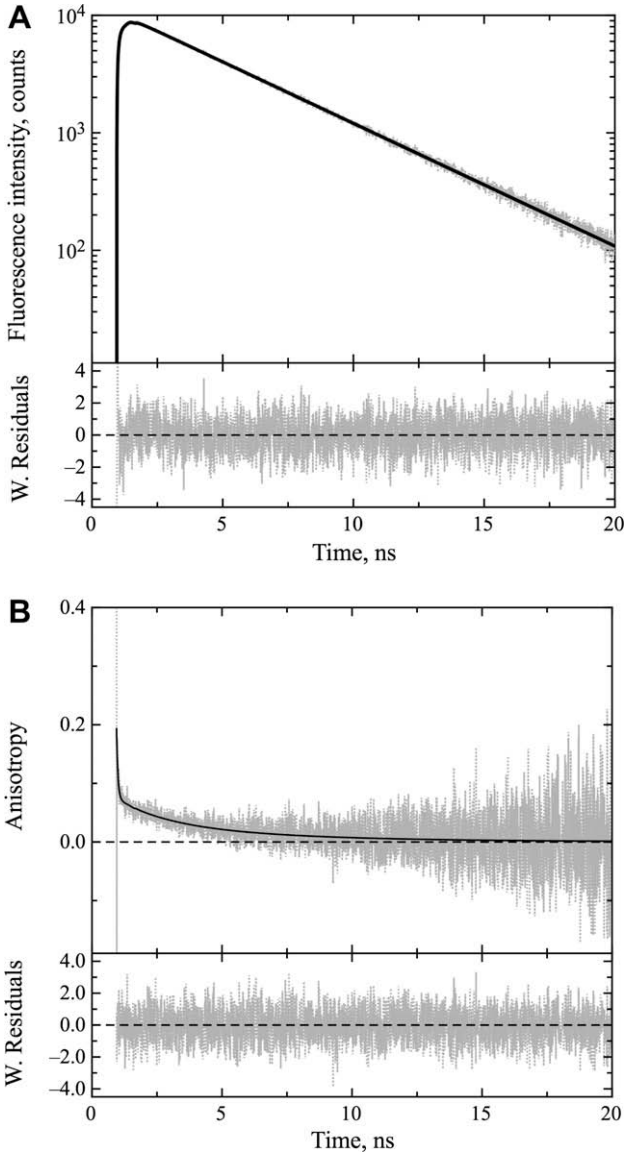

Fig. 3. Polarized time-resolved fluorescence of 4 μM native 5-FTrp apoflavodoxin in 100 mM potassium pyrophosphate, pH 6.0, 25 °C. Excitation wavelength is 308 nm and emission is detected at 363 nm. (A) Total fluorescence decay (grey) and associated fit (black). The optimized, single lifetime $\tau = 4.14 \pm 0.01$ ns. The goodness-of-fit parameter $\chi^2 = 1.00$. (B) Fluorescence anisotropy decay (grey) and associated fit (black). The fluorescence anisotropy decay is fitted to: $\{\beta_{T1}\exp(-t/\phi_{T1}) + \beta_{T2}\exp(-t/\phi_{T2}) + \beta_r\}\exp(-t/\phi_r)$, in which the rotational correlation time of the protein ϕ_r is fixed to 10.4 ns (for details concerning the parameters such as amplitudes β_T and transfer correlation times ϕ_T see [9]). The optimized fitted parameters are: $\phi_{T1} = 0.06 \pm 0.03$ ns ($\beta_{T1} = 0.085 \pm 0.035$), $\phi_{T2} = 3.3 \pm 0.1$ ns ($\beta_{T2} = 0.052 \pm 0.006$) and $\beta_r = 0.010 \pm 0.005$, goodness-of-fit parameter $\chi^2 = 1.00$. To illustrate the quality of the fits the weighted residuals are shown in panels A and B.

transfer from 5-FTrp74 to 5-FTrp167 and is shorter than the corresponding value observed for native apoflavodoxin (i.e., 6.9 ns) [9]. Apparently, in native apoflavodoxin excitation energy is more rapidly transferred from 5-FTrp74 to 5-FTrp167 than it is from Trp74 to Trp167 (as the transfer rate equals the reciprocal of the transfer correlation time). This more rapid transfer of energy can be ascribed to the 2.05 times larger overlap integral between absorption and emission spectra of native 5-FTrp apoflavodoxin, which amounts to 4.91×10^{12} nm^4 M^{-1} cm^{-1}, compared to the corresponding value obtained for native apoflavodoxin, which equals 2.40×10^{12} nm^4 M^{-1} cm^{-1}. Indeed, since the rate of energy transfer is directly proportional to the integral of the spectral overlap, we experimentally observe that energy transfer from 5-FTrp74 to 5-FTrp167 is 2.09 times faster than energy transfer from Trp74 to Trp167 (i.e., 0.303 ns^{-1} versus 0.145 ns^{-1}).

The data presented here show that replacement of tryptophan residues in a protein by their 5-fluoroTrp analogs preserves the three-dimensional structure of the protein involved. This conclusion is drawn because native apoflavodoxin contains three tryptophans that have specific relative positions and orientations in the hydrophobic core of the molecule. These positions and orientations must remain unaltered upon incorporation of 5-fluoroTrp analogs to explain the time-resolved fluorescence data obtained. Far-UV CD spectra of flavodoxin and 5-FTrp flavodoxin are comparable (see Supporting information) and thus secondary structure content of both proteins is similar. The 5-FTrp containing protein is isolated and purified in its holo form, which is yellow due to non-covalent binding of FMN. Tight binding of FMN to 5-FTrp apoflavodoxin reveals additional proof for preservation of the three-dimensional structure of apoflavodoxin upon replacement of tryptophan residues by their 5-FTrp analogs. Firm binding of FMN occurs primarily through a very specific combination and geometry of hydrogen bonds and aromatic interactions of FMN with apoflavodoxin, implying that the three-dimensional structures of apoflavodoxin and 5-FTrp apoflavodoxin must be similar. The incorporation of 5-FTrp analogs has the advantage that now both NMR and fluorescence spectroscopy can be used to extract information about the submolecular properties of a protein molecule. In case of apoflavodoxin, although the chemical environment of the three tryptophan residues in apoflavodoxin is shown to be different, the corresponding fluorescence decay of the protein turns out to be mono-exponential. This observation supports that indeed excitation energy is transferred in a radiationless manner to the excited state of 5-FTrp128, which subsequently emits detectable fluorescence.

In case of single-tryptophan containing protein molecules, the combination of NMR and fluorescence spectroscopy enables one to directly correlate the signature of the fluorescence lifetime to the chemical environment of the particular tryptophan residue involved. The use of this dual, non-perturbing probe also holds great promise for studies of protein stability and protein folding as recently reviewed for ^{19}F NMR applications [12].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.022.

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