Dielectric relaxation in a single tryptophan protein

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Abstract Although dielectric relaxation can significantly affect the intrinsic fluorescence properties of a protein, usually it is fast compared to fluorescence timescales and needs to be slowed down by adding viscogens or lowering temperature before its impact on fluorescence can be studied. We report here a remarkable blue shift in fluorescence upon bimolecular quenching in the single-tryptophan thermostable protein Bj2S, the 2S seed albumin from *Brassica juncea*, at ambient temperature and viscosity. The magnitude of the blue shift (~5 nm at 50% quenching by acrylamide) is striking in a single-tryptophan protein and is attributed to a slowly relaxing dielectric environment in Bj2S from red edge excitation, steady-state polarization and time-resolved fluorescence experiments. Our results have important implications on interpretation of fluorescence of proteins with highly constrained backbones and in designing model systems for studying slow protein solvation dynamics using Trp fluorescence as the reporter probe. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Tryptophan fluorescence; Dielectric relaxation; Red edge excitation shift; Seed protein; *Brassica juncea*

1. Introduction

Tryptophan (Trp) fluorescence is one of the most widely used tools to probe the structure, dynamics and folding/unfolding properties of proteins. This is primarily due to the extreme sensitivity of Trp fluorescence to its electronic surroundings [1]. Despite its simplicity, a detailed analysis of fluorescence spectra of proteins is often complicated by the presence of multiple Trp residues, each present in a slightly different electronic environment. Extensive fluorescence study of single-Trp proteins is therefore essential in understanding and establishing unambiguous correlation between fluorescence properties and equilibrium structure/dynamics of a protein. Keeping this in mind we investigated the fluorescence of Bj2S, a 2S seed albumin from *Brassica juncea*, a single-Trp protein consisting of two chains (29 and 86 residues long) that are constrained by two inter- and two intra-disulfide bonds [2]. We observed a remarkable fluorescence blue shift (~5 nm at 50% quenching) upon bimolecular quenching, observed rarely [3,4] for a single-Trp protein. This blue shift is attributed to an effect influencing the slowly relaxing protein–water dielectric environment surrounding the Trp residue. The characteristic timescale of protein relaxation is distributed over a wide range (ps–ns) and is usually fast compared to Trp fluorescence lifetimes (ns). As a result, often protein relaxation needs to be slowed down with added viscogens or by lowering temperature before the relaxation and the intrinsic fluorescence timescales become comparable [5]. However, for Bj2S, protein relaxation was found to be slow (comparable to fluorescence lifetime) under ambient conditions. The disulfide-constrained backbone of Bj2S, giving rise to its extreme thermostability, is the most likely candidate for the unusually sluggish dynamics. Apart from being a rare example of slow protein relaxation under ambient conditions, our results have implications on fluorescence-detected structural changes [6] of backbone-constrained proteins. It also provides inputs for designing model systems where solvation dynamics [7], appropriate for complex systems like proteins [8], can be studied [5,9].

2. Materials and methods

2.1. Materials and experiments

Bj2S was obtained and purified as described elsewhere [10]. Absorption spectra were recorded in a Shimadzu 2040 spectrophotometer and the spectra were corrected for light scattering by extrapolation of a linear fit of the logarithm of optical density and wavelength for $\lambda > 320$ nm. Circular dichroism (CD) spectra were recorded on a Jasco J-600 spectropolarimeter using a cuvette with a path length of 1 mm. Steady-state fluorescence experiments were performed on a Hitachi F-4500 spectrofluorimeter, in a water-circulated thermostated cell adjusted to required temperatures. Spectra were collected at various excitation wavelengths with a band pass of 5 nm or 2.5 nm. Steady-state fluorescence polarization experiments were performed in the standard L-format method. Protein concentrations used were 7–10 μM for all spectroscopic measurements. Fluorescence lifetime experiments were performed on a FLA-900 Edinburgh Analytical Instruments spectrophotometer operated in the time-correlated single photon counting mode with a thyraton-gated nanosecond nitrogen flash lamp (repetition rate 25 kHz; FWHM ~0.57 ns) and a 4 nm slit width.

2.2. Analysis

Lifetimes (τ_i) were estimated from multi-exponential fits to the fluorescence decay curves after suitable deconvolution of the lamp profile as

$$I(t,\lambda_{em}) = \sum_i \alpha_i(\lambda_{em}) \exp\left(\frac{-t}{\tau_i(\lambda_{em})}\right) \quad (1)$$

with

$$\sum_i \alpha_i(\lambda_{em}) = 1.$$

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The time-resolved emission spectra were reconstructed as

$$F(t,\lambda_{em}) = \left\{\frac{F_{eq}(\lambda_{em})}{\sum_i \alpha_i(\lambda_{em})\tau_i(\lambda_{em})}\right\}\left\{\sum_i \alpha_i(\lambda_{em})\exp\left(\frac{-t}{\tau_i(\lambda_{em})}\right)\right\} \quad (2)$$

where $F_{eq}(\lambda_{em})$ is the normalized steady-state fluorescence spectrum. The fluorescence spectra as a function of added quencher [Q] were generated after suitable correction for inner filter effect and analyzed for a two-state ground state conformational heterogeneity (GSCH) model according to

$$\frac{F(\lambda_{em})}{F_0(\lambda_{em})} = \frac{f_1(\lambda_{em})}{1+K_{SV1}[Q]} + \frac{f_2(\lambda_{em})}{1+K_{SV2}[Q]} \quad (3)$$

where K_{SV1} and K_{SV2} are the Stern–Volmer constants while $f_1(\lambda_{em})$ and $f_2(\lambda_{em})$ are the fraction of total fluorescence at λ_{em} from components 1 and 2 respectively.

Homology modeling of Bj2S was performed using Insight II (version 2.0, Biosym Technologies) on a Silicon Graphics Indigo workstation using the standard Homology module and the mutation criteria as the scoring matrix. The sequence of the template used for modeling (pdb code: 1pnb) had high overall similarity (~50% in case of chain A and ~55% in case of chain B) with the sequence of Bj2S.

3. Results and discussion

The Trp residue in Bj2S is significantly solvent-shielded as indicated by the fluorescence emission maximum (λ_{max} = 336 nm at λ_{ex} = 295 nm). Although λ_{max} is a fairly good indicator of Trp solvent accessibility, it is actually correlated to the local effective dielectric constant [11] rather than the degree of Trp burial. On the other hand, acrylamide-induced bimolecular quenching constant (k_q) is correlated directly to Trp solvent accessibility [12]. Acrylamide quenching of Bj2S fluorescence (λ_{ex} = 295 nm) yielded λ_{em}-dependent Stern–Volmer plots. The emission spectra showed a remarkable blue shift with progressive quenching (Fig. 1; ~5 nm at 50% quenching) bringing about the observed λ_{em} dependence. The blue shift was maintained with KI quenching as well showing that

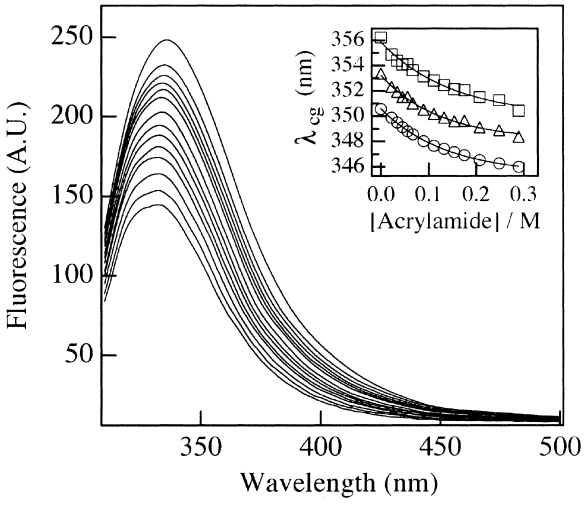

Fig. 1. Acrylamide-quenched fluorescence spectra of Bj2S (295 nm excitation) showing a progressive blue shift in λ_{max} with increasing concentration of acrylamide (acrylamide concentration for the most quenched spectrum is 0.26 M). Center of gravity of emission, λ_{cg} (integrated λ_{em}-weighted normalized fluorescence intensity), as a function of acrylamide concentration for 295 (○), 298 (△) and 300 (□) nm excitation are shown in the inset.

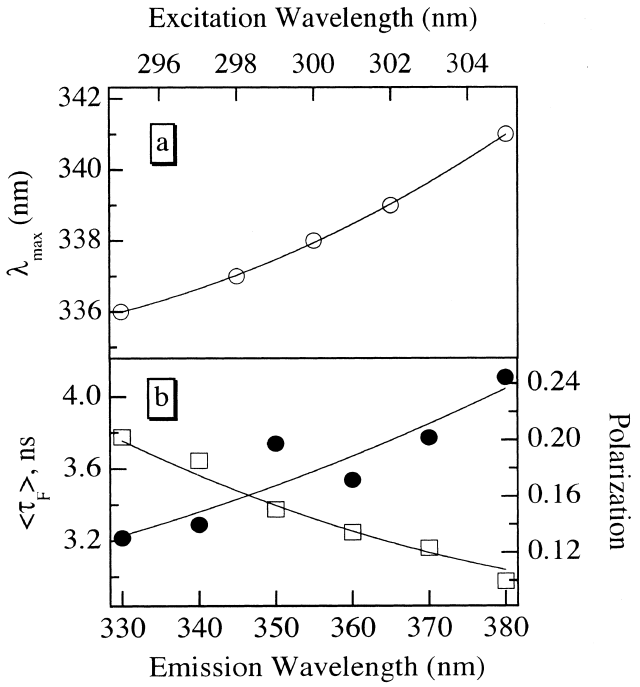

Fig. 2. a: Red shift of λ_{max} (○) as a function of λ_{ex}. (b) Fluorescence polarization (□) and mean fluorescence lifetimes (●) as a function of λ_{em} at pH 7.4.

the shift is not due to any specific interaction of the protein and acrylamide, rather it is an intrinsic property of the protein.

Multi-Trp proteins often exhibit a blue shift in fluorescence upon bimolecular quenching, arising from Trp residues with different emission maxima (λ_{max}) and quenching rates [13]. Although such a blue shift can also potentially occur in single-Trp proteins, arising from GSCH, the magnitude is much less and the phenomenon rare [12]. In the GSCH model the solvent-exposed conformer ($\lambda_{max} \approx 350$ nm) is preferentially quenched over the buried conformer. Individual emission spectra from the conformers can be estimated by analyzing Stern–Volmer plots over the entire emission wavelengths [14]. A global analysis of quenching data according to Eq. 3 yielded emission wavelength-dependent (increasing with increasing λ_{em}) Stern–Volmer quenching constants (K_{SV}) for the two conformers, not expected from a simple GSCH model. The estimated λ_{max} values for the two conformers, obtained from a global analysis, were found to be too widely separated (λ_{max} = {~329 and ~360 nm}; K_{SV} = {~10 and ~1 M^{-1}}), unlikely for Bj2S which showed extreme thermostability and invariance of λ_{em} with increasing temperature as discussed later. This by itself is not evidence against the GSCH mechanism but it makes a simple two-state GSCH model inconsistent with the observed quenching-induced blue shift.

The other mechanism that can give rise to the blue shift is slow solvent relaxation (SSR). When the relaxation rate of the dielectric environment (the protein–water 'solvent') surrounding the excited indole ring is comparable to the rate of collisional quenching, the red-shifted fluorescence, emitted at the final stages of solvent relaxation, is selectively quenched over the more blue-shifted fluorescence, produced at the early stages of solvent relaxation. This results in a net blue shift

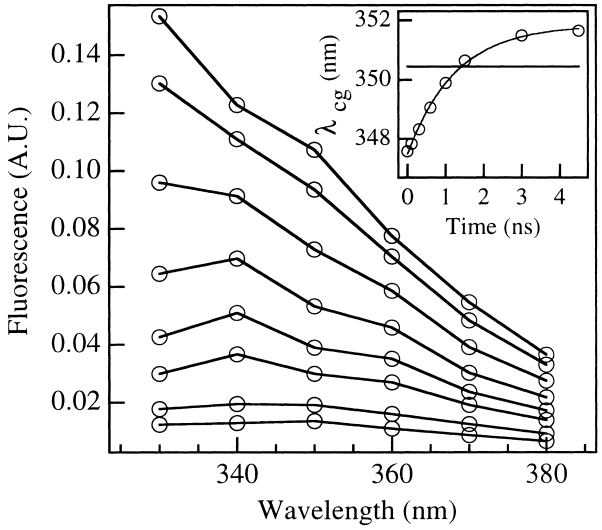

Fig. 3. Fluorescence intensity of Bj2S {λ_{ex} = 297 nm} following excitation at 0.0, 0.1, 0.3, 0.6, 1.0, 1.5, 3.0 and 4.5 ns (in descending intensity order). The progressive red shift in λ_{cg} with increasing time is shown in the inset (the horizontal line denotes λ_{cg} for the steady-state spectrum). The red shift in λ_{cg} exhibits a characteristic rise time of 1.25 ns.

in the observed equilibrium fluorescence spectrum as a function of increasing quencher concentration, as has been suggested [12]. Presence of SSR is characterized by red edge excitation shift (REES) [15–17] – a concomitant red shift in λ_{max} as the λ_{ex} is shifted along the red edge of the absorption spectrum. With increasing λ_{ex}, Bj2S exhibited emission red shift as reflected in center of gravity of emission, λ_{cg} (inset to Fig. 1), and λ_{max} (Fig. 2a) values. The observed red shifts are considerable, indicating that the relaxation time of the dielectric micro-environment of Trp in Bj2S is comparable to the fluorescence lifetime supporting the SSR model. The red shift is also evidence against an exclusive GSCH model. In the GSCH model, compared to the solvent shielded conformer, fluorescence and the absorption of the solvent-exposed conformer are red- and blue-shifted respectively. As a result, with progressive red edge excitation, the intensity of the blue-shifted fluorescence of the buried conformer should have been amplified.

Although rarely observed for proteins, the direct evidence for the SSR model is the growth of fluorescence at early times, monitored at the red edge of the emission spectrum [18]. Fluorescence decay curves (up to λ_{em} = 380 nm) for Bj2S did not show any early growth. This does not rule out the SSR model, since spectral overlap between the relaxed and the unrelaxed emission at the red side would make it undetectable. Time-dependent spectral shift of intrinsic protein fluorescence is

Table 1
Fluorescence lifetime components of Bj2S as a function of λ_{em} (λ_{ex} = 297 nm)

λ_{em} (nm)	τ_1 (ns)	τ_a (ns)	α_1	α_2	$\langle\tau\rangle$ (ns)
330	0.48	4.25	0.77	0.23	3.21
340	0.74	4.35	0.71	0.29	3.29
350	0.49	4.41	0.65	0.35	3.74
360	0.66	4.19	0.59	0.41	3.54
370	0.47	4.19	0.53	0.47	3.77
380	0.55	4.57	0.52	0.48	4.11

another method of detecting SSR [5,9]. In Fig. 3 the evolution of the emission spectra as a function of time from 330 to 380 nm excitation is shown. The emission center of gravity λ_{cg} shows a clear red shift (Fig. 3, inset) supporting the SSR model. The spectral shift arises due to the increase of mean fluorescence lifetime ($\langle\tau_F\rangle = \Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$) as a function of increasing λ_{em} (Fig. 2b and Table 1) resulting from SSR. The bi-exponentiality observed in the emission spectra may not originate from a simple GSCH or SSR model since at 7 M urea, when Bj2S was found to be completely unfolded as detected by CD and fluorescence, the bi-exponentiality {1.8 (60%), 4.4} persisted. The characteristic rise time of λ_{cg} is 1.25 ns which reflects the timescale of dielectric relaxation assuming that the spectral shift is exclusively from the SSR model. It should be pointed out that the λ_{cg} values used in this analysis neglect emission below 330 nm and therefore the shift is slightly underestimated for early times. Therefore the estimated characteristic rise time is only the upper limit to the true value. In any case, as expected, the order of magnitude of the timescale is comparable to the mean fluorescence lifetime ($\sim$3.5 ns) or the inverse of bimolecular quenching rate k_q[Q] ([$\sim 10^{-9}$ M^{-1} s^{-1}][0.1 M]). In addition, the steady-state fluorescence polarization is expected to decrease in the SSR model as a function of increasing λ_{em}. The emission wavelength dependence of polarization is shown in Fig. 2b. The results agree well with the SSR model.

As with other 2S albumins [19] Bj2S showed extreme temperature stability as judged from both CD and fluorescence experiments. Temperature stability (25–75°C) of Bj2S was attributed from the invariance of fluorescence λ_{max} (Fig. 4) and CD signal (invariance of spectral shape with a slight increase in negative intensity; data not shown). The stability of the protein backbone (CD results) is expected from a disulfide-constrained protein. The invariance of λ_{max} with increasing temperature could arise from a number of factors. With increasing temperature solvent relaxation should speed up accompanied by a red shift of λ_{max} in the SSR model. However, if the relaxation process has a high activation free energy (compared to RT), it will only be mildly sensitive to changes

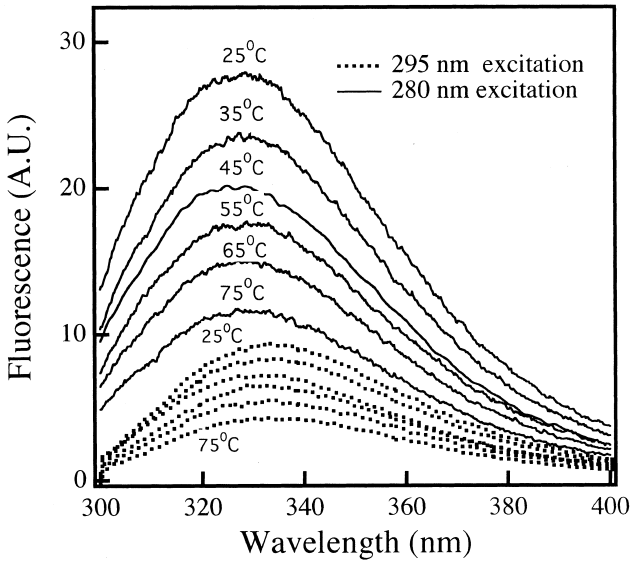

Fig. 4. Temperature-dependent Bj2S fluorescence spectra excited at 280 nm (solid line) and 295 nm (broken line).

in temperature. Alternatively if the fluorescence yields (inversely related to fluorescence lifetimes) of the blue and the red emission decrease with unequal rates with temperature increase, $\Delta\lambda_{max}$ will not directly reflect changes in solvent relaxation rates. In any case, the CD-detected thermostability of Bj2S indicates a very constrained protein backbone and that could make it predisposed to SSR.

The observed blue shift can arise from a constrained environment surrounding the Trp residue which also has to be polar enough to differentially solvate the ground and the excited states of the indole ring. In the absence of any X-ray or NMR structure of Bj2S, we homology-modeled the structure of 2SBj using the NMR-derived structure of the closely related protein BnIb [20]. The modeled structure agrees very well with steady-state fluorescence and CD data, especially with respect to a buried Trp, its proximity to the Tyr residue and the overall secondary structural content. In Bj2S the Trp residue (A20) occurs in a helical region of the short chain with the indole ring buried at the interface of the short (A) and the long (B) chains. The environment is dominated by non-polar residues except for a Lys (A23) and a His (B63) residue. Apart from the peptide dipoles, the restricted relaxation of either of these two (or both) along with bound water molecules must be the origin of the spectral shift.

4. Conclusion

We report here an unusually large quenching-induced blue shift in fluorescence λ_{max} in Bj2S, a single-Trp protein with constrained backbone. Two underlying mechanisms, SSR and GSCH, can give rise to the observed blue shift. Although the co-presence of GSCH could not be ruled out and is only shown to be unlikely, presence of SSR was confirmed in Bj2S from REES studies with strong supporting evidence from steady-state polarization and time-resolved fluorescence experiments. The estimated rate of dielectric relaxation in Bj2S was about 1 ns, comparable to the fluorescence lifetime of Trp and that characteristic time of bimolecular quenching. Understanding SSR in protein systems is of fundamental interest [5]. SSR can also control elementary biological processes like charge transfer reactions [21]. However, under ambient conditions SSR in proteins is usually much faster than typical fluorescence timescales. It is in this context that the presence of SSR in Bj2S at ambient temperature and viscosity is remarkable. It remains to be seen if SSR is special in Bj2S or if it is often present in other proteins with constrained backbone.

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