Global and local dynamics of the human U1A protein determined by tryptophan fluorescence

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(RECEIVED March 15, 1999; ACCEPTED July 5, 1999)

Abstract

Tryptophan residues have been introduced into two domains of the human U1A protein to probe solution dynamics. The full length protein contains 282 residues, separated into three distinct domains: the N-terminal RBD1 (RNA Binding Domain I), consisting of amino acids 1–101; the C-terminal RBD2, residues 202–282; and the intervening linker region. Tryptophan residues have been substituted for specific phenylalanine residues on the surface of the β -sheet of either RBD1 or RBD2, thus introducing a single solvent exposed tryptophan as a fluorescence reporter. Both steady-state and time-resolved fluorescence measurements of the isolated RBD domains show that each tryptophan experiences a unique environment on the β -sheet surface. The spectral properties of each tryptophan in RBD1 and RBD2 are preserved in the context of the U1A protein, indicating these domains do not interact with each other or with the linker region. The rotational correlation times of the isolated RBDs and the whole U1A, determined by dynamic polarization measurements, show that the linker region is highly flexible such that each RBD exhibits uncorrelated motion.

Keywords: RBD; tryptophan fluorescence; U1A

The human U1A snRNP protein is one of three specific proteins associated with the U1 snRNP (Scherly et al., 1989), one of the U snRNPs involved in pre-mRNA splicing. The protein has three distinct domains: the N-terminal RNA binding domain (RBD1 (RNA Binding Domain I), residues 1–101); the C-terminal RBD2 (residues 202–282); and the intervening 100 amino acid sequence, which has a high proline and lysine content (26% and 6%, respectively) (Sillikens et al., 1987). RBD1 binds tightly and specifically to the U1 snRNA (Boelens et al., 1991), and also to a conserved sequence found in the 3' UTR (UnTranslated Region) of its pre-mRNA (van Gelder et al., 1993). However, the function of RBD2 is unknown, for it does not appear to bind RNA (Lu & Hall, 1995). There is one U1A protein in the U1 snRNP, but two U1A proteins associate with the UTR structure where both are required to regulate polyadenylation (van Gelder et al., 1993; Gubser & Varani, 1996; Gunderson et al., 1997). Since U1A is bound to the RNAs through the interaction with RBD1, the remaining 20 kDa of protein (linker plus RBD2) could be available for interactions with other components of the splicing machinery when bound in U1 snRNP or with components of the polyadenylation apparatus when bound at the 3' UTR.

The role of the U1A protein in splicing is unknown. While some experiments suggest that it is a nonessential component of the U1 snRNP (Will et al., 1996), other experiments suggest that it may be

functionally important for splicing (Tang & Rosbash, 1996) or other cellular processes (Salz & Flickinger, 1996). Many RBD-containing proteins contain multiple RBD domains, where it is clear that the several RBDs together can regulate the RNA binding specificity (e.g., *sxl*, hnRNP A1). In the case of U1A, however, RNA binding is mediated by RBD1 alone. The spatial proximity of the two U1A RBDs in solution is unknown, although the long linking sequence between them is predicted by PHD (Rost & Sander, 1993; Rost et al., 1994; Rost & Sander, 1994) to have no particular structure. The position of RBD2 in the snRNP or in the UTR complex is likewise unknown, although RBD2 in the U1 snRNP is a primary target of autoimmune antibodies (Klein Gunnewiek et al., 1997), indicating that it is exposed in the particle. Thus one feature of the U1A geometry that may be critical for any function of RBD2 is the relative flexibility of the two RBDs.

To probe the conformational flexibility of the U1A protein, we have introduced a single tryptophan residue in place of phenylalanine at specific sites on each RBD. RNA Binding Domains adopt an α/β sandwich global fold containing a four-stranded antiparallel β -sheet. In RBDs, the surface of the β -sheet is the platform for RNA binding and contains two consensus sequences, the RNP-2 hexamer (β 1) and RNP-1 octamer (β 3) (Bandziulis et al., 1989). One conserved feature of RBDs is the presence of two or three aromatic amino acids displayed on the surface of the sheet: in the case of U1A RBD1, it is Tyr13 on β 1, and Phe56 on β 3; in RBD2, it is Phe211 on β 1, and Phe248 on β 3 (see Fig. 1). In our constructs, Phe56, Phe211, and Phe248 have individually been replaced with tryptophan. Using tryptophan fluorescence, we are

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Fig. 1. **A:** Structure of wild-type RBD1 obtained from the crystal structure of the protein complexed with a 21 nucleotide RNA hairpin (Oubridge et al., 1994; RNA atoms have been removed). **B:** NMR solution structure of RBD2 (Lu & Hall, 1997). The phenylalanine residues at positions 56 in RBD1 and positions 211 and 248 in RBD2 are displayed in ball-and-stick format.

able to determine the solution properties of the single RBD domains and the U1A protein.

In addition to acting as a probe of the dynamic behavior of U1A, the tryptophans on the surface of the β -sheets have also served to

report on their local environments. The fluorescent properties of each tryptophan are found to be unique, indicating that although these are solvent exposed residues, their behavior is dependent on their context. Replacement of a phenylalanine by a tryptophan with its larger heterocyclic side chain and amide group may lead to rearrangement of the amino acid interactions on the surface of the sheet, and so the unique environments observed for these three tryptophan residues may not reflect the standard state of the protein. A further consideration is that specific amino acids on the RNA binding surface can determine RNA binding affinity and specificity (Kranz & Hall, 1999). Therefore, it is important to note that the RBD1 F56W retains its affinity and specificity for RNA, and thus the surface environment reported by this tryptophan is that of a competent domain (Trp56 occurs naturally in the potato U1A protein (Simpson et al., 1995)). These individual RBD domains and the U1A protein containing single tryptophan residues are used here in a series of both steady-state and time resolved fluorescence experiments designed to compare the solution properties of the proteins.

Results

Circular dichroism of RBDs

The circular dichroism (CD) spectra of wild-type RBD1 and the Trp-containing mutants RBD1 F56W, RBD1(93) F56W, RBD2 F211W, and RBD2 F248W are shown in Figure 2. The spectrum of RBD1 F56W is similar to that of the wild type RBD1 protein in the 200–250 nm region where backbone transitions dominate, indicating substitution of Trp for Phe on the β -sheet introduces little, if any, perturbation of the secondary structure of the protein. The reduced signal at 208 nm in the spectrum of RBD1(93) F56W reflects the loss of the C-terminal α -helix (α 3). Similarly, the spectra of the RBD2 F211W and RBD2 F248W are nearly identical to that of the wild-type protein in the backbone region (Lu & Hall, 1995). Despite nearly identical secondary structures, substan-

Fig. 2. UV CD spectra of wild-type RBD1 and Trp-containing RBD1 and RBD2 proteins at $T = 25^\circ\text{C}$. (□) Wild-type RBD1; (○) RBD1 F56W; (△) RBD1(93) F56W; (▼) RBD2 F211W; (---) RBD2 F248W. Inset: Expanded view of the near-UV region.

tial differences exist in the spectra of RBD1 and RBD2 below 230 nm.

Aromatic side chains generally display weak CD signals in the 260–300 nm region. RBD1 has four tyrosines and five phenylalanines; however, the lack of any signal in this region in wild-type RBD1 suggests the differences observed in the spectra of RBD1 F56W and RBD1(93) F56W in this region arise from slight variations in the electronic environment around the Trp side chain. The RBD2 mutants also show weak induced CD signals in this region, which can be assigned to Trp211 or Trp248.

Steady state fluorescence and quenching of RBD proteins

Fluorescence spectra of the four RBD mutants obtained using 300 nm excitation are shown in Figure 3. The fluorescence spectrum of each mutant is broad with no discernible structure, characteristic of L_a state emission and a polar environment (Callis, 1997). The emission maxima vary from 332 nm for RBD1 F56W to 344 nm for RBD2 F211W (see Table 1). The RBD1 F56W spectrum is blue shifted from those of the other proteins. Truncation of $\alpha 3$ in this protein shifts the maximum from 332 to 338 nm. The fluorescence spectra of U1A F56W, U1A F211W, and U1A F248W are identical to the corresponding RBDs (data not shown).

The dependence of the fluorescence spectrum of each protein on excitation wavelength was also determined. Emission spectra of RBD1 F56W and U1A F56W obtained using 295, 300, and 305 nm excitation are shown in Figure 4 along with those of RBD1(93) F56W and U1A F248W for comparison. The emission spectra of Trp211 (data not shown) and Trp248 in the context of both the single domain and the whole protein are independent of excitation wavelength. In contrast, Trp56 displays a substantial red edge excitation effect in both RBD1 and U1A. Varying the excitation wavelength from 295–305 nm results in a continuous shift of $\lambda_{\max}$ for these two proteins from 332 to 336 nm with some spectral narrowing in the case of U1A. The magnitude of the red-edge spectral shift in RBD1 decreases slightly over the temperature range 4–35 °C (data not shown). In contrast to the results from the full length RBD1, the spectrum of RBD1(93) F56W is indepen-

Fig. 3. Steady state fluorescence spectra of Trp-containing RBD1 and RBD2 proteins at $T = 25^\circ\text{C}$ using $\lambda_{\text{exc}} = 295\text{ nm}$: (—) RBD1 F56W; (---) RBD1(93) F56W; (···) RBD2 F248W; (-·-) RBD2 F211W.

Table 1. Steady state fluorescence parameters for Trp-containing RBD and U1A proteins^a

Protein	$\lambda_{\max}$ (nm)	K_{SV} (M^{-1})	V (M^{-1})	k_q ($\text{M}^{-1}\text{s}^{-1}$)
RBD1 F56W	332	2.4	2.0	5.8×10^8
RBD1(93) F56W	338	5.4	2.2	1.1×10^9
U1A F56W	332	—	—	—
RBD2 F211W	344	7.4	2.2	1.1×10^9
U1A F211W	344	—	—	—
RBD2 F248W	340	7.9	2.8	1.3×10^9
U1A F248W	340	—	—	—

^a $\lambda_{\max}$ is the wavelength of maximum emission; K_{SV} and V are the dynamic and static Stern–Volmer quenching constants; k_q is the bimolecular collisional quenching constant determined from $k_q = K_{SV}/\langle\tau\rangle$, where $\langle\tau\rangle = \sum_i f_i \tau_i$ is the intensity weighted average lifetime.

dent of excitation wavelength. The emission maximum of this protein is similar, though still red shifted, to that of RBD1 F56W obtained at the reddest excitation wavelength.

The range of emission maxima observed for the RBD mutants suggests varying degrees of solvent accessibility of the Trp side chains, though internal electrostatic fields arising from nearby polar or charged residues can also have an effect on protein Trp emission properties (Callis & Burgess, 1997). Supporting evidence for variable solvent exposure comes from the acrylamide quenching data shown in Figure 5. For each RBD mutant both static and dynamic quenching were observed and values for the corresponding quenching constants are shown in Table 1. A useful measure of Trp side-chain solvent accessibility is the bimolecular quenching constant k_q , which can be determined from the dynamic quenching constant K_{SV} , and the intensity-weighted fluorescence lifetime (vide infra). Values of k_q for the various Trp-containing RBD proteins are shown in Table 1. Comparison of the bimolecular quenching constants of the two RBD domains shows that the Trp56 in RBD1 F56W is more sequestered than either of the Trp side chains on the β -sheet of RBD2 and notably also of Trp56 in RBD1(93), which has no $\alpha 3$. The increased accessibility of Trp56 in RBD1(93) is consistent with the observed red shift of the fluorescence of this protein compared to RBD1 F56W.

Fluorescence decay kinetics of RBD and U1A proteins

Fluorescence decays for each of the single Trp-containing RBD1 and U1A proteins were measured using 300 nm excitation. Three to five data sets were collected for each protein and fit simultaneously using the Globals Unlimited program. Decay parameters for each protein and the intensity weighted average lifetimes are shown in Table 2.

The decay law for each Trp is multiexponential and contains a short (~ 0.5) ns component, which accounts for $<5\%$ of the total intensity. Heterogeneous Trp fluorescence decays in proteins are generally taken to reflect the presence of different orientations of the side chain (rotamers) that experience different quenching rates, although slow dipolar relaxation of the surroundings may also be a factor. A subnanosecond component is common in proteins and may result from efficient quenching of rotamers that have their indole side chain proximate to a proton donor or electron acceptor.

Fig. 4. Steady state fluorescence spectra at $T = 25\text{ }^{\circ}\text{C}$ obtained using (—) $\lambda_{\text{exc}} = 295\text{ nm}$; (---) $\lambda_{\text{exc}} = 300\text{ nm}$; (+ + +) $\lambda_{\text{exc}} = 305\text{ nm}$. (A) RBD1 F56W; (B) RBD1(93) F56W; (C) U1A F56W; (D) RBD2 F248W.

Table 2. Time-resolved fluorescence parameters for Trp-containing RBD and U1A proteins ^a

Protein	f_1	τ_1 (ns)	f_2	τ_2 (ns)	f_3	τ_3 (ns)	$\langle\tau\rangle$ (ns)	χ^2
RBD1 F56W	0.04	0.38 (+0.11,−0.12)	0.33	2.72 (+0.45,−0.46)	0.63	5.30 (+0.43,−0.26)	4.3	1.20
RBD1(93)F56W	0.02	0.35 (+0.05,−0.07)	0.25	2.91 (+0.41,−.38)	0.73	5.50 (+0.23,−0.22)	4.7	0.21
U1A F56W	0.05	0.58 (+0.10,−0.12)	0.36	2.87 (+0.42,−0.36)	0.59	5.38 (+0.43,−0.26)	4.2	0.12
RBD2 F211W	0.02	0.47 (+0.13,−0.13)	0.15	3.25 (+0.45,−0.66)	0.83	7.75 (+0.50,−0.25)	6.9	1.14
U1A F211W	0.02	0.38 (+0.10,−0.09)	0.14	3.01 (+0.65,−0.40)	0.84	7.65 (+0.43,−0.26)	6.8	1.08
RBD2 F248W	0.02	0.98 (+0.21,−0.22)	—	—	0.98	6.45 (+0.02,−0.01)	6.3	1.34
U1A F248W	0.03	0.75 (+0.11,−0.10)	—	—	0.97	6.30 (+0.02,−0.02)	6.1	1.78

^a f_i and τ_i are the fractional intensity and lifetime of the i^{th} emission component. $\langle\tau\rangle$ is the intensity-weighted average lifetime, $\langle\tau\rangle = \sum_i f_i \tau_i$. Numbers in parentheses represent the 67% confidence limits for that parameter determined by correlated error analyses. Protein concentrations were 20–30 μM in 20 mM Tris (pH 8), 200 mM NaCl, 2 mM EDTA, 10% glycerol.

Fig. 5. Quenching of RBD Trp fluorescence by acrylamide (Q) at $T = 25^\circ\text{C}$. (●) RBD1 F56W; (■) RBD1(93) F56W; (×) RBD2 F211W; (▲) RBD2 F248W. Dynamic and static quenching constants are shown in Table 1.

In addition to the ubiquitous short component, the fitted decays for the Trp56 and Trp211 in both the RBD and U1A are characterized by intermediate and long lifetime (nanosecond) components. Attempts to fit the data from either mutant to a two exponential decay law gave significantly larger values for χ^2 . In contrast, the decays for RBD2 F248W and its corresponding U1A fit quite well to a double exponential with $\sim 97\%$ of the steady state intensity contained in a ~ 6.3 ns component. Addition of a third exponential to the decay law for these proteins resulted in a similar χ^2 value and yielded nearly equally weighted decay components of approximately 5.0 and 7.0 ns.

The fluorescence decay laws and average lifetimes of the tryptophan residues vary considerably, which reflects the different quenching rates at each site. The intensity weighted average lifetimes of the two RBD2s are similar, though the RBD2 F211W decay is considerably more heterogeneous. The decay laws for RBD1 F56W and RBD1(93) F56W are similar; however, the average lifetime of the mutant lacking the C-terminal helix (4.7 ns) is slightly longer than that of the full-length mutant (4.3 ns).

While the decay properties vary among the RBD Trps, it is evident that the relative amplitudes and time constants of the various components of a given Trp are substantially unchanged in going from an isolated domain to the intact U1A protein. For each Trp, the difference in the average lifetime in the single domain and in the whole protein is less than 0.2 ns.

Steady state and time-resolved anisotropies of RBD and U1A proteins

Steady state measurements

To compare Trp side-chain motions and rotational dynamics among RBD domains and between a given RBD and its corresponding U1A, steady state fluorescence anisotropies were measured at the fluorescence maximum for each protein from 8 to 40°C . Temperature-dependent CD spectra showed no conformational transitions over this range of temperature. Perrin plots con-

structed from the temperature-dependent anisotropy data are shown for both the individual domains and whole proteins in Figure 6. In each case the data fit well to a linear function (i.e., Equation 5), consistent with the lack of a temperature-induced conformational change. Estimates of the order parameter for each Trp obtained from the limiting anisotropy r_0 and the true initial anisotropy $r(0)$ using Equation 6 are shown in Table 3.

Anisotropies for each RBD were also measured as a function of emission wavelength over the range 340–375 nm using 300 nm excitation. Results for the two RBD1 proteins are shown in Figure 7 along with those from RBD2 F248W for comparison. The anisotropy values at each wavelength have been scaled to the value obtained at 340 nm to illustrate the different wavelength dependences of the various Trps. For each Trp, the anisotropy decreases with increasing wavelength. The largest change is observed for RBD1 F56W. For this domain, the anisotropy decreases by nearly 30% as the wavelength is varied from the center of the emission band to the red edge. In contrast, the anisotropy of the truncated form of this domain changes by only 15% over the same range, similar to the result obtained for RBD2 F248W. The observed decrease in the steady state anisotropy across the emission spectrum suggests that the effective lifetime for each Trp increases with longer wavelength.

Fig. 6. Perrin plots of the temperature-dependent fluorescence anisotropy of RBD and U1A proteins. **A:** (●) U1A F56W; (▲) RBD1 F56W; (×) RBD1(93) F56W. **B:** (●) U1A F248W; (◆) RBD2 F248W; (▲) U1A F211W; (■) RBD2 F211W.

Table 3. Anisotropy parameters for Trp-containing RBD and U1A proteins^a

Protein	ϕ_S (ns)	ϕ_R (ns)	S^2 (ss)	S^2 (tr)	χ^2
RBD1 F56W	0.18 (+0.03, -0.03)	8.66 (+0.25, -0.16)	0.87	0.89 (+0.01, -0.01)	0.56
RBD1(93) F56W	0.20 (+0.06, -0.06)	7.93 (+0.29, -0.27)	0.81	0.88 (+0.02, -0.01)	1.03
U1A F56W	0.21 (+0.05, -0.06)	12.85 (+0.51, -0.39)	0.93	0.88 (+0.01, -0.01)	0.66
RBD2 F211W	0.36 (+0.05, -0.04)	5.21 (+0.27, -0.25)	0.80	0.68 (+0.02, -0.02)	1.80
U1A F211W	0.27 (+0.06, -0.05)	8.10 (+0.61, -0.50)	0.80	0.70 (+0.02, -0.02)	3.25
RBD2 F248W	0.56 (+0.09, -0.08)	6.50 (+0.48, -0.30)	0.89	0.91 (+0.03, -0.03)	1.70
U1A F248W	0.45 (+0.19, -0.20)	9.36 (+0.55, -0.42)	0.91	0.89 (+0.01, -0.04)	1.27

^a ϕ_S and ϕ_R are the local and global rotational correlation times. The reported values of ϕ_R correspond to a solution viscosity of 1.0 cP. $S^2(ss)$ and $S^2(tr)$ are the order parameters obtained from fits of the steady state and time-resolved data, respectively. Numbers in parentheses are the 67% confidence limits for each parameter. Protein concentrations were 20–30 μ M in 20 mM Tris (pH 8), 200 mM NaCl, 2 mM EDTA, 10% glycerol.

Time-resolved measurements

The Trp rotational dynamics in both the isolated domains and the U1A protein were obtained from time-resolved anisotropy measurements. The local and global rotational correlation times (ϕ_S and ϕ_R , respectively) and order parameters (S^2) obtained from fitting the differential phase and modulation data to a two-component model are shown in Table 3. The reported rotational correlation times, determined from the Stokes–Einstein relation, have been corrected for the effect of the 10% glycerol on the solution viscosity.

The rotational correlation time of RBD1(93) F56W (7.93 ns) is less than the 8.66 ns time measured for RBD1 F56W, which is

consistent with the $\sim 8\%$ difference in the molecular weight resulting from truncation of $\alpha 3$. The value for RBD1 F56W is slightly longer than the 7.6 ± 0.5 ns reported from NMR ^{15}N relaxation measurements of wt RBD1(101) (Kranz & Hall, 1999). The rotational correlation time measured for RBD2 was found to be 5.21 ns using Trp211 as the probe and 6.50 ns using Trp248. The latter value agrees well with that reported from NMR measurements (6.6 ± 0.4 ns; Lu & Hall, 1997). The dependence of the measured rotational time on Trp probe could be due to anisotropic tumbling of RBD2. Projections of the transition dipole moment in each Trp onto different inertial axes of the RBD2 could account for the difference in the correlation time.

The measured rotational correlation times of the U1A protein depend on the probe and range from 8.10 ns for the Trp211 mutant to 12.85 ns for the Trp56 mutant (Table 3). While these times vary significantly, in each case the value of ϕ_R is approximately 45–55% longer than the value obtained for the corresponding RBD. Depending on the degree of rigidity of the linker region connecting the two RBDs, the rotational dynamics of U1A might be better described by two (or more) correlation times reflecting diffusional motion about axes with substantially different moments of inertia. However, attempts to fit the U1A anisotropy data to a three component decay law did not improve the quality of the fit. The observation of a single global rotational correlation time in U1A is consistent with the linearity of the Perrin plots for these proteins over a wide range of T/η values.

The order parameters obtained from fits to the time-resolved data are shown in Table 3. These values agree reasonably well ($<8\%$ difference) with those obtained from the steady state measurements with the exception of the Trp211, which shows the greatest degree of segmental motion. The discrepancy in this case ($\sim 15\%$) may result from the approximations assumed in the determination of the order parameter from the intercept of the Perrin plot. For example, temperature and anisotropy effects on the seg-

Fig. 7. Emission wavelength-resolved steady state anisotropy at $T = 25^\circ\text{C}$. (Δ) RBD1 F56W; (○) RBD1(93) F56W; (×) RBD2 F248W. For each protein, the anisotropy at each wavelength has been normalized to its value at $\lambda = 340$ nm.

mental component are ignored in Equation 5. The values of the order parameters obtained from the time-resolved measurements indicate that the side chain of Trp56 is quite hindered ($S^2 = 0.89$ in the case of RBD1 F56W) and that truncation of the C-terminal helix does not result in increased side-chain mobility. The lack of Trp56 segmental motion in RBD1 could arise from stacking of its indole ring with Tyr13, analogous to the Phe56Tyr13 geometry in the wild-type RBD1 crystal structure (Nagai et al., 1990), although it may participate in a hydrogen bonding interaction that restricts the side-chain motion. Similarly, Trp248 ($S^2 = 0.91$ for RBD2) also exhibits little local motion. In the NMR structure of wild-type RBD2, Phe248 packs with Ile209, and such an interaction involving Trp248 is consistent with the high value of S^2 observed in this protein. However, Phe211 packs with Ile246 in wild-type RBD2, yet Trp211 has a lower order parameter ($S^2 = 0.68$) perhaps indicating a rearrangement of the surface residues. The order parameter for each Trp is unchanged in going from the isolated domain to the intact U1A protein.

Segmental motion timescales were found to vary among the RBD tryptophans. The fastest side-chain motion occurs in Trp56, which was found to be ~ 0.2 ns. The correlation times for the RBD2 Trps are approximately 0.3 and 0.5 ns for Trp211 and Trp248, respectively. In each case, the timescale of the local motion was found to be the same (within experimental error) in the RBD and corresponding U1A protein.

Discussion

Emission characteristics of RBDs and orientation of the C-terminal tail in RBD1

Tryptophan fluorescence in proteins can range from ~ 308 – 350 nm (Lakowicz, 1983; Eftink, 1991) and provides a sensitive probe of the local environment. The magnitude of the Stokes shift is generally taken to reflect the extent of solvent exposure of the Trp side chain; however, the local electric field provided by the protein environment and its dynamical properties also plays an important role in determining the emission characteristics. Except for the structured L_b emission from the buried Trp in Azurin ($\lambda_{\max} = 308$ nm), the fluorescence spectra of protein Trps are dominated by emission from the L_a state (Callis, 1997). The large change in the permanent dipole moment accompanying this transition accounts for the sensitivity of the spectral characteristics to the local environment. The Stokes shift is dynamic and, thus, it is useful to consider the contributions to the local field in terms of their relaxation timescales. Relaxation of the reaction field due to nearby water molecules is generally complete within a picosecond or less, which is much faster than the average lifetime of the Trp L_a state (i.e., several nanoseconds). Slower relaxing or static components of the local field may originate from nearby polar or charged amino acid side chains with limited mobility or from water that is partially constrained by protein structural elements or side chains. If the relaxation timescale of these contributions is comparable to or longer than the excited state lifetime, then the steady state spectrum receives a substantial contribution from excited Trps that are not fully "solvated." Hallmarks of a slowly relaxing environment include fluorescence lifetimes that increase at longer emission wavelengths and fluorescence maxima that increase at longer excitation wavelengths (red edge excitation effect) (Lakowicz & Keating-Nakamoto, 1984).

The fluorescence maxima and bimolecular quenching constants for Trp211 and Trp248 in RBD2 and for Trp56 in the truncated RBD1 protein indicate that these residues are substantially solvent exposed. As noted earlier, in the crystal structure of wild-type RBD1, Phe56 stacks with the Tyr13 on $\beta 1$ (Nagai et al., 1990). A stacking or hydrogen bonding interaction between Trp56 and Tyr13 could influence the local field around the Trp and/or lead to partial sequestration of the side chain. This is supported by the observation that the RBD1(93) F56W spectrum is slightly blue shifted from those of the RBD2 proteins and that the Trp56 side chain undergoes little local motion in either of the RBD1 constructs.

The C-terminal tail of RBD1 is known to play an important role in RNA binding and specificity by anchoring the Thr89Asp90Ser91 sequence, and thus its orientation and dynamics are of considerable interest. The differences in steady state spectral properties and quenching results between RBD1 F56W and RBD1(93) F56W are dramatic and clearly show that residues 94–101 partially sequester the Trp56 from the solvent. A recent NMR study of an RBD1 construct containing residues 2–117 suggested that in solution the C-terminal α -helix lies across the β -sheet surface with residues Ile93, Ile94, and Met97 in the helix in contact with Leu44, Phe56, and Gln58 (Avis et al., 1996). These results were obtained at sufficiently high concentrations (2.5 mM) that one cannot rule out intermolecular interactions. Recent NMR data on RBD2, for example, showed evidence for aggregation at concentrations greater than 1.0 mM (Lu & Hall, 1997). The sensitivity of fluorescence techniques allows us to work at micromolar concentrations where such problems are alleviated, thus the results presented here provide clear evidence for interaction of $\alpha 3$ with the Trp56 on the β -sheet surface; however, while it is clear that $\alpha 3$ is in close proximity to the surface of the β -sheet in RBD1, amide relaxation measurements (Kranz et al., 1996) show that the residues in the C-terminal helix are more disordered than those in $\alpha 1$, $\alpha 2$, and the β -sheet, which suggests the interaction between $\alpha 3$ and the β -sheet is weak. This conclusion is in accord with the time-resolved fluorescence data, which shows that the decay of Trp56 is largely unaffected by the presence of $\alpha 3$. A strong interaction between Trp56 and one or more residues in the tail would be expected to significantly shorten the lifetime.

The C-terminal helix in RBD1 contains several charged and polar residues, which may contribute to the local electric field felt by the Trp side chain. In addition, the helix itself has a permanent dipole moment arising from the peptide backbone. The red-edge excitation effect and increased lifetime at longer wavelengths, as determined from the wavelength-resolved steady state anisotropy data, suggest that in RBD1 F56W there is a slowly relaxing component of the local field. The absence of the red-edge effect in the truncated RBD1 and reduced dependence of lifetime on emission wavelength observed for this construct show that the slow relaxation in RBD1 F56W can be attributed to motion of the C-terminal helix on the nanosecond or longer timescale. This is consistent with the low values for the backbone order parameters of the C-terminal tail determined from ^{15}N NMR relaxation data (Kranz et al., 1996).

Flexibility of the linker region

Comparison of the spectral and polarization properties of the RBDs with their corresponding U1As allows us to assess the flexibility of the 95 amino acid linker region connecting the two RBDs in the intact U1A protein. This information is crucial in understanding

the orientation of the RBD2 domains in the U1A:UTR complex and has important implications in the interaction between U1A and poly(A) polymerase (Gunderson et al., 1997). For each of the correspondingly-labeled RBD/U1A pairs, the global rotational correlation time of the U1A was found to be approximately 50% longer than that of the isolated domain. The degree to which the linker region is flexible can be qualitatively determined by comparing the ratio of measured U1A and RBD correlation times with that predicted for a rigid U1A structure obtained from a hydrodynamic treatment of rotational diffusion of a dumbbell consisting of two spheres (domains) of identical radius σ_1 connected by a rigid rod (linker) of radius σ_2 and length L (Garcia de la Torre & Bloomfield, 1981). These authors modeled the rod as a collection of collinear spherical elements, each of which acts as a frictional center, and calculated the diffusion constant for rotation about the symmetry axis perpendicular to the rod. Their result, expressed here as the ratio of the correlation times for perpendicular rotational diffusion ($\phi_\perp$) and rotational diffusion of the isolated domain (ϕ_D), is

$$\frac{\phi_\perp}{\phi_D} = \left\{ 0.2675 \exp \left[\sum_i \sum_j c_{ij} \left(\frac{L}{\sigma_1} \right)^i \left(\frac{\sigma_1}{\sigma_2} \right)^j \right] \right\}^{-1}. \quad (1)$$

The domain radii of RBD1 F56W and RBD2 F248W obtained from the rotational correlation times in Table 3, and the Stokes–Einstein formula are 20 and 18 Å, respectively. We thus choose the average of these two, 19 Å, for σ_1 . The presence of amino acid side chains of varying size makes the radius of the linker an ill-defined quantity, thus we arbitrarily choose a value for σ_2 of 2.5 Å. The interpolative polynomial derived by Garcia de la Torre and Bloomfield (1981) is valid for $L/\sigma_1 < 10$, thus we are limited to a rod length of less than 190 Å, which corresponds to approximately 50 amino acids (half the number of residues in the linker region of U1A). Using this value for L and the values of the polynomial coefficients c_{ij} , found in Garcia de la Torre and Bloomfield (1981), the calculated ratio of correlation times is found to be 5.4. Varying the radius of the linker from 1.5 to 3.5 Å changes this ratio by less than 15%. However, the measured ratio for U1A/RBD pairs is only ~ 1.5 , substantially less than this limiting value. This ratio predicts, based on the measured rotational correlation times for the isolated domains, that the perpendicular rotational correlation time for a rigid U1A structure would be in the range of 45–70 ns. No evidence for anisotropy decay components on this timescale was observed in the dynamic polarization experiments on any of the Trp-containing U1A proteins.

It is important to note that the limitation imposed on the length of the linker by the range of validity of Equation 1 makes the result obtained from the chosen parameters a lower limit for the correlation time of a rigid U1A protein. A longer rigid linker would lead to slower rotational diffusion about the perpendicular axis. The substantial difference between the measured U1A correlation time and that predicted from the hydrodynamic treatment of a rigid model for U1A leads us to conclude that the linker is highly flexible, and thus the rotational dynamics of RBD2 are essentially uncorrelated from those of RBD1 in the U1A protein.

Given the length of the linker, its high degree of flexibility, and the fact that the β -sheet surfaces of the two domains are highly nonpolar, we next address the possibility of domain–domain interactions in U1A that involve the β -sheet surfaces. The presence of such interactions would be expected to lead to a blue shift in the

U1A Trp fluorescence spectra compared to those of the corresponding RBD, changes in the order parameter for the Trp side chain, and increased quenching (decreased lifetime) of the Trp emission. None of these effects is seen in comparing the steady state and time-resolved emission properties of the RBDs and intact U1A proteins. These results clearly show that even while the U1A protein is highly flexible, there are no interactions between the two RNA binding domains.

Conclusions

Time-resolved and steady-state fluorescence measurements on the RBD1 and RBD2 domains of the human U1A protein in which tryptophan residues have been substituted for phenylalanine residues on the β -sheet surface show that each tryptophan experiences a unique environment on the β -sheet surface. The spectral properties of the Trp56 residue in the full length RBD1 domain and an RBD1 construct missing the C-terminal α -helix show that the helix weakly associates with the β -sheet surface and suggest that it is mobile on the nanosecond or longer timescale. The spectral properties of each tryptophan in RBD1 and RBD2 are preserved in the context of the U1A, indicating these domains do not interact with each other or with the linker region. The rotational correlation times of the isolated RBDs and the whole U1A protein show that the linker region is flexible such that the global motions of the individual RBDs are substantially uncorrelated.

Materials and methods

Site-directed mutagenesis

Mutations in RBD1, RBD2, and U1A were generated by site-directed mutagenesis with PCR methods using the wild-type RBD1 and RBD2 genes derived from the U1A construct (Sillikens et al., 1987). Phenylalanine mutations at positions 56 (RBD1) and 211 and 248 (RBD2) were made from polymerase chain reaction (PCR) primers containing the mutations. The truncated RBD1 mutant (residues 1–93) was made from a PCR primer corresponding to the new 3' end.

The linker region of the U1A protein (residues 102–195) contains a site at Lys122Lys123 that is readily cleaved by proteases. Lys122 and Lys123 were therefore substituted with threonine and alanine, respectively, and the proteins expressed in *Escherichia coli* BL21 (Grodberg & Dunn, 1988). This strain lacks the endoprotease OmpT, which cleaves between LysArg and LysLys. The lysine mutations at positions 122 and 123 were made from PCR primers for all U1A constructs.

PCR used standard procedures (Sambrook et al., 1989) and products were cloned into a plasmid under control of the tac promoter (Hall & Stump, 1992). All mutations were verified by sequencing of the DNA plasmid.

Protein purification

The RBD mutants were overproduced and purified following a standard procedure (Hall & Stump, 1992). Cells were grown at 37 °C in LB media to an A_{600} of 0.7 before induction with isopropyl- β -thiogalactoside (IPTG), and then allowed to grow for an additional 4 h. The cells were then centrifuged and lysed by French pressing, followed by centrifugation to remove the cell debris. The

supernatant was fractionated with 30% (w/v), then 60% ammonium sulfate to collect the protein. After overnight dialysis, the proteins were purified over a CM-Sepharose column, followed by ammonium sulfate precipitation. The RBD1 proteins were microdialyzed against the storage buffer (10 mM sodium cacodylate, pH 6, 50 mM NaCl, 10% glycerol). The RBD2 mutants were dialyzed overnight against a DEAE column buffer (20 mM Tris, pH 8), purified over the DEAE columns, followed by ammonium sulfate precipitation and microdialysis against the storage buffer. All RBDs were >95% pure as determined by silver staining for RBD1 and Coomassie staining for RBD2 (this domain is not stained by silver). The RBDs were stored at 4 °C in 50 mM NaCl, 10 mM sodium cacodylate, pH 6, 10% glycerol.

The U1A mutants were isolated from *E. coli* BL21 grown in LB media at 37 °C and induced in mid-log phase ($A_{600} = 0.7$) with 1 mM IPTG. The cells were allowed to grow an additional 4 h and then centrifuged. The pellet was washed with 20 mM Tris, 20 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 and stored at -70 °C until lysis. The cells were resuspended in 50 mM Tris, 20 mM NaCl, 2 mM EDTA, pH 7.5, and incubated on ice with lysozyme (0.32 mg/mL), followed by addition of sodium deoxycholate (20 min each) in the presence of protease inhibitors leupeptin, pepstatin, and phenylmethyl sulfonyl fluoride. The cells were sonicated and then centrifuged at 4 °C at $13,000 \times g$ for 30 min to remove the cell debris. DNA was precipitated by titrating with protamine sulfate to a final concentration of 2 mg/mL. The supernatant, obtained after centrifugation at 4 °C at $13,000 \times g$ for 15 min, was fractionated with 30% ammonium sulfate to collect the U1A protein. After overnight dialysis against 10 mM potassium phosphate, 50 mM NaCl, 2 mM EDTA, 10% glycerol, pH 8 (buffer C), the dialysate was loaded on a freshly prepared P-11 phosphocellulose column (Whatman, Clifton, New Jersey). The U1A protein was eluted off the column in a gradient from 150 to 650 mM NaCl in buffer C. After a 30% ammonium sulfate precipitation, the U1A proteins were microdialyzed against buffer C. The purity was assayed by a silver-stained SDS-PAGE gel.

All protein concentrations were determined spectroscopically at 280 nm using the extinction coefficients based on the Tyr/Trp/Phe content.

CD measurements

CD measurements of the RBD proteins were performed at 20 °C on a JASCO J-600 Spectropolarimeter. A 0.1 cm path length cuvette was used for measurements from 250–200 nm, and a 1.0 cm cell was used in the 350–250 nm region. Samples were obtained by diluting the protein stock solution into 50 mM NaCl, 30 mM sodium cacodylate, pH 7. Concentrations were 10 μ M for the RBD1 proteins and 15 μ M for the RBD2 proteins.

Steady state fluorescence spectral measurements

Steady state fluorescence spectra were recorded on a SLM 8100 spectrofluorometer with an excitation bandwidth of 2 nm and an emission bandwidth of 4 nm. All spectra were corrected using subtraction of a buffer blank. Samples were obtained by diluting the protein stock solutions into a 20 mM Tris, 200 mM NaCl, 2 mM EDTA, 10% glycerol, pH 8 buffer to a final concentration of 10 μ M. The temperature was 20 °C for all measurements.

Quenching of tryptophan fluorescence was determined using 295 nm excitation and a 4 nm emission bandwidth centered at the

peak of the fluorescence spectrum. Aliquots of 8 M acrylamide were added to 2 mL of 5 μ M RBD solution and to a buffer blank. The fluorescence intensity was corrected using background subtraction of the blank and for dilution effects. The quenching data were fitted to a modified form of the Stern–Volmer equation (Lakowicz, 1983), which includes the effect of static quenching to second order in the acrylamide concentration,

$$\frac{I_0}{I} - 1 = (K_{SV} + V) + \left(K_{SV}V + \frac{V^2}{2} \right) [Q]. \quad (2)$$

Here I and I_0 are the fluorescence intensity in the presence and absence of the acrylamide, respectively; $[Q]$ is the molar concentration of the acrylamide; and K_{SV} and V are the dynamic and static quenching constants, respectively. Values for the quenching constants were determined from the slope and intercept of plots of the left-hand side of Equation 2 vs. acrylamide concentration. The bimolecular quenching constants k_q were determined from the intensity weighted average lifetimes $\langle \tau \rangle$ (vide infra) using the relation

$$K_{SV} = k_q \langle \tau \rangle. \quad (3)$$

Steady state anisotropy measurements

Steady state anisotropies were determined using Equation 4:

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (4)$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the fluorescence components polarized parallel and perpendicular, respectively, to the vertically-polarized excitation beam. The intensities were measured using the L-format method. The G -factor, which corrects for the polarization-dependent properties of the detection system, was obtained by measuring the ratio of horizontally and vertically polarized intensities with horizontally polarized excitation. The excitation wavelength was 300 nm with a 2 nm bandwidth and a 4 nm emission bandwidth. Each anisotropy measurement was repeated until the standard deviation of the measurement was <0.002.

Anisotropies for each protein were measured at the emission maximum as a function of temperature over the range 8–40 °C and fit to the Perrin equation (Equation 5)

$$\frac{1}{r} = \frac{1}{r(0)} \left[1 + K \frac{T}{\eta} \right] \quad (5)$$

where $K = k_B \tau / V$ with k_B the Boltzmann constant, τ the excited state lifetime, η the solution viscosity, V the hydrodynamic volume of the protein, and $r(0)$ the initial anisotropy. Equation 5 is derived assuming (1) the decay of the initial anisotropy is characterized by a single exponential representing the rotational diffusion of the protein, (2) the rotational correlation time ϕ_R obeys the hydrodynamic relation $\phi_R = \eta V / k_B T$, and (3) the excited state decay is monoexponential. The depolarization of tryptophan in most protein environments receives contributions from local (segmental) motion of the Trp side chain in addition to rotational diffusion of the protein. The presence of a fast (compared to the excited state lifetime) component in the anisotropy decay, corresponding to fluctuations of the Trp side chain, results in an extrapolated value for

the initial anisotropy that is lower than the true value. In this case, the apparent initial anisotropy (r_0) represents the limiting anisotropy resulting from hindered local motion. The limiting anisotropy obtained from the Perrin plots was used to estimate the order parameter for side-chain mobility S^2 using the relation

$$S^2 = \frac{r_0}{r(0)}. \quad (6)$$

Steady state anisotropies were also measured as a function of emission wavelength over the range 340–375 nm at 20 °C using 300 nm excitation. In these experiments, the emission bandwidth was 4 nm and signal averaging was performed until the standard deviation of each measurement was <0.001.

Fluorescence decay kinetics

Fluorescence lifetime measurements were carried out at the Laboratory for Fluorescence Dynamics at the University of Illinois using the multifrequency phase-modulation technique. The excitation source was a cavity-dumped Rhodamine 590 dye laser (Coherent Model 700) synchronously pumped by the second harmonic of a modelocked Coherent Antares Nd:YAG laser. Frequency modulation of the excitation was realized using the harmonic content method (Gratton et al., 1984). The 300 nm excitation was obtained by frequency doubling of the 600 nm dye laser fundamental using KDP. After passing through a polarizer oriented at 54.7°, the fluorescence was observed through both a Corion U340 broad band-pass filter and a Schott WG335 high pass filter to avoid scattered light and detected by a Hamamatsu R928 photomultiplier tube. The frequency-dependent phase and modulation of the fluorescence were measured relative to the values for p-terphenyl in ethanol ($\tau = 1.05$ ns) at 16 modulation frequencies over the range 3.8 to 251.5 MHz using ISS data acquisition software. At each modulation frequency, the emission signal was averaged until the standard deviations in the phase shift and modulation factor were less than $\pm 0.2^\circ$ and 0.004, respectively.

Samples were prepared in 200 mM NaCl, 20 mM Tris, pH 8, 2 mM EDTA, 10% glycerol with protein concentrations ranging from 20–30 μ M. Background buffer fluorescence and scattering accounted for less than 2% of the counts. The sample temperature was maintained at 20 °C using a recirculating water bath.

Anisotropy decays

Dynamic polarization data for each protein were obtained on the same instrument as above by measuring the frequency-dependent differential phase and modulation ratio for emission polarized perpendicular and parallel to the vertically-polarized ultraviolet excitation using an L-format geometry. The polarization components were selected using Glan Thompson polarizers. For all measurements, the excitation wavelength was 300 nm. The error tolerances for the differential phase and modulation ratio and the modulation frequency range were the same as those used for the lifetime measurements.

Data analysis

Analyses of the lifetime and polarization data were carried out using the Globals Unlimited program (Beechem, 1992). Lifetime

parameters were obtained by fitting the frequency-dependent phase and modulation data to theoretical expressions relating these observables to the sine and cosine transforms of the intensity decay function (Lakowicz, 1983), which was taken to be a sum of exponentials:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}. \quad (7)$$

The parameters α_i and τ_i are the amplitude and lifetime, respectively, of the i^{th} decay component. The χ^2 analyses were performed assuming a constant standard deviation of 0.2° for the phase and 0.004 for the modulation factor. The assumption of a frequency-independent standard deviation in the statistical analysis of the data disregards variations in fluorescence intensities from sample to sample as well as laser power fluctuations from measurement to measurement. This will occasionally lead to overestimation of the error for a particular sample with the result that the χ^2 value is <1.0. In such cases, the χ^2 statistic serves primarily to judge the performance of various decay laws in accurately fitting the data. The intensity weighted average lifetime is given by

$$\langle \tau \rangle = \sum_i f_i \tau_i \quad (8)$$

where $f_i = \alpha_i \tau_i / \sum_i \alpha_i \tau_i$ is the fractional intensity contained in the i^{th} decay component.

Dynamic polarization data were fit to a model in which a two component anisotropy decay function, which can be written in the form

$$r(t) = r(0)[S^2 + (1 - S^2)e^{-t/\phi_S}]e^{-t/\phi_R}, \quad (9)$$

is associated with each lifetime component. As noted earlier, S^2 is the order parameter for Trp side-chain fluctuations, and ϕ_S and ϕ_R are the correlation times for segmental and global rotational motion, respectively. The use of a single (slow) component to describe the overall tumbling of the protein is, in principle, not warranted given that RBD1 and RBD2 are not perfect spheres; however, the inertial axial ratios are sufficiently similar (0.67:1:1 for RBD1; Lu & Hall, 1997) that resolution of the individual rotational correlation times is not feasible.

The value for $r(0)$ for Trp is excitation wavelength dependent due to ultrafast electronic relaxation of the L_a and L_b states; however, for >300 nm excitation the value plateaus to ~ 0.31 (Ruggiero et al., 1990). The value of $r(0)$ obtained from the nonlinear least-squares fitting procedure typically ranged from 0.30–0.34. Repeating the analyses with $r(0)$ held fixed at 0.31 resulted in minor changes in the values of ϕ_S and had little effect on the values of ϕ_R and S^2 . The anisotropy parameters reported here are the values obtained with $r(0)$ fixed at 0.31.

Acknowledgments

We thank the Laboratory for Fluorescence Dynamics at the University of Illinois, Urbana-Champaign for use of the phase-modulation fluorometer, Dr. T.L. Hazlett for advice and technical assistance, and Prof. Timothy Lohman for use of the steady state fluorometer. This work is supported by the NIH (GM46318).

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