Fluorescence studies on the role of tryptophan in heterogeneous nuclear ribonucleoprotein particles of HeLa cells

Johannes SCHENKEL,*†§ Isolde APPEL,† Ralph SCHWARZWALD,‡ Ekkehard K. F. BAUTZ,*
Jürgen WOLFRUM‡ and Karl Otto GREULICH‡

*Molekulare Genetik der Universität Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg,

†Institut für Immunologie und Genetik Abteilung 740, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, and ‡Physikalisch-Chemisches Institut der Universität Heidelberg, Im Neuenheimer Feld 253, D-6900 Heidelberg, Federal Republic of Germany

The 40 S heterogeneous nuclear ribonucleoprotein (hnRNP) particles from HeLa cells reveal tryptophan fluorescence with a bi-exponential decay, indicating that only a few of the 'core' proteins contain tryptophan residues. The presence of tryptophan residues distinguishes hnRNP particles from nucleosomes, with which they otherwise share a number of properties. This difference, however, is not essential for protein–RNA binding, as the fluorescence decay remains unchanged when hnRNP particles are dissociated into protein and RNA. However, the Stern–Volmer quenching constant is doubled upon salt dissociation, i.e. tryptophan residues become more accessible to solvent. Thus tryptophan quenching is a useful parameter for monitoring protein–protein interactions in hnRNP particles.

INTRODUCTION

Immediately after transcription, eukaryotic hnRNA is complexed with a set of primarily basic proteins to form hnRNP particles [1–3]. The assembly of hnRNP particles seems not to be sequence-specific [4–6], but Beyer *et al.* [7] concluded from electron-microscopic observations that these proteins bind to the primary transcript at various distances that are constant for each transcript. hnRNP particles are involved in the splicing procedure (for example see ref. [8]). Best described are particles sedimenting with *s* values of 30–50 S in sucrose gradients, resulting after cleavage of high-molecular-mass hnRNA by endogenous nucleases ([1,3,9–12]; for reviews see also refs. [13] and [14]). In HeLa cells the most important hnRNP-particle proteins, the 'core' proteins, have molecular masses of 32 kDa (*A*₁), 34 kDa (*A*₂), 36 kDa (*B*₁), 37 kDa (*B*₂), 42 kDa (*C*₁) and 44 kDa (*C*₂). Group C proteins are the hnRNP-particle proteins that bind best to the RNA [1,15], and they play an important role in the splicing procedure [16]. Core protein *A*₁ is clearly involved in connective-tissue diseases such as systemic lupus erythematosus or rheumatoid arthritis, as indicated by the presence of autoantibodies against protein *A*₁ in these diseases [17]. Depending on the method of particle isolation, in total up to 40 proteins have been reported to be involved in the formation of hnRNP particles. In almost all of them, small amounts of tryptophan have been detected by amino acid analysis (from 0.1 to 1.0 mol/100 mol of residues) [18]. By cDNA cloning it was shown that core protein *A*₁ contains a tryptophan residue [19], whereas core proteins *C*₁ and *C*₂ do not contain tryptophan [20]. These data are partly contrary to data obtained by amino acid analysis.

Knowledge concerning the presence of tryptophan in hnRNP particles is of interest for two reasons. First, it would distinguish hnRNP particles from nucleosomes, with which they otherwise share a number of properties,

such as the mostly basic character of the core proteins or the relative lack of cysteine residues and thus the lack of stabilizing disulphide bridges [21,22]. Secondly, the large aromatic tryptophan residue could be involved in protein–protein and protein–RNA interactions and may thus be functionally important. If, for example, tryptophan residues are involved in protein–RNA interactions [23], this would indicate that the mechanism of stabilization of hnRNP particles is basically different, and that the analogy between hnRNP particles and nucleosomes, which is often implied by naming the proteins core proteins, is superficial. With this in mind we have studied the possible role of tryptophan residues by using fluorescence spectra, lifetimes and solute quenchers of fluorescence.

MATERIALS AND METHODS

Isolation of hnRNP particles

HeLa S-3 cells were grown in Belco spinner culture in minimal essential medium (Gibco/BRL, Eggenstein, Germany) containing 8% (v/v) fetal-calf serum, up to a density of 0.5×10^6 – 1×10^6 cells/ml [6].

Then 1×10^9 cells were suspended in 30 ml of TSS buffer (50 mM-Tris/HCl buffer, pH 7.0, containing 10 mM-MgCl₂ and 25 mM-KCl) containing 250 mM-sucrose (RNAase-free) and 1 mM-phenylmethanesulphonyl fluoride and homogenized by 20 strokes in a Potter homogenizer (1000 rev./min) at 4 °C. Nuclei and cytoplasm were separated by centrifugation at 2600 *g* for 10 min. The nuclear pellet was dissolved in 250 ml of NHS buffer (20 mM-Tris/HCl buffer, pH 7.0, containing 1.5 mM-MgCl₂ and 0.02% Triton X-100), homogenized twice in a Potter homogenizer and pelleted by centrifugation at 400 *g* for 10 min. Finally, the nuclei were dissolved in ice-cold double-distilled water and sedimented by centrifugation at 650 *g* for 15 min.

Abbreviation used: hnRNP, heterogeneous nuclear ribonucleoprotein.

§ To whom correspondence should be sent, at the Deutsches Krebsforschungszentrum address.

The nuclei were left to stand for 45 min in 3 ml of ice-cold pH 8 buffer (10 mM-Tris/HCl buffer, containing 140 mM-NaCl and 1 mM-MgCl₂), and the hnRNP particles were then extracted by three sonications (5 s each). The nuclear debris was removed by centrifugation at 16 500 *g* for 5 min, and the supernatant was incubated for 30 min at 37 °C (to activate endogenous nucleases). The 40 S hnRNP particles were isolated after centrifugation at 285 000 *g* for 8 h on a 10–40 % (w/v) sucrose gradient in a Beckman SW40 rotor. The gradients were fractionated by means of an ISCO density-gradient fractionator, and the peak fractions containing 40 S hnRNP particles were pelleted by centrifugation at 215 000 *g* for 7 h in a Beckman SW60 rotor [1,3,6].

The hnRNP particles were dissociated into protein and RNA in the presence of 1 M-NaCl [1].

The different batches of hnRNP were checked by means of SDS/polyacrylamide-gel electrophoresis [24]. In each batch the same protein pattern was found and no significant differences in the concentrations were detectable.

Fluorescence experiments

Fluorescence spectra were taken with a Perkin-Elmer LS 3 spectrometer. The excitation wavelength throughout this work was 280 nm. Solutions of 5 M-KI and 5 M-CsCl were used for quenching studies, where small amounts (10–50 μ l) of quencher solutions were added to 2 ml solutions containing hnRNP particles.

Fluorescence decay curves were measured by exciting the samples with laser pulses of 15 ps half-width of a peak. The pulses were generated by an excimer laser (Lambda Physik EMG 103MSC), which pumps a two-stage laser to shorten the pulses. The first stage is a quenched-transient dye laser, which generates a sub-nanosecond pulse. Its pulse is amplified and used to pump a distributed-feedback dye laser [25,26]. After amplification and frequency doubling (generation of second harmonic) to 280 nm the pulse excites the sample. The temporal behaviour of the fluorescence was recorded with a streak camera (Hamamatsu C1966). A silicon-intensified-target camera was used to store the image and to send it to the temporal analyser (Hamamatsu TA C2200). Since the excitation pulse is about two orders of magnitudes smaller than the shortest lifetime reported in this paper, no mathematical deconvolution is necessary. Thus the fluorescence decay curves shown in Fig. 2 are logarithmic representations of raw data (except for shadowing correction allowing for inhomogeneities of the silicon-intensified-target camera). For a detailed description of the equipment see refs. [27] and [28].

The experiments were repeated several times with different batches of hnRNP particles. For all fluorescence measurements hnRNP particles containing 0.1 μ g of protein were used.

RESULTS

hnRNP particles show a tryptophan-specific fluorescence with a bi-exponential decay

When excited at 280 nm, hnRNP particles fluoresce with an emission maximum at 337 nm typical for proteins containing tryptophan residues in a hydrophobic environment [29] (Fig. 1). This is of interest in view of the

Fig. 1. Fluorescence spectrum of hnRNP particles

hnRNP particles were excited at 280 nm, and the emission spectrum was measured. The arrow shows the fluorescence maximum at 337 nm, typical for tryptophan residues in a hydrophobic environment [29].

fact that earlier publications on the amino acid composition of the core proteins in HeLa cells [1] did not indicate the presence of tryptophan in hnRNP particles.

The fluorescence decay (Fig. 2a) of the hnRNP-particle protein can be described to a good approximation by two exponents with fluorescence lifetimes $T_1 = 1.5$ ns and $T_2 = 3.4$ ns. Such a simple fluorescence decay might appear to be surprising in the view of the fact that hnRNP particles are complexes of many proteins and contain tyrosine residues as well as tryptophan residues. However, as with many proteins containing tyrosine and tryptophan residues, hnRNP particles reveal a pure tryptophan fluorescence spectrum, with no shoulder in the tyrosine emission region at 305 nm. In addition, fluorescence decays of peptides can become simpler with increasing length [28], and tryptophan-containing proteins can reveal pure mono-exponential decay even when complexed with nucleic acids [30]. Under the same conditions, as for hnRNP particles, we find for free tryptophan a decay with a lifetime of 3.5 ns. A bi-exponential decay can best be explained by a small number of tryptophan residues in the hnRNP particle.

Intact and dissociated hnRNP particles have the same fluorescence characteristics

In 1 M-NaCl hnRNP particles dissociate [1]. The fluorescence decay under this condition is identical with that at physiological salt concentration (Fig. 2b), and the fluorescence intensity as a function of salt concentration is almost constant up to 2 M-NaCl (Fig. 3), i.e. none of the tryptophan residues changes its microenvironment. This supports our suggestion that only very few of the core proteins contain tryptophan, since it is unlikely that

Fig. 2. Fluorescence decay curves of tryptophan residues in hnRNP particles

hnRNP particles were excited at 280 nm, and the signal was integrated over the whole frequency spectrum from 300 to 380 nm. hnRNP particles were used in the native form (a) and in the presence of 1 M-NaCl (b).

Fig. 3. Salt-dependence of the fluorescence intensity of hnRNP particles

hnRNP particles were treated with NaCl and excited at 280 nm. The fluorescence intensity was measured at the emission maximum (338 nm).

all of a large number of tryptophan residues remain in their microenvironment upon dissociation. From the dissociation experiment it becomes also clear that tryptophan residues are not involved in protein-RNA contact, since then the fluorescence would increase upon dissociation as the result of a release of fluorescence energy transfer from tryptophan to RNA [31].

Tryptophan residues of hnRNP protein become more accessible to solvent upon dissociation

The accessibility of tryptophan to solvent can be tested

Table 1. Stern-Volmer constants (K_{SV}) of free tryptophan and hnRNP particles

	$K_{SV} \text{ (mol}^{-1}\text{)}$	
	Cs ⁺ quenching	I ⁻ quenching
Free tryptophan	2.38	16.7
hnRNP particles at physiological salt concentrations	0.45	2.2
Dissociated hnRNP particles after 1 M-NaCl treatment	0.83	4.3

by quenching experiments with I⁻ (as KI) or Cs⁺ (as CsCl), which both reduce the fluorescence of tryptophan residues if they are accessible to the solvent. Since, as described above, the microenvironment of tryptophan residues in hnRNP is stable, quenching experiments give information on the geometric accessibility. Fig. 4 shows plots based on the Stern-Volmer equation [32]:

$$F_0/F = 1 + K_{SV} \cdot [Q] = 1 + k_q \cdot T_0 \cdot [Q]$$

where F_0 and F are the fluorescence intensities at 337 nm without and with quencher at a concentration $[Q]$, K_{SV} is the slope of the theoretically straight line, k_q is the bimolecular quenching constant and T_0 is the fluorescence lifetime of the sample without quencher. If the fluorescence emission of a molecule is heterogeneous, e.g. if tryptophan residues in different environments are contributing to fluorescence, the theoretically straight Stern-Volmer lines are curved (for details on the interpretation of curved Stern-Volmer plots see ref. [32]). Fig. 4(a) gives a Stern-Volmer plot at physiological condition, and Fig. 4(b) that at 1 M-NaCl, where hnRNP particles are dissociated [1].

Generally it is k_q and not K_{SV} that allows statements on the accessibility of a fluorophore to the solvent and to the solute quenchers. K_{SV} can be used directly only if the fluorescence lifetimes of the molecules to be compared with each other (free tryptophan, free core protein and

Fig. 4. Microenvironment of hnRNP particles: Stern-Volmer plots

Fluorescence intensities of free tryptophan ($\square$), of hnRNP particles ($\circ$) and of hnRNP particles treated with 1 M-NaCl ($\bullet$) are shown. (a) Quenching with I^- : the slope of the quenching curve of hnRNP particles is 13% of that of free tryptophan; salt-treated hnRNP particles have a slope that is 26% of that of free tryptophan. (b) Quenching with Cs^+ : the slope of the quenching curve of hnRNP particles is 19% of that of free tryptophan; salt-treated hnRNP particles have a slope that is 35% of that of free tryptophan. The difference in the scales reflects the different quenching efficiencies of Cs^+ and I^- .

core protein of the intact hnRNP particles) are similar. The fluorescence lifetimes, though somewhat different, are similar enough to allow an accessibility study on the basis solely of K_{SV} , i.e. using the slopes of the Stern-Volmer plots. Table 1 summarizes the results for free tryptophan as calibration substance for hnRNP particles at physiological conditions and for hnRNP particles at 1 M-NaCl, where dissociation into proteins and RNA has taken place.

DISCUSSION

The decay of tryptophan fluorescence in hnRNP particles of HeLa cells is bi-exponential and is not changed by dissociation in 1 M-NaCl. The accessibility of tryptophan residues to the surface quenchers KI and CsCl is doubled upon dissociation. From this, several conclusions can be drawn, as follows.

(1) The accessibility of tryptophan residues in core protein of hnRNP particles (as compared with free tryptophan) is similar for the positive surface quencher Cs^+ and the negative surface quencher I^- . Thus tryptophan residues in hnRNP particles are in an electroneutral environment.

(2) Tryptophan residues are not involved in protein-RNA interactions, since then one would expect an increase in fluorescence due to release of fluorescence

quenching by RNA. Thus, in that respect, tryptophan residues are not functional, and the analogy of hnRNP particles to nucleosomes remains valid in spite of the fact that only the former contain tryptophan. The data concur with the consensus sequence of the RNA-binding domain of several RNA-binding proteins as published in refs. [8], [33] and [34]. These consensus sequences do not contain tryptophan. Since the fluorescence measurements show no participation of tryptophan residues in protein-RNA interactions in hnRNP particles, these data argue for the existence of the RNP1/RNP2 domain [8] in all RNA-binding hnRNP-particle proteins.

(3) The hnRNP particle contains tryptophan residues in one or two microenvironments. It is unlikely that tryptophan residues present in a large number of core proteins do not change their fluorescence behaviour upon dissociation of hnRNP particles. The most probable explanation for the fluorescence results is that the hnRNP particles contain only a limited number of tryptophan residues. Sequencing data show that core protein A_1 contains a tryptophan residue [19] and that core proteins C_1 and C_2 do not [20]. Therefore we expect that most of the non-sequenced hnRNP proteins are tryptophan-free, in analogy to the core proteins of nucleosomes.

(4) In core proteins dissociated from RNA tryptophan residues are partly accessible to solvent. In intact hnRNP particles the accessibility is smaller by a factor of 2-fold.

The change in accessibility may be caused by a structural transition of one of the proteins during dissociation of the complex, upon which the accessibility, however, would probably change the fluorescence spectrum or the fluorescence decay curve. Since this is not the case, we suggest that the proteins do not change their structure and that the lower accessibility in the complex is due to steric hindrance. Thus tryptophan fluorescence quenching can be used to monitor protein-protein contacts in hnRNP particles. In addition, there remains the possibility that tryptophan residues are involved in stabilization of protein-protein interactions.

We thank Dr. K. Kleinermanns for stimulating discussion, Professor F. A. Bautz for the HeLa cells and F. Hug for performing control experiments. This work was supported by the Stiftung Volkswagenwerk and the Bundesminister für Forschung und Technologie (Grant BCT 381-5).

REFERENCES

- Beyer, A. L., Christensen, M. E., Walker, B. W. & Le Stourgeon, W. M. (1977) *Cell* **11**, 127-138
- Pederson, T. (1974) *J. Mol. Biol.* **83**, 163-183
- Louis, C. & Sekeris, C. E. (1976) *Exp. Cell Res.* **102**, 317-328
- Pullman, J. M. & Martin, T. E. (1983) *J. Cell Biol.* **97**, 99-111
- Wilk, H. E., Angeli, G. & Schäfer, K. P. (1983) *Biochemistry* **22**, 4592-4600
- Schenkel, J., Sekeris, C. E., Alonso, A. & Bautz, E. K. F. (1988) *Eur. J. Biochem.* **171**, 565-589
- Beyer, A. L., Miller, O. L. & McKnight, S. L. (1980) *Cell* **20**, 75-84
- Dreyfuss, G., Swanson, M. S. & Pinol-Roma, S. (1988) *Trends Biochem. Sci.* **13**, 86-91
- Economides, I. V. & Pederson, T. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1599-1602
- van Eekelen, C. A. G. & van Venrooij, W. J. (1981) *J. Cell Biol.* **88**, 554-563
- Guialis, A., Arvanitopoulou, A., Patrino-Georgoula, M. & Sekeris, C. E. (1983) *FEBS Lett.* **151**, 127-133
- Flytzanis, C., Alonso, A., Louis, C., Krieg, L. & Sekeris, C. E. (1978) *FEBS Lett.* **96**, 201-206
- Jacob, M., Devilliers, G., Fuchs, J. P., Gallinaro, H., Gattoni, R., Judes, C. & Stevenin, J. (1981) in *The Cell Nucleus*, vol. 8 (Bush, H., ed.), pp. 194-246, Academic Press, New York
- Samarina, O. P. & Krichevskaya, A. A. (1981) in *The Cell Nucleus*, vol. 9 (Bush, H., ed.), pp. 2-48, Academic Press, New York
- Schenkel, J. & Kloetzel, P. M. (1989) *FEBS Lett.* **247**, 51-54
- Goswami, P. & Goldenberg, C. J. (1988) *Nucleic Acids Res.* **16**, 4995-5011
- Astaldi Ricotti, G. C. B., Bertagno, M., Cerino, A., Negri, C., Caporali, R., Cobiachi, F., Loughi, M. & Montecucco, C. M. (1989) *J. Cell. Biochem.* **40**, 43-47
- Wilk, H. E., Werr, H., Friedrich, D., Kiltz, H. H. & Schäfer, K. P. (1985) *Eur. J. Biochem.* **146**, 71-81
- Buvoli, M., Biamonti, G., Tsouflas, T., Bassi, M. T., Ghatti, A., Riva, S. & Morandi, C. (1988) *Nucleic Acids Res.* **16**, 3751-3770
- Swanson, M. S., Nakagawa, T. Y., LeVan, K. & Dreyfuss, G. (1987) *Mol. Cell. Biol.* **7**, 1731-1739
- Sperling, R. & Wachtel, E. J. (1981) *Adv. Protein Chem.* **34**, 1-60
- Pederson, D. S., Thoma, F. & Simpson, R. T. (1986) *Annu. Rev. Cell Biol.* **2**, 117-147
- Helene, C. & Lancelot, G. (1982) *Prog. Biophys. Mol. Biol.* **39**, 1-68
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Bor, Zs., Müller, A., Racz, B. & Schäfer, F. P. (1982) *Appl. Phys. B* **27**, 9-14
- Bor, Zs., Müller, A., Racz, B. & Schäfer, F. P. (1982) *Appl. Phys. B* **27**, 77-81
- Schwarzwald, R., Monkhouse, P. & Wolfrum, J. (1987) *Chem. Phys. Lett.* **142**, 15-18
- Schwarzwald, R. & Greulich, K. O. (1989) *Ber. Bunsen-Ges. Phys. Chem.* **93**, 342-346
- Burstein, E. A., Vedenkina, N. S. & Ivkova, M. N. (1973) *Photochem. Photobiol.* **18**, 263-275
- Greulich, K. O., Wijnaendts van Resandt, R. & Kneale, G. C. (1985) *Eur. Biophys. J.* **11**, 195-201
- Stryer, L. (1978) *Annu. Rev. Biochem.* **47**, 819-846
- Eftink, M. & Ghiron, C. (1981) *Anal. Biochem.* **114**, 199-227
- Chan, E. K. L., Sullivan, K. F. & Tan, E. M. (1989) *Nucleic Acids Res.* **17**, 2233-2244
- Bandziulis, R. J., Swanson, M. S. & Dreyfuss, G. (1989) *Genes Dev.* **3**, 431-437

Received 28 March 1989/26 June 1989; accepted 4 July 1989