RNA Conformation and Folding Studied with Fluorescence Resonance Energy Transfer

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Fluorescence resonance energy transfer (FRET) results from nonradiative coupling of two fluorophores and reports on distances in the range 10–100 Å. It is therefore a suitable probe to determine distances in RNA molecules and define their global structure, to follow kinetics of RNA conformational changes during folding in real time, to monitor ion binding, or to analyze conformational equilibria and assess the thermodynamic stability of tertiary structure conformers. Along with the basic principles of steady-state and time-resolved fluorescence resonance energy transfer measurements, approaches to investigate RNA conformational transitions and folding are described and illustrated with selected examples. The versatility of FRET-based techniques has recently been demonstrated by implementations of FRET in high-throughput screening of potential drugs as well as studies of energy transfer that monitor RNA conformational changes on the single-molecule level.

INTRODUCTION

RNA is an extremely versatile biomacromolecule that fulfills a broad range of crucial biological functions. Apart from the rather “passive” role of mRNAs and tRNAs in mediating gene expression, the rRNAs seem to have an “active” role on the way from the gene to the protein. RNAs regulate the expression of virus genomes, and a wide variety of chemical reactions can be catalyzed by ribozymes [for a recent review see (1, 2)]. With improved techniques to prepare and manipulate RNA molecules on a large scale, the three-dimensional structures of several RNAs have been solved, among them tRNAs, rRNAs, ribozymes, aptamers, and RNA–protein complexes, revealing principles governing RNA tertiary structure [for a review see (3–5)]. The unique features of RNAs are exploited in therapeutic efforts like antisense approaches to silence the expression of certain disease-associated genes or in studies to interfere with gene expression in vivo by directing the catalytic activity of ribozymes to the respective targets [reviewed in (6–8)].

In vivo RNAs, similar to proteins, are released after synthesis from the RNA polymerase as linear biopolymers that need to adopt a defined three-dimensional structure to attain biological function. RNA folding is a prerequisite for the formation of active sites or recognition sites for proteins and other ligands. While protein folding has been studied extensively using a plethora of different methods, the processes involved in RNA folding have emerged mainly within the last decade. Distinct steps in the folding of certain RNA molecules could be monitored and basic principles of folding have been extracted [for a review see (9–15)]. Although structural, thermodynamic, and kinetic information on various RNAs is available, there are only a few examples where the nature of conformational changes on the folding pathway has been characterized.

Fluorescence spectroscopy is a powerful tool to study the structure and dynamics of biological macromolecules. Fluorescence is the emission of light from electronically excited singlet states. The properties of the emitted light reflect the molecular environment of the fluorophore. As the lifetime of excited states is on the order of nanoseconds, fluorescence
is sensitive toward processes occurring on this time scale and allows for resolution of conformational changes that are time-averaged in other techniques. Fluorescence measurements can be readily performed under a variety of solvent conditions and have the additional advantage of high sensitivity and excellent time resolution.

Fluorescence resonance energy transfer (FRET) is the result of a long-range nonradiative coupling between two fluorophores (16), termed donor and acceptor, that is dependent on the interfluorophore distance and therefore allows for distance mapping, typically over a range of 10 to 100 Å. In contrast to conventional structural biology techniques that usually do not report on conformational changes, FRET provides structural, thermodynamic, and kinetic information on conformational transitions of flexible molecules in solution and is suitable even for large disordered complexes that are not amenable to study by nuclear magnetic resonance (NMR) spectroscopy or X-ray crystallography. Whereas steady-state FRET is limited to average distances, time-resolved FRET (trFRET) provides more dynamic information and can be used to distinguish between different conformers in equilibrium and thereby quantify free energy differences between conformers.

In this review, the requirements and underlying principles of steady-state and time-resolved FRET are explained. Approaches to study RNA conformation and folding with FRET, including distance mapping, kinetics and thermodynamics, ion binding, and conformational equilibria, are described in principle and illustrated with selected examples.

**DESCRIPTION OF METHOD**

**Fluorophores and Labeling**

In FRET experiments changes in donor fluorescence in the absence and presence of acceptor are measured. If the acceptor is fluorescent, induced fluorescence (sensitized emission) can be monitored instead of donor quenching. As the common bases in RNA do not exhibit fluorescence, attachment of extrinsic fluorophores is usually necessary. Site-specific labeling with fluorescent probes is most readily achieved through direct chemical synthesis of RNA. Donor and acceptor probes can be directly incorporated during oligonucleotide synthesis using suitably derivatized phosphoramidites or attached postsynthetically to specifically incorporated reactive functional groups (usually primary amino groups). End-labeling is most common, either with amino linkers introduced at the 5’ and/or 3’ end during solid-phase synthesis and postsynthetic attachment of the fluorescent moiety or introduction of the fluorophore on the solid support on the 5’ end. Reactive groups can also be introduced elsewhere in the sequence for attachment of fluorescent probes at internal positions.

Fluorescein and cyanine dyes are commercially available as phosphoramidites and are compatible with the conditions during solid-phase synthesis and deprotection, whereas rhodamine dyes are not stable under these conditions and thus have to be attached postsynthetically (17). Fluorophores are attached to amino groups (5’ or 3’ end, or internal) as succinimidyloxystyryl or isothiocyanate derivatives. Common suppliers for fluorophores are Molecular Probes (Eugene, OR), Amersham (Arlington Heights, IL), and Sigma-Aldrich (St. Louis, MO).

Often an n-carbon linker (n = 2-12) separates the fluorescent moiety from the RNA to allow for free rotation of the dye, thereby minimizing uncertainties in the orientation factor $k^2$ (see below). Linkers also minimize dye quenching in a hydrophobic environment due to stacking on helix ends.

Selectivity of labeling is usually achieved due to the limited number of reactive groups. Likewise, the choice of the labeling position is more straightforward compared with protein labeling as potential interference with folding is predictable. End labeling and internal labeling of RNA with fluorescent moieties has been reviewed recently (17). Coupling of a fluorophore to the 2’ position of the ribose has also been described (18). Reliable results were obtained with FRET and a DNA containing a single 2’-modified ribose (19). It remains to be seen if this labeling procedure is applicable to RNA.

Common donor-acceptor pairs are fluorescein and tetramethylrhodamine, or fluorescein and the cyanine dye Cy3 (see examples), though various pairs have been used for certain systems (20). Apart from the appropriate $R_0$ value (see later) for the distances to be determined, high photostability and selective excitation of the donor without interference from other chromophores are desired properties.

**Steady-State FRET**

In the simplest implementation of FRET, the donor is excited and the distance between donor and
acceptor is calculated from changes in the steady-state fluorescence emission intensity of the donor in the absence and presence of the acceptor. Steady-state fluorescence spectrometers equipped with xenon lamps for continuous excitation are commercially available from various manufacturers. The steady-state emission is an intensity-weighted average of the underlying decay processes and is related to the average fluorescence lifetime by

$$I = \int_0^\infty l(t) \exp \left(-\frac{t}{\tau}\right) dt = I_0 \tau,$$  \[1\]

where $l_0$ is a parameter that depends on the fluorophore concentration and instrument settings (sensitivity) and $\tau$ is the average fluorescence lifetime:

$$\tau = \frac{1}{k_r + k_{nr} + k_p + k_{et} + k_q [Q]}.$$  \[2\]

$\tau$ depends on the rate constants for radiative and nonradiative deactivation of the excited state, $k_r$ and $k_{nr}$, respectively, the rate constant for photochemical processes starting from the excited state, $k_p$, the rate constant of energy transfer, $k_{et}$, and the bimolecular rate constant for collisional quenching, $k_q$, when an exogenous quencher is present at concentration [Q]. The different processes contributing to $\tau$ cause its dependence on the molecular environment. Equations [1] and [2] show that in the presence of an acceptor, the donor fluorescence lifetime will be reduced as a result of the additional term $k_{et}$ in the denominator, leading to a decrease in the steady-state fluorescence intensity.

The energy transfer rate constant $k_{et}$ depends on the inverse sixth power of the distance between the fluorophores involved in the dipolar coupling,

$$k_{et} = \frac{1}{\tau_D \left(\frac{R_0}{R}\right)^6},$$  \[3\]

where $\tau_D$ is the donor fluorescence lifetime, $R_0$ the characteristic transfer distance or Förster distance (16), and $R$ the actual distance between donor and acceptor. For the FRET efficiency $E$, the following dependence on the distance between donor and acceptor can be derived:

$$E = \frac{R_0^6}{R_0^6 + R^6}.$$  \[4\]

$R_0$ is a characteristic of the particular donor-acceptor pair and defines the distance at which the transfer efficiency is 0.5. The value is determined by the spectral properties of the respective donor-acceptor pair and can be calculated from their spectra (see below).

Experimentally, the FRET efficiency is calculated according to:

$$E = 1 - \frac{I_{DA}}{I_D}.$$  \[5\]

$I_{DA}$ and $I_D$ are the normalized donor intensities in the presence and absence of acceptor, respectively. This normalization to the same donor concentration is not required in time-resolved measurements, where the FRET efficiency can be directly deduced from the amplitude-averaged lifetimes of the donor in the presence ($\langle \tau_{DA} \rangle$) and absence ($\langle \tau_D \rangle$) of the acceptor:

$$E = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_D \rangle}.$$  \[6\]

Thus, inaccuracies due to erroneous concentration determinations are avoided in trFRET.

The concentrations of the fluorophores, however, may be critical in some cases. Intermolecular energy transfer can occur if a critical concentration of the acceptor, $[A]_{crit}$ (in M), is exceeded. It can be estimated according to:

$$[A]_{crit} = \frac{447}{R_0^3}.$$  \[7\]

when the critical transfer distance $R_0$ (in Å) is known. For common values of $R_0$, however, $[A]_{crit}$ will be in the millimolar range and is rarely exceeded in biological samples.

As the steady-state emission represents an intensity-weighted average of the individual decays, distances obtained from steady-state FRET experiments are weighted averages of all distances existing in the sample. Therefore results have to be interpreted carefully in terms of actual intramolecular distances. FRET is a very useful probe, however, to monitor ligand binding in equilibrium titrations or kinetic experiments, to compare mutant variants of a particular RNA molecule, or in cases where the existence of multiple conformers can be excluded.
Time-Resolved FRET

A considerable amount of additional information is available from time-resolved measurements of FRET. In this approach, information on the donor-acceptor distance is obtained from analysis of the time-resolved decay of the donor emission. Additionally, because time-resolved fluorescence spectroscopy monitors events occurring during the lifetime of the excited state (few picoseconds to hundreds of nanoseconds) that are averaged over steady-state experiments, trFRET allows for simultaneous detection of two or more conformations of an RNA molecule.

There are two alternative approaches to time-resolved fluorescence spectroscopy: impulse response (time-domain) and harmonic response (frequency-domain) experiments. For time-domain experiments, pulsed excitation is necessary. Picosecond pulses may be obtained from mode-locked argon ion, dye, or solid-state lasers (Ti-sapphire) with fixed or tunable excitation wavelengths. The emission is measured with rapid detection techniques such as time-correlated single photon counting and microchannel plate detectors that provide picosecond resolution. As no commercially available setup for these types of experiments exists, it has to be assembled from the individual components. A detailed description of time-resolved fluorescence instrumentation can be found in (21–24).

In frequency-domain time-resolved fluorescence spectroscopy, the sample is excited with sinusoidally modulated light at a certain frequency f. The phase shift \( \varphi \) of emitted light relative to the excitation reports on changes in lifetime, as does the reduced modulation depth m:

\[
\tan(\varphi) = f \tau, \quad [8]
\]

\[
m = \frac{1}{\sqrt{1 + f^2 \tau^2}}. \quad [9]
\]

If more than one lifetime is present, a range of frequencies are required. Excitation is done with arc lamps or continuous-wave lasers modulated with Pockels cells, acoustooptic modulators, or Kerr cells. Integrated systems are commercially available from various manufacturers. While impulse response and harmonic response methods are equivalent in principle, they may differ with respect to sensitivity, precision, and statistical quality of the data.

In principle, donor quenching and sensitized acceptor emission are equivalent probes for FRET. However, complications may arise in cases where the acceptor shows some absorbance at the excitation wavelength of the donor. In this laboratory, trFRET experiments are analyzed in terms of the nanosecond emission decay kinetics of the donor, thereby avoiding potential complications due to direct excitation of the acceptor fluorophore. From the fluorescence decay of a sample labeled only with the fluorescence donor \( I_D(t) \), the intrinsic donor decay parameters are extracted according to:

\[
I_D = \sum \alpha_i \exp \left( -\frac{t}{\tau} \right), \quad [10]
\]

where \( \alpha_i \) is the decay amplitude associated with the fluorescence lifetime \( \tau \). A multiexponential decay of a single fluorophore may arise from heterogeneity due to different microenvironments. These could be different conformations of the molecule or excited state reactions like exciplex formation or excited state proton transfer.

The decay of donor fluorescence in the presence of acceptor \( I_{DA}(t) \) is described in terms of contributions from one or more D–A species, as in:

\[
I_{DA} = \sum_k f_k \int P_k(R) \sum \alpha_i \exp \left[ -\frac{t}{\tau} \left( 1 + \frac{R^6}{R^6} \right) \right] dR, \quad [11]
\]

where \( f_k \) is the fractional population of species \( k \), \( P_k(R) \) is the corresponding distance distribution, and \( R \) is the donor-acceptor distance. The distance distribution \( P_k(R) \) for each species is modeled by a Gaussian profile,

\[
P_k(R) = 4\pi R^2 c \exp \left[ -a_k(R - b_k)^2 \right], \quad [12]
\]

where \( a_k \) and \( b_k \) describe the shape and c is a normalizing factor. Equations [11] and [12] are used to fit experimental data by adjusting the values of \( f_k \), \( a_k \), and \( b_k \) for each D–A species. Usually, experimental data describe the average donor-acceptor distance better than the shape of the distribution, though distance information is lost if the distance is either too long or too short. The shape tends to be better defined if the mean distance is less than \( R_0 \), whereas the average distance is measured more accurately when slightly larger than \( R_0 \) (25).
The lifetimes $\tau_i$ obtained from a time-resolved fluorescence experiment are related to the average lifetime ($\tau$) from the corresponding steady-state experiment by

$$\tau_{\text{ave}} = \sum_i w_i \tau_i.$$  \[13\]

Due to the finite duration of the excitation pulse, measured fluorescence decays $I(t)$ are a convolution of the true decay of the fluorophore $F(t)$ and the exciting pulse filtered by the electronic response of the instrument, $R(t)$:

$$I(t) = R(t) \otimes F(t),$$  \[14\]

where the operator $\otimes$ denotes the convolution of the two functions. The instrument response $R(t)$ can be measured from scattering solutions that do not exhibit fluorescence, e.g., dilute suspensions of silica or non-dairy creamer. The convolution operation specified in Eq. [14] must be applied when fitting either $I_D(t)$ or $I_{DA}(t)$.

**Determination of $R_0$**

The Förster distance or characteristic transfer distance $R_0$ determines the range of distances that can be assessed by FRET using a particular donor-acceptor pair. It can be calculated from the spectral properties of the donor and acceptor according to

$$R_0 = 8.785 \times 10^{-5} \frac{\kappa^2 \phi_D J}{n^4},$$  \[15\]

where $\kappa^2$ is the orientation factor, $\phi_D$ the quantum yield of the donor fluorescence, and $J$ the overlap integral between donor emission and acceptor absorbance. The refractive index of medium ($n$) is usually 1.4 for aqueous solutions. The overlap integral $J$ can be calculated from the normalized emission spectrum of the donor and the absorbance spectrum of the acceptor:

$$J = \int F_D(\lambda) e_A(\lambda) \lambda^4 \, d\lambda.$$  \[16\]

With the extinction coefficient $e$ in $M^{-1} \text{cm}^{-1}$ and $\lambda$ in nm, substitution of the value for $J$ into Eq. [15] yields the Förster distance $R_0$ in Å.

The quantum yield $\phi_D$ of the donor emission can be measured in comparison to a reference substance with known quantum yield $\phi_{\text{ref}}$.

$$\phi_D = \frac{I_D \phi_{\text{ref}} A_{\text{ref}}}{A_D I_{\text{ref}}}$$  \[17\]

$A_D$ and $I_D$ are the absorbance and emission intensity of the donor, respectively, and $A_{\text{ref}}$ and $I_{\text{ref}}$ the corresponding values for the reference fluorophore.

A list of Förster distances of various compounds can be found in (26). They range from 10 to 70 Å, which allows the determination of distances up to 120 Å. The highest $R_0$ value (80 Å) has been reported for the rhodamine B–malachite green pair (27). From Eq. [4] it becomes obvious that a donor–acceptor pair instrument, $R(t)$:

$$\phi_D = \frac{I_D \phi_{\text{ref}} A_{\text{ref}}}{A_D I_{\text{ref}}}$$  \[17\]

$A_D$ and $I_D$ are the absorbance and emission intensity of the donor, respectively, and $A_{\text{ref}}$ and $I_{\text{ref}}$ the corresponding values for the reference fluorophore.

FRET efficiency is dependent not only on the inter-fluorophore distance, but also on the relative orientation of donor and acceptor fluorophores. This is accounted for by introducing the orientation factor $\kappa^2$ (Eq. [15]). As $\kappa^2$ can lead to a multiplication of the distance by a factor between 0 and 1.26 (25) it is crucial to know its value to determine a meaningful donor–acceptor distance. From fluorescence anisotropy measurements, an angle for a cone describing the fluorophore mobility can be derived to narrow the range for $\kappa^2$. Only in two cases can an exact value for $\kappa^2$ be assigned: nonrestricted motion of the fluorophores leads to the dynamically averaged $\kappa^2$ of 2/3, whereas completely restricted motion requires the transition dipoles of donor and acceptor fluorophores to be known in both length and direction to calculate $\kappa^2$ correctly. Transition dipole directions are rarely known, however, while the former case can be achieved using appropriate linkers for the attachment of fluorophores.

**APPLICATIONS OF FRET TO RNA**

FRET has been applied to nucleic acids as a qualitative probe (30–49), to obtain average distances (50–69) and distance distributions (70–74). Apart from long-range distance information, FRET can be used as a probe to detect conformational changes that are induced by ligand binding and to assess the affinity for these ligands. Kinetics of structural transitions can be monitored by FRET with donor
FIG. 1. Applications of FRET to map distances and to monitor conformational kinetics and ion binding to RNA. (A) Distance mapping. From a series of experiments with molecules labeled with donor and acceptor fluorophores at different sites, a set of intramolecular distances can be derived that defines the global structure and yields information on stacking of helical arms. The distance pattern shown in the center of the figure corresponds to the X-shape conformation of the four-way junction shown on the right. This rationale was applied to different RNA four-way junctions (68, 69) and in a more quantitative way to the hammerhead ribozyme (64). (B) Kinetics of conformational changes. With donor and acceptor fluorophores attached to different sites on the molecule that experience a change in distance on the conformational transition to be studied, the donor fluorescence can be used to monitor the folding kinetics. The reaction is initiated by rapid mixing of the RNA with a ligand that induces the transition. In the example depicted, the decrease in donor fluorescence and the concomitant increase in acceptor fluorescence report on a shortening of the interfluorophore distance and allow for continuous real-time monitoring of the conformational transition. Examples where FRET has been used as a probe to monitor kinetics comprise substrate binding, cleavage, and product release by the hammerhead and hairpin ribozymes (45, 49). (C) Ion binding. Metal ions stabilize RNA tertiary structures. As a result, ion binding is frequently linked to a conformational change. Placing donor and acceptor fluorophores on parts of the RNA that differ in distance in the two conformers allows for monitoring ion binding with FRET. In the schematic example shown, a magnesium ion binds to a three-way junction, resulting in a shortening of the distance between two of the helices. The titration curves can be analyzed in terms of the appropriate binding model (93) using the FRET efficiency either directly or after calculating the fraction of RNA-ion complex from the fluorescence. Similarly, these data can be obtained from trFRET measurements that allow for quantification of the two conformers in equilibrium under all ion concentrations. Metal binding to the hammerhead (94) and hairpin ribozymes (74) has been studied using steady-state and trFRET, respectively.
and acceptor fluorophores in suitable positions. Fluorescence assays generally benefit from being continuous and provide the same information as discontinuous sampling and analysis that can be tedious and time consuming. Insights that can be gained from trFRET measurements include the identification and quantification of multiple tertiary structure conformers as well as sampling of (slightly) different species under different solution conditions. Equilibrium constants for interconverting conformers contain information on the underlying thermodynamics, rendering FRET useful as a probe to determine enthalpic and entropic contributions to structural transitions. In certain cases, it is possible to determine changes in heat capacity (ΔC_p) from samples too dilute for a direct measurement using calorimetry (75).

Distance Mapping

FRET provides distance information in the 10–100 Å range and thus is an ideal probe to analyze the global structure of RNA. Using a series of doubly labeled RNA molecules with the probes attached to multiple sites and the corresponding molecules with only the donor, a set of intramolecular distances can be determined that define the global structure or can be used as structural constraints for modeling (Fig. 1A). In steady-state FRET experiments, the donor fluorescence intensity of each construct is measured in the absence and presence of the acceptor and the FRET efficiency is calculated according to Eq. [5]. From Eq. [4], the average interfluorophore distance can be calculated from a known R_0. Similarly, the distances can be determined with trFRET experiments from the decay of the donor fluorescence in the presence and absence of the acceptor (Eqs. [6] and [8]–[13]).

Global structures reveal the overall arrangement of helices and other structural elements. They can be deduced from relative distances and therefore do not necessarily require knowledge of the exact orientation factor χ^2. In contrast, the value for χ^2 has to be known accurately to determine reliable distances as constraints for modeling. Tuschl and co-workers (64) derived a three-dimensional model for the hammerhead ribozyme from steady-state FRET measurements interpreted on the basis of vector algebra analysis. The Y-shape deduced from FRET was confirmed by the crystal structure (76).

Though FRET methods cannot provide high-resolution structures, the technique is extremely useful for molecules that are not amenable to NMR or X-ray crystallography. This is illustrated by the wealth of information that was gained on ribosome structure using distance mapping with FRET. Early FRET experiments on RNA were performed mainly with tRNAs containing nonstandard bases that were amenable to site-specific chemical modification and introduction of fluorophores. The intrinsic fluorescence of the natural Y base was exploited as a donor to determine the distance of the anticodon in tRNA_Phe from the 3’-CCA terminus (50). The terminal ribose on the 3’-CCA end was oxidized to provide a functional group for coupling acceptor fluorophores (acridine, proflavine, and acridine). From steady-state FRET efficiencies the donor–acceptor distance was determined to be >40 Å, which allowed exclusion of some of the proposed tertiary structure models. A more extensive study was performed on tRNA^{Met}, introducing fluorophores on the 5’ and 3’ ends as well as reacting pseudouridine and dihydrouridine to define the global structure (51). The results obtained from lifetime measurements of the donor (dansyl and anthraniloyl) fluorescence in the presence and absence of the acceptor (proflavine and acridine) were consistent with the proposed cloverleaf structure that was confirmed in the tRNA_Phe crystal structure (77). The excellent agreement of the FRET-derived distances and the respective distances in the crystal structure proves that FRET is a powerful tool for structural studies on biomolecules.

The arrangement of rRNAs in the ribosome was probed with FRET (53). With rRNAs labeled on the 3’ end, ribosomes were assembled and distances between the 3’ ends were determined. Robbins and colleagues addressed the question of the relative position of tRNA_Phe on the ribosome using FRET (54). 16S rRNA was isolated, oxidized at the 3’ end, and labeled with fluorescein and eosine, and 30S subunits were reconstituted. tRNA_Phe was labeled at three different sites chosen from the crystal structure to be well-separated reference points. The labeled tRNA was bound into the peptidyl site of 70S ribosomes and the distances between the 3’ end of the 16S rRNA and the labeled positions on the tRNA were determined from FRET efficiencies calculated from changes in fluorescence lifetimes. The FRET results were consistent with the tRNA position estimated from affinity immunoelectron microscopy (80). However, one distance within the tRNA was significantly shorter than estimated from the crystal structure, indicating that the conformation of tRNA bound
to the ribosome might be altered to some extent compared with free tRNA. The distance between tRNAs bound at the ribosomal A and P sites was estimated using FRET between fMet-RNA\textsuperscript{fMet-AEDANS} (donor) and fluorescein-labeled Phe-tRNA\textsuperscript{Phe} [acceptor, (56)] or between two Phe-tRNA\textsuperscript{Phe} molecules, one containing the natural Y base or labeled with proflavine (donor), the second molecule labeled with proflavine or ethidium [acceptor, (55)]. Harmonic response FRET studies on the aminoacyl-tRNA-EFTu-GTP complex (with donor fluorescein and acceptor rhodamine attached to the aminoacyl-tRNA and the GTP ribose moiety, respectively), in combination with crosslinking results, limited the possible orientations of tRNA with respect to EFTu to a small subset (65).

The hairpin ribozyme from tobacco ringspot virus satellite RNA has been investigated using FRET to address a variety of questions. A minimal hairpin ribozyme consists of two independent helix–loop–helix elements with catalytically important residues in the two loops. Cleavage occurs in loop A and the substrate is bound to the ribozyme via base-pairing interactions within the two adjacent helices. The global fold of a hairpin ribozyme with loop A and loop B on different arms of a four-way helical junction that was labeled with fluorescein and Cy3 has been derived from all interarm distances in steady-state FRET experiments (69). The results showed that as a prerequisite for catalytic activity, a Mg\textsuperscript{2+}-induced conformational change brings the loops into close proximity. Characterization of the global fold of two other RNA four-way junctions in an experimentally similar way (68) revealed that both junctions show pairwise coaxial stacking of helices. Only one of the junctions, however, undergoes a Mg\textsuperscript{2+}-induced conformational change into an antiparallel structure, indicating that different structures can be formed by the same structural motif.

The influence of bulges on kinking of DNA and RNA helices has been investigated with FRET (63). Bulges of up to nine unopposed adenine nucleotides were introduced into a 18-bp RNA helix that was end-labeled with fluorescein and tetramethylrhodamine, and the bending angles due to the bulges were estimated from FRET efficiency. In these constructs, no heterogeneity of conformations was expected, providing a well-suited system for steady-state FRET with the apparent distances being real measures for the end to end distances. The results were consistent with a bending of DNA and RNA helices that is greater for larger bulges.

Kinetics of RNA Conformational Transitions

To follow the kinetics of a conformational transition, the RNA is labeled with donor and acceptor fluorophores at two sites that are expected to experience a change in distance during the transition. Using a stopped-flow mixing device attached to a steady-state fluorometer or a commercially available integrated stopped-flow spectrometer, the RNA is mixed rapidly with a ligand that induces the conformational transition, and the change in donor fluorescence is monitored over time. Rate constants can be extracted by fitting the transient fluorescence with a single exponential function, or a sum of exponentials functions if multiple kinetic phases are observed. The acceptor signal change should be an inversion of the donor fluorescence change and yield the same rate constants (Fig. 1B).

Again, an early example for this type of experiment involves ribosomes (30). In stopped-flow experiments, the energy transfer between wybutine- and proflavine labeled tRNAs bound to the ribosome was measured during translocation. The three kinetic phases observed were attributed to an ordered movement of the tRNAs between the ribosomal A, P, and E sites.

Since the discovery of catalytic function of certain structured RNAs (79–81), there has been ongoing interest in the structure and mechanism of catalytic activity of ribozymes. The hammerhead ribozyme was a major object of interest for FRET studies on RNA [for a review see (82)]. Although the global structure deduced from FRET (64) and the crystal structure (76) defined the Y-shape of the molecule and gave insights into stabilizing interactions, open questions remained on cleavage kinetics, conformational changes related to activity, and folding. The kinetics of the hammerhead ribozyme–substrate duplex formation have been investigated with FRET using a ribozyme with a rhodamine label attached to the 5' end (42). The substrate was labeled with fluorescein on the 3' end that, in the ribozyme–substrate complex, is adjacent to the rhodamine and therefore efficiently quenched. From the donor quenching on duplex formation it was found that the dissociation of the complex is faster than cleavage, which requires multiple interactions of substrates with the ribozyme to undergo cleavage. Cleavage was enhanced, however, by adding facilitator nucleotides that stabilize the duplex by accelerating its formation. Singh and co-workers (49) devised a system
for real-time monitoring of hammerhead ribozyme-catalyzed reactions under single- and multiple-turnover conditions. The substrate was labeled with fluorescein and tetramethylrhodamine on opposite ends, allowing for efficient FRET that was alleviated on cleavage and product dissociation. Fluorescence assays not only allow for continuous monitoring of reactions, but also avoid the time-consuming procedures of sampling, gel electrophoresis for separation of substrates and products, and quantification that are required in standard activity assays employing radiolabeled oligonucleotides.

The kinetics of substrate binding to the hairpin ribozyme and the subsequent cleavage and dissociation steps have been monitored in real time through base-specific quenching of a substrate that was fluorescein-labeled on its 3' end (43). When the substrate was bound to the ribozyme, a guanine added on the 5' end of the ribozyme led to quenching of the fluorescein emission, and a subsequent dequenching was observed on cleavage and dissociation. As product dissociation had been shown to be at least 10 times faster than transesterification steps (83), the cleavage step was rate-limiting and thus determined the observed rate of dequenching. Thus, the second-order binding constant for substrate and the rate constant for cleavage could be determined using this assay. The rate constant obtained from the fluorescence dequenching (2.5 \times 10^{-3} \text{s}^{-1}) showed excellent agreement with the value from a discontinuous measurement using radiolabeled substrate (1.9 \times 10^{-3} \text{s}^{-1}).

Analysis of Ion Binding to RNA

Metal ions influence the stability of RNA tertiary structures (for a review see (90)). Binding of divalent ions like Mg^{2+} is usually linked to a conformational transition:

$$\text{conformer}_1 + n\text{Me}^{2+} \rightleftharpoons \text{conformer}_2 \cdot n\text{Me}^{2+}.$$  \[18\]

With RNA that has donor and acceptor fluorophores attached to sites that change distance on the conformational transition, FRET can be used to monitor ion binding to RNA in equilibrium metal ion titrations (Fig. 1C). The fraction of the ion-bound form can be derived from the donor intensity. In the simplest case of a single class of metal binding sites, the titration data can be analyzed using the Hill equation yielding an apparent dissociation equilibrium constant,

$$f = f_{\text{max}} \frac{[\text{Me}^{2+}]^n}{[\text{Me}^{2+}]^n(K_d)^n},$$  \[19\]

with the actual fluorescence signal f at the respective metal ion concentration, [Me^{2+}], the amplitude of the fluorescence change f_{\text{max}}, the apparent dissociation equilibrium constant K_d as defined by Eq. [18], and the Hill coefficient n.

In a more general approach, the influence of metal ions on RNA tertiary structure can be explained by a differential binding of the ion to the two conformers. A stabilizing effect is the result of an ion binding more tightly to the folded than to the unfolded RNA conformer, resulting in a thermodynamic linkage of ion binding and the conformational transition (91). Thus, the equilibrium constant for the conformational change depends on the metal ion concentration according to

$$\ln K_{\text{obs}} = \ln K_0 + \ln Z_f - \ln Z_u,$$  \[20\]
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\[ K_{\text{dock}} = \frac{\text{fraction docked}}{\text{fraction undocked}} \]

\[ \Delta G_{\text{dock}} = -RT \ln K_{\text{dock}} \]

Diagram showing:
- Donor decay parameters: \( \alpha_i, \tau_i \)
- \( R_{\text{ave}} \), FWHM
- Fraction docked, fraction undocked
where $K_{\text{obs}}$ denotes the equilibrium constant at a certain metal ion concentration, $K_0$ is the equilibrium constant in the absence of metal ions, and $Z_i$ and $Z_o$ are the binding polynomials for the two RNA conformers (92). These binding polynomials are dependent on the metal ion concentration and the precise functional form depends on the model that describes the ion binding (93). Two general models to be distinguished are specific binding of one or more ions to specific sites that are present only in the folded conformation, and nonspecific binding to the same type of sites in both the folded and the unfolded conformers (usually phosphates) with different affinities. These models predict a different dependence of $K_{\text{obs}}$ on the metal ion concentration and, along with more sophisticated models that take into account several independent or coupled binding sites, as well as a mixture of specific and nonspecific binding sites, are described in (93).

The ion-induced folding of the hammerhead ribozyme has been characterized with steady-state FRET (94). Fluorescein (donor) and Cy3 (acceptor) were placed on the ends of the helical arms I and II, I and III, or II and III of the three-way helical junction, respectively, and the change in FRET efficiency on titration with Mg$^{2+}$ was measured. Using the FRET efficiencies as a relative measure for the interarm distances, the existence of an intermediate conformation at low Mg$^{2+}$ concentrations was inferred, in which the helical arms I and III are in close proximity and II and III are coaxially stacked. At higher Mg$^{2+}$ concentrations a conformational transition brings arms I and II into proximity while II and III remain stacked. Both transitions are linked to binding of a single Mg$^{2+}$ ion with apparent association constants of 10,000 and 1100 M$^{-1}$, respectively.

Using the hairpin ribozyme labeled with fluorescein and Cy3 on the arms carrying the loops, the interarm distances were determined in titrations with different metal ions (95), revealing that Mg$^{2+}$, Ca$^{2+}$, and Sr$^{2+}$ are capable of inducing the formation of the catalytically active conformer of the hairpin ribozyme with the loops in close proximity. In this case, the data obtained were best described assuming the cooperative binding of two ions being linked to folding.

Conformational Equilibria

Only in selected cases do RNA molecules exist as a single conformer in solution. Especially small structural motifs used as model systems to study RNA conformational changes and folding often form alternative conformations, giving rise to two or more donor–acceptor distances in FRET experiments. In these cases, steady-state FRET experiments yield only an average of the donor–acceptor distances in the different conformers, weighted by their relative population under the conditions of the measurement, and thus do not reflect true intramolecular distances.

Provided that there is a measurable difference in the donor–acceptor distances in the conformers present, with trFRET a heterogeneous conformer distribution can be identified and the relative populations of two or more conformers can be determined. These fractions immediately define the equilibrium constants and the corresponding differences in free energy between the conformers. Figure 2 illustrates as an example the procedure to determine free energy differences between the active (docked) and extended (undocked) conformers of the hairpin ribozyme. From the decay of the fluorescein-labeled ribozyme, the donor-only decay parameters are extracted. In the same ribozyme labeled with fluorescein (donor) and tetramethylrhodamine (acceptor), the donor fluorescence decays more rapidly due to energy transfer.

**FIG. 2.** Illustration of the procedure to determine the conformer distribution in equilibrium from trFRET data. Docking of the hairpin ribozyme decreases the distance between fluorescein (F, donor) and tetramethylrhodamine (T, acceptor) attached to the helical arms of the ribozyme, thereby increasing the FRET efficiency. The movement around the hinge of the helical junction is indicated by the double-headed arrow. The donor fluorescence decay is accelerated in the presence of the acceptor. This effect becomes more pronounced if the ribozyme exists mainly in the docked conformation. From ribozymes labeled with fluorescein only, the intrinsic donor decay parameters ($\alpha$, $\tau$) can be measured. With the doubly labeled ribozyme constructs, the decays in the presence of the acceptor can be analyzed in terms of two conformers with different distance distributions that are modeled with Gaussian profiles (see Description of Method). From this analysis, a mean distance ($R_{\text{ave}}$) and a width (FWHM) for each conformer are obtained. The mean distance reflects the average interfluorophore distance, whereas the width contains contributions from the mobility of the fluorophore and the internal dynamics of the conformer. The area under the distribution curve is proportional to the fraction of the respective conformer and affords the equilibrium constant, $K_{\text{dock}}$, that is related to the free energy of docking, $\Delta G_{\text{dock}}$. Thus, thermodynamic information on conformational changes can be derived from changes in FRET. This approach has been used to characterize tertiary structure formation in the hairpin ribozyme (74).
Evaluation of the decay according to Eqs. [10]–[12] yields the distance distribution for the donor–acceptor distances in the conformers. The distributions are defined by a mean distance and a width (FWHM) that reflects the mobility of the fluorophore and the internal dynamics of the molecule. Together, these parameters define the area under the curve, which is proportional to the population of this conformer in equilibrium. From the ratio of the fraction of docked and undocked ribozymes, $K_{dock}$ can be calculated, which is related to the free energy $\Delta G_{dock}$ that stabilizes the docked conformer relative to the extended ribozyme.

Using trFRET to study the formation of hairpin ribozyme tertiary structure with ribozymes labeled with fluorescein and tetramethylrhodamine on the helical arms, different propensities for the docked conformer could be determined that depend on the nature of the helical junction (74). In the four-way junction the docked conformer is highly favored (95%), whereas only 65% of ribozymes containing a two-way junction or 40% of those with a three-way junction are docked. These results suggest that the four-way junction stabilizes the docked conformation, presumably by prearranging the two loops in proximity to each other or by providing additional favorable interactions between the helical arms. In contrast, the three-way junction may impose structural constraints on the relative orientation of the two arms carrying the loops that actually interfere with docking.

By applying the same approach to modified ribozymes it could be demonstrated that several nucleotide alterations that have been reported to cause a drop in ribozyme activity actually render the ribozyme impaired in docking (74). Similarly, trFRET revealed the different requirements for Mg$^{2+}$ on docking of the two-way, three-way, and four-way junction ribozymes. The apparent dissociation constants for Mg$^{2+}$, determined from Eq. [19], were 3.3 mM (two-way junction), 0.6 mM (three-way junction), and 0.1 mM (four-way junction), and implied that under physiological Mg$^{2+}$ concentrations of $\sim$1 mM only the four-way junction is completely folded and catalytically active. These results suggest that a four-way junction ribozyme may be more promising for therapeutic applications than a minimal two-way junction hairpin ribozyme that has been the basis for strategies of applications in gene therapy (96).

Thermodynamics

The dependence of the equilibrium constants on temperature provides information on enthalpic ($\Delta H^0$) and entropic ($\Delta S^0$) contributions to the free energy ($\Delta G^0$) of a conformer. As illustrated in the previous section, conformer distributions in equilibrium can be quantified with trFRET to yield equilibrium constants. From measurements performed at different temperatures within the spectroscopically accessible range of 4 to 90°C, thermodynamic data can be derived by fitting $\Delta H^0$ and $\Delta S^0$ according to the van’t Hoff equation

$$\ln K = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R}, \quad [21]$$

where $K$ is the equilibrium constant at temperature $T$, and $R$ is the general gas constant (8.3143 J mol$^{-1}$ K$^{-1}$). A curved van’t Hoff plot ($\ln K$ vs $1/T$) indicates that $\Delta H^0$ and $\Delta S^0$ are temperature-dependent due to a change in heat capacity associated with the transition. In this general case, the curvature defines $\Delta C_p^0$, and the applicable equations are

$$\ln K = \frac{\Delta C_p^0}{R} \left( \frac{T_H}{T} - \ln \frac{T_S}{T} - 1 \right), \quad [22]$$

$$\Delta H^0 = \Delta C_p^0(T - T_H), \quad [23]$$

$$\Delta S^0 = \Delta C_p^0 \left( \ln \frac{T}{T_S} \right) \quad [24]$$

where $\Delta C_p^0$ is the change in heat capacity, and $T_H$ and $T_S$ are the temperatures at which $\Delta H$ and $\Delta S$ are zero.

Accordingly, the dependence of the hairpin ribozyme docking equilibrium constant on temperature potentially provides information on the enthalpic and entropic contributions to the stabilization of the docked conformer and should reveal the thermodynamic reason for the different docking propensities of the two-, three-, and four-way junction. trFRET measurements in the temperature range 10 to 60°C showed that the two-way and three-way junction ribozymes exhibit some degree of undocking at elevated temperature under standard conditions of 12 mM Mg$^{2+}$, while the four-way junction ribozyme remains stably docked up to 60°C (74). Consequently,
no comparative thermodynamic analysis could be performed. We recently succeeded in characterizing the docking thermodynamics of all three ribozymes in detail under a physiological Mg²⁺ concentration of 1 mM (75) where the four-way junction ribozyme is sufficiently destabilized to undergo undocking within the spectroscopically accessible temperature range. UV melting curves, however, showed that the tertiary structure transition overlaps with the disruption of secondary structure. Combining the information from UV melting (secondary structure transition) and trFRET (tertiary structure transition), both structural changes could be thermodynamically characterized simultaneously by evaluating the FRET data with a three-species model. This shows that trFRET is a powerful tool to investigate conformational equilibria, even in complex cases when supplementary data can be obtained with other methods.

CONCLUDING REMARKS

FRET techniques have provided a wide variety of information on nucleic acid conformation in the past. In addition to structural information, thermodynamics and kinetics of conformational changes involved in RNA folding and activity can be investigated with FRET. Due to more versatile labeling techniques and ongoing interest in the important biological functions of RNA, more results are to be expected. FRET turned out to be extremely powerful, especially if combined with other methods. More and more atomic resolution structures of RNAs are becoming available and provide well-characterized systems, where FRET can be applied to study details of conformational changes and dynamics. Suitable positions for fluorescent labels can be rationally chosen on the basis of the three-dimensional structure of the RNA and the insight gained with FRET provides complementary information on thermodynamics of structural transitions and their kinetics in real time. Development of new fluorophores with microsecond lifetimes and tunable emission wavelengths based on lanthanides (97) will extend the applications of FRET and potentially alleviate requirements on instrumentation for fluorescence detection in the nanosecond regime.

FRET is a sensitive probe that has recently been exploited in high-throughput screening with fluorescently labeled DNAs to identify inhibitors of DNA helicase activity (98) and will be useful for drug development involving RNA targets (99–102). On the other hand, recent single molecule studies on freely diffusing DNA molecules have demonstrated that mean distances determined from FRET between single donor–acceptor pairs qualitatively agree with predictions from Förster theory (103). Ha and coworkers (104) monitored the Mg²⁺-induced conformational changes in the S15 binding site of 16S rRNA on a single-molecule level with an immobilized RNA three-way junction and showed that the motion of a single molecule can be tracked with FRET. The average behavior over a large number of molecules agreed with results previously obtained with different techniques. These encouraging results illustrate that the extension of FRET to the single molecule level will contribute substantially to our knowledge of RNA conformational changes in the near future.

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