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Minireview

Fluorescence resonance energy transfer in studies of inter-chromophoric distances in biomolecules*

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Fluorescence resonance energy transfer (FRET) is a technique widely used in studies of interchromophoric distances in biomolecules such as peptides, proteins and nucleic acids. FRET is especially useful in determination of conformational changes caused by a solvent, presence of denaturing agents, diffusion and other external factors. Precision of interchromophoric distances obtained using the FRET technique is comparable with that of low-resolution X-ray diffraction and NMR data. Comparison of FRET results with the crystal structure for several proteins is reviewed. Moreover, the effect of the orientation factor κ^2 value on FRET results and determinants of κ^2 are discussed.

Trapping of electronic excitation by an acceptor has been intensively studied theoretically and experimentally for at least five last decades. Fluorescence resonance energy transfer (FRET) has found numerous applications in studies of biological macromolecules.

Fluorescence resonance energy transfer is the result of a distance dependent, through-space and very week (often 2–4 cm^{-1} compared to the spectroscopic energies that are interchanged, 15000–40000 cm^{-1}) dipole-dipole interaction which occurs between an excited donor and an unexcited acceptor. A physical theory predicting a distance dependence of energy transfer was proposed by Förster [1–4]. Very good descriptions of the theory of FRET were also given by: Turro [5], Speiser [6] and Clegg [7].

The process of energy transfer can be depicted mechanistically as a transition between two chromophores (donor (D*) and acceptor (A))

$$(D^*, A) \xrightarrow{k_T(r)} (D, A^*) \qquad (1)$$

where k_T is the rate constant of FRET between a particular D–A pair.

Förster theory predicts that energy could be transferred by the resonance dipole-dipole mechanism over a distance of 10–100 Å, de-

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DNS, 5-[dimethylamino]naphthalene-1-sulfonyl chloride; FRET, fluorescence resonance energy transfer; POPC, 1-palmitoyl-2-oleoyl-L-α-phosphatidylcholine; XRD, X-ray diffraction technique.

pending on the spectroscopic parameters of D and A; usually D and A should not be closer than 10 Å to avoid strong ground-state D–A interactions or energy transfer by exchange mechanism [5, 6, 8].

Förster theory describes the rate of energy transfer by the following equation:

$$k_T = 1/\tau_D\,(R_0/r)^6 \tag{2}$$

where: τ_D is the donor lifetime in the absence of acceptor, r is the donor-acceptor distance and R_0 (the Förster critical distance) is a distance calculated from spectroscopic data and mutual dipole orientation of D and A. R_0 defines the distance between the donor and acceptor chromophores at which the probability of donor deexcitation by energy transfer is equal to the sum of probabilities of all other deactivation processes that occur in the absence of the acceptor (from Eqn. (2) for $r = R_0 \Rightarrow k_T = 1/\tau_D$). The Förster critical distance is defined by equation:

$$R_0^6 = \frac{9(\ln 10)\kappa^2 Q_D J}{128\pi^5 n^4 N} \tag{3}$$

where: κ^2 is the orientation factor for dipole-dipole interaction determined by the angle between the donor and acceptor dipoles, Q_D is the fluorescence quantum yield of the donor in the absence of the acceptor, n is the refraction index of the medium between the donor and the acceptor, N is Avogadro's number and J is the normalized spectral overlap integral, given by

$$J = \frac{\int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int F_D(\lambda)d\lambda} \tag{4}$$

where: $F_D(\lambda)$ is the fluorescence intensity of the donor in the absence of the acceptor at wavelength λ, and $\varepsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor at λ.

The efficiency of the energy transfer, E_T, is a quantitative measure of the number of quanta that are transferred from D to A. From a dynamic point of view, E_T is the ratio of k_T to the total sum of rate constants of all processes by which an excited D molecule can return to its ground state, including k_T

$$E_T = \frac{k_T}{k_T + \sum_{i \neq T} k_i} = k_T \tau_{DA} \tag{5}$$

where subscript i refers to the different pathways of deactivation from the D* state, and τ_{DA} is the measured fluorescence lifetime of D* in the presence of A. E_T can be measured in numerous ways; steady-state and time-resolved measurement are employed. The choice of the measurement method depends on the application and the information desired. E_T is usually determined by measurements of:

- 1) enhanced fluorescence of the acceptor (acceptor must fluoresce for this method),
- 2) decreased fluorescence quantum yield of the donor,
- 3) decrease of the donor fluorescence lifetimes,
- 4) change in the fluorescence anisotropy of the donor and acceptor,
- 5) donor [9, 10] or acceptor [11] photobleaching.

Detailed descriptions of these methods can be found in many monographs [7, 12–21].

From the efficiency of energy transfer, E_T, the distance r between the donor and the acceptor may be calculated using the equation:

$$E_T = \frac{R_0^6}{R_0^6 + r^6} \tag{6}.$$

This predicted distance dependence was verified by fluorescence studies of donor-acceptor pairs separated by known distances in well-defined model systems. Stryer & Haugland [22] suggested that energy transfer could be used as a "spectroscopic ruler" within the 10–60 Å range to measure distances in biological macromolecules. The practical importance of R_0 is that it provides a tool for estimating the range of distances for which FRET can be observed for any given probe pair. Steady-state FRET experiments

can be carried out within the value of ±50% of R_0. Outside of these distance limits it is still possible to estimate r using fluorescence lifetime determination but the uncertainty is increased, and in any case the limits should not exceed ±85–90% of R_0. It is therefore helpful to have some estimate of the distance of interest before a FRET measurement is undertaken.

The values of R_0 for various donor-acceptor pairs can be found in many publications [12, 16, 18, 21, 23, 24]. The highest R_0 do not exceed 60 Å. The largest R_0 = 90 Å was obtained for a D–A pair in which europium cryptant was the donor and allophycocyanin was the acceptor [25].

Perhaps the greatest impact of FRET has resulted from its application to biological macromolecules. An outstanding feature of all biological systems is the intricate and interdependent organization of their various structures. FRET is unique in its capacity to supply accurate spatial information about molecular structures at distances from approx. 10 to about 100 Å. In many cases, geometrical information can also be obtained [20, 26–29]. This possibility of detecting and quantifying molecular proximities decided that the FRET method has been used very extensively in biology over the past 30 years and continues, at present, to evoke a vivid and increasing interest.

The question arises whether the FRET method is good enough to determine absolute interchromophoric distances with the precision comparable to that of X-ray diffraction technique. To answer that question one has to consider parameters that influence a measured interchromophoric distance. The energy transfer is usually measured in a solution. The chromophores: donors or acceptors of energy are connected covalently to a studied molecule. These chromophores are mainly of other than protein origin. Only tryptophan and more rarely tyrosine exhibit fluorescence that is intensive enough, and can be used as energy donors. Phenylalanine, because of a small molar absorption factor and a low quantum yield of fluorescence, is used in FRET measurements very seldom. External chromophores (a large selection of them one can find in many papers [30–34]) are coupled to specific sites in peptides or proteins *via* a spacer so that the interchromophoric distance is greater from that between the selected sites by about 5–10 Å, although this is not true for metal cations which can be used as donors or acceptors. FRET occurs between delocalized electrons in the probes rather than between the side chains of the amino-acid residues. This also leads to errors in distance determination.

Peptides, polypeptides, and proteins (to some extent) have some conformational freedom connected with flexibility of the peptidic backbone. Application of time-resolved fluorescence spectroscopy allowed to determine not only the mean interchromophoric distance, like in steady-state measurements, but also the distance distribution as well (for steady-state measurements it is also possible to determine the distance distribution using an external quencher or D–A pairs with various R_0 values [35–38]). Such measurements were performed for peptides exhibiting different levels of structural arrangement in a solution [39–44], and proteins like myosin [45], bovine pancreatic trypsin inhibitor (BPTI) [28, 29, 46, 47], troponins [48, 49], nucleases [50, 51], ribonuclease A [52, 53] and glycopeptides [54, 55]. For biomolecules showing a well-defined structure in solution like melittin in methanol, proteins, and glycopeptides the function describing the distance distribution (Gauss's [28, 29, 39–53] or Lorentz's [54, 55]) is characterized by small half-width, and its most probable value (a mean value of a distribution) can be compared with the average value obtained from steady-state measurements. From FRET measurements with the use of time-resolved spectroscopy it is possible to estimate not only the distance distributions but also coefficients of mutual diffusion of chromophores connected with a flexible fragment of the studied molecule [41, 43, 56, 57].

The coefficients of mutual diffusion of chromophores measured with the use of FRET, are lower by at least one order of magnitude than diffusion coefficients observed for unconstrained chromophores in the same conditions [57]. For large biomolecules such as proteins, or for measurements in a viscous solution using energy donors with lifetimes

in a nanosecond range it is not possible to determine accurately the diffusion coefficients. It is only possible to establish the upper limits of the coefficient values [57]. If during the calculation of the distance distribution function, diffusion is not taken into account this leads to a decrease of the half-width of the distribution and to a shift of its maximum to lower values of interchromophoric distances [58]. There are some well-known difficulties in determining accurate values of donor-acceptor distances from the energy transfer measurement. R_0, defined by Eqn. (3), must be known to calculate r. The correct determination of orientation factor κ^2 is often considered to be the limiting factor for estimating an accurate D–A distance from FRET data. κ^2 is varying from 0 to 4, although the actual value of κ^2 rarely, if ever, assumes these limiting values. In general, there is no sufficient information (often none at all) regarding D–A orientations, and approximations must be made. There is an extensive literature dealing with the so-called κ^2 problem [7, 20, 59–62]. The correct average value for κ^2 depends not only on the form of the orientational distribution, but also on the rate of transition between various orientations. The general case is complex. However, a few simple cases exist, $\langle\kappa^2\rangle = 2/3$ for "dynamically rapid averaging" of the donor and the acceptor.

The term "dynamically rapid averaging" means that the angular distributions of both D and A are implicitly assumed to be uniformly distributed all the time. This is only possible if both the donor and the acceptor molecules achieve isotropic rotational equilibrium in a time much shorter than the emission lifetime of the donor. This case of "dynamically rapid averaging" of both the donor and the acceptor is the customary assumption in the FRET calculations.

Another phenomenon that can lead to the $\langle\kappa^2\rangle$, value of 2/3, even if the donor and the acceptor are oriented nonrandomly, is the effect of mixed polarizations in the spectral bands of the chromophores. The overlap integral in Eqn. (4) involves an integration over the entire frequency interval where the emission of the donor and absorption of the acceptor overlap. If the electronic absorption and emission involve more than a single molecular transition, and if the different transitions have incoherent dipoles oriented differently in a molecule, the actual κ^2 value will be averaged over the overlap integral [23, 62]. It is possible to minimize the uncertainty of $\langle\kappa^2\rangle$, estimation using chromophores exhibiting mixed polarization. In the case of organic chromophores the mixed polarization of a donor emission does not reduce the space of uncertainty of κ^2 because the energy transfer occurs from the relaxed, lowest vibrational level of the excited state connected with the single transition dipole. The chromophores having the spherical symmetry with three, perpendicular one to each other axes of transition dipoles (isotropic oscillators like Tb^{3+}, Eu^{3+}, Nd^{3+}, Co^{2+}), used either as donors or acceptors, decrease substantially the uncertainty of $\langle\kappa^2\rangle$, estimation. When such isotropic oscillators are used as a donor and an acceptor at the same time, $\kappa^2 = 2/3$ [63]. The same $\kappa^2 = 2/3$ is observed in the case of random dynamic and isotropic rotational averaging of the acceptor or the donor, even with no rotational averaging of the other dipole if the angular orientation of the isotropic dipole relative to the fixed one corresponds to the "magic angle" (54.74°) [7, 60].

The assumption that $\langle\kappa^2\rangle = 2/3$ is often made for two reasons: either (i) because sufficient orientation information is lacking, and one hopes that the relative orientations of the transition dipoles of D and A do not have a significant effect, or (ii) simply because subsequent FRET efficiency calculations and distance estimates are usually greatly simplified if κ^2 is assumed to be constant for all D–A pairs.

Correlation of the actual value for $\langle\kappa^2\rangle$, with the error of r determination due to the general use of $\langle\kappa^2\rangle = 2/3$ can be calculated from the equation:

$$\kappa^2 = \frac{2}{3}\left(\frac{r}{r_{2/3}}\right)^6 \qquad (7).$$

Therefore, on the assumption that $\kappa^2 = 2/3$ leads to the errors in distance r determination not exceeding ±20%, the actual values of

κ^2 lie within the limits of $0.22 < \kappa^2 < 2.54$. Similarly, if $0.38 < \kappa^2 < 1.25$, the error is lower than ±10%. Several approaches have been made to estimate the errors resulting from using the value of 2/3 in calculating the D–A distance. The general consensus is that the most cautious approach to the problem is to establish the minimum and maximum values of the average orientation factor $\langle\kappa^2\rangle$, based on observed depolarization parameters of both donor and acceptor [59–61, 64]. This approach imposes the lower and upper limits on r and provides information about the range of actual values to be expected. For some simple models the level of κ^2 uncertainty was calculated [7, 23, 59–61]. In our review we present only a few most representative calculations that have been used in practice. More information can be found in papers by Dale and coworkers [59, 60].

For rapid and complete random dynamic orientation of either A or D with a unique orientation of the complementary chromophore D or A, respectively, the value of $\langle\kappa^2\rangle$, depends in a very simple way on the angle (θ) between the fixed transition dipol moment and a vector joining the D–A pair:

$$\langle\kappa^2\rangle = \cos^2\theta + 1/3 \qquad (8)$$

$\langle\kappa^2\rangle$, has the maximum of 4/3 at $\theta = 0°$ and minimum of 1/3 at $\theta = 90°$ [23, 59, 60]. The completely random dynamic orientation of one of the dipoles has reduced the difference between κ^2 min-max values pertaining to two fixed dipoles from 0 (at $\theta = 90°$) and 4 (at $\theta = 0°$) to 1/3 (at $\theta = 90°$) and 4/3 (at $\theta = 0°$), however, the average value of $\langle\kappa^2\rangle$, still depends on θ (at $\theta = 54.74°$ κ^2 equals 2/3). The error in calculating an R_0 value can be quite small, even though κ^2 varies strongly throughout the sample. The corresponding min-max errors in the R_0 are $0.88\ R_0^{(2/3)} < R_0^{(2/3)}\ 1.12\ R_0^{(2/3)}$. The 12% error in this case is the maximal possible error in R_0 determination and it is often much lower than errors connected with fluorescence measurements. A ±100% error in the estimated value of κ^2 leads to only a 12% error in the values of R_0. The min-max values refer only to the cases when $\theta = 90°$ and $\theta = 0°$, however, very seldom θ attains one of these extreme values. Usually θ is distributed over a wide range of values.

In biomolecules a chromophore does not have an absolutely rigid conformation but very often has, to some extent, conformational freedom. Let's make an assumption that one of the transition dipoles has a rapidly changing and completely random dynamic orientation. The second one can rotate rapidly and freely within the constrains of a cone the axis of which forms an angle φ with the vector r joining the donor and the acceptor. The maximum angular deviation of the transition dipole in the cone is described by the cone half-angle φ (volume averaging) [60].

In this case, one can expect that the difference between the minimal and the maximal values of $\langle\kappa^2\rangle$, decreases as the cone angle φ increases. At $\varphi = 90°$ the dipole is completely randomly orientated and $\langle\kappa^2\rangle = 2/3$ for all θ. For $\theta < 54.74°$ the limits are $4/3 > \langle\kappa^2\rangle > 2/3$ $(1.12 > R_0/R_0^{(2/3)} > 1)$; for $\theta > 54.74°$ the limits are $1/3 < \langle\kappa^2\rangle < 2/3$ $(0.88 < R_0/R_0^{(2/3)} < 1)$. The maximum values of κ^2 are reached for $\theta = 0°$ and $\theta = 90°$. Estimation of φ can be made from fluorescence polarization measurement [60]. Physically, volume averaging corresponds to the transition dipole with increased rotational freedom. If a probe is in a lipid membrane or attached to a protein, *via* a covalent bond and the motion of the chromophore takes place in a rigid region of protein, the transition dipole can rotate rapidly only on the surface of a cone (surface averaging). In this case the orientation factor has the limit: for $\theta < 54.74°$ $4/3 > \langle\kappa^2\rangle > 1/3$ $(1.12 > R_0/R_0^{(2/3)} > 0.88)$; for $\theta > 54.74°$ $1/3 < \langle\kappa^2\rangle < 5/6$ $(0.88 < R_0/R_0^{(2/3)} < 1.04)$. For $\theta = 54.74°$, similarly as for the example previously mentioned, $\langle\kappa^2\rangle = 2/3$. For $\varphi = 0°$ (no rotational freedom) we have the same situation as in the example of the static transition dipole described above. If $\varphi = 90°$, the cone becomes a circular plane and $1/3 < \langle\kappa^2\rangle < 5/6$. From these examples one can see that the freedom of dipole orientations must extend in the three-dimensional space (not only in plane) if κ^2 is expected to be close to 2/3 for all values of θ.

Most frequently both chromophores (donor and acceptor) can rotate rapidly and freely within the constraint of a cone [59–61]. For a single donor-acceptor pair, it is best to use fluorophores that satisfy volume averaging and satisfy the condition that either the donor or the acceptor has the cone half-angle $\varphi > 60°$ (20% error, if $\varphi > 30°$). For the surface averaging this condition will be satisfied, if both the donor and the acceptor cone half-angles lie between 45° and 135° [61].

If the motion of chromophoric moiety is influenced by fluctuation of the surrounding structure the transition dipole is not necessarily restricted to a conical surface and can be found anywhere within the volume of the cone. In the case of two amino-acid residues with full conformational freedom we can assume that the range of the κ^2 values can be determined by volume averaging. The conformational freedom of four amino-acid residues leads to the dynamically averaged value of $\kappa^2 = 2/3$ [65–67]. Volume averaging, therefore, provides the model which is a more general description of the dipole movement and may be more appropriate to apply in the absence of specific information.

In the case of completely static distribution of mutual D–A orientations (chromophores do not undergo Brownian motion (translation or rotation) at all in the time scale of fluorescence decay) with the random distributions of the free donor and acceptor, $\langle\kappa^2\rangle = 0.476$ [68]. When donor and acceptor molecules are bound to macromolecules with static but random orientations and distances, no average $\langle\kappa^2\rangle$ independent of r does exist [60, 62, 69]. The $\langle\kappa^2\rangle$ for the D–A pairs that are separated by the distance $r \leq R_0$ tends to zero as r tends to approach zero. As r increases, with $r > R_0$, $\langle\kappa^2\rangle$ approaches the value of 2/3 [69]. Wu & Brand [70] have considered the effect of a static distribution of D and A orientations on the calculated distance distribution. They concluded that if $r > R_0$ $\langle\kappa^2\rangle$ approaches 2/3 and the error in the estimation of the mean distance is small (the distribution can be fitted to a symmetrical function). However, if $r < R_0$ the error made in determining the distance distribution function by assuming only the distribution of r becomes substantial (the width of the calculated distance distribution function is often considerable, even if only a single distance between donor and acceptor is present). Then a skewed distribution is required. When dealing with the distribution of distances between D and A the assumption that $\langle\kappa^2\rangle = 2/3$ is almost always made, usually because otherwise the calculation would be very complex [66, 67, 70–73].

In this part of our paper we would like to compare structural parameters (especially interchromophoric distances) determined using the FRET with those obtained by other techniques, mostly XRD (X-ray diffraction), just to show that even in spite of very often discussed difficulties in establishing the exact κ^2 values and the conformational freedom of chromophore linkers, the FRET method gives interchromophoric distances comparable with those determined by the XRD. One of the first observations of the non-radiative energy transfer between metal cations in a protein was that of the excitation transfer from Tb^{3+} situated in the calcium-binding site S1 of thermolysin to Co^{2+} at the active Zn^{2+}-binding site of the enzyme [74]. The distance between these cations was found to be 13.7 Å, in perfect agreement with the Ca^{2+}–Zn^{2+} distance found in the crystal structure [75]. Another, worth mentioning, example of intermetal ion measurements was described by Horrocks & Tingey [76] for calmodulin. Calmodulin was treated with 19:1 Nd^{3+}-Eu^{3+} mixture to saturate the four calcium-binding sites (Eu^{3+} is the energy donor and Nd^{3+} is the most efficient acceptor). Not going into details of this useful strategy, we should add that the authors were able to determine the distance between sites I and II in the first domain, and between sites III and IV in the second globular domain. The intersite distances measured by the FRET technique were: 12.1 ± 0.5 Å and 11.6 ± 0.8 Å, respectively. Almost identical distances have been obtained by X-ray diffraction [77] — the distance I–II was found to be 11.9 Å and III–IV 11.5 Å. Intermetal ion distance measurements have been made to date for a number of protein systems (and other biomolecules), including thermolysin [78], parvalbumin [79], human Factor Xa [80], and other compounds [63].

A quite different approach was applied in conformational studies of RNase A [81]. The authors measured the interchromophoric distance between two extrinsic chromophores attached to the protein. A nonfluorescent acceptor probe (2,4-dinitrophenyl, DNP) was covalently attached to the α-amino group and then a fluorescent donor (ethylenediamine monoamide of 2-naphthoxyacetic acid, ENA) was linked in the vicinity of residue 50 (75% at Glu-49 and 25% at Asp-53). The interprobe separation was estimated to be 35 ± 2 Å under folding conditions. Using of the 1.45 Å r.m.s. X-ray coordinates [82] and taking into account the labeling distribution, the average distance separating the α-amino nitrogen (N-terminus) from the carboxylate carbon (in the vicinity of residue 50) has been calculated to be 28 Å. Moreover, taking into account the size of the linkages connecting the probes to the protein the calculated average interprobe distance was found to be 36 Å which is in excellent agreement with the FRET experimental results reported by McWherter *et al.* [81] for the folded state of RNase A.

A more advanced approach was used in the studies of conformational parameters of BPTI [28, 29]. A series of four BPTI derivatives, site specifically labeled by (2-methoxy-1-naphthyl)methyl at the N-terminal amino group and by [7-(dimethylamino)-coumarin-4-yl]acetyl at one of the four ε-amino groups (Lys-15, 26, 41 and 46), was prepared. Analysis of the experimental decay curves yielded the detailed intramolecular distance distribution functions for each pair of labeled sites. The averages of the calculated distance distribution functions are close to the values obtained for BPTI in crystalline state [83]. Comparison of the above data is presented in Table 1.

Table 1. Comparison of intramolecular distances for BPTI obtained from FRET and X-ray experiments

Labeled sites in BPTI	Average distance Å FRET measurements	Distance Å X-ray diffraction
1–15	31 ±1	31.7
1–26	20 ±1.5	16.8
1–41	19 ±1.5	19.1
1–46	21 ±1.5	21.7

One of the protein systems the most intensively studied to date using the FRET methods is the myosin-actin system [20, 26]. In 1987 Dos Remedios *et al.* [27] reviewed the available FRET publications related to actin and myosin, and they even constructed a model of this protein system using the known at that time 62 interchromophoric distances determined by FRET. The crystal structure of actin [84] has revealed that, with the exception of Cys-10 location, the model of actin, and the interchromophoric distances presented by Dos Remedios *et al.* [27] are in good agreement with the definitive XRD data. This reasonable agreement between the FRET and XRD data was later confirmed by Miki *et al.* [85] and O'Donoghue *et al.* [86]. Since then, other high resolution crystal structures of actin have been published [87–89] and also in these cases a good agreement between the crystal data and the FRET determinations has been found. To illustrate this comparison we present in Table 2 some distances determined by FRET and XRD. The complete comparison and all available data can be found in the literature [20, 26, 90].

The examples presented above testify that FRET can provide information about interchromophoric distances with the accuracy comparable with the data from low-resolution XRD measurements. Because the fluorimetric measurements of the energy transfer are performed in solution, the native environment of biomolecules, the FRET method is very often the only one allowing to obtain an information about a process of conformational changes in solution. Moreover, FRET is the only technique able to measure structural changes of a studied biomolecule in the nanosecond time scale. To study conformational changes of biomolecules in solution it is not necessary to know the absolute value of an interchromophoric distance. So, the previously discussed limitations of accuracy of measurements of interchromophoric distances become, to a large extent, less important or even meaningless. FRET experiments give not only infor-

Table 2. Comparison of some XRD and FRET inter-actin distances [26]

Distance [Å] between	XRD	FRET
Cys-1 — Tyr-69	17	27
Cys-10 — Divalent cation site	11	30
Lys-61 — Nucleotide site	25	33
Lys-61 — Divalent cation site	26	28
Tyr-69 — Cys-374	28	25
Tyr-69 — Nucleotide site	15	21
Nucleotide site — Divalent cation site	12	10
Cys-374 — Nucleotide site	30	29

mation about conformational changes but also tell us whether the interchromophoric distances increase or decrease. This advantage is clearly useful in testing hypotheses on the mechanism of conformational changes. There are several good examples of this approach in the literature [28, 29, 45, 48, 49, 64, 81].

Melittin can serve as a very good example of application of the FRET technique to studies of conformational changes. It was found from FRET measurements, performed for the melittin monomer in the Mops buffer (interaction between Trp-10-donor and DNS, an acceptor covalently connected to the N-terminal amino group) that the function describing the distance distribution possesses a large half-width typical for a disordered polypeptide conformation. When denaturing conditions were applied (6 M guanidinium hydrochloride) the half-width of the distribution increased even a little more. These data (the half-width of the distribution in the buffer and in the denaturing environment) suggest the existence of a residual ordered conformation of melittin in the aqueous solution.

Very similar results were obtained for melittin from ^{1}H-NMR measurements performed in water [91]. It was found that melittin is predominantly in an extended, flexible form, with fragments 5–9 and 14–20 more highly structured than the rest of the amino--acid sequence. The change of solvent from water to methanol causes a shift of the average value of the distance distribution in the direction of larger interchromophoric distances and, at the same time, a decrease of the half-width of the distribution. These effects (the shift and decrease) are growing with the increasing content of methanol up to 60%. At the methanol concentrations above 60% the distance distribution function does not change its shape any longer. The changes of this type in the shape of the distance distribution function suggest that the conformation of melittin is changing from disordered to constrained and well-ordered one (the small half-width of distribution). CD and ^{1}H-NMR measurements performed for melittin in methanol showed that the peptide structure is mainly helical and similar to that determined in crystals from diffraction data. Residues 2–11 and 13–26 form regular α-helices linked by a "hinge" between residues 11–12 [92]. Studies of the distance distributions between Trp and DNS obtained for melittin (FRET measurements) in water, in the presence of calmodulin, troponin I, or POPC revealed that melittin has, in these conditions, a highly ordered and rigid conformation, as it was also proved by NMR studies [93, 94].

The FRET technique has been widely used to estimate interchromophoric distances in biomolecules. The similar data obtained by other experimental techniques commonly used in structural studies of proteins (nuclear magnetic resonance, NMR; and X-ray diffraction, XRD) are less or more comparable to FRET results. NMR and XRD both

produce almost complete structural information but, on the other hand, they require large quantities of a studied compound. Moreover NMR is limited to relatively small molecules, it is time consuming and the spectrometers are expensive. X-ray is not limited to small molecules but one can face, in many cases, problems with getting crystals. In addition, NMR and XRD are limited to *in vitro* measurements. The FRET method can be applied to almost all biomolecular systems. So, in many cases FRET is often used to obtain some initial structural information when the complete structure is not known from NMR and/or XRD experiments.

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