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# The Versatility of Combining FRET Measurements and Molecular Mechanics Results for Determining the Structural Features of Ordered Peptides in Solution

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Combination of fluorescence resonance energy transfer (FRET) measurements with molecular mechanics results makes it possible to determine the most relevant structural features of a series of short, ordered L-(αMe) Val-based peptides [(αMe) Val = $C^{\alpha}$-methylvaline] in methanol solution.

**KEY WORDS:** Foldamers; FRET measurements; molecular mechanics calculations; $3_{10}$-helical peptides.

## INTRODUCTION

One of the main goals pursued in our laboratories in the past years was the determination of the structural features of sterically constrained peptides (foldamers) [1] in solution, making use of a combined approach of FRET [2] experiments and molecular mechanics calculations [3]. This approach implies that the samples investigated have to carry two fluorophores, which is often the case, e.g., in a number of bioactive peptides or drugs [4], one acting as acceptor (A) and the other one as donor (D). Then, where long-range energy transfer occurs (D* → A), one can evaluate both the energy transfer efficiency (that depends on both the interprobe distance and mutual orientation, according to the Förster model [2]) and population of the species in solution exhibiting that efficiency. If comparison between these data and those calculated from the low-energy conformers, as obtained by molecular mechanics, is satisfactory, a good piece of structural information is obtained. Alternatively, the experimental results may be used as constraints for structural calculations. This method is applied below to a series of L-(αMe) Val-based peptides:

$$F\text{-}[(\alpha Me)\ Val]_r\text{-}T\text{-}[(\alpha Me)\ Val]_2NHtBu$$

where *F* is fluoren-9-ylmethoxy-carbonyl (Fmoc) and *T* is 2,2,6,6-tetramethyl-piperidine-1-oxyl-4-amino-4-carboxylic acid (Toac), a fluorophoric $N^{\alpha}$-protecting group and a nitroxide-based α-aminoacid quencher, respectively, and *r* is 0–3.

## EXPERIMENTAL

The syntheses of the Fmoc/Toac peptides were performed as already described [3]. Spectrograde solvents (Fluka) were always used in the optical measurements. Steady-state fluorescence spectra were recorded on a SPEX Fluoromax spectrofluorimeter, operating in SPC mode. Nanosecond decays were measured by a CD900, SPC lifetime apparatus from Edinburgh Instruments. The decay curves were fitted by a non-linear least squares

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analysis to exponential functions through an iterative deconvolution method. All solutions were pre-bubbled for 20 min with ultrapure argon.

## RESULTS AND DISCUSSION

Steady-state fluorescence spectra of F-[(αMe) Val]$_r$-T-[(αMe) Val]$_2$NH*t*Bu in methanol ($\lambda_{ex}$ = 264 nm) show that the Fmoc singlet emission is strongly quenched by Toac, the quenching efficiency $E = 1 - (\Phi/\Phi_0)$ being, for instance, 0.94 and 0.84 for the peptides at the extremes of the series examined, i.e., $r = 0$ and 3.

The fluorescence time decays ($\lambda_{em}$ = 320 n*M*) are found to be well fitted by a bi- or three-exponential, according to the following expression $I(t) = \Sigma_i \alpha_i \exp(-t/\tau_i)$, whilst the reference decay is strictly monoexponential ($\tau_0 = 8.7 \pm 0.2$ ns, in methanol), as shown in Fig. 1. The lifetimes distribution analysis of the experimental decays gives rise to narrow distributions, whose parameters are in excellent agreement with those obtained from the discrete model. For instance, for the $r = 0$ peptide, one obtains $\tau_1 = 0.31$ ($\alpha_1 = 0.94$) and $\tau_2 = 6.3$ ns ($\alpha_2 = 0.06$) from the discrete model ($\chi^2 = 1.12$), compared to 0.28 (0.94) and 6.2 ns (0.06) from the lifetime distribution analysis ($\chi^2 = 1.17$), while for the $r = 3$ peptide, one obtains $\tau_1 = 0.70$ ($\alpha_1 = 0.76$), $\tau_2 = 1.9$ ($\alpha_2 = 0.18$) and $\tau_3 = 8.2$ ns ($\alpha^3 = 0.06$) from the discrete model ($\chi^2 = 0.98$), compared to 0.82 (0.90), 3.0 (0.05), and 8.5 ns (0.05) from the lifetime distribution ($\chi^2$ = 0.96). These findings imply that even the $r = 0$ peptide is structured in methanol, despite the shortness of the backbone chain [3] and make it reasonable to assign each decay component to one conformer, characterized by a different center-to-center distance and mutual orientation of the chromophores, whose amount is measured by the preexponent $\alpha_i$.

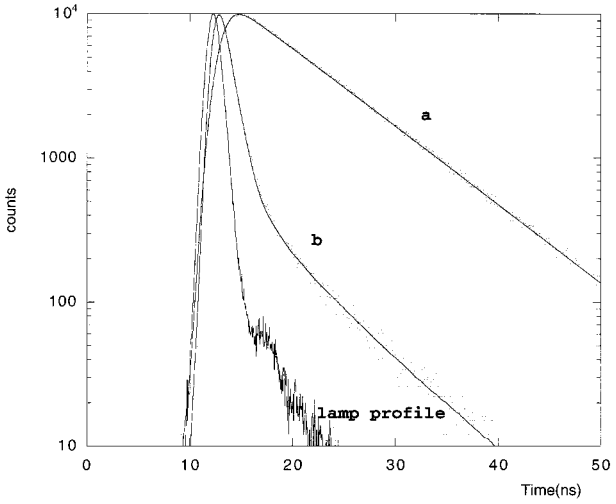


**Fig. 1.** Typical fluorescence time decay of excited Fmoc ($\lambda_{ex}$ = 264, $\lambda_{em}$ = 320 nm) in methanol, referring to F-[(αMe) Val]-T-[(αMe) Val]$_2$-NH*t*Bu (a) and the reference F-[(αMe) Val]-OH (b). The full lines represent the best fit to the experimental data by a bi- (a) and mono-exponential (b) decay. The lamp profile is also shown.

Molecular mechanics calculations were performed [5] to get the low-energy structures, which are characterized by a backbone chain in a (right-handed) $3_{10}$-helical conformation [3], as expected for the relevant presence of the L-(αMe) Val aminoacid. From the computed structures the quenching efficiency $E_m$ (assuming a Förster energy transfer model for the reason that will be apparent shortly) [2,3] for each conformer of a given peptide can be evaluated, according to the following expression:

$$E_{m,calcd} = \frac{1}{1 + \left[\frac{2}{3\,\kappa_m^2}\left(\frac{R_m}{R_0}\right)^6\right]} \qquad (1)$$

where $R_m$ is the distance between the probes in the mth conformer, $\kappa_m^2$ is a dimensionless geometric factor determining the relative orientation of the donor and acceptor transition dipoles, and $R_0$ is the Förster radius, i.e., the distance at which 50% transfer of excitation energy occurs. Then the values of $E_m$ can be compared with those experimentally determined from time-resolved fluorescence measurements, i.e., $E_i = [1 - (\tau_i/\tau_0)]$. For instance, for the most favored conformer of the $r = 0$ peptide in methanol, one obtains $E_m = 0.97$ vs. $E_i = 0.96$, while for the two most populated conformers of the $r = 3$ peptide, one has $E_m = 0.93$ and 0.80 vs. $E_i = 0.92$ and 0.79. It is worth noting that there is a minor fraction ($\leq$10%) of the $r = 2$ and 3 peptides exhibiting an experimental efficiency of nearly zero. This finding implies that the transition dipole moments of the probes experience a perpendicular arrangement that prevents energy transfer from occurring, provided that the probes are frozen on the time scale of the donor lifetime [2,3], and confirms the idea that a Förster energy transfer is the leading quenching process in the peptides examined. Indeed, this model comprises the $\kappa^2$ orientation factor, ranging from 0 to 4 [6], which makes it possible to find conformers with a negligible transfer efficiency, even if the probes are close to each other.

By comparing the calculated population of the conformers $P_m$, as obtained from the Boltzmann distribution, with that experimentally determined from time-resolved measurements ($\alpha_i$), a rather satisfactory agreement is observed, which represents an additional and independent support to the computed structures. Indeed, $P_m$ has exactly

the same meaning as $\alpha_i$, provided that static quenching is absent [7], as is here the case. For the most favored conformer of F-T-[(αMe) Val]$_2$NH*t*Bu in methanol, one obtains $P_m = 0.89$ as compared to $\alpha_i = 0.94$, while for the two most populated structures of F-[(αMe) Val]$_3$-T-[(αMe) Val]$_2$NH*t*Bu $P_m = 0.52$ and 0.32 as compared to $\alpha_i = 0.76$ and 0.18. Even if in this latter case the relative deviations are as high as 30–40%, we consider the result satisfactory, in view of both the larger experimental errors than those of quenching efficiencies and the empirical nature of the force field employed. Figure 2 illustrates the molecular model of the most favored conformer of F-[(αMe) Val]-T-[(αMe) Val]$_2$NH*t*Bu.

Theoretical and experimental quantities compare satisfactorily for the other peptides examined, too, as shown in Figs. 3 and 4, where both the calculated and experimental quenching efficiencies and populations of a number of peptides or bioactive pseudo-peptides in solution (mainly in methanol or water/methanol), carrying different kind of probes, are also reported [5,7,8]. The agreement between the values of $E_m$ and $E_i$ is very good only when the $\kappa^2$ orientation parameter, as obtained by the theoretical conformations in the deepest energy minimum, is used. By contrast, where freely rotating probes are considered, i.e., $\langle\kappa^2\rangle = 2/3$ [6], the two parameters diverge significantly, as shown in Fig. 3. On the other hand, not unexpectedly, the scattering of the points in Fig. 4, where $P_m$ is reported against $\alpha_i$, is much higher than that in Fig. 3 for the aforementioned reasons.

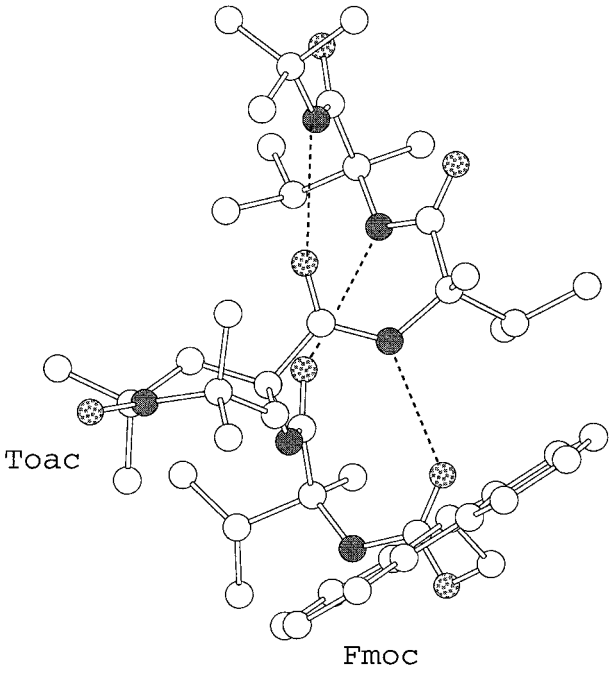


**Fig. 2.** Molecular model of the deepest energy minimum conformer of F-[(αMe) Val]-T-[(αMe) Val]$_2$NH*t*Bu, with the backbone chain in the (right-handed) $3_{10}$-helix, viewed perpendicularly to the helix axis. Nitrogen atoms are in black, oxygen atoms are dotted, and hydrogen atoms are omitted for clarity. The broken lines indicate the intramolecular hydrogen bonds. The calculated efficiency and population from this structure are $E_m = 0.91$ and $P_m = 0.98$, respectively, compared to the same experimentally determined quantities $E_i = 0.90$ and $\alpha_i = 0.98$.

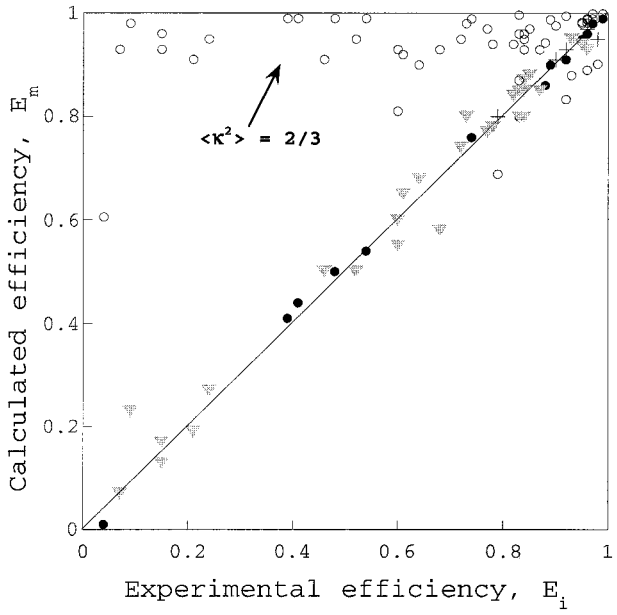


**Fig. 3.** Comparison between experimental (from time-resolved measurements) and calculated (from the deepest energy minimum structures) quenching efficiencies of a number of foldamers, carrying different probes [5,7,8], by using the orientation parameter ($\kappa^2$), as obtained by the theoretical conformations (full symbols), or by $\langle\kappa^2\rangle = 2/3$ (empty symbols), corresponding to freely rotating probes. The full line is not the best fit to the data but simply the diagonal. The reversed triangles refer to peptides other than F-[(αMe) Val]$_r$-T-[(αMe) Val]$_2$ NH*t*Bu (represented by + symbols), carrying Toac as acceptor. Conformers populated more than 10% are only reported.

Two further points deserve a few comments. First, it is thought that one of the major pathways of nitroxides quenching of singlet state is the intersystem crossing to the triplet induced by electron exchange [9,10], or an internal conversion to ground state [11], both mechanisms being not mutually exclusive. In other instances, however, charge transfer [12] or Förster energy transfer was found to be important [13], so that the mechanism of nitroxide quenching is still uncertain. In the last years we have examined a number of D-spacer-A assemblies (where A and D are acceptor and donor, respectively), carrying Toac as acceptor, short peptides as spacer and tryptophan [7], binaphthyl [7] or fluorene as donor, always finding that the quenching mechanism could be correctly described by the Förster model, on which the data of Fig.

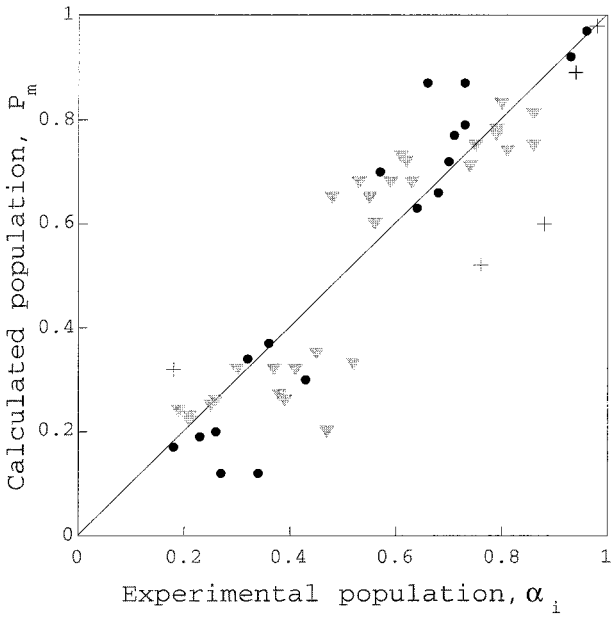


**Fig. 4.** Comparison between experimental (from time-resolved measurements) and calculated (from the deepest energy minimum structures) populations of a number of foldamers, carrying different probes [5,7,8]. The full line is not the best fit to the data but simply the diagonal. The reversed triangles refer to peptides other than F-[(αMe) Val]$_r$-T-[(αMe) Val]$_2$ NH*t*Bu (represented by + symbols), carrying Toac as acceptor. All data are taken for those peptides in which static quenching is absent [7] and refer to conformers that are populated more than 10%.

3 are based. This gives further support to the idea that a dipole-dipole interaction holds in F-[(αMe) Val]$_r$-T-[(αMe) Val]$_2$NH*t*Bu, too. Second, the results reported in Figs. 3 and 4 refer to peptides that have a common feature, namely that of being conformationally constrained by the presence of both $C^\alpha$-disubstituted amino acids and suitable chromophores in the chain [5,7,8]. Therefore, they were particularly amenable to being treated by our method, combining time-resolved FRET measurements with molecular mechanics data, because of the exact knowledge of the mutual orientation of the chromophores, which are conformationally frozen on the time scale of the energy transfer process [see Eq. (1)].

Despite the foregoing considerations and the excellent agreement between theoretical and experimental quenching efficiencies, we checked whether a Dexter formulation of exchange interaction [14,15] would contribute to the process investigated, chiefly in the first terms of the series examined because of their relatively short chain length. The Dexter quenching efficiency is exponentially dependent on the interchromophoric distance, through the electronic matrix coupling element, i.e.,

$$V_m = [K \exp(-2R_m/L)]^{1/2} \qquad (2)$$

where $K$ is a constant corresponding to the electronic matrix coupling at orbital contact, $R_m$ is the interprobe center-to-center distance in the mth conformer, and $L$ is the average radius involved in the initial and final states [14]. By using the same L = 2.89 Å value for the van der Waals radius of the probes in all peptides examined, it turns out that the values of K are around $10^{-14}$ erg, i.e., more than 4 orders of magnitude higher than those reported for electronic energy transfer via exchange interaction in bichromophoric molecules [15]. This clearly indicates that the intramolecular energy transfer in F-[(αMe) Val]$_r$-T-*x*[(αMe) Val]$_2$NH*t*Bu is not controlled by the Dexter mechanism, a finding not surprising also because the wave functions of the probes are unable to overlap for structural reasons. In fact, the helical periodicity is such that the NO and Fmoc groups lie approximately on the opposite sides of the helical backbone in the $r$ = 0 and 1 peptides, as shown in Fig. 2 for the the latter compound, while in the $r$ = 2 peptide there are two methyl groups of Toac inbetween NO and Fmoc that prevent a close approach of the chromophores, making an exchange interaction very unlikely. Finally, the deepest energy minimum conformers of the $r$ = 3 peptide exhibit $R_m$ values higher than 8.5 Å, which makes it unfeasible to accomplish such mechanism.

In conclusion, combination of time-resolved FRET measurements with molecular mechanics calculations appears suitable to tackle the problem of a quick identification of the most relevant structural features of relatively small compounds in solution, as the peptides examined. The overall findings provide great confidence in the energetically most favored computed structures, and make it reasonable to consider them as a good representation of the conformations populating the methanol solution.

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