# Distance Measurements by Fluorescence Energy Homotransfer: Evaluation in T4 Lysozyme and Correlation with Dipolar Coupling between Spin Labels

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ABSTRACT We demonstrate the feasibility and practical limitations of using steady-state anisotropy to determine distances from fluorescence homotransfer in the context of a protein of known crystal structure. Eight double mutants of T4 lysozyme spanning the distance range between 20 Å and 50 Å were labeled with a methanethiosulfonate derivative of fluorescein. The measured distances in liquid solution are in agreement with those determined from dipolar coupling between spin labels in the frozen state. They can be interpreted in the context of the crystal structure after accounting for the probe linking arm. Overall, the results establish the necessary calibration for this spectroscopic ruler. The measurement of similar distance trends using independent probes sets the stage for the complementary use of homotransfer and dipolar coupling in the determination of static structures and detection of conformational changes.

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The measurement of distances in proteins provides the major source of geometric constraints in the process of structure determination by spectroscopic methods. In addition, distance constraints can be used to interpret cryo-EM density maps of protein complexes in terms of detailed models (1) and to determine the amplitude of conformational transitions during function (2,3).

In probe-based approaches such as spin labeling electron paramagnetic resonance (EPR) and fluorescence spectroscopies, distances can be measured up to 100 Å (3,4). Because there are few limits on the size of the protein or its environment, spin and fluorescence labeling are unique tools to map conformational transitions in membrane proteins. However, the distances determined are between pairs of optical or paramagnetic probes projected from the backbone by a linking arm. This reduces the quality and accuracy of the constraints by increasing the upper and lower bounds that bracket the distances, which ultimately compromises the resolution of the derived models. The orientation of the probe relative to the $\alpha$-carbon can be constrained by distance measurements using two probes with different linking arm structures and/or using different techniques. EPR and fluorescence offer complementary advantages in terms of distance range and accuracy, but their combined use requires calibration in a model system.

Distance measurement between two optical probes is based on nonradiative resonance energy transfer. The most common application of this technique involves energy transfer between probes of different photophysical properties. In general, the introduction of two different probes in the same protein is challenging particularly when it relies on the same reactive chemical group such as the free sulfhydryl of cysteine residues. Two approaches have been developed to circumvent this problem. The use of chelated lanthanides as donors in the context of luminescence resonance energy transfer enhances the selectivity of the signal even in the presence of donor-only or acceptor-only labeled proteins (3). Alternatively, energy transfer can be measured between identical chromophores that have a limited Stokes shift and is referred to as homotransfer (5). The theoretical analysis of homotransfer is intrinsically complex since it can only be detected by depolarization experiments (6–8).

We are using homotransfer and spin labeling EPR to measure distances in transporters with the goal of evaluating the compatibility of the measured distances in liposomes with crystal structures and to determine the amplitude of conformational changes during the transport cycle. Therefore, we sought to evaluate the accuracy and precision of distances calculated from steady-state anisotropy (SSA) and analyze their correlation with distances determined from dipolar coupling between spin labels. Although the use of time-resolved anisotropy to measure distances is well established (8), to our knowledge, there has been no systematic calibration of homotransfer detected by steady-state anisotropy.

We selected T4 lysozyme (T4L) (Fig. 1) as the protein model system because of the wealth of crystallographic and spectroscopic data available. T4L has been used previously in the context of the development of site-directed spin labeling (9), particularly to demonstrate the use of pulsed dipolar EPR spectroscopy for long-range distance measurements between spin labels (4). Fluorescein is an ideal probe for homotransfer: it has a small Stokes shift, high quantum


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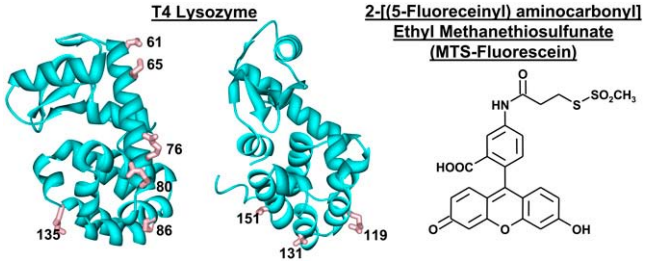


**FIGURE 1 Ribbon representation of T4L along with the structure of methanethiosulfonate-fluorescein.**

yield, and a large extinction coefficient. Furthermore, we took advantage of a methanethiosulfonate-linked fluorescein (Toronto Research Chemicals, North York, Ontario, Canada), which is expected to enhance reactivity compared to substituents that couple to the cysteine via a thioether bond (Fig. 1).

Pairs of cysteines were introduced at the solvent exposed surface of T4L as highlighted on the structure in Fig. 1. The samples were expressed, purified, and labeled as previously described (10). Of all the pairs of mutants (Table 1), only 65/80 became insoluble after reaction with fluorescein. Inclusion of 20% glycerol during labeling improves protein stability and yield.

Two approximate expressions have been derived for SSA in the presence of distance-dependent energy transfer and in the limit where the back transfer term is neglected (7,11). The interprobe distance $R_{av}$ is given by

$$R_{av1} = R_0 \times [0.5 \times (2r - r_1)/(r_1 - r)]^{1/6} \quad (1)$$

$$R_{av2} = R_0 \times [(2r - r_1)/(r_1 - r)]^{1/6}. \quad (2)$$

Equations 1 and 2 also assume a random relative orientation of the two probes i.e., $\kappa^2$ value of 2/3. $R_0$ is the critical distance, which for fluorescein is 44 Å. Calculation of $R_{av}$ requires two measurements of SSA: one in the presence of energy transfer, $r$, and one in its absence, $r_1$. Therefore, for each double mutant, we collected SSA for a stoichiometrically labeled sample as well as an underlabeled sample. The latter serves as a reference wherein the SSA reflects the intrinsic reorientation of the probe. The underlabeled sample was prepared by adding 0.2 mol of fluorescein per mol of T4L followed by addition of fivefold molar excess of (1-acetyl-2,2,5,5-tetramethyl 3-pyrroline-3-methyl) methanethiosulfonate, a diamagnetic analog of the spin label, to block unreacted cysteines (12). Analysis of labeling efficiency is described in the Supplementary Material. Fig. 2 *A* illustrates the difference in emission intensity between fully and underlabeled samples for the 65/135 pair.

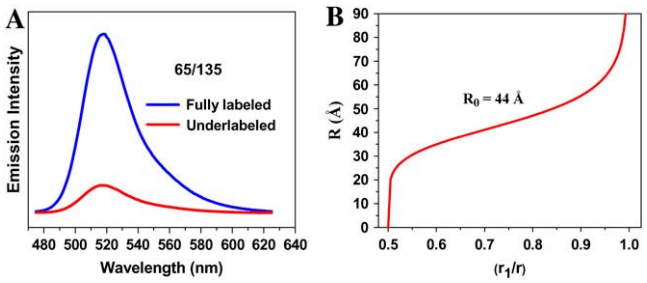


**FIGURE 2 (*A*) Emission spectra of 65/135. (*B*) Dependence of $R_{av}$ on $r/r_1$ based on Eq. 1.**

**TABLE 1 Steady-state anisotropy values for T4L mutants**

| | 30% (w/w) sucrose | | | | 33% (w/w) ficoll | |
|---|---|---|---|---|---|---|
| Mutant | $r_1$ | $r$ | $r_1$ | $r$ | $r_1$ | $r$ |
| 61/80 | 0.234 | 0.157 | 0.237 | 0.155 | 0.265 | 0.154 |
| 65/76 | 0.254 | 0.129 | 0.252 | 0.127 | 0.254 | 0.128 |
| 65/86 | 0.222 | 0.151 | 0.218 | 0.148 | 0.236 | 0.154 |
| 61/135 | 0.259 | 0.179 | 0.261 | 0.182 | 0.265 | 0.172 |
| 65/135 | 0.248 | 0.170 | 0.246 | 0.171 | 0.258 | 0.183 |
| 80/135 | 0.251 | 0.157 | 0.248 | 0.155 | 0.270 | 0.166 |
| 131/119 | 0.212 | 0.119 | 0.213 | 0.119 | 0.231 | 0.123 |
| 131/151 | 0.264 | 0.134 | 0.264 | 0.133 | 0.261 | 0.134 |

Table 1 shows SSA values for each of the pairs obtained at room temperature in the presence of either sucrose or ficoll added to eliminate the contribution of protein tumbling to depolarization. In all cases, the SSA of the underlabeled samples are larger than the ones of the stoichiometrically labeled samples. The fluorescence lifetimes for all the samples are within 10% of 4 ns (data not shown), consistent with homotransfer being the mechanism of added depolarization in the fully labeled samples.

The interprobe distances were calculated using Eqs. 1 and 2. Table 2 reports the average distances and the standard deviations calculated from three independent measurements along with the corresponding separation between the $\alpha$- and $\beta$-carbons of the original residues determined from the crystal structure. The difference between $R_{\alpha\alpha}$ and $R_{\beta\beta}$ is an indication of the orientation of the secondary structures relative to the interprobe vector $R_{av}$. For pairs along the same helix, $R_{\alpha\alpha}$ and $R_{\beta\beta}$ are similar. For pairs on different secondary structures,

**TABLE 2 Distances between fluorescein probes, $R_{av1}$ and $R_{av2}$**

| Mutant | $R_{av1}$ (Å) | $R_{av2}$ (Å) | $R_{\alpha\alpha}$ (Å) | $R_{\beta\beta}$ (Å) | $R_{sl}$ (Å) |
|---|---|---|---|---|---|
| 61/80 | 37.1 ± 3.2 | 41.7 ± 3.5 | 28.7 | 28.8 | 34 / 29* |
| 65/76 | 20.5 ± 1.4 | 23 ± 1.6 | 16.7 | 16.6 | 21.5 |
| 65/86 | 39.4 ± 1.3 | 44.3 ± 1.0 | 28.9 | 31.2 | 37.4 |
| 61/135 | 39.9 ± 1.5 | 44.8 ± 1.7 | 37.7 | 40.4 | 47.2 / 41.8* |
| 65/135 | 40.9 ± 0.7 | 46 ± 0.8 | 34.3 | 36.6 | 46.3 |
| 80/135 | 36.4 ± 0.4 | 41 ± 0.4 | 26.7 | 27.4 | 36.8 |
| 131/119 | 30.5 ± 1.9 | 34 ± 2.2 | 13.2 | 15 | 23 |
| 131/151 | 21.9 ± 2.3 | 24.6 ± 2.6 | 10.4 | 10 | 8 |

$R_{\alpha\alpha}$ and $R_{\beta\beta}$ are the distances between the $\alpha$-carbons and the $\beta$-carbons. $R_{sl}$ is the distance between two spin labels at the same sites (4).
*Two-component distance distributions.

$R_{\beta\beta}$ is larger, which implies that the linking arm may add significantly to $R_{av}$.

For the pairs 61/135, 65/86, 65/135 and 80/135, we find that the distances determined by homotransfer are in reasonable agreement with the distances measured between spin labels (4). Both report longer distances than $R_{\alpha\alpha}$ and $R_{\beta\beta}$, and the difference can be rationalized by the projection of the probes away from the backbone.

In the lower range of distances explored, the deviation between $R_{av}$ and $R_{sl}$ is substantial for the 131/151 pair. One key contributing factor is likely to be incipient back transfer neglected in Eqs. 1 and 2. In addition, these equations predict loss of sensitivity, as $2r$ asymptotically tends toward $r_1$ around 0.5 $R_0$ (Fig. 2 *B*). Practically, it is difficult to obtain meaningful results since small errors may render $2r-r_1$ negative. These factors effectively limit the shorter range of measurable distances to ~20 Å.

Unlike the 131/151 pair, the measured distance between 119 and 131 is within the optimum range. Its deviation from the $R_{\beta\beta}$ distance reproduces that observed using spin labels. It is likely that the probes at these sites project away from each other. The larger size of fluorescein compared to the spin label and the extension of its linker by two bonds account for the 7 Å difference between the two distances.

In summary, the data show that distances can be readily extracted from SSA and that the assumptions intrinsic to Eqs. 1 and 2 do not lead to substantial errors in the range between 25 Å and 60 Å. The finding of similar distance trends from spin and florescence labels sets the stage for the concerted use of these independent probes to map structures and determine amplitude of conformational changes. Homotransfer has the advantage that long-range distances can be measured at physiological temperatures whereas the use of spin labels overcomes issues of net orientation. Because the molar volumes of spin labels tend to be smaller than their fluorescence counterpart, they are less perturbing. In contrast to homotransfer, dipolar coupling can be measured in the 5–20 Å range (13), a range that encompasses the packing of neighboring helices in a protein. An alternative fluorescence approach to obtain proximity in this range has been developed based on the quenching of bimane fluorescence by tryptophan (14).

Although SSA technology is more accessible than time-resolved anisotropy and its interpretation can be relatively simple, it is subject to a number of experimental caveats. A small fraction of unreacted probes can introduce errors. Multiple rounds of desalting were required to remove unreacted fluorescein in our samples. Different probes and/or linking arms can overcome these issues and also serve to reduce differences with distances calculated from spin labels. In the same context, the presence of multiple distances due to protein conformational flexibility or probe repacking is masked and an average distance is obtained. Similarly, a fraction of singly labeled protein will increase the SSA and the calculated distance. Thus, it is advisable that these measurements be confined to pairs introduced at solvent-exposed sites and that the probes used be highly reactive.

## SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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