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Development of an spFRET method to measure structure changes in ion exchange proteins

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Abstract

Aim: Understanding the mechanism of tightly coupled ion exchange proteins, important effectors of cell volume regulation and other physiologically important transport processes requires means to observe dynamic changes in structure during the transport cycle. As a step towards this goal, we have applied single-pair fluorescence resonance energy transfer to a monomeric bacterial oxalate-formate exchanger (OxlT).

Methods: A His-9 tagged OxlT mutant containing two cysteines at positions 17 and 224 was labelled with cyanine dye maleimides (Cy3 donor and Cy5 acceptor) and attached to glass coverslips for measurements of donor and acceptor emission from single molecules, as described (P. Pal *et al. Biophys J* **89**, L11, 2005).

Results: Time-series data from 20 spots containing donor and acceptor provided evidence for single-pair energy transfer. From the efficiency of energy transfer, the mean donor–acceptor distance was determined to be 44.2 Å. Considering the size of the probes, this is in good agreement with the Cα distance of 39.6 Å for the corresponding sites found in the OxlT structural (homology) model (Q. Yang *et al. Proc Natl Acad Sci* **102**, 8513, 2005).

Conclusion: These results demonstrate the feasibility of single-pair fluorescence resonance energy transfer to measure distances between known sites in single OxlT molecules. This technique provides a potential means to test models for transport-related conformational changes, as well as to detect real-time structure alterations during the catalytic transport cycle.

Keywords bacterial transporter, major facilitator superfamily, membrane transport, membrane transporter, OxlT, single molecule, spFRET, transport protein.

Tightly coupled ion exchangers, such as the AE anion exchange systems and the NHE Na^+/H^+ exchange systems, are important mediators of cell volume regulation, as well as other physiologically essential cell functions (Jennings 1992, Hunte *et al.* 2005). Most evidence favours a model in which these transporters function by a ping-pong lock-carrier mechanism (Gunn & Fröhlich 1979, Knauf & Pal 2003, Hunte *et al.* 2005), in which the protein contains a substrate site that can be either accessible from the external medium in the Eo conformation or accessible from the cytoplasm in the Ei conformation (Fig. 1). The tight one-for-one coupling of ion flows in opposite directions, essential for the thermodynamic gradient of one ion to drive the other ion in the opposite direction, is explained in this model by proposing that the conformational change from Ei to Eo or

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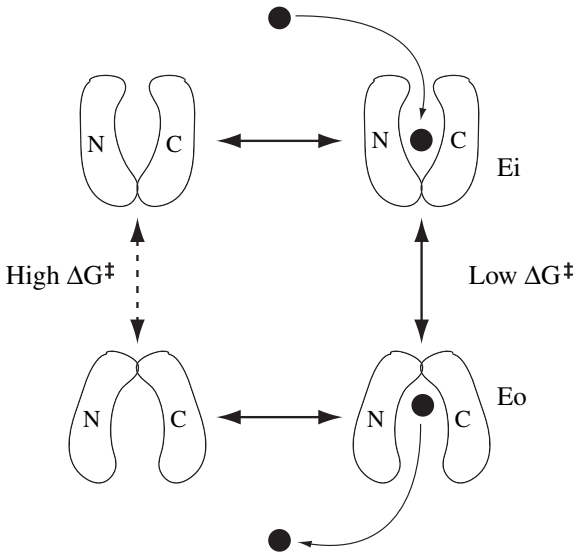

Figure 1 Model of OxlT transport mechanism with Ei representing the form with the binding pocket accessible to the cytoplasm and with Eo representing the conformation with the binding pocket accessible to the periplasm. The change from Ei to Eo is caused by reorientation of the C-terminal 6 transmembrane helices (labelled C) with respect to the 6 N-terminal transmembrane helices (labelled N). When substrate (e.g. oxalate) is bound, the transition state free energy for the transition from Ei to Eo or *vice versa*, $\Delta G^{\ddagger}$, is greatly reduced, thus facilitating the conformational change.

vice versa can only take place at an appreciable rate if a suitable substrate ion is bound to the transport site. Binding of substrate lowers the transition-state free energy, $\Delta G^{\ddagger}$, for the Ei↔Eo conformational shift (Fig. 1). Thus, after transport of an ion outward, the system is left in the Eo state, and can only return to the Ei state by transporting another ion inward.

Structural information is necessary to understand how such a mechanism can work at the molecular level; in particular, how substrate binding and ion translocation alter the protein structure. Low-resolution electron diffraction structures have been reported for the AE1 human Cl^-/HCO_3^- exchanger (Wang *et al.* 1994) and for a bacterial Na^+/H^+ exchanger (Williams 2000). These, however, lack sufficient resolution to identify the transport site or to provide much information about the relation of structure to function. Recently, the X-ray structure of GlpT, an *Escherichia coli* protein that exchanges glycerol-3-phosphate (G-3-P) for phosphate (Pi), has been obtained with sufficient resolution to provide a basis for mechanistic models (Huang *et al.* 2003). A crystal structure for a functionally analogous bacterial Na^+/H^+ exchanger, NhaA, has also been reported (Hunte *et al.* 2005).

In the case of GlpT, the protein has two positively charged arginines facing a central hydrophilic cavity, which form the putative anion binding site. In the crystal structure, this site is accessible to hydrophilic molecules from the cytoplasmic side, but not the external (periplasmic) side, indicating that it represents an Ei form. Based on this structure, Wang *et al.* (Huang *et al.* 2003) have proposed a model in which a rocking motion of the 6 N-terminal transmembrane segments of GlpT, relative to the C-terminal 6 transmembrane segments, causes a change in conformation so that the anion binding site becomes accessible from the outside, but not from the inside, thus adopting an Eo conformation (Fig. 1). Binding of substrate is proposed to alter the structure so as to facilitate this change. Like the ping-pong model of which it is a subspecies, this model implies a very strong similarity between ion exchange systems and ion channels. Unlike ion channels, which usually have a single gate and which permit free diffusion of ions when the gate is open, the ping-pong lock-carrier has two gates in series, structured such that the two gates are never open at the same time so that the system can never permit free diffusion of ions across the membrane.

The analogous OxlT transport system, which exchanges oxalate for formate, has the advantage that the crystallized form, although solved at a lower resolution than that of GlpT, includes substrate (Heymann *et al.* 2001), and that a cysteineless form retains function (Fu & Maloney 1998). In this case, the anion binding site is formed by Lys-355 and Arg-272 (Yang *et al.* 2005), analogous to the two Arg residues in GlpT. Maloney and collaborators have made a homology model for the Ei form of OxlT (Yang *et al.* 2005), based on the GlpT structure and other evidence, as well as a model for the Eo form (P. Maloney, personal communication).

To test these models and to better understand the dynamics of ion exchange systems, it is necessary not only to have static crystal structures, but also some means of measuring changes in structure as the proteins go through their catalytic cycle. For large populations of transporters, any such changes would be hidden by ensemble averaging, as the catalytic cycles for individual transporters are not synchronized. To avoid this problem, we have turned to single-molecule techniques, recently made possible through advances in laser and fluorescence detection technology (Ha 2001).

This paper describes the first attempt to apply the technique of single-pair fluorescence energy transfer (spFRET) to single molecules of OxlT, attached to glass coverslips by a method described previously (Pal *et al.* 2005). The results show the feasibility of such measurements, and open up the possibility of further single-molecule experiments to examine the dynamics of this and other ion exchange proteins.

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Materials and methods

Mutant and purification

Cysteine-less OxlT (Fu & Maloney 1998) was used as parent for generation of the double-cysteine variant (W17C and V224C) using standard techniques of site-directed mutagenesis (Fu & Maloney 1998). The double-cysteine variant was purified by NTA Ni^{2+}-chelate affinity chromatography as described (Heymann *et al.* 2001) and stored frozen as a concentrated stock (approx. 1 mg mL^{-1}) in buffer (pH 4.5) containing 20% glycerol, 0.4% *E. coli* polar lipid extract (Avanti, Alabaster, AL, USA), 0.25% diheptanoyl phosphatidylcholine (Avanti), 100 mM potassium oxalate, 50 mM potassium acetate.

Labelling with Cy3 and Cy5

The experimental buffer contained 50 mM potassium phosphate, pH 7.5, and 10 mM potassium oxalate. The buffer was supplemented with 0.4% *E. coli* polar lipid extract dissolved in 1.5% *N*-octyl-β-D-glucopyranoside (Calbiochem, San Diego, CA, USA), and passed through a 0.22 μm filter before use. OxlT was labelled at 5 μM with 50 μM each of Cy3 and Cy5 (GE Healthcare, Piscataway, NJ, USA) overnight at 4 °C. Reactions were stopped with 1 mM dithiothreitol, and passed though two successive Sephadex G-25 size exclusion columns (Pro-Spin; Princeton Separations, Adelphia, NJ, USA) to remove unlabelled dyes.

Attachment to coverslips

Coverslips were cleaned and coated with a poly-ethyleneglycol (PEG)/PEG-biotin mixture as previously described (Pal *et al.* 2005). Immobilization was performed as described (Pal *et al.* 2005), with some modifications. All solutions were made in the labelling buffer described above. A coated coverslip was mounted on a cooled metal holder and covered with a plastic chamber, allowing solutions to be exchanged while maintaining a temperature of approx. 8 °C. Non-specific interactions were blocked with 0.2% albumin for 20 min. The coverslip was washed briefly, and incubated with 3.3 μM streptavidin (Invitrogen, Carlsbad, CA, USA) for an additional 20 min. After extensive washing, 20 nM biotinylated anti his-tag antibody (Abcam, Cambridge, MA, USA) was incubated for 20 min. The coverslip was then washed extensively and 5 nM labelled OxlT 17/224 was added and incubated for 45 min before data collection.

Laser confocal microscopy

Confocal scanning optical microscopy was performed with a Nikon Eclipse TE 300 inverted microscope (Micro Video Instruments, Avon, MA, USA). The sample was placed above an oil immersion Nikon 100× Plan Apo NA = 1.4 objective. An Nd:YVO laser's 532 nm emission was circularly polarized and used to produce a diffraction limited focal spot. The sample was scanned through this spot using an x–y piezoelectric transducer (Mad City Labs, Madison, WI, USA). The emitted fluorescence was collected through the same objective, after which donor and acceptor fluorescence were separated using a 645 nm dichroic beamsplitter (Chroma, VT, USA). The fluorescence then passed through band pass filters (Chroma) appropriate to the donor and acceptor dyes (HQ590/75 m for donor and HQ700/75 m for acceptor). The light was then focused onto two separate single-photon avalanche photodiodes.

Data analysis

Single molecule intensity traces for both the donor and the acceptor were recorded with 1 ms precision and binned to 20 ms in order to improve the signal to noise ratio. Relative distances were calculated for each molecule using a maximum likelihood algorithm (Watkins & Yang 2004) which returns the most probable distance based on the donor and acceptor intensities. The intensities used in the distance calculations were taken from the start of a trace to the point of acceptor photobleaching. Backgrounds for each molecule were determined after photobleaching of both donor and acceptor dyes. The distances measured for each molecule were converted into histograms with area normalized to unity. These individual histograms were then summed to produce the histogram shown in Figure 4.

Results

Labelling of single OxlT molecules with donor (Cy3) and acceptor (Cy5) requires that each OxlT molecule have two reactive SH groups. For this study, we have used a mutant of OxlT with cysteines inserted at residues Trp-17 and Val-224. Because the cyanine maleimides may react with either cysteine, after labelling with Cy3 and Cy5 some of the molecules will contain only Cy3 donor, some only Cy5 acceptor, and in some cases one SH will be labelled with Cy3 and the other with Cy5. These donor–acceptor labelled molecules are the only ones suitable for spFRET.

Figure 2 shows a raster scan of a 30 μm × 30 μm area of a coverslip to which have been attached OxlT molecules labelled with Cy3 and Cy5. Panel A shows the fluorescence intensity measured at the donor emission wavelength, with laser excitation at 532 nm. Bright spots indicate the position of molecules containing a Cy3 (donor) label. Panel B shows the fluorescence at the

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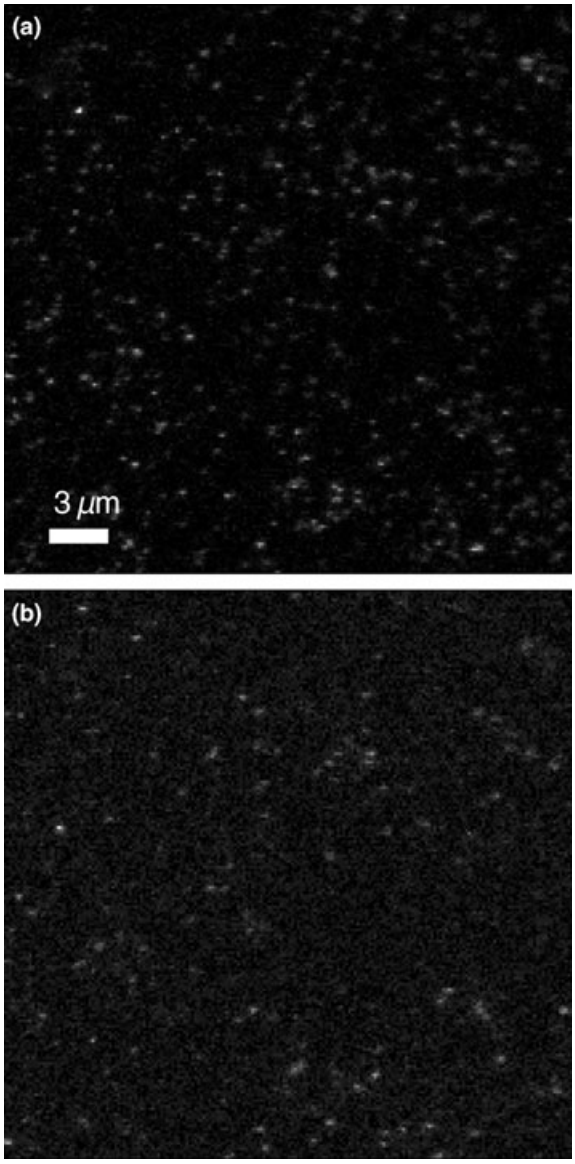

Figure 2 Raster scans of 30 × 30 μm areas showing (a) donor fluorescence; and (b) acceptor fluorescence. Laser light at 532 nm was used, which excites the donor but not the acceptor, so spots in B likely represent molecules containing both a donor and an acceptor, in which acceptor excitation is caused by energy transfer from the donor.

acceptor emission wavelength, again with excitation at a wavelength (532 nm) which causes donor excitation but which does not measurably excite the acceptor (Cy5). Thus, the spots that appear in the acceptor channel are most likely due to energy transfer from Cy3 to Cy5 in molecules containing both labels, and are candidates for spFRET measurements. The positions of these spots in the acceptor channel were determined, and the sample was then moved so that a fluorescence time series could be recorded for each of the spots.

A typical time series for OxlT is shown in Figure 3. Fluorescence at the donor wavelength is shown in green and that at the acceptor wavelength in red. Initially, acceptor fluorescence is high and donor fluorescence is low, indicating possible energy transfer. At the point shown by the arrow marked A, the acceptor fluorescence suddenly decreases to background, due to photobleaching. The fact that the entire decrease in acceptor fluorescence occurs suddenly rather than gradually provides evidence that the acceptor fluorescence prior to photobleaching arises from only a single dye molecule. After the acceptor is photobleached, the donor fluorescence jumps up to a much higher level, again in a single step, as expected if there was energy transfer from the donor to a single acceptor molecule prior to acceptor photobleaching. From the donor and acceptor fluorescence intensities before and after photobleaching, the energy transfer efficiency, E, can be calculated. If the fluorescence is measured for a sufficiently long period, the donor eventually photobleaches, again in a single step marked by the arrow B, indicating that the spot contained only a single donor fluorophore. Only spots which satisfied the criterion of having single step photobleaching of both donor and acceptor were used for data analysis.

Figure 4 shows a histogram of energy transfer data for 20 spots that met the above criteria for spFRET. The mean of a Gaussian fit to the data gives a donor-acceptor distance of 0.935 R_0. Since the Förster distance, R_0 (the distance at which energy transfer is 50%), for the Cy3–Cy5 pair is calculated to be 47.3 Å, this gives a mean distance between donor and acceptor of 44.2 Å. For comparison, the OxlT homology model for the Ei form predicts a distance between the corresponding Cα atoms of positions 16 and 223 (the first Met residue is absent in the OxlT homology model) of 39.6 Å (Fig. 5). Considering the length of the linkage between the Cα and the Cy3 and Cy5 fluorophores, together with the tendency of the bulky fluorophores to extend away from the compact protein structure, the difference between the spFRET and the Cα distances is within expected limits. This problem of the location of the fluorophores relative to the Cα position along with errors in the calculation of R_0 limit the accuracy of spFRET distance measurements, but should not affect the ability of this method to detect *changes* in distance, which are the most important parameter for testing models for the conformational change and for observing the dynamics of transitions between different conformations.

Discussion

The data presented above represent the first application of spFRET to obtain structural information in an ion

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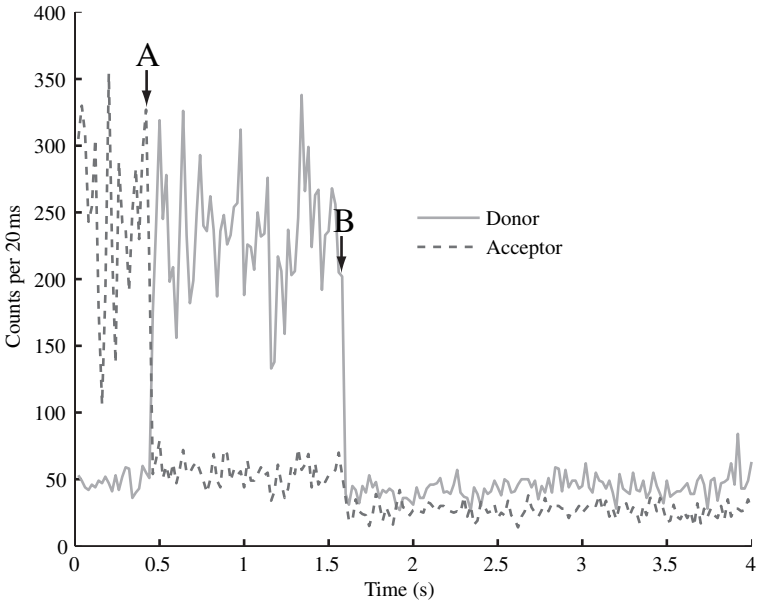

Figure 3 A typical fluorescence time series showing fluorescence at the donor wavelength in a solid line and at the acceptor wavelength in a dashed line. Single step photobleaching of acceptor (arrow A) and donor (arrow B) dyes is observed, indicating the presence of an OxlT molecule labelled with one donor and one acceptor molecule. Donor fluorescence is quenched up to (a), the point of acceptor photobleaching, due to energy transfer. Backgrounds in the donor and acceptor channels are calculated from the intensities after (b), the photobleaching of the donor, for each OxlT molecule.

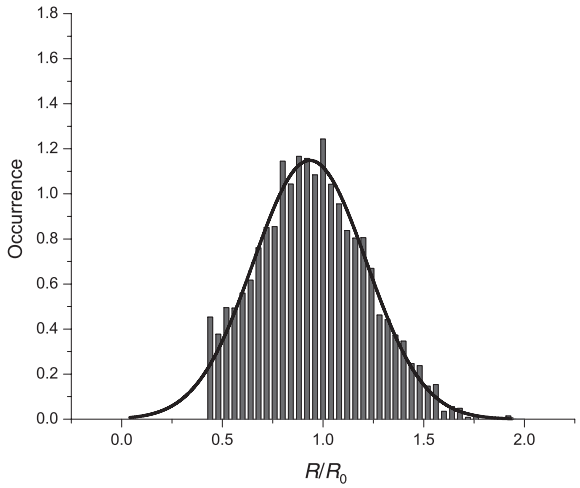

Figure 4 A relative distance histogram of 20 OxlT molecules which met the criteria for labelling by a single donor molecule and a single acceptor molecule.

exchange protein. The specificity of attachment of the labelled OxlT proteins to the coverslip surface, and the ease of detecting spots containing single donor and acceptor molecules, demonstrates the feasibility of this approach. Furthermore, the good agreement between the homology model for the Ei form of OxlT and the spFRET distance provides evidence that the spFRET method can yield useful information on distances in ion exchange proteins.

It should be noted that the distance values are based on the assumption that the orientation factor, κ^2, is 2/3, expected if donor and acceptor are free to rotate randomly within the lifetime of the donor. This is probably a reasonable assumption, in light of the flexible attachment between the fluorophores and the protein backbone, but it has not yet been tested experimentally. The refractive index of $n = 4/3$ for water was used in calculating R_0, but this index may be higher due to the presence of the protein or the proximity of the glass coverslip. Thus, the actual distance between the fluorophores could be somewhat higher or lower than is estimated on the basis of random orientation and $n = 4/3$, but these errors are associated with R_0 and not the relative distance R/R_0, the experimental value which is determined through spFRET.

The distribution of distances may be broad because the signal to background is only 2–5 and the presence of background reduces the accuracy of the distance measurement. The broad distribution might also reflect the presence of different OxlT conformations, but this cannot be determined at present because of the effects of background on the measurements. The data were binned to 20 ms in order to improve the signal to noise ratio, but OxlT may have a turnover which is faster than 20 ms under the experimental conditions. In order to produce distance measurements which monitor real time changes in OxlT, the binning time needs to be reduced, but this would in general reduce the signal to noise ratio and with present conditions this would broaden the distance distribution even more. Currently, modifications to the data collection apparatus are being made to increase the signal to background ratio to thereby achieve both a higher time resolution and a more accurate distance measurement.

The ultimate purpose of such spFRET measurements is to detect changes in conformation during the transport cycle. The cysteine substitutions at positions 17 and 224;

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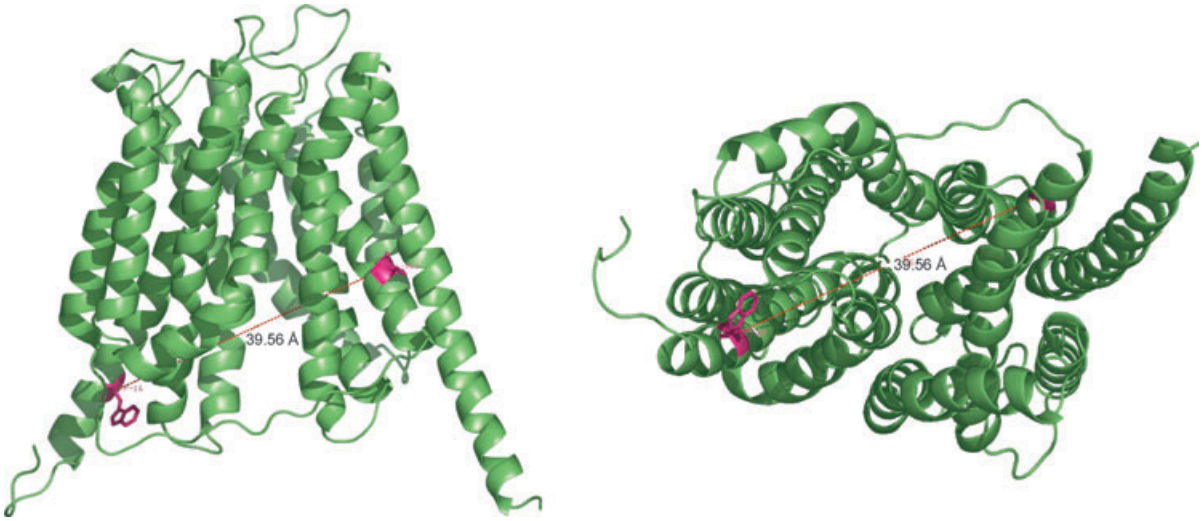

Figure 5 The OxlT homology model (Yang *et al.* 2005) for the inward facing Ei form showing a predicted distance between the Cα atoms for the residues labelled in the experiment of 39.6 Å. Left, side view of molecule from the lipid bilayer; right, top view of the molecule from the cytoplasmic side.

however, are unlikely to provide such information; since the expected changes in distance based on the models of the Maloney group would be approx. 2 Å, probably too small to be detected by this method. Nevertheless, the results show in principle that measurements with cysteines substituted at different positions should be able to provide such information. Calculations based on the models indicate that for some double cysteine substitution positions, distance changes >7 Å are expected (P.A. Venkataraman, personal communication), which should be easily measurable by this spFRET technique. Such measurements will test hypotheses regarding the structure of the Eo form and, with improved time resolution, may permit observation of real-time changes in OxlT structure during the transport cycle.

In general, the spFRET method has the great advantage that individual molecular conformations can be measured (Lipman *et al.* 2003), which otherwise would be hidden by averaging in ensemble measurements. It does have certain disadvantages. The first of these is the low intensity of the measured fluorescence, which restricts both the time and distance resolution of the method. The second is that exogenous fluorophores must be reacted with the protein of interest, and these may interfere with the functionally important changes in conformation. The magnitude of this problem can be assessed by making functional measurements on the labelled material, and choosing labelling sites where little effect on function is observed. Another problem is that the exogenous fluorophores are attached to the protein by flexible linkers, so the distance between fluorophores may not accurately reflect the distance changes of the amino acids to which they are attached. Nevertheless, it is highly likely that changes in fluorophore distances will be sensitive to conformational changes that affect the distance between the α-carbon atoms of the residues to which they are attached. Thus, spFRET can provide unique and useful information that complements ensemble measurements and can provide insights into molecular mechanisms of transport.

Conflict of interest

There are no conflicts of interest.

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