Thinking outside the crystal

Complementary approaches for examining transporter conformational change

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As the number of high-resolution structures of membrane proteins continues to rise, so has the necessity for techniques to link this structural information to protein function. In the case of transporters, function is achieved via coupling of conformational changes to substrate binding and release. Static structural data alone cannot convey information on these protein movements, but it can provide a high-resolution foundation on which to interpret lower resolution data obtained by complementary approaches. Here, we review selected biochemical and spectroscopic methods for assessing transporter conformational change. In addition to more traditional techniques, we present 19F-NMR as an attractive method for characterizing conformational change in transporters of known structure. Using biosynthetic labeling, multiple, non-perturbing fluorine-labeled amino acids can be incorporated throughout a protein to serve as reporters of conformational change. Such flexibility in labeling allows characterization of movement in protein regions that may not be accessible via other methods.

High-resolution structures of membrane proteins are now emerging at an exponential rate. While these structures provide molecular details fundamental to understanding membrane protein function, alone they cannot convey any dynamic information. Therefore, integration of static structural data with measurement of protein movement is essential to understanding function at a molecular level. While most (if not all) integral membrane proteins must undergo some type of conformational change to carry out their specific tasks, the necessity for such change in transporter proteins is particularly obvious. In order to shuttle their cargo across the membrane, transporters must orchestrate a series of conformational changes for every round of ion transport. In the case of coupled transporters, these conformational changes are driven by an energy source (either light, ATP hydrolysis, or a transmembrane ion gradient), which enables these proteins to move solutes against their electrochemical gradients.

This is in contrast to ion movement through channels, where—upon opening of the channel gates—passive diffusion occurs through aqueous pores. Although this gating can itself involve substantial conformational changes, such movement is not required for each cycle of ion transport. In transporters, the coupling of conformational change with substrate translocation highlights the importance of identifying stable transporter conformations and determining the mechanism of conversion between these conformations.

Here, we review selected biochemical and spectroscopic methods for assessing transporter conformational change, discussing specific examples that best illustrate the type of information that can be learned from each technique. We begin with crystallography, which permits extrapolation of movement based on observation of multiple static structures. We then move to techniques that provide complementary information on protein conformations and dynamics that may be inaccessible via crystallography alone. This includes biochemical approaches for assessing changes in residue accessibility as well as fluorescence techniques for characterizing substrate-induced conformational change. When viewed on its own, information provided by these techniques can be of relatively low resolution. However, when seen through the lens of an available high-resolution crystal structure, such data can take on more substantial meaning. Finally, we discuss 19F-NMR as a well-suited but underutilized method for characterizing conformational change in transporters of known structure. Here, multiple non-perturbing labels can be biosynthetically incorporated throughout the protein, allowing characterization of movement in regions that may not be accessible via other methods.

Crystallographic Filmstrips

Just as the illusion of motion can be achieved on film using a series of static pictures, protein motion can be inferred through observation of multiple conformational states. Occasionally, such conformations can be observed within a single X-ray crystallographic snapshot, as in the case of the AcrB subunit of the E. coli AcrA/AcrB/TolC drug resistance pump. The structure of the trimeric AcrB protein depicts each of the three monomers in different conformations, and implies a peristaltic pump mechanism for drug transport. Unfortunately, such serendipity in crystallization is rare, and additional protein conformations must usually be identified independently. In cases where conformational states have been defined functionally, clever biochemical “tricks” can be employed to capture and crystallize the
protein in these conformations. The nature of transporters lends itself well to such a strategy, since conformational change is propagated by binding and release of substrates. Therefore, crystallographic access to certain species can be granted (in theory) by manipulating substrate availability.

Perhaps the most stunning “crystallographic filmstrip” is that of the P-type Ca\textsuperscript{2+}-ATPase, SERCA1a. Prior to the collection of any high-resolution structural data, several functional aspects of SERCA1a had been well defined. SERCA1a transports calcium from the cytosol of skeletal muscle cells into the sarcoplasmic reticulum store, hydrolyzing ATP and counter-transporting protons in the process.\textsuperscript{3-8} In a P-type ATPase mechanism, this process must involve transition between several conformations (Fig. 1). One conformation must allow Ca\textsuperscript{2+} to bind on one side of the membrane, and another must allow Ca\textsuperscript{2+} to be released on the opposite side.\textsuperscript{9} The first high-resolution crystal structure of SERCA1a depicted the protein with Ca\textsuperscript{2+} tightly bound at two locations deep within the transmembrane domain.\textsuperscript{10} Following observation of this conformation, previous functional characterization of SERCA1a was exploited to obtain additional protein snapshots. Based on the knowledge that transport is driven by phosphorylation and substrate binding, subsequent crystallographic studies used an extensive array of ligands, metals, substrate analogs, and inhibitors to stabilize SERCA1a in various conformational states.\textsuperscript{11-16} This strategy yielded impressive results: since publication of the first structure of SERCA1a in 2000, over 20 crystal structures have been reported that depict the protein in nine different conformational states.\textsuperscript{17}

The extensive crystallographic coverage of SERCA1a reaction intermediates has allowed construction of a fairly detailed picture of the molecular movements that occur as the protein hydrolyzes ATP and translocates ions across the membrane. Although static crystal structures alone cannot depict such movement, they provide an invaluable high-resolution basis on which dynamics can be projected. Molecular dynamics simulations,\textsuperscript{18,19} which combine available structural data and thermodynamic constraints to compute likely protein movements, were used to investigate the functional roles of several critical residues in SERCA1a.\textsuperscript{20} Combined with other biochemical and biophysical techniques, these molecular simulations have allowed the static crystal structures of SERCA1a to be transformed into a dynamic functional model.\textsuperscript{17}

**Biochemical Topographic Maps**

Long before the arrival of X-ray crystallography to the membrane protein scene, probes of solvent accessibility aided in determining topology and constraining conformational models of protein movement. More recently such approaches have been recognized as powerful complementary techniques for accessing crystallographically inaccessible protein conformations, as these normally low-resolution data on solvent accessibility gain vivid new meaning when viewed in light of a high-resolution structural model. In the commonly used “cysteine-scanning mutagenesis” strategy, residues in the protein are systematically replaced with cysteine and then tested for reactivity with an exogenously added probe molecule. The availability of each residue for modification may be measured in the presence of substrate, ligand, or other modulators, thus allowing for comparisons of conformational changes induced by these molecules. In the case of the *E. coli* Na\textsuperscript{+}/H\textsuperscript{+} antiporter NhaA, cysteine-scanning analysis was used to reveal the opening of a cytoplasmic funnel first identified in the crystal structure. Several residues in this funnel were inaccessible to reaction at the acidic pH used for crystallization but were accessible to reaction at a more alkaline pH. This implies that at the higher, physiologically relevant pH the funnel widens to facilitate ion transport.\textsuperscript{21}

The most comprehensive example of how cysteine scanning can help define functionally relevant (but crystallographically inaccessible) states is seen in the galactoside/H\textsuperscript{+} symporter LacY. LacY, a member of the major facilitator superfamily of transporters, utilizes the energy released from downhill transmembrane transport of protons to drive the uphill transport of galactoside sugars.\textsuperscript{22} Despite crystallization of both mutant and wild-type proteins under a variety of conditions, all available structures of LacY depict the protein in an inward facing conformation in which a hydrophilic cavity is open to the cytoplasmic side (Fig. 2A).\textsuperscript{23-25} In the structure where a bound sugar is observed, this molecule is buried deep within the transmembrane domain of LacY.\textsuperscript{24} This suggests that in order for this sugar to be translocated, a relatively large conformational change must occur that opens a pathway towards the periplasm while closing off cytoplasmic access. Support for such an “alternating access” mechanism...
It is obvious from the data presented in Figure 2B that residues lining the cytoplasmic hydrophilic cavity and putative periplasmic cavity are not the only regions subject to changes in NEM reactivity.\(^{35}\) This suggests that a more global change to LacY structure occurs upon substrate binding in addition to those directly involved in alternating substrate access. However, the features of such global change cannot be defined by chemical modification techniques alone. Such techniques are insensitive to any conformational change that does not result in a change to residue reactivity, and thus will not report on residues that remain buried deep within the protein or occluded by lipid. Therefore, while biochemical data provide convincing support for the LacY alternating access transport scheme, the precise nature of the conformations adopted during this process must be defined by other means.

**Fluorescent Reporters**

Although we cannot directly see conformational change on an atomic scale, we can see the evidence of such movement once it is amplified and translated from motion into an observable metric. Proteins can communicate a great deal of information about their movements using fluorescent molecules as translators. Since fluorescent properties (quantum yield, emission and excitation spectra) change in response to local environment, fluorophores can act as sensitive reporters of conformational change. It is even possible to examine conformational changes in a protein using the built-in fluorescent properties of aromatic amino acids. In cases where higher sensitivity is needed, exogenous fluorescent labels with higher quantum yields can be used. The simplicity and flexibility of many fluorescence techniques have contributed to their continued popularity.

The physiological importance of tryptophan residues in transporter function make intrinsic tryptophan fluorescence an attractive method for examining conformational change and substrate binding.\(^{36-39}\) Tryptophan is both hydrophobic and capable of forming hydrogen bonds, so these residues are often located in the binding pocket of transporters with hydrophobic or planar substrates as well as at the protein/lipid interface, stabilizing the protein through interaction with lipid headgroups.\(^{40}\) Because of these important functional roles, monitoring changes in the environment of Trp residues can deliver information regarding transporter conformational change. Often, water-soluble fluorescence quenchers such as acrylimide are used to measure the solvent accessibility of tryptophan residues. Any observed changes in accessibility upon substrate addition can be attributed to conformational changes resulting from binding or to direct interaction of the substrate with tryptophan. In cases where a high-resolution structure is known for one conformation, this accessibility data can be more specifically interpreted. Such an approach was used to examine substrate-binding effects in the *E. coli* ABC transporter, MsbA. Based upon the crystal structure of MsbA, it was hypothesized that in order for ATP hydrolysis to occur, there must be a significant rearrangement of the protein that brings together two protein domains separated by ~50 Å in the absence of substrate.\(^{41}\) However, addition of ATP to MsbA did not change the Trp-quenching ability of acrylimide, and addition of the MsbA substrate lipid A caused only a mild decrease in Trp accessibility.\(^{37}\) These data indicate that conformational changes induced by substrate binding are either small, or do not affect the local environment of

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**Figure 2. Substrate-induced structural rearrangements in LacY are detected through changes in cysteine-reactivity.** (A) Crystal structure of LacY depicting the cytoplasmic hydrophilic cavity with the bound lactose analog TDG in black (pdb 1PV7). (B) Cysteine-replaced residues subject to an increase (gold) and decrease (blue) in NEM reactivity upon TDG binding. (B) reproduced with permission.\(^{35}\) © 2007 National Academy of Sciences, USA.
Trp residues. These results do not rule out the possibility that a large conformational change that does not alter the solvent accessibility of tryptophan residues, such as domain rotation.

Although tryptophan fluorescence studies offer the ability to examine conformational changes in a protein with minimal perturbation to function or structure, this is achieved at the expense of the sensitivity offered by exogenous fluorescent moieties. In addition to their higher quantum yield, exogenous labels provide increased flexibility in labeling location since they can be covalently attached to any accessible residue that has been mutated to a cysteine. The use of exogenous, environmentally sensitive fluorescent probes has been applied to examine conformational change in all three major classes of membrane proteins, including receptors, channels, and transporters. In the case of transporters, the fluorescence of protein regions involved in conformational change is expected to depend on binding of the substrate that drives the conformational changes. An example of such behavior is seen for the chloride proton antiporter ClC-ec1. Fluorescent labels were placed along the R-helix, a position which lies near the bound Cl- ions and is expected to change solvent exposure during the transport cycle. Proton-dependent spectral changes were observed for all four positions tested along helix R, and the magnitude of these changes was modulated by chloride. Interestingly, addition of a mutation that removes the effect of pH on ClC-ec1 function did not abolish the pH-sensitivity of the fluorescence spectra. This indicates that the pH-dependent conformational change persists even in the absence of proton transport and suggests that additional residues facilitate the conformational change.

Voltage Clamp Fluorimetry (VCF) is an interesting twist on simple fluorescent analysis, which allows real-time monitoring of fluorescence measurements in concert with electrophysiological recordings. This allows protein rearrangements to be correlated with voltage-dependent events such as substrate binding and gating. VCF has been used to examine conformational changes in many transporters, including the glucose transporter SGLT1, the GABA transporter GAT1, and the serotonin transporter SERT. More recently, simultaneous measurements of both fluorescence and current allowed visualization of transition between conformational states in the glutamate transporter EAAT3. Here, the protein was expressed in oocytes and labeled with an environmentally sensitive fluorescent maleimide on an outer membrane loop. As the voltage was held constant, current was recorded and fluorescence changes were monitored by microscopy. Using this method, it was demonstrated that the substrates L-glutamate and D-aspartate induce protein rearrangements during both forward and reverse transport. Due to the temporal resolution allowed by VCF, an additional Na+-dependent conformational step preceding glutamate binding was also detected.

Fluorescent Rulers

Fluorophores can be exploited not only for mere observation of conformational change but also for quantification of these changes. A common spectroscopic tool for this purpose is Förster resonance energy transfer (FRET). In a typical FRET system, two chromophores are attached to the protein at locations that are suspected to undergo movement relative to each other during conformational changes. Attachment can be accomplished covalently through cysteine modification or thorough the incorporation of genetically encoded fluorescent tags such as CFP and YFP. The donor chromophore is excited at a particular wavelength (typically, one that will minimize direct excitation of the acceptor chromophore), and the fluorescence transfer to the acceptor chromophore is detected and quantified. Typically, a useful amount of energy transfer occurs between chromophores when they are between 10–75 Å apart. Since the efficiency of energy transfer between donor and acceptor molecules depends upon the inverse sixth power of the distance, FRET measurements can be used to calculate distance.

When made within populations of proteins, FRET measurements are well suited to placing lower limits on protein movement or determining relative conformational changes induced by substrate binding. Such measurements proved useful in studies of substrate-induced conformational changes occurring in the human anion exchanger, hAE1. hAE1 exists as a dimer, and acts in human erythrocytes to carry out the electroneutral exchange of chloride for bicarbonate. During this exchange, the protein cycles through two main conformations where a single anion-binding site is exposed to either the cytoplasmic or extracellular side. Energy transfer between two labels placed on opposite monomers was found to increase in the presence of chloride or bicarbonate, but not citrate. This FRET increase indicates that substrate binding to hAE1 is capable of inducing significant protein rearrangements. Further quantification of the conformational change demonstrated that a relative inter-subunit movement of at least 7 Å occurs upon substrate binding.

More recently, many FRET experimentalists have capitalized on single-molecule technology, which allows observation of individual proteins over time instead of simultaneous observation of many protein molecules. The smFRET approach removes the ensemble averaging that occurs when observing multiple conformations, and thus allows for more reliable measurements of distance to be made for any single protein conformation. Experimentally, observation of a single molecule for FRET is accomplished by immobilizing labeled proteins at a low density on a glass surface or by monitoring single molecules as they diffuse through an area of excitation. Such smFRET techniques have provided additional information concerning galactopyranoside-induced closing of the cytoplasmic cavity of LacY.

In addition to providing more reliable distance measurements of static conformational states, smFRET also opens the door towards protein dynamics. smFRET studies of the F$_0$F$_1$ ATP synthase provide an excellent example of how such measurements can be used to assess separate conformational states together with kinetic parameters of conversion between these states. In these experiments, the FRET donor molecule was placed on the γ-subunit, while the acceptor molecule was bound to the b-subunit (Fig. 3A). Previous biochemical and computational experiments suggested that the γ-subunit rotates during ATP synthesis and hydrolysis while the b-subunit remains static. Measurement of the FRET efficiency between the labeled residues over time provides a striking picture of three separate intensity levels corresponding to three distinct residue positions (Fig. 3B). Calculation of the distance between FRET molecules confirmed that these positions corresponded to that expected for a rotating γ-subunit (Fig. 3C). The step-wise jump between these states indicates that the transition between conformers occurs in discrete steps instead of a continuous rotation. Analysis of many smFRET records under conditions promoting either ATP hydrolysis or synthesis allowed the
Histogram of FD/FA measurements reveals that as ATP hydrolysis progresses, a single F0F1 ATP synthase molecule reconstituted into liposomes. (A) Model of F0F1 ATP synthase in side and top-down views. The FRET donor (green) is attached to a β-subunit, and the FRET acceptor (red) is attached to the γ-subunit. Rotor subunits are shown in blue, and stator subunits are shown in orange. (B) FRET measurement (F donor/F acceptor) during ATP hydrolysis for a single F0F1 ATP synthase molecule reconstituted into liposomes. (C) Histogram of Fd/Fa measurements reveals that as ATP hydrolysis progresses, three distinct donor positions (R DA) are observed. Figure reproduced with permission.54

authors to further conclude that during synthesis the γ-subunit turns in a clockwise manner while ATP hydrolysis causes rotation in the opposite direction.

Digging Deeper

The methods discussed so far are well suited for study of transporters like LacY, OxtT and GlpT whose mechanisms involve alternating access of large aqueous cavities between sides of the membrane.24,55,56 However, transporters need not undergo such large conformational changes to coordinate movement of their cargo. In order for a transporter to function properly, protein movement must be orchestrated in such a way that substrate does not have simultaneous access to both sides of the membrane. While this can be accomplished by pivoting the protein around the bound substrate molecule, opening and closing of gates on opposite sides of the membrane can also be accomplished by much smaller protein movements. Such conformational changes may easily go undetected by methods that rely on differential labeling by large modifying reagents.

Small conformational changes have been proposed to underlie ion transport facilitated by CLC Cl-/H+ antiporters, including the E. coli CLC homolog ClC-ec1. Despite crystallization under a variety of conditions, to date only one conformation has been observed for wild-type ClC-ec1.57-61 This structure depicts the protein in an “occluded” state in which bound chloride ions do not have access to exits on either side of the protein. Clearly, in order for antiport to occur, additional conformations must exist that allow the protein to pick up and deliver ions from one side of the membrane to the other. Recent work has shown that whatever the ClC-ec1 antiport mechanism may be, it does not involve a large quaternary rearrangement of the subunits.62 This was demonstrated by restraining protein movement through covalent cross-linking of residues across the ClC-ec1 dimeric interface. The ability of such “straight jacketed” proteins to facilitate Cl-/H+ antiport indicates that any movement of the subunits relative to each other must be small.

So how can we examine conformational changes without biasing ourselves towards more traditional transporter movements involving large domain rearrangements or changes in solvent accessibility? Crystallography has the potential to provide snapshots of the resolution necessary to observe subtle differences in protein conformations, but (as discussed above) such filmstrips are not always available. Protein structures can also be obtained using solution-state NMR techniques, but NMR is limited considerably by the size of the molecule being examined. This is of particular concern with integral membrane proteins, whose effective size is increased significantly with the addition of detergent needed for stabilization. Nevertheless, for proteins in which the X-ray crystallographic structure of one conformation is already known, NMR has the potential to identify and characterize regions of conformational change even in those cases where it cannot define additional high-resolution structures on its own.

We propose 19F-NMR as an ideal (but as yet untapped) method for identifying moving regions in transporters of known structure. The 19F chemical shift is highly sensitive to the environment, making it an excellent probe for conformational changes of proteins.63-66 As these changes occur, the chemical environment surrounding certain amino acids will be altered, resulting in changes in the 19F NMR spectrum of the labeled protein. When evaluated in light of high-resolution structural data, the shift in chemical environment of labeled residues will identify which portions of the protein move as the transporter catalyzes ion transport. This concept was illustrated years ago with the light-activated receptor rhodopsin. Residues in the putative intracellular loops of this protein were mutated to cysteine and reacted with a fluorinated label.67 The observation of light-induced 19F chemical shifts for all but two of the residues tested (Fig. 4A and B) led to the conclusion that a large portion of the cytoplasmic face of rhodopsin undergoes structural changes upon activation. Since publication of this study, high-resolution crystallographic data has become available for bovine rhodopsin.68 Mapping of the 19F-labeled residues onto this structure provides additional insight into the nature of the conformational change observed through 19F-NMR. Figure 4C illustrates that the two residues that do not experience light-induced changes (shown in yellow) face the outside of the helical bundle. The residues that do experience a change in chemical environment are located on (or face towards) regions that had previously been shown to move upon light activation.69,70
To fully exploit the potential of 19F-NMR, it will be necessary to examine probes placed throughout the protein. Although the rhodopsin experiments described above demonstrate the feasibility of acquiring 19F-NMR spectra of a relatively large membrane protein, they suffer the same limitation as fluorescence approaches in that they relied on the generation of cysteine mutants and their accessibility to labeling reagents. This limitation is easily overcome, however, as biosynthetic labeling of proteins with fluorine can be accomplished by supplementing the growth media with a fluorinated amino acid while excluding the unlabeled amino acid. The physical size of the fluorine nucleus (1.35 Å, comparable to the 1.2 Å proton) gives it a major advantage over labeling with the traditionally bulky chemical or fluorescent moieties, which are more likely to cause artificial changes to protein conformation.63 Using biosynthetic labeling, multiple, non-perturbing fluorine-labeled amino acids can be incorporated throughout a protein to serve as reporters of conformational change. Such labeling can be accomplished regardless of the solvent accessibility of residues.

Although 19F-NMR has primarily been used to examine conformational change in soluble proteins,63,71 a number of studies have demonstrated that reasonable 19F signal can be achieved with membrane proteins as large as 65 kDa.72-76 Experiments with these labeled membrane proteins have so far addressed primarily basic structural questions concerning topology and insertion depth of the labels. However, we see no reason why these labels could not also be used as reporters of conformational change. We envision that once regions of interest are identified, 19F-NMR techniques can also be used to investigate more specific aspects of conformational change. For example, paramagnetic broadening can be used to determine the solvent or membrane accessibility of different residues.73 Since the ability of a paramagnet to broaden the 19F resonance depends on the inverse sixth power of the distance between the probe and the 19F nucleus, they can also be used to measure distances.63,75 NMR can also be used to extract kinetics of protein movement.63,77 As a fluorine-labeled residue moves between different local environments during conformational change, its NMR frequencies will shift and broaden upon the speed of this movement relative to the NMR timescale. Examination of these frequencies then allows for determination of the rate of interconversion between conformational species.

The number of high-resolution structures available for transporters is growing rapidly. With this new information comes an increased need for methods to translate these static pictures into dynamic functional models. Together with tried and true biochemical and fluorescence spectroscopic techniques, 19F-NMR should be a valuable addition to our arsenal of complementary methods to study transporter conformational changes. When viewed in light of available crystallographic data, such methods will facilitate the characterization of protein movement necessary for achieving cohesive molecular models of transporter mechanism that are not biased towards particular types of conformational change.

Figure 4. 19F-NMR reveals light-induced structural changes in mammalian rhodopsin. (A) Secondary structural model of bovine rhodopsin showing the location of residues examined using 19F-NMR. Red shading indicates solvent accessibility of residues. (B) Light-induced structural changes are indicated by changes in the 19F-NMR spectra of fluorine-labeled rhodopsin upon transition from dark (red) to light (blue). (C) Three-dimensional structure of bovine rhodopsin (pdb 1GZM) highlighting the 19F-NMR results. Residues subject to light-induced chemical shifts are in red, while light-insensitive residues are in yellow. (A and B) reproduced with permission. © 1999 National Academy of Sciences, USA.
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