A Fluorescence-Detection Size-Exclusion Chromatography-Based Thermostability Assay for Membrane Protein Precrystallization Screening

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SUMMARY

Optimization of membrane protein stability under different solution conditions is essential for obtaining crystals that diffract to high resolution. Traditional methods that evaluate protein stability require large amounts of material and are, therefore, unsuited for medium- to high-throughput screening of membrane proteins. Here we present a rapid and efficient fluorescence-detection size-exclusion chromatography-based thermostability assay (FSEC-TS). In this method, the target protein is fused to GFP. Heated protein samples, treated with a panel of additives, are then analyzed by FSEC. FSEC-TS allows one to evaluate the thermostability of nanogram-to-microgram amounts of the target protein under a variety of conditions without purification. We applied this method to the Danio rerio P2X4 receptor and Caenorhabditis elegans GluCl to screen ligands, ions, and lipids, including newly designed cholesterol derivatives. In the case of GluCl, the screening results were used to obtain crystals of the receptor in the presence of lipids.

INTRODUCTION

Growth of membrane protein crystals for the purpose of determining the underlying atomic structure traditionally requires a labor-intensive screening approach to identify conditions that stabilize the protein of interest. This “precrystallization” screening is usually performed with purified protein, which places a requirement for large amounts of pure material and, necessarily, the ability to purify the protein of interest. Membrane proteins, particularly those from eukaryotes, are typically expressed at low levels and are often unstable during and after purification. The challenge of eukaryotic membrane protein crystallization bears itself out in the Protein Data Bank, where <1% of the deposited structures are from this elusive category. The degree to which one can identify, in advance of protein purification, conditions and compounds that stabilize a given protein candidate, the higher the likelihood that protein purification will proceed smoothly and that well-diffracting crystals will be obtained. Atomic structures of membrane proteins are of tremendous interest from both basic science and drug development perspectives; relatively little structural information exists for this class of proteins, molecular mechanisms of signal transduction across cell membranes are not well understood, and drug design for targets of many currently prescribed therapeutics (Zambrowicz and Sands, 2003) is rendered relatively blind.

One useful technique for precrystallization screening of membrane proteins is fluorescence-detection size-exclusion chromatography (FSEC) (Kawate and Gouaux, 2006). In this method, the target protein is expressed as a GFP fusion, and the SEC profile of the resulting fusion protein is monitored by fluorescence spectroscopy. This simple method allows for rapid evaluation of protein expression level and monodispersity of the target protein using nanograms to micrograms of unpurified material from whole-cell extracts. This method has proved to be a powerful tool for screening panels of membrane protein orthologs, detergent, and conformationally sensitive antibodies, and thus has led to the recent determination of a number of membrane protein structures from this laboratory, including LeuT, ApcT, ASIC, P2X, GluA2, and GluCl (Hattori and Gouaux, 2012; Hibbs and Gouaux, 2011; Jasti et al., 2007; Kawate et al., 2009; Krishnamurthy and Gouaux, 2012; Shaffer et al., 2009; Sobolevsky et al., 2009; Yamashita et al., 2005).

There are several methods to evaluate protein stability that have already been described, but these techniques often require large amounts of pure protein and, therefore, are unsuitable for high-throughput screening of eukaryotic membrane proteins. Fluorescent dye-based thermal stability assays using a thiol-reactive fluorophore N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) (Alexandrov et al., 2008) have been developed to evaluate the thermostability of membrane proteins in a high-throughput manner. Although the method requires only microgram samples of protein, because of the high sensitivity of the CPM dye, it requires free cysteine residues embedded in the protein core. A thermostability assay that relies upon analytical-size-exclusion chromatography was used effectively to screen compounds and conditions for stabilizing membrane proteins for crystallization (Czyzewski and Wang, 2012; Mancusso et al., 2011), but this latter method requires a relatively large amount of purified sample because of the low sensitivity of UV absorbance.
In the current study, we present a FSEC-based thermostability assay (FSEC-TS). In this method, nanogram-to-microgram quantities of purified or unpurified proteins are incubated over a range of temperatures and then are applied to a SEC column in line with a fluorescence detector to monitor GFP or tryptophan fluorescence. The results from FSEC-TS provide a denaturation or “melting” temperature (T_m), which is used as a reference point to test the degree of protein thermostabilization by a panel of small molecules. We applied this simple and rapid method to the D. rerio P2X4 receptor and the C. elegans GluCl channel to screen ligands, divalent cations, and lipids, including new synthetic cholesterol derivatives. We find that thermostabilization of both proteins by different compounds is not qualitatively altered by the presence of GFP and that, in proof-of-principle experiments, T_m by FSEC-TS agrees well with T_m determined by a radioligand binding assay. The results are useful in identifying compounds for stabilizing the proteins during purification and in crystallization and, in the case of GluCl, have been used to obtain a distinct, lipid-dependent crystal form.

RESULTS

Validation of FSEC-TS

To validate our assay, we used three proteins: EGFP, D. rerio P2X4 (zfP2X4) receptor, and C. elegans glutamate-gated chloride channel α (GluCl). Protein samples were incubated over a range of temperatures for 10 min using a thermal cycler (Figure 1), followed by ultracentrifugation to remove precipitated material. The supernatant was then applied to a SEC column attached to a fluorescence detector to monitor GFP or trypto-phan fluorescence (Figure 1). In these experiments, we used detergents that we knew from previous experience would stabilize the native oligomeric state of the receptors.

As shown in Figure 2A, peak profiles from EGFP samples incubated between 4°C and 65°C were sharp and symmetrical, indicating that the protein remained monodisperse. In contrast, a clear thermal shift of the peak profiles was observed from the samples incubated at temperatures near to and above 70°C (Figure 2A). Aggregation and precipitation after heating resulted in a significant decrease in the peak height, and a melting curve, from the fluorescence signal intensity at the respective temperatures, was observed (Figure 2B). The calculated T_m is 76°C, which is consistent with the reported melting temperature for GFP (76°C), the temperature where one half of the intrinsic fluorescence is lost (Bokman and Ward, 1981; Ward, 1981).

P2X receptors are nonselective cation channels gated by extracellular ATP and are implicated in diverse physiological processes, such as synaptic transmission, inflammation, and taste and pain sensing (Jarvis and Khakh, 2009; North, 2002; Surprenant and North, 2009). Seven P2X receptor subtypes assembled into either homomeric or heteromeric trimers (Jarvis and Khakh, 2009; North, 2002). Recently, our group reported the crystal structure of zfP2X4 in an apo, closed-channel conformation (Kawate et al., 2009). Precrystallization screening of P2X orthologs by FSEC had identified zfP2X4 as a promising target for the crystallization trials.

The well-behaved, crystallized construct of an N-terminal EGFP fusion to zebrafish P2X4 (ΔP2X4-A) (Kawate et al., 2009) was expressed with an octa-histidine affinity tag and purified for the assay (see Figure S1A available online). FSEC peak profiles from the EGFP-tagged ΔP2X4-A samples incubated between 4°C and 40°C were sharp and symmetrical (Figure 2C); these peaks correspond to the functional P2X receptor trimer. A clear thermal shift of the peak profiles was observed from the samples incubated around 50°C (Figure 2C). Protein denaturation led to a significant decrease in the trimer peak height and concomitant increase in the putative monomer peak height. A clear melting curve from the trimer peak height at the respective temperatures was observed (Figure 2D). The calculated T_m is 49°C, on the basis of the trimer peak height.

To verify that the result from FSEC-TS is relevant to binding-competent receptor, we measured the ATP binding activity of the heat-treated samples (Figure S1B). The experiment was performed at 100 nM ATP concentration, approximately 5-fold above the K_d value of 19.6 ± 3.8 nM (Figure S1C). The calculated T_m is 47.8°C ± 0.3°C, consistent with FSEC-TS (49°C). To assess the effect of the GFP-tag on T_m, we also obtained a melting curve from EGFP-free ΔP2X4-A samples by monitoring tryptophan fluorescence from the endogenous Trp residues in ΔP2X4-A (Figure 2D); the estimated T_m is 50°C, not significantly different from the T_m for EGFP-tagged ΔP2X4-A (49°C).

GluCl is a pentameric chloride channel gated by glutamate and ivermectin (Cully et al., 1994) and is a member of the Cys-loop receptor superfamily (Thompson et al., 2010). This receptor family also includes the ion channels activated by acetylcholine, serotonin, γ-aminobutyric acid, and glycine. GluCl was identified as a promising target for crystallization by FSEC screening of Cys-loop receptor orthologs, which subsequently led to the determination of the structure of a GluCl-Fab complex with the
allosteric agonist ivermectin bound and the channel in an open conformation (Hibbs and Gouaux, 2011).

We carried out FSEC-TS with GluCl to test the validity of the assay and to identify compounds for stabilizing the receptor in a resting, closed channel state. The crystallized construct of EGFP-free GluCl (GluCl cryst) was purified (Figure S1D) and incubated at 4–80°C and applied to a SEC column, with the eluted material monitored by tryptophan fluorescence (Figure 2E). We used the TSKgel SuperSW2000 column for GluCl, which requires only 15 min per sample, to test many different lipid combinations in a high-throughput manner, as described later. A melting curve based on the peak height of the pentameric species was observed (Figure 2F), with a calculated Tm of a remarkably high 59°C. Determination of Tm based on a radioligand binding experiment using 3H-ivermectin yields a similar melting temperature of 58°C (Figure S1E).

Evaluation of Ligands for Cocrystallization by FSEC-TS

To test whether FSEC-TS can be used to estimate the effect of ligands on membrane proteins for crystallization, we evaluated

![Figure 2. Thermostability Profiles of zfP2X4 and GluCl](image-url)

(A, C, and E) Representative FSEC profiles from EGFP (A), EGFP-ΔP2X4-A (C), and GluCl cryst (E), heated at the respective temperatures, detected by GFP fluorescence (A and C) or by Trp fluorescence (E).

(B, D, and F) Representative melting curves of EGFP (B), EGFP-tagged and EGFP-free ΔP2X4-A (D), and GluCl cryst and GluCl-EGFP (F). Melting temperatures (Tm) were determined by fitting the curves to a sigmoidal dose-response equation. The fitted curves are shown as a black line.

See also Figure S1.
the thermostabilizing effects of ATP on zfP2X4 and of ivermectin on GluCl<sub>cryst</sub>. Both ligands bind to their cognate receptors with high affinity, and, thus, we can use them to examine whether we can pick up stabilizing effects of tightly bound ligands. In FSEC-TS, both ATP and ivermectin had dramatic stabilizing effects, increasing the calculated \( T_m \) by 21°C for ΔP2X4-A and by 22°C for GluCl (Figures 2D and 2F, respectively). In accordance with these results, crystallization of zfP2X4 with ATP yields crystals that diffract to 2.8 Å resolution (Hattori and Gouaux, 2012), and crystals of the GluCl-ivermectin complex diffract to 3.3 Å resolution (Hibbs and Gouaux, 2011).

**FSEC-TS with Unpurified Protein**

One of the advantages of FSEC using GFP-tagged protein is that unpurified protein can be analyzed because of the wide spectral separation between GFP fluorescence and the fluorescence intrinsic to most proteins. To test whether unpurified protein can be analyzed using FSEC-TS, EGFP-tagged ΔP2X4-A and GluCl obtained from whole-cell lysates were heat-treated in the presence and absence of ATP and ivermectin, respectively, and were applied to a SEC column after centrifugation.

For EGFP-ΔP2X4-A, the calculated \( T_m \) values in the absence and presence of ATP are 52°C and 69°C, respectively (Figure 2D), consistent with the \( T_m \) values from the purified EGFP-fusion ΔP2X4-A, which are 49°C in the absence of ATP and 70°C in the presence of ATP.

For GluCl-EGFP, the apparent \( T_m \) values in the absence and presence of ivermectin are 41°C and 53°C (Figure 2F), respectively. These values are significantly different from the apparent \( T_m \) values from the purified GluCl<sub>cryst</sub> (58°C in the absence of ivermectin and 81°C in the presence of ivermectin). There are two likely sources of the difference in \( T_m \) between GluCl-EGFP and GluCl<sub>cryst</sub>. First, unlike in zfP2X4, wherein EGFP is positioned at the receptor terminus, in GluCl, the EGFP is inserted into an internal loop, between the third and fourth transmembrane helices. We suggest that insertion of GFP into this loop destabilizes the protein. Second, GluCl-EGFP contains the full-length, wild-type sequence of the GluCl gene, whereas GluCl<sub>cryst</sub> is a truncated construct used for crystallization. Regardless of the differences in \( T_m \), however, the qualitative effect of ivermectin on GluCl thermostability remains unchanged. Taken together, we conclude that FSEC-TS can be used to analyze unpurified GFP-tagged protein.

**Effect of Divalent Cations on zfP2X4**

The effects of three different divalent cations (Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Zn<sup>2+</sup>) on zfP2X4 thermostability were tested by FSEC-TS (Figure 3A). EGFP-tagged ΔP2X4-A samples in a series of buffers containing the respective additives were heat-treated at 50°C for 10 min, which is close to the calculated \( T_m \) of apo protein. As in the case for other P2X receptors, ATP-responses at P2X4 receptors can be modulated by divalent cations, such as Zn<sup>2+</sup> (Garcia-Guzman et al., 1997; Wildman et al., 1999) and Mg<sup>2+</sup> ions (Negulyaev and Markwardt, 2000). The significant divalent cation-induced stabilization of the trimer peak is observed in the presence of all tested divalent cation (Figure 3A). Consistently, best crystals of zfP2X4 in an apo, closed state (Kawate et al., 2009), as well as crystals in the presence of ATP (Hattori and Gouaux, 2012) were obtained in presence of Mg<sup>2+</sup>.

**Effect of Ligands on zfP2X4**

The effects of different P2X ligands, 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP) (antagonist) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (antagonist) on the thermostability of GFP-fusion ΔP2X4-A were tested by FSEC-TS (Figure 3A). TNP-ATP stabilized the zfP2X4 trimer to a similarly strong extent as was observed with ATP (Figure 3A). PPADS, by contrast, destabilized the protein (Figure 3A). In correlation to the effects of ligands on thermostability, we have successfully crystallized zfP2X4 in the presence of TNP-ATP but have not been able to produce crystals with PPADS.

**Effect of Lipids on zfP2X4**

Purification of membrane proteins from their native environment requires the use of detergents. However, detergent solubilization of membrane proteins often leads to aggregation, because membrane proteins tend to be less stable in detergent micelles than in the lipid bilayer. To remedy this behavior, the addition of lipids during purification or crystallization can be used to improve membrane protein behavior for the purposes of...
biophysical characterization, crystallization, and structure determination (Guan et al., 2006; Hattori et al., 2007; Hibbs and Gouaux, 2011; Krishnamurthy and Gouaux, 2012; Lemieux et al., 2003; Sobolevsky et al., 2009; Zhang et al., 2003).

The effects of ten different lipids on the thermostability of GFP-fusion ∆P2X4-A were examined by FSEC-TS (Figure 3A) and also by ATP binding assay (Figure S2). Among the tested lipids, phosphocholine hydroxyethyl cholesterol (CH-PC), maltose side hydroxyethyl cholesterol (CH-maltoside), and a hexaethyleneglycol hydroxyethyl cholesterol (CH-HEG) are newly designed cholesterol derivatives (Figures 3B–3D). Hydroxyl groups of cholesterol derivatives as well as other lipids by FSEC-TS.

The crystallized construct of zfP2X4 (Hibbs and Gouaux, 2011). MO also stabilized the trimer peak of zfP2X4 by FSEC-TS but was not better than other “good” lipids requires only nanogram-to-microgram quantities of protein and allows for screening of different stabilizing conditions in an efficient manner with both purified and unpurified protein. Using this method, we screened various conditions, such as ligands and lipids, to stabilize zP2X4 and GluCl. Ligand screening demonstrated that ATP, a biological agonist of P2X, and its synthetic analog, NTP-ATP, dramatically stabilized zP2X4, suggesting that these are suitable ligands for cocrystalization. Lipid screening showed that our designed cholesterol derivatives and many other lipids significantly stabilized zP2X4 or GluCl (Figures 3 and 4). Employing the ligands and lipids identified in this study has led to a distinct well-diffracting crystal form of GluCl.

Figure 4. Effects of Lipids on the Thermostability of GluCl
The normalized peak heights from GluCl crystalline heat-treated at 57°C for 10 min, in the presence of the respective lipids. The peak heights were normalized to that from the sample at 4°C without any additive. See also Figure S3 and Table S2.

### Effect of Lipids on GluCl

We previously found that either POPC or DPPC was required for growth of well-diffracting crystals of the GluCl-Fab-ivermectin complex (Hibbs and Gouaux, 2011). To screen lipids for a more stabilizing effect on GluCl, we tested 24 different lipid combinations using FSEC-TS with pure protein (Figure 4). GluCl crystalline samples in a series of buffers containing the respective lipids were heat-treated for 10 min at 57°C, which is close to the calculated Tm of apo protein. Most lipids had some significant stabilizing effect on GluCl, whereas total soy lipid, brain ceramide, and DMPE did not stabilize GluCl (Figure 4). Among tested lipids, sphingomyelin and PO-containing lipids consistently had strong stabilizing effects, and the addition of CHS often further increased thermostability. The lipids identified in this experiment have been fed into crystallization trials of GluCl with the goal of obtaining well-diffracting crystals in an ivermectin free condition, and a new, to our knowledge lipid-dependent crystal form of GluCl has been obtained (Figure S3A). These crystals are from the C2 space group, currently diffract X-rays to between 3 and 4 Å (Figure S3B), and are a promising lead in obtaining the structure of a closed-channel conformation of a eukaryotic Cys-loop receptor.

### Conclusions

In this study, we applied FSEC-TS to zP2X4 and GluCl. This method carries over the advantages of conventional FSEC: it requires only nanogram-to-microgram quantities of protein and allows for screening of different stabilizing conditions in an efficient manner with both purified and unpurified protein. Using this method, we screened various conditions, such as lipids and lipids, to stabilize zP2X4 and GluCl. Ligand screening demonstrated that ATP, a biological agonist of P2X, and its synthetic analog, TNP-ATP, dramatically stabilized zP2X4, suggesting that these are suitable ligands for cocrystalization. Lipid screening showed that our designed cholesterol derivatives and many other lipids significantly stabilized zP2X4 or GluCl (Figures 3 and 4). Employing the ligands and lipids identified in this study has led to a distinct well-diffracting crystal form of GluCl.

### EXPERIMENTAL PROCEDURES

#### Expression and Purification

The crystallized construct of zP2X4 (∆P2X4-A) was expressed as an N-terminal EGFP fusion and was purified as described previously (Kawate et al., 2012). The FXS-Cys-loop receptor was expressed and purified using the His-tag purification strategy as described previously (Johansson et al., 2009). MO also stabilized the trimer peak of zfP2X4 by FSEC-TS but was not better than other “good” lipids.
et al., 2009). The EGFP-tagged and EGFP-free purified proteins were concentrated and dialyzed overnight at 4°C against three changes of buffer I (20 mM HEPES-NaOH [pH 7.0], 80 mM NaCl, 20 mM KCl, and 2 mM cysteine), and then were stored at −80°C before use. The crystalized construct of C. elegans GluCl (GluCl<sub>cond</sub>) with a C-terminal 8x-His tag was expressed in Sf9 cells and purified as described previously (Hibbs and Gouaux, 2011), omitting the addition of Fab before the SEC purification step.

For GluCl experiments in crude cell extracts, a fusion of GluCl with EGFP was made with GFP inserted into the full-length, wild-type C. elegans GluCl<sub>cond</sub> receptor between Aes392 and Sce939 in the mature protein sequence. Sf9 cells at a density of 3–4 × 10⁶ cells per ml were infected with baculovirus encoding GluCl-EGFP and were harvested 72 hr later by centrifugation; cell pellets were stored at −80°C.

Radioligand Saturation Binding Assay for zfP2X4 Measurements of total ATP binding was obtained by adding GFP-fusion ΔP2X4-A to a final concentration of 30 nM in 250 μl buffer I containing 0–1000 nM radiolabeled ATP where the hot ATP was diluted with cold ATP in a ratio of 1:4, yielding a final specific activity of 6 Ci/mmol. Reactions were incubated at 4°C overnight and then were terminated by filtering through GSWP 02500 nitrocellulose membranes pre-equilibrated with buffer I containing 100 μM cold ATP. Filters were washed three times with 2 ml of buffer I, placed in 6 ml of Ultima Gold scintillation cocktail, and counted after 1 hr. Estimates of nonspecific binding were obtained by reactions in the presence of 100 μM cold ATP. The experiment was performed in triplicate, and the data were fit to a rectangular hyperbola using the GraphPad Prism 4 program.

FSEC-TS with Purified Proteins For EGFP, 500 ng of EGFP in 50 μl of buffer II (20 mM Tris-HCl [pH 8.0] and 150 mM NaCl) were incubated at 4°C for 10 min, using a thermal cycler and then were centrifuged at 87,000 × g for 20 min. A fraction of the supernatant (25 μl) was loaded onto a SEC column (Superose 6 10/300 GL, GE Healthcare) pre-equilibrated with buffer II and run at a flow rate of 0.5 ml/min.

For zfP2X4, 1 μg of GFP-fusion and GFP-free ΔP2X4-A in 50 μl of buffer II was incubated at 4–80°C and 4–70°C for 10 min and was centrifuged at 87,000 × g for 20 min. Twenty-five microliters of the supernatant were loaded onto a SEC column (Superose 6 10/300 GL) pre-equilibrated with buffer II (20 mM HEPES-NaOH [pH 7.0], 80 mM NaCl, 20 mM KCl, and 1 mM n-dodecyl β-D-maltoside, C12M) and run at a flow rate of 0.5 ml/min.

For GluCl, 2 μg of GFP-free GluCl<sub>cond</sub> in 100 μl of buffer IV (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 2 mM C12M) were incubated at 4–80°C for 10 min, and centrifuged as described above. Twenty-five microliters of the supernatant were loaded onto a TSKgel SuperSW2000 column (TOSOH Bioscience), pre-equilibrated with buffer IV, and run at a flow rate of 0.35 ml/min.

The eluent from the SEC column was passed through a fluorometer (Kawate and Gouaux, 2006) (excitation, 480 nm; emission, 510 nm for GFP fluorescence). Data were normalized and fit to the Hill equation, as described above.

FSEC-TS Screening of Additives To determine the effect of lipids on the stability of zfP2X4, 5 μg of GFP-fusion ΔP2X4-A in 250 μl of buffer I were rotated at 4°C for 1 hr after addition of 2.5 μl of a 100× lipid suspension (20% DMSO and 80% buffer I). The mixture was next incubated at 50°C for 10 min and then centrifuged. Twenty-five microliters of the supernatant of the sample used was added to FSEC as described above. The rest of the sample was stored at 4°C for use in binding assays; 100× additive solutions were added to GFP-fusion ΔP2X4-A at the respective final concentrations shown in Table S1.

For GluCl, 850 ng of GluCl<sub>cond</sub> in 10 μl of buffer IV were rotated at 4°C for 40 min after the addition of 0.1 μl of lipid stock solution in buffer IV containing 40 mM C12M. The mixture was next incubated at 50°C for 10 min and then centrifuged. A 25 μl aliquot of the sample was used for FSEC as described above. Lipids were added to GluCl<sub>cond</sub> at the respective final concentrations as shown in Table S2.

Binding Assay of Heat-Treated ΔP2X4-A These experiments were performed as described above except that the binding was initiated by adding heat-treated GFP-fusion ΔP2X4-A to a final concentration of 30 nM in 250 μl of buffer I containing 100 nM ATP (1:4 ³H;H; 6 Ci/mmol final specific activity). The entire experiment was performed in triplicate.

Binding Assay of Heat-Treated GluCl<sub>cond</sub> Binding of ³H-ivermectin to GluCl<sub>cond</sub> after heat treatment was performed using a scintillation proximity assay (SPA). A solution of pure GluCl<sub>cond</sub> was made in buffer IV at a subunit concentration of 200 nM, and 70 μl were aliquotted into thin-walled PCR tubes and incubated for 10 min at temperatures ranging from 4°C to 80°C. A suspension was made containing 2 mg/ml SPA beads (YSI copper his tag, GE Healthcare) and 100 mM ivermectin (1:9 ³H:H; 5 Ci/mmol final specific activity; ³H-ivermectin purchased from American Radiolabeled Chemicals and ³H-ivermectin purchased from Sigma) in buffer IV. The SPA suspension was mixed 1:1 (50 μl to 50 μl) with the protein solution by pipetting in a 96-well microtiter plate, and the cpm data were collected after 18 hr of incubation at room temperature.

SUPPLEMENTAL INFORMATION Supplemental Information includes three figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.str.2012.06.009.

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