Point of VIEW

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Staples, Tape Measures, and Bungee Cords: A Variety of Bifunctional Reagents for Understanding and Controlling Ion Channels

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ABSTRACT Chemical modification of proteins with bifunctional reagents has become a widely used technique for analyzing protein structure and dynamics. A new era is emerging, and scientists can now actually control the function of proteins by tethering molecular switches at a desired position. In a new paper, researchers stretch the technique a bit further by using a reactive derivative of a peptide toxin to probe the subunit composition of a voltage-gated K^+ channel.

Understanding the structural basis of ion channel function has been a long quest, and X-ray crystallographic information has been the Holy Grail. We finally have real answers about ion selectivity, ligand binding sites, and gating through the discovery of high-resolution structures of a handful of channels and receptors, but crystallizing membrane proteins is by no means a routine matter. In the meantime, chemical biologists have been busy building and using an abundance of bifunctional reagents to probe and manipulate channels. This approach has evolved from the initial invention of affinity labeling, which sheds light on primary structure, to the analysis of aqueous accessibility with cysteine-reactive probes, which helps scientists understand secondary and tertiary structure. In the paper on page 469 of this issue, Morin and Kobertz (*1*) move this approach into the realm of understanding quaternary structure by using a novel type of bifunctional reagent that spans two proteins within a heteromultimeric complex.

The concept of affinity labeling was developed half a century ago, with the aim of elucidating the structure of ligand binding sites in enzymes and receptors (*2*). In affinity labeling, a ligand first binds reversibly to an active binding site in a protein and then covalently modifies amino acids in this site; a chemical landmark is thus provided for localizing the binding site. Because affinity labeling requires reactive amino acids, which are abundant in catalytic sites of enzymes but rare in receptors, clever tricks were needed to "staple" affinity labels to ion channels. Photoaffinity labels are chemically inert compounds that become hyper-reactive upon light exposure, and this enables their attachment to any of the 20 amino acids present in proteins (*3*). In addition to providing a static picture, photoaffinity labels can be used in combination with time-resolved techniques to map a receptor's binding site in different conformations (*4*).

With the development of cloning and molecular biology techniques, reactive amino acids could be incorporated into the polypeptide chain. Cysteine is ideal for site-directed labeling. It is rare (2.3% genome-wide), its moderate size and hydrophobicity minimally perturb protein structure and function, and its high reactivity toward electrophiles ensures rapid and efficient labeling. Chemists have designed sulfhydryl-reactive compounds with all sorts of physicochemical properties (size, charge, hydrophobicity, fluorescence, photosensitivity, and further reactivity) that can modify the structure and function of proteins, beyond what is possible by simply substituting one of the natural amino acids for another.

In the substituted-cysteine accessibility method (SCAM) developed by Karlin and Akabas (*5*), single-cysteine mutations are assayed for their reactivity toward small

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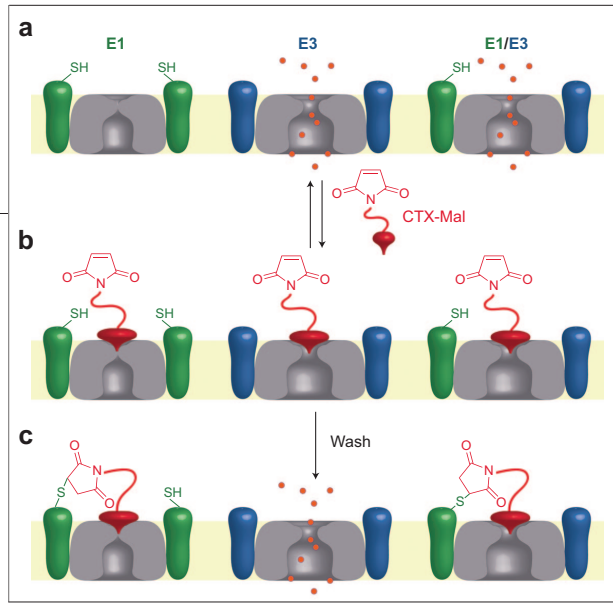

Figure 1. The strategy for identifying the subunit composition of a K^+ channel heterocomplex with a bifunctional cross-linking reagent. a) The starting point. Channels containing E1 or E3 subunits can be distinguished because they have different voltage sensitivities. A cysteine added to E1 and removed from E3 allows selective attachment of the cross-linker. Hypothetical E1/E3 hybrids are shown in the right panel. b) Addition of the bifunctional reagent. The peptide toxin moiety initially binds to the pore, reversibly blocking the channel. c) Covalent attachment to the channel. The maleimide moiety reacts with cysteines, permanently tethering the toxin to channels that contain E1.

nonspecific hydrophilic thiol reagents, and this provides a way to map protein amino acid residues that are water-accessible. In the absence of high-resolution structures, SCAM studies are valuable for defining membrane topology, pore-lining residues, and ligand-binding pockets. SCAM reagents containing quaternary ammoniums (QAs), which act as agonists on the nicotinic acetylcholine receptor, have been used to label cysteines in the acetylcholine binding site and to characterize the ligand orientation requirements for receptor activation (*6*). A change in the microenvironment surrounding a cysteine can cause a change in the SCAM reagent reaction rate, and this provides a way to probe conformational changes in the protein (*5*). Environment-sensitive probes tethered to a cysteine, like a spin label (*7*) or a fluorophore (*8*), also have been used to examine local movements of ion channels.

The advantages of SCAM and affinity labeling can be combined by using a bifunctional affinity reagent and substituting residues in the hypothetical binding pocket, one at a time, with a cysteine (*9*). This strategy has been applied to $GABA_A$ and *N*-methyl-D-aspartic acid receptors to identify residues that are part of the binding cleft and to map reliably the position of the ligand in its binding site. Like SCAM, this strategy can be used to probe local conformational changes of receptors (*10*).

Affinity labeling can also be used to label a cysteine located outside of the binding site, *via* a technique called "exo-mechanism proximity-accelerated alkylation" (*11*). This strategy has been elegantly applied to a voltage-gated K^+ channel, as a way to measure distances between engineered cysteines and the binding site for QA blockers (*12*). The authors used a library of bifunctional reagents that vary in length and are composed of a thiol-reactive maleimide group on one end and a QA on the other end. These molecular *tape measures* tether to the channel *via* affinity labeling, in which QA binding drives the maleimide to react with an engineered cysteine (*13*). Other bifunctional molecular *tape measures* have been synthesized to measure distances within or between sites in ion channels and receptors. These include polymer-linked ligand dimers for spanning agonist binding sites in homotetrameric cyclic nucleotide-gated channels (*14*), a heterobifunctional reagent for measuring between residues on neighboring subunits of nicotinic acetylcholine receptors (*15*), and a DNA-containing cross-linker to measure intramolecular distances in the pore-forming bacterial toxin α-hemolysin (*16*).

New high-resolution structures are leading to a further step in the evolution of bifunctional reagents. We can now re-engineer protein function by attaching to channels and receptors molecular switches that can bring about a conformational change in response to an external stimulus. Long before any high-resolution model of the nicotinic receptor structure was available, Lester and colleagues performed *avant-garde* work, tethering the agonistic photoswitch 3-(α-bromomethyl)-3′-[α-(trimethylammonium) methyl]azobenzene bromide (QBr) in the acetylcholine binding site. QBr is a reactive reagent that possesses a QA agonist, a cysteine-reactive bromomethyl phenyl, and a photosensitive azobenzene moiety. Light acts by photoisomerizing the azobenzene part of QBr, thereby presenting or withdrawing the agonist from its binding site (*17*). In the last couple of years, many other ion channels have been rendered light-sensitive (for review, see ref 18). Bifunctional photoswitches that contain a thiol-reactive group were nonspecifically introduced onto an engineered cysteine in the lumen of transmembrane channels such as the mecanosensitive MscL channel (*19*) or α-hemolysin (*20*). Photoswitch affinity molecules that attach in a more specific manner to channel proteins can also be used to produce light-sensitive ion channels. Such bifunctional photoswitches have been used to generate a light-activated K^+ channel called synthetic photoisomerizable azobenzene regulated K^+ (SPARK) (*21*) and a light-activated glutamate receptor (LiGluR) (*22*). These synthetic photoswitches are composed of three elements: a maleimide group for covalent conjugation to a cysteine residue, a reversible azobenzene photoswitch, and a specific ligand (agonist glutamate for LiGluR or channel blocker QA for SPARK). Upon exposure to the appropriate wavelength of light, the azobenzene photoisomerizes, and the tethered ligand (glutamate or QA) binds to or unbinds from the active binding site, thereby activating or inhibiting the ion channel.

In this issue, Morin and Kobertz (*1*) add a new twist to the affinity labeling approach. They used a bifunctional reagent consisting

of charybdotoxin, a peptide toxin from scorpion venom, and a cysteine-reactive maleimide group. They used this molecule to probe the subunit composition of an important class of voltage-gated K^+ channels that is involved in regulating cardiac rhythmicity as well as many other functions. K^+ channels contain a core group of four subunits surrounded by ancillary "β" subunits that control their membrane trafficking and modulate their gating. A multiplicity of core KNCQ and ancillary KCNE subunits have evolved to produce a wide diversity of heterocomplexes needed to carry out the many different functions of K^+ channels. The authors asked whether the situation is even more complicated: is an individual KCNQ core devoted to a specific KCNE type, or is it part of a plural marriage, involving multiple KCNE types?

To answer this question, the authors incorporated their bifunctional reagent on KCNE1, E3, and E4 subunits, one by one. The covalent attachment of charybdotoxin–maleimide (CTX-Mal) is driven by the high affinity of the toxin for the ion pore. What is unique here is that the affinity of the ligand for the core protein results in the labeling of the ancillary protein. In essence, the two proteins are tied together by a molecular *bungee cord*. The maleimide end remains attached to the KCNE subunit, and the toxin end is permanently available for binding to the pore and blocking ion conduction. Taking advantage of the various gating properties of the KCNQ1/E complexes, the authors were able to selectively and irreversibly block KCNQ channels containing E1/E3, E1/E4, and E3/E4 subunits (Figure 1). In each experiment, they fingerprinted which specific complex was present, thereby demonstrating the formation of hybrid complexes.

Nevertheless, questions concerning these molecular assemblies remain unanswered. How many KCNE subunits assemble with a KCNQ channel? How are the KCNE subunits positioned with respect to the KNCQ channel? The strategy presented here by Morin and Kobertz could be extended to provide important insights into these two unsettled issues. The first question could be investigated by measuring the kinetics of CTX-Mal tethering. Indeed, as demonstrated previously by Blaustein for K^+ channels (*13*), the time constant for covalent attachment of maleimide-containing compounds depends on the number of cysteines available. The second issue, that is, the distance between the different subunits in the complex, could be explored by varying the length of the flexible linker or the attachment site on the KCNE peptide, in a return to the *molecular tape measure* idea (*12*). The methodology developed by Morin and Kobertz is versatile. It can be used to address many problems and can be extended to a wide range of ion channels containing ancillary subunits.

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