Salk scientists add new bond to protein engineering toolbox

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Proteins are the workhorses of cells, adopting conformations that allow them to set off chemical reactions, send signals and transport materials. But when a scientist is designing a new drug, trying to visualize the processes inside cells, or probe how molecules interact with each other, they can't always find a protein that will do the job they want. Instead, they often engineer their own novel proteins to use in experiments, either from scratch or by altering existing molecules.

Engineered proteins can be drugs that turn on or off signaling pathways in the body, imaging agents that light up other molecules or processes, or enzymes that produce molecules with commercial value. Now, researchers at the Salk Institute for Biological Studies have developed a new tool for such protein engineering: a way to add strong, unbreakable bonds between two points in a protein or between two proteins. The new technique was published August 4, 2013, in the journal *Nature Methods*.

"Even though you could modify proteins in lots of different ways, adding a new bond into a protein was not possible before this," says senior study author Lei Wang, an associate professor in Salk's Jack H. Skirball Center for Chemical Biology and Proteomics and holder of the Frederick B. Rentschler Developmental Chair.

When a protein folds from a loose chain of amino acid building blocks into its active three-dimensional structure, bonds and chemical interactions naturally form between different parts of the chain to keep the structure assembled. Most are relatively weak, driven by the

electrochemical charges of different amino acids. Stronger bonds, called disulfide bridges, occur between pairs of cysteines, one particular amino acid. But for protein engineers, either type of bond has its own deficiencies. So linking two parts of a protein in a predictable and permanent way had been notoriously hard.

Wang and his collaborators wanted to be able to add strong, irreversible bonds —— called covalent bonds—— to proteins to alter their shape, make them more stable, or attach them to one another. They knew that cysteine amino acids reacted not only with other cysteines to make disulfide bridges, but with many other chemicals as well. So they began trying to create a new amino acid, different from the 20 that exist naturally, that cysteine would covalently bind to. They needed just the right compound, one that didn't bind to cysteine too quickly but also didn't bind too weakly.

"If you introduce something into a protein that forms bonds very easily, then it will bind to everything and make a big mess," Zheng Xiang, a postdoctoral fellow on Wang's team says. "But if it doesn't bind easily enough, then you won't be guaranteed the bond you want."

Xiang created dozens of possible amino acids, using basic laws of chemistry to design molecules that would potentially react with cysteine. Then, they tested each by mixing it with a solution of cysteine molecules to see if it bound with just the right strength. After a series of initial tests, Wang and Xiang settled on a newly created amino acid called p-2-fluoroacetyl-phenylalanine, or Ffact. To test whether Ffact, when integrated into proteins, would work as well as it did loose in solution, Wang and postdoctoral fellows Haiyan Ren and Irene Coin next designed three proteins using the new amino acid in their sequences.

Their first goal was to create a bond between an "affibody," an engineered protein similar to an antibody, and the molecule that it

recognizes, called Z protein. The affibody and Z protein naturally associate, but usually come apart after some time. By engineering them to bind permanently together whenever they interact, scientists can more easily detect whether the interaction occurs or whether a solution contains both molecules. So Wang and his colleagues engineered the affibody to contain an Ffact amino acid in a spot that aligned perfectly with a cysteine in the Z protein. When the researchers combined the engineered affibody and the Z protein, the two proteins successfully formed a covalent bond between the two amino acids when they got close.

"Because the interaction is now irreversible, the affinity between the two proteins is much higher," Ren explains. "If you apply this to diagnostic tests, it means you can detect a lower concentration of a substrate."

In a similar test of the bond's strength, Coin engineered a G protein coupled receptor and the signaling molecule that turns it on to form a bond when they associate. Once again, the amino acids became covalently attached.

To illustrate that Ffact and cysteine could also bind within a single protein's structure, Wang designed a version of a fluorescent protein in which the cysteine and Ffact were opposite each in different arms of the structure. With the help from Assistant Professor Hu Cang and Ying Hu, a Salk research associate, they found that the number of photons one can get out of the protein almost doubled because of the increased stability caused when a covalent bond formed. Such an improvement is a boon to those using fluorescence to visualize the movement or arrangement of molecules in a cell on the single molecule level, as it can increase the resolution and duration of imaging.

"I think anyone who is working on proteins, or anything related to proteins, could make use of this new technology," says Wang. "It can

provide a novel way to control proteins or design proteins to study basic biology."

In the future, Wang's team hopes to design additional amino acids that can be integrated into protein structures to form other kinds of bonds. The more bonds that are in the protein engineering toolkit, the more diverse proteins can be designed, he says.

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