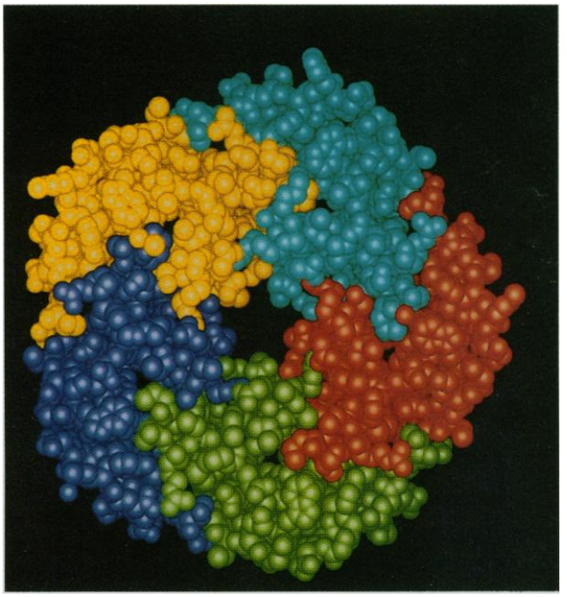
New 3-D Protein Structures Revealed

The Shape of Cholera

CHOLERA SOUNDS LIKE A 19TH-CENTURY DISEASE—something the heroine of an Emily Bronte novel dies of. But it's not. The debilitating diarrhea caused by the *Vibrio cholerae* bacterium, which has created seven pandemics in the last 150 years, is on the rise again in South America, causing 2000 deaths already this year and tens of thousands every year in the Indian subcontinent. What's needed to stop this plague is a better artificial vaccine and new therapies to replace the inactivated cholera-toxin vaccines, which are relatively poor at disease prevention. Producing such a vaccine depends on knowing the structure of key bacterial proteins, the ones that produce the symptoms, but those proteins have proved frustratingly difficult to crystallize and analyze. Now, concluding a 14-year search, Rongguang Zhang and Edwin Westbrook at Argonne National Laboratory and their colleagues at Yale and Boston University have solved the structure of the cholera toxin, which is the first step toward better vaccines and drug treatments. They plan to announce their results this week at the annual meeting of the American Crystallography Association in Toledo, Ohio.



Poisoned doughnut. *The B subunit of the cholera toxin.*

Just as exciting as the discovery of the structure of the cholera toxin is the fact that it's only one breakthrough in the rapidly moving field of enterotoxins—a family of large bacterial proteins that also includes the lethal diphtheria toxin. In May, a team of researchers from the University of Groningen in the Netherlands, publishing in *Nature*, described the crystal structure of the *Escherichia coli* toxin, which has a three-dimensional structure almost identical to that of the cholera toxin and causes a milder form of diarrhea that often afflicts travelers. And another team is hinting that it has uncovered the structure of the diphtheria toxin.

The hunt for the structure of the cholera toxin got off to a fast start. In 1977, biologist Paul Sigler at the University of Chicago was pleased at how quickly his lab was able to grow the first crystals of the deadly toxin. "We were able to grow a crystal right off the bat. We thought we were golden," recalls Sigler, now a professor of molecular biophysics and biochemistry at Yale. But then the research hit a brick wall: Sigler couldn't produce crystals that were good enough for x-ray crystallography, and he moved on to other areas of research. Fortunately, two members of his lab—Westbrook (then a graduate student) and Zhang (then a postdoc)—took up the challenge and followed it through.

The Sigler group was drawn to the cholera toxin because of interest in several aspects of its mechanism. The toxin was one of the first proteins identified that attaches itself to the intestinal wall of its victims by binding to receptors known as gangliosides. These receptors, called GM1, are found in the membrane of the cells that line the gut. The researchers were also intrigued, Sigler says, by the way the toxin is able to cross the cell membrane without destroying the cells. Finally, the researchers wanted to know how the toxin poisons the cells.

But they couldn't elucidate how the toxin works without finding out what its structure is—and that meant overcoming the shortage of high-quality, uniform crystals. To get around that obstacle, Westbrook and Zhang, who moved to Argonne after they left the Sigler lab, began racing against several other groups to grow crystals. At first, the results were frustrating—the crystals were poorly ordered, which meant that the diffraction pattern was useless. But, with the help of Graham Shipley at Boston University, eventually they got a pure enough version of the cholera toxin to grow well-ordered crystals. In the end, more than 25,000 diffraction measurements of the crystals were entered into a computer to produce the colorful images of the cholera toxin.

Those images reveal in new detail that the cholera toxin has a skeletal backbone consisting of two parts—the A subunit and the B subunit. The B subunit is made of five parts, each with a molecular weight of about 11,000, arranged in a pentagonal ring. This doughnut-shaped ring is attached to the much smaller A subunit, which has a molecular weight of about 29,000. The resolution is so good (2.4 angstroms) that Westbrook says he can locate each and every atom accurately.

By examining this structure, the researchers think they now understand how the toxin debilitates its victims. The key is a nefarious kind of molecular teamwork. The doughnut-shaped B subunit anchors itself to the intestinal cell wall by binding to the GM1 receptor. Then, the doughnut shoves the smaller A subunit into the cell. Once inside the cell, the A subunit functions enzymatically to initiate a chain of events that poisons the cell by permanently turning on the regulatory proteins known as G proteins. That leads to overproduction of cyclic AMP, which stimulates the enzyme cyclic AMP-dependent kinase, ultimately causing the intestinal cells to secrete copious amounts of water. The end result: diarrhea.

The same mechanism apparently is employed by the *E. coli* toxin, which is called LT. That might be expected because the two toxins are about 80% similar. But the 20% difference is significant, because it explains, says biophysicist Westbrook, "why LT makes you sick and cholera kills you." It turns out that the cholera toxin stimulates far more cyclic AMP, which causes the poisoned cells to pump out as much as 9 gallons of water a day in the form of diarrhea.

By combining the Argonne images with those of the Netherlands team, the groups hope to get a better handle on the shape and function of the entire family of enterotoxins. "All the bacterial toxins act in the same way; one component of the toxin is an enzyme that does the dirty work inside the cell, while the other component works to get it inside," says Westbrook. This indeed appears to be the case with the diphtheria toxin, whose

structure is about to be revealed by researchers at Harvard University and the University of California at Los Angeles.

As researchers close in on the structure of these enterotoxins, they are gaining ideas for the design of new drugs—some of which could have applications far beyond cholera. For example, some investigators are considering the disarmed toxins as vectors for launching other drug payloads into cells. That might be a way to destroy cancer cells. While such drugs still are distant prospects, researchers are pleased that after decades of work they are finally deciphering the elegant way in which nature designed these toxins to poison cells. "The toxin figured out a long time ago how to block the regulatory proteins inside the cell," says Sigler. "And now, we're following its work. This [result] was long overdue." ■ ANN GIBBONS

First Protein Kinase Structure

OVER THE PAST TWO DECADES, FEW ENZYME families have achieved the preeminence of the protein kinases. The enzymes in this large family (cell biologists have identified some 200 members) play key roles in many of the pathways by which hormones, growth factors, neurotransmitters, and toxins, such as the cholera toxin, have their effects. What's more, several protein kinases that transmit growth signals can, if they malfunction, contribute to cancer development. For these reasons, the protein kinases have been intensely studied. Yet all the while, researchers have had to work in the dark, without a good picture of the kinases' three-dimensional structures, information that could help them understand how the enzymes function.

Now come Susan Taylor, Daniel Knighton, Janusz Sowadski, and their colleagues at the University of California, San Diego, who report on pages 407 and 414 that they have for the first time solved the three-dimensional structure of a protein kinase. The achievement "is a monumental piece of work," says Edwin Krebs of the University of Washington in Seattle, the leader of the team that in 1968 isolated the kinase whose structure was just determined.

And the work's significance is not limited to the specific kinase in question, which goes by the name cyclic-AMP dependent protein kinase. On the contrary, the discovery should lead to a better understanding of the structures of all the family members. Several years ago, Tony Hunter of the Salk Institute in La Jolla, California, showed that, based on similarities in their amino acid sequences, the catalytically active portions of all the protein kinases ought to have similar three-dimensional structures. The new structure means, Hunter says, that "it is possible to model almost all of the eukaryotic protein kinase structures now." That will not only help scientists pin down the molecular mechanisms of kinase action, Krebs notes, but it may have practical consequences as well—providing targets for drugs to treat a variety of conditions, ranging from cancer and high blood pressure to cholera.

Although the cyclic-AMP dependent protein kinase itself has many important functions—for example, it transmits signals for the neurotransmitter norepinephrine, a major regulator of blood pressure and heart rate— biochemist Taylor says she and her x-ray crystallographer colleagues Knighton and Sowadski chose to study that enzyme mainly because of its relative simplicity.

All protein kinases consist of two parts. One is a regulatory subunit that enables them to sense incoming signals. The cyclic-AMP dependent protein kinase, for example, is so called because its regulatory subunit responds to cyclic AMP, formed as a "second messenger" when certain receptors are activated by hormone binding. The other component, the catalytic subunit, is the business end of the enzyme: It takes phosphate groups from adenosine triphosphate (ATP) and adds them to other proteins, altering the targets' activity and ultimately producing cellular responses.

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All in the family. *The new protein kinase structure, shown here with an inhibitory peptide* (right)*, may help decipher the structures of related enzymes.*

In most of the kinases, the regulatory and catalytic components are functional elements of one large protein. But in cyclic-AMP dependent protein kinase, they are separate. Taylor and her colleagues were thus able to study the catalytic protein without the added complication of the regulatory subunit.

Even so, Taylor says, the project, which took 6 years from start to finish, posed tough technical challenges. She and her colleagues had no problems getting crystals of the protein good enough for x-ray diffraction studies. But they ran into trouble when it came to interpreting the data those studies produced.

A three-dimensional structure is built up by using x-rays to "photograph" a crystal at different angles and then superimposing the resulting electron maps on one another. But in order to orient each image with respect to the others, crystallographers have to incorporate a heavy atom into the crystallized protein to serve as a reference point. And try as they might, Taylor and her colleagues couldn't get a heavy atom into crystals of the catalytic subunit of cyclic-AMP dependent protein kinase. "We couldn't interpret the data from our initial studies at all," she recalls.

The turning point came, Taylor says, when her group managed to incorporate a heavy atom into a crystal containing a smaller peptide bound to the kinase protein. Collecting x-ray diffraction data on the two together gave the researchers the reference point they needed to go back and interpret all the data they couldn't use before, and it provided some additional data as well. Since the peptide is part of a natural inhibitor of the kinase that acts by binding to the enzyme's active site, it helped the researchers define what that site looks like.

The picture that finally emerged shows that the catalytic subunit of cyclic-AMP dependent protein kinase has two lobes, one somewhat larger than the other. The smaller lobe binds ATP, while the larger one binds the protein that the enzyme will phosphorylate. The actual phosphate transfer occurs in the cleft between the two lobes.

Seeing this active sight, Krebs says, will help to work out a precise description of how the reaction occurs, identifying which amino acids on the enzyme bring about the phosphate transfer and which help it recognize the correct protein targets. The new structure will be, Taylor predicts, a virtual "Rosetta stone" that will allow researchers to decipher many of the remaining mysteries about the family of protein kinases.

Getting a clearer picture of the kinase structures and molecular mechanisms should in turn, Krebs says, aid in the rational design of drugs that either inhibit or augment kinase activity, depending on what's needed to treat conditions such as cancer and high blood pressure. And that prospect should give some sense of the power that comes from understanding a protein's three-dimensional structure. ■ MICHELLE HOFFMAN