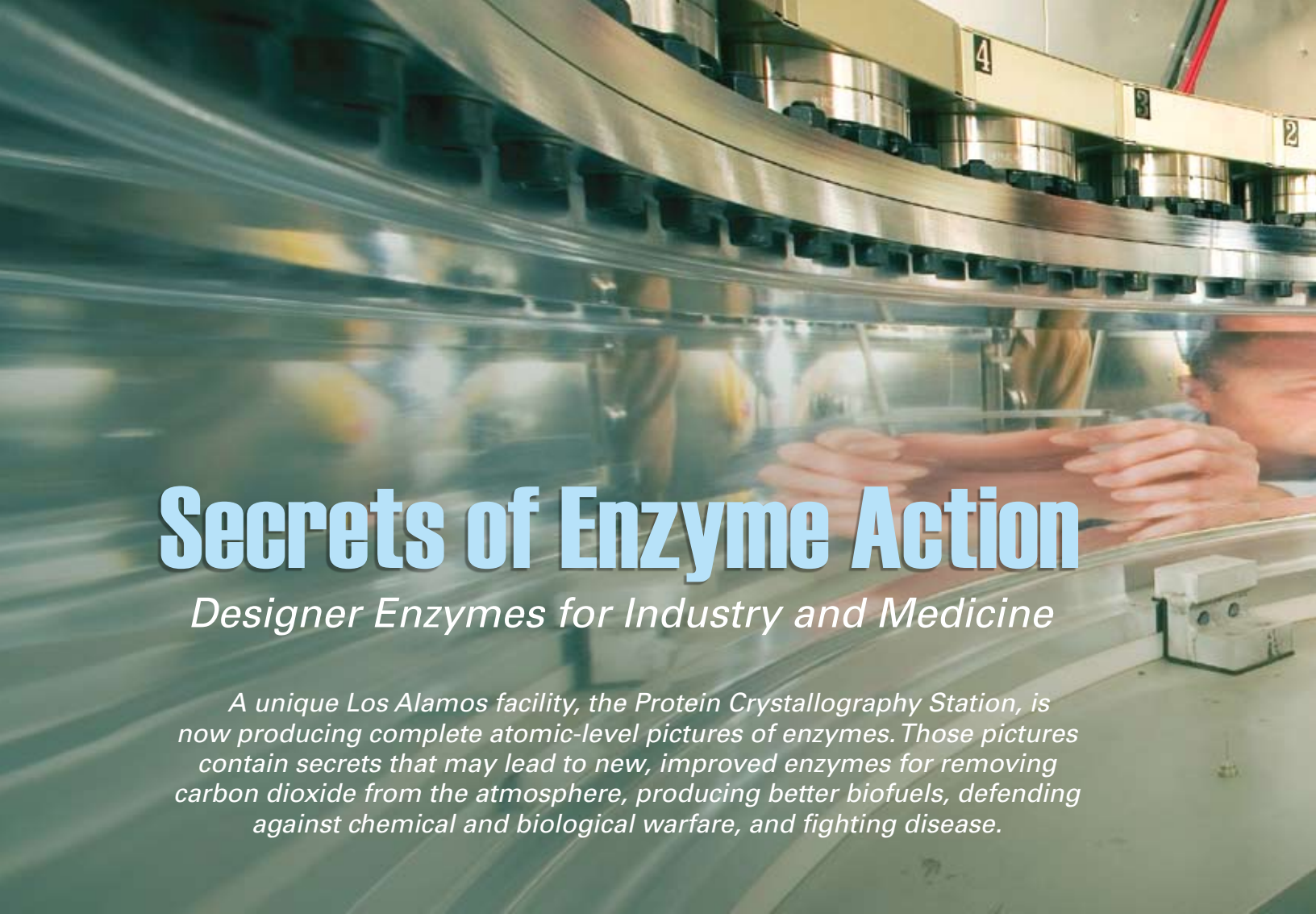


Secrets of Enzyme Action

Designer Enzymes for Industry and Medicine

A unique Los Alamos facility, the Protein Crystallography Station, is now producing complete atomic-level pictures of enzymes. Those pictures contain secrets that may lead to new, improved enzymes for removing carbon dioxide from the atmosphere, producing better biofuels, defending against chemical and biological warfare, and fighting disease.

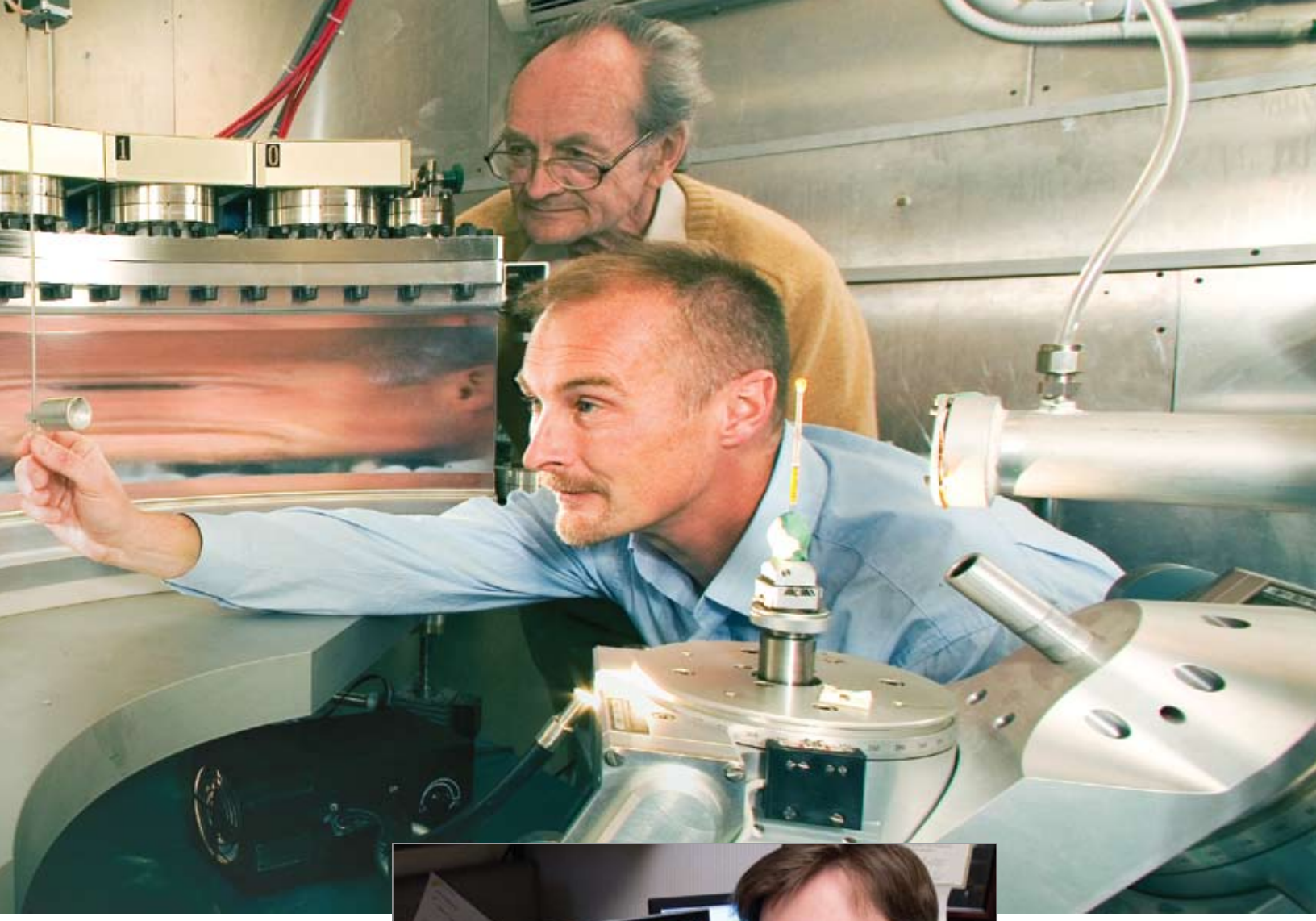
Andrey Kovalevsky, postdoctoral fellow at the Los Alamos Protein Crystallography Station (PCS), stares intently at a computer screen filled with dark dots on a white background. It looks like the old photographic plates of stars in the night sky. But Kovalevsky is not looking for astronomical objects. He's trying to infer the positions of atoms in a crystallized enzyme. He hopes to uncover the complete structure and operation of xylose isomerase, an important enzyme that catalyzes the conversion of one type of sugar into another.

Enzymes are large protein molecules made by living organisms. They are the most-powerful catalysts known on Earth, speeding up biochemical reactions that, in their absence, would never occur. How is that possible? At the PCS, Kovalevsky and other young scientists hope to learn the enzymes' secret ways and then re-engineer them to do new tasks or perform old ones more efficiently.

Until the PCS opened 5 years ago at the Los Alamos Neutron Science Center (LANSCE), most scientists used only x-ray crystallography to learn the atomic-level structure of proteins. Researchers would shine an x-ray beam on a crystallized protein and record the diffraction "peaks"—intense spots created when x-rays diffract (scatter coherently) from the crystal's orderly array of atoms. From the pattern and intensity of those peaks, scientists could deduce the three-dimensional arrangement of atoms making up the protein's structure.

An astonishing 60,000 proteins have been analyzed this way, but for enzymes, the resulting structural models have one serious limitation—the hydrogen atoms are missing. X-ray diffraction peaks from hydrogen are usually too faint to see.

Diffraction peaks are created when x-rays scatter from the electron cloud surrounding an atomic nucleus. Hydrogen has only one electron in its cloud, so it scatters x-rays very weakly. With 50 percent of the atoms in an enzyme being hydrogen, x-ray crystallography can provide only an incomplete picture



of an enzyme's structure.

Marc-Michael Blum, a German researcher using the PCS, explains, "Some enzymes are like Swiss army knives. Their surfaces look almost alike, but they contain very different tools. Only by knowing the atomic arrangement of those tools, including the positions of the hydrogen atoms, can you figure out how an enzyme really works."

The positions of the hydrogen atoms are especially important because they invariably get shuffled about during enzyme-catalyzed reactions. The hydrogen positions give critical cues about how the reaction moves forward.

"Researchers now have a way around the hydrogen problem," says Paul Langan, team leader of the PCS. "Here at the PCS, they can use neutrons, not x-rays, to do the crystallography and within a few weeks have enough data to locate the hydrogen positions relative to the other atoms in an enzyme." (See "Birth of a New Technique.")

Unlike x-rays, neutrons scatter as strongly from hydrogen as from other elements because they scatter from the atomic nucleus rather than the electron cloud. But the scattering strength depends on the nuclear composition. By replacing a crystal's hydrogen with the isotope deuterium, researchers can add a distinct

Above: Paul Langan (foreground) and Benno Schoenborn aligning the beamstop that protects the gleaming neutron detector of LANSCE's Protein Crystallography Station. The detector, developed by Brookhaven National Laboratory, is a major advance and makes the PCS a practical tool. Inset: Andrey Kovalevsky working on an atomic-level model of the enzyme xylose isomerase. PHOTO BY DIXON WOLF

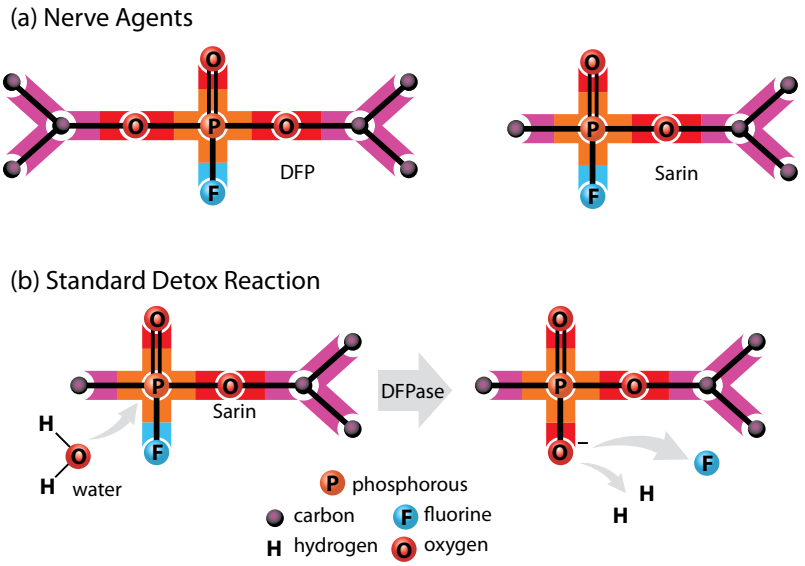
DFPase, an enzyme that destroys nerve agents, was discovered accidentally in squid during early experiments on nerve conduction.

A New Defense against Nerve Agents

For many years now, a German team has been studying an obscure enzyme found in squid. Why? Because the enzyme could serve as a defense against nerve agents. Called DFPase because it catalyzes the destruction of the nerve agent DFP (di-isopropyl fluorophosphate), this enzyme is effective against a number of phosphorus-based nerve agents, including Sarin, the substance that killed 12 and sickened thousands in the Tokyo subway attacks of 1995.

Two members of the team, Blum, who has been working with the medical branch of the German army, and Julian Chen, a California transplant and an assistant professor of biophysical chemistry at Goethe University in Frankfurt, recently came to the PCS to clinch their findings on the inner workings of DFPase. The new findings, published early this year in the *Proceedings of the National Academy of Sciences* (PNAS), not only have overthrown past ideas about how the enzyme works, but also have led to an engineered version of the enzyme, speeding its activity rate to the point that it can safely decontaminate exposed skin and sensitive surfaces on optics and electronics in less than 10 minutes. The engineered enzyme also works on another nerve agent, VX, which was immune to the action of DFPase.

All these nerve agents act by quickly blocking the nervous system's "off switch" for muscles and glands, leaving them stuck in a semi-active state. As a result, they may tire, and in the case of large doses, the body can lose the ability to sustain breathing.



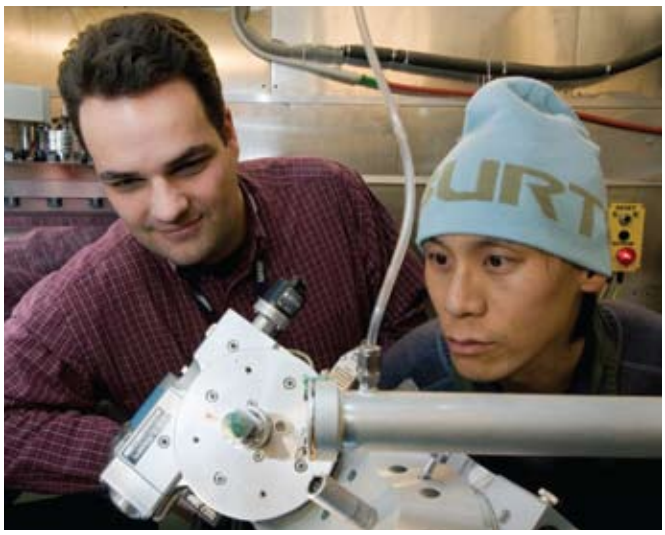
signal component that helps them locate the positions of the replaced atoms. Conveniently, Mary Jo Waltman, a crackerjack technologist, is available to help PCS users grow deuterated crystals in special deuteration laboratories.

Kovalevsky adds, "If we can crystallize the enzyme at various catalytic stages, we can visualize, step by step, how it changes shape and shuffles hydrogens to different locations."

Seeing this level of detail has reveled a big surprise: some enzymes take a much more active role in the reaction chemistry than commonly thought.

That conclusion is based on x-ray structures, the results of biochemical experiments in solution, theoretical calculations, and more. "The neutron work merely 'dots the i's,'" comments Benno Schoenborn, inventor of neutron protein crystallography. "But that final level of information keeps the theorists honest."

It can also provide a firm basis for engineering an improved version of an enzyme, as a group of German researchers is showing.

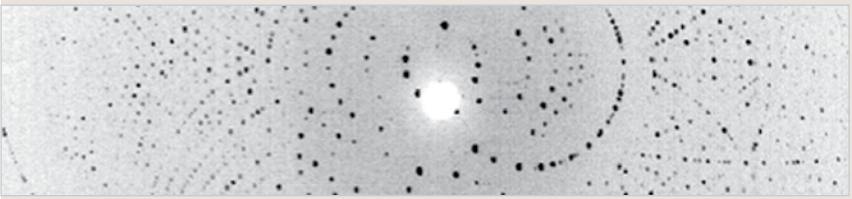


Marc-Michael Blum (left) and Julian Chen examining the PCS's sample holder. PHOTO BY DIXON WOLF

Birth of a New Technique

Amidst all the excitement and bustle at the Los Alamos Protein Crystallography Station (PCS) is an intense, urbane gentleman, Benno Schoenborn, giving very-specific advice on how to get the most out of the instrument he and his colleagues have designed. Schoenborn first dreamt of making neutron crystallography a tool for biology more than 40 years ago.

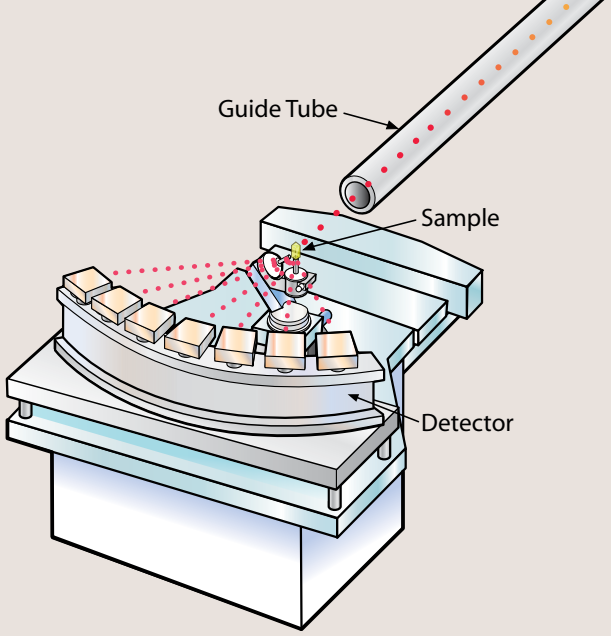
Back then, neutrons were available only at nuclear reactors, and safe reactor operations put a firm lid on neutron intensity (the number of neutrons per second, per square centimeter, coming out of the reactor). Although Schoenborn used reactor neutrons to prove that neutron crystallography would work on proteins, he saw it as a labor of love, taking many, many months to collect enough data for a single protein structure.



A neutron diffraction pattern.

The intensity lid came off with the advent of the spallation neutron source at the Los Alamos Neutron Science Center (LANSCE). A high-intensity pulse of neutrons is produced each time a proton pulse from the LANSCE accelerator hits a tungsten target. Some of those neutrons are directed down an evacuated tube to the crystal mounted in front of the PCS neutron detector.

Benno Schoenborn



A neutron pulse striking and diffracting from a sample at the PCS.

Like runners coming out of the gate, the neutrons stretch out according to speed along an evacuated guide tube, the fastest arriving at the crystal sample first and the slowest 10 milliseconds (1/1000th of a second) later. Each neutron's arrival is like a time stamp that announces its speed, or energy. Neutrons of different energies are diffracted by different sets of planes in the crystal. The PCS records the diffraction patterns created at 100 different energies (times) within the duration of each 10-millisecond pulse.

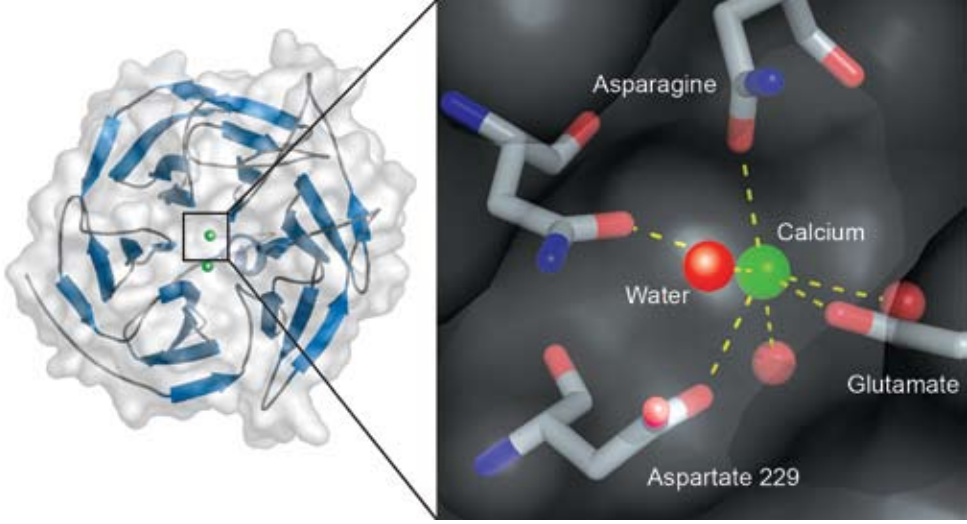
Schoenborn, with colleagues Eric Pitcher, Phil Ferguson, and later Paul Langan, designed every component of the PCS to maximize the neutron intensity and reduce background scattering so scientists could collect excellent data in 15 to 30 days of beam time using much-smaller crystals. The big payoff is that the larger proteins, for which large crystals are nearly impossible to grow, have become eligible for neutron crystallography.

The original belief was that DFPase did its defensive work by catalyzing a reaction between water and the nerve agent. As shown in the diagram on the facing page, the standard picture was that oxygen from the water attacked by binding to the phosphorus in the nerve agent. That attack caused the fluorine that is bound to the phosphorus to be jettisoned, thereby detoxifying the nerve agent. (Without the fluorine, the agent no longer binds strongly to the critical off switch.) This detox reaction was presumably catalyzed in the area of the enzyme in which chemical reactions take place, DFPase's active site.

Before testing that theory, DFPase research was focused on just isolating the enzyme, that is, getting enough of it to work on. "At one point my Ph.D. advisor's group came back from the fish market with a couple hundred squid," says Blum. "We needed that many just to get micrograms of the enzyme." Fortunately, that amount was enough to determine the unique sequence of 314 amino acids that form the precious enzyme.

With the sequence in hand, the researchers could exploit gene technology to manufacture the enzyme in much larger quantities and pursue a slew of different

Diagram, facing page: (a) DFP, Sarin, and other phosphorus-based nerve agents typically have a fluorine atom (blue) bound to a phosphorus atom. (b) It was thought that DFPase catalyzed a reaction in which the fluorine was jettisoned and an oxygen from a water molecule swooped in to replace it. The research reported here shows otherwise.



Top view of the enzyme DFPase (white) shows six sections (the groupings of blue ribbons), arranged in a circle around a central tunnel containing two calcium ions (green). The central calcium holds the enzyme together; the upper calcium is in the enzyme's active site. Blowup: In the x-ray structure of DFPase's active site, four amino acids and three oxygen atoms (red) —presumably water molecules—surround and are bound to the calcium ion. Without this calcium ion, the enzyme is not active against nerve agents.

techniques to probe its mode of operation.

X-ray crystallography revealed the structure of the enzyme (minus the hydrogen atoms, of course). The active site, where the enzyme presumably holds the nerve agent and the water in close proximity, turned out to be an indentation at the top where four of the enzyme's amino acids and three isolated oxygen atoms (red) were all loosely bound to a central calcium ion (see blowup in the figure above). The oxygen atoms were presumably water molecules (with their hydrogen atoms invisible) that had been frozen into position during the crystallization of the enzyme in the presence of water.

Blum says, "It was not clear which amino acid or water molecule would act to attack a nerve agent's phosphorus atom."

To uncover the active player, the team co-crystallized the enzyme with a nerve agent surrogate and again used x-rays to determine the combined structure.

To everyone's surprise, the nerve agent surrogate pushed the topmost water molecule out of the active site rather than interacting with it. Moreover, the surrogate had bound to the calcium in an unexpected and very exacting configuration. It looked as if one of the amino acids in the active site, rather than water, could easily provide the oxygen atom for detoxifying the nerve agent.

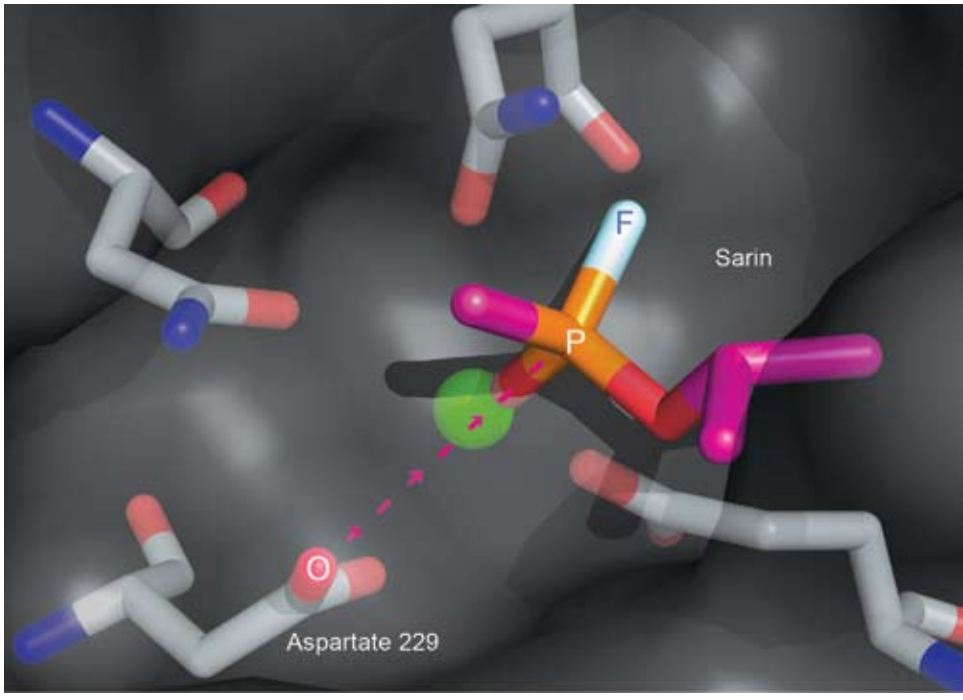
Blum and Chen began to suspect that, contrary to previous assumptions, a catalyzed reaction with water is not what destroys a real nerve agent. They proposed, instead, that a negatively charged oxygen from the enzyme's amino acid aspartate 229, is what binds to the nerve agent, causing the ejection of a bound fluorine atom (see figure at right). A water molecule later replaces the enzyme's oxygen.

To verify this radical proposal, the researchers did a series of tracer experiments in which the enzyme and the nerve agent DFP were surrounded by water in which oxygen-16, the common oxygen isotope, had been replaced by the heavier oxygen-18. By showing that the oxygen-18 atoms in the water showed up in the reaction *not initially* but only *after* a given DFPase molecule had acted multiple times, Blum and Chen were able to demonstrate that DFPase was indeed the source of oxygen that detoxified the nerve agent.

But the critics remained unconvinced, claiming that an invisible hydrogen ion was present on the aspartate 229's oxygen, neutralizing its negative charge and thereby preventing it from making a successful attack. These critics also claimed that the necessary oxygen came instead from a hydroxide ion bound to the calcium in the enzyme's active site.

To find out who was right, Blum and Chen abandoned x-ray crystallography and mounted a PCS neutron crystallography experiment to pinpoint the hydrogen atoms' positions in the DFPase structure.

Chen came to Los Alamos from Frankfurt to grow



Blum and Chen's newly discovered detox mechanism. An oxygen from aspartate 229 lines up with the fluorine-phosphorus chemical bond on the nerve agent Sarin. It then attaches to the phosphorus and jettisons the fluorine (not shown), destroying the nerve agent's toxicity.

the needed crystals of DFPase and some weeks later returned for the delicate task of mounting a large, soft 2.4-mm-long crystal in a capillary tube. Enzyme crystals are typically soft and delicate because they are about 50 percent water.

Blum and Chen used this crystal to record 37 neutron diffraction images during one month. They then took advantage of new computer programs developed by Langan, Marat Mustyakimov (staff member at Los Alamos), and colleagues from Lawrence Berkeley Laboratory to compare x-ray diffraction data with the new neutron diffraction data and determine the most-likely structure for DFPase crystallized in the presence of water.

Zoe Fisher, with her baby Owen, asks Marat Mustyakimov to admire the model of carbonic anhydrase, her favorite enzyme.

The neutron work unequivocally showed that the topmost oxygen atom in DFPase's active site had two hydrogen ions bound to it. It was indeed water, not a hydroxide ion. Also, there was no hydrogen ion on aspartate 229's active oxygen; it was negatively charged. All objections to the new theory about the detox mechanism had been knocked down.

"Our practical goal was to design better versions of DFPase," says Blum. "With proof for the new detox mechanism in hand, we redesigned the shape of the enzyme's active site so that the most-toxic versions of each nerve agent would naturally bind in the best orientation for an aspartate 229 attack. Our best new version of DFPase worked 2 to 10 times faster on all the known nerve agents than the original enzyme did, and it will likely become an important defense for first responders in the case of a nerve agent attack."

In the Wings

The DFPase success story may soon be repeated with other enzymes being studied at the PCS.

Kovalevsky is focused on improving the effectiveness of xylose isomerase (XI) because it's used in the food industry to convert glucose from starch into the much-sweeter fructose. XI is also used in the biofuel industry to convert xylose, a sugar derived from woody plants, into xylulose, a sugar that microorganisms more readily convert into ethanol.

XI has been studied for 30 years, but it took the PCS to locate the hydrogens. Kovalevsky has found them not only in the native enzyme but also in the enzyme crystallized with an intermediate form of the sugar. An unexpected amino acid, far from the purported active site, had removed a hydrogen from the sugar, not donated one, as expected for this type of reaction. The reaction seems to be proceeding in new way, one first intuited from x-ray and solution experiments. Kovalevsky needs neutron diffraction results from two more stages of the reaction to pin down the exact mechanism.

Also under study at the PCS is a type of carbonic anhydrase (CA) found in humans—HCAII—the fastest acting of all the enzymes that catalyze the conversion of carbon dioxide to bicarbonate. HCAII is being extensively developed for potential use in the sequestration of carbon—capturing and chemically changing carbon dioxide for underground burial. The rate of catalysis is limited by how fast the enzyme moves hydrogen ions away from its active site. Using recent PCS neutron data, Los Alamos postdoctoral fellow Zoe Fisher and researchers from the University of Florida hope to map out this transfer pathway, information that could lead to improving this enzyme's performance.

Impact on the Biological Community

At this point the PCS is a unique instrument with many more subscribers than can be handled during the year.

Schoenborn believes this is just the beginning for neutron crystallography. "We've only scratched the surface. The information we gather will increase in value as scientists, especially the drug design people, need more details about enzyme action."

Langan agrees. "The greater the demand for new, improved enzymes to address new challenges in renewable energy, the environment, chemical and biological threat reduction, and therapeutics, the more scientists will turn for answers to neutron crystallography and the greater the need will be for new instruments like the PCS." ❖

—Necia Grant Cooper