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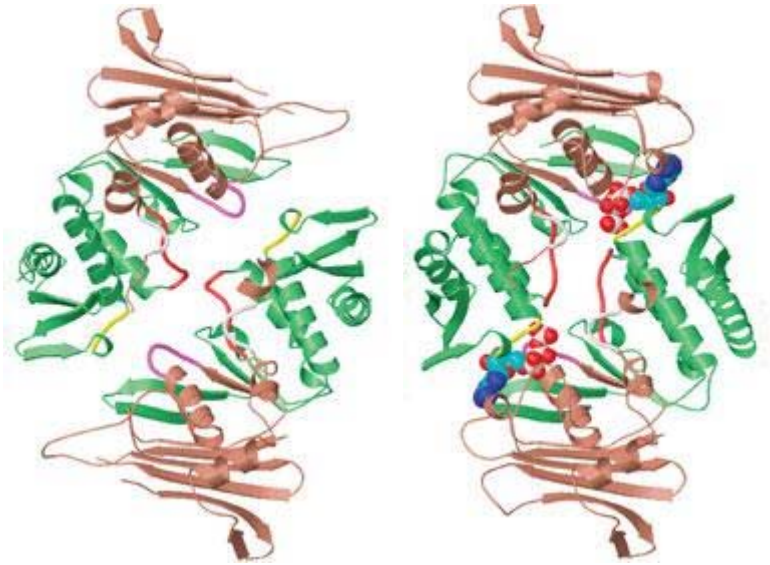
FROM THE ACS MEETING

ENZYMES IN MOTION

Energy from phosphoryl-transfer reactions can fuel a rich choreography of enzyme motions that help regulate catalytic activity

REBECCA RAWLS, C&EN WASHINGTON



CLOSING IN **Binding of ATP to catalytic portion of DNA binding protein called Rad50, at right, pulls two halves of enzyme together and rearranges many individual domains. Rad50 without ATP is at left.**
STRUCTURES COURTESY OF JOHN A. TAINER

Enzymes fascinate chemists because they combine dramatic catalytic capacity with exquisite control of chemical reactivity. And no enzymes are more impressive in their ability to bring both of these capacities together than some of the ones that move phosphoryl groups from one molecule to another.

In living systems, phosphoryl transfer in the form of

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hydrolysis of triphosphates of adenosine or guanosine frequently supplies the energy that drives otherwise thermodynamically unfavored chemical reactions. The resulting nucleotide monophosphate is itself a reactant in several important classes of enzyme-directed reactions, such as DNA synthesis and repair. In other cases, such as kinase activation, it's the phosphoryl group that the enzyme attaches to a target molecule. In one guise or another, phosphoryl transfer is probably involved in more than half of the enzyme-catalyzed reactions biochemists and molecular biologists study, suggests Stephen R. Sprang, Howard Hughes Medical Institute investigator and professor of biochemistry at the University of Texas Southwestern Medical Center, Dallas.

A picture is beginning to emerge of how some of these enzymes manage to combine catalytic function with regulation and control. Some of the research that is bringing that picture into focus was showcased at a symposium sponsored by the Division of Physical Chemistry, organized by Sprang and Jan Florián, a computational biochemist and assistant research professor at the University of Southern California.

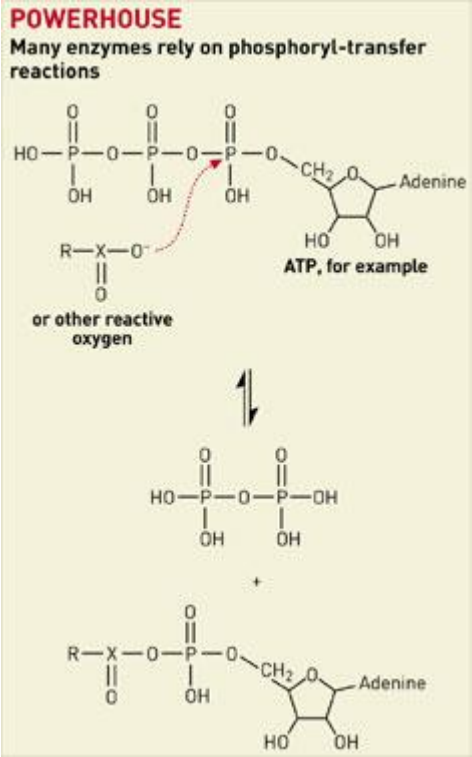
Many phosphoryl-transfer enzymes have evolved not just to be efficient catalysts, but also to perform their catalytic chemistry only in a regulated way when activated by an external "signal," Sprang explained. Often the enzyme has to move into an appropriate configuration to perform its catalysis, and those conformational changes seem linked to regulating the enzyme's activity.

"One of the things that we are trying to understand is the coupling between the interaction of these enzymes with their substrates and the dynamical events that allow the enzyme to carry out catalysis," Sprang said. "A more complex agenda for many of the enzymes considered in the symposium includes regulation or assembly as well. Enzymes that might otherwise simply turn over a substrate are not biologically free to do that until they have assembled themselves to make a complex. Somehow catalytic activity is coupled to that process."

Sprang continued: "The overall problem that the enzyme faces and we would like to understand is how to trigger the system in such a way that the trigger only goes off when everything is assembled."

MUCH OF THE TIME "enzymes are not just performing functional group chemistry at the active site," said John A. Tainer, professor of molecular and chemical biology at

Scripps Research Institute. "They also have to be coordinated with other enzymes in a given pathway, and the pathways have to be regulated, too.



"Of course, there are external things that can help regulate enzyme function," Tainer explained, "but there's an emerging idea, heard clearly in the symposium, that different pathways are coordinated and regulated, in part, by the structural chemistry of the enzymes themselves. Regulation is emerging as a property of the overall architecture and structure of an enzyme and of its ability to switch conformation."

Tainer has studied several phosphoryl-transfer enzymes involved in DNA replication or repair. There is something very special about these processes, he noted, that makes the enzymes that carry out these functions particularly good ones to study for insight into how enzymes regulate their own activity. In both replication and repair, enzymes in the first steps of the process change the cell's DNA in ways that increase the damage the DNA could cause the organism. Because it's critically important to the survival of the organism that these early changes be carefully coordinated with those that follow, the cell is willing to pay a high energetic cost for this coordination, Tainer suggested. That means the structural changes involved in this coordination may be more pronounced and therefore easier to study.

"What's happened over evolution is the selection for a structural chemistry that allows coordination and coupling of events, as well as chemistry," Tainer said.

In one example Tainer has studied, the adenosinetriphosphatase (ATPase) known as Rad50 works in conjunction with an endonuclease called Mre11 to repair double-strand breaks in DNA. Crystal structure data from Tainer's lab reveal Rad50 to be a lollipop-shaped molecule, in which the two ends of the enzyme come together to form the catalytic portion--the candy--with an extremely long middle section that forms a coiled-coil "stick" that extends out from the main body of the enzyme for several hundred angstroms. Mre11 binds to Rad50 at a site on the coiled coil adjacent to

the catalytic domains.

Crystal structures of Rad50 with and without ATP bound to it show that ATP binding and subsequent hydrolysis cause substantial changes in the conformation of the enzyme. ATP binding causes dimerization of the two Rad50 catalytic domains and brings the two halves of each catalytic domain closer together. Some domains rotate as much as 30° to create an electrostatically positive channel in which DNA can bind. The reconfiguration also helps bring the two Mre11 endonucleases into the right orientation to cleave the DNA. When the ATP is hydrolyzed, the structure opens to release the DNA and to allow the enzyme complex to shift into position for the next step in the repair process. This change in the structure of Rad50 "coordinates and regulates double-strand break repair," Tainer said.

A NUMBER OF enzymes that play a role in processes that involve both strands of DNA have long coiled-coil regions like Rad50 does as part of their structures, Tainer noted. The long stick of the lollipop provides a way for chemical reactions taking place on DNA strands up to 1,000 Å apart to be coordinated with one another, he suggested.

"We have structures that show how two independent DNA-binding heads, each bound to a sister strand of DNA, could communicate over this 1,000-Å distance by ATP-mediated conformational switching," Tainer said. As the ATP-bound form of Rad50 binds to DNA and the hydrolyzed form releases it, the conformation change shifts a domain of the enzyme that is directly connected to the coiled coil, causing the whole lolliop stick to move. Tainer proposed a dynamic interaction at the far end of coiled coils from two such molecules, each interacting with a different strand of DNA. Such an interaction could signal the binding state of one enzyme to the other.

Enzymes like these, Tainer said, "are not multipurpose tools. They are complex machinery that has to be coordinated. They are more like your car than the tools you use to work on your car."

Another phosphoryl-transfer enzyme much studied because of its exquisite selectivity is DNA polymerase b. The enzyme is involved in repairing damaged DNA in a process known as base-excision repair. Its job is to fill a one-nucleotide gap in DNA by inserting the correct base to complement the base in the opposite DNA strand.

The enzyme inserts the correct base into the gap at a rate that's "something like 100,000 times faster than for incorrect bases," Samuel H. Wilson explained. Wilson is principal investigator in the laboratory of structural biology at the National Institute of Environmental Health Sciences in Research Triangle Park, N.C. How the enzyme is able to

distinguish so precisely between nucleotide bases has drawn the attention of many researchers.

Tsai
PHOTO BY LI ZHAO

Tainer
SCRIPPS RESEARCH INSTITUTE PHOTO

Goldsmith
PHOTO BY GARY SIDLE

OVER THE PAST several years, Wilson and his collaborators, along with other crystallographers, have captured the structure of the enzyme alone and bound to one or both of its substrates, which are DNA and the triphosphate form of the nucleoside that the enzyme will insert into the DNA. Such structures represent different points along the DNA synthesis pathway, and by comparing them, researchers have developed a model for the enzyme's activity that links conformational changes in the enzyme to its specificity.

"We saw a so-called closed, active conformation by this approach, plus an open, inactive conformation," Wilson explained. The proposal was that, when the right substrates are in place, segments of the enzyme close in to achieve the active conformation that would lead to the transition state in which the phosphoryl transfer occurs. But crystal structure data can only provide snapshots of enzyme motion. "We had never been able to directly demonstrate segmental motion within the enzyme needed for the crystal structure interpretation," he said.

At the ACS meeting, Wilson presented data from experiments using a fluorescence tag--the amino acid tryptophan--inserted into the protein in the segment that crystal structure data suggest moves to produce the catalytically active form of the enzyme. The researchers could excite the tryptophan with a pulse of laser light and, by monitoring the fluorescence decay, watch the segment move relative to the rest of the protein.

The motions they saw confirmed that the enzyme undergoes a conformational change in order to achieve the precise structure necessary to allow transition-state development for DNA synthesis, Wilson said. However, the experiments also delivered a surprise.

"Earlier proposals had been that this motion would be the rate-limiting factor for DNA synthesis, but it's not," Wilson said. The segmental movement takes only a few nanoseconds, but the rate of DNA synthesis is about 10 per second. "The segmental motion is much, much, much faster than the rate-limiting step for synthesis."

In kinetic terms, an enzyme's selectivity is determined by its relative synthesis rates: It's because the enzyme inserts the

correct base so much faster than an incorrect one that it almost always makes the right product. Thus, finding that relatively large conformational changes taking place in the enzyme are not what determines the rate of the reaction "is really interesting," Wilson said.

The finding means, he suggested, that the reaction rate is determined either by the chemistry itself--the actual phosphoryl-transfer reaction--or else by some microscopic structural adjustments in the immediate vicinity of the reaction site. An example might be rotation of an amino acid side chain. "I think we can't know at this point which of those possibilities will be true," Wilson noted. "But these results certainly point in a different direction, in terms of allowing us to understand the rate-limiting step for DNA synthesis for this polymerase."

One possibility, which Wilson and his colleagues will be investigating, is that incorporation of incorrect bases, which does occur but much more slowly, follows a different pathway to the transition state and perhaps even a different mechanism for the phosphoryl transfer itself. "We're trying to understand these proposed alternate pathways for developing the transition state, which could be quite different for the correct and incorrect incoming nucleotide," Wilson said.

Page: 1 | 2 | Next Page

Top

Back Issues
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CENEAR 80 19 pp. 41-44
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[continued from previous page]

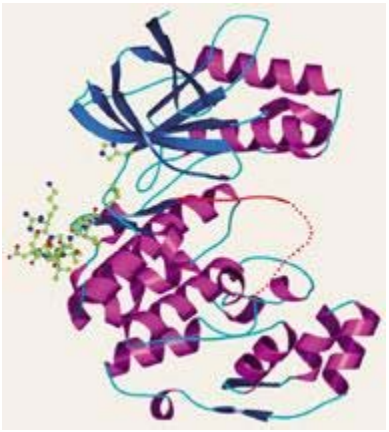
Ming-Daw Tsai, professor of chemistry and biochemistry at Ohio State University, also investigates the selectivity of DNA polymerase β. Using a different experimental approach, Tsai and his colleagues also conclude that the major conformational change that takes place in this enzyme is too fast to be the rate-limiting step for the reaction.

Using stopped-flow fluorescence techniques, Tsai found that the enzyme-catalyzed reaction proceeds in two steps, the first of which is faster than the second. By substituting chromium in place of one of the magnesium ions that the enzyme requires for its activity, Tsai and collaborator Michael K. Chan obtained a crystal structure of the enzyme after the first, rapid step but before the second, slower one. "We were expecting this complex to still be in the open form, because it is before the slow step," Tsai said. "But the crystal structure shows that it is already in the closed form. That's when we realized that the slow step doesn't have anything to do with the domain-changing conformational change." Subsequent stopped-flow experiments clearly suggest that the rate-limiting step is the chemical step, Tsai said.

DNA polymerase β is known as a high-fidelity enzyme because it inserts the appropriate base into the DNA strand it is repairing so reliably. But not all polymerases are this reliable, and Tsai and his colleagues are particularly interested in understanding why.

"SOME PEOPLE would say low-fidelity polymerases don't have good control over fidelity because they have loose active sites," Tsai said. "But I don't think so. I think low-fidelity enzymes have active sites that are optimized for low fidelity."

Recognizing that the fidelity of these enzymes is controlled by the chemical



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step rather than conformational change may make it easier to understand how different polymerases have evolved to have different degrees of fidelity, Tsai suggested.

sequence of target protein (shown in yellow at the left of the structure) wraps around and binds to the outside of MAP kinase in a signaling cascade, it changes the conformation of MAP kinase in a way that makes its active site (dotted red line at right) accessible for phosphorylation. STRUCTURE COURTESY OF ELIZABETH J. GOLDSMITH

Tsai also studies a very small polymerase, known as DNA polymerase X, found in the African swine fever virus. This enzyme is about half the size of polymerase [redacted], which is itself one of the smallest DNA polymerases. From solution structure data his lab solved recently, he finds that the tiny viral polymerase resembles the C-terminal half of polymerase [redacted]. However, the half of DNA polymerase [redacted] that primarily binds DNA is missing from polymerase X. Using different residues, the small polymerase manages to bind DNA as tightly as DNA polymerase [redacted] does, Tsai said.

In earlier work, Tsai and his colleagues showed that, although polymerase X inserts nucleoside bases into DNA according to the standard rules of complementary base pairing, it also recognizes another pairing possibility. This polymerase will insert guanine opposite guanine, when the rules of base-pairing specify that the "correct" base in such a position should be cytosine (C&EN, March 12, 2001, page 60).

At the ACS meeting, Tsai described a second DNA repair enzyme, a ligase, from the same virus that tolerates this guanine-guanine mismatch. The finding "suggests that the specificity of DNA polymerase X toward the guanine-guanine mismatch has biological relevance," Tsai said. "We hypothesize that the polymerase and the ligase work together in a 'mutagenic' repair pathway. When the virus senses DNA damage, instead of fixing it accurately, as polymerase [redacted] does in mammalian systems, it repairs it with a mutation." The resulting increase in genome variability could well be advantageous to a virus, Tsai suggested.

Phosphoryl-transfer enzymes involved in DNA synthesis and repair are not the only ones where researchers are finding connections between structural changes in the enzyme and control of its chemistry. Elizabeth J. Goldsmith, professor of biochemistry at the University of Texas Southwestern Medical Center, sees a similar connection in signaling enzymes known as MAP (mitogen-activated protein) kinases.

Kinases are enzymes that transfer phosphoryl groups to a target molecule. In the case of MAP kinases, they often form elaborate cascades in which each kinase transfers a phosphoryl group to another enzyme that is itself a kinase. The transfer activates the second kinase, enabling it to bind to its target enzyme, transfer a phosphoryl group to activate that

enzyme, and thus form a cascade. Signals from receptors on the surface of cells trigger these cascades, which, after a series of steps, phosphorylate proteins in the nucleus to produce changes in protein synthesis. The cascade allows signals from outside the cell to be amplified and coordinated, but the activity of each individual kinase needs to be carefully regulated so that the right signal reaches the nucleus.

MAP kinases phosphorylate target proteins at serine and threonine residues that are followed by proline, Goldsmith explained. However, short peptides containing this motif don't bind to the kinase. Instead, the target protein must contain a docking-site sequence for the kinase to phosphorylate it. Goldsmith and her colleagues determined the structure of MAP kinase p38 in the presence of docking-site sequences previously identified by several laboratories.

THE DOCKING SEQUENCES bind outside of the enzyme's active site in a groove on the enzyme's surface, Goldsmith found. This binding causes "unexpected conformational changes, both locally in the peptide-binding groove and in the active site," Goldsmith said. By wrapping an "arm" around the outside of the enzyme, the target protein in some way seems to cause the active site to shift to become more accessible.

The arm doesn't actually have to be attached to the target enzyme, Goldsmith noted. The presence of a short peptide containing the docking sequence improves the phosphorylation of substrates lacking the docking site.

It's not just the substrate that docks into the kinase in a groove on its surface, Goldsmith said. The enzyme that activates the MAP kinase docks to the same site as well. Thus the enzymes that precede and follow the kinase in a particular cascade both interact with it in the same location. "It's a very interesting way to preserve pathway specificity," Goldsmith said.

As Tainer remarked, "Proteins like these are chemomechanical devices. They have the functional group chemistry that does reactions, but the rest of the protein is important, too."

FIGHTING DISEASE

Understanding Enzyme Action May Lead To Better Drugs

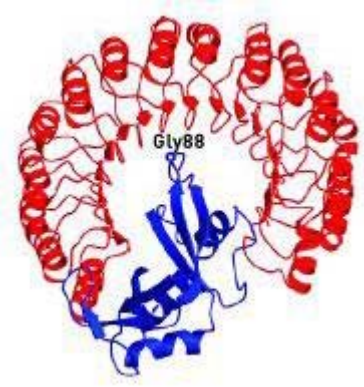
Promising approaches to fighting HIV and cancer were among the goals of research described at a symposium on structure-function correlations in phosphoryl-transfer enzymes.

The anti-HIV drug known as AZT must first be

phosphorylated by cellular enzymes into its triphosphate form before it can inhibit transcription of viral RNA into DNA, a key step in the life cycle of retroviruses such as HIV. One of these enzymes, thymidylate kinase, is inefficient when the AZT metabolite is its substrate, said Roger S. Goody, senior investigator at the Max Planck Institute of Molecular Physiology in Dortmund, Germany.

By examining the three-dimensional structure of human thymidylate kinase at several intermediate stages in the reaction, Goody and his colleagues identified an unusual conformational change that is an essential step for catalysis. Further structural studies showed that the AZT metabolite, AZT monophosphate, contains a bulky azido group that gets in the way of this conformational change.

Goody and his collaborators used their X-ray crystal structure data for human, yeast, and bacterial forms of thymidylate kinase to design and construct mutant forms of the human enzyme. Their mutants can phosphorylate AZT monophosphate up to two orders of magnitude more rapidly than the natural enzyme. In cell culture experiments using human cells, they see a dramatic increase in the amount of AZT triphosphate, the active form of the drug, produced when the cells are exposed to AZT.



IN CHECK Bovine pancreatic ribonuclease, blue, is ordinarily surrounded by and tightly bound to its inhibitor, red. Designed mutations of a single amino acid at glycine 88 release the enzyme to kill cancer cells.
STRUCTURE COURTESY OF RONALD T. RAINES

A possible application of these results to AIDS therapy would be to transfect cells specifically targeted by the HIV virus with these mutant forms of human thymidylate kinase, Goody suggested. Such an approach "would have the potential advantage of allowing targeting of the very cells that are also the target of HIV, thus only leading to improved phosphorylation of AZT in those cells," he pointed out.

Ronald T. Raines, professor of chemistry and biochemistry at the University of Wisconsin, Madison, and his colleagues are trying to take a different human phosphoryl-transfer enzyme, pancreatic ribonuclease, and design changes into it that will convert it into an anticancer drug.

The work began with the discovery in the 1990s by researchers at Alfacell, a biopharmaceutical company in Bloomfield, N.J., that the equivalent ribonuclease from the

northern leopard frog is naturally cytotoxic. This compound, under the name of Onconase, is now in Phase III clinical trials for the treatment of a type of lung cancer.

In humans, Raines noted, the dose of Onconase that patients can tolerate is limited by toxic effects on the kidneys. That's not surprising, he pointed out, because it is an amphibian protein, and many foreign proteins accumulate in the kidneys. Raines reasoned that a human, or at least mammalian, form of the protein might overcome this problem. However, neither the human nor bovine forms of the enzyme show the same ability to kill cells.

The reason, Raines finds, is a second protein, ribonuclease inhibitor. Humans and other mammals produce large amounts of this inhibitor in their cells. Crystal structure data reveal that the inhibitor wraps tightly around the ribonuclease, keeping its activity in check. Onconase, though only slightly different in its structure from mammalian ribonucleases, doesn't bind to this inhibitor protein.

In studies with bovine pancreatic ribonuclease, Raines and his colleagues found that, by changing only one of the 124 amino acid residues of the molecule, they could engineer the protein to evade ribonuclease inhibitor protein. More recent work using the human form of the enzyme shows that it, too, can be engineered to evade the inhibitor protein.

"In other words, we can take the ribonuclease that is already in our bodies, change a few amino acids, and make it toxic for cancer cells," Raines said.

All ribonucleases destroy RNA by cleaving its phosphodiester backbone. The frog ribonuclease and its mammalian equivalents selectively target the RNA of cancer cells, and Raines and his colleagues want to understand the reason for this selectivity. One possibility is that the ribonucleases can distinguish the carbohydrates that are displayed on the surface of cancer cells. The carbohydrate "fingers" known as gangliosides that coat the surface of cancer cells are richer in sialic acid than the gangliosides of normal cells. "We have demonstrated that these ribonucleases have good affinity for sialic-acid-rich gangliosides," Raines noted. "We believe this affinity for sialic acid could play a role in targeting cancer cells."
