Hot-Blooded Proteins

Heat-loving enzymes stay cool under stress

By CORINNA WU

As the saying goes, if you can't take the heat, get out of the kitchen. That ultimatum doesn't apply universally, however. Some creatures not only take the heat, they thrive in it. In recent years, scientists have discovered many such organisms—ranging from microbes to fuzzy, colorful worms—living comfortably in boiling-hot geysers or in steam vents on the ocean floor.

How do these beings keep themselves from getting cooked? Part of the answer lies in the structure of their proteins, which don't unravel even in temperatures approaching 100° Celsius, which would cause most proteins to fall apart.

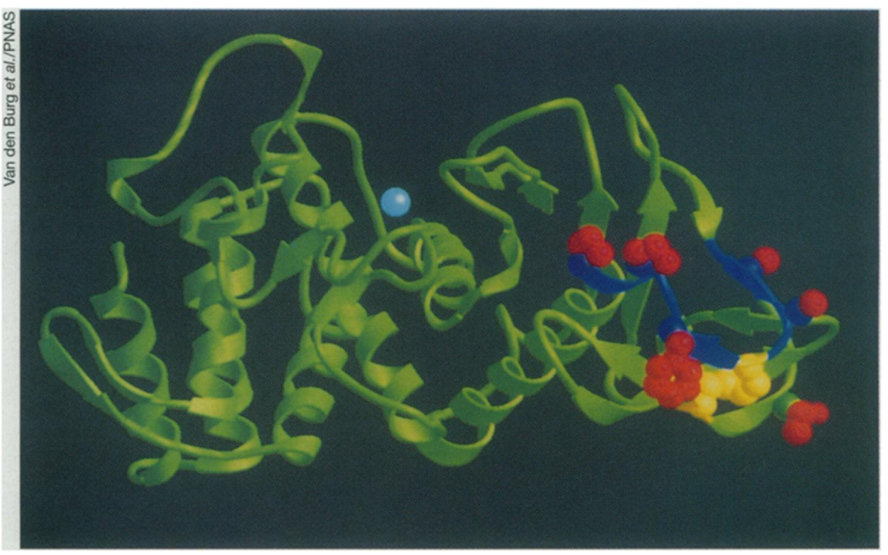
Scientists have been hard-pressed to figure out exactly what makes these proteins so heat-resistant. "No one really understands the molecular basis of thermal stability," says Frances H. Arnold of the California Institute of Technology in Pasadena. "People who have stared at protein sequences from nature for years will now readily admit that there are no general rules for stabilizing proteins."

Even without any rules to guide them, researchers have had great success in synthesizing thermophilic, or heat-loving, proteins. They use several techniques to either change the structure of existing proteins or design new ones.

Proteins, especially enzymes, that can tolerate heat have lots of potential industrial applications because high temperatures speed reactions and enhance solubility. Such enzymes can purify wastewater, help laundry detergents work better, and aid the synthesis of drugs, for example. Heat-stable enzymes also have a long shelf life, a characteristic that can reduce the ultimate cost of a chemical process.

Enzymes that withstand the assault of high heat—or acidity, alkalinity, and salt—could improve upon many of the inorganic catalysts now used in chemical manufacturing. Those catalysts work in harsh conditions that would destroy most enzymes, but they also tend to be less specific than enzymes and therefore produce more unwanted by-products. Enzymes also do their job in water, whereas the catalysts used in many standard industrial processes require toxic organic solvents.

In trying to understand heat tolerance, some scientists have come to the conclusion that the earliest proteins worked in high heat and that only after millions of years of evolution have proteins acquired the ability to function in cooler conditions. As Arnold states in the March 3 PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (PNAS), "Perhaps we should be wondering why mesophilic [moderate-temperature-loving] enzymes are so unstable, rather than why thermophilic ones are so stable."



Van den Burg *et al.*/PNAS

Mutations in a small region (dark blue, yellow, and red) of this bacterial enzyme significantly boost its stability in heat. The changed amino acids (red) form a link (yellow) that keeps the enzyme intact even at boiling temperatures. The enzyme binds a zinc atom (light blue) that helps catalyze reactions.

A protein is essentially a long strand of amino acids that twists and curls upon itself into a specific three-dimensional structure. The amino acids interact with each other at every turn, forming bonds and fitting neatly into the spaces created by the folds. Adding heat provides energy to break the bonds and thus unravels most proteins. For thermophilic proteins, though, even temperatures near 100°C don't provide enough energy to break those bonds.

Scientists do have a few clues about what makes a protein thermophilic, says Bertus Van den Burg of the University of Groningen in the Netherlands. For example, the amino acid proline adds stiffness to its section of the protein chain and thus some rigidity to a protein's overall structure. Also, amino acids that contain sulfur can form disulfide bonds with each other—again, locking the chain into place. Finally, charged amino acids can attract each other, forming a bond known as a salt bridge. Nevertheless, these features don't guarantee heat resistance, and many thermophilic proteins don't possess any of them.

Using elementary ideas of this sort, Van den Burg and his colleagues recently modified an enzyme from the soil bacterium *Bacillus stearothermophilus* to make it hyperthermophilic. They found that changing just eight amino acids in its sequence boosted the enzyme's heat

tolerance from about 86°C to 100°C. They describe their achievement in the March 3 PNAS.

The unmodified enzyme shares about 85 percent of its amino acid sequence with a well-characterized, heat-loving enzyme called thermolysin, which is produced by a different *Bacillus* bacterium. The two enzymes differ by only 43 amino acids. Thermolysin is used in the synthesis of the artificial sweetener aspartame, says Van den Burg.

The researchers used computer simulations to identify the amino acids important for stabilization, and then they methodically replaced individual amino acids in the *B. stearothermophilus* enzyme with their thermolysin counterparts.

"We didn't mutate all 43," says Van den Burg. "Quite soon, we noticed that all the big differences in thermostability were located in a small, defined part of the protein. Once we identified the location of the weak spot, we combined several mutations in that particular region."

At high temperatures, that region probably is one of the first to unfold, Van den Burg explains. The enzyme is a protease, whose job is to snip apart proteins. Once it starts to unfold, it becomes vulnerable to attack by its fellow enzymes.

At 100°C, the mutated protein is 340 times more stable than the original and more stable than thermolysin. Moreover, it is just as active at lower temperatures—no small feat. "Most things that have evolved to be thermophilic are most active at high temperatures," says Arnold.

The enzyme needs a certain amount of flexibility in order to work properly, says Van den Burg. Making it too rigid robs it of its function. Even noting that the eight mutations were quite far from the part of the enzyme that binds reactants, Van den Burg says, "it was very surprising that none of the mutations we made had any effect on activity."

Satisfied with the enzyme's stability, the researchers have already moved on to tweaking its other properties. Ultimately, they would like to see whether the enzyme works better than thermolysin for synthesizing aspartame.

Looking at thermophilicity is also a useful way for scientists to delve into the connection between protein structure and function, says Van den Burg. "It gives us a nice tool to study activity over a long range of temperatures."

Van den Burg and his coworkers took what chemists call a rational approach to endowing their enzyme with heat resistance, using theoretical arguments to design and test systematic changes. Arnold and her group at Caltech think they have an easier, faster way to accomplish the same goal. Instead of picking and choosing which amino acids to replace, they let ordinary enzymes evolve in a test tube until they acquire thermophilic properties (SN: 8/7/93, p. 90).

In this process, known as directed evolution, the researchers introduce random mutations into the gene that encodes the protein, thereby creating a library of genes that encode proteins with single amino acid changes. They move each gene into a bacterium that produces the modified protein. Once they identify a better protein, they take its gene and repeat the process. "We'll repeat it 10 times if necessary to accumulate the amino acid substitutions necessary to convert the protein into its thermophilic counterpart," Arnold says.

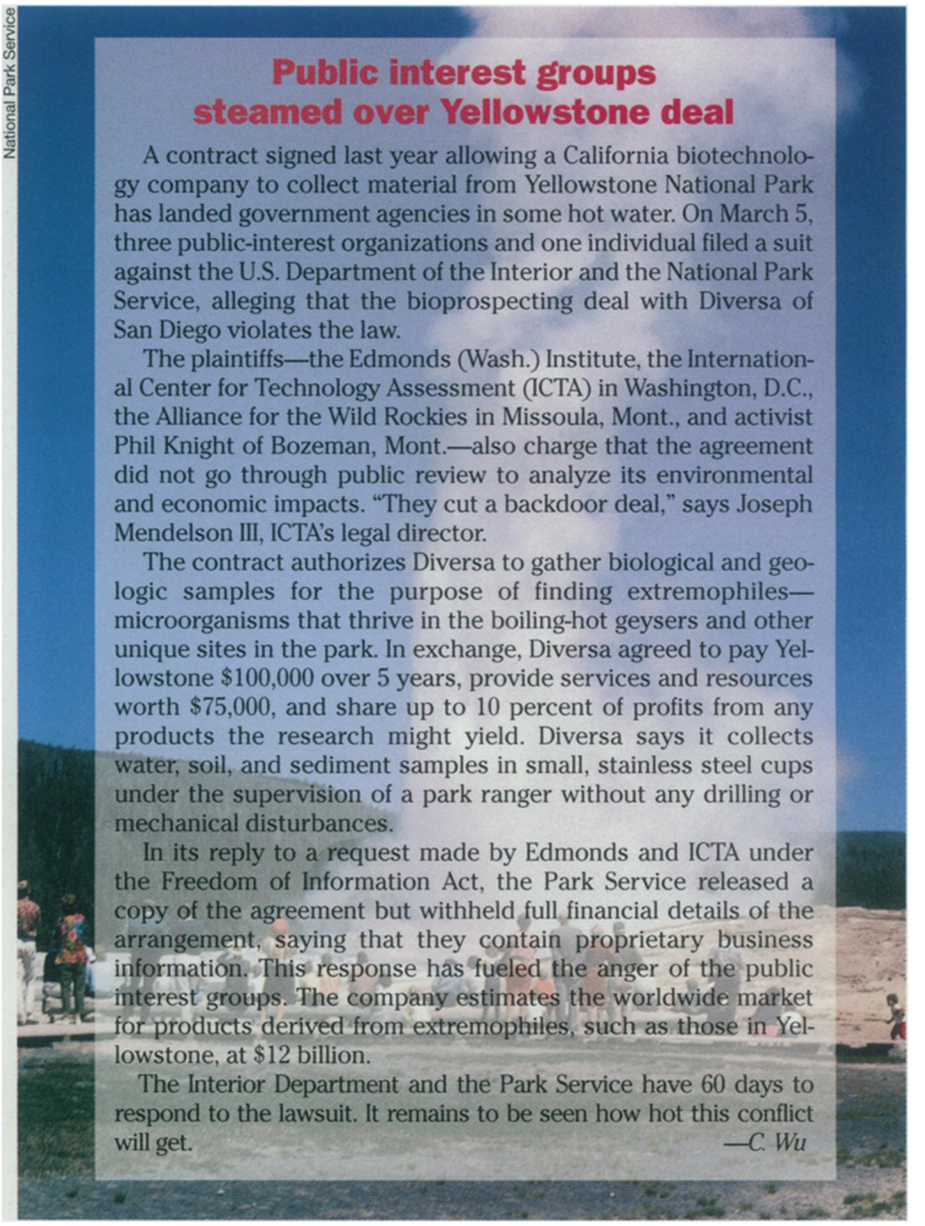
Arnold is studying several different enzymes, some in collaboration with companies such as Procter & Gamble, British Petroleum, and Eli Lilly. It doesn't take long to get results using directed evolution. She and her colleagues found that five rounds of screening of the enzyme subtilisin increased its heat tolerance 18°C. Six repetitions increased the limit of an esterase 15°C.

Arnold favors directed evolution because ordinary and thermophilic proteins often "differ in 100 or more amino acids. There's absolutely no way to test all of those." In a sense, this approach is "irrational," Arnold says in the March ACCOUNTS OF CHEMICAL RESEARCH. The scientist doesn't need to worry about specifying the exact changes, he or she just needs to make sure that each subsequent protein is better than the last.

"We recognize that enzymes are complex beasts," says Arnold. "Our knowledge base is puny compared with what we'd have to know in order to stabilize them rationally."

Arnold also points out that Van den Burg and his colleagues had thermolysin to use as a road map for their amino acid substitutions. Those counterparts don't exist for most enzymes of interest to industry, she claims.

Van den Burg agrees, but he thinks that directed evolution isn't the best


National Park Service

Public interest groups steamed over Yellowstone deal

A contract signed last year allowing a California biotechnology company to collect material from Yellowstone National Park has landed government agencies in some hot water. On March 5, three public-interest organizations and one individual filed a suit against the U.S. Department of the Interior and the National Park Service, alleging that the bioprospecting deal with Diversa of San Diego violates the law.

The plaintiffs—the Edmonds (Wash.) Institute, the International Center for Technology Assessment (ICTA) in Washington, D.C., the Alliance for the Wild Rockies in Missoula, Mont., and activist Phil Knight of Bozeman, Mont.—also charge that the agreement did not go through public review to analyze its environmental and economic impacts. "They cut a backdoor deal," says Joseph Mendelson III, ICTA's legal director.

The contract authorizes Diversa to gather biological and geologic samples for the purpose of finding extremophiles—microorganisms that thrive in the boiling-hot geysers and other unique sites in the park. In exchange, Diversa agreed to pay Yellowstone $100,000 over 5 years, provide services and resources worth $75,000, and share up to 10 percent of profits from any products the research might yield. Diversa says it collects water, soil, and sediment samples in small, stainless steel cups under the supervision of a park ranger without any drilling or mechanical disturbances.

In its reply to a request made by Edmonds and ICTA under the Freedom of Information Act, the Park Service released a copy of the agreement but withheld full financial details of the arrangement, saying that they contain proprietary business information. This response has fueled the anger of the public interest groups. The company estimates the worldwide market for products derived from extremophiles, such as those in Yellowstone, at $12 billion.

The Interior Department and the Park Service have 60 days to respond to the lawsuit. It remains to be seen how hot this conflict will get.

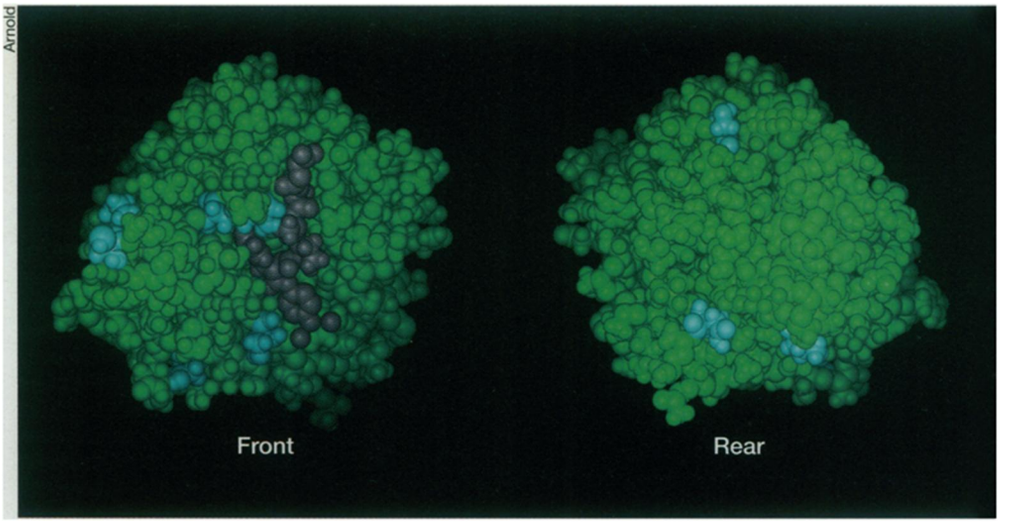
—*C. Wu*

technique for increasing the heat resistance of some enzymes, especially proteases.

Directed evolution studies also shed light on how protein stability evolved in nature, Arnold says. When early forms of life first appeared on Earth, the world was most likely much hotter than it is now. "Somewhere, our distant past proteins were thermophilic. If our proteins have lost that capability, what is the process by which it was lost?" she wonders.

If the ability to function at low temperatures resulted from random genetic drift—essentially the reverse of what the Caltech scientists do in the lab—"then staring at sequences of our ancestors isn't going to tell us anything about the mechanism of thermophilicity. It'd be virtually impossible to trace it back."

According to Arnold, one of her students described the issue this way: There's no Holy Grail for thermophilicity, but there's a holy approach, and that's evolution.



Laundry detergents contain the enzyme subtilisin (front and rear views) to help break down peptides (gray). Eight amino acid substitutions (blue), incorporated through directed evolution, improved the heat stability of subtilisin by 18°C.

While the Caltech researchers direct evolution in the test tube, or in vitro, scientists at Rutgers University in Newark, N.J., do the same "in computo." Ramy S. Farid and his colleagues have developed a computer program called CORE to design proteins that are stable at high temperatures (SN: 3/8/97, p. 146).

The researchers figure out what overall shape a desired enzyme must have, as well as the amino acid sequences of its active regions. Starting with this information, CORE tries to find the best sequence of amino acids for the inner core, which holds the enzyme together. It starts off with "a terrible sequence," Farid says, and tests how well the amino acids fit together. As it tests additional sequences, the program asks if each new choice is better or worse than the previous one. Over the course of the computer simulation, the program gets pickier about what it considers acceptable, and in this way, the protein evolves.

CORE considers only one aspect of what makes a protein hold together well: how tightly the hydrophobic, or water-avoiding, amino acids in the interior of the enzyme pack together. Using only that characteristic is controversial, Farid concedes, but CORE arrives at the same answers as other, lengthier computer simulations. "We intend to consider other methods," he notes, "but we haven't needed to yet."

Farid and his group are interested in using CORE to modify existing enzymes to work at high temperatures, but they also want to design entirely new proteins for other purposes. Whatever the purpose, the novel molecule must be stable. "If the protein comes out to be hyperthermophilic, it suggests that we've designed it right," he explains.

Both Arnold and Farid are collaborating with Diversa in San Diego, a company interested in developing thermophilic proteins for industrial uses. "They have great technology for isolating DNA from the environment," Arnold says. The company's robotic methods can screen 100,000 samples per week, says Dan E. Robertson, Diversa's director of enzymology. Last year, Diversa signed a controversial bioprospecting agreement with Yellowstone National Park in Wyoming, which allows the company to collect thermophilic organisms from the park's geysers in return for a share of any profits that result (see sidebar).

Scientists are looking to these organisms for clues to recapturing an old property that many proteins lost millions of years ago. The result may be heat-loving enzymes that never existed before—enzymes whose purpose is not to help critters survive in their hellish environments but to aid humans in their urge to improve their world. □