BIOCHEMISTRY

Resurrected enzymes

A paleoenzymology approach yields a thermostable thioredoxin enzyme that is functional at acidic pH; the underlying method could prove effective for generating other enzymes with such properties.

"Wouldn't you want to try wine made with 4-billion-year-old enzymes?" asked Julio Fernandez, a biophysicist at Columbia University, when discussing his recent paleoenzymology work. Though the wine is just an idea for now, the collaboration that originated after Fernandez read a paper on a subject far from his primary research interest has yielded thought-provoking results.

In that paper, Eric Gaucher's group at the Georgia Institute of Technology reported the reconstruction of ancient protein sequences. Using sequences from diverse species alive today, they constructed a phylogenetic tree and predicted protein sequences in long-extinct ancestors.

In stark contrast, Fernandez's group probes chemical reactions of enzymes, and they were studying an essential enzyme, thioredoxin (Trx). Because it is a well-studied enzyme, there were many Trx sequences available from all domains of life, making it amenable to Gaucher's analysis.

Upon seeing the reconstructed sequences, however, Fernandez's initial reaction was complete disbelief: "The sequences are up to 60% different from the modern ones, and I was not ready to believe that these extrapolated sequences would actually result in functioning enzymes." Nevertheless, they selected seven sequences from ancestors ranging from about 1.37 billion years ago to the very oldest bacterial, archaeal and eukaryotic common ancestors from about 4.2–3.5 billion years ago. When expressed in *Escherichia coli*, all seven enzymes were functional.

To estimate the temperatures at which these proteins operate, the researchers measured their denaturation temperatures using differential scanning calorimetry. The value for the oldest enzymes was 113 °C, which is 25 °C higher than for today's *E. coli* Trx.

Next the researchers used a single-molecule force spectroscopy assay they developed to investigate the chemical mechanism of Trxs—disulfide bond reduction. A synthetic substrate containing a series of disulfides is stretched using an atomic force microscope. If active, Trxs in the surrounding solution reduce the exposed disulfides, and resulting substrate-lengthening 'steps' are detected. By varying the force applied on the substrate, the chemical mechanisms can be distinguished.

The researchers speculated that the chemistry was initially primitive and the enzymes acquired sophistication later. But all the ancient enzymes acted through the same three-step mechanism as the current ones. And unlike modern Trxs, which optimally function at neutral pH, the ancient enzymes maintained function at pH 5, consistent with the likely acidic environment on early Earth.

As Fernandez notes, "Clearly the enzymes have adapted, because their thermal stability was very high and lowered as the Earth cooled, and the optimal pH for these enzymes became more neutral as the Earth switched from a CO_2-rich atmosphere to include oxygen, … but the chemistry was already established 4 billion years ago. How do you explain that? To me, it seems like a big mystery."

Though answers to these questions are still far off, he hopes this approach will enable major improvements in enzymes that will have practical applications. For example, the researchers are collaborating to improve cellulases used in the production of biofuels. And as mentioned, Fernandez sees a possibility of improving enzymes used in wine-making: "We are hoping we can develop a single-molecule assay for the substrates and activities of β-glucosidases, and also improve these enzymes so that they can work at more acidic pH, which [would make] them ideal for wine-making."

Delivering drastic changes in sequence, this paleoenzymology approach is more effective than any currently available technologies for increasing thermostability and pH range while preserving the chemistry of the enzyme. To reconstruct the enzymes, all one needs is enough sequences of different modern enzymes. With next-generation sequencing technologies, exponentially more genetic data are being generated for a broad range of species. "I think five years from now you will be able to do this with just about any protein you can think of," Fernandez predicts.

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RESEARCH PAPERS

Perez-Jimenez, R. *et al.* Single-molecule paleoenzymology probes the chemistry of resurrected enzymes. *Nat. Struct. Mol. Biol.* **18**, 592–596 (2011).