Backbone extension acyl rearrangements edit cellular proteins to install β-, γ- and δ-backbones

We present a versatile, chemo-ribosomal approach to generating isolable quantities of protein-derived biopolymers containing site-specific backbone modifications, including β-, γ- and δ-peptide linkages. Two of these extended backbones would be difficult to establish by alternative strategies in folded, full-length proteins generated in cells.

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The problem

Proteins and peptides are remarkable molecules: encodable, programmable, designable and even predictable. But their versatility is limited by a polypeptide backbone composed of only α-amino acid building blocks. Myriad societally impactful materials make use of alternative building blocks, including fabrics (Nylon-6), peptide-derived natural products (Taxol) and bio-plastics (polyhydroxyalkanoates). Evidence exists that engineered proteins whose backbones contain even a few alternative building blocks can exhibit desirable activities or reveal new insights into protein structure, function and evolution[1]. Although thousands of cellular proteins have been engineered to contain one or more non-proteinogenic α-amino acids[2], there are only four examples in which any building block other than an α-amino or α-hydroxy acid has been introduced into a protein in a cell[3]. The problem is that cellular translation is complex and depends on parts whose identities have been optimized over billions of years to assemble proteins containing only L-α-amino acids. We set out to identify a general strategy to circumvent this obstacle to produce proteins that contain alternative backbones that avoids complicated and difficult-to-reproduce synthetic biology tools.

The solution

To achieve this goal, we developed a post-translational chemical reaction that converts a protein containing only an α-backbone into one that contains a β-, γ- or δ-backbone. The general strategy relied on an intramolecular backbone extension acyl rearrangement (BEAR) reaction that post-translationally edits the backbone to introduce a new functional unit (Fig. 1). The substrate for a BEAR reaction is a protein — in our case, one expressed in cells — that contains a single backbone ester (X = O) whose side chain carries a masked nucleophile. The reaction begins after the addition of a post-translational trigger that removes the mask to reveal a nucleophile (Fig. 1; an amine, in this case); the amine then attacks the ester in an intra-molecular reaction to extend the backbone. Although elements of this reaction have been known for decades[4] and can establish new backbones in short peptides in vitro[5], it was not obvious that such a rearrangement could be set up and occur within a folded protein.

We encountered two challenges during this work whose solutions may have broad utility. First, under physiological conditions, the widely used reducing agent tris(2-carboxyethyl)phosphine did not cleanly remove the mask to generate the amine nucleophile, producing unacceptable levels of an alcohol byproduct that limited the BEAR reaction yield. However, 1,3,5-triaza-7-phospha-adamantane cleanly removed the mask to generate the amine at exceptional yield. The second challenge was establishing a workflow to prove that the BEAR reaction had occurred, as the products and starting materials are isobaric. Here, we partnered with experts at Amgen to perform high-resolution peptide mapping, which provided clear evidence for the BEAR transformation.

The implications

BEAR reactions have several advantages over existing methods for generating proteins and peptides with site-specific backbone edits. First, they rely only on the ability to incorporate α-hydroxy acid building blocks into proteins, which are known to be excellent substrates for translation. Although many unusual ribosome reactions can be performed at a small scale in the context of short unstructured peptides using chemically acylated tRNAs and costly in vitro translation reagents, BEAR can be scaled up readily and can affordably provide isolable quantities of materials whose properties can be studied using all manner of biochemical and biophysical tools.

Second, although the BEAR strategy proceeds without modifications to a cell's translation machinery, it does demand active and orthogonal aminoacyl tRNA synthetase and tRNA pairs, alongside ester-containing proteins that resist hydrolysis until the masked nucleophile is revealed. It also suffers from the same two limitations that plague the introduction of diverse non-canonical α-amino acids into proteins: the absence of clear rules for where stop codon suppression will proceed at high yield, and limited robust genomes and organisms that support the simultaneous incorporation of multiple different building blocks with high fidelity.

To us, many next steps are obvious. Even with the molecules in hand, including proteins with β-, γ- and δ-peptide linkages, we can begin to establish design rules to predict where extended backbones are stabilizing or destabilizing or imbue altered function. At the same time, we will seek to introduce more highly functionalized α-hydroxy acid building blocks to diversify the chemical potential of the resulting BEAR peptide and protein products.

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FROM THE EDITOR

"Polypeptide polymers containing monomers with an extended backbone could have a variety of useful properties; however, the protein synthesis machinery has evolved to be highly selective and is difficult to reengineer. This Article stood out, as the rearrangement reaction is a very neat approach to circumventing this challenge."
Russell Johnson, Chief Editor, *Nature Chemical Biology*.

FIGURE

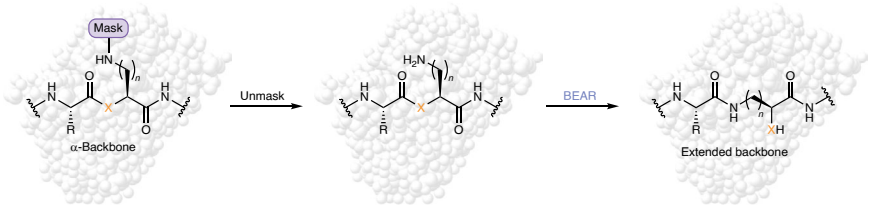

Fig. 1 | The BEAR reaction. A BEAR reaction begins with a full-length protein (background image, Protein Data Bank accession code 2B3P (ref. 6)) containing an α-hydroxy acid building block (X = O) whose pendant side chain carries a masked nucleophile (in this case, an amine). In this work, the ester-containing protein was generated in *Escherichia coli* cells that contain only wild-type ribosomes. Treatment of this protein with an unmasking agent (light or a reducing agent) provides the post-translational trigger to reveal the amine nucleophile. The resulting BEAR results in a protein that contains a modified (extended) backbone — in this case, a β-, γ- or δ-peptide linkage. Molecular graphics and analyses performed with UCSF ChimeraX.

BEHIND THE PAPER

Although the fundamental concepts of genetic code expansion were established decades ago, there are remarkably few examples in which anything other than an α-amino or α-hydroxy acid building block has been introduced into a protein in a cell. Some of the reasons are now clear: neither EF-Tu nor the ribosome always functions adequately when the A-site acyl-tRNA substrate is loaded with an alternative monomer, and these deficiencies limit biopolymer yield and fidelity. Although these two problems may very well yield solutions in the fullness of time, the 'name of the game' today is making molecules with novel structures and functions at scale. An alternative approach was needed. BEAR reactions provide a generalized chemo-ribosomal solution to this challenge — and one that succeeds without ribosome engineering. **A.S.**

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