Protein engineering

Boersma, Arnold J.; Roelfes, Gerard

Published in:
Nature Chemistry

DOI:
10.1038/nchem.2220

a gradual inversion of the CD signal, from positive to negative, indicative of the formation of either ADP or AMP (this first-generation supramolecular polymer cannot distinguish between them). Increasing the concentration of enzyme or the temperature of the experiment (up to 40 °C) resulted in faster inversion kinetics as a direct result of ATP hydrolysis; in the fastest case a steady negative CD signal was reached in approximately 10 minutes. Further hydrolysis resulted in a disappearance of optical activity, highlighting that the phosphate groups are essential to the observation of a CD signal induced by the chirality of the ribose unit.

The present strategy based on the observation of CD absorption spectroscopy is not the most sensitive that one could envisage. Indeed, synthetic molecular systems that monitor fluorescence are more sensitive[2,3], and supramolecular pores have also been used to sense enzyme activity[8]. Nevertheless, the inversion of chirality could be monitored in fluorescence mode, and the promise of using either spectroscopy or even microscopy based on this kind of system is an attractive prospect when it comes to the observation of processes at the single-cell level.

Sensing molecular species in biological systems based on responses of self-assembled synthetic systems is a compelling paradigm. It begs important questions. Could entirely man-made supramolecular constructs be used to monitor and regulate functions within living cells? What might be the consequences of this intrusion on the living matter? Given the success of the modelling in defining the chirality in this particular system, could modelling help predict new supramolecular systems capable of specifically detecting all three APs? Whatever the answers, using the chemistry of aggregates to probe biological function has taken an intriguing step forward that promises to lead to exciting new knowledge that may not be accessible through other routes. ❐

David B. Amabilino is in the School of Chemistry at the University of Nottingham, Nottingham NG7 2RD, UK.
e-mail: david.amabilino@nottingham.ac.uk

References

1. Moro, A. J., Cywinski, P. J., Körsten, S. & Mohr, G. J. *Chem. Commun.* **46,** 1085–1087 (2010).
2. Butler, S. J. *Chem. Eur. J.* **20,** 15768–15774 (2014).
3. Tang, J. L., Li, C. Y., Li, Y. F. & Zou, C. X. *Chem. Commun.* **50,** 15411–15414 (2014).
4. Imamura, H. *et al. Proc. Natl Acad. Sci. USA* **106,** 15651–15656 (2009).
5. Kumar, M. *et al. Nature Commun.* **5,** 5793 (2014).
6. Palmans, A. R. A. & Meijer, E. W. *Angew. Chem. Int. Ed.* **46,** 8948–8968 (2007).
7. Aida, T., Meijer, E. W. & Stupp, S. I. *Science* **335,** 813–817 (2012).
8. Das, G., Talukdar, P. & Matile, S. *Science* **298,** 1600–1602 (2002).

PROTEIN ENGINEERING

The power of four

Supramolecular assembly has been used to design and create new proteins capable of performing biomimetic functions in complex environments such as membranes and inside living cells.

Arnold J. Boersma and Gerard Roelfes

The high activity and accuracy demonstrated by proteins in processes such as catalysis and transport relies on the precise positioning of residues in three dimensions for every chemical step. This requires the folding of the peptide chain using a myriad of non-covalent interactions; a process that, despite decades of research and undeniable progress, is still not understood to a level that allows the *de novo* design of functional proteins. The fact that even nature simplifies the folding problem as much as possible illustrates the challenges involved. For example, nature often recycles successful protein folds and equips them with a repurposed active site[1]. Also, instead of using a single large peptide chain, complex protein structures are frequently created by supramolecular assembly of two or more proteins, which is a relatively simple and economical way to achieve complexity[2,3]. Indeed, many proteins even have active sites that comprise residues from multiple subunits. Similar strategies, combining the use of existing proteins and protein fragments and supramolecular assembly, are also applied in the laboratory for the design of new proteins. For many years, the workhorse for protein design has been the coiled coil, which is a supramolecular assembly of α-helical peptides[4]. These are relatively simple structures that can be designed by

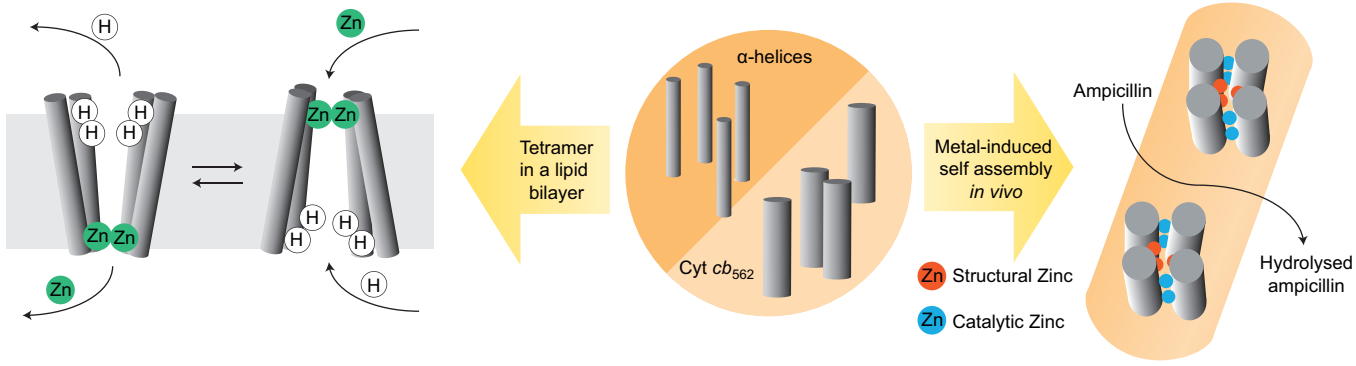

Figure 1 | Supramolecular assembly of four components can be used to design proteins with a diverse range of biomimetic functions. Left, the assembly of four α-helices to create a membrane channel that is capable of transporting Zn^{2+} across a membrane whilst antiporting protons. Right, the assembly of a tetramer that can hydrolyse the antibiotic ampicillin in *E. coli*.

computation with great accuracy. More recently, oligomeric proteins have been created by using metal coordination as the 'glue' between individual folded proteins[5]. These efforts have given rise to stable and well-defined oligomeric designer protein structures, some of which were even shown to exhibit catalytic activity *in vitro*[6–8].

Now two teams, both writing in *Science*, have taken this bio-inspired approach to protein design to the next level. In one example Grigoryan, DeGrado and co-workers report the design of a functional transmembrane channel capable of transporting Zn^{2+} ions, while antiporting protons[9]. In a second report by Song and Tezcan, supramolecular assembly is used to fabricate a new enzyme capable of catalysing the hydrolysis of an antibiotic within bacteria[10].

To design their channel protein, the team led by Grigoryan and DeGrado started by creating two metal-coordinating sites at the interface of a dimer of dimers, with each dimer comprised of two antiparallel peptides. Each metal binding site consists of four glutamate (Glu) and two histidine (His) residues, with at least one residue provided by each of the four peptides. Collectively these side-chains enable the binding of four Zn^{2+} ions — two Zn^{2+} in each binding site. The coiled coil sequence was calculated using the Crick equations to ensure proper orientation of the residues for the coordination of Zn^{2+} and the coordination sites were placed at both ends of the helix bundle, to facilitate the binding of Zn^{2+}.

A significant step towards creating a zinc transporting channel was achieved by destabilizing the symmetric coiled coil — which was cylindrical — in favour of a cone-shaped conformation that could oscillate dynamically between the two states and which only allowed the coordination of two zinc ions at a time. Thus the two Zn^{2+} ions can only be bound at one site if the Zn^{2+} ions bound at the other site are released. This cone shape was generated by straightening the helices. The final structure of the dimer of dimers, which they called 'rocker', was characterized by X-ray crystallography, and a variety of NMR techniques, which, in combination with molecular dynamic simulations, indicated that the peptide assembly did indeed form a tetramer that spanned the membrane as designed.

The function of the rocker assembly was investigated by inserting it into large unilamellar vesicles (LUVs). This enabled Zn^{2+} efflux from the vesicles to be observed using a fluorescent dye. The channel was found to transport Zn^{2+}, as well as Co^{2+}, but not Ca^{2+}. Furthermore, it was shown that substituting the Glu residues prevents transport of Zn^{2+}, which proves that the coordination sites are required for transport. Interestingly, they found that protons are antiported, even against a pH gradient, as Zn^{2+} is exported. The team proposes that this occurs because the Zn^{2+} and H^{+} ions compete for the same binding sites, although diffusion of protons through the Zn^{2+} bound rocker also occurred.

The significance of this study is that, aided by computational design, Grigoryan, DeGrado and co-workers were able to create a dynamic supramolecular assembly of α-helices that is capable of carrying out a biomimetic function — in this case the transport of ions across a membrane. Even though the observed transport rates are much lower than those of natural transporters, this represents a milestone in protein design, which is still mainly focused on creating stable, static structures. The fact that a functional transporter can be designed and assembled from four identical small peptides will yield a great deal of information on the fundamental aspects underlying transport in nature. While this approach may be further extended to the design of other transporters for applications *in vitro*, it is difficult to envision that this design can be extended to function *in vivo*. However, another team based in the US is working to design proteins that function in cells.

In a key advance Song and Tezcan, demonstrated that metal-mediated self-assembly of well-defined oligomeric proteins can be used to create a novel metalloenzyme that displays enzymatic activity *in vivo*[10]. The new enzyme was designed using a previously introduced strategy called 'metal templated interface redesign'. In this approach a small number of surface mutations are introduced to create a metal binding site at the interface between two proteins. This approach was used to convert a cytochrome cb_{562} into a mutant that self-assembles into a tetramer using the coordination of four equivalents of Zn^{2+} to cross-link one pair of interfaces, and the introduction of stabilizing hydrophobic interactions and the formation of disulfide bonds, to crosslink the other pair of interfaces. Further His and Glu residues were also introduced at judicious positions in the latter interfaces so that, in addition to the structural Zn sites, four catalytic Zn sites were created. The design was then optimized by removing a lysine residue that was erroneously binding to the catalytic Zn sites.

The artificial zinc enzyme is an efficient catalyst for the hydrolysis of activated esters, even showing Michaelis–Menten kinetics — which is characteristic for enzyme-like catalysis. This prompted Song and Tezcan to question whether the enzyme could catalyse an important reaction for *in vivo* applications: the hydrolysis of ampicillin, a lactam antibiotic. Testing the enzyme *in vitro* showed that it possessed considerable activity although in this case no saturation behaviour was observed, indicating that there are no significant interactions between the antibiotic and the protein scaffold.

To test the *in vivo* catalytic activity in *Escherichia coli* cells, they used a variant of the enzyme that is directed to the periplasm, as this environment would enable the disulfide bonds to form readily and the protein was expected to be able to compete for the Zn^{2+} ions required for assembly and catalysis. However, a method of detecting whether the enzyme was active within *E. coli* was still needed. To address this they used a survival assay to show whether the enzyme could deactivate the antibiotic ampicillin, and thereby enable the *E. coli* cells to grow. It was found that *E. coli* cells containing the artificial zinc enzyme were able to grow despite the presence of ampicillin, whereas controls containing variants without the catalytic Zn^{2+} sites could not grow under the same conditions. This clearly demonstrated that the designed enzyme was active *in vivo*.

The enzyme was further optimized by saturation mutagenesis at selected positions, using the frequency of colonies containing a mutation that was expressed under these conditions, as a readout. Some mutations were found to occur more frequently than others, albeit that there was in general only a weak correlation between the prevalence of certain mutations and the inherent catalytic activity. Nevertheless, the two most frequently found mutants, Gly 57 and Thr 105, also proved to be the most active in catalysis, with the latter even showing emerging saturation behaviour, although the measured K_m was very low. The Gly 57 variant also accepted larger substrates. Based on X-ray structural information, this was attributed to a widening of the interfaces and the emergence of a flexible loop that was suggested to be capable of interactions with the antibiotic, in a fashion analogous to natural β-lactamases. This is particularly noteworthy given that that this designed lactamase was created by introducing as few as 16 mutations in cytochrome cb_{562}, which is a totally unrelated oxidase enzyme.

Both the report by Grigoryan, DeGrado and co-workers and the work of Song and Tezcan are impressive demonstrations of how far the field of protein design has advanced. They show convincingly how the concept of supramolecular assembly can be used to create ever more sophisticated protein structures, capable of performing a biomimetic function in a complex environment such as a membrane or even a

living cell. Both designs share some common features, including localization of the active site on the protein–protein interface and that both proteins are tetramers, which is an intriguing detail in view of the fact that the average oligomerization state of proteins in nature is tetrameric[2].

The designs are not optimized yet: the activity and substrate binding affinity of both proteins clearly need further improvement. Nevertheless, achieving this should be feasible via a combination of the increasingly powerful computational techniques that are now available, and evolutionary approaches that select for improved function[1]. These two studies show that a future in which proteins are routinely designed for specific functions *in vitro* and *in vivo* is becoming ever more likely. ❐

Arnold J. Boersma is in the Groningen Biomolecular Sciences and Biotechnology Institute and Gerard Roelfes is in the Stratingh Institute for Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, the Netherlands.
e-mail: a.j.boersma@rug.nl; j.g.roelfes@rug.nl

References
1. Kiss, G., Çelebi-Ölçüm, N., Moretti, R., Baker, D. & Houk, K. N. *Angew. Chem. Int. Ed.* **52,** 5700–5725 (2013).
2. Ali, M. H. & Imperiali, B. *Bioorg. Med. Chem.* **13,** 5013–5020 (2005).
3. Marianayagam, N. J., Sunde, M. & Matthews, J. M. *Trends Biochem. Sci.* **29,** 618–625 (2004).
4. Bryson, J. W. *et al. Science* **270,** 935–941 (1995).
5. Sontz, P. A., Song, W. J. & Tezcan, F. A. *Curr. Opin. Chem. Biol.* **19,** 42–49 (2014).
6. Zastrow, M. L., Peacock, A. F. A., Stuckey, J. A. & Pecoraro, V. L. *Nature Chem.* **4,** 118–123 (2012).
7. Der, B. S., Edwards, D. R. & Kuhlman, B. *Biochemistry* **51,** 3933–3940 (2012).
8. Faiella, M. *et al. Nature Chem. Biol.* **5,** 882–884 (2009).
9. Joh, N. H. *et al. Science*, **346,** 1520–1524 (2014).
10. Song, W. J. & Tezcan, F. A. *Science* **346,** 1525–1528 (2014).

SURFACE CHEMISTRY

A step in the right direction

Identifying the contribution of different surface sites to the overall kinetics of molecular desorption from solid surfaces is difficult even when using single crystals. A new technique that combines molecular beams with UV–UV double resonance spectroscopy resolves this problem for the case of carbon monoxide on Pt(111).

Francisco Zaera

Reactions on solid surfaces are ubiquitous in nature and central to many chemical processes such as catalysis, electrochemistry and film deposition, yet the bonding involved is not as well understood as in homogeneous systems. This is not for a lack of trying, it is just that chemistry at interfaces is much more difficult to investigate.

First, there are far fewer surface atoms than those in the bulk of a given material, so special techniques are necessary to selectively probe bonds and chemistry at those surface sites. A revolution in surface science in the late twentieth century led to the development of a plethora of new surface-sensitive techniques[1], but the information that they have provided remains somewhat limited, particularly when compared with spectroscopies designed to study bulk samples. Second, bonding to solid surfaces is more complex and more difficult to describe than in discrete molecules. This is particularly true on metals, where the electronic structure is delocalized and extends over large atomic distances[2]. Finally, it is quite difficult to prepare surfaces with only one type of bonding, since adsorbates can coordinate in many different ways to the various ensembles of surface atoms exposed by the solid. Surface scientists deal with this latter problem by using specific facets of single crystals[1], which exhibit well defined coordination environments, but even those can feature enough defects — typically low-coordination, highly-reactive atoms in steps, kinks, and other unique arrangements — to dominate the overall chemistry and skew the results of kinetics measurements.

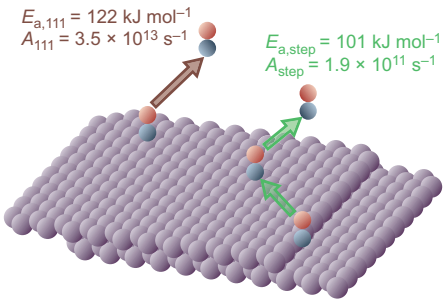

Figure 1 | Schematic of the CO desorption processes identified by Bartels and colleagues. The addition of UV–UV double resonance spectroscopy to molecular beam experiments on surfaces allowed the identification of two desorption processes. The desorption of CO from terrace sites occurs rapidly (brown arrow), yet a slower process involving the diffusion of CO from step sites to terraces sites before subsequent desorption (green arrows) dominates at low coverages (reaction rate constant = $Ae^{(-E_a/RT)}$).

It is this third limitation that has been addressed by Bartels and colleagues writing in the *Journal of the American Chemical Society*[3]. They report a new technique to estimate the binding energy of carbon monoxide on the flat, hexagonal close-packed, (111) facet of platinum — a prototypical system often used by surface scientists as a benchmark. There are three basic ways of measuring binding energies of molecules on surfaces. The most common is temperature programmed desorption, a technique where the temperature at which molecules desorb from a surface as it is heated is used to estimate desorption activation energies. The strength of this technique, and the reason for its wide use, is its simplicity, but its poor accuracy is a significant weakness[4]. Kinetic data can be better measured isothermally by directing pulsed beams of molecules toward the surface of interest and detecting and analysing the angular and energy distributions of the scattered molecules. However, such molecular beam experiments are difficult to carry out and often suffer from problems of sensitivity and time resolution. Finally, new differential microcalorimeters have been developed recently to directly measure small heat changes, and thus adsorption energies, on single-crystal surfaces.

Bartels and colleagues now incorporate UV–UV double resonance spectroscopy into a molecular beam setup to detect the desorbing molecules. This involves an arrangement in which the CO molecules leaving the Pt(111) surface are first excited to a metastable state by means of the adsorption of a UV photon, and then detected following a second excitation, triggered by a second UV photon, at