BIOPHYSICS

Breaking out of the cage

Activating caged reactive sites in proteins using mechanical force provides a powerful approach in the study of chemical reactions, and provides greater insight into which reactions are possible and their rates.

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Chemical bonds vary greatly in strength: just compare, say, the covalent bonds in diamond with the extremely weak van der Waals interactions between layers of graphite, in the two most common allotropes of carbon. These different bonds play a key role in regulating the properties of functionally diverse protein materials in nature. Strong covalent bonds form a permanent connection between atoms in the backbone of polypeptide chains. Weaker non-covalent bonds, such as hydrogen bonds, play an important role in defining a protein's three-dimensional structure.

Intermediate between these strong and weak interactions is the disulfide bond, which serves as a flexible connection on account of its variable bond strength in different chemical microenvironments[1]. Disulfide bonds weaken in the presence of reducing agents (approaching that of hydrogen bonds) and are strengthened in an oxidizing environment (approaching that of other strong covalent bonds). They can thus act as an effective switch to stabilize or weaken a folded protein structure, or even to rearrange a protein's geometry altogether. These mechanisms enable a protein with disulfide bonds to provide distinct biological functions, a phenomenon called mutability, and also a critical feature in molecular sensing and signalling. The change in the strength of disulfide bonds is similarly important in the context of disease, where a failure to form the correct disulfide bond or excessive strengthening under oxidative stress can lead to malfunction through protein misfolding or aggregation, as seen in inflammation, cardiovascular disease, amyotrophic lateral sclerosis or cataract formation[2–5].

Protein disulfide bonds are typically formed through the oxidization of thiol groups in two cysteine residues. On the other hand, disulfide bonds can be broken by a reducing thiol–disulfide exchange reaction in which a thiolate anion attacks an existing disulfide bond and replaces a sulfur atom of the original disulfide bond to form a new bond. This reaction can also occur intramolecularly, resulting in

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disulfide bond reshuffling. In biology, such an intramolecular thiol–disulfide exchange reaction represents a mechanism to repair incorrectly formed disulfide connections and to adjust a protein's folded geometry based on external signals. Because the number of protein atoms remains constant during the reshuffling of disulfide bonds, this process is called disulfide bond isomerization.

Now, writing in *Nature Chemistry*[6], Jorge Alegre-Cebollada, Julio Fernández and co-workers present the first direct observation of the kinetics of disulfide bond isomerization in a protein. They designed a protein molecule that contains two disulfide bonds A and B (Fig. 1a). Disulfide A serves as a 'cage', which prevents either of its cysteines from reacting with a second hidden disulfide, B.

Once disulfide A is broken, secondary reactions associated with disulfide B are possible. Caged structures can be found in many natural systems, for example in caged molecules that can be photoactivated, where ultraviolet light serves as the trigger to allow reactions at a hidden site[7]. In the present study, instead of light, a mechanical force, applied using an atomic force microscope, is used as a signal. The advantage of this nanomechanical approach compared with using light is that it allows a detailed analysis of the steps in the chemical reactions through the availability of time-resolved force–displacement data, which can be directly associated with the molecular mechanisms of different chemical reactions.

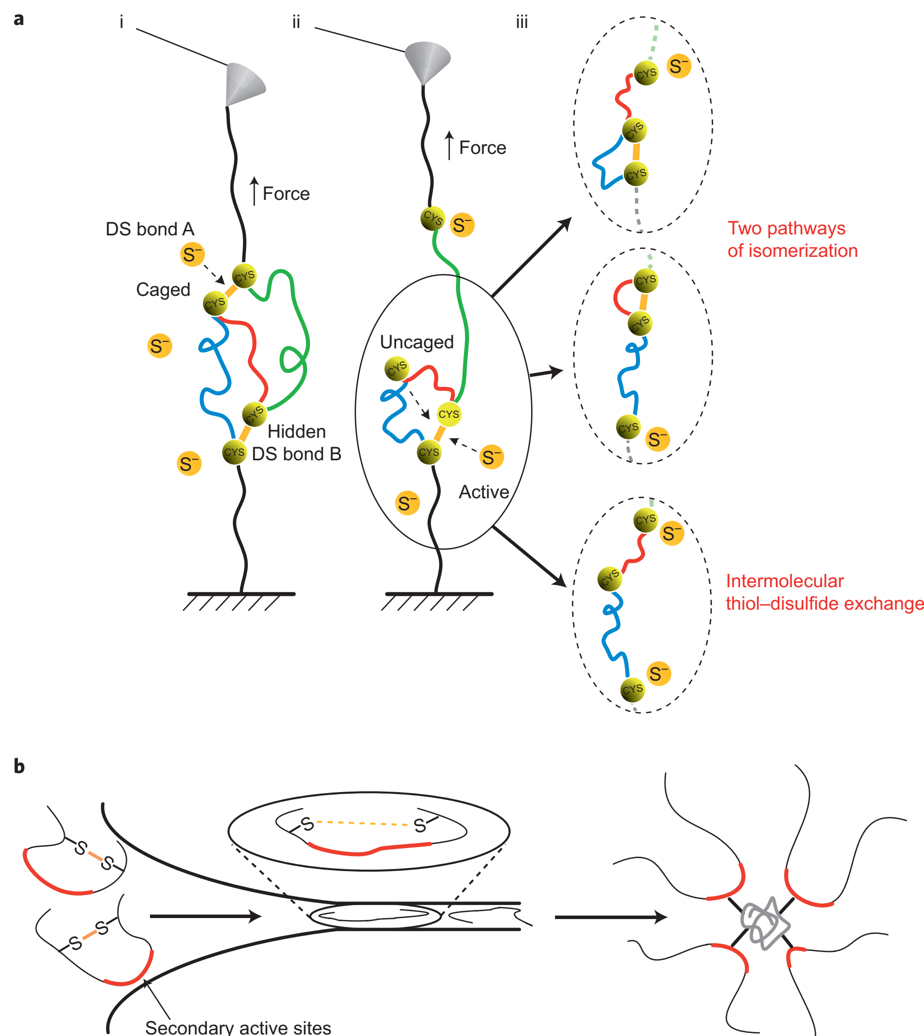

Figure 1 | Reactions of caged disulfide (DS) bonds assessed through a nanomechanical approach, and potential applications in a microfluidic device. **a**, In step i, the applied force causes disulfide A to break when it is exposed to the reducing agent. In step ii, one of the cysteine (CYS) residues produced by breaking disulfide A reacts with the hidden disulfide B. This enables the reactions in step iii, which can follow three possible pathways as shown. There are different numbers of amino acids in the red and blue loops that are released on increase of the force, so the force–displacement measurements display a distinct elongation on breaking of the disulfide bond. This allows the specific pathway of isomerization or intermolecular thiol–disulfide exchange to be identified directly from the analysis of the nanomechanical behaviour. **b**, A potential application of force-activated caged disulfide bonds in flow-driven self-assembly of materials. Elongational flow exposes molecules to mechanical force sufficient to break disulfide bonds and allow reaction at secondary sites that are unchanged in this process. These secondary reactions could be used to control the self-assembly of biopolymers into micelles or other fibre-like structures, including crosslinked polymer networks.

The different reactions that occur are detected as distinct extensions of the protein (Fig. 1a). In the presence of a reducing agent (L-cysteine), disulfide A is the first to break. The applied force means that the two cysteine residues are immediately separated so that recombination is impossible, but this 'uncaging' leaves one residue near to disulfide B, which becomes the site of a secondary reaction. Disulfide B can be broken by three competing pathways — two different isomerization reactions in which one of the two cysteine residues in B reacts with the uncaged cysteine residue from A, or a third pathway in which disulfide B directly interacts with the surrounding reducing agent. The geometry of the protein and the number of hidden amino acids confined by the disulfide bond determines the magnitude of elongation[8,9]. Hence, the pathway of the reaction can be directly inferred from the recorded force–displacement data after the secondary reaction and the rate from the frequency at which such an elongation occurs.

An interesting finding from these experiments[6] is that the kinetics of disulfide isomerization are highly dependent on the concentration of the reducing agent. An increase of this concentration leads to a decrease in the rate of isomerization reactions and a dominance of the thiol–disulfide exchange. Notably, the two pathways of isomerization open to disulfide B show different reaction rates, suggesting a regiospecificity of disulfide isomerization. Current theoretical models[10] do not provide a thorough explanation of this regiospecificity, pointing out a need for further investigation.

The nanomechanical approach used here provides an exciting opportunity to study the reshuffling behaviour of disulfide bonds in the context of various diseases or engineered biomaterials, using force–displacement measurements as a powerful approach to elucidate the kinetics and mechanisms of chemical reactions. What's more, the changes in bonding within a protein during isomerization form an example of the universality–diversity paradigm of nature, where different functional states arise from the same building blocks and the structure controls the function[5]. The concept of a force-activated hidden site opens new avenues for designing materials. In existing

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applications, disulfide bonds are already used in the design of micelles for gene delivery and self-assembled monolayers because of the reversibility of crosslinking[11].

The idea of force-activated caged structures also hints at a potential use as a flexible building block inside microfluidic channels. In elongational flow, molecules can be stretched and this can trigger secondary reactions that cause specific self-assembly mechanisms (Fig. 1b) — like the protein assembly that controls the formation of silk fibres in a spider's spinning duct. Potential applications of mutable disulfide bond arrangements also exist in the design of structural materials, where weak bonds can enhance mechanical resilience by sacrificially dissipating large deformation forces, leading to great fracture toughness or ductility[12]. Moreover, controlling the arrangement of disulfide bonds through force-activated isomerization could lead to new materials with mutable fracture properties, where isomerization processes at high-stress sites such as crack tips could be controlled by external signals — for example, reducing agents or light — and thereby used to mitigate the risk of crack propagation when needed. ❐

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