Allosteric Control through Mechanical Tension

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Conformational changes of proteins modulate their function. In allosteric control, the conformational change is induced by the binding of a signaling molecule. Here we insert a "molecular spring" on the enzyme guanylate kinase, to control the conformation of this protein. The stiffness of the spring can be varied externally, which allows one to exert a controlled mechanical tension between the two points on the protein's surface where the spring is attached. We show that by applying and releasing the tension we can reversibly turn the enzyme off and on.

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Most enzymes in the cell are directly regulated by allosteric control. Unlike the control of gene expression, this mechanism provides fast response and is crucial in signaling pathways [1]. In allosteric control [2], the signaling molecule binds to the enzyme at a site (A) distinct from the substrate's binding site (S). Separating A and S is an essential design feature because the substrate and the controlling molecule can then be unrelated chemically. Qualitatively, the local binding force at A produces a stress that is believed to propagate through the protein, modifying the conformation at S. Here we construct such a mechanism, through a new protein engineering approach, which allows us to exert a controlled mechanical tension between any two chosen points on a protein's surface [3]. We show on the example of the enzyme guanylate kinase that by applying and releasing the tension we can control the enzymatic activity.

Guanylate kinase (GK) is an essential enzyme that catalyzes the transfer of a phosphate from adenosine triphosphate (ATP) to guanosine monophosphate (GMP) [4]; here we work with the 24 kDa protein from *Mycobacterium tuberculosis*. The structure, shown in Fig. 1 [Protein Data Bank (PDB) entry 1S4Q], resembles a vice; when the substrates ATP and GMP bind inside the cavity, the "jaws" of the vice close through a ~1 nm conformational change [5]. We reasoned that a mechanical tension favoring the "open" conformation of the enzyme would lower the binding affinity for the substrates, the rate of catalysis, or both. We accordingly constructed a chimera where the mechanical tension is provided by the elasticity of a second polymer coupled to the enzyme: in this realization, a DNA oligomer. To couple the DNA, we modified GK through site-directed mutagenesis (Thr 75 → Cys; Arg 171 → Cys) introducing a Cys residue on each of the two lobes of the molecule (Fig. 1). To those "chemical handles" the two amino-modified ends of a single stranded (ss) DNA 60mer were covalently coupled through a cross-linker, resulting in the chimera shown in Fig. 1. By gel electrophoresis, the chimera can be easily distinguished from other species present in the samples, such as uncoupled GK, GK dimers (encouraged by the Cys modification), and DNA-coupled dimers. Partially coupled chimeras (where the DNA is attached by one end only) are, however, not distinguished on the gels. From the gels, the measurements below, and measurements on a different chimera which we report elsewhere, we estimate the yield p of correct chimeras in our samples to be somewhere between 50% and 70%, depending on the sample.

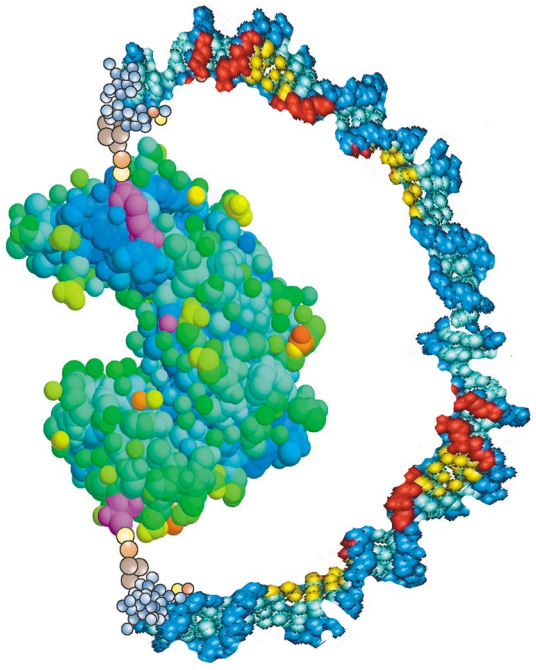

FIG. 1 (color). The protein-DNA chimera. The GK structure is PDB entry 1S4Q. The locations of the Cys mutations are shown in magenta. The distance between these two groups is 4.5 nm.

With this construction, we can exert a controlled mechanical tension between the two spots on the protein's surface where the DNA is attached. Namely, the ss DNA 60mer of the chimera is a flexible polymer, and exerts no tension on the protein; however, it rigidifies upon hybridization with a complementary strand (the persistence length of ss DNA is $\ell_{ss} \approx 1$ nm or ~ 3 bases, while $\ell_{ds} \approx 50$ nm or ~ 150 bp). The rigid double stranded (ds) DNA has to bend in order to maintain the end-to-end distance imposed by the attachment points on the protein; therefore it exerts a mechanical stress on the protein, similar to the tension exerted by a bow on its string. This mechanical stress on the enzyme in solution can be controlled externally, by adding DNA partially or totally complementary to the DNA of the chimera. In this report, we show that we can thus modulate the enzyme's activity through the presence of a specific DNA sequence in solution. Furthermore, we obtain new insight into the architecture of this molecule by measuring how a mechanical stress applied between two specific residues far away from the active site alters the enzymatic activity.

Our first construct with this approach utilized the maltose binding protein from *E. coli* (which is not an enzyme). By applying a mechanical tension, we could lower the binding affinity for maltose at least twofold [3], and possibly much more, the measured effect being limited by the yield of correct chimeras in the samples [6]. Here we apply, for the first time, this approach to allosterically control an enzyme.

Results.—The materials and methods, including the mutagenesis, and the synthesis of the chimera, are described in [7]. GK activity was measured using the luciferase chemoluminescent assay, which monitors the conversion of ATP (see methods). A calibration curve was obtained (data not shown) to relate the measured luminescence [in relative light units (RLU)] to the concentration of 100% active GK; in the following, "effective [GK]" means RLU transformed into [GK] using the calibration curve. The enzyme activity of the mutant is about 20% lower than the wild type, and the activity of the ss chimera is indistinguishable from that of the mutant. Figure 2(a) shows the reduction in kinase activity of the chimera upon introducing the complementary DNA 60mer in solution, for different samples. The magnitude of the observed effect appears limited by the yield of correct chimeras. Figure 2(b) shows that the reduction in activity is reversible: it disappears if the mechanical tension is released.

Finally, the data in Fig. 3 give insight into the structure-function relation for this enzyme, as follows. Within the MM description, catalysis occurs in two steps:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \overset{K_2}{\rightarrow} R + E \tag{1}$$

(E, S, ES, R: enzyme, substrate, intermediate complex, product), characterized by the MM constant of the intermediate complex, $K_M = (K_{-1} + K_2)/K_1$, and the rate of the catalytic step, K_2 [8]. The speed of the reaction is

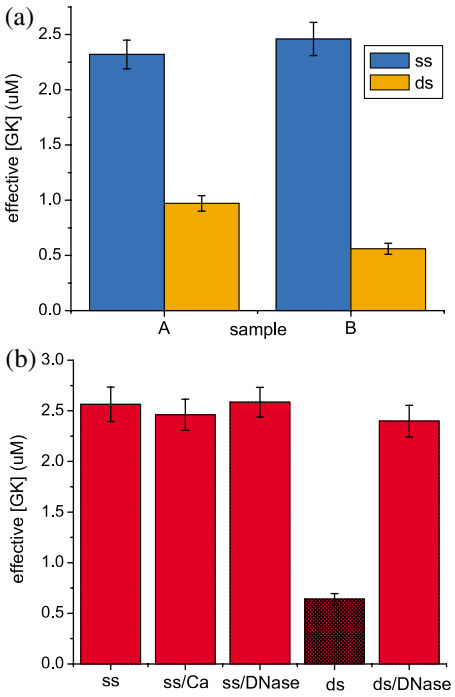

FIG. 2 (color). Reduction in GK activity upon hybridization of the chimera. Data are the average of 4–5 experiments; the error bars are ± 1 standard deviation (SD). (a) Sample A shows a 2-fold effect, which rises to a 4-fold effect (sample B) upon purification on a sulfhydryl column, which retains molecules with unreacted Cys. (b) The 4-fold decrease in enzymatic activity going from ss to ds chimera, is reversed after adding DNAse, which degrades the DNA of the chimera, releasing the tension. The columns ss/DNAse and ss/Ca are controls.

$$\frac{d[R]}{dt} = P(\text{on})[E]_{\text{tot}} K_2, \tag{2}$$

where $P(\text{on})$ is the probability that the enzyme has a bound substrate:

$$P(\text{on}) = \frac{1}{K_M/[S] + 1}. \tag{3}$$

With two substrates G (for GMP) and A (for ATP) the same approach (2) and (3) leads to

$$\frac{d[R]}{dt} = \frac{[E]_{\text{tot}} K_2}{(K_G/[G] + 1)(K_A/[A] + 1)}. \tag{4}$$

We performed measurements not directly of $d[R]/dt$, but, instead, of the product formed after a time τ, for varying initial GMP concentrations $[G(0)]$, at fixed initial ATP concentration $[A(0)]$. More precisely, in the experiments we measure the ATP concentration $[A(\tau)]$ remaining after the time τ. The data are shown in Fig. 3, for the ss and ds chimera. The assay conditions are such that $K_A/[A] > 1$ and there is excess GMP over ATP; with the approximations $K_A/[A] + 1 \approx K_A/[A]$, $[G] \approx \text{const} = [G(0)]$, and since $d[R]/dt = -d[A]/dt$, Eq. (4) becomes

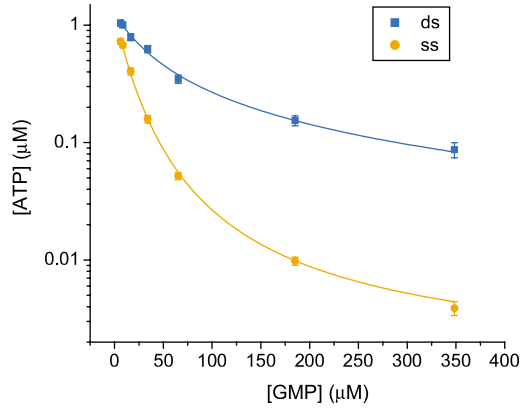

FIG. 3 (color). The concentration of ATP remaining after a time τ, $[A(\tau)]$, vs the initial GMP concentration $[G(0)]$, for the ss and ds chimera. $[A(\tau)]$ is a measure of the speed of the enzymatic reaction. Each experimental point represents the average of 4–6 measurements; error bars are ± 1 SD. The ss data are fitted using Eq. (6), the ds data using (9); the corresponding parameter values are listed in Table I.

$$-\frac{d[A]}{dt} = \frac{[E]_{\text{tot}} K_2/K_A}{1 + K_G/[G(0)]}[A] \tag{5}$$

with the solution

$$[A(\tau)] = [A(0)]e^{-ht}, \qquad h = \frac{[E]_{\text{tot}} K_2/K_A}{1 + K_G/[G(0)]}. \tag{6}$$

We use (6) to fit the ss data in Fig. 3, and extract the parameters $(K_2/K_A)^{\text{ss}}$ and K_G^{ss} (Table I). Under these assay conditions, it is not possible to extract K_2 and K_A separately. For the ds chimera, we must take into account that the experimental samples consist of a yield p $(0 < p < 1)$ of correct chimera, plus a fraction of enzyme $(1 - p)$ which is functionally unmodified. Equation (5) then becomes

$$-\frac{d[A]}{dt} = (1 - p)h^{\text{ss}}[A] + ph^{\text{ds}}[A], \tag{8}$$

where

$$h^{\text{ds}} = \frac{[E]_{\text{tot}}(K_2/K_A)^{\text{ds}}}{K_G^{\text{ds}}/[G(0)] + 1}, \qquad h^{\text{ss}} = \frac{[E]_{\text{tot}}(K_2/K_A)^{\text{ss}}}{K_G^{\text{ss}}/[G(0)] + 1}.$$

The solution is

$$[A(\tau)] = [A(0)]\exp\{-[(1 - p)h^{\text{ss}} + ph^{\text{ds}}]\tau\}. \tag{9}$$

We use this form (with the ss parameters determined above) to fit the ds data in Fig. 3, obtaining the yield $p \approx 0.7$ and the ds parameters listed in Table I. The yield $p \approx 0.7$ is consistent with the evidence from the gels and the maltose binding protein (MBP) measurements mentioned earlier, both of which indicate typical yields of our synthesis in the range $0.5 < p < 0.7$. It is possible that $p < 0.7$ in the present case; then the effect on the ds parameters (Table I) would be even bigger. The final result is that the mechanical tension, exerted between Thr 75 and Arg 171 (Fig. 1), i.e., between helices $\alpha 2$ and $\alpha 6$ (Fig. 4), increases the MM constant for the substrate GMP, K_G, at least 10-fold, while the ratio (K_2/K_A) decreases by less than a factor of 2. A big effect on K_G, a smaller effect on K_2 and/or K_A.

TABLE I. The values of the parameters extracted from the fits in Fig. 3.

K_G^{ss} (μM)	82 ± 2
K_G^{ds} (μM)	788 ± 13
$(K_2/K_A)^{\text{ss}}$	0.26 ± 0.02
$(K_2/K_A)^{\text{ds}}$	0.17 ± 0.01

Discussion.—The static picture is that the applied mechanical stress significantly deforms (''opens'') the GMP binding pocket, without disrupting too much the ATP binding site [disruption of the ATP binding site would increase K_A, while K_2 would decrease or stay the same, so the ratio (K_2/K_A) would decrease]. Future independent measurements of K_A will settle this question, but for the moment we note that this correlates with the known structures of the open (ligand-free) conformation of GK [5 and PDB entry 1EX6] vs the closed conformation (with GMP bound [5 and PDB entry 1GKY], or both substrates bound [9 and PDB entry 1LVG]). Figure 4 shows that the whole domain consisting of helix $\alpha 2$ and strands $\beta 3$, $\beta 4$, $\beta 5$, $\beta 6$ swings to ''closed'' upon GMP binding [5], with less drastic changes for the ATP binding domain. Our mechanical tension, applied between $\alpha 2$ and $\alpha 6$, presumably reverses this conformational change and deforms the GMP binding site. Such structural deformations could be investigated experimentally by fluorescence energy transfer measurements, and computationally by molecular dynamics simulations [9].

The dynamic picture (reviewed in [10]) could be quite different. A dynamical mechanism for allostery has been suggested whereby binding of the regulatory molecule alters the spectrum of long-wavelength elastic excitations of the protein; this translates into an entropic contribution to the free energy of substrate binding [11–13]. Our molecular spring affects both the statics and the dynamics, and, in fact, it could be used to test specific predictions of the Cooper and Dryden dynamical mechanism. Similarly, even small, single-domain proteins exist in a statistical ensemble of conformational substates [14], which can be functionally distinct. In this language, allostery arises because ligand binding alters the energy landscape and thus the statistical weights of the substates [15,16]. The ''molecular spring'' offers a practical implementation for this mechanism, vindicating the view that any protein is—or can be—allosterically controlled [15,17]. Clearly through this method we can ask new questions about the structure-function relation.

It seems likely that, given a large enough mechanical tension, the enzyme could be completely shut off, because eventually the protein will unfold. The tension in the present construct can be estimated from the bending modulus of DNA [3]; we find that the DNA stores an elastic energy of order $W \sim 25kT_{\text{room}}$ and provides a force of order $F \sim 10$ pN. This is an upper bound, as it does not

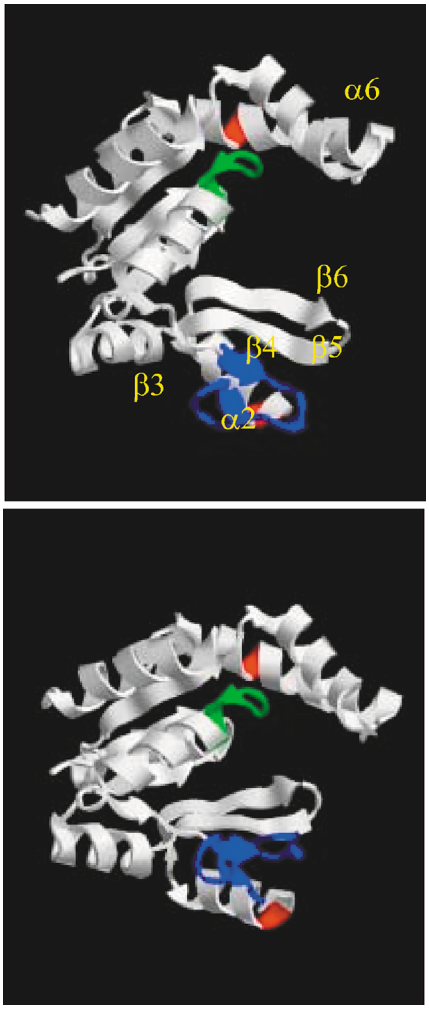

FIG. 4 (color). The conformational change between the open (top: ligand-free) and closed (bottom) conformation of GK, from the PDB structures 1EX6 and 1GKY. While these structures are of the yeast protein, the structure of the TB protein used in this study is essentially identical. The structural location of the Cys mutations, where the mechanical stress is applied, is shown in red. The GMP binding site is colored blue, the ATP binding site green. Inspection of the structures suggests that the applied mechanical stress, between helices $\alpha 2$ and $\alpha 6$, favors the open conformation, and probably deforms the GMP binding site more than the ATP one. This correlates with the measured change in the binding constants for the two substrates upon applying the mechanical stress (see Table I).

take into account force-limiting effects such as, for instance, bubble formation in the DNA.

Our approach differs from previous work on artificial allostery, where metal ion binding sites were engineered into a protein to control a conformational change [18,19]; it is also conceptually different from recent, innovative work where external control is achieved by blocking the active site [20]. Important features of our ''spring-loaded'' molecules are the externally controlled mechanical tension, which can be continuously modulated, and the possibility of applying the tension between any two chosen points on the protein's surface. The present study suggests that allostery can be explained or mimicked in terms of mechanical tension originating from local binding forces. Perhaps allosteric proteins are similar to our chimera, with part of the polypeptide chain playing the role of the DNA, but all integrated in the protein structure. For the enzyme GK, we have measured how a specific mechanical stress affects substrate binding. Further studies for varying ATP concentration and different application points for the stress will reveal new details. Finally, we foresee exciting biotechnology applications for these chimeras, as amplified molecular probes and possibly ''smart'' drugs.

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