Allosteric Control of Guanylate Kinase Using Molecular Springs

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ABSTRACT In protein dynamics, allosteric regulation is a mechanism in which chemical interaction at an allosteric site, which is to say a site other than the catalytically active protein site, affects the conformation and subsequent function of the protein. Allosteric control is a fundamental control mechanism in cells, especially with regards to enzymes. We use mechanical forces applied to allosteric sites on proteins to mimic a conformational change typically attributed to chemical reactions. In this way, we control the function of the protein with mechanical forces instead of chemical forces. We have attached a semi-rigid DNA polymer to a kinase protein that performs a simple catalytic reaction. This DNA, which acts as a molecular spring, is attached on opposite sides of the soft protein and exerts an outward force on the protein that results in an "open" and functionally incapacitated conformation. This paper investigates how a DNA-binding agent, ethidium bromide, affects the rigidity of our DNA spring. Previous molecular spring experiments with our DNA-protein chimera recorded inhibition of the protein function by single-stranded (ss) DNA interaction with the protein active site. This inhibitory effect lessens our ability to investigate the relationship between mechanical stress and protein function. This paper also inspects the effect that histones have on removing this functional inhibition.

Introduction—Since the advent of Koshland's induced fit theory (1), understanding the relationship between protein form and function has been of fundamental importance to research scientists. We use mechanical stresses applied to guanylate kinase (GK) to deform the conformation of the protein, which affects its function. We are able to use mechanical stress to deform the geometry of proteins because their physical properties resemble soft condensed matter (2). By controlling the mechanical forces we exert on the protein, and using this method with a protein that performs a relatively simple catalytic reaction, we are able to better understand the complex relationship between form and function, while at the same time developing a method to "probe" molecules using mechanical forces. In the long run, the benefits of being able to, in essence, turn a protein on or off, has numerous applications and is of incalculable biological importance.

Allosteric regulation is the regulation of a protein by an effector molecule at an allosteric site—that is, a site other than the catalytically active site. We implement a mechanical analog to traditional allosteric control. Previous experiments that used mechanical forces to change protein conformation and function have shown to be successful (3,4,5). Instead of a natural chemical reaction causing the protein's conformational change, we use mechanical stress caused by the tension stored in a rigid polymer, in this case double-stranded (ds) DNA. We are able to use the energy stored in this molecular spring because of GK's geometry. A simplified model of GK structure can be identified as three distinct parts—LID, CORE, and NMP-BD—that move with respect to one another (figure 1); this relative motion characterizes changes in the protein conformation. The three parts form a U-shaped structure with the active site located on the inside of the structure (6). Because GK is a soft macromolecule, it naturally fluctuates between different conformations; the forces we apply simply bias the probability of a particular conformation. We favor the open conformation, attaching the dsDNA to the outside of the protein, on opposite sides of the active site, and the dsDNA acts like a bent spring and exerts an outward force of the GK, which changes the GK's conformation. This "open" conformation is functionally inactive, contrary to the functionally active "closed" conformation.

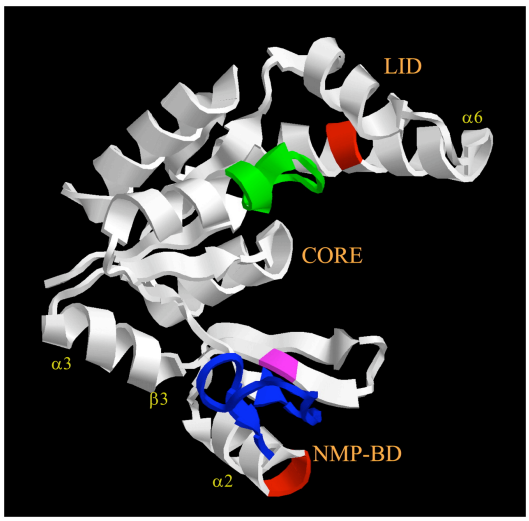


Figure 1: Cartoon of GK structure. The red structures indicate where the outward mechanical forces are applied (Cys mutations). Blue: GMP-binding site. Green: ATP binding site. PDB structure 1S4Q. Image from (3).

Because GK has a relatively simple relationship between form and function, we are able to inspect how the application of different mechanical forces affects the conformation of the GK.

GK catalyzes the reversible phosphoryl transfer from ATP to GMP, producing ADP and GDP. Activity of GK is associated with large conformational changes, about 1 nm. Substrate binding to the GK results in the conformational changes that move as rigid bodies connected by hinges (6). GMP binding induces a large change that brings the NMP-binding region and LID region closer together. Subsequent ATP binding increases the closure on a smaller scale (6). This is a good example of the induced fit mechanism that was mentioned previously.

In previous experiments involving the allosteric mechanical control of GK, it was shown that GMP-binding is not necessary for ATP to bind; however, for the phosphoryl transfer to occur, GMP must certainly be bound (3). In this way, GMP controls the reaction of GK through an induced fit mechanism. Without GMP bound to bring the NMP-binding region and LID region closer together, without GMP changing the conformation of GK, the protein function is nonexistent.

We control GK by counteracting the work performed by GMP chemically instigating an induced fit mechanism. As mentioned previously, GMP binding is coupled with a large conformational change that "closes" the U-shaped GK. Similarly, the binding affinity of GMP is drastically reduced when GK is in the open conformation (3). If we are able to control the geometry of GK, which is a soft protein that is structurally deformed by simple thermal agitation (2), and hold the GK in the open configuration, we will be able to directly control the reaction rate enzymatically controlled by the protein.

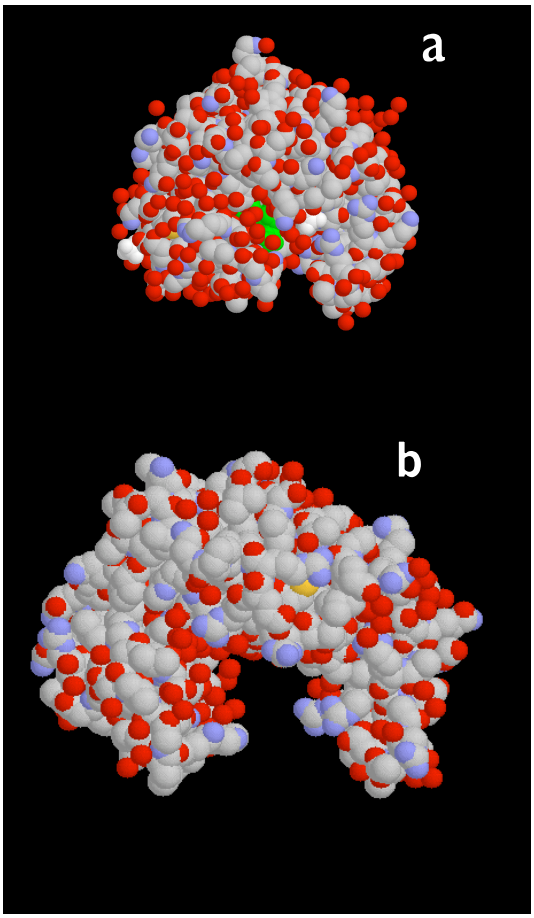


Figure 2: (a) GK in the closed conformation with GMP(green) bound (PDB structure 1EX7). (b) GK in the open conformation (PDB structure 1S4Q)

We use dsDNA to apply mechanical forces to the GK to change its conformation. The dsDNA acts as a molecular spring attached to opposite sides of the GK. Previous experiments with a 60mer dsDNA attached to GK (figure 3) showed two-fold inhibition that resulted from the protein being held in the open configuration by the mechanical force exerted by the DNA. The force exerted by the DNA is calculated by the work required to bend it (7):

$$\frac{W}{s} = \frac{1}{2} B_0 \frac{1}{R^2}$$

where B_0 is the bending modulus of dsDNA, s is the contour length of the dsDNA, and R is the radius of curvature of the bent spring. The preparation of the DNA attachment to GK is detailed in (8).

Our project is an extension of the experiment that showed two-fold inhibition by 60mer dsDNA attached to GK (3).

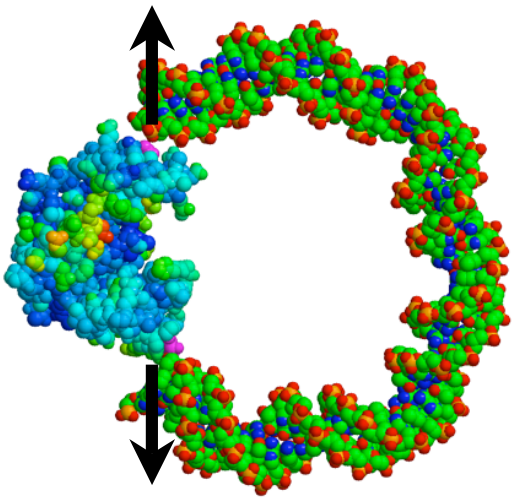


Figure 3: 60mer dsDNA exerts an outward mechanical force on GK, biasing the open, or inactive, conformation.

Project 1

Initially, we looked at the effects that a DNA-intercalator would have on the rigidity of DNA. The motivation of this project was to increase the dynamic range of mechanical forces that we could exert on proteins. We also were experimenting with adding a second layer of control to the initial allosteric spring probe experiment. The first layer of control is using different lengths of dsDNA as a molecular spring, and altering the physical properties of this spring is the second layer of control.

Ethidium Bromide—We looked at how ethidium bromide (EB) affects the rigidity of dsDNA. Ethidium bromide is well known to intercalate between base pairs of dsDNA, The planar intercalating molecule sliding in between base pairs of dsDNA characterizes intercalation. This interaction results via interaction of the aromatic ring systems of the intercalator with the π-bonds of adjacent base pairs (9), hydrogen bonding, and even covalent binding involving side drug chains (10). In general, intercalators are good electron acceptors, and DNA base pairs are good electron donors (10). In order for intercalators to slide into the DNA double helical structure, DNA base pairs separate by approximately 3.4 angstroms (11,12). This separation is coupled with an unwinding of the DNA double helix (11). For EB, the insertion of its phenanthradinium ring results in an unwinding of 26° (11). Obviously, the separation of base pairs and unwinding of DNA's helical structure can affect the physical properties of dsDNA, but the effects on its rigidity or elasticity is not clear.

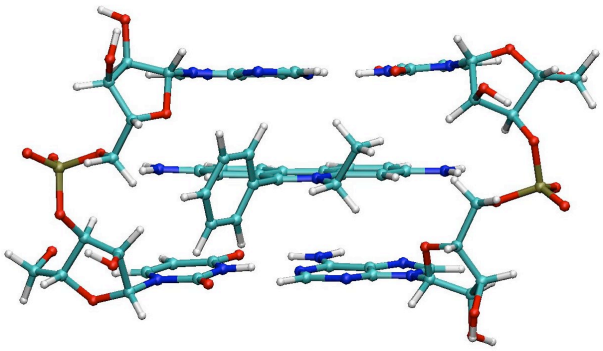


Figure 4: Ethidium Bromide between DNA base pairs.

One study found that dsDNA rigidified at low concentrations of EB, but increased in flexibility at slightly higher concentrations (13). The authors of this study suggested that this unexpected result was due to the higher concentration of EB neutralizing the negative charge of the DNA polymer. Because the initial chimera construction (fig) failed, we used a different construction that exploited the fact that ssDNA inhibits GK function. In an earlier experiment performed by our lab, a 60mer ssDNA was attached to opposite sides of the GK, and then it was hybridized with different lengths of complementary DNA, from 12 to 60 bases (3). With complete hybridization, a two-fold inhibition of GK was recorded. However, by hybridizing cDNA to the ssDNA to form intermittent chains of dsDNA, a construction that does not exert tension on the GK, a two-fold activation was still recorded. This shows that the hybridization removed the inhibition that was a result of ssDNA interacting with the protein active site.

Single-stranded DNA is a flexible polymer and its nucleotide sequence readily binds to the active site of GK. The phosphate group associated with the substrates of GK interact with the active site of GK (6), and DNA polymers are composed of a phosphate and sugar backbone (7).

Method and Materials—The chimera we used to examine the effects of ssDNA inhibition on GK was obtained by covalently attaching a 20mer ssDNA (Operon, Huntsville, AL) to GK. Using site-directed mutagenesis (Thr-75→Cys; Arg-171→Cys), we create a cysteine residue on opposite sites of the GK. The chimera construction with DNA attached to opposite sides of the GK (pic) also utilized this specific mutagenesis. The addition of cysteine establishes chemically active sites for DNA attachment. A 5′-amino modified 20mer ssDNA is covalently coupled to GK through a cross-linker. Our sample included GK with DNA arms attached on either the 75 or 171 cysteine, but not both. We then introduce two different cDNA bridges that hybridize with the ssDNA to form dsDNA and a different strand of ssDNA that is constrained at a further distance from the GK active site. (figure 5).

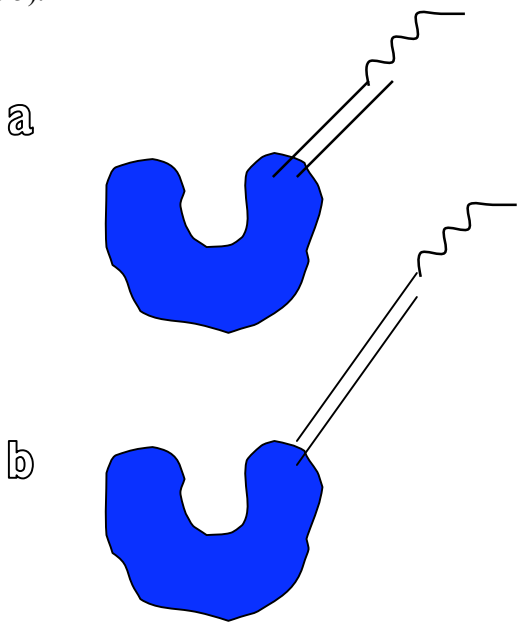


Figure 5: a) Bridge bA attached hybridized to the ssDNA has a 20mer ds and 20mer ss DNA. b) Bridge B has 40mer ds and 20mer ss DNA

Bridge bA is a 40mer ssDNA with 20 base complementarity to the chimera DNA. Bridge B consists of two 40mer ssDNA with 20 base complementarity to each other (figure 5). Physically, the only difference between the bridges is that bridge B keeps its portion of ssDNA further away from the GK active site. The ssDNA chimera is incubated with four-fold excess bridge DNA strands for two hours at ~37°C.

Assay—Our assay uses fluorescence to measure the reaction speed of GK phosphorylation. GK binds GMP and ATP and transfers a phosphate group from ATP to GMP, producing ADP and GDP. However, it is not easy to directly measure the concentration of these products. Instead, we integrate ADP and GDP into a coupled chemical reaction:

ATP + GMP ↔ ADP + GDP

ADP/GDP + 2PEP ↔ ATP/GTP + 2 pyruvate

$$2\ \text{pyruvate} + 2\ \text{NADH} + 2H^+ \leftrightarrow 2\ \text{lactate} + 2\ NAD^+$$

Equal volumes of 2mM ATP, 10mM GMP, 100mM phospho(enol)pyruvate (PEP), 106 units/mL pyruvate kinase, 133 units/mL lactate dehydrogenase, 1.5mM NADH, 1M Tris·HCl buffer (pH 7.5), 1M KCl, and 0.1M $MgCl_2$ are added together (Sigma, St. Louis, MO); this base solution is GKRM. NADH, a product that is consumed in the third step of the reaction, is a fluorescent molecule that has peak absorption at 340 nm and emits wavelengths at 465 nm. We use a Beckman-Coulter DTX 800 Multimode Detector that shines light of wavelength 365 nm into our sample and records the resulting fluorescence of the sample at 465 nm. We record the change in fluorescence over time; the slope of this change is a direct indication of how quickly ATP is hydrolyzed and GMP is phosphorylated by GK. We are able to extract the reaction speed of our GK population using this method because the first step of the coupled chemical reaction, the one dependent on a functionally active GK, is significantly slower than the next two steps of the reaction. There is also an abundance of necessary reactants for the other two steps to occur.

Equal parts of the chimera (GK+ssDNA arm), chimera hybridized with bridge bA, and chimera hybridized with bridge B are combined in wellplates with 40uL of GKRM. The final concentration of the different chimeras is typically between .2 and .5μM. EB was also added to the GKRM plus chimera solutions.

EB Assay Results—As shown in Table 1, the hybridized bridges remove the inhibition caused by the 20mer ssDNA. Bridge B exhibits ~25% activation in comparison with bridge bA. This shows us that bridge bA does not fully remove the inhibition caused by the ssDNA. Because bridge B has twice the length of rigid dsDNA holding the ssDNA strand further from the GK active site (40bp~13nm). A 20mer ssDNA has a contour length of approximately 14 nm (7). Clearly, a 20mer of flexible ssDNA is long enough to interact with the GK active site when held by only 20 bp of dsDNA (~7 nm) away from the GK active site. This accounts for the fact that bridge bA does not completely remove the ssDNA inhibtion. We also added EB to the system to observe how it affected the flexibility of the dsDNA. Once again, this construction is not ideal for testing how EB affects the physical properties of the DNA spring, and our data is inconclusive. Table 1 shows an apparent inhibition factor with EB present, but EB also inhibits GK function when no DNA is present in the sample. Most likely, this apparent inhibition results from an experimental error in our fluorescent reading. EB is a fluorescent molecule and may absorb the 365 nm incident light shone on the sample or affect the amount of fluorescence that the machine records from NADH emission. NADH emits light at 465 nm, and this wavelength lies on EB's absorption spectra (14). EB could absorb this NADH fluorescence and re-emit light at a longer wavelength that the machine does not bias in its fluorescence readings. The dsDNA spring chimera construction would be more sensitive to detecting EB affect on dsDNA rigidity. In this construction, a change in the DNA spring constant would directly change the mechanical stress applied on the GK and alter the GK conformation under basic mechanical dynamics. If DNA became more rigid, the resulting higher tension in the DNA would increase the outward force exerted on the GK, which would bias the open and inactive

Table 1: EB effect on different chimera

	GK	GK + ss arm	GK + bridge B	GK + bridge bA
No EB	794	137	714	560
5uM	654	114	606	442
No EB/5uM	1.21	1.20	1.18	1.27

conformation. The coupled chemical reaction rate would change as a direct result of the GK conformation, with a slower reaction indicating a greater force applied by the dsDNA.

Project 2

We turned our attention to the problem of ssDNA inhibiting GK function by interacting with the catalytically active site on GK. Of course, hybridizing ssDNA with cDNA to form rigid dsDNA, which is not flexible enough to bend into the GK active site, removes the ssDNA inhibition. However, for our allosteric spring probe construction, this idea is not beneficial. As mentioned previously, when we hybridize ssDNA to form dsDNA, the tension of the newly formed spring exerts an outward force that opens the GK and inhibits its function. Simultaneously, though, the formation of dsDNA removes the inhibition that was already present due to the ssDNA attached to the cysteine residues on opposite sides of the GK (figure 6).

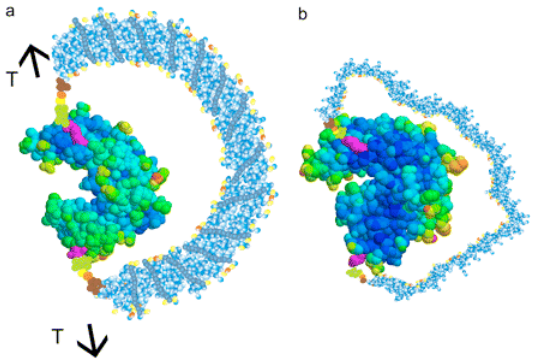


Figure 6: a) The dsDNA spring favors the open conformation of GK, inhibiting its function. During hybridization, the protein reaction rate is increased as the inhibition due to ssDNA (b) is removed. ssDNA is flexible and able to interact with GK;s active site (see figure 1) . Image from (3).

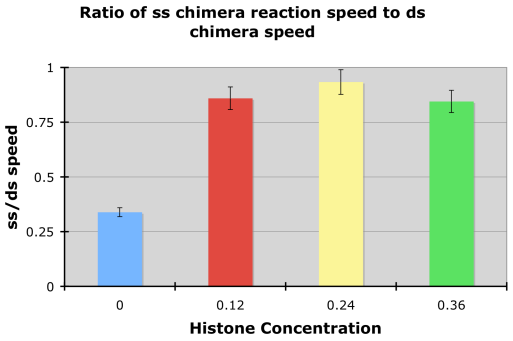
The removal of this ssDNA inhibition has an activation factor, while the tension has an inhibition factor. It appeared that the tension of the DNA spring produced a two-fold net inhibition, but if the removal of the ssDNA inhibition produces two-fold activation, the allosteric spring probe on this chimera actually produces a four-fold inhibition. We need to remove the ssDNA inhibition from our samples to get an accurate description of how mechanical stress on the GK is actually affecting its conformation and subsequent function.

Method and Materials—We experimented with histones to try to remove the ssDNA inhibition. Histones are small proteins whose functional importance in cells is binding to DNA to assist in the higher-order structuring of DNA, such as that necessary in the formation of chromosomes (7). In binding to the ssDNA, perhaps the histones will make ssDNA too bulky to interact with the GK active site, or will shield the interaction between the nucleotides of ssDNA and the catalytic reaction site of GK. Of course, we must also make sure that histones do not prevent the hybridization of dsDNA when cDNA is introduced into the sample. Our goal is to uncouple the activation and inhibition factors that are both present when ssDNA becomes dsDNA. We need to be confident that the force resulting from the formation of the dsDNA spring is the only variable responsible for a change in GK function—that the only difference between the ss chimera and histone sample and the sample containing ds chimera and histones is the new molecular force instantiated by the DNA hybridization.

Three concentrations of histone from calf thymus, type II-S (Sigma)—.12, .24, and .36 μM—were added to samples of ss chimera and ss chimera hybridized with bridge bA.

Results—As shown in graph 2, the histones successfully remove most of the inhibition caused by ssDNA. The first bar in the graph shows that the ss chimera has a much slower reaction speed because of the inhibition by ssDNA interacting with the protein active site. However, when histones are added to the samples containing both ss and ds chimera, the relative reaction rates are well over 80%. With histones present, the reaction rates are similar, which means that the GK are functioning at an equal yield.

This shows that ssDNA is no longer inhibiting GK function because the histones are bound to the ssDNA and preventing the interaction between ssDNA nucleotides and the GK active site which is phosphate specific.



Graph 2: Histone effects on relative reaction speeds of ds and ss chimera samples.

Conclusions—Our chimera construction was not ideal for retrieving data that would give insight into the physical effects that ethidium bromide has on dsDNA. With proper construction, we would be able to see if the intercalation of EB between DNA base pairs altered the stiffness of our DNA spring probe. If a change was realized, we would have identified a compound that is able to add a second layer of control to our allosteric spring probe; the first layer, of course, is hybridizing different lengths of cDNA to ssDNA that is attached to allosteric sites on the protein, creating different lengths of a stiff dsDNA spring. If EB stiffened the spring, we will have access to a greater dynamic range of force that we can apply to proteins to alter their geometry and function—i.e., we will be control in affecting the functional expression of proteins.

The removal of inhibition that we see from the presence of histones is important for accurately interpreting the relationship between mechanical stress and protein function that our allosteric spring probe employs. We must be sure that any protein function changes are due solely to the mechanical forces we exert in the protein. Only then will we be able to dissect the details of how protein function is related to protein conformation and potentially gain an ability to control protein function by exploiting weaknesses in structural hierarchies.

The novelty of our approach is that the allosteric spring probe can be applied to virtually any protein to allows us to study the fundamental mechanics of allostery.

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