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Mimicking cAMP-Dependent Allosteric Control of Protein Kinase A through Mechanical Tension

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Abstract: We report the activation of an enzyme complex by mechanical tension. Protein kinase A, a tetrameric enzyme that, in the cell, is allosterically controlled by cAMP, has been modified by the insertion of a "molecular spring" on the regulatory subunit. The spring is made of DNA, and its stiffness can be varied externally by hybridization to a complementary strand. This allows us to exert a controlled mechanical tension between the two points on the protein's surface where the spring is attached. We show that upon applying the tension, we can activate the enzyme with efficiency comparable to the activation by its natural regulatory molecule, cAMP.

Introduction

An astounding aspect of the living cell is the extraordinary extent of control exercised in its chemical processes. The corresponding regulatory mechanisms invariably rely on the intimate connection between the structure and function of proteins, exploited through allosteric control. First proposed by Monod, Changeux, and Jacob,[1] allostery is the mechanism by which binding of a controlling molecule (activator or inhibitor) at a site A on a protein's surface induces a conformational or dynamic change in the structure that affects a distant active site B, thus turning the activity of the protein on or off. The microscopic picture (reviewed in ref 2) is that binding at the allosteric site produces stresses that are propagated through the globular structure, resulting in a conformational change at a distant site. Alternatively,[3,4] the stress due to binding of the effector could produce a change in flexibility of parts of the protein, changing the dynamics rather than the statics of the structure. This can also cause the chemical affinity for the substrates to change. In either case, our understanding remains qualitative due to the stress in the experimental systems being neither controlled nor measured.

Although many experimental results on allostery can be understood in terms of a thermodynamic model[5] first introduced by Monod, Wyman, and Changeux,[6] and later modified in different ways,[7] here we are concerned with building a microscopic understanding of allostery from "microscopic" experimental measurements. We ask what stresses, and applied where on the protein's surface, result in what kind of modulation in function. We present an experimental approach where the stress is controlled: we control the points of application of the stress, and we can externally vary the magnitude of the stress semicontinuously. We measure the response of the protein in terms of its function (enzymatic activity). We do not yet measure the response in terms of conformation.

Our experimental system is protein kinase A (PKA). PKA is allosterically regulated by cAMP (cyclic adenosine monophosphate); it is, in fact, the primary receptor for cAMP in eukaryotic cells,[8,9] playing a crucial role in signaling pathways and numerous metabolic processes. It is a tetramer composed of two regulatory (RS) and two catalytic (CS) subunits, which form a catalytically inept tetrameric holoenzyme complex. The RS binds to the CS through a surface of contact that includes the catalytic site,[10] which is thus not accessible in this state. Upon cAMP binding (in the presence of Mg^{2+} [11]) the RS undergoes a conformational change, which causes the CS to dissociate from the complex, activating catalysis. The process can thus be summarized by the following chemical equation:

$$R_2C_2 + 4\mathrm{cAMP} \leftrightarrow 2C + R_2(\mathrm{cAMP})_4$$

The free CS catalyzes the phosphoryl transfer from an adenosine triphosphate (ATP) to a Ser/Thr residue on target proteins.

In the present work, we obtain allosteric control of PKA by directly applying a controllable stress between two chosen points on the surface of the regulatory subunit. We choose the application points of the stress on two elements of the protein's secondary structure that are known to move with respect to each other in the cAMP-induced conformational change; our applied

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stress goes in the direction of favoring this motion. We find that the mechanical stress is roughly as effective as cAMP in turning on kinase activity. The mechanical stress is exerted by a "molecular spring" made of a short piece of DNA, which we chemically couple to the regulatory subunit by attaching the ends of the DNA to Cysteine residues introduced at specific locations by site-directed mutagenesis. The stiffness of the DNA spring can be varied externally by hybridization with complementary DNA of varying lengths, providing external control over the mechanical stress. This process is summarized by the chemical equation:

$$R^*_2C_2 + 2\ DNA \leftrightarrow 2C + R^*_2\ (DNA)_2$$

where R* is the chimera regulatory subunit (i.e. the RS with the ss DNA spring); in the following we also consider activation by both cAMP and the molecular spring, i.e.:

$$R^*_2C_2 + 2\ DNA + 4\ cAMP \leftrightarrow 2C + R^*_2\ (cAMP)_4\ (DNA)_2$$

These processes are also graphically summarized in Figure 5.

Experimental Section

PKA Mutagenesis, Expression, and Purification. The plasmids pRSET (regulatory subunit − RIα) and pET15B (catalytic subunit) were gifts of Prof. Susan Taylor (UCSD) and prepared as in refs 12, 28, and references therein. The QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) was used to introduce the S145C/G235C mutations in the RIα. The recombinant proteins were expressed at approximately 8 mg/L/OD_{600nm} (RS) and 8 mg/L/OD_{600nm} (CS) in BL21(DE3) (Stratagene) *Escherichia coli* in enriched, buffered LB medium (10 g NaCl, 40 g tryptone, 20 g yeast extract/L of medium, 5% glycerol, 10mM MOPS, pH 7.0). The mutagenesis was confirmed by sequencing. The his-tagged CS, RS, and the S145C/G235C RS mutant (herein referred to as simply SG) were purified using a Superflow Ni−NTA column (Qiagen). SDS−PAGE of the proteins showed a single, well-defined band (purified protein was ≥90% pure). The protein was expressed at ∼10 mg/L culture/OD_{600nm} for both the SG and CS.

Chimera Construction. A 60-base ss DNA oligomer (Operon), 5′ and 3′ amino modified, was conjugated to the cross-linker NHS−PEO_2−Maleimide (Pierce) by incubating together for 1 h at 150 μM and 15 mM, respectively. Next, a final concentration of 90 mM Tris was added to quench any free NHS−esters of the cross-linker. SG was reduced with DTT (Sigma) and EDTA (Sigma) at 75 μM, 50 mM, and 1 mM for 30−40 min. Protein desalting spin columns (Pierce) were used to remove excess NHS−PEO_2−Maleimide and DTT. The DNA is rendered reactive to the sulfhydryls (Cys) of SG (via the cross-linker maleimide group). The SG and DNA were incubated together (pH ∼6.7) at final concentrations of 60 and 100 μM, respectively, for 3−4 h at 21 °C and then 16 h at 4 °C.

Oligomer Sequences for Chimera. See Supporting Information.

Chimera Purification. The SG + DNA mixture was incubated with Ni−NTA agarose beads (Qiagen); chelation of the hexahistadine of SG to the Ni−NTA allows unattached DNA to be washed away and the SG + DNA complex (chimera) can be eluted using imidazole. To purify the ss chimera from SG entities with free Cys, we used SulfoLink coupling gel (Pierce), which is reactive toward sulfhydryls. The ss chimera was concentrated to a final concentration of ∼20 μM.

PKA Holoenzyme Formation. CS and a 1.4 molar excess of ss chimera (or SG) were incubated together in holoenzyme formation buffer (HFB) (3 mM $MgCl_2$, 75 mM NaCl, 2 mM b-Me, 10 μM ATP) with final concentrations of 0.6 and 0.85 μM, respectively. This mixture of the two subunits in HFB, called the holoenzyme formation solution (HFS), is incubated overnight (∼16 h) at 4 °C.

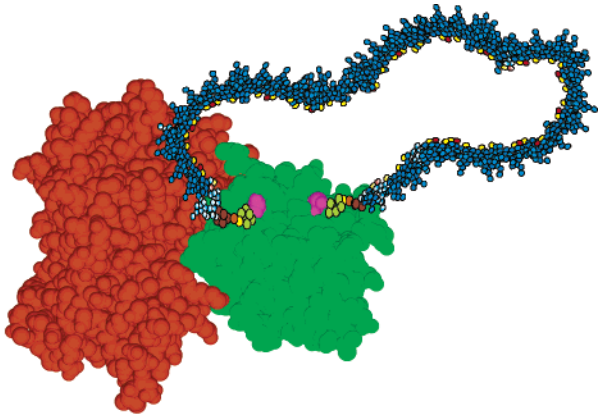

Figure 1. Cartoon of the chimeric holoenzyme (ss chimera + CS, shown in dimer form). The PKA holoenzyme complex is from the PDB structure 1U7E. It consists of the regulatory subunit (green) bound to the catalytic subunit (orange). The DNA molecular spring (blue) is 60 bases long. Native PKA is a tetramer of two regulatory and two catalytic subunits; the protein structure shown is a deletion mutant (D(91−244)) of the regulatory subunit, which does not allow for two regulatory subunits to be linked. However, in the experiments, we use the full regulatory subunit. The locations of the Cys mutations (spring attachment points) on the regulatory subunit are shown in magenta. The distance between these two sites is approximately 2.3 nm. Note that the attachment sites of the ss DNA are distant from the contact surface between the two subunits.

Enzyme Activity Assay. Kinase activity was measured using the Kinase-Glo (Promega) luminescence assay. Catalysis was initiated by adding peptide (kemptide) LRRASLG (BioPeptide) into prepared HFS aliquots (see above) at final concentrations of 35 mM and then adding an allosteric activator (DNA or cAMP). For DNA activation (dissociation) of the PKA holoenzyme, the final concentrations of ss chimera (or SG), kemptide, ATP, and complementary DNA used in the assay were 0.6, 6−350, 2, and 35 μM, respectively. For cAMP (Sigma) activation (dissociation) of the PKA holoenzyme, the final concentrations remained the same, except DNA was replaced by cAMP at a final concentration of 30−40 μM. For simultaneous activation (DNA + cAMP), both DNA and cAMP were present at their aforementioned concentrations. The reaction time allotted for PKA to catalyze phosphoryl transfer was 6−7 min, whereas the subsequent reaction with Kinase-Glo reaction buffer was 12 min. The luminescence measurements were performed on a Lumat LB 9507 luminometer.

The DNA, represented schematically by the blue ribbon, is presumably unstructured, i.e., fluctuating between all conformations compatible with the fixed ends. The figure also implicitly conveys the assumption that the ss DNA does not significantly adsorb on the surface of the protein. In our measurements and controls, we do not find evidence for a significant nonspecific protein−DNA interaction of this sort in this system.

Results and Discussion

The conformational changes associated with the cAMP-dependent allostery of PKA have been studied extensively in the lab of Susan Taylor at UCSD. Here we work with the same recombinant form of PKA as in those studies: the RS (which is a RIα isomer) is from *mus musculus*, and the CS is from *bos taurus*. In fact, for the mutagenesis, we used plasmids kindly provided by Prof. Taylor. The structure of the CS + RS holoenzyme is shown in Figure 1. Comparison of the CS-bound state (PKA holoenzyme) against the dissociated (cAMP-bound) state reveals a significant conformational change in the RS,[12] particularly in the drastic movement of the αA:A (α-helix of

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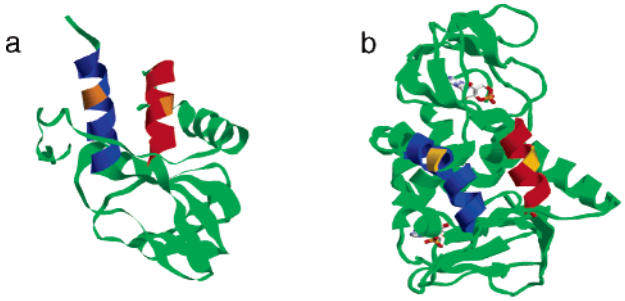

Figure 2. Highlights of the conformational change of the regulatory subunit induced by cAMP binding (which is the natural allosteric control mechanism of PKA). (a) In the PKA holoenzyme (RS + CS, in the absence of cAMP; the structure shown is PDB entry 1U7E with the C subunit removed for clarity), the αA:A and αC:A helices of the R subunit (colored *red* and *blue*, respectively) are aligned in close proximity and nearly parallel. (b) RIα isomer of the regulatory subunit (RS) with cAMP bound (PDB entry 1RGS) shows a large relative displacement of the two alpha helices of interest, αA:A and αC:A, with respect to the structure in (a). Highlighted in *yellow* are the spring attachment sites.

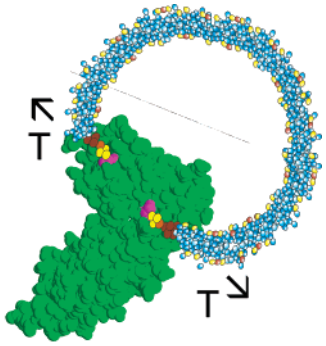

Figure 3. After hybridization with a complementary strand, the DNA "molecular spring" is much stiffer. It has to bend because of the constraint of the fixed end points, so it exerts a force on the attachment points on the protein's surface, directed along the line joining the attachment points and tending to pull them apart (arrows). The regulatory subunit in the cartoon is from PDB entry 1RGS.

domain A of RIα) with respect to αC:A, as shown in Figure 2 and Figure 5. Although this description of the aforementioned motion is based on the crystal structure of a double deletion mutant Δ(91−244),[10] we worked with the full RS, where separate experiments with the full RS using amide hydrogen/deuterium exchange mass spectroscopy also indicate the conformational change in this region.[13,14] We accordingly assumed that the relative displacement of the two α-helices depicted in Figure 2 represents an important part of the cAMP-induced conformational change thatcauses the subunits to dissociate, and this provided the rationale for the choice of our spring-attachment points (colored yellow in Figure 2).

The "molecular spring" is made of a short (60 bases) piece of DNA and exploits the property that the persistence length of ds DNA ($l_{ds} \approx 50$ nm or 150 bp) is much larger than the persistence length of ss DNA ($l_{ss} \approx 1$ nm or 3 bases); therefore, at length scales between l_{ss} and l_{ds}, ds DNA is much stiffer than ss DNA. The idea of using this property to construct a controllable spring has been exploited before in several clever configurations, notably in the molecular beacons,[15] and in a creative experiment where an inhibitor is removed from an enzyme (thus activating catalysis) by literally pulling it off by means of such a spring.[16] In our case, we covalently attach the ends of a ss DNA 60mer to two Cys residues introduced by mutagenesis on the RS (Ser 145 → Cys; Gly 235 → Cys) at the positions marked in yellow in Figure 2. This mutant is referred to in the following as the SG. Coupling of the amino-modified ends of the DNA to the Cysteines was obtained via a heterobifunctional cross-linker. A cartoon of the resulting RIα−DNA holoenzyme chimera is shown in Figures 1 and 5.

Referring to Figure 1, the DNA "molecular spring" in the ss form is very flexible (as its length is about 20 times l_{ss}); it exerts only a small entropic compression on the protein (i.e., a small force tending to push the attachment points toward each other). The magnitude of this small compression force can be estimated from the Flory theory of polymer elasticity,[17] and it is <1 pN in the present case. In the following, we call this construction of SG + ss DNA the "ss chimera".

If a complementary strand is hybridized to the DNA of the ss chimera, the resulting ds 60mer can be thought of roughly as a semiflexible rod (having a contour length of about 1/3 l_{ds}), which (for a fixed protein conformation) has to bend (Figure 3). The ds DNA, therefore, exerts a large average force on the attachment points on the protein, directed along the line joining the attachment points and tending to separate them (i.e., an extension). One can think of the ds DNA as a strung bow, where the "string" connecting the ends of the bow is therefore under tension. This mechanical tension can be estimated from the bending modulus of ds DNA, and it is large: on the order of ∼10 pN.[18] The corresponding elastic energy is also large (∼40 kT_{room}).

In summary, the configuration of Figure 1 allows us to exert a controlled mechanical tension between the yellow residues, up to values that will strongly affect the conformation of the subunit. Upon hybridization of a complementary strand, the mechanical stress tends to favor the displacement of the α-helices shown in Figure 2, which is part of the cAMP-induced conformational change of the SG that results in dissociation of the CS. We have shown previously that within this approach, the mechanical tension can be varied semicontinuously by hybridizing complementary sequences of varying lengths to the DNA of the chimera.[18,19]

In the experiments, we reconstitute the holoenzyme with the ss chimera as the regulatory subunit and measure kinase activity of this "chimeric" holoenzyme (Chi), the holoenzyme + complementary DNA (Chi + DNA), the holoenzyme + cAMP (Chi + cAMP), the holoenzyme + complementary DNA + cAMP (Chi + DNA + cAMP), and various controls. Kinase activity is measured using the Luciferase assay, which monitors the disappearance of ATP.

The SG mutant showed >80% of the inhibitory effect compared to the wild-type RS (see Supporting Information, Figure S1), whereas the ss chimera showed roughly 80% of the inhibitory effect of the SG (Figure 4b). It should be noted

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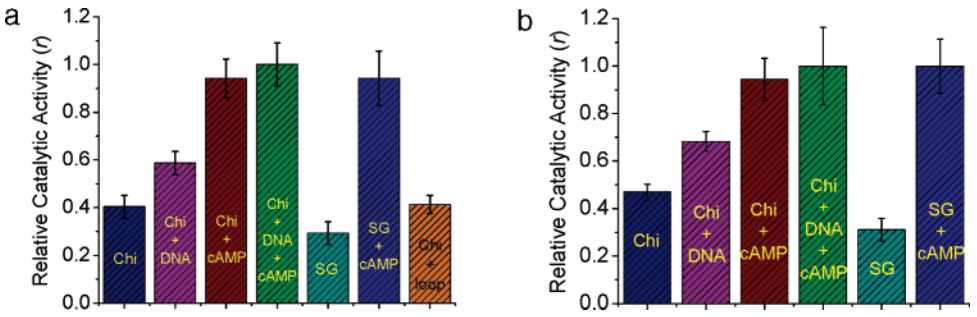

Figure 4. Experimental results for the activation of the PKA chimera by mechanical tension (through hybridization of a complementary DNA strand) compared to activation by cAMP. We plot, for the different samples, a relative measure of kinase activity (r) based on the rate of ATP disappearance, with the activity in the presence of cAMP and DNA normalized to 1. The samples are: Chi: ss chimera; Chi + DNA: ss chimera in the presence of the complementary DNA (forming the ds chimera); Chi + cAMP: ss chimera in the presence of cAMP; Chi + DNA + cAMP: ss chimera in the presence of the complementary DNA and cAMP; Chi + loop: ss chimera in the presence of a partially complementary DNA, which binds to the DNA of the chimera but does not produce mechanical tension (this is a control). The data are the average of 5−6 experiments; the error bars are ±1 standard deviations (SD). (a) By DNA hybridization (mechanical tension), PKA activity increased approximately by a factor 1.5; with cAMP, by a factor 2.3. We believe this difference merely reflects the finite yield of correctly constructed chimeras in the samples. The effects of mechanical tension and cAMP are noncumulative, because r(Chi + DNA + cAMP) = r(Chi + cAMP) within error. The control Chi + loop, showing r(Chi + loop) = r(Chi) within error, supports the picture that the activation effect of the complementary DNA is due to mechanical tension. (b) For this second, independent synthesis batch (which in general will have a somewhat different yield of correct chimeras), kinase activity increased by a factor 1.6 with mechanical tension and by a factor 2 with cAMP. SG refers to the holoenzyme constructed with the Cys mutant but with no DNA coupled. We see that the cAMP activation (the dynamic range) is somewhat larger for the SG (3-fold difference) than for the ss chimera (2-fold difference). In this plot, both r(Chi + cAMP + DNA) *and* r(SG + cAMP) are normalized to 1.

that a different mutant (Ser 110 → Cys and Ser 245 → Cys, herein referred to as SS) had ∼70% the inhibitory ability as the wild-type RS (see Supporting Information, Figure S1), and thus, the SG was used in the experiments (as the working dynamic range was greater). In conclusion, in the experiments, we prepare the chimera using the SG mutant, which is a (>80%) competent regulatory subunit. In Figure 4, we present the results from two different batches (independent synthesis) of the chimera, in the form of "kinase activity" A, quantified by the amount of ATP remaining in the enzymatic reaction mixture (containing the combination specified on the abscissa, ATP, and a synthetic peptide substrate for PKA, see Experimental Section) a fixed time after adding the substrate. In Figure 4a, the total concentration of ss chimera enzyme (holoenzyme + dissociated enzyme) is the same for all samples, by construction. We have normalized the activity A(Chi + DNA + cAMP) to 1, so all other activities are relative to this. The first result is that the chimeric holoenzyme, formed with the ss chimera and CS (referred to in the figures as Chi), is still a competent cAMP controlled allosteric enzyme. Starting from the chimeric holoenzyme (Chi), addition of cAMP increases kinase activity by a factor ∼3. The second result is that adding the complementary DNA, i.e., turning on the mechanical tension, also activates the enzyme: kinase activity increases by a factor ∼2. The statistical significance of these results is as follows. The activities A reported in Figure 4a,b are:

$$\begin{aligned} \mathrm{A(Chi)} &= 0.429 \pm 0.047 \\ \mathrm{A(Chi + DNA)} &= 0.624 \pm 0.049 \end{aligned} \qquad \text{(a)}$$

$$\begin{aligned} \mathrm{A(Chi)} &= 0.469 \pm 0.034 \\ \mathrm{A(Chi + DNA)} &= 0.683 \pm 0.041 \end{aligned} \qquad \text{(b)}$$

where the errors are ± one standard deviation (SD). Thus, for both samples, A(Chi + DNA) is more than 3 SD above A(Chi), i.e., the data show that DNA has an activation effect with more than 99.7% confidence. Similarly, a simple statistical t-test between Chi and Chi + DNA shows a very highly significant difference between the activities with and without DNA. The DNA activation of PKA varies for different synthesized batches of chimera (as shown in the data above), but the effect is always present with better than 99.7% confidence for all batches.

The same data could be presented differently, by subtracting from all columns the "background activity" r(Chi); then we would say that the complementary DNA is about half as effective in activating the enzyme as cAMP. We believe this difference is mostly or even entirely due to the finite yield of correctly constructed chimeras (CCC) in the samples (i.e., chimeras with both ends of the DNA attached at the correct sites), as we argue below. In fact, the best possible yield that we can expect with our protocol, determined by the synthesis and purification processes, is 70% (see Supporting Information). The main reason is that it is difficult to distinguish and separate a chimera with the DNA strand attached by one end versus both ends, as the two species have identical molecular weights. For this reason, we do not yet have a good method to measure the yield directly. We can, however, measure the yield indirectly from the titration curves, as we detail below. Our typical yields thus measured are, for 3 different proteins, consistently in the range 40−70%.[18,19] In the present case, when the yield is taken into account, we find that the complementary DNA is roughly as effective as cAMP in activating the enzyme. Thus, the main result is that an opportunely placed mechanical tension can mimic the natural, cAMP-induced allosteric activation. For comparison, cAMP activation of the SG holoenzyme (SG + CS, referred to as SG in the figures) is shown to increase activity by more than a factor of 3.

Figure 4b shows an independent set of measurements on a different chimera synthesis batch, where we again compare the cAMP-induced activation of the holoenzyme (SG) with the holoenzyme complexes formed with the ss chimera. The SG and SG + cAMP columns are the same data as in Figure 4a, displayed here again for ease of comparison. In Figure 4a,b the total enzyme concentration is the same by construction within all the "Chi" columns, and within the two "SG" columns, but it is not necessarily identical between the "Chi" and the "SG" columns, the samples coming from different purification histories. The total enzyme concentration is approximately the same on the basis of the Bradford assay; however, this leaves

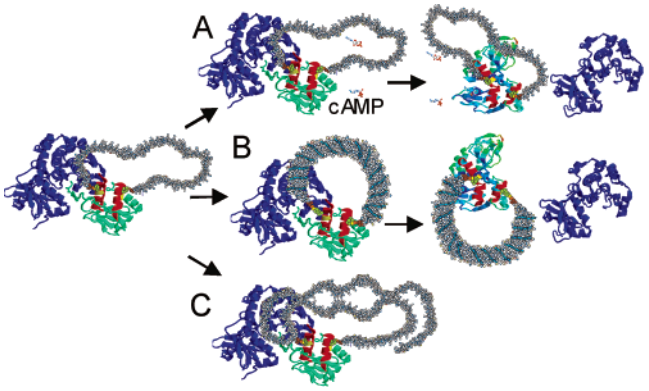

Figure 5. (A) Cartoon showing the activation of the chimeric holoenzyme by cAMP (small molecules in figure), where the CS (blue in the holoenzyme) is dissociated from the ss chimera (green) and activated to perform catalysis. (B) Tension of the ds DNA causes a similar movement of the helices (red) as occurs for cAMP activation. Shown for simplicity is a dimer of the ss chimera + CS, whereas the actual holoenzymes of PKA are tetramers. (C) Chimeric holoenzyme intact even after hybridization of the loop complement (control), which causes essentially no tension on the helices.

room for differences. Therefore, the data are presented with *both* columns (SG + CS + cAMP) and (Chi + cAMP) normalized to 1, and what is to be compared is the dynamic range of allosteric control for the SG case and for the Chi case. In Figure 4b, we see that in the case of the (SG), cAMP activation increases kinase activity by a factor of approximately 3, whereas for the chimera, cAMP activation increases activity by a factor of about 2. These data are consistent (within error bars) with the measurements of Figure 4a, although the latter shows a somewhat larger dynamic range of allosteric control for the ss chimera (close to a factor 3). Finally, the complementary DNA in Figure 4b has an apparent effect that is about half that of cAMP, but as before, we believe this merely reflects a yield of correct chimeras in the samples of order 50%.

Figure 4a shows a control measurement (column "Chi + loop") where a DNA 60mer, which is only partially complementary to the DNA of the chimera, is hybridized to the chimera; this "loop" complement leaves large single-stranded bubbles between double-stranded segments (Figure 5C). The bubbles act as hinges that release the tension, resulting in a configuration that is very close to the ds chimera as far as the possibility of the DNA interacting with the protein's surface is concerned, but with no (or much reduced) mechanical tension. The purpose is to demonstrate that the mere presence of a second DNA oligomer in close proximity to the ss chimera does not by itself cause activation of PKA, supporting our view that it is the mechanical tension that is responsible for the observed activation of the chimera upon hybridization with the true complementary. Several other "Chi + loop" controls were performed on independent batches of chimeras (data not shown), always obtaining zero effect within the errors. A schematic representation of the activation of the chimeric holoenzymes by cAMP, DNA, and the control experiment with the "loop" complement is shown in Figure 5.

In our previous work,[19] we have obtained (indirect) measurements of the yield of correct chimeras obtained from our coupling and purifying procedure (using the same chemistry but a different protein); this yield typically lies somewhere between 0.4 and 0.7. To take into account a finite yield in the present case, we use the following simple model.

The rate of consumption of ATP is given by:

$$\frac{\mathrm{d}}{\mathrm{d}t}[\mathrm{ATP}] = -\,r[\mathrm{ATP}] \tag{1}$$

where r is the catalytic activity. Thus:

$$[\mathrm{ATP}](t) = [\mathrm{ATP}](0)\,\mathrm{e}^{-rt} \tag{2}$$

where $[\mathrm{ATP}](t)$ is the concentration of ATP at time t.

In the experiments, we measure the ratio between the initial and final concentration of ATP after a fixed time τ; i.e., we obtain the catalytic activity from:

$$r = \frac{1}{\tau}\ln\left\{\frac{[\mathrm{ATP}](0)}{[\mathrm{ATP}](\tau)}\right\} \tag{3}$$

The best resolution in the measurements is obtained by choosing $\tau \approx 1/r$. In the following, we call A(ss), A(ds), A(ss, cAMP) etc. the enzymatic activities of the ss chimera, ds chimera, ss chimera + cAMP, etc. We describe the increase in activity due to allosteric activation caused by either the complementary DNA or the cAMP by introducing parameters γ_{DNA}, γ_{cAMP} as follows:

$$A(\mathrm{ds}) = \gamma_{\mathrm{DNA}}\,A(\mathrm{ss})$$
$$A(\mathrm{ss,cAMP}) = \gamma_{\mathrm{cAMP}}\,A(\mathrm{ss}) \tag{4}$$

This Ansatz is correct if all measurements are done at a fixed total (= undissociated + dissociated) concentration of PKA. In general, the more fundamental description would be in terms of the dissociation constant for the holoenzyme, which is different in the presence or absence of the allosteric activators. The factors γ above are functions of the two dissociation constants (with and without activator) and the total concentration of PKA. The functional form can be determined easily, and in principle, the values of the two dissociation constants can be determined by using this functional form to fit titration curves of the activities vs the total concentration of PKA. We postpone this considerable work until a future study.

For the moment, we consider instead that in the experimental samples there is a finite fraction ("yield") p ($0 < p < 1$) of "correct" chimeras, i.e., chimeras with the DNA correctly attached at both ends. In fact, we know from the synthesis and purification procedure (see Supporting Information) that $p \leq p_{\max} = 0.7$. For the *measured* kinase activity r (operationally defined above), we can then write:

$$r(\mathrm{ds}) = p\,\gamma_{\mathrm{DNA}}\,A(\mathrm{ss}) + (1-p)\,A(\mathrm{ss})$$
$$r(\mathrm{ss,cAMP}) = \gamma_{\mathrm{cAMP}}\,A(\mathrm{ss})$$
$$r(\mathrm{ds,cAMP}) = p\,\max\{\gamma_{\mathrm{DNA}},\gamma_{\mathrm{cAMP}}\}\,A(\mathrm{ss}) + (1-p)\,\gamma_{\mathrm{cAMP}}\,A(\mathrm{ss}) \tag{5}$$

The term max $\{\gamma_{DNA},\gamma_{cAMP}\}$ means we take the parameter with the higher value. These equations express the fact that cAMP affects *all* the holoenzymes in the sample, whereas the complementary DNA affects *only the fraction p* of holoenzymes that have the DNA correctly "installed". Further, $A(\mathrm{ss}) \equiv r(\mathrm{ss})$, and there is an assumption in the third equation that the effect of the two different activators is "not cumulative". A sufficient

condition for this is, for instance, that at least one of the activators leads to nearly 100% dissociation of the holoenzyme. This assumption is consistent with the experimental data on the DNA activator (Figure 4), which show that r(ds, cAMP) = r(ss, cAMP). The corresponding data on the cumulative effect of cAMP show, however, r(ds, cAMP) > r(ds) because of the yield $p < 1$.

Now there are two cases: $\gamma_{DNA} > \gamma_{cAMP}$ or $\gamma_{DNA} < \gamma_{cAMP}$. If we assume $\gamma_{DNA} \geq \gamma_{cAMP}$, we can extract all three parameters (p, γ_{DNA}, γ_{cAMP}) from the data. If we assume instead $\gamma_{DNA} < \gamma_{cAMP}$, we can obtain only lower and upper bounds for γ_{DNA} and p from the data. We assume first $\gamma_{DNA} \geq \gamma_{cAMP}$. Solving the system of linear eqs 5 for p, γ_{DNA}, γ_{cAMP} in the case max $\{\gamma_{DNA}, \gamma_{cAMP}\} = \gamma_{DNA}$, we obtain:

$$1 - p = \frac{r(\text{ds,cAMP}) - r(\text{ds})}{r(\text{ss,cAMP}) - r(\text{ss})}$$

$$\gamma_{cAMP} = \frac{r(\text{ss,cAMP})}{r(\text{ss})}$$

$$\gamma_{DNA} = \frac{1}{p}\frac{r(\text{ds})}{r(\text{ss})} - \frac{1-p}{p} \qquad (6)$$

Using the data displayed in Figure 4a, we obtain:

$$p = 0.35 \pm 0.06$$

$$\gamma_{DNA} = 2.7 \pm 0.5$$

$$\gamma_{cAMP} = 2.3 \pm 0.3 \qquad (7)$$

i.e., $\gamma_{DNA} = \gamma_{cAMP}$ within experimental error, consistent with the initial assumption $\gamma_{DNA} \geq \gamma_{cAMP}$. The computed yield is consistent with our estimates from previous work.[19]

If we assume instead max $\{\gamma_{DNA}, \gamma_{cAMP}\} = \gamma_{cAMP}$ in eq 5, then the equations are consistent if $r(ds, cAMP) = r(ss, cAMP)$, which is the case in the experiments, and either of the last two equations determines γ_{cAMP}, whereas the first eq gives a relation between p and γ_{DNA}:

$$\gamma_{cAMP} = \frac{r(\text{ss,cAMP})}{r(\text{ss})}$$

$$p\,(\gamma_{DNA} - 1) = \frac{r(\text{ds})}{r(\text{ss})} - 1 \qquad (8)$$

Because we know that $0.35 < p < p_{max} = 0.7$ (the lower limit coming from the treatment of the opposite case $\gamma_{DNA} > \gamma_{cAMP}$, the upper limit from the preparation methods), we can use the data and eq 8 to obtain the limits:

$$\gamma_{cAMP} = 2.3$$

$$1.6 < \gamma_{DNA} < 2.3 \qquad (9)$$

In summary, the data show that the mechanical tension is, if not just as efficient, then almost as efficient as cAMP in activating PKA.

Conclusion

We have achieved external control of protein conformation by applying a controlled mechanical stress on the structure. In the case of PKA, we show that mechanical tension can mimic the activation effect of the natural allosteric activator, cAMP.

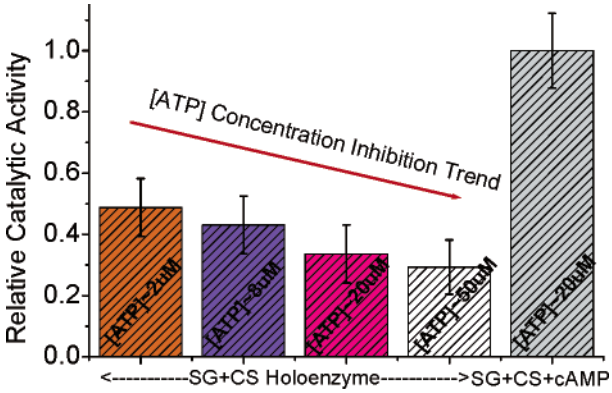

Figure 6. The efficiency of inhibition of the SG of PKA here is shown to be dependent on the concentration of ATP present. The activity of PKA is measured at 2 μM ATP, which allows for the best sensitivity within the assay, whereas the largest inhibition (dynamic range) is shown for 20 μM ATP.

Namely, the mechanical tension influences the conformation of the regulatory subunit, which in turn controls the dissociation of the catalytic subunit from the complex and, thus, the activity of the protein. In our artificial allosteric mechanism, the mechanical tension is derived from a "molecular spring" made of DNA.

The raw data show an increase in the activity of PKA induced by stiffening the molecular spring of more than 50%, but this is limited by the yield of the synthesis and purification of the chimeras. We believe that a sample of 100% correct chimeras would show an efficiency of activation upon stiffening the spring similar to that induced by cAMP. Indeed, it is intuitive that a large enough stress will eventually be at least as effective as any activator binding in altering the structure of the RS enough that the CS will dissociate—eventually, a large enough mechanical tension will even unfold the RS.

The choice of parameters (i.e., concentrations) in the assay reflects a compromise: relatively low ATP concentration is desirable to maximize the sensitivity of the assay, but relatively high ATP concentration promotes association of the holoenzyme and thus a larger dynamic range in the measurements. Figure 6 suggests a trend of inhibition as the concentration of ATP is increased. To quantitate the statistical significance of this trend, we used an analysis of variance (ANOVA) to compare the four measurements in the figure—the 2, 8, 20, and 50 μM ATP concentration activities. What is calculated is an F-value, which is the ratio of the variance between measurements to the overall variance, where finally the calculated F-value is compared to an F-value tabulated at a given confidence level. In our case, we calculated F(calc) = 4.52, where for our system of four measurements, repeated six times each, we have an F(tab) = 4.5 for a confidence level of 95%. In other words, the ATP inhibition trend is statistically significant with a confidence level of 95%. Therefore, the possibility of utilizing a future assay in which we can both maximize sensitivity in the assay and incur a large dynamic range in measurements would better manifest the effects of this artificial allosteric activation of PKA. This plot simply suggests that higher concentrations of ATP lead to more efficient holoenzyme formation, justifying further the assay conditions for holoenzyme formation in previous experiments.[14,20]

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The artificial (DNA-based) allosteric module does not interfere dramatically with the natural (cAMP-dependent) allosteric mechanism, but some interference is present. Indeed, it would be surprising if the presence of a 20 kD piece of DNA attached to the SG (in the ss chimera) did not interfere at all with the association of the holoenzyme. Figure 4b shows that, assuming the DNA on the chimera does not interfere with cAMP binding (which we confirmed by cAMP titration experiments, see following paragraph), and if we accordingly normalize both r(SGCS + cAMP) and r(Chi + cAMP) to 1, then SG is more competent than the ss chimera in associating into the holoenzyme complex (because r(SG) < r(Chi)), but not dramatically so.

Another question is whether the ss chimera and the ds chimera, which is under tension, have the same binding affinity for cAMP, and whether this is the same as the binding affinity of the SG**.** We have performed cAMP titration measurements of the ss and ds chimera and of the SG, obtained by exploiting the Tryptophan fluorescence quenching associated with cAMP binding (Supporting Information). The result is that the binding affinity for cAMP is the same in the three cases, within error, and consistent with previous measurements by the Taylor group.[20]

In a previous study, we showed[19] how through mechanical tension one can turn off the activity of an enzyme; here we achieve, on the contrary, activation of the enzyme PKA. Demonstrating allosteric control of PKA using our "mechanical" approach represents a significant step with respect to our previous work on Guanylate Kinase (GK), also because the multimeric PKA enzyme is considerably more complex than the monomeric GK. Taken together, our results argue that the concept of controlling protein conformation by mechanical stress is general, applicable to virtually any protein or protein complex. One could speculate what interesting observations this approach might lead to when applied to complex molecular machinery such as the ribosome. Even molecular motors could be studied from a new angle, by imposing stresses within the motor, instead of between the motor and the tracks.[21]

Our approach differs from previous work on artificial allostery, where metal ion binding sites were engineered into a protein to control a conformational change;[22,23] it is also conceptually different from recent, innovative work where inhibition is achieved by blocking the active site,[24] and activation is achieved by mechanically removing an inhibitor.[16] The strength of our approach is that it allows one to investigate the very mechanism of allostery, i.e., how a mechanical stress applied at specific sites is transported through the complex structure of the protein to affect the static or dynamic conformation at distant sites. It is a general approach that is not restricted to blocking the active site or removing an inhibitor from the active site.

In the language of statistical mechanics, proteins exist in a statistical ensemble of conformational substates,[25] which can be functionally distinct. Allostery arises because ligand binding alters the energy landscape and, thus, the statistical weights of the substates.[26,27] The "molecular spring" approach offers a practical implementation for this mechanism.

In more mechanistic terms, the conformational change of the RS of PKA underlying the natural allostery mechanisms is complex: it involves many more rearrangements than the displacement of the blue α-helix in Figure 2. With this experiment, we pose the question: if we try to mechanically force just this part of the conformational change, by pulling on the attachment points shown in Figure 2, what is the effect? The answer is, the effect is the same as that induced by cAMP: dissociation of the catalytic subunit from the complex (with similar efficiency).

This is one of a series of experiments by which we aim to build a microscopic understanding of allostery, by probing which application points for the stress result in allosteric behavior similar to the natural one (elicited by cAMP in the case of PKA) and which do not. We believe that our approach, where the mechanical stress is controlled, will prove a more incisive research tool for understanding allostery than mutagenesis studies alone.

One important limitation at present is that we measure the effect of the stress on the protein's function, but not on the structure. Although what ultimately matters is controlling the function, knowledge of the structural changes is necessary to build a microscopic understanding. For instance, one can pose the question of which parts of the complex conformational change of the RS are sufficient and/or necessary to elicit the functional response (dissociation of the CS in the present case). So far, we have shown that the displacement of the helices (Figure 2) is sufficient to elicit the response. In addition to the movements of the helices, there is also a long "arm" in the RS that is floppy (unstructured) in the dissociated form but becomes ordered in the holoenzyme and participates in PKA holoenzyme formation.[10] The mutant SS (S110C/S245C), which appears in Supporting Information, Figure S1, was in fact engineered specifically to disrupt this "arm", where one of the mutations (S110C) is within the arm. Indeed, already the Cys mutation on this arm results in less inhibition by the RS (Supporting Information, Figure S1). However, we have not yet performed the corresponding experiments with the DNA spring.

Thus, introducing structural probes is an important next step and not necessarily an easy one. There are different possibilities. Major structural changes could be detected in a relatively simple way by CD spectroscopy. Local information on structural changes can, in principle, be obtained by fluorescence energy transfer (FRET) methods, both from ensemble and single-molecule measurements. The main difficulty here is that the corresponding experimental system needs four specific labels on the same protein: two attachments for the spring and two attachments for the FRET labels; to be specific, the attachment chemistry must be different for the two sets. In the end, the most practical way of obtaining structural information could be through NMR; also, crystallizing at least some chimeras,

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and obtaining the structure from X-ray scattering, though probably difficult, cannot be ruled out.

On the plus side, one important merit of our approach is its generality: the principle is manifestly general, and we have so far demonstrated it experimentally on three different proteins. We believe that we can make an allosteric control module for virtually any protein. Although it is not the subject of this paper, we mention in passing that as a consequence of this generality, we believe this approach will also lead to exciting biotechnology applications, specifically the construction of new amplified molecular probes, and actively controlled ("smart") drugs.

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Supporting Information Available: Additional calculations on the maximal chimera yield as well as the results of the cAMP titration fluorescence experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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