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Protein–DNA chimeras: synthesis of two-arm chimeras and non-mechanical effects of the DNA spring

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Abstract

DNA molecular springs have recently been used to control the activity of enzymes and ribozymes. In this approach, the mechanical stress exerted by the molecular spring alters the enzyme's conformation and thus the enzymatic activity. Here we describe a method alternative to our previous one to attach DNA molecular springs to proteins, where two separate DNA 'arms' are coupled to the protein and subsequently ligated. We report certain non-mechanical effects associated with the DNA spring observed in some chimeras with specific DNA sequences and the nucleotide binding enzyme guanylate kinase. If a ssDNA 'arm' is attached to the protein by one end only, we find that in some cases (depending on the DNA sequence and attachment point on the protein's surface) the unhybridized DNA arm inhibits the enzyme, while hybridization of the DNA arm leads to an apparent activation of the enzyme. One interpretation is that, in these cases, hybridization of the DNA arm removes it from the vicinity of the active site of the enzyme. We show how mechanical and non-mechanical effects of the DNA spring can be distinguished. This is important if one wants to use the protein–DNA chimeras to quantitatively study the response of the enzyme to mechanical perturbations.

1. Introduction

Nucleic acid–protein complexes occur naturally in important biological processes and structures, such as protein-primed replication of viral DNA [1], gene regulation [2, 3], and translation at the ribosome, where they play essential roles. On the other hand, artificial protein–DNA conjugates, or protein–DNA chimeras, could serve as powerful tools for the study of conformational transitions and for the development of new bio-techniques and smart drugs. Protein–DNA chimeras were recently constructed where the DNA functions as a controllable molecular spring which affects the conformational state of the protein. The elastic energy of the spring can be used to control binding of an inhibitor to the enzyme's active site [4], or to directly control the conformation of a protein [5–8] as well as a ribozyme [9–11].

DNA is an interesting choice as a molecular spring because of its elastic properties and because it can be addressed by a nucleotide sequence, i.e. a chemical code. Single stranded DNA (ssDNA) is a flexible polymer, with a persistence length of ∼1 nm or ∼3 bases, while double stranded DNA (dsDNA) has a persistence length of ∼50 nm or ∼150 base pair (bp) [12], and is thus 50 times more rigid. This property can be exploited in various configurations; for example, if the ends of a single DNA strand (of length intermediate between the persistence length of ss and dsDNA) are held close together, upon hybridization with the complementary strand this 'spring' tends to pull the endpoints apart, as it costs elastic energy to bend the dsDNA. This effect is used in the molecular beacons [13], and in the molecular spring constructions mentioned above. Another possible configuration is to use the strand hybridization energy to pull two distant points together [10]. In all these applications, the molecular spring is reversibly addressable by a specific nucleotide sequence and competitor sequence.

In order to attach a DNA molecular spring to a protein, different schemes are possible; two are shown in figure 1(a). In scheme A, both ends of a 60mer (for example) DNA strand

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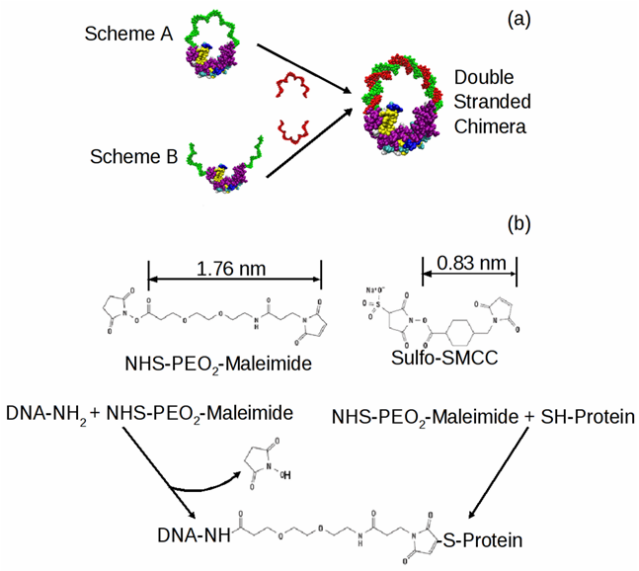

Figure 1. (a) Schemes for attaching a DNA molecular spring to a protein. In scheme A, the two ends of a DNA 60mer strand are attached to Cys residues on the surface of the protein. Upon hybridization with the complementary DNA strand ('double stranded chimera'), the double stranded molecular spring exerts a mechanical stress on the protein which tends to move the attachment points apart. In scheme B, two distinct DNA 30mer strands are attached by one end to the Cys residues. One strand is attached by the 5′ end and the other by the 3′ end; upon hybridization with the complementary 60mer we obtain again the double stranded chimera, except that now there is a nick in the molecular spring. This relaxes part (but not all) of the tension, however the nick can subsequently be ligated. The advantage of this scheme is that this synthesis uses DNA modified at one end only. (b) Cross-linkers used in the synthesis of the chimeras and the coupling chemistry. We have made constructions with either of two different cross-linkers, NHS-PEO_2-Maleimide (Pierce) and Sulfo-SMCC (Pierce). The spacer groups of these two cross-linkers are different in both structure and length, but the coupling chemistry is the same: one end reacts with a primary NH_2 group at the end of the modified DNA, the other end reacts with a free SH group on the surface of the protein [17–19].

(This figure is in colour only in the electronic version)

are attached to two sites on the protein's surface (we call this construction the 'doughnut chimera'). With complementary DNA present and under hybridization conditions, the DNA spring becomes double stranded and exerts a mechanical stress on the protein, pulling the attachment points apart. The tension can be modulated by adding complementary DNA of different lengths. In addition, the complementary DNA can be removed by a competitor strand; therefore, application of the stress on the protein is reversible. However, one problem with this scheme is that in the synthesis other species are constructed beside the doughnut chimera, in particular 'tadpole' chimeras (only one end of the DNA attached to the protein). These are difficult to remove, so that in the end the yield of doughnut chimeras is low. The reasons why there are tadpole chimeras in the final samples are that: (1) not all the DNA has reactive groups at both ends; (2) protein polymerization by disulfide bond formation is a competitive process during conjugation.

An alternative strategy is scheme B, where two different DNA 30mers are attached to the protein, one by the 5′ end and the other by the 3′ end. We call the resultant construct the 'croissant chimera'. After hybridization with the complementary DNA, we obtain a molecular spring with a nick. However, the nick can subsequently be ligated. The advantage of this construction is that it uses DNA functionalized at one end only. Moreover, the yield of end-functionalized DNA is not critical: 'defective' DNA (not properly end-functionalized because either the cross-linker is missing or its maleimide group has hydrolysed, for example) does not attach to the protein at all. By contrast, with scheme A the yield of DNA functionalized at both ends (already a stronger requirement) is critical, because DNA functionalized at one end only can still attach to the protein by that one end, leading to defective chimeras. On the other hand, scheme B has its own complications, such as elasticity-driven polymerization [14] at high enough concentrations, if the spring is not ligated.

In the course of these experiments, we were led to purposefully synthesize chimeras where the DNA strand is attached to the protein by one end only ('tadpole' chimeras). For some of these chimeras, we observe non-mechanical effects stemming from protein–DNA interactions. With the nucleotide binding enzyme guanylate kinase (GK) as the protein, we find that certain DNA sequences can inhibit the enzyme, possibly by partially blocking the active site, which leads to an observed activation effect upon hybridization of the DNA. We report measurements of this 'non-mechanical' effect, and show that it can be distinguished from the mechanical effect of the DNA spring through suitable control experiments. This is important if one wants to study the mechanical response of the protein using a DNA spring.

The method of controlling an enzyme's activity through a DNA spring is in principle general (i.e. applicable to any protein), as it is independent of the specific chemistry at the active site. The elastic energy of the DNA spring can be estimated or measured [14], and it is of order $10kT$ (where T is room temperature). This is also the order of the free energy stabilizing the folded conformation of a protein domain, so we expect that, in general, one can significantly perturb a protein's conformation with the DNA spring. On the other hand, the whole approach may be unsuitable for certain classes of proteins, such as DNA binding proteins or proteins with a very basic isoelectric point: in both cases one expects that protein–DNA interactions will substantially interfere with the method.

The optimized conditions to attach the DNA spring to a protein, as well as the 'non-mechanical' protein–DNA interactions, will of course be different for different proteins. In this paper we focus on those aspects of both the construction and the measurements which are likely to be generally relevant, for example the competitive reaction of protein dimerization by disulfide bond formation if one uses Cys as the specific attachment points, and how to subtract 'non-mechanical' effects from the measurements.

Table 1. DNA sequences used in the experiments. [AminoC6] is the amino terminal modification for labelling of the 5′ end while [AmC7A1-Q] is the amino-modification for the 3′ end. [Phos] is the phosphate modification. Complementary DNA (cDNA) and competitor strand (pDNA) are not modified.

DNA ID	DNA sequence
N5L15	[AminoC6]GCCCGCAGTAGACCA
N5L20	[AminoC6]GCCCGCAGTAGACCACAGAC
N5L20G	[AminoC6]TTTTTTTTTTTTTTTTTGGG
N5L20T	[AminoC6]TTTTTTTTTTTTTTTTTTTT
N5L20GA	[AminoC6]CTTCCTTCCTCCGAGCCAGA
N5L60N3	[AminoC6]GACCCTTACCACTGTCTAATCGTAGCAGCGTCGGCTTTATTTCCATACTCTTTGCCCAGG[AmC7A1-Q]
N5L60N3-B	[AminoC6]GGCTCCCGATGCGGTCAGACCTGCTCTGCACTCCCCAGTACGTGCGGGCTGTCACTCGGT[AmC7A1-Q]
N5L30	[AminoC6]GAGTGTGGAGCCTAGACCGTGAGTTGCTGG
P5L30N3	[Phos]CAGTGGTGCGACCGACGTGGAGCCTCCCTC[AmC7A1-Q]
cDNA15f1	CCTGGGCAAAGAGTA
cDNA15f2	AAAGCCGACGCTGCT
cDNA15f3	ACAGTGGTAAGGGTC
cDNA30	TGGAAATAAAGCCGACGCTGCTACGATTAG
cDNA60	CCTGGGCAAAGAGTATGGAAATAAAGCCGACGCTGCTACGATTAGACAGTGGTAAGGGTC
pDNA	GACCCTTACCACTGTCTAATCGTAGCAGCGTCGGCTTTATTTCCATACTCTTTGCCCAGG

2. Materials and methods

2.1. DNA, protein, and cross-linker

DNA oligonucleotides were ordered from Operon, with amino-modifiers on one or both ends, depending on the purpose of conjugation. The DNA sequences were generated randomly with an assigned GC content and then refined by hand in order to remove unwanted secondary structures, using MFOLD [15]. The DNA sequences used in the experiments are listed in table 1.

Our model protein is guanylate kinase (GK), an essential enzyme that catalyses the transfer of a phosphate from adenosine triphosphate (ATP) to guanosine monophosphate (GMP) [16]. The GK gene (Rv1389c) was cloned into plasmid pET22-*b*(+) (EMD Chemicals Inc., San Diego, CA), which adds a hexa-histidine tag (His-Tag) to the expressed protein. The expressed His-Tag proteins can be purified conveniently by a Ni-NTA column. GK was mutated using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene) to remove the two internal cysteines (replaced with serines) and add cysteine(s) at specific surface location(s) to form attachment point(s) for the DNA. The GK mutants utilized in the experiments and their descriptions are listed in table 2.

The end-modified DNA was covalently coupled to the Cys residues on the mutated protein by cross-linkers. We made constructs with two different cross-linkers, NHS-PEO_2-Maleimide (Pierce) and Sulfo-SMCC (Pierce), shown in figure 1(b). Both cross-linkers can react with primary NH_2 groups and free SH groups [17–19]. However, the spacer groups of these two cross-linkers are different in both structure and length. The spacer of NHS-PEO_2-Maleimide is twice as long as that of Sulfo-SMCC. NHS-PEO_2-Maleimide is more convenient to use in the assays due to its higher solubility in both water and DMSO.

2.2. Other chemicals

Phosphate buffered saline (PBS) was purchased from Pierce in pack-form. Ethylenediaminetetraacetic acid (EDTA) was ordered from EMD. Tris(2-Carboxyethyl)phosphine (TCEP) was obtained from Pierce. Dimethyl sulfoxide (DMSO), sodium chloride (NaCl), sodium hydroxide (NaOH), tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), tris(hydroxymethyl)aminomethane (Tris), β-mercaptoethanol (BME), potassium chloride (KCl), magnesium chloride ($MgCl_2$), adenosine 5′-triphosphate disodium salt (ATP), guanosine 5′-monophosphate disodium salt hydrate (GMP), pyruvate kinase/lactic dehydrogenase enzymes (PK/LDH), phospho(enol)pyruvic acid trisodium salt hydrate (PEP), β-nicotinamide adenine dinucleotide (NADH), L-glutathione (GSH) and iodoacetamide (IAN) were purchased from Sigma-Aldrich and used without further purification.

Table 2. GK mutants utilized in the experiments.

GK mutant name	Description
GK2075171	Internal cysteines of guanylate kinase are kept, and residues 075 and 171 are replaced by cysteines
icrGK	The two internal cysteine of guanylate kinase are removed (substituted by serine)
icrGK1075	The two internal cysteine of guanylate kinase are removed (substituted by serine), and the residue 075 is replaced by cysteine
icrGK1171	Internal cysteines of guanylate kinase are removed, and site 171 is replaced by cysteine
icrGK2075171	Internal cysteines of guanylate kinase are removed, and residues 075 and 171 are replaced by cysteines
icrGK2112144	Internal cysteines of guanylate kinase are removed, and residues 112 and 144 are replaced by cysteines

2.3. GK-NADH activity assay

Activities of GK mutants and GK chimeras were measured by a coupled enzymes assay (‘GK-NADH activity assay’) introduced by Agarwal *et al* [20]. The coupled enzymatic reactions involve pyruvate kinase and lactate dehydrogenase, and what is finally measured is the conversion of NADH

to NAD^+, which we monitor through the fluorescence at 465 nm [21].

The chimeras synthesized and purified with the protocol described in the sections 2.7 and 2.8 were typically divided into aliquots to form a reference sample (Chimera only), a sample of Chimera + complementary DNA (Chimera + cDNA), obtained by mixing the chimera with 1–5 fold excess complementary DNA, and incubating at room temperature for 1–2 h before the measurements, and a strand-displaced sample, where Chimera + cDNA is mixed with 50 fold excess competitor DNA (pDNA) and incubated at room temperature for 30 min, to remove the cDNA from the chimera. Hybridization of the chimeras with the cDNA was checked by gel electrophoresis, the hybridized form showing lower mobility on a native polyacrylamide gel. The spectroscopic measurements were made on a Beckmann Coulter DTX 800 Multimode Detector. Temperature control is not critical because chimeras, Chimeras + cDNA and Chimeras + cDNA + pDNA are measured simultaneously on the same plate. Each sample contained 100 mM Tris HCl, 100 mM KCl, 10 mM $MgCl_2$, 1.5 mM NADH, 10 mM PEP, 10 units ml^{-1} pyruvate kinase, 13.2 units ml^{-1} lactate dehydrogenase, 0.2 mM ATP and 1 mM GMP. GK chimeras to a final concentration of ∼50 nM were added to the aforementioned mixture (guanylate kinase reaction mixture with GMP: GKRM + GMP). The fluorescence of NADH (EX = 365 nm, EM = 465 nm, integration time = 0.5 s) was monitored, starting immediately after addition of GK chimeras to the samples. The enzymatic activity was determined from the slope of the fluorescence intensity versus time curve, the above conditions being chosen to operate in the linear region of the calibration curve. The relative activities of chimera + cDNA and chimera + cDNA + pDNA were calculated by normalizing the activity of the reference chimera to 1.

2.4. Effectiveness of TCEP as a reducing agent

TCEP is used before conjugation with the DNA, to break up the protein polymers which form through protein–protein disulfide bonds. Because TCEP interferes with the conjugation reaction, it must be subsequently removed. We investigated the minimum concentration of TCEP necessary for efficient reduction of protein–protein disulfide bonds, using the one Cys GK mutant and different TCEP concentrations; the amount of monomers and dimers was quantified by SDS-PAGE. We found that 1 mM TCEP is sufficient in this case, and normally used 5 mM TCEP in our protocol.

We also investigated the maximum concentration of TCEP which can be tolerated in the subsequent conjugation reaction, by conjugating glutathione (GSH) to the DNA + crosslinker in the presence of different concentrations of TCEP; the DNA, DNA + crosslinker, and DNA + crosslinker + GSH species are separated on a TBE-Urea DNA gel. We find that TCEP concentrations <10 μM are safe, so if one uses ∼5 mM TCEP in the reduction step, the subsequent purification should decrease the TCEP concentration by at least a factor 1000. We use HPLC for this purification step, with a size exclusion column (Bio-Sil® SEC Column).

2.5. Timescales of polymerization versus conjugation

Two competitive processes are present during the conjugation of the DNA to the protein, namely protein polymerization due to the formation of disulfide bonds between the Cys residues of different protein monomers, and the coupling of DNA–cross-linker constructs to the protein. It is necessary to find conditions under which the latter overwhelms the former. We investigated the rate of protein dimerization and the rate of protein–DNA conjugation under different conditions, using gel shift assays and the single-Cys mutant icrGK1075. The dimerization was stopped after a given time by addition of Iodoacetamide (IAN), which blocks the cysteines; DNA conjugation was stopped with GSH.

In the presence of EDTA, the dimerization process is largely suppressed: for example, no dimers are observed after 70 min with a protein concentration of 72 μM. We find that with 1 mM EDTA the rate of cross-linker–protein coupling is much larger than that of dimerization under our protocol conditions.

2.6. Chemical reactivity of the cross-linker and yield of doubly amino-modified DNA

The reactive groups of the cross-linker have a limited lifetime, which is dependent on the conditions (Pierce Manual). We developed an assay to determine the integrity of the cross-linker, which consists of coupling a short piece of one-end-modified DNA (N5L15, table 1) with glutathione through the cross-linker. 100 fold molar excess cross-linker is reacted with N5L15 at room temperature for 1 h. After removing the excess cross-linker with HPLC, freshly made glutathione solution (100 mM in CBB (100 mM PBS, 1 mM EDTA, pH 7.0; 0.15 M NaCl)) is added to the DNA–cross-linker construct to the 1 mM final concentration. After incubation at room temperature for 80 min, the samples are run on a Ready Gel® 10% TBE-Urea precast polyacrylamide gel, where one can distinguish the three species: DNA, DNA + crosslinker, DNA + crosslinker + glutathione. We find that essentially 100% of the cross-linkers which coupled to the DNA are still competent to conjugate with the Cys residues, under the conditions of our protocol. This is seen by comparing lanes a and g in figure 2 (the assay is conducted with excess GSH).

For the construction of scheme A (figure 1), it is essential that the amino-modification is present at both ends of the DNA. To assay the yield of doubly modified DNA, we use the single-Cys mutant and couple the double modified DNA to two GK monomers. DNA with amino groups at both ends gives rise to GK–DNA–GK 'dumbbells', while DNA with only one reactive end gives rise to GK–DNA 'tadpoles'; the two species are distinguished by SDS-PAGE (figure 3). The conjugation is as described in section 2.7, with a protein–DNA ratio around 6. In principle, several factors could affect the results of figure 3, namely, the yield of doubly modified DNA, the coupling efficiency of the DNA and cross-linker, and the availability of free sulfhydryl groups on the protein. However, from the above results for the reactivity of the cross-linker, with 100 fold excess cross-linker and 6 fold excess protein,

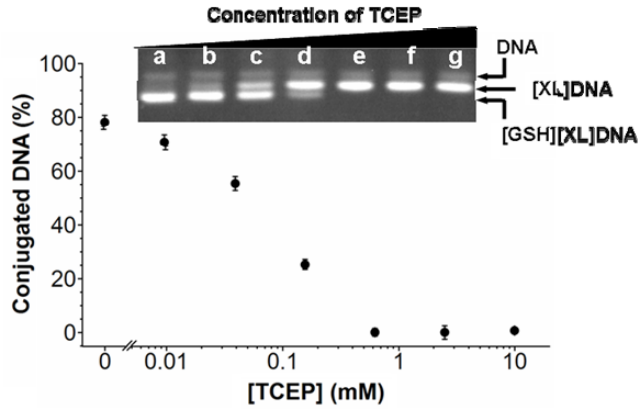

Figure 2. This TBE-Urea DNA gel shows the inhibitory effect of TCEP on the conjugation between the sulfhydryl group (of glutathione, in this case) and the cross-linker. The top band is the amino-modified DNA, the middle band the DNA + crosslinker ([XL]DNA), and the bottom band is the conjugated glutathione–DNA chimera ([GSH][XL]DNA). GSH is in 34 fold molar excess with respect to the DNA. The concentration of TCEP in the different lanes is as follows. Lane a: [TCEP] = 0 mM; b: [TCEP] = 0.0098 mM; c: [TCEP] = 0.039 mM; d: [TCEP] = 0.156 25 mM; e: [TCEP] = 0.625 mM; f: [TCEP] = 2.5 mM; g: [TCEP] = 10 mM. Lane b shows a negligible effect of TCEP, so we conclude that TCEP needs to be removed before conjugation to levels below about 10 μM.

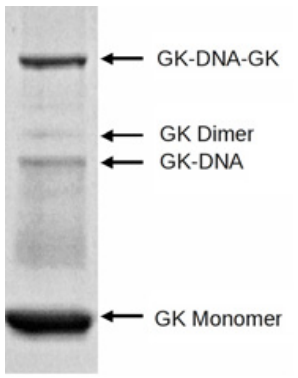

Figure 3. Assay to verify the yield of double modified DNA, represented by the GK–DNA–GK 'dumbbells' band. DNA with only one reactive arm gives rise to the GK–DNA 'tadpole' band instead. For this assay, the single-Cys mutant icrGK1075 is used. To extract the molar ratio of dumbbells to tadpoles, the intensity of the dumbbell band has to be divided by 2 because a dumbbell contains 2 proteins. For this sample, we measure 57% yield of double modified DNA. The gel is SDS-PAGE. The GK band is much darker due to the fact that 6 fold excess of protein is used in this assay.

the assay of figure 3 mainly reflects the yield of double-end-modification for this batch of DNA. From the ratio of GK–DNA–GK dumbbells to GK–DNA tadpoles in figure 3 (note that the intensity of the GK–DNA–GK band has to be divided by 2 because what is detected is the amount of protein) we find a yield of double amino-modification of 57%. This is the main factor that limits the final yield of correct 'doughnut' chimeras in scheme A.

2.7. 'Doughnut' chimera construction (scheme A)

The amino-modified DNA was dissolved in CBA (100 mM PBS, 0.15 M NaCl, 3 mM EDTA, pH 7.5; the pH is adjusted with NaOH) at 1 mM concentration. Cross-linker stock solution (1 M in DMSO) was added to a 100 mM final concentration (100 fold molar excess). The mixture was incubated at room temperature for about 1 h, followed by HPLC purification with a UNO™ Q1 ion exchange column (Bio-Rad). The blended buffer used in the HPLC is CBB (100 mM PBS, 1 mM EDTA, pH 7.0; the NaCl gradient concentration ranges from 0 to 1 M). Flow rates ranged from 1 to 4 ml min^{-1}. Fractions of the cross-linker–DNA construction from HPLC were concentrated using Amicon Ultra-15 Centrifugal Filters (Millipore). The GK mutant was first reduced in 5 mM TCEP (Pierce) at room temperature for 30 min, and then passed through a Bio-Sil® SEC Column (Bio-Rad) to remove the TCEP. The concentrated DNA solution was immediately mixed with the reduced protein from the HPLC, in a molar ratio which depended on the purpose of the conjugation. The reaction mixture was incubated at room temperature for 2–3 h and then at 4 °C overnight. The crude sample was first purified using a Ni-NTA column (Qiagen) to remove uncoupled DNA, followed by HPLC UNO™ Q1 ion exchange column purification, to remove uncoupled protein. The last purification step was with the sulfo-link gel (Pierce).

2.8. Two-arm ('Croissant') chimera construction (scheme B)

We construct the croissant chimera in two sequential steps, attaching first one and then the other of two different DNA strands (N5L30 and P5L30N3) to the double-Cys mutant icrGK2075171. In the first step, we construct a tadpole chimera following the DNA attachment protocol above. Since there are two reactive Cys on the protein, unavoidably some two-arm chimeras (with identical arms) are also obtained, as well as protein dimers (bound by one disulfide bond) with one or two DNA arms; these give rise to tadpoles and protein monomers upon TCEP reduction. To improve the yield of desired tadpole chimeras, a 2:1 protein-to-DNA ratio is used in this synthesis step. After this first step, and in the reduced state, we typically obtain at least five times more tadpole chimeras than the unwanted two-arm species (figure 4(a), lane 2). Next the sample is reduced with TCEP and the tadpole chimeras are purified from the other species using ion exchange HPLC with a salt gradient (figure 4(b)).

For the second step in the synthesis, an excess of the second DNA arm is reacted with the purified tadpole (typically a 5:1 molar ratio of DNA to tadpole chimera). Finally, the uncoupled DNA is removed by Ni-NTA chromatography.

2.9. Ligation of the croissant chimera

To ligate the two DNA arms of the Croissant chimera, we employ T4 DNA ligase (New England Biolab) to link the 5′ end phosphate of P5L30N3 to the 3′ end of N5L30. 2 μM of croissant chimera and 2 μM of 18mer complementary DNA (ℓ = 18), which serves as a splint to hold the ends of the chimera DNA together, are incubated with ligase at a final

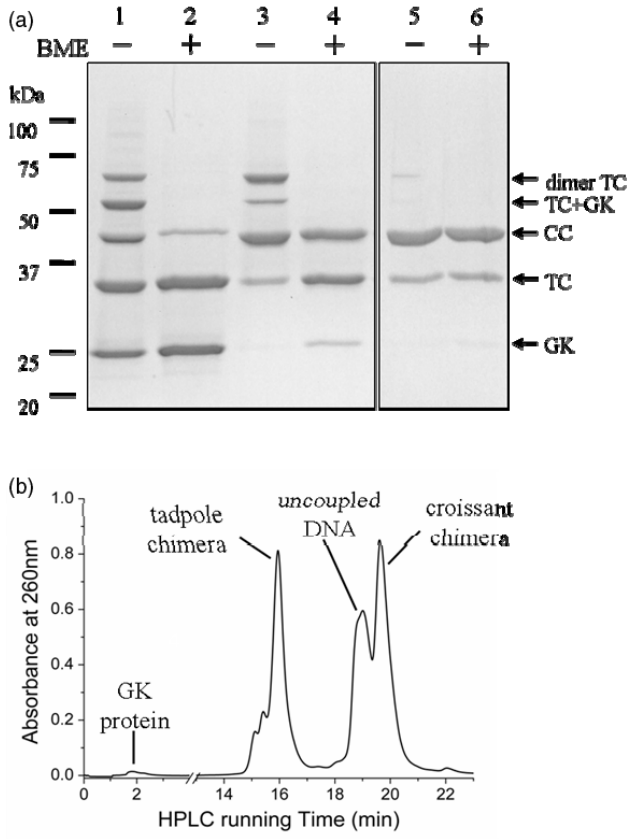

Figure 4. Sequential construction of two-arm chimera. (a) SDS-PAGE with (+) and without (−) reducing agent, BME, and stained for protein with Coomassie stain. Lane 1 and 2 show the various products after the attachment of the first DNA strand. The molecular weight of the GK protein is 25 kDa and the single DNA strand is 10 kDa. Therefore, bands with higher MW than the protein, from low to high, are the tadpole chimera (TC, 35 kDa), croissant chimera (CC, 45 kDa), tadpole chimera plus one protein (TC + GK, 60 kDa) and dimer of tadpole chimera (dimer TC, 70 kDa). With BME reduction, only the tadpole chimera and croissant chimera are left. Lanes 3–6 show products after the addition of the second DNA strand. In 3 and 4 the purified tadpole chimera is concentrated before attachment while in 5 and 6 (from a different experiment) a low tadpole chimera concentration (∼10 μM) is used. Clearly, a lower concentration improves the yield of desired croissant chimeras. (b) A representative example of HPLC purification of tadpole chimera. Assignment of peaks to various species in the sample is determined by analysing the corresponding fractions with SDS-PAGE (not shown). The weakly charged GK does not interact with the ion exchange column and thus elutes first. The large peak at 16 min is the tadpole chimera, which is well separated from the uncoupled DNA (19 min) and two-identical-arm croissant chimera (20 min).

concentration of 4 units μl^{-1} in the reaction buffer provided by the manufacturer of ligase for 2 h at 16 °C and then overnight at room temperature. The result is shown on the SDS-PAGE of figure 5: the ligated croissant chimera (which is the same as the doughnut chimera) is the majority species, but there are other linear polymers as well. Because the doughnut chimera is circular, under denaturing conditions we expect it to have lower mobility than the linear 'croissant' chimera with the same molecular weight. Therefore, the doughnut chimera was identified on the gel as the only band which cannot be

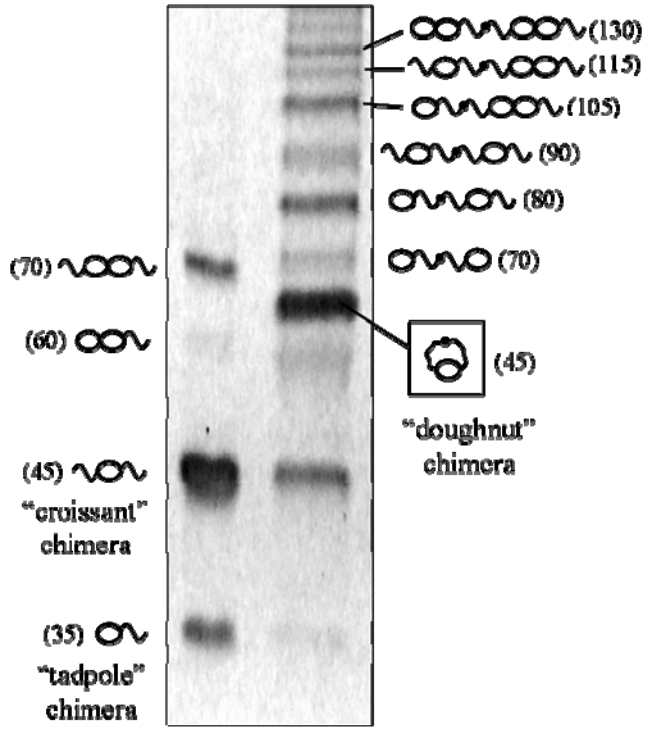

Figure 5. Ligation of croissant chimera to form doughnut chimera. Modified SDS-PAGE (without BME) with SyBr gold DNA staining of the sample before (left lane) and after (right lane) ligation. The presumed corresponding molecular species is drawn on the side. The molecular weight of the band (in kDa) is shown in parenthesis, estimated from the comparison with a protein molecular weight standard. The linkage of two DNA strands after ligation is represented as a small dot in the configuration. Before ligation (left), the croissant chimera (45 kDa) is the dominant species. Tadpole chimera (35 kDa) and its dimer (70 kDa) are still detectable while the band of tadpole chimera plus one GK (60 kDa) is negligible. After ligation (right), many different polymers are produced, some of which can be identified by their molecular weight. The only band which does not correspond to a simple combination of constituents is the doughnut chimera, which is 45 kDa but shows lower mobility due to its circular conformation. If the protein, instead of DNA, is stained, the yield of doughnut chimera can be estimated.

explained as a possible linear ligation product in the system. The mobility of the ligated croissant chimera is about the same as a 65 kDa protein.

3. Results

The gel of figure 6 shows a scheme A sample (60mer double modified DNA N5L60N3 and the double-Cys mutant icrGK2075171) after Ni-NTA and HPLC purification, but before sulfo-link purification. The different bands in the gel are identified by comparison with our own standards of tadpole chimera, dumbbell chimera, etc. The majority population is the doughnut chimera we want to construct, but still it represents only about 25% of the GK in the sample. Note that even this consists, strictly speaking, of two different isomers, because we do not control which Cys gets which end of the DNA. The 'ideal' mechanical effect is the same for the two isomers. (By ideal we mean the effect produced by the force applied by the

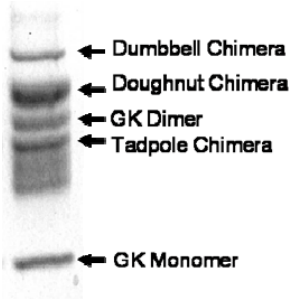

Figure 6. SDS-PAGE of the chimera construction before sulfo-link purification. The doughnut chimera, which is what we want, is the majority species but still constitutes only 25% of the total protein present. The smear in front of the tadpole band is caused, we believe, by the impurities in the synthetic DNA samples used to construct the chimeras (shorter DNA strands not completely removed by the HPLC purification), which give rise to tadpole chimeras with shorter DNA. The gel is running downwards.

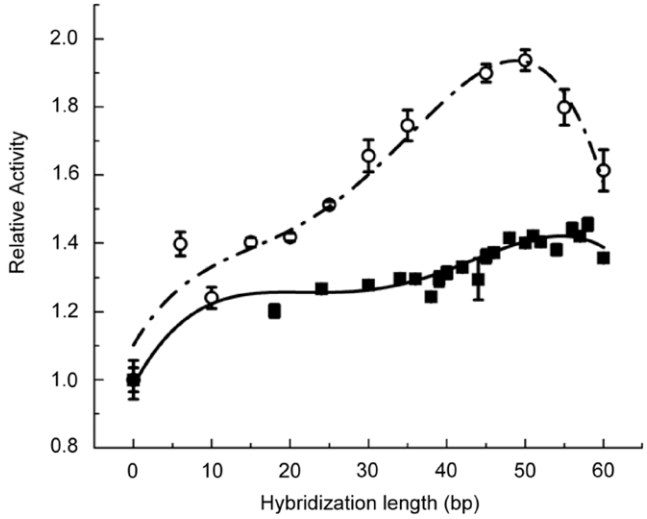

Figure 7. Relative activity versus hybridization length for two different chimeras. The filled squares represent the sample of figure 6. The two chimeras are based on different GK mutants (icrGK2112144 (open circles) and icrGK2075171 (filled squares)), different DNA strands (N5L60N3 and N5L30/P5L30N3, respectively), and different construction schemes (scheme B, and scheme A, not sulfo-link purified). The magnitude of the activation effect is different in the two cases, but the effects are qualitatively similar.

DNA ends on the attachment sites on the protein, in the absence of other interactions between the nucleotides and the protein. By Newton's laws, the force exerted by the DNA at one end is then equal in magnitude and opposite in direction to the force at the other end. Therefore in this context the orientation of the DNA spring does not matter.) However, non-mechanical effects are, we believe, related to interactions between the nucleotides of the DNA and the protein. We find (see below) that these effects in general depend both on the DNA sequence and the attachment point on the protein's surface (for DNA attached at one end only); thus in principle non-mechanical effects can be different for the two different isomers.

We assayed the enzymatic activity for this sample and were surprised to find an *activation* effect upon hybridization with the complementary 60mer (figure 7). The activity increases by about a factor 2. This is in contrast to our previous results on GK (GK2075171 in table 2) with the same attachment points of the molecular spring [6], also using a 60mer DNA (N5L60N3-B in table 1), where we found inhibition of the enzyme under stress (i.e. upon hybridization of the DNA spring). The differences between the two experiments lie in the yield of doughnut chimeras (the measurements of figure 7 are before sulfo-link purification), the removal of the two internal Cys in the present experiment and a different sequence for the 60mer DNA spring. After the series of experiments described below, we conclude that the origin of this activation effect lies in a 'non-mechanical' interaction between certain DNA sequences and the enzyme. By non-mechanical we mean an effect which is distinct from the effect of the mechanical stress exerted by the molecular spring. Because we are interested in using the molecular spring approach to measure the effect of a mechanical perturbation on the enzyme, it is important to be able to distinguish mechanical from 'non-mechanical' effects in the measurements. In the following we show how to separate the two.

First we confirmed by native gel electrophoresis that the cDNA does indeed hybridize to the chimera. Next, a similar 'activation' effect was also observed with samples constructed with a different two-Cys mutant (i.e. different attachment points for the DNA spring). On the other hand, samples constructed with the first mutant, but a different DNA sequence, showed no activation. By now our hypothesis was that certain DNA sequences could, in the ss form, interfere with the enzyme, while hybridization with the complementary would remove the DNA from the surface of the protein and 'activate' the enzyme. We set out to explore this hypothesis using tadpole chimeras, where the DNA is attached only on one side and thus does not exert the mechanical stresses envisioned in figure 1. The tadpole chimeras were constructed using various single-Cys mutants, and also different DNA arms.

Table 3. Cys mutant location and DNA arm sequence combinations used in the tadpole chimera activity experiments.

Cys mutant location	Sequence name	Activity ratio of ds chimera to ss chimera
icrGK1075	N5L15	2.04 ± 0.02
icrGK1075	N5L20	1.70 ± 0.34
icrGK1171	N5L20	2.60 ± 0.03
icrGK1075	N5L20GA	1.03 ± 0.04
icrGK1171	N5L20GA	1.35 ± 0.03
icrGK1171	N5L20G	1.10 ± 0.03
icrGK1171	N5L20T	1.04 ± 0.04

The different configurations used are reported in table 3, together with the observed activation effects. The DNA arm N5L15 is a shorter version of N5L20 (with the last 5 bases deleted), and on the icrGK1075 mutant both lead to similar activation effects of about a factor 2. The same N5L20 attached on the opposite lobe of the GK (the mutant icrGK1171) shows a somewhat larger activation effect (a factor 2.6). On the other hand, the N5L20GA DNA, which is a different 20mer sequence, shows no activation on the icrGK1075 mutant and little activation on the icrGK1171 mutant.

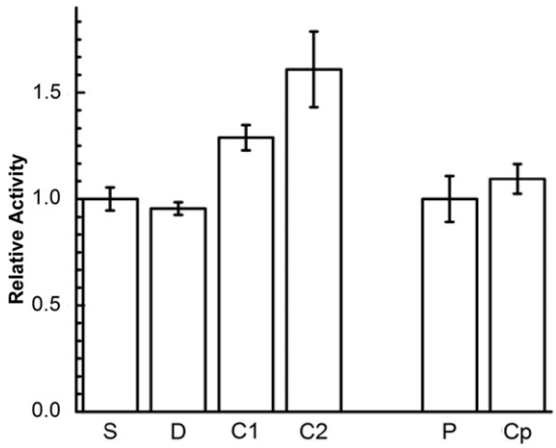

Figure 8. Relative activities for the sample of figure 6, but after sulfo-link purification, for different hybridization states. D is sample S hybridized with a complementary DNA of length 60; C1 and C2 are controls. C1 is S hybridized with three 15mer fragments (cDNA15f1, cDNA15f2, cDNA15f3), for a total hybridized length of 45; C2 is S hybridized with one 30mer fragment (cDNA30). For both configurations we expect no tension because of the ss gaps. Both show activation. On the other hand, D shows no change in activity with respect to S. This result is in contrast to the same sample before the sulfo-link purification, which showed activation (figure 7, filled squares). We interpret this result as arising from the combination of non-mechanical activation and mechanical inhibition. C1 and C2 are two control experiments which support this view. P and Cp represent a further control (column P: protein mutant icrGK; column Cp: protein mutant icrGK + cDNA60 in solution at ∼12 μM concentration). This control indicates that DNA by itself does not activate the protein.

In summary, certain sequences for the chimera DNA show a non-mechanical activation effect upon hybridization, at least when they are attached on one side only; the magnitude of the effect depends on the sequence and also on the attachment point on the protein; some sequences show no effect at all.

We performed GMP and ATP titration experiments to further investigate the activation effect. Without entering into details, we just mention that the results are consistent with the 'activation' being due to an increase of the apparent binding affinity of ATP upon hybridization of the chimeras (the binding affinity for GMP being instead unaffected).

3.1. Scheme A samples: distinguishing mechanical and non-mechanical effects

For the sample of figure 6, we tried to isolate the doughnut chimera by purification on the sulfo-link gel (Pierce), which retains species with free sulfhydryls; this should remove tadpoles etc. This was the procedure adopted in our previous work [6]; its shortcoming is that the yield of this purification is small (i.e. a lot of sample is retained whether it has free sulfhydryls or not). We obtained enough purified sample to run an assay, but not enough to run a gel. The result of the enzymatic assay is that upon hybridization with the complementary 60mer there is now no change in activity (figure 8, S and D). This is in contrast to the same sample before sulfo-link purification, which shows a factor 2 activation (figure 7). Our interpretation is that, for this sample, we see the combination of two effects. One is the activation effect when the ssDNA is hybridized; this is present for all the different species (tadpoles, doughnuts, etc) although possibly with different amplitudes. The second effect is the inhibition due to the mechanical stress, and this is present only for the doughnuts (the correct construction).

Table 4. Fractions of the different species for the sample of figure 6, estimated from the intensity of the bands on the gel.

Species	Fraction (%)
Tadpole	44.9 ± 0.3
Doughnut	24.8 ± 0.6
Dumbbell	5.6 ± 0.1
Others	24.7 ± 0.4

We know that the activation effect is present for the tadpole construction with N5L20, from the experiments discussed earlier. In order to confirm that there are two opposite effects at play in the sample 'D' of figure 8, we performed two control experiments, where the sample 'S' of figure 8 is partially hybridized with DNA oligomers which leave ss gaps in the DNA spring. In this configuration there is no mechanical tension (because of the ss gaps), and indeed we see activation (figure 8, C1 and C2).

We now wish to correct the measurement D of figure 8 by subtracting the non-mechanical effects in order to isolate the effect of the mechanical tension provided by the DNA spring. The DNA in solution, at the concentrations used in the experiments (∼12 μM), has no effect on the enzyme, as shown in figure 8, column P and column Cp. We denote the fractions of the species present by P_{tadpole}, P_{dumbbell}, P_{doughnut}, and lump everything else (monomers, dimers, ...) into P_{others}. From the gel figure 6 we obtain the fractions listed in table 4.

We describe the effects of non-mechanical interaction and mechanical tension on the enzymatic rate with the coefficients V_{s} ('steric') and V_{m} ('mechanical'), respectively. Assuming all species apart from doughnut chimeras are removed by the sulfo-link gel, two relations are obtained as follows:

$$P_{\text{tadpole}} V_{\text{s}} + P_{\text{doughnut}}(V_{\text{s}} V_{\text{m}}) + P_{\text{dumbbell}} + P_{\text{others}} = V_{\text{before}}$$
$$V_{\text{s}} V_{\text{m}} = V_{\text{after}}. \tag{1}$$

Here, the V's are relative changes of enzymatic rates between hybridized and non-hybridized samples; V_{before} refers to the sample before sulfo-link purification and V_{after} to the sample after purification. This is of course only an estimate, as it includes a number of assumptions. We assume that there is no activation effect for dumbbells (because of the constraints on the DNA conformations imposed by the presence of the two proteins; however, since P_{dumbbell} is only 5%, this particular assumption has little effect on the final result). We also assume that the activation effect is the same for tadpoles and doughnuts, and that mechanical and non-mechanical effects are independent. With these caveats, from (1), the mechanical and

non-mechanical effects are:

$$V_{\rm m} = \frac{V_{\rm after} P_{\rm tadpole}}{V_{\rm before} - P_{\rm doughnut} V_{\rm after} - P_{\rm others} - P_{\rm dumbbell}}$$
$$V_{\rm s} = \frac{V_{\rm after}}{V_{\rm m}}. \qquad (2)$$

Based on figure 6 we get $V_{\rm m} \approx 0.2$; $V_{\rm s} \approx 5.3$. Thus the corrected value of the measurement D of figure 8 is $V_{\rm m} = 0.2$; i.e. the mechanical effect of the DNA spring is to suppress activity to 20% of the original. This is qualitatively consistent with our previous measurements [6] with a different sequence for the DNA spring.

3.2. Two-arm ('Croissant') chimera construction (scheme B)

In figure 4 we show various stages of the construction scheme B. In the end, the overall yield of chimeras is poor, typically ∼10% of the initial amount of modified DNA oligomer used, where sample loss is mainly due to the HPLC purification of the tadpole chimeras. However, the purity of croissant chimera in the final sample (with respect to impurities, which are mainly unreacted tadpole chimeras and its dimer) is quite acceptable (>85%, estimated from figure 4(a), lane 5). Note that here also, the croissant chimera is actually a mixture of two DNA arm swapped isomers.

The key to a high yield of croissant chimeras is to minimize the dimerization of tadpole chimeras through protein–protein disulfide bonds, which can be achieved by simply lowering the concentration of tadpole chimeras in the second attachment step. This is shown in figure 4(a) by the comparison of lanes 3, 4 with lanes 5, 6. For the former, the purified tadpole was concentrated before conjugation with the second DNA arm, leading to a larger amount of tadpole chimera dimer (dimer TC, lane 3) and thus a lower ratio of croissant to tadpole chimera in the final sample (compare lanes 4 and 6). We find ∼10 μM tadpole chimera (thus ∼50 μM P5L30N3) is a good compromise, since even lower concentrations would lengthen the total time needed to complete the reaction and increase the loss of reactive groups of cross-linkers.

3.3. Enzymatic activity of scheme B samples before and after ligation

Figure 9 shows the evolution of the enzymatic activity of the sample of figure 5 for increasing hybridization length ℓ. Before ligation (figure 9 filled triangles; figure 5 left lane) we observe activation for increasing ℓ, while after ligation (figure 9 filled diamonds; figure 5 right lane) we observe inhibition.

Now we use the measurements on the non-ligated (NL) sample to correct the measurements on the ligated (L) sample for non-mechanical effects. Because the actual quantity of chimeras used in establishing L and NL are not the same, NL has to be normalized with a scaling factor, which is derived from a linear fit of the ratio of the activity of NL at $\ell = 18, 24$, and 30 to that of L at the same lengths. The reason for using these three data points is because, for $\ell < 30$, there should be no tension on the molecular spring (thus modulations of the activity are due to purely non-mechanical effects) and there should be no differences in activity between ligated and non-ligated croissant chimeras. The rescaled activity curve is RNL ('rescaled-non-ligated') in figure 9(a).

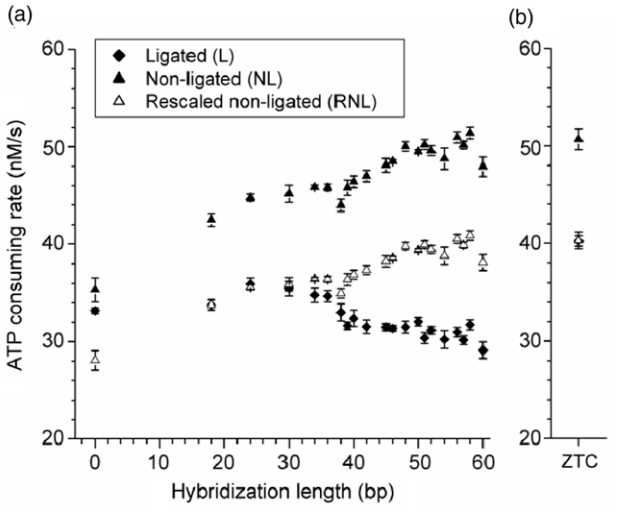

Figure 9. (a) Enzymatic activity versus hybridization length for the chimera sample of figure 5, before ligation (solid triangles, corresponding to figure 5 left lane) and after ligation (solid diamonds, corresponding to figure 5 right lane). To directly compare the non-ligated (NL) curve to the ligated (L) one, we rescale NL with a factor, which is estimated from the ratio of the activity of NL to that of L for $\ell = 18$, 24, and 30 (where we expect no mechanical tension); the rescaled curve (RNL) is represented by the open triangles. (b) Activity of the zero tension negative controls (ZTC) of the ligated and non-ligated samples. The same symbols as in (a) are used. ZTC is prepared by hybridizing the sample with the 30mers complementary to N5L30 and P5L30N3. This does not apply a stress on the protein. The open triangle represents the value of the filled triangle rescaled by the same factor as in part (a) of the figure; the fact that this rescaled activity is the same as the activity of the ligated sample (filled diamonds) suggests that the nick in the molecular spring eliminates most of the mechanical effect on the enzymatic activity. Therefore, RNL basically represents the non-mechanical effect. All error bars are ±1 SD estimated from 6 experiments.

On the right-hand side of the graph of figure 9 we also report the activity of a zero tension negative control (ZTC) for the same samples. In the ZTC, we hybridize the sample with two separate DNA 30mers which are complementary to the two arms of the croissant chimera (i.e. the symbols on the right in the figure represent the activity of the corresponding sample in the presence of 50 fold molar excess of two 30mers complementary to N5L30 and P5L30N3, respectively). The filled triangle is the non-ligated sample, the solid diamond the ligated sample. The open triangle is the filled triangle rescaled by the same factor as was applied to the data on the rest of the graph. It is apparent that this control is consistent with the normalization of RNL (the ZTC of the ligated sample comes out the same as the rescaled activity of the $\ell = 60$ unligated sample); it also shows that the molecular spring with the nick has no measurable effect on the activity of the enzyme. This notwithstanding the fact that the elastic energy of the protein–DNA chimera with the nick is substantial: in a separate experiment we measure it to be about $9kT$ [14]. With

the nick ligated, the elastic energy is presumably even larger, and there is an evident effect on the enzymatic activity as we detail below.

To isolate the mechanical effect, we proceed as in the previous section: we consider the activation effect and the heterogeneity of the samples, and write for the curves of figure 9:

$$\mathrm{L}(\ell) = A_0 P_{\mathrm{doughnut}}[V_{\mathrm{s}}(\ell)V_{\mathrm{m}}(\ell)] + A_0(1 - P_{\mathrm{doughnut}})V_{\mathrm{s}}(\ell)$$
$$\mathrm{RNL}(\ell) = A_0 V_{\mathrm{s}}(\ell) \tag{3}$$

where, as before, V_{s} and V_{m} represent the non-mechanical ('steric') and mechanical effects as multiplicative factors ($V_{\mathrm{s}} > 1$, $V_{\mathrm{m}} < 1$), A_0 is the native activity of GK, and P is the yield for the corresponding chimera. For simplicity we have assumed that, for $\ell \neq 0$, V_{s} is the same for all different species (doughnut chimera, croissant chimera, and other polymers present in the sample).

From (3) we obtain the relative activity due to mechanical effects only:

$$V_{\mathrm{m}}(\ell) = \frac{1}{P_{\mathrm{doughnut}}}\left(\frac{L(\ell)}{RNL(\ell)} + P_{\mathrm{doughnut}} - 1\right). \tag{4}$$

Using $P_{\mathrm{doughnut}} = 0.6$ (estimated from the gel figure 5), the result is plotted in figure 10 and compared with our previous result published in [6], where the chimera was synthesized according to scheme A. Qualitatively, the two curves are fairly similar, although very different approaches are used to synthesize the doughnut chimera. Both curves show an onset length for mechanical inhibition around $\ell = 40$, but the largest inhibition effect in the current work ($V_{\mathrm{m}}(60) = 0.6$) is smaller than our previous result. However, a number of factors could account for this difference. First, different assay conditions were used in the two cases to measure 'enzymatic activity', and the temperature for the two experiments may have differed by several degrees, as there was no temperature control beyond the air conditioning of the room; both factors could affect the measured dynamic range of control. Then the present chimera is actually a slightly different molecule from the one of [6], because (1) here we have removed the internal cysteines, and (2) the present cross-linker is longer than the one used in [6] so that, if the protein was perfectly rigid, the end-to-end distance (EED) of the DNA spring in the present construction would be 2 nm longer than in the former construction. We thus consider figure 10 as evidence that both scheme A and scheme B are viable strategies to synthesize these chimeras for the purpose of probing the response of the enzyme to mechanical perturbations.

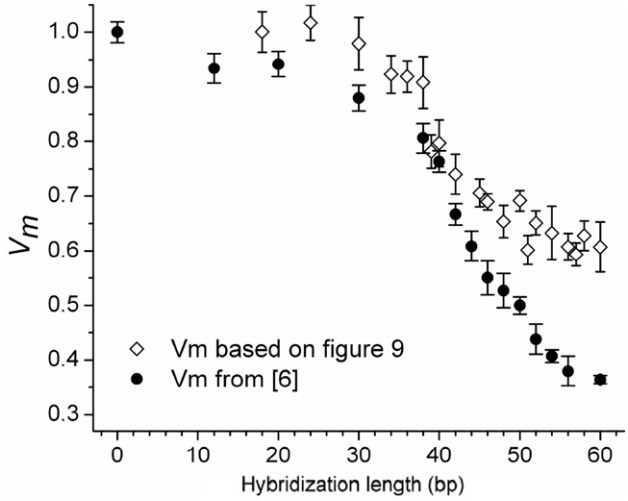

Figure 10. Two different measurements of the relative enzymatic activity versus hybridized length of the molecular spring. The result from the present experiment (open diamonds) is derived from the data represented by the filled diamonds in figure 9 corrected to subtract the non-mechanical effects using RNL in figure 9 and the measured yield of doughnut chimeras (see text). Error bars are ±1 SD estimated from 6 experiments. The result published previously in [6] (solid circles), where the doughnut chimera was constructed according to scheme A, is also shown here for comparison. Both curves show the onset of inhibition around $\ell = 40$. The overall inhibitory effect is smaller in the present experiments. This could be due to (1) errors in the corrections applied to the present measurements; (2) different conditions for the enzymatic assay; (3) the fact that the present chimera is a slightly different molecule from the chimera of [6] (see text).

4. Conclusions

One objective of this paper is to describe a new 'two-arm' protein–DNA chimera construction (scheme B) for the purpose of attaching a DNA 'molecular spring' to a protein. Compared to our previous construction method (scheme A), this is easier to control and leads to better yields.

We also report new observations on 'non-mechanical' interactions between the DNA molecular spring and the protein. In the case of GK, we find that some (but not all) DNA sequences, when end-attached to the protein (and therefore at a high 'effective concentration' in the vicinity of the protein's surface), can have an inhibitory effect in the ss form. Hybridization to the ds form removes the inhibition. It is not difficult to imagine mechanisms whereby hydrogen bonding of unpaired (i.e. ss) DNA bases with residues on the enzyme's surface could interfere with the active site or otherwise constrain the enzyme's conformations, leading to inhibition. However, other mechanisms for this effect are also possible.

Whatever the origin of this 'non-mechanical' effect, we show how to subtract it from the measurements in order to obtain the modulation of the enzymatic activity due to the mechanical stress alone. This is important if, for example, one wants to study how the response of the enzyme differs if one applies a relatively small mechanical perturbation (one which does not completely kill the enzyme) at different points. Thus from the point of view of using the enzyme–DNA chimeras for studying the response of the enzyme to mechanical perturbations, it is important to know that 'non-mechanical' effects may arise, that they are enzyme and DNA sequence specific, but that they can be detected and separated from the direct effect of the mechanical stress with appropriate control experiments.

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