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Review

Probing DNA–peptide interaction forces at the single-molecule level

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Abstract: The versatility of chemical peptide synthesis combined with the high sensitivity of AFM single-molecule force spectroscopy allows us to investigate, quantify, and control molecular recognition processes (molecular nanotechnology), offering a tremendous potential in chemical biology.

Single-molecule force spectroscopy experiments are able to detect fast intermediate transition states, details of the energy landscape, and structural changes, while avoiding multiple binding events that can occur under ensemble conditions. Dynamic force spectroscopy (DFS) is even able to provide data on the complex lifetime. This minireview outlines the biophysical methodology, discusses different experimental set-ups, and presents representative results in the form of two case studies, both dealing with DNA-binding peptides. They may serve as model systems, e.g., for transcription factors or gene transfection agents. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: DNA; peptide; transcription factor; molecular recognition; single-molecule force spectroscopy; atomic force microscopy; optical tweezers

INTRODUCTION

Regulation of all events in living organisms relies on a complex interplay between molecules. Molecular recognition consequently can be regarded as a major precondition of cellular processes. For instance, gene expression is controlled on the transcriptional level by the specific interaction between proteins (transcription factors) and regulatory DNA sequences. The discovery of the molecular structure of DNA can be considered as the birth of modern molecular life sciences [1].

Structural methods are gaining increasing importance in this context, and numerous structures of complexes between DNA and proteins or small-molecule ligands are available in the RCSB Protein Data Bank. Investigations on the interaction between DNA and its binding partners that comprise a broad variety of chemical entities from small molecules to proteins is of interest for the understanding of processes such as unspecific or specific DNA binding as well as transcription. Small-molecule ligands that bind to DNA without sequence specificity are known both from natural sources and of synthetic origin. They are frequently used, e.g., in cancer therapy. Molecular recognition of DNA by proteins, peptides, and other effector molecules is usually governed by a combination of electrostatic, hydrophobic, and dipole–dipole interactions as well as hydrogen bonding. Cationic amphiphilic peptides, in which the positive charge is predominantly located on one face of the helix, bind to DNA by interaction with the negatively charged phosphate groups. DNA may also function as a template and induce helical structures in basic peptides. Besides these non-specific interactions, amphiphilic helices form specific contacts and serve as recognition elements in protein–DNA contacts as found in transcription factors.

In this minireview, the biophysical techniques atomic force microscopy (AFM) and optical tweezers (OT) together with their implications in single-molecule force spectroscopy will be briefly introduced and two case studies will be presented in order to illustrate the potential of the methodology employed: First, non-specific binding of amphiphilic model peptides to DNA will be discussed and then the interaction of DNA with an amphiphilic α-helical epitope of a transcription factor will be presented. It can doubtlessly be regarded one of the major strengths of peptide synthesis that practically any chemical modification can be performed. Either tailor-made probes to tackle with a specific biological and biochemical phenomenon or specific therapeutic agents to address certain pathological settings may be obtained in a straightforward manner.

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BIOGRAPHY

Norbert Sewald

was born in 1961 in Munich (Germany) He obtained his Diploma degree in chemistry and his PhD in organic chemistry at the Technical University of Munich. After a postdoctoral fellowship with Prof. J. E. Baldwin at the Dyson Perrins Laboratory, University of Oxford, he started independent research at the Technical University of Munich and later at the University of Leipzig. In 1998 he finished his habilitation, and was appointed as a full Professor of Organic and Bioorganic Chemistry at Bielefeld University in 1999. His research interests in general comprise organic chemistry at the interface to biology and medical sciences. Special focus is placed on the development of synthetic methods, the isolation, structure elucidation, and total synthesis of bioactive natural products, studies regarding the interaction of peptides with proteins or DNA, analysis of the solution structure of peptides using NMR, and the development of novel molecular tools for biochemical research.

AFM AND OT – POWERFUL SINGLE-MOLECULE TECHNIQUES

Besides the thermodynamic and kinetic parameters of non-covalent biomolecular contacts, knowledge on the complex interplay of these interactions is necessary to gain insight into structure–activity relationships. With surface plasmon resonance and microcalorimetry, different analytical methods are available to characterize such interactions on a functional ensemble level. In the case of protein–DNA contacts, the biochemical methods gel-shift assay and DNAase foot-printing are also widely used to detect such phenomena.

However, these methods provide a view on the behaviour of an ensemble of molecules. In contrast to such well-established ensemble measurements, experiments handling single molecules are capable of detecting fast intermediate transition states, details of the energy landscape, structural changes, and, consequently, 'individual' behaviour. With the modern biophysical methods AFM [2] and OT [3,4], the interaction force between molecules becomes directly observable and can be used to describe the interaction between different molecules at the single-molecule level. The force response is recorded while a complex formed by two molecules is separated, or a single chain-like molecule is stretched. In AFM, the bending of a microfabricated cantilever is mostly detected by the deflection of a laser beam [5]. Cantilever spring constants around 10^{-3}–100 N/m and deflection sensitivities in the subnanometre range allow for measurements of forces between 10 pN and 10 μN. During the last 15 years, ultrasensitive techniques have been developed that permit measurements of inter- and intra-molecular forces at the single-molecule level [6].

OT systems are characterized by superior force sensitivity when compared to AFM. A micrometre-bead is trapped in the electric field gradient of a laser focus by optical forces [3,4]. Displacements of the bead from the centre of the laser focus are proportional to the forces exerted. OT possess superb force resolution in the sub-piconewton range, but the maximum force is smaller than 200 pN owing to the low spring constants of the optical trap (<1 pN/nm). OT may be used for measurements of elastic responses, e.g., of immobilized single or double-stranded DNA molecules, also in the presence of binding ligands.

Numerous basic mechanical experiments have been performed at the single-molecule level. Pioneering work on the mechanics of double-stranded DNA molecules have used magnetic [7] and optical tweezers [8] as well as AFM [9].

Correlation of the retraction force with the molecular extension may provide information on intra-molecular structural transitions, which were observed, e.g., for DNA. A highly cooperative transition from B-DNA to an overstretched conformation termed *S-DNA*, which is 1.7 times as long as B-DNA, was reported [10].

AFM especially has been used for the investigation of mechanical properties of ligand–receptor interactions, such as ligand–DNA interactions, in force–extension measurements. Groundbreaking investigations have been dealing with ligand–receptor interactions such as that between biotin–streptavidin [11] or antigen–antibody [12–14] or even low-affinity binding events in supra-molecular chemistry [15,16]. Others probed the molecular adhesion between two complementary DNA strands [17] or the elasticity of DNA [7]. It could be shown that the mechanical properties of double-stranded DNA observed in AFM experiments provide unambiguous information on the binding mode of small molecules that associate with to DNA without any sequence specificity [18–21].

Direct force measurements by mechanically unbinding sequence-specific protein–DNA complexes have been performed at the single-molecule level with OT in the case of binding of the restriction endonucleases *Bso*BI, *Xho*I, and *Eco*RI [22] and with AFM, e.g., in the case of transcriptional regulator proteins [23]. In the latter case, three DNA fragments comprising the target sequences were selected for the force spectroscopy measurements and attached covalently across a long flexible polymer linker to the AFM tip while the protein was anchored covalently on the surface [23]. A representative experimental set-up is shown in Figure 1.

Dynamic force spectroscopy (DFS) records binding forces for different loading rates (loading rate =

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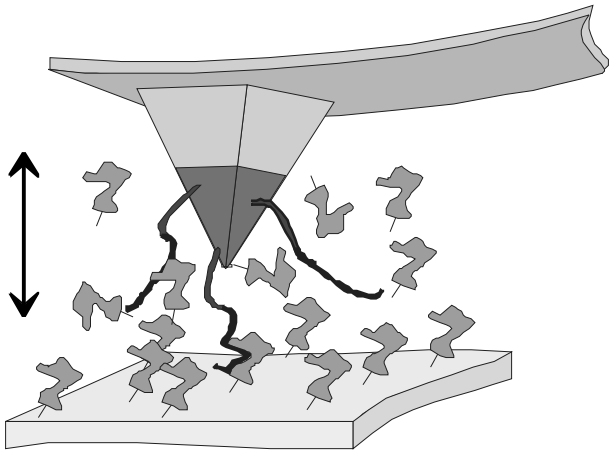

Figure 1 Schematic experimental set-up of an AFM force spectroscopy experiment on DNA–peptide/protein interaction with a dsDNA fragment immobilized on an Si_3N_4 AFM tip and a peptide or protein on a mica or gold substrate (adapted from [23]).

retract velocity × molecular elasticity) [24]. In a thermodynamically driven system, the measured separation forces depend on the loading rate that is exerted on the bound complex. A slowly increasing load provides ample time for thermal fluctuations to drive the system over the energy barrier of the binding potential, resulting in small unbinding forces. DFS experiments allow for the determination of the biomolecular complex lifetimes, τ, thermal off-rates k_{off}, and the detection of inner barriers in the energy landscape [25]. Consequently, force spectroscopic experiments not only provide information on the interaction forces but also on selected kinetic parameters at the single-molecule level. Additionally, a new data analysis method for DFS experiments has been proposed recently [26].

Case Study I: Amphiphilic Model Peptides

Amphiphilic helical peptides are characterized by unilateral alignment of, e.g., polar, positively charged amino acid side chains (e.g. of lysine or arginine), while on the other faces of the helix hydrophobic side chains are presented. Amphiphilic helices are often found in ribosomally synthesized anti-microbial peptides, in ion channel peptides, and, in the form of short to medium epitopes, in DNA-binding proteins such as transcription factors. The binding of ligands to DNA changes the overall mechanical response of the dsDNA molecule. This fundamental property can be used for discrimination and identification of different binding modes. An α-helix in fact is a 3.6_{13} helix with 3.6 amino acids per turn and a 13-membered 'ring' formed by the hydrogen bond from the NH of the amino acid in position $i+4$ to the CO of amino acid in position i. Consequently, in an amphiphilic model, approximately every fourth or fifth amino acid in the peptide sequence should bear a basic residue that is positively charged under physiological conditions. In a similar fashion, an amphiphilic 3_{10} helix should contain a basic side chain in every fourth amino acid. As such a helix comprises exactly three amino acids per turn, the side chains are arranged in a collinear manner.

For studies on the DNA-binding properties of such artificial model peptides, the sequences Ac-(Leu-Ala-Arg-Leu)$_3$-NHR **1** and Ac-(Aib-Leu-Arg)$_4$-NHR (R = $CH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$) **2** were employed. Peptide design was based on the peptide Ac-(Leu-Ala-Arg-Leu)$_x$-NHMe (x = 1–4), which had previously been described [27,28] with respect to its amphiphilic properties and gene-transfer abilities. The *C*-terminal amide was modified by an ethylene glycol-type linker (1,8-diamino-3,6-dioxaoctane) with an amino group at the other end to allow for further immobilization. Both peptides were obtained by solid-phase peptide synthesis (SPPS) [29]. Two different approaches were followed for the SPPS of Ac-(Leu-Ala-Arg-Leu)$_3$-$NHCH_2CH_2OCH_2CH_2OCH_2CH_2NH_2$: first, synthesis on an aliphatic safety-catch resin (4-sulfamylbutyryl-type) [30] with subsequent activation by treatment with iodoacetonitrile and aminolysis using a mono-Boc-protected linker, and second, synthesis on the 1,8-diamino-3,6-dioxaoctane-loaded 2-chlorotrityl resin [31]. The method involving the safety-catch resin requires the synthesis of the mono-Boc-protected linker, together with the somewhat tedious loading and cleavage procedures. The 2-chlorotrityl resin is easier to handle and more convenient to use, as monitoring of the reaction progress by MALDI–ToF MS using a small sample after cleavage from the resin is more straightforward than for the safety-catch resin because activation prior to cleavage is not necessary and the cleavage time is short.

The influence of the amphiphilic model peptides **1** and **2** on poly(dG-dC) was investigated using AFM force spectroscopy, which allowed the distinction of different binding modes of ligands interacting unspecifically with dsDNA. Additionally, the behaviour was compared to that of free dsDNA and dsDNA in the presence of, e.g., daunomycin. In the 'tapping mode', the AFM tip was allowed to grasp one end of dsDNA–or dsDNA–peptide complexes deposited on the substrate, while the other end was still 'sticking' to the substrate, and the mechanical behaviour was recorded. The AFM measurements on free dsDNA (Figure 2(A)) basically match the previously reported results [9,32]. There is a clearly visible plateau at 75 pN owing to overstretching of the dsDNA to >170% of the B-DNA contour length. Force-induced double helix melting is initiated at an extension of 550 nm up to a force of 300 pN, followed by single-strand stretching. Finally, the single strand is detached from the tip at a force exceeding 540 pN and an extension of 660 nm, while the cantilever relaxes.

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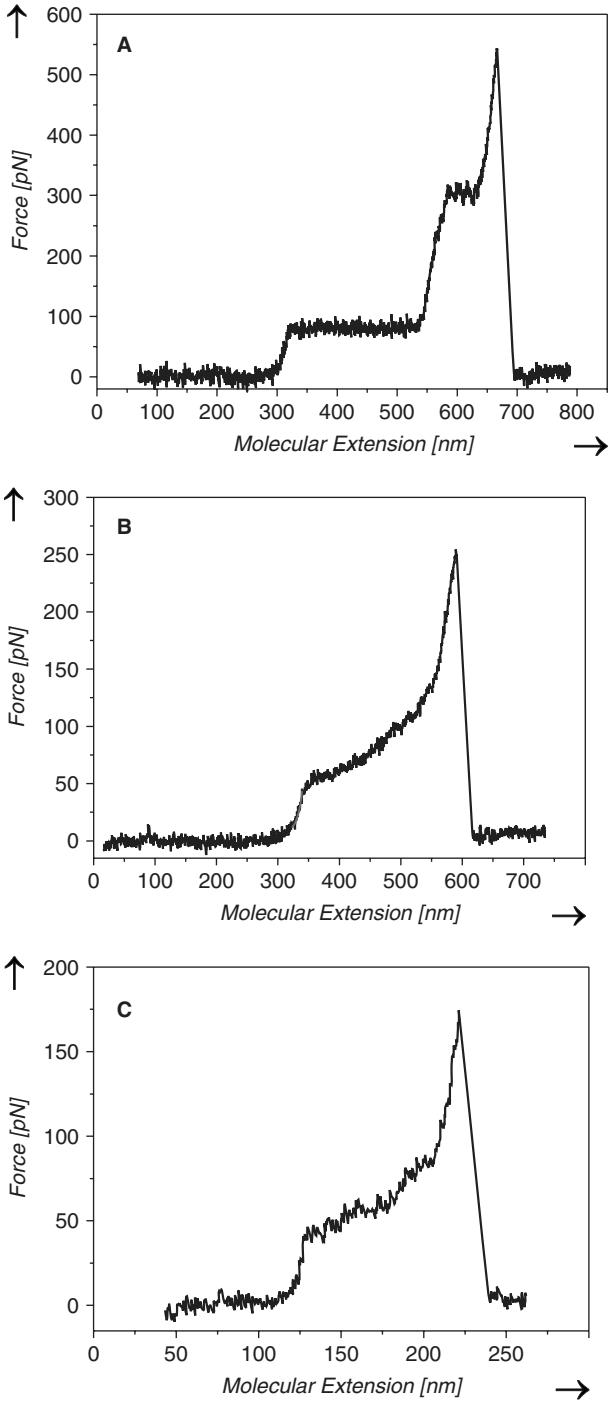

Figure 2 (A) AFM force–extension trace of free poly(dG-dC) dsDNA; (B) poly(dG-dC) double-stranded DNA in complex with the major groove binding α-helical peptide **1**; (C) poly(dG-dC) double-stranded DNA in complex with the 3_{10}-helical peptide **2** [33].

In the presence of either the α-helical peptide **1** or the 3_{10}-helical peptide **2,** the B–S transition of double-stranded poly(dG-dC) is shifted towards lower forces and cannot be observed separately from the melting transition (Figure 2(B),(C)), while the point of maximum B-DNA elongation can still be distinguished. Hence, the force–extension characteristics of these two peptides clearly differ from the one for the minor groove binder distamycin A [20]. As the binding of the peptide helices to DNA should be based on unspecific electrostatic interactions between the guanidino groups of the peptide and the negatively charged DNA backbone, it is likely to occur in the major groove of the double helix [27]. Hence, the force spectroscopy data support the assumption that peptides **1** and **2** bind to the major groove.

Peptide **1** was also employed in studies with OT [34]. The elastic response curve of λ-DNA (51% GC pairs) in complex with the α-helical peptide **1** is characterized by an intersected transition (between 17 and 22 mm) between the elastic stretching of B-DNA at low forces and the less pronounced overstretching transition (22–27 mm) at 80–85 pN, which had not been found in the AFM studies with poly(dG-dC) dsDNA. The different binding behaviour of **1** towards GC-rich and AT-rich regions might be the reason for this observation.

Case Study II: Peptide Epitopes of the Transcription Factor PhoB

Chemically synthesized peptides have an enormous potential for retrieving the key interaction sites in protein–biomolecule contacts. The synthetic methodology is well established and straightforward. Modification or immobilization sites and non-natural building blocks may be incorporated into synthetic peptides without nearly any restriction. This was shown, e.g., for protein–protein interaction [35], protein–DNA interaction [20,29,33,34], and also protein–carbohydrate interaction [36]. Such an approach is especially powerful in combination with single-molecule force spectroscopy in order to reveal the contribution of a single helix or a single amino acid to the binding forces.

Amphiphilic helical epitopes serve as recognition and binding elements in protein–DNA contacts as found in transcription factors. The protein PhoB occurs in *Escherichia coli* and closely related bacteria and is a signal transduction response regulator. It is responsible for triggering expression of the genes involved in phosphate metabolism. When the phosphate concentration is low, PhoB gets phosphorylated, which increases the binding affinity of PhoB to the phosphate regulon *pho*. The PhoB protein from *E. coli* [37] comprises an *N*-terminal regulatory phosphorylation domain [PhoB (1–127)] and a *C*-terminal DNA-binding domain [PhoB (128–229)]. The DNA-binding domain has been characterized structurally by NMR spectroscopy – also in combination with binding studies [38]. The structure of a complex of two molecules of the DNA-binding domain of PhoB and one double-stranded DNA has been unravelled by X-ray analysis (Figure 3) [39].

The DNA-binding domain structurally belongs to the family of *winged helix-turn-helix*-transcription factors of

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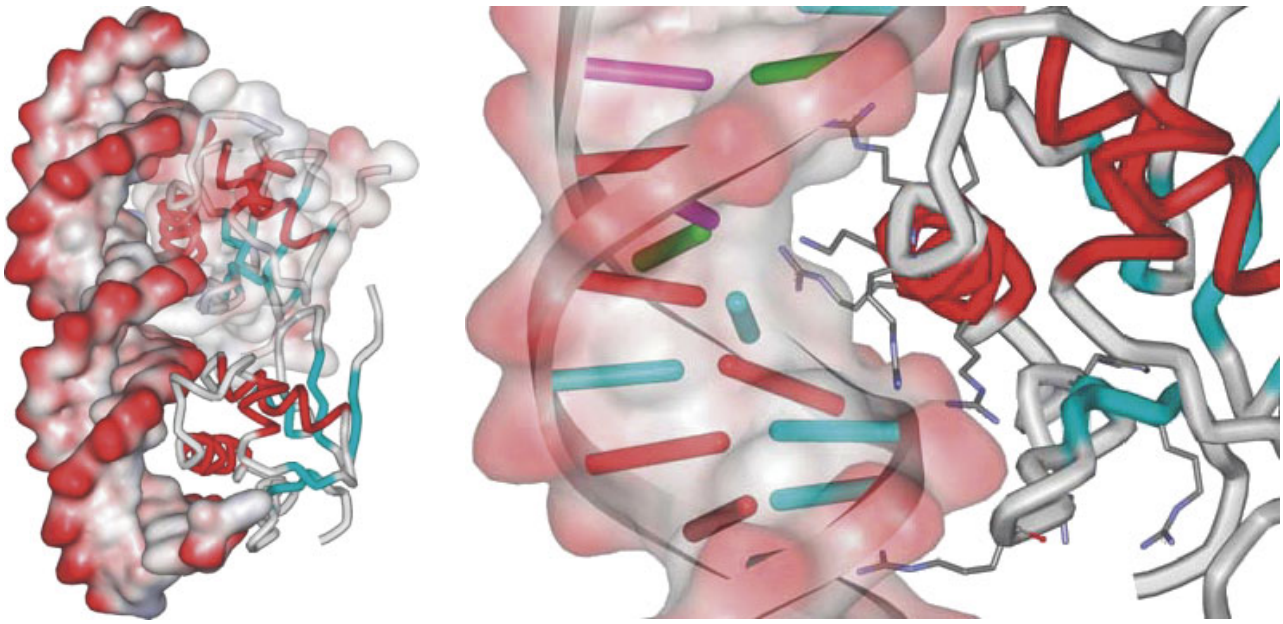

Figure 3 Left: Molecular structure of the complex between the dsDNA fragment of the *pho* box and two molecules of the DNA-binding domain of PhoB according to the X-ray analysis [39]; Right: View along the helix axis of the amphiphilic helix α^3 of PhoB bound to the dsDNA fragment of the *pho* box [39].

Table 1 Sequences, k_{off}, τ, and x_β values of the native peptide epitope PhoB (190–209) **3** and of three mutated peptides **4**–**6** [33]

Peptide	Sequence	k_{off} (s^{-1})	τ (s)	xβ[Å]
PhoB (190–209) **3**	VEDRTVDVHIRRLRKALEPG	3.1 ± 2.1	0.32 ± 0.22	6.8 ± 1.2
PhoB (190–209) R193A **4**	VEDATVDVHIRRLRKALEPG	0.071 ± 0.053	14.1 ± 10.5	9.3 ± 2.6
PhoB (190–209) H198A **5**	VEDRTVDVAIRRLRKALEPG	49.5 ± 21.2	0.020 ± 0.008	7.2 ± 3.5
PhoB (190–209) R203A **6**	VEDRTVDVHIRRLAKALEPG	No binding	No binding	No binding

the topology β^1-β^2-β^3-β^4-α^1-β^5-α^2-α^3-β^6-β^7 (Figure 3). The amphiphilic helix α^3 is the DNA recognition epitope, with the two helices α^2 and α^3 being separated by a loop instead of a tight turn. Additionally, the β-hairpin β^6-β^7 is considered as the *recognition wing* that also contributes to specific binding.

A series of amphiphilic helical peptides (Table 1) were designed on the basis of the primary structure of the helix α^3 and synthesized by Fmoc SPPS on Barlos resin (2-chlorotrityl resin). The resin was pre-loaded with 1,8-diamino-3,6-dioxaoctane as the linker moiety to allow *C*-terminal attachment to the AFM surface [33]. Genomic DNA from *E. coli* that contained the *pho* box with the recognition motifs TGTCA was amplified by PCR with a 5′-thiol-modified deoxyribose and immobilized to the AFM tip across a bifunctional polyethylenglycol linker with a medium length of approx. 30 nm. Basic Arg and His residues in strategic positions were replaced by Ala to reveal the contribution of the single amino acids. The forces involved were investigated in detail at the single-molecule level by atomic force spectroscopy.

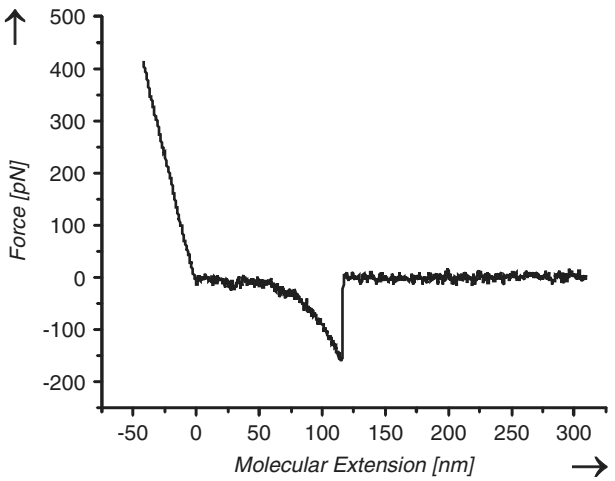

Figure 4 Typical force–distance curve for the PhoB (190–209)–DNA interaction. dsDNA is attached to the AFM tip and the peptide is immobilized to the surface. While increasing the distance between the points of attachments with sub-nanometre accuracy, the restoring force is detected.

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A representative AFM experiment is displayed in Figure 4. DNA was covalently attached to a silanized AFM tip by reacting the 5′-thiol group with a maleimide-derivatized 3.4 kDa PEG linker, while the peptide was immobilized on the silanized mica across the *C*-terminally attached linker. The experiment starts at piezo z-extension = 0. In the first phase of the experiment, the force slowly increases until the interaction suddenly breaks. A force histogram can be established after a sufficient number of experiments (Figure 5). It could be shown in single-molecule competition experiments that both free, non-immobilized peptide and free DNA drastically reduce the number of binding events [33].

For the DNA–peptide complexes, the loading rate dependence of the most probable bond rupture forces was measured in DFS. Extrapolation to zero external force ($F = 0$) gives the thermal off-rate. Typically 2000 force–distance curves were recorded at eight different retraction velocities ranging from 10 to 6000 nm s^{-1}. Peptide **4** most strongly binds to the genomic DNA fragment followed by the wild-type peptide PhoB (190–209) **3** and the mutant PhoB (190–209) H198A **5**, while all other modified peptides did not display significant affinity to DNA. The higher affinity of **4** compared to **3** is puzzling, as the Arg residue that was replaced by Ala would be expected to establish an ionic guanidinium–phosphate interaction with DNA.

Similar single-molecule force spectroscopy experiments on the interaction of a complete transcription factor of the helix-turn-helix family (ExpG from *S. meliloti*) with different DNA fragments gave k_{off} values in the range of 10^{-3} s^{-1} corresponding to complex lifetimes τ of about 1000 s [23]. As to be expected because of the multivalency of the protein–DNA interaction, the k_{off} values of most peptides were much higher and the complex lifetimes much shorter. Interestingly enough, the k_{off} value of peptide **4** approaches the range that

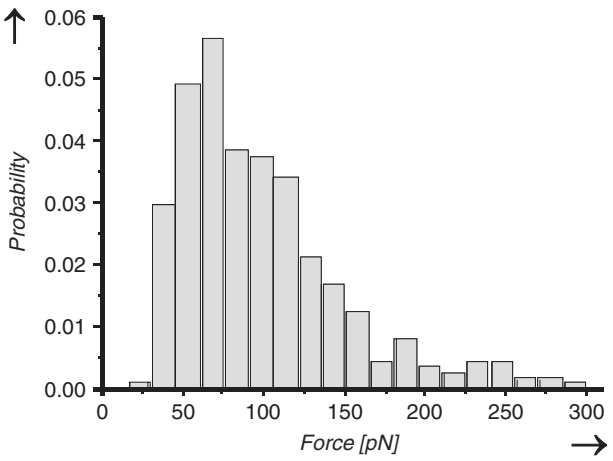

Figure 5 Force histogram of an AFM experiment with peptide **3**.

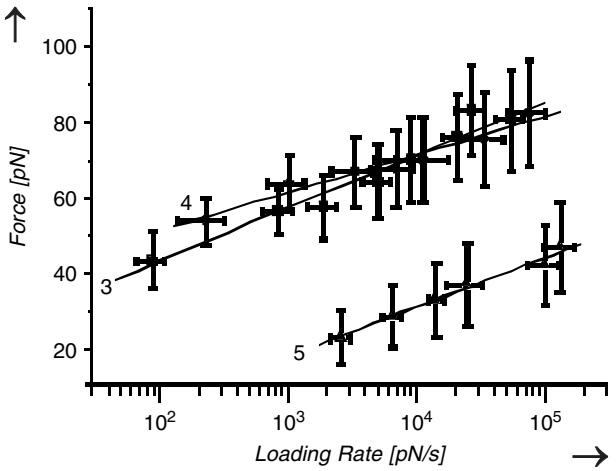

Figure 6 Dynamic force spectroscopy with peptides **3**–**5** by variation of the loading rate, allowing the determination of k_{off}, τ, and x_β values (Table 1).

is usually observed for protein–DNA interaction. Additionally, a molecular reaction length parameter x_β can be obtained from the inverse slope of the linear data fit (Figure 6; Table 1). The value for x_β corresponds to the location of the final activation barrier along the reaction coordinate. For peptide PhoB(190–209) R193A **4**, which exhibits the longest complex lifetime among the peptides examined, the largest value of x_β was found (Table 1). This suggests the possibility for the complex to re-associate (assuming microscopic reversibility) over a larger distance along the reaction coordinate. Further experiments will have to show whether this also would imply the predominance of unspecific binding.

The data compiled here show that the specific interaction of synthetic peptides comprising only the recognition helix of a transcription activator with DNA can be investigated at the single-molecule level. The molecular binding forces observed for single peptide–DNA complexes upon induced dissociation not only provide k_{off} values, but the experimental setup also allows competition studies and a direct affinity ranking of synthetic peptides with single-point mutations.

Acknowledgements

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