Detailed studies of the binding mechanism of the *Sinorhizobium meliloti* transcriptional activator ExpG to DNA

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The exopolysaccharide galactoglucan promotes the establishment of symbiosis between the nitrogen-fixing Gram-negative soil bacterium *Sinorhizobium meliloti* 2011 and its host plant alfalfa. The transcriptional regulator ExpG activates expression of galactoglucan biosynthesis genes by direct binding to the expA1, expG/expD1 and expE1 promoter regions. ExpG is a member of the MarR family of regulatory proteins. Analysis of target sequences of an ExpG(His)_6 fusion protein in the exp promoter regions resulted in the identification of a binding site composed of a conserved palindromic region and two associated sequence motifs. Association and dissociation kinetics of the specific binding of ExpG(His)_6 to this binding site were characterized by standard biochemical methods and by single-molecule spectroscopy based on the atomic force microscope (AFM). Dynamic force spectroscopy indicated a distinct difference in the kinetics between the wild-type binding sequence and two mutated binding sites, leading to a closer understanding of the ExpG–DNA interaction.

**INTRODUCTION**

The Gram-negative soil bacterium *Sinorhizobium meliloti* has the ability to fix molecular nitrogen in a symbiotic interaction with plants of the genera *Medicago*, *Melilotus* and *Trigonella*. The initiation of this symbiosis is a highly specific and complex developmental process, in which both partners undergo differentiation in a concerted way. In the early stages of this interaction, flavonoids in the root exudates induce the production of Nod factors by the bacteria which, in turn, trigger a developmental programme leading to the formation of root nodules (reviewed by Long, 2001; Spaink, 2000). The bacteria infect the nodule through infection threads and colonize the cytoplasm of plant cells. Once inside the plant cell, the bacteria differentiate into nitrogen-fixing bacteroids (reviewed by Oke & Long, 1999).

Bacterial exopolysaccharides (EPSs) are important for nodule infection. *S. meliloti* is able to synthesize two acidic EPSs, succinoglycan (EPS I) and galactoglucon (EPS II). Infection of *Medicago sativa* root nodules by *S. meliloti* depends on low-molecular-mass forms of EPS I or EPS II (Glazebrook & Walker, 1989; Gonzalez et al., 1996; Wang et al., 1999). EPS II is composed of alternating glucose and galactose residues which are decorated by acetyl and pyruvyl groups (Her et al., 1990). The biosynthesis of EPS II is directed by the 30 kb exp gene cluster, containing 22 genes organized in four operons (Becker et al., 1997; Rüberg et al., 1999).

Under standard culture conditions in a complex medium, wild-type strain *S. meliloti* 2011 synthesizes EPS I and only traces of EPS II. The biosynthesis of EPS II is increased by phosphate-limiting conditions (Zhan et al., 1991) or a mutation in either of the regulatory genes mucR (Keller et al., 1995; Zhan et al., 1989) and expR (Glazebrook & Walker, 1989; Peloń et al., 2002), which are unlinked to the exp gene cluster. Extra copies of the regulatory gene expG located in the exp gene cluster (Astete & Leigh, 1996; Becker et al., 1997; Rüberg et al., 1999) stimulate transcription of the expA, expD and expE operons (Rüberg et al., 1999). Under phosphate-limiting conditions the enhanced transcription of these operons requires expG, implying that ExpG acts as a transcriptional activator of exp gene expression (Astete & Leigh, 1996; Rüberg et al., 1999).

ExpG was grouped into the MarR family of regulatory proteins (Becker et al., 1997). Like many other transcriptional regulators MarR-type regulators bind DNA through a helix–turn–helix (HTH) motif (Cohen et al., 1993; Sulavik et al., 1995). An assortment of biological functions, e.g. the expression of resistance to multiple antibiotics, detergents

**Abbreviations:** AFM, atomic force microscopy; EMSA, electrophoretic mobility shift assay; EPS, exopolysaccharide; EPS I, succinoglycan; EPS II, galactoglucon; HTH, helix–turn–helix; k_on, on-rate; k_off, off-rate; K_d, dissociation constant.

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and oxidative stress agents, organic solvents and pathogenic factors, is controlled by members of the MarR family (Aleksun & Levy, 1998; Miller & Sulavik, 1996). Most members act as repressors and only a few as activators (Egland & Harwood, 1999; Komeda et al., 1996; Oscarsson et al., 1996). Recently, we showed that ExpG itself exerts positive regulation of exp gene expression by binding to the expA1, expG/expD1 and expEl promoter regions in the exp gene cluster (Bartels et al., 2003).

In this paper we describe three distinct DNA sequence elements of the ExpG binding sites and their contribution to the specific binding process. Association and dissociation kinetics were characterized by ensemble and single-molecule methods.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *Escherichia coli* SURE (Young & Davis, 1983), used as expression strain, was cultivated in SB (Super broth) medium (32 g tryptone l⁻¹, 20 g yeast extract l⁻¹ and 5 g NaCl l⁻¹, adjusted to pH 7.6) at 37°C. Plasmid pHisGC31 was constructed by insertion of a 589 bp EcoRI–EclI6II fragment carrying expG of *S. meliloti* 2011 (Casse et al., 1979) into vector pEXP2 (MBBL, Bielefeld, Germany). This fragment was amplified by PCR using the primers expGEXP2.5 (5'-AAAA-GAATTCCAAAAACACAGATTACTCTATCCG) and HisGEXP2.3 (5'-AAAAGAGGCTCTCAGtgattgttgtgtgtgtgtgATCGGGTAGCCTCGGCGG) (EcoRI and EclI6II restriction sites are underlined, the antisense sequence of the stop codon is printed in bold face and antisense His codons are in lower case) and pAR11s as template (Becker et al., 1997). Expression of the hybrid gene resulted in a fusion protein consisting of ExpG from Asn-2 to Ile-190, with a (His)n C-terminal tag and ten additional N-terminal amino acids (MAIFEMLRNS).

**Proteins.** Expression of recombinant ExpG(His), fusion protein was performed essentially as described previously (Bartels et al., 2003). Purification was carried out by Ni-NTA affinity chromatography (Qiagen). Purified fusion protein was concentrated using an Ultrafree 4 centrifugal concentrator (Millipore), resuspended in buffer (250 mM NaCl, 10 mM Tris, 1 mM DTT and 50% v/v, glycerol) and stored at −20°C. The concentration of purified protein was determined by using the Bio-Rad Protein Assay (Bradford, 1976).

**DNA fragments.** DNA fragments I, II and III (see Fig. 2a) and competitor fragments for electrophoretic mobility shift assays (EMSAs) were generated by PCR as described previously (Bartels et al., 2003). Following hybridization of the oligonucleotides (Fig. 2b) and their respective antisense oligonucleotides (synthesized by Qiagen) as described by Bertram-Drogatz et al. (1998), the double-stranded hybridization products were inserted into pUC18 (Yanisch-Perron et al., 1985). The resulting plasmids were used as templates for amplification of fragments KF-A1c, d, e, f and h, KF-Ge and KF-E1e by PCR (for fragment lengths, see Fig. 2b) employing primers M13uni (5’-CGCCAGGGTTTCCAGTCACGC-3’) and M13rev (5’-AGGCGATAACATTTTCAACAGGA-3’). These plasmids were also used for amplification of DNA fragments KF-A1c, f, g and h for atomic force microscopy (AFM) force spectroscopy employing primers M13uni (see above) and 5’SH-labelled primer M13rev (see above). DNA fragments for AFM imaging were amplified by PCR with primer ExpG1 (5’-AAAACCTAAGATGTCTTTATACCGG-3’) and M13uni (see above) from pPGG3/41 (pUC18 carrying the 348 bp EcoRI–HindIII fragment that comprises the intergenic region between expD1 and expG).

**EMSA analysis.** Cy3-labelled DNA fragments I, II and III (see Fig. 1a) in various concentrations were mixed with purified ExpG(His)n (0.013 µg µl⁻¹) in a reaction buffer containing 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.1 mM MgSO₄, 5% glycerol, 0.05 mg sonicated herring testis DNA ml⁻¹ and 0.5 mg bovine serum albumin ml⁻¹ and were subjected to EMSAs (Bartels et al., 2003). In competition assays, protein was added to Cy3-labelled DNA fragments in the presence of various concentrations of competitor DNA fragments. After incubation at 20°C for 15 min, the reaction was loaded onto a 2% non-denaturing agarose gel prepared in gel buffer (40 mM Tris base, 10 mM sodium acetate and 1 mM EDTA adjusted to pH 7.8 with acetic acid) and electrophoresed at 4°C in gel buffer at 4.5 V cm⁻¹ for 2 h. Gel images were acquired using a Typhoon 8600 Variable Mode Imager (Amersham Bioscience).

**Determination of on- and off-rates.** Association kinetics for the binding reactions of ExpG(His)n to fragments I, II and III (Fig. 2a) were followed by loading samples taken after various time intervals between 0 and 15 min from a standard assay on an already electrophoresing agarose gel (Fried & Brothers, 1981; Lane et al., 1992). For determination of the on-rate (k_on) of the reaction, initial rate data were evaluated by calculating k_on = [dI/(P–N)] ln[N(P–PN)/(PN–PN)]/dt (Lane et al., 1992), where P is the concentration of free protein, PN the concentration of the protein–DNA complex, N the concentration of free DNA substrate, and t the time of sampling. The kinetics of dissociation of ExpG(His)n from fragments I, II and III (Fig. 2a) were investigated by adding a 50-fold excess of unlabelled competitor fragment to a mixture containing protein–DNA complexes and determining the degree of competition after various time intervals, as described above. The off-rate (k_off) was determined by calculating k_off = −d[PN(PN–P)]/dt (Lane et al., 1992), where PN is the concentration of the protein–DNA complex at sampling point after the addition of competitor, PN0 represents the concentration of the protein–DNA complex directly before the addition of competitor, and t is the time of sampling. The dissociation constant (K_d) of the protein–DNA complex was calculated as the ratio of the off-rate (k_off) over the on-rate (k_on) (Bisswanger, 1994; Lane et al., 1992). Experiments to determine the on- and off-rates were repeated three times.

**HPLC gel-permeation chromatography of ExpG(His).** Size distribution of ExpG(His)n was measured by gel-permeation chromatography on a TSK Gel G2000SW column (TosoHaas) with a flow rate of 0.5 ml min⁻¹ (elucent: 50 mM sodium phosphate buffer, pH 7.0). Calibration was performed with an LMW Gel Filtration Calibration Kit (Amersham Biosciences). Protein absorbance was measured at 280 nm.

**AFM imaging.** DNA fragments for AFM imaging consisted of 158 bp of the expG promoter region and a non-binding sequence of 990 bp. DNA (0.2 ng µl⁻¹) and ExpG protein (0.2 ng µl⁻¹) were mixed in buffer solution (50 mM NaCl, 4 mM NiCl₂, pH 8.3) and left to incubate for 5 min before being brought to a freshly cleaved mica surface (Procav AG, Balzers, Liechtenstein) which was then immediately installed under the AFM liquid cell. The concentration of Ni²⁺ counter-ions was optimized to yield a flexible immobilization of the DNA to the mica surface by electrostatic attraction (Hansma & Laney, 1996). Protein–DNA complexes were investigated at 25°C with a commercial AFM (Multimode, Veeco Instruments) in tapping mode, using oxide-sharpened Si₃N₄ cantilevers (Veeco Instruments) at a resonance frequency of about 28 kHz. Images were taken at a scan rate of 2 Hz, while the setpoint was kept at 0.2 V. Amplitude and phase were recorded simultaneously (from the signal in trace/retrace direction) to distinguish between DNA and protein (Lysetska et al., 2002).

**Sample surface and AFM tip modification.** For force spectroscopy measurements, sample surfaces and AFM tips were prepared involved.
ExpG(His)6 fusion protein was expressed and purified in competition experiments. For this purpose, an ExpG in moter regions in the protein is required for binding of the ExpG. Modified surfaces were stable for at least 2 days if stored at 4°C for 1 h at 4°C, with the calculated molecular mass of 23 kDa which was detected using an anti-His-tag antibody (data not shown). This corresponds well with the calculated molecular mass of 23-2 kDa. An apparent molecular mass of the ExpG(His)6 protein of 44-4 kDa in a 50 mM sodium phosphate buffer (pH 7-0) in non-denaturing conditions was determined by gel permeation chromatography (data not shown). This major peak probably represented a dimer. In addition to this peak, three higher molecular mass forms that may include a tetramer were detected in lower concentrations. This leads to the speculation that ExpG forms dimers and binds to its target DNA at least as a dimer.

The topography of the binding site was investigated by AFM in buffer solution. The 158 bp promoter region of expG (Bartels et al., 2003) was extended by a 990 bp non-binding sequence at one end, resulting in a 390 nm DNA fragment suitable for AFM imaging. In accordance with this experimental setup, bound proteins were observed at only one end of a given DNA fragment, confirming the binding to the promoter region. Furthermore, AFM revealed a change in DNA conformation during the process of unbinding (Fig. 1), with a different curvature of the promoter region. Such a structural change was a recurring motif and has been observed for at least eight different DNA fragment–protein complexes. Proteins binding at other DNA sites or without structural transition have not been observed. Although the unbound and bound state was not directly observed in reverse order, it can be assumed that the DNA acquires its characteristic bend during the formation of the protein–DNA complex.

A conserved 21 bp region with a palindromic sequence which may constitute the binding site of ExpG was recently found in the promoter regions of expA1, expG, expD1 and expE1 (Bartels et al., 2003; Lloret et al., 2002). In addition to this conserved sequence two further regions in the exp promoter fragments, box 1 and box 2, share similarities (Fig. 2b). Eight different competitor fragments (Fig. 2b) were designed to test the importance of these two boxes and the palindromic sequence for binding of ExpG(His)6. The double-stranded hybridization products from 28 bp to 80 bp (see Methods and Fig. 2b) were not effective competitors in EMSA experiments. To exclude the possibility that the competitor fragments were too short for protein binding, although they may carry the specific binding-site sequence, these fragments were cloned into the pUC18 vector. Flanking sequences derived from the pUC18 vector added 102 bp to the specific sequences from the exp promoter regions so that the fragments measured from 130 bp to 182 bp (Fig. 2b).

Competitor fragments KF-A1e, g and h, KF-Ge and KF-E1e contained the three motifs of the expA, ExpG/expD and expE promoter regions, respectively. Fragments KF-A1e (Fig. 3a), KF-Ge (Fig. 3b) and KF-E1e (Fig. 3c) with the wild-type sequence were effective competitors for the binding of ExpG(His)6 to DNA fragments I, II and III containing the expA, ExpG/expD and expE promoter regions, respectively. KF-A1d, containing only the conserved palindromic region (Fig. 2b) did not compete out binding of ExpG(His)6 (Fig. 3a). This was also the case for fragment KF-A1c, which

**RESULTS AND DISCUSSION**

**A palindromic sequence in the exp promoter regions is required for binding of the ExpG protein**

Recently, we demonstrated the binding of ExpG to promoter regions in the exp gene cluster (Bartels et al., 2003). To localize the DNA region recognized by ExpG more precisely, we tested the specificity of DNA binding by ExpG in competition experiments. For this purpose, an ExpG(His)6 fusion protein was expressed and purified by Ni-NTA affinity chromatography. In SDS-PAGE the purified protein exhibited a major band migrating at approximately 23 kDa which was detected using an anti-His-tag antibody (data not shown). This corresponds well with the calculated molecular mass of 23-2 kDa. An
**Fig. 1.** AFM reveals a change in DNA conformation during unbinding of the protein–DNA complex. DNA fragments 390 nm long, containing the expG promoter region at one end, were imaged in buffer solution (50 mM Tris, 25 mM NaCl, 4 mM NiCl₂, pH 8.3) in the presence of ExpG(His)₆ proteins. Images on the left show the topography (z range 3-5 nm), while the corresponding phase information (z range 8-0°) is presented on the right. Height and phase signal were recorded simultaneously to distinguish between protein and DNA. The protein exhibits a negative phase shift (i.e. appears darker in the phase image) while the DNA shows a positive phase shift (i.e. appears brighter) (Lysetska et al., 2002). Images (a) and (b) show two DNA fragments (probably stabilizing each other by the medium of counter-ions), one of which carries a bound protein. When the protein (red arrow) breaks away (time between images: 9 min), the curvature of the DNA binding region changes (c and d). The bound state and the position of the two DNA fragments are shown in cartoon form in (e).

**Fig. 2.** (a) The exp gene region from expA1 to expE2 (10 330 nt) of the exp gene cluster of S. meliloti 2011 (Casas et al., 1979). Transcriptional units are marked by arrows. I, II and III are DNA fragments for EMSAs. Residues identical in the expA1, expG and expE1 promoter regions are boxed and shaded. Mutations in box 1, the conserved palindrome region and box 2 are in bold. Inverted arrows indicate a palindrome found within the conserved region. AGCT, HindIII overhang; AATT, EcoRI overhang. *Fragment length after amplification by PCR; length of the native S. meliloti sequence is given in parentheses. See Fig. 3.
included box 1, the palindrome region and 3 bp of box 2 (Figs 2b and 3a), suggesting that at least the palindrome region and box 2 are required for binding of ExpG.

Exchange of five nucleotides in the palindrome of the KF-A1e fragment resulted in fragment KF-A1f (Fig. 2b), which did not affect binding of ExpG(His)$_6$ to the wild-type sequence in the competition experiments (Fig. 3a). This indicates that the conserved palindromic sequence is essential for binding of ExpG(His)$_6$. Neither a mutation in box 1, KF-A1g (Fig. 2b), nor a mutation in box 2, KF-A1h (Fig. 2b), completely blocked binding of ExpG(His)$_6$ in the competition experiment (Fig. 3a). However, competition with fragments KF-A1c, g and h resulted in an incomplete shift (Fig. 3a), indicating a lower binding affinity of ExpG(His)$_6$ to these fragments. An EMSA using the eight specific competitor fragments as Cy3-labelled DNA fragments confirmed that the palindrome is essential for binding of ExpG(His)$_6$. ExpG(His)$_6$ completely reduced the electrophoretic mobility of each fragment except for the KF-A1f fragment, which contains the mutated palindrome (Fig. 4). Nevertheless, the failure of the fragments containing only the palindrome region (KF-A1d) or the palindrome region and box 1 (KF-A1c) in competition showed that the additional motifs box 1 and box 2 influence binding of ExpG(His)$_6$ as well. Possibly the change of 1 or 2 bp in box 1 and box 2 was not sufficient to destroy the function of these motifs.

The above results were confirmed by AFM force spectroscopy experiments. In this direct approach, binding of the ExpG(His)$_6$ protein to different DNA fragments can be observed on a single-molecule basis (Bartels et al., 2003). By covalently attaching the binding partners to the AFM tip and the sample surface, respectively, unbinding forces of the protein–DNA complex can be measured during multiple approach–retract cycles of tip and surface. The unbinding forces under a single retract velocity show a nearly Gaussian distribution around the most probable unbinding force (Fig. 5). Again, mutations in the palindrome (fragment KF-A1f) led to no recognizable binding (Fig. 5b), whereas the fragments with mutations in box 1 (KF-A1g) or box 2 (KF-A1h) reached almost the same binding probability as the wild-type fragment (KF-A1e).

The function and structure of DNA-binding transcription factors of the MarR family are well investigated but little is known about their binding sites. We were able to narrow down the region required for binding of ExpG(His)$_6$ to a 63 bp region for the expA$_1$ promoter, a 62 bp region for the expG promoter, and a 72 bp region for the expE$_1$ promoter. We suggest that the 21 bp conserved sequence within the different exp promoter regions is the core region required for the binding of an ExpG dimer and the additional motifs box 1 and/or box 2 enable the complete DNA–protein interaction. A similar situation was suggested for LysR-type regulators (LTTR). A typical LTTR
binds to a sequence of approximately 50–60 bp, which contains two distinct sites, a recognition-binding site (RBS) recognized primarily by the LTTR, and an activation-binding site (ABS) (Schell, 1993). The long DNA sequence stretch containing the RBS and ABS and the size range of active LTTRs suggest that in vivo these regulators are multimeric and bind probably as dimers or tetramers (Henikoff et al., 1988).

ExpG, which contains a HTH-MarR motif at the C-terminus (residues 70-164), is a member of the MarR family, which belongs to a supergroup of eight regulator families sharing a conserved extended sequence including the classical HTH motif (Perez-Rueda & Collado-Vides, 2001). The HTH motif is one of the most common DNA-binding motifs in proteins that control transcription

![Fig. 5. Single-molecule force spectroscopy. Unbinding of the protein-DNA complex was measured in buffer solution (50 mM Tris/HCl, 100 mM NaCl, 0.1 mM NiCl₂, pH 8.3) for the ExpG protein and different DNA fragments: the wild-type sequence (KF-A1e) and fragments with mutations in the palindrome (KF-A1f), box 1 (KF-A1g) or box 2 (KF-A1h). Evidently, the palindrome was necessary for binding (b), while mutations in the box 1 and box 2 regions only slightly reduced the probability of binding (c–d) with respect to the wild-type sequence (a).](image)

![Fig. 6. EMSA with purified ExpG(His)₆ fusion protein of increasing concentration and DNA fragment II (see Fig. 2a). DNA was added to all reactions to give a final DNA concentration of 3.7 ng μl⁻¹.](image)

![Fig. 7. On-rates (k_on) of the (a) ExpG–expD/expG, (b) ExpG–expE and (c) ExpG–expA protein–DNA complexes: association kinetics, represented by a plot of 1/(P–N) ln[N(P–PN)/P(N–PN)] versus time. The ExpG protein concentration in the assay was calculated to be (a) 2.8 × 10⁻⁸ M, (b) 4.5 × 10⁻⁸ M and (c) 5.6 × 10⁻⁸ M. DNA was added to give the following final concentrations: (a) 3.64 × 10⁻⁸ M, (b) 1.6 × 10⁻⁸ M and (c) 4.68 × 10⁻⁸ M. The slope represents k_on. The insets show the original data obtained from an EMSA.](image)
initiation (Sauer et al., 1982). In repressor proteins the HTH binding motif is predominantly situated at the N-terminus, whereas activators mainly contain this motif at the C-terminus (Perez-Rueda & Collado-Vides, 2001). This observation is in agreement with the C-terminal position of this motif in the transcriptional activator ExpG.

### Binding kinetics of the ExpG–DNA complexes

With this competition assay available, we aimed to determine the on- and off-rates of the ExpG(His)$_6$–DNA complexes of the expD/expG, expE1 and expA1 promoter fragments (see Methods). To determine the protein concentration for analysis of the binding kinetics we carried out EMSAs with increasing protein concentrations and DNA fragment II (Fig. 6), DNA fragment I and DNA fragment III (data not shown). Only at a protein concentration of 0.28 μg μl$^{-1}$ (1.2 × 10$^{-5}$ M) was the electrophoretic mobility of the protein–DNA complex more strongly reduced in comparison to the lower protein concentrations. This may indicate the formation of a protein tetramer–DNA complex compared to a protein dimer–DNA complex that is probably formed at lower protein concentrations (Fig. 6). The protein concentration used in the EMSAs to investigate the binding kinetics of ExpG and the different exp promoter fragments was in the range 6.5 × 10$^{-4}$ to 0.013 μg μl$^{-1}$ (2.8 × 10$^{-8}$ M to 5.6 × 10$^{-7}$ M).

On-rates ($k_{on}$) measured for the binding reaction of ExpG(His)$_6$ to the different promoter fragments (Fig. 7) are summarized in Table 1. Since the results of the GPC analysis support the assumption that ExpG binds the DNA as a dimer we also calculated the on-rates for this DNA–dimer protein interaction (Table 1).

The dissociation kinetics of the ExpG(His)$_6$–DNA complexes were investigated by using the unlabelled fragments I, II and III (Fig. 2a) as competitors (Fig. 8). This revealed off-rates ($k_{off}$) at the lower limit of the mean off-rate $k_{off} = (1.2 ± 1.0) × 10^{-3}$ s$^{-1}$ for all three DNA target sequences which were previously obtained by AFM force spectroscopy experiments (Bartels et al., 2003). From the on- and off-rates the different dissociation constants $K_d$ were estimated for the complexes between the ExpG(His)$_6$ protein and the corresponding promoter fragments (Table 1). Many other transcriptional regulators, e.g. PcaU, MucR or Lrp, are characterized by quite similar dissociation constants in the nanomolar range (Bertram-Drogatz et al., 1997; Calvo & Matthews, 1994; Popp et al., 2002).

#### Table 1. On-rates ($k_{on}$), off-rates ($k_{off}$) and dissociation constants ($K_d$) of the ExpG(His)$_6$–DNA complexes

<table>
<thead>
<tr>
<th>Promoter region</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>expD/expG</td>
<td>1.7 × 10$^5$</td>
<td>3.7 × 10$^3$</td>
<td>5.4 × 10$^{-9}$</td>
</tr>
<tr>
<td>expE1</td>
<td>1.3 × 10$^5$</td>
<td>5.0 × 10$^3$</td>
<td>2.2 × 10$^{-9}$</td>
</tr>
<tr>
<td>expA1</td>
<td>5.0 × 10$^4$</td>
<td>1.0 × 10$^3$</td>
<td>1.3 × 10$^{-9}$</td>
</tr>
</tbody>
</table>

#### Fig. 8. Off-rates ($k_{off}$) of the (a) ExpG–expD/expG, (b) ExpG–expE and (c) ExpG–expA protein–DNA complexes: dissociation kinetics, represented by a plot of $-\ln(PN/PO)$ versus time. The ExpG protein concentration in the assay was calculated to be (a) 2.8 × 10$^{-7}$ M, (b) and (c) 5.6 × 10$^{-7}$ M. The slope represents $k_{off}$. DNA was added to give the following final concentrations: (a) 3.64 × 10$^{-8}$ M, (b) 1.06 × 10$^{-7}$ M and (c) 4.68 × 10$^{-8}$ M. The insets show the original data obtained from an EMSA. The data obtained from the sample taken at 0 min (directly before the addition of competitor) was used to calculate $PN_0$. 

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We investigated the details of ExpG(His)$_6$ binding by single-molecule AFM force spectroscopy under different retract velocities. By varying the time-dependency of the external forces, i.e. the loading rate (loading rate = retract velocity × molecule elasticity), natural thermal off-rates can be measured (Bartels et al., 2003; Evans & Ritchie, 1997; Merkel et al., 1999; Schwegsinger et al., 2000; Strunz et al., 1999). Dynamic force spectroscopy (Fig. 9) revealed a distinct difference in the kinetics between the wild-type sequence (KF-A1e) and its two mutated but still binding derivates (KF-A1g and KF-A1h). When the unbinding forces are plotted against the corresponding loading rates on a logarithmic scale, the experimental data can be fitted to a linear function according to the formula given by Strunz et al. (1999):

\[ F = \frac{k_B T}{x_\beta} \ln \left( \frac{x_\beta r}{k_B T k_{\text{off}}} \right) \]

where \( F \) is the most probable unbinding force, \( k_B T = 4\cdot114 \text{ pN nm} \) (at 298 K) is a Boltzmann factor, \( x_\beta \) is the distance between the minimum of the potential well of the bound state and the maximum of the energy barrier separating the bound state from the free state along the reaction coordinate, \( r \) is the loading rate, and \( k_{\text{off}} \) is the thermal off-rate under zero load. The natural thermal off-rate \( k_{\text{off}} \) can be derived by extrapolating the linear fit to the state of zero external force.

We measured an off-rate \( k_{\text{off}} = (4\cdot4 \pm 2\cdot5) \times 10^{-4} \text{ s}^{-1} \) for the binding of ExpG(His)$_6$ to the wild-type DNA fragment, but \( k_{\text{off}} = (5\cdot3 \pm 1\cdot5) \times 10^{-3} \text{ s}^{-1} \) for fragment KF-A1h with a mutation in box 2 and even \( k_{\text{off}} = (1\cdot3 \pm 0\cdot2) \times 10^{-2} \text{ s}^{-1} \) for fragment KFA1g with a mutation in box 1. Thus, mutations in the box 1 and box 2 regions resulted in DNA sequences still capable of binding to ExpG(His)$_6$ but at the expense of a higher off-rate (i.e. a lower lifetime of the bond). We reason that the motifs box 1 and box 2 complement the 21 bp core region by fulfilling an important structural function in the binding of the ExpG(His)$_6$ protein, namely to stabilize the protein–DNA complex.

**Conclusions**

Our experiments demonstrate that the palindromic sequence within the expA promoter region is essential for binding of ExpG(His)$_6$ and suggest that the flanking sequence elements box 1 and box 2 contribute significantly to an efficient DNA–protein interaction. These findings were not only confirmed by standard ensemble methods, but also supported by data from AFM single-molecule force spectroscopy. AFM imaging explored the topography of the binding site in buffer solution conditions and suggests a change in DNA conformation upon binding of ExpG(His)$_6$. The dissociation constants \( K_d \) determined for the complex of ExpG(His)$_6$ and the corresponding promoter fragments are in good agreement with \( K_d \) values of other transcriptional regulators. The \( k_{\text{off}} \) rates obtained by the EMSA competition assay are at the lower limit of the mean off-rate for the expA1, expD/expG and expEI promoter regions previously measured by AFM force spectroscopy using a N-terminal (His)$_6$ExpG fusion protein (Bartels et al., 2003). This makes it unlikely that the DNA-binding activity was severely affected in the same way by both tags. Furthermore, dynamic force spectroscopy reveals a distinct difference in the kinetics of the wild-type binding sequence and the fragments containing mutations within the box 1 and box 2 motifs. Whereas the EMSA experiments detected binding or non-binding of the DNA–protein complex, the analysis at single molecule level showed that the mutated sequences of box 1 and box 2 lead to a higher off-rate. The ExpG(His)$_6$ binding sites characterized in this study overlap with the putative PHO boxes previously predicted in the exp promoter regions (Ruberg et al., 1999), suggesting an interference of PhoB and ExpG binding in the regulation of exp promoter activities.
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