The EMBO Journal Vol.18 No.22 pp.6472–6480, 1999

Hinge-helix formation and DNA bending in various *lac* repressor–operator complexes

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The hinge-region of the *lac* repressor plays an important role in the models for induction and DNA looping in the *lac* operon. When *lac* repressor is bound to a tight-binding symmetric operator, this region forms an α-helix that induces bending of the operator. The presence of the hinge-helices is questioned by previous data that suggest that the repressor does not bend the wild-type operator. We show that in the wild-type complex the hinge-helices are formed and the DNA is bent, similar to the symmetric complex. Furthermore, our data show differences in the binding of the DNA binding domains to the half-sites of the wild-type operator and reveal the role of the central base-pair of the wild-type operator in the repressor–operator interaction. The differences in binding to the operator half-sites are incorporated into a model that explains the relative affinities of the repressor for various *lac* operator sequences that contain left and right half-sites with different spacer lengths.

Keywords: cooperativity/DNA bending/*lac* operator/*lac* repressor/NMR

Introduction

The *Escherichia coli lac* operon is now a well understood example of gene expression and its regulation. The *lac* repressor is the protein responsible for switching the expression of the *lac* genes on or off. The tetrameric repressor can be viewed as a dimer of dimers, where each dimer can bind one *lac* operator DNA sequence with its two DNA binding domains or headpieces. The headpieces are connected to the inducer binding core domain via a hinge-region, which is known to play a crucial role in the induction of the *lac* operon. X-ray and NMR structural studies have shown the formation of α-helices (residues 50–58) in the hinge-region when *lac* repressor binds to a symmetrical *lac* operator [the 22 bp SymL(–1) operator, Figure 1] (Lewis *et al.*, 1996; Spronk *et al.*, 1996, 1999). The hinge-helices interact in the minor groove of the SymL(–1) operator and cause a bending of ~45° in the center of the operator. When an inducer molecule binds to the core domain this induces a separation of the two hinge-helices, causing them to unfold and the affinity for *lac* operator to decrease.

The bending of *lac* operator by *lac* repressor plays an important role in a model proposed for DNA looping and the *lac* repressor–CAP interaction (Lewis *et al.*, 1996), where it presumably facilitates the formation of the DNA loop. However, one of the objections raised against this DNA looping model is that previous data indicate that the wild-type operator is not bent by *lac* repressor (Wu and Crothers, 1984; Zinkel and Crothers, 1987; Fried and Hudson, 1996; Lewis, 1996; Perros and Steitz, 1996), although protein-induced DNA bending of this operator has been suggested by Adhya and co-workers (Zwieb *et al.*, 1989). The presumed absence of repressor-induced DNA bending further questions the role of the hinge-helices in DNA binding in the wild-type complex and therefore the validity of the model describing the induction of the wild-type *lac* operon (Lewis *et al.*, 1996; Spronk *et al.*, 1996).

In contrast, the effect of changes in the spacing of the *lac* operator half-sites in *in vivo* affinity studies suggests that interaction between the hinge-regions of the two headpieces does contribute to the binding of repressor to the wild-type operator (Betz *et al.*, 1986; Sasmor and Betz, 1990). When *lac* repressor binds to operators containing two right half-sites, the affinity of the repressor for the DNA increases with increased spacing between the binding sites. The increase in affinity is largest when the spacing is 1 bp larger than that in the wild-type operator, yielding the 24 bp SymR(+1) operator (Figure 1). Operators that contain two left halves of the wild-type operator show the opposite effect: the affinity for repressor is increased when the spacing between the two binding sites is smaller than the wild-type spacing. Furthermore, when the right half-site of the wild-type operator is replaced by the sequence of the left half-site the affinity for the repressor is lowered (Betz *et al.*, 1986; Sasmor and Betz, 1990). This suggests decreased cooperativity between the headpieces since the intrinsic affinity for the left half-site is higher than for the right half-site (Horton *et al.*, 1997). In the complex of the isolated headpieces (headpiece 62, HP62) with the SymL(–1) operator this binding cooperativity is caused by the formation of hinge-helices, which are stable only when protein–protein interactions between the two hinge-regions in a dimeric unit can occur (Spronk *et al.*, 1996). To elucidate the structural basis of the relative *in vivo* affinities of the repressor for the differently spaced *lac* operator half-sites we have measured relative *in vitro* binding affinities and repressor-induced DNA bending. In addition, we have investigated the presence of the hinge-helices in complexes of HP62 with the wild-type and SymR(+1) operators. Furthermore, we have used NMR spectroscopy to address the effect of replacing a left half-site by a right half-site on the binding

Fig. 1. The *lac* operator sequences used for the present NMR studies. For the two symmetric operators the spacing of the two half-sites relative to the wild-type operator is indicated in parentheses.

affinity by investigating complexes of HP62 with operators derived from the right half-site of the wild-type operator (Figure 1).

The results we have obtained in the present study are consistent with a model where a proper spacing of the headpieces on the operator contributes to binding affinity by allowing protein–protein interactions to occur. Protein–protein interactions favor the formation of the hinge-helices and their interactions with the minor groove of the DNA. The opposite effect of the spacing of the binding sites on the affinities of the repressor for operators containing two left- or right-halves can be explained by a shift in the binding position of the headpieces towards the center of the operator when they bind to the right half-site. The latter result contrasts with previous suggestions that the binding of the headpieces to the right half-site occurs further away from the center of the operator (Horton *et al.*, 1997).

Results

DNA bending and relative in vitro affinities

We have probed the protein-induced DNA bending using the circular permutation method of Wu and Crothers (1984) on complexes of a variety of differently spaced *lac* operators derived from the wild-type, SymL(–1) and SymR(+1) sequences (Table I). In addition, we have used heterologous competitive binding- and electrophoretic mobility shift assays to determine the relative affinities of the repressor for the different operators. All experiments were performed with both the HP62 construct and the intact *lac* repressor. Control experiments were performed using operators where half-sites are scrambled, yielding the L11, R11 or the non-operator DNA (NOD) sequences.

As shown in Figure 2, and in agreement with the *in vivo* affinities determined by Sasmor and Betz (1990), the highest relative affinities of the repressor are found for the SymL(–1) and wild-type operators, while a 2- to 3-fold lower affinity was found for the SymR(+1) operator (Figure 2A and B; Table I). All other sequences show low or negligible affinities compared with the wild-type sequence, as determined in competition experiments or in binding affinity experiments (Figure 2C; Table I). Similar results were obtained in competitive binding experiments using HP62 (data not shown). Figure 3 shows the results of the bending experiments on the SymL(–1), wild-type and SymR(+1) operators with both HP62 and intact *lac* repressor. It is clear that intact *lac* repressor bends all three operators, whereas HP62 only bends the SymL(–1) and wild-type operators. No detectable binding of HP62 to the SymR(+1) operator was observed. These results suggest that the core domain of the intact repressor modulates the interaction of the headpieces with the operator, presumably by facilitating the proper positioning of the headpieces on the operator. The effect is seen in both the SymR(+1) operator as well in the spacing derivatives of the wild-type, SymL(–1) and SymR(+1) operators. It is interesting to note here that in the homologous purine repressor inter-domain hydrogen bonds between the core and the DNA binding domains are required for the stability of the hinge-helices (Schumacher *et al*., 1994; Lu *et al*., 1998) and the isolated DNA binding domains do not bind specifically to the *pur* operator (Nagadoi *et al*., 1995). Although the isolated headpieces of the *lac* repressor do bind the high affinity *lac* operators specifically, extra stability of the hinge-helices in the intact *lac* repressor–operator complex may be acquired by similar inter-domain contacts as seen in the *pur* repressor–operator complex. The data from the bending experiments listed in Table I further suggest that the *lac* repressor appears to accommodate different spacings in the SymR sequences more easily than those in the SymL sequences, which may be explained by the less tight binding of the headpieces to the right half-site as compared with the binding to the left half-site.

The DNA bending observed in the various operators indicates that the hinge-regions in these complexes form similar minor-groove binding α-helices as seen in the structures of the repressor–SymL(–1) and HP62–SymL(–1) complexes (Lewis *et al.*, 1996; Spronk *et al.*, 1996, 1999). Furthermore, the experiments with the operators where half-sites are scrambled indicate that the bending we observe is a specific effect of *lac* operators containing two suitably spaced binding sites for the headpieces. It must be noted, however, that when intact repressor is added to a 100-fold excess, all operators containing two headpiece binding sites, including the SymL(+1) sequence, show bending. It appears that binding of the low affinity operators still involves the specific binding mode, similar to observations in a purine repressor mutant that binds specifically to the *pur* operator with very low affinity (Glasfeld *et al*., 1996). In agreement with this, addition of excess *lac* repressor to the L11, R11 and NOD sequences apparently results in binding in a non-specific mode, since bending is not observed in these complexes. Finally, it is important to note here that the observation of HP62-induced bending in the SymL(–1) and wild-type operators shows that the observed bending is a local effect of the interaction of the headpieces with the DNA and does not originate from wrapping of the DNA around the core of the protein.

Hinge-helix formation

As mentioned above, DNA bending observed in the complexes of *lac* repressor with various operators indicates the presence of the minor-groove-binding α-helices. To obtain direct evidence for hinge-helix formation in the wild-type complex we investigated the complex of HP62 with the wild-type operator using NMR. The formation of the hinge-helices was probed by recording ^{1}H-^{15}N HSQC spectra of the free and complexed forms of HP62 and monitoring the

Table I. *lac* repressor and HP62 induced bending of various *lac* operators[a]

Operator	Sequence (top strand)	HP62-induced bend (°)	Lac-induced bend (°)	IC_{50}/IC_{50wt}	$K_d/K_{d,wt}$
Wt(–1)	GAATTGTGAGCGATAACAATTT	N.d.b.	43	>100	N.s.r.
Wt	GAATTGTGAGCGGATAACAATTT	28	68	1	1
Wt(+1)	GAATTGTGAGCCGGATAACAATTT	N.d.b.	47	>100	31 ± 13
SymL(–1)	GAATTGTGAGCGCTCACAATTC	39	72	0.6 ± 0.1	0.8 ± 0.3
SymL	GAATTGTGAGCGGCTCACAATTC	N.d.b.	64	>100	24 ± 16
SymL(+1)	GAATTGTGAGCCGGCTCACAATTC	N.d.b.	N.d.b.	>>100	N.s.r.
SymR(–1)	AATTGTTATCGATAACAATT	N.d.b.	44	>>100	N.s.r.
SymR	AATTGTTATCGGATAACAATT	N.d.b.	46	>100	31 ± 21
SymR(+1)	AATTGTTATCCGGATAACAATT	N.d.b.	56	2.9 ± 0.7	2.4 ± 2.2
L11	GAATTGTGAGCGAAATCTTAGAT	N.d.b.	N.d.b.	>>100	N.s.r.
R11	GCGGATAGATTGGATAACAATTT	N.d.b.	N.d.b.	>>100	N.s.r.
NOD	GCGGATAGATTGAAATCTTAGAT	N.d.b.	N.d.b.	>>100	N.s.r.

[a]The various *lac* operators that were used for DNA bending experiments. The specific half-sites are shown underlined (left half-site, solid underlined; right half-site, dotted underlined). N.d.b., no detectable binding of operators in the bending experiments. The standard deviation in the bending angles determined in repeated experiments is ~10%. IC_{50} (inhibitory concentration 50%) values of the intact *lac* repressor for the various operators were determined from the quantification of four independent experiments using a curve fitting program, and are scaled to the value found for the homologous binding competition experiment for the wild-type operator. >>100, no competition was detected for these sequences. >100, although competition was observed, exact determination of the IC_{50} value was impossible due to incomplete competition. K_ds were determined from the quantification of 5–6 independent experiments using curve fitting and scaled to the value found for the wild-type operator. N.s.r., no saturation could be reached at the highest tested concentration (100 nM) and therefore no relative binding affinity could be determined.

chemical shift changes for the residues in the hinge-region. Figure 4 shows the spectra of free HP62 and its complexes with the SymL(–1) and wild-type operators. When HP62 is complexed to the SymL(–1) operator, the hinge-helix formation is expressed in clear chemical shift changes in the resonances of residues 50–58 (Figure 4A and B). The NMR spectrum of the wild-type complex suffers from unfavorable chemical exchange processes, which result in very broad NMR lines in addition to the doubling of the resonances caused by the asymmetry of the complex. Inspection of the spectrum shows resonances of the hinge-region that remain relatively sharp and do not shift significantly upon binding of the wild-type operator, indicating that this region is unfolded. The spectra further show the presence of two additional low intensity peaks in the upper right part of the spectrum (Figure 4C). Upon binding HP62 to the SymL(–1) operator, Gly58 shows a very pronounced shift from its random coil position to the same part of the spectrum, suggesting that the two corresponding peaks in the wild-type complex may originate from two additional folded states for Gly58. Indeed these peaks can be identified as resonances corresponding to different states of Gly58 since they turn out to be in slow chemical exchange with the random coil resonance of Gly58. The exchange is observed when ^{1}H-^{15}N HSQC spectra are recorded with an additional mixing time to allow magnetization transfer between the different states (Wider *et al.*, 1991). This yields a spectrum with cross-peaks between the exchanging states in addition to the auto-correlation peaks (Figure 4D). Based on the similarity with the spectrum of the SymL(–1) complex these resonances correspond most likely to Gly58 in two asymmetrical hinge-helices in the wild-type complex. Similar exchange peaks between folded and unfolded states are seen for hinge-region residues Ala53 and Ala57. Note that very weak cross-peaks are also observed between the two shifted Gly58 resonances as a result of exchange between binding to the right and left half-sites. This exchange process may involve flipping of the headpieces on one operator or exchange of headpieces between different operators.

The presence of significant equilibrium amounts of unfolded or partly unfolded hinge-helices as seen in our NMR experiments may be a characteristic of the complex of the intact repressor with the wild-type operator and explain the differences found in the number of residues involved in the folding transition when *lac* repressor binds to the wild-type and SymL(–1) operators (Spolar and Record, 1994). An alternative explanation may be that fewer residues are involved in the coil-to-helix transition in the wild-type complex. However, our NMR data show that the effect of folding still extends to residue Gly58, the same as in the SymL(–1) complex, suggesting that the latter explanation is not valid.

It is interesting to note that the second residue of the recognition helix, Gln18, which is involved in important interactions with the major groove of the DNA, appears to show similar exchange processes as seen in the hinge region. This glutamine shows two pairs of shifted NH_2 resonances when the complex is formed. The two states presumably correspond to the asymmetrical interactions taking place in the left and right half-sites of the operator. In addition, an exchange of these states is seen towards yet two other states of the Gln18 side chains, as shown by the occurrence of the cross-peaks present in the exchange-correlation experiment. A similar behavior was

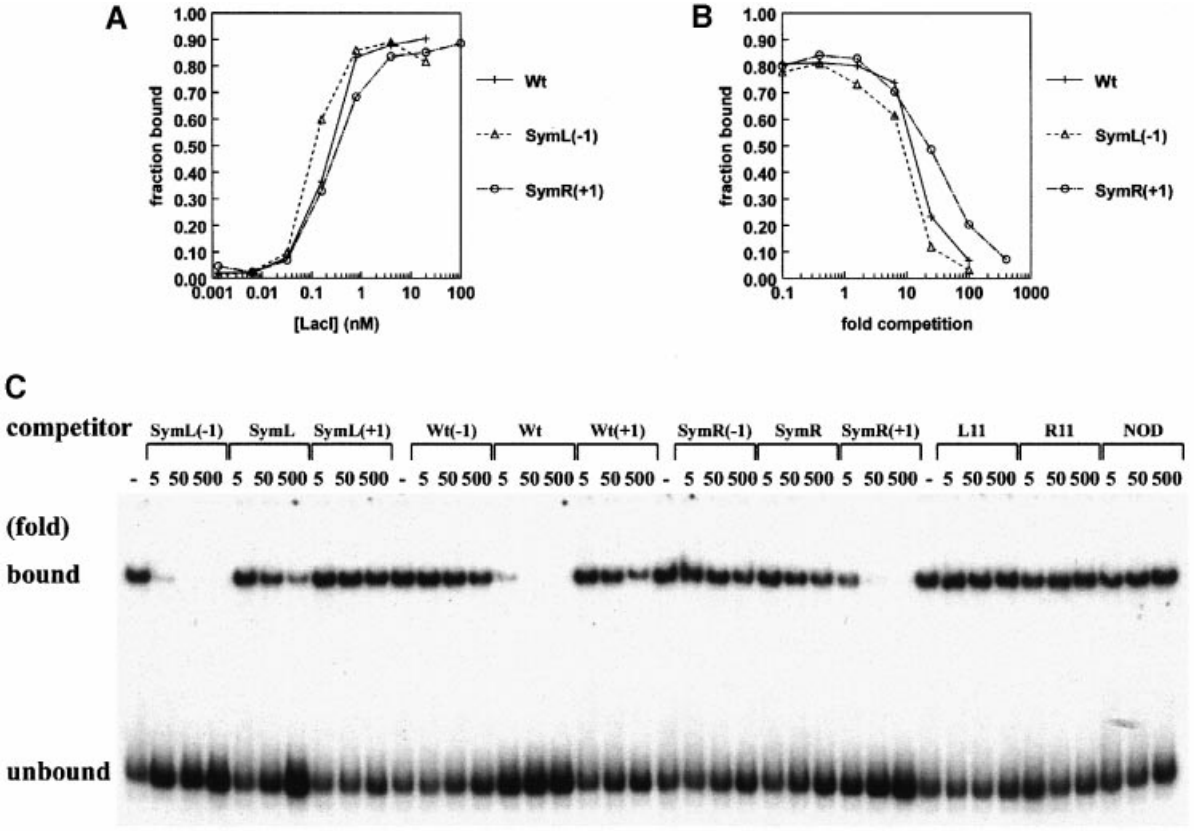

Fig. 2. Relative binding affinity of the various *lac* operators. (**A**) Quantification of electrophoretic mobility shift assays of titrations of *lac* repressor to the wild-type, SymL(–1) and SymR(+1) *lac* operators (1 fmol). The fractions of probe bound are plotted as a function of the *lac* repressor concentration. (**B** and **C**) Competitive electrophoretic mobility shift assay using various *lac* operator sequences (listed in Table I) as competitor. *Lac* repressor (0.4 nM) and 0.5–1 fmol wild-type *lac* operator were mixed with increasing amounts of unlabeled competitor sequences (molar fold excess). Quantification of an experiment is shown in (B). A representative experiment is shown in (C); the unbound probe is indicated as 'unbound', the *lac* repressor–operator complex is labeled 'bound', the fold excesses of the various operators are depicted at the top of the figure and '–' represents the complex in the absence of competitor.

seen in the SymL(–1) complex, where at least two states were distinguished for Gln18. It is not clear yet whether the exchange processes seen in the hinge-region and the recognition helices are correlated. The interaction of the hinge-helices with the minor groove of the DNA will cause changes on the surface of the major groove (Spronk *et al.*, 1999). When the hinge-helices unfold, the effect of the loss of the contacts with the minor groove of the operator may be transferred to the side chains of the recognition helix by the changes in the major groove of the DNA.

Position of binding of lac headpieces to lac operator

The observation that the hinge-helices are formed in the wild-type complex indicates the presence of protein–protein interactions between the two headpieces and thus that the presence of the central base-pair is compensated for in the binding of the headpieces. The relative affinities of repressor for the different operators suggest that this compensation is achieved by a shift in the binding towards the center of the wild-type operator to include interaction with the central base-pair when the repressor binds to the right half-site. To test this hypothesis we investigated complexes of HP62 with the SymR(+1) and R14 operator sequence. These sequences contain the right half-site and overlap the center of the wild-type *lac* operator. The NOESY spectra of the HP62–R14 complex immediately revealed specific interactions between HP62 and the operator (Figure 5). Some of the NOEs we observe are similar to those found in the complex with the left half-site of *lac* operator (Boelens *et al*., 1987; Lamerichs *et al.*, 1989, 1990; Chuprina *et al.*, 1993). These NOEs involve short proton–proton distances between His29 ring-protons and Ade20 sugar-protons, between the Tyr7 ring-protons and the Gua12 base H8, and between the Tyr17 ring-protons and the Thy14 methyl group (data not shown). Important, however, are the NOEs we observe here between the ring-protons of Tyr7 to the H8 and H1′ protons of Gua11. These indicate a shift in interaction towards the center of the operator and were also found in a complex of HP62 with the SymR(+1) operator (data not shown). Note that the observation of the NOEs between Tyr7 to Gua12 and Tyr17 to Thy14 is not in disagreement with a shift of binding towards the center of the operator for two reasons: the large size of the tyrosine rings and a dynamic equilibrium between different binding positions at a half-site, which is likely to be present in the complex, make observation of these NOEs also possible when the interaction is shifted toward the center of the operator. The reason for the observed shift in binding position may be largely explained by the loss of the important specific

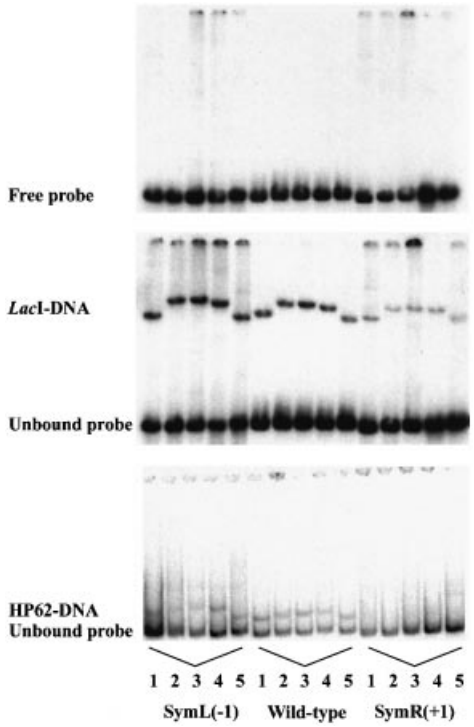

Fig. 3. Electrophoretic mobility shift assays of intact *lac* repressor and *lac* repressor HP62 on different circularly permuted *lac* operator sites. The upper panel shows the results obtained for the free SymL(–1), wild-type and SymR(+1) operators without protein added, the middle panel shows the results obtained for the complexes with the intact *lac* repressor and the lower panel shows the results obtained for the complexes with HP62. The numbers indicated correspond to restriction enzymes used to obtain the circularly permuted DNA fragments: *Mlu*I (1), *Nhe*I (2), *Xho*I (3), *Stu*I (4) and *Bam*HI (5).

interaction of Gln18 with base-pair 15 of the wild-type and R14 operators, and base-pair 16 of the SymR(+1) operator. It has been shown in several studies (Ebright, 1986; Lamerichs *et al.*, 1990; Chuprina *et al.*, 1993) that in the left half-site Gln18 is involved in specific contacts to Cyt7, which is replaced by an adenine in the right half-site.

The shift in binding position of HP62 in the complexes with the R14 and SymR(+1) operators suggests that the spacing between the hinge-regions in the SymR(+1) complex is similar to that in the SymL(–1) complex. Therefore, we have investigated the presence of the hinge-helices in the HP62–SymR(+1) complex by NMR spectroscopy. We have found no indications from ^{1}H-^{15}N HSQC spectra that the hinge-helices are formed in this complex, which is in agreement with the results from the DNA bending experiments. Without the core domain the headpieces may not be bound tightly enough to the major groove to allow the formation of a stable protein–protein interface and thus the formation of the hinge-helices in this complex.

Furthermore, it must be mentioned here that the NOEs of His29 to the sugar-protons of Ade20, which are the same as those found in the complex of HP56 with the left half-site, appear to argue against a mere shift in binding position. It has been noted, however, that in DNase I cleavage experiments on a number of different *lac* operators, including those used in this NMR study, the same end-to-end protection is observed (Sasmor and Betz, 1990). In these experiments, the only differences were found in the specific protection of the left and right half-sites. These results indicate that the repressor may adapt itself to the DNA sequence to form the most optimal protein–DNA interactions. To keep the interactions with the ends of the operators the repressor may adjust the conformation of the flexible loop between the second and the third helix of the headpieces. Such an adaptation would result in a somewhat stretched headpiece interacting with the DNA. Alternatively, variations in the DNA bend angle of the different operators (see Table I and Figure 3) and the flexibility of the AT tracts at the operator ends are mechanisms to adapt and increase the complementarity of the protein and DNA surfaces.

Discussion

Induction and DNA looping

The results presented in this paper provide a better understanding of the interactions occurring in the complexes of *lac* repressor with the wild-type operator and operator variants. Although previous data have suggested that the *lac* repressor does not bend the wild-type operator (Wu and Crothers, 1984; Zinkel and Crothers, 1987), our results show that the hinge-helices do induce bending of this operator. The discrepancy may be a result of differences in experimental set-up and DNA sequences used. The data presented here indicate that the model of the induction derived from the studies on the symmetrical complexes is also valid for the wild-type *lac* operon. Furthermore, the presence of the hinge-helices in the wild-type repressor–operator complex has important consequences for the model for DNA looping and the *lac* repressor–CAP interaction in the wild-type *lac* operon. The directions of bending imposed by *lac* repressor and CAP on the *lac* operon DNA both favor the formation of loops of the type proposed by Lewis *et al.* (1996) and are unfavorable for the types of loops involving extended *lac* repressor conformations (Friedman *et al.*, 1995).

Model of headpiece binding to lac operators

In contrast to previous suggestions that the binding of the headpiece to the right half-site of the wild-type operator occurs further away from the center of the operator (Horton *et al.*, 1997), we have provided evidence that the binding to this site is shifted toward the center. The central base-pair in the wild-type *lac* operator is needed to accommodate this different binding pattern such that the two headpieces can form the protein–protein interface needed for the stability of the hinge-helices. In addition we propose a model for the binding of the headpieces of the intact repressor to the operator variants used in this study and by Sasmor and Betz (1990) (Figure 6) that explains the observed relative *in vivo* and *in vitro* affinities. Central to the model is the ability of the repressor to form the hinge-helices for which protein–protein interactions are necessary. The hinge-helices, which contribute to the binding affinity, can only form when the headpiece binding sites are properly spaced.

The tightest binding operator is the SymL(–1) operator, which contains the two strongest binding half-sites and has the optimal spacing for the formation of the protein–

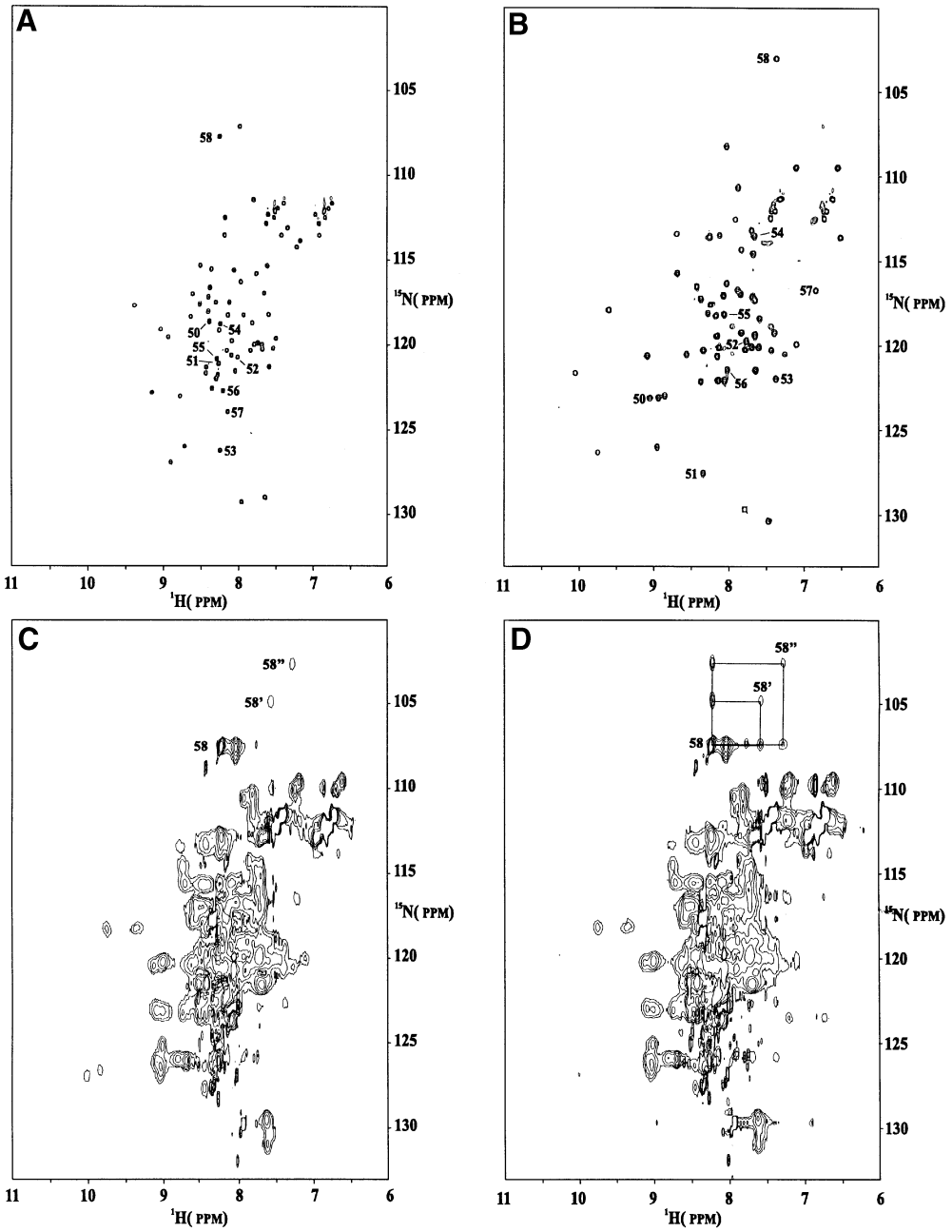

Fig. 4. ^{1}H-^{15}N-HSQC spectra of (**A**) free HP62, (**B**) the HP62–SymL(–1) complex and (**C**) the HP62–wild-type DNA complex. Peaks corresponding to residues involved in the folding transition in the hinge-region upon DNA binding are labeled in (A) and (B). In (C) and (D), the peaks corresponding to the different states of Gly58 are indicated where 58' and 58'' are the folded states. (**D**) Spectrum acquired on the HP62–wild-type DNA complex using the modified ^{1}H-^{15}N-HSQC sequence to allow the observation of correlation peaks between slowly exchanging states. The correlations between the different states of Gly58 are indicated by the rectangles.

protein interface. Increase of the spacing of the two binding sites leads to disruption of the protein–protein interactions in the hinge-region and decreased affinity. The core domain of the intact repressor can modulate the binding of the headpieces to the SymL operator such that the hinge-helices are still formed and some of the affinity is retained. Further increase of the spacing, yielding the SymL(+1) operator, completely disrupts the protein–protein interface in the hinge-region and the affinity of the repressor for the operator becomes very low.

As discussed, the binding of the headpiece to the right half-site is shifted towards the center of the operator. For optimal interaction with operators containing two right half-sites, therefore, two extra base-pairs are needed in the center as compared with the optimal spacing for the operators containing two left halves of the repressor. The SymR(–1) operator lacks both these base-pairs, which results in partial overlap of the two headpiece binding sites. Owing to a steric clash the two hinge-helices cannot be formed completely, causing the repressor largely to lose its affinity for the operator. Increasing the spacing leads to a decrease of the steric clash, more favorable interactions between the hinge-regions and tighter binding of the repressor.

The third *lac* operator that has an optimal half-site spacing for formation of the hinge-helices and interaction

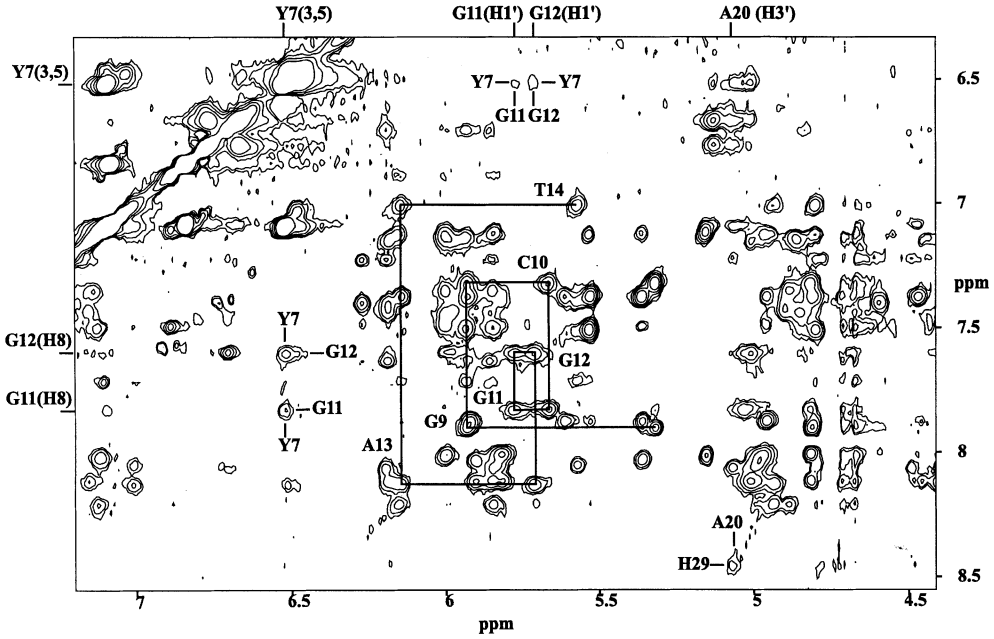

Fig. 5. Part of a 150 ms 2D-NOE spectrum of the HP62–R14 complex in D_2O recorded at 750 MHz. The sequential assignment of the base H6/H8 protons to the ribose H1′ protons is indicated for the nucleotides close to the center of the wild-type operator. Several important protein–DNA NOEs are indicated.

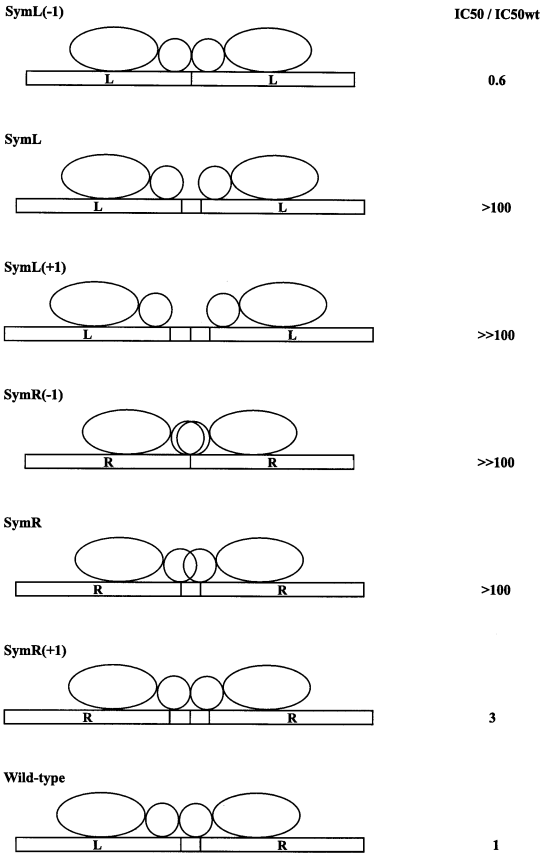

Fig. 6. Model for the binding positions of the *lac* repressor headpieces to different *lac* operators. The hinge-regions are represented by small circles, the globular major-groove-binding subdomains are represented by large ovals. The relative affinities of the repressor for the different operators are given on the right. Essential to the relative binding affinities of the different operators are the positions of the hinge-regions within the dimer. Optimal spacing of the headpieces needed for hinge-helix formation occurs in SymL(–1), wild-type and SymR(+1) binding of repressor.

with the DNA is the wild-type operator. The affinity of the repressor for the wild-type operator is lower than for the SymL(–1) operator due to the presence of the intrinsically weaker binding right half-site, but higher than for the SymR(+1) operator, which contains two weaker binding sites.

An important implication of the data presented here is that the models for the induction and DNA bending derived from the structural data on the symmetrical complexes can be extrapolated to the wild-type *lac* repressor–operator complex. The data further provide the basis for a detailed understanding of the role of the hinge-regions of the *lac* repressor in DNA binding and the importance of spacing for the binding affinity. Furthermore, the high sequence homology in the hinge-region among the members of the *Lac*I–*Gal*R family of repressors strongly suggests that these proteins utilize similar DNA binding mechanisms.

An interesting exception in the *Lac*I–*Gal*R family is the *Cyt*R repressor, which deviates from the other repressors in the residues responsible for the tight interaction of the hinge-region with the minor groove of the DNA. The crucial leucine at position 56 in the *lac* repressor is a valine in the *Cyt*R repressor, which, based on molecular modeling studies, is unable to intercalate at the central CpG base-pair step with its side chain (Arvidson *et al*., 1998). The function of this substitution is demonstrated in recent work by Valentin-Hansen and co-workers who show that *Cyt*R requires a flexible hinge-region to enable it to bind to operators with half-site spacings varying from 1 up to 31 bp (Pedersen and Valentin-Hansen, 1997; Jørgensen *et al*., 1998). Although the *Cyt*R hinge-region is largely devoid of structure, tryptic cleavage experiments suggest that some residual secondary structure is still present. This residual secondary structure may account for the observation that *Cyt*R binds preferentially to half-sites with small spacings. Similar to what is seen for the *lac* repressor, protein–protein interactions between ordered

or partially ordered hinge-regions could enhance the binding in the *Cyt*R–DNA complexes when proper half-site spacings are present.

Materials and methods

NMR sample preparation

Cloning, expression and purification of *lac* HP62 were performed analogously to the method described by Slijper for *lac* HP56 (Slijper, 1996). All *lac* operator DNA fragments were purchased at Carl Roth GmbH (Germany) and further purified on a Q-Sepharose (Pharmacia) column. The following samples were used for NMR measurements: (i) 3 mM ^{15}N-labeled HP62; (ii) 3 mM ^{15}N-labeled HP62, 1.5 mM 22 bp SymL(–1) operator DNA; (iii) 3 mM ^{15}N-labeled HP62, 1.5 mM 23 bp wild-type operator DNA; (iv) 2 mM ^{15}N-labeled HP62, 1 mM 24 bp SymR(+1) operator DNA; and (v) 4 mM ^{15}N-labeled HP62, 4 mM 14 bp R14 operator DNA. The free HP62 sample contained 0.4 M KCl, 0.06 M potassium phosphate buffer pH 4.5. The R14 complex sample contained 0.2 M KCl, 0.01 M potassium phosphate buffer pH 6.1. All other samples contained 0.02 M KCl, 0.01 M potassium phosphate buffer pH 6.1. The R14 and SymR(+1) complexes were dissolved in either 95% H_2O/5% D_2O or in 100% D_2O. Trace amounts of NaN_3 were added to all NMR samples as a preservative.

NMR spectroscopy

NMR spectra were recorded on Varian Inova 750 MHz and Bruker DRX-600 spectrometers equipped with triple-resonance gradient probes. NMR spectra were recorded at 25, 42, 27, 27 and 25°C for the free HP62 and the SymL(–1), SymR(+1), R14 and wild-type DNA complexes, respectively. Sequential assignments of the DNA spectra were performed in a straightforward manner based on 2D-TOCSY (Griesinger *et al.*, 1988) and 2D-NOESY (Jeener *et al.*, 1979) spectra using the method described by Wüthrich (1986). Protein–DNA interactions were identified using 2D-NOESY spectra with mixing times of 50, 100 and 150 ms of the complexes dissolved in D_2O. ^{1}H-^{15}N HSQC spectra of the wild-type complex were recorded with and without an additional mixing time between the first two 90° pulses of the reversed-INEPT building block, similar to the method described by Wider *et al.* (1991). The mixing time varied from 5 to 20 ms, all sufficient to observe cross-peaks between the different states of HP62, which are in slow chemical exchange. All NMR spectra were processed using the NMRPipe software package (Delaglio *et al.*, 1995) and analyzed with the NMR analysis program REGINE on Silicon Graphics workstations.

DNA bending and binding affinities

DNA bending was determined using the circular permutation method as described by Wu and Crothers (1984), using the pBend2 vector (Kim *et al.*, 1989). Oligonucleotides containing the different *lac* operators (Table I) were cloned in the *Xba*I site of pBend2 and verified by DNA sequencing. Further details on the cloning are available upon request. The plasmids containing the various *lac* operators were digested with different restriction enzymes to create permuted DNA fragments. Labeling of the DNA fragments was performed by end-labeling using T4 polynucleotide kinase and [γ-^{32}P]ATP or by PCR amplification in the presence of [α-^{32}P]dCTP. Electrophoretic mobility shift assays were performed essentially as described by Fried and Crothers (1984). Purified *lac* HP62 (0.3 mM) was incubated for at least 2 h at 4°C in 20 µl of reaction buffer containing 10 mM Tris pH 8.1, 50 mM KCl, 1 mM EDTA, 5% (v/v) glycerol, 0.1 mg/ml bovine serum albumin and 500–1000 c.p.m. of end-labeled PAA-gel purified probes (specific activity: 1×10^6–1×10^7 c.p.m./µg DNA). Subsequently, reaction mixtures were loaded on a pre-run 7% (w/v) polyacrylamide (30:1) gel containing 0.5× TBE as running buffer and electrophoresed at 150 V for 4–5 h at 4°C. Gels were vacuum dried and exposed against Fuji RX films for 2–10 days at –80°C using an intensifying screen. The experiments with full-length *lac* repressor were performed in the same manner as for HP62, except that the samples contained 0.06 nM protein and the gels contained 6% polyacrylamide. The overall angle of bending was determined by the ratio of the migrated distances of the slowest (µM) and the fastest (µE) migrating complexes in an electrophoretic mobility assay, according to the equation $\mu M/\mu E = \cos(\alpha/2)$, where α is the bend angle.

Competition experiments were performed under conditions where ~50% of the wild-type operator containing DNA fragment was bound to repressor. For these experiments increasing amounts of non-labeled PCR fragments containing any of the operator sequences were mixed with the labeled wild-type operator containing PCR fragments, followed by addition of the protein. The reaction mixtures were incubated and loaded on 6% polyacrylamide (30:1) gels containing 0.5× TBE as running buffer and electrophoresed at 150 V for 4–5 h at 4°C. Complexes were quantified with a phosphorimager using ImageQuant software (Molecular Dynamics). The IC_{50} values (inhibitory concentration 50%) were obtained from plots of the amount of the wild-type complex versus the concentration of PCR fragments added. The affinities of *lac* repressor for the various *lac* operators were determined relative to the affinity of the repressor for the wild-type operator using heterologous competitive binding experiments.

Binding constants were determined under the same conditions as the competition experiments. Increasing amounts of repressor was added to probes containing operator DNA. The dissociation constants were determined using a curve-fitting procedure of the binding curves and assuming a 1:1 binding stoichiometry. Given the limitations of the method applied here, the K_ds are represented as apparent K_ds, which are used for a relative comparison of the affinities of *lac* repressor for the different *lac* operators. In contrast to the values in the picomolar range obtained using filter-binding assays (O'Gorman *et al*., 1980 and references therein), the apparent K_ds that were measured here for the wild-type and SymL(–1) sequences are in the nanomolar range (0.2–4 nM). However, the concentrations of protein and DNA used in this study to bind 50% of the DNA are similar to those used in gel-shift experiments performed by Müller-Hill and co-workers (Krämer *et al*., 1987; Eismann and Müller-Hill, 1990). Furthermore, the *in vivo* affinity of repressor for DNA containing only the wild-type O1 operator is estimated to be in the nanomolar range (Oehler *et al*., 1990). We attribute the differences in absolute values of K_ds found in this study with those reported earlier to differences in the methods used. For an accurate determination of absolute values of K_ds of *lac* repressor–operator complexes we refer to the method used by Levandoski *et al*. (1996).

Acknowledgements

We especially thank Daniel J.Felitsky, Dr Ruth Saecker and Dr M.Thomas Record, Jr, of the Department of Chemistry, University of Wisconsin-Madison, Madison, for kindly providing intact *lac* repressor and for fruitful discussions regarding the DNA bending experiments. This work was financially supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO).

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Received June 10, 1999; revised September 22, 1999; accepted September 23, 1999