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The LexA Repressor and Its Isolated Amino-Terminal Domain Interact Cooperatively with Poly[d(A-T)], a Contiguous Pseudo-Operator, but Not with Random DNA: A Circular Dichroism Study†

Serge Hurstel, Michèle Granger-Schnarr, and Manfred Schnarr*

Institut de Biologie Moléculaire et Cellulaire, CNRS LP 6201 Affiliated to INSERM and Université Louis Pasteur, 15 rue René Descartes, 67084 Strasbourg, France

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ABSTRACT: The interaction of the entire LexA repressor and its amino-terminal DNA binding domain with poly[d(A-T)] and random DNA has been studied by circular dichroism. Binding of both protein species induces an about 2-fold increase of the positive circular dichroism band at about 270 nm of both polynucleotides, allowing a precise determination of the principal parameters as a function of mono- and divalent salt concentration and pH. Both proteins interact much more strongly (about 2000-fold) with poly[d(A-T)] than with random DNA as expected from the homology with the specific consensus binding site of LexA (CTGTATATATATACAG). For both LexA and its DNA binding domain we find that the interaction with poly[d(A-T)] is cooperative with a cooperativity factor ω of about 50–70 for both proteins over a wide range of solvent conditions, suggesting that the carboxy-terminal domain of LexA is not involved in this type of cooperativity. On the contrary, no cooperativity could be detected for the interaction of the LexA DNA binding domain with a random DNA fragment. The overall binding constant $K\omega$ (or simply K in the case of random DNA) depends strongly on the salt concentration as observed for most protein–DNA interactions, but the behavior of LexA is unusual in that the steepness of this salt dependence ($\delta \log K\omega/\delta \log [NaCl]$) is much more pronounced at slightly acidic pH values as compared to that at neutral or slightly alkaline pH. The behavior is not easily understood in terms of the current theories on the electrostatic contribution to protein–DNA interactions on the basis of polyelectrolyte theory. A comparison of the overall binding constant $K\omega$ of the entire LexA repressor and its DNA binding domain reveals that LexA binds only 20–50-fold stronger under a wide variety of salt and pH conditions. This result tends to demonstrate further that the additional energy due to the dimerization of LexA via the carboxy-terminal domain should be rather weak as expected from the small dimerization constant of LexA (2×10^{-4} M^{-1}).

The SOS network of *Escherichia coli* consists of a family of about 20 unlinked genes involved mainly in DNA repair of damages caused to DNA by chemical and radiative carcinogens. These SOS genes include those responsible for excision repair of DNA adducts (uvrA,uvrB, uvrD), repair by recombination (recA, recN, and probably ruv), and SOS mutagenesis (umuDC, mucAB, recA) as well as inhibition of cell division (sulA) [for reviews see Little and Mount (1982) and Walker (1984)]. In the absence of DNA damage the expression of these genes is inhibited by the LexA repressor, a protein of 202 amino acids, which consists of two structural domains linked by a flexible "hinge" region (Little & Hill, 1985).

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* To whom correspondence should be addressed.

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The carboxy-terminal domain of the LexA repressor is involved in the weak dimerization of the molecule (Schnarr et al., 1985, 1988) as well as in the specific cleavage reaction between Ala-84 and Gly-85. Under in vivo conditions this cleavage reaction requires an activation of the RecA protein by an inducing signal that is thought to be single-standed DNA. Under alkaline pH conditions the same cleavage reaction occurs in vitro in the absence of RecA (Little, 1984). Numerous mutants within or in close proximity of the cleavage site were found to inactivate cleavage (Lin & Little, 1988), whereas a replacement of Ala-84 by a cysteine does not inhibit cleavage as judged from the induction of the SOS system in vivo (Granger-Schnarr et al., 1988a). Both self-cleavage and RecA promoted cleavage of LexA are dependent on the amino acids Ser-119 and Lys-156 which seem to form the catalytic center of the protein, suggesting that LexA belongs to the family of the serine proteases (Slilaty & Little, 1987).

The amino-terminal domain of LexA is in direct contact with the DNA as shown mainly by studies on the isolated domain ranging from amino acid 1 to amino acid 84. This domain forms essentially the same contacts with the DNA as the entire protein within the major groove as shown by methylation protection studies (Hurstel et al., 1986) and with the backbone of the DNA as shown by hydroxyl radical footprinting and ethylation interference studies (Hurstel et al., 1988). The isolated domain retains substantial DNA binding affinity as shown for the recA and the uvrA operators, since its equilibrium association constant is only 10–20-fold smaller than that of the entire repressor (Bertrand-Burggraf et al., 1987; Hurstel et al., 1988). This domain has been shown by two-dimensional NMR spectroscopy to contain three α helices across amino acids 8–20, 28–35, and 41–54. Helix 2 and helix 3 were found to form a "helix–turn–helix"-like motif reminiscent of those found for other transcription factors, albeit with a conformation different from the usual one (Lamerichs et al., 1989).

Upon binding to operator DNA the LexA repressor is able to induce a substantial bending of the DNA. This has been shown in detail for the colicin A operator. This overlapping tandem operator forms two different complexes with LexA (Granger-Schnarr et al., 1988b), and DNA bending takes place in both complexes (Lloubès et al., 1988). Bending in these complexes has been tentatively attributed to T_n and A_n tracts situated on both sides of the recognition sequence, since those tracts are known for their ability to undergo intrinsic curvature.

In most cases LexA exerts repression through binding to a single operator situated close to the promoters of the different SOS genes. The sequence of these operators varies slightly, allowing a variable degree of repression from one gene to the other. The palindromic consensus sequence derived from these operators (Wertman & Mount, 1985) is

CTGTATAT|ATATACAG

This sequence is closely related to a simple alternating poly[d(A-T)][1] polymer that matches 12 of 16 base pairs of the consensus operator. An earlier study on the interaction of LexA with poly[d(A-T)] has shown that this interaction was surprisingly strong for a purely nonspecific binding mode (Schnarr & Daune, 1984). The homology between poly[d(A-T)] and the consensus sequence makes it more likely that poly[d(A-T)] represents in fact not a true nonspecific target for the LexA repressor but rather what may be called a "pseudo-operator" (Berg, 1988). This means that some of the thermodynamic parameters governing specific binding (like salt and pH dependence of the association constants, cooperativity of binding, electrostatic and hydrophobic energy contributions) may be inferred from interaction studies with poly[d(A-T)]. This interaction may be investigated by circular dichroism measurements, since the binding of LexA induces a conformational change of both poly[d(A-T)] and random DNA leading to a 2–3-fold increase in the positive CD band of both polynucleotides (Schnarr & Daune, 1984; Hurstel et al., 1986).

In this work we compare the binding of LexA and its amino-terminal domain to both poly[d(A-T)] and random DNA. Binding to poly[d(A-T)] is found to be about 2000-fold stronger than binding to random DNA. Part of this higher binding affinity to poly[d(A-T)] is due to the cooperativity of the interaction, whereas the bulk of random DNA interacts essentially not cooperatively. The isolated amino-terminal domain shows essentially the same degree of cooperativity as the entire repressor. Both proteins bind poly[d(A-T)] more strongly when the pH is lowered, a quite common behavior for DNA binding proteins; however, none of the current models used to explain these effects may be applied in the case of LexA. Furthermore, the salt dependence of the equilibrium binding constants is much steeper at slightly acidic pH values.

Materials and Methods

Circular Dichroism Measurements. Circular dichroism measurements were done on a Jobin-Yvon Mark III dichrograph at 20 °C using cylindrical cells (1 cm) with DNA or poly[d(A-T)] concentrations of typically 30 μM (in base pairs). Concentrated stock solutions of LexA or its amino-terminal domain were added stepwise until a final concentration of about 30–40 μM. The binding of both proteins was monitored by the increase of the CD signal at 263 nm for poly[d(A-T)] and at 275 nm for DNA. Complete CD spectra in the wavelength range from 330 to 250 nm were taken in duplicate at regular intervals during those titrations. In this wavelength range the contribution of both proteins may be neglected with respect to the CD signal of the DNA. After each addition of protein or of concentrated salt solution, a few minutes were necessary until the CD signal was fully stabilized. All calculations were done on a Macintosh microcomputer using Turbo Pascal programs. Visual comparison of theoretical and experimental binding isotherms was performed by using the program Cricket Graph.

Proteins and DNA. The LexA repressor of *E. coli* and its amino-terminal DNA binding domain were purified as described earlier (Schnarr et al., 1985; Hurstel et al., 1986). The concentration of LexA was determined by using ϵ_{280nm} = 7300 M^{-1} cm^{-1} (Schnarr & Daune, 1984), and that of the amino-terminal domain was determined at 205 nm according to the method of Scopes (1974).

A linear DNA fragment of 1100 base pairs arising from plasmid pBR345 (Schnarr et al., 1983) and polydAT from Boehringer (Mannheim) were used for these measurements. All nucleic acid samples were extensively dialyzed against a low ionic strength buffer (1 mM potassium phosphate, 0.1 mM EDTA, pH 7.25). The polynucleotide concentration was determined from ϵ_{260nm} = 6630 M^{-1} cm^{-1} for DNA and from ϵ_{260nm} = 6650 M^{-1} cm^{-1} for poly[d(A-T)].

A 175 base pair 5′ end labeled DNA fragment harboring the recA operator was used for the band shift competition experiments of the entire LexA repressor with poly[d(A-T)]

[1] Abbreviations: poly[d(A-T)], poly(deoxyadenylate-thymidylate); LexA, LexA repressor; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

and random DNA (Schnarr et al., 1985).

Theoretical Background

Determination of Binding Parameters. The binding of a protein to a homogeneous nucleic acid lattice can be described by three parameters: n, the binding site size (in base pairs); K, the equilibrium association constant for an isolated binding site; and if necessary ω, the cooperativity parameter (the relative affinity of an incoming ligand for a contiguous binding site—elongation—as compared to an isolated binding site).

Determination of $K\omega$ by Interpolation at Half-Saturation. If the binding is cooperative, the product $K\omega$ of the intrinsic binding constant and the cooperativity parameter is directly related to $L_{\text{free }1/2}$, the concentration of free protein if the DNA lattice is half-saturated with bound ligand (which in turn is simply related to the total protein concentration by $L_{\text{free}} = L_{\text{total}} - (\Theta P)/n$, where P is the total polynucleotide concentration expressed in base pairs and Θ the fractional saturation of the DNA lattice), by

$$K\omega = \tau(1/L_{\text{free }1/2}) \quad (1)$$

where $\tau = \omega/(ABn)$ is a correction factor that deviates from unity only if ω is small (see eq 3 for the expression of A and B). This correction necessitates an iterative determination of ω determined in a first step by preliminary curve fitting of binding isotherms using the uncorrected $K\omega$ value, before the same isotherms are fitted with the corrected $K\omega$ value.

Calculation of Noncooperative Binding Isotherms. Noncooperative theoretical binding isotherms are generated according to

$$\frac{\Theta}{nL_{\text{free}}(\Theta,n,K)} = K(1-\Theta)\left[\frac{1-\Theta}{1-(n-1)\Theta/n}\right]^{n-1} \quad (2)$$

The K value giving rise to the best fit of the experimental binding curve was determined by visual inspection.

Calculation of Cooperative Binding Isotherms for the Determination of K and ω. In the cooperative binding case a great number of theoretical isotherms were generated by holding the experimental value of $K\omega$ constant and varying K and ω reciprocally according to

$$\Theta/nL_{\text{free}}(\Theta,n,K) = KAB(1-\Theta) \quad (3)$$

with

$$A = \left[\frac{1-(n+1)\Theta/n+R}{2(1-\Theta)}\right]^2$$

$$B = \left[\frac{(2\omega-1)(1-\Theta)+(\Theta/n)-R}{2(\omega-1)(1-\Theta)}\right]^{n-1}$$

$$R = \{[1-(n+1)\Theta/n]^2 + 4\omega\Theta(1-\Theta)/n\}^{1/2}$$

Again the best fit of the experimental isotherms was determined by visual inspection.

Equations 2 and 3 (McGhee & von Hippel, 1974) allow determination of theoretical binding isotherms upon stepwise variation of Θ and calculation of the corresponding value of $L_{\text{free}}(\Theta,n,K,\omega)$.

Determination of K and ω by Linearization of the Binding Data. Schwartz and Watanabe (1983) have introduced a very useful expression of Θ versus L_{free} which, although entirely valuable in the highly cooperative binding case only ($\omega \gg n$), can nevertheless be used for a quantitative analysis of moderately cooperative interactions in the range of fractional lattice saturations from 20% to 80%. This analysis involves the simplified binding equation

$$\frac{2\Theta-1}{[\Theta(1-\Theta)]^{1/2}} = [(\omega/n)^{1/2}K\omega]L_{\text{free}} - (\omega/n)^{1/2} \quad (4)$$

A straight line that intersects the ordinate at $-(\omega/n)^{1/2}$ with slope $K\omega\,(\omega/n)^{1/2}$ results from a plot of $(2\Theta-1)/[\Theta(1-\Theta)]^{1/2}$ versus L_{free}. As for the isotherm curve fitting procedure, overall binding constants from this analysis have to be corrected for moderate cooperativity. In this case the correction factor τ is determined by using the general binding equations of Schwartz and Watanabe (which cannot be written in a closed form). The values obtained for τ are identical with those calculated with the isotherm analysis.

Analysis of the Data According to the Method of Record and Collaborators. Record and collaborators (Record et al., 1976; deHaseth et al., 1977; Lohman et al., 1980) have developed a general formalism to explain the salt and pH dependence of protein–DNA interactions in terms of counterion and anion release upon binding of the protein. A fairly general description of this formalism may be given by

$$\log K = A + B + C + D \quad (5)$$

with

$$A = \log K_0$$

$$B = -r \log\{(1 + K_{\text{H}}[\text{H}^+])/K_{\text{H}}[\text{H}^+]\}$$

(protonation necessary for binding)

$$C = -a \log(1 + K_x[\text{X}^-])$$ (anion release from the protein)

$$D = -Z\Psi \log[\text{M}^+]$$ (cation release from the DNA)

where Z may be a function of pH: $Z = [Z_0 - \sum_{i=1,m}(1 + K_i[\text{H}^+])^{-1}]$. K represents the equilibrium association constant (either for an isolated binding site or for an overall binding constant $K\omega$), r is the number of titrable protein groups that must be protonated to allow for DNA binding, K_{H} is the intrinsic binding constant of these protons, a the number of anions released from the protein upon binding, K_x is the intrinsic binding constant of these anions, $[\text{X}^-]$ and $[\text{M}^+]$ are, respectively, the anion and cation concentrations, Z is the number of ion pairs formed between the protein and the DNA, Ψ is the number of cations per phosphate bound to the DNA (0.88 for monovalent ions). The slope of a log K versus log $[\text{M}^+]$ plot (log–log plot) is given by

$$-\partial(\log K)/\partial(\log[\text{M}^+]) = Z\Psi + a \quad (6)$$

Z and a can be determined separately from additional binding isotherms in a buffer containing divalent cations like Mg^{2+}. In this case the slope of a log–log plot is given by

$$-\partial(\log K)/\partial(\log[\text{M}^{2+}]) = Z\Phi + a \quad (7)$$

with $\Phi = 0.47$ Mg^{2+} ion bound per phosphate.

Results

The association of the LexA repressor and its amino-terminal domain to either synthetic poly[d(A-T)] or random DNA can be followed by the induced increase of the positive circular dichroism band of the polynucleotides. As shown earlier both poly[d(A-T)] (Schnarr & Daune, 1984) and random DNA (Hurstel et al., 1986) undergo about 2-fold and 3-fold, respectively, increases of the entire CD band between 300 and 250 nm upon addition of LexA without substantial deformation of the spectral shape (Schnarr & Daune, 1984; Hurstel et al., 1986). For the evaluation of the binding pa-

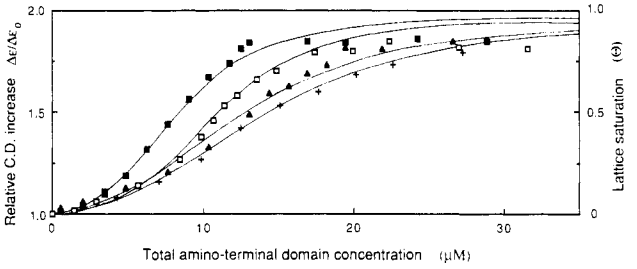

FIGURE 1: Binding isotherms of the amino-terminal fragment of the LexA repressor with poly[d(A-T)] plotted as the relative increase of the circular dichroism signal of the polynucleotide ($\Delta\epsilon/\Delta\epsilon_0$), or its saturation with protein (Θ), as a function of the total amino-domain concentration. Binding isotherms were determined at 20 °C under the following conditions: 295 mM NaCl, 10 mM sodium phosphate, pH 6.7 (+); 230 mM NaCl, pH 7.2 (▲); 136 mM (■) and 160 mM (□) NaCl, pH 8.0. The total poly[d(A-T)] concentration was 30 μM (in base pairs). The full lines through the data points are the best fitting theoretical binding curves.

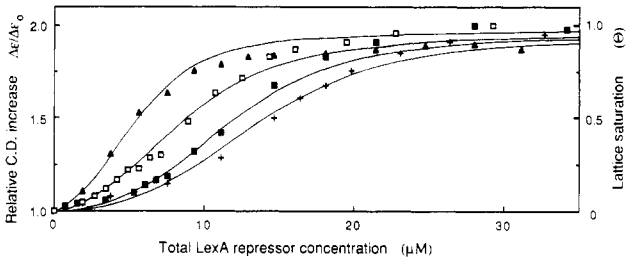

FIGURE 2: Binding isotherms of the entire LexA repressor with poly[d(A-T)] plotted as the relative increase of the circular dichroism signal of the polynucleotide ($\Delta\epsilon/\Delta\epsilon_0$), or its saturation with protein (Θ), as a function of the total LexA repressor concentration. The corresponding buffers are 407 mM NaCl, 10 mM sodium phosphate, pH 7.2 (▲), pH 7.6 (□), pH 8.0 (■), and 485 mM NaCl and 10 mM sodium phosphate, pH 7.2 (+). The total poly[d(A-T)] concentration was 30 μM (in base pairs).

rameters reported below we have quantified the CD changes at 263 nm in the case of poly[d(A-T)] and at 275 nm in the case of random DNA, because these wavelengths are situated close to the maxima of the corresponding CD bands, giving thus rise to the greatest changes upon addition of LexA. The binding parameters reported below are, however, independent of the exact wavelength chosen to monitor LexA binding. Further, the contribution of the protein to the CD signal may be neglected at these wavelengths.

Binding isotherms can be determined upon addition of increasing amounts of protein to a constant concentration of polynucleotides by assuming that the change in circular dichroism is linearly related to the fractional saturation of the lattice with bound ligand. This assumption is always necessary if protein–DNA interactions are monitored via a physicochemical property of the nucleic acid (fluorescence in most cases). If the number of nucleobases perturbed by the binding of the ligand is greater than the binding-site size n, this assumption may lead to a deformation of the binding isotherms at a high degree of lattice saturation Θ (Charlier & Maurizot, 1983; Schnarr et al., 1983). However, if Θ is smaller than 0.5 or if the interaction is cooperative, no major deformation of the binding isotherms will take place.

The Amino-Terminal Domain Interacts Cooperatively with Poly[d(A-T)] but Not with Random DNA. Figure 1 shows some of the binding isotherms determined for the association of the amino-terminal domain of LexA with poly[d(A-T)] as a function of the total protein concentration. All binding isotherms show a more or less pronounced sigmoidal shape, indicating that the amino-terminal domain interacts cooperatively with poly[d(A-T)]. Essentially the same behavior is

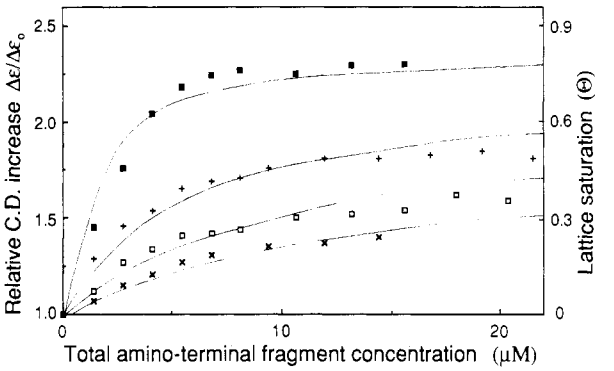

FIGURE 3: Binding isotherms of the amino-terminal domain of the LexA repressor to pBR345 DNA. Buffer conditions are 10 mM sodium phosphate, pH 6.7, 20 mM NaCl (■), 48 mM NaCl (+), 63 mM NaCl (□), and 87 mM NaCl (×). The total DNA concentration was 30 μM (in base pairs).

observed for the entire repressor as shown in Figure 2. Both protein species give rise to about the same cooperativity factor ω (see below). This result is somewhat unexpected since in the case of the phage λ repressor, for example, that shows a two-domain organization similar to that of LexA and some homology in the carboxy-terminal domain, binding cooperativity between OR1 and OR2 is conferred by the carboxy-terminal domain (Johnson et al., 1979).

On the contrary, the binding of the amino-terminal domain to random DNA (a 1100-bp restriction fragment of pBR345) is essentially noncooperative as seen from Figure 3. In this case the binding isotherms are not sigmoidal and full saturation is more difficult to achieve due to the excluded site effect (McGhee & von Hippel, 1974; Kowalczykowski, 1986). Similar measurements with random DNA could not be conducted for the entire LexA repressor due to precipitation of the complex under salt conditions suitable for equilibrium binding measurements.

Determination of the Overall Binding Constant $K\omega$ for Poly[d(A-T)]. According to eq 1 the overall binding constant $K\omega$ is easily determined from the free protein concentration at half-saturation of the polynucleotide lattice. The fractional saturation Θ of the lattice is given by $\Theta = (\Delta\epsilon - \Delta\epsilon_0)/(\Delta\epsilon_{max} - \Delta\epsilon_0)$, where $\Delta\epsilon_{max}$ is the final height of the CD signal if the polynucleotide is fully saturated with bound ligand. Throughout this study we have used the $\Delta\epsilon$ values measured close to the maxima of the CD spectra, that is, 263 nm for poly[d(A-T)] and 275 nm in the case of random DNA. For both LexA and the amino-terminal domain we have used a binding-site size n of 8 base pairs per monomer as determined earlier for LexA (Schnarr & Daune, 1984). During this study it turned out that $K\omega$ depends not only on the salt concentration of the solution, an effect observed to varying degrees for all protein–DNA interactions, but also on the pH value. Since each binding isotherm was measured at a constant pH value, it was necessary to adjust the salt concentration to work under near-optimal equilibrium conditions. To our experience this means that $L_{free\,1/2}$ should not be smaller than 2 μM and not exceed 20 μM since otherwise it is difficult to achieve full lattice saturation. This means, for example, that measurements with LexA at pH 8.0 had to be done close to 380 mM NaCl (150 mM for the amino-terminal domain), whereas measurements at pH 5.6 were done close to 750 mM (475 mM). The corresponding $K\omega$ values are listed in Table I. The detailed salt and pH dependence of these overall binding constants will be discussed below.

Separate Determination of K and ω. As outlined under Theoretical Background two different approaches have been

Table I: Equilibrium Parameters in Buffers Containing NaCl Only[a]

pH	[NaCl] (mM)	$K\omega$ ($\times 10^{-6}$ M^{-1})	ω^b	K ($\times 10^{-3}$ M^{-1})	$(\omega/n)^{1/2}$	ω^c	$-(\partial \log K\omega/\partial \log [NaCl])$	log ($K\omega$) (at 1 M NaCl)	Z^d
				LexA Repressor: Poly[d(A-T)]					
5.6	728	0.620 ± 0.180	20	31	1.8	25			
	795	0.185 ± 0.050	50	3.7	2.5	50	11.0 ± 1.9	4.2 ± 0.2	12.5 ± 2.0
5.9	727	0.180 ± 0.050	50	3.6	2.5	50	8.7 ± 2.3	4.1 ± 0.2	9.9 ± 2.4
6.2	631	0.140 ± 0.040	50	2.8	2.8	60			
	665	0.125 ± 0.033	25	5.4	2.1	35	6.9 ± 1.8	3.8 ± 0.3	7.8 ± 1.9
6.7	548	0.140 ± 0.033	50	2.8	2.6	50			
	573	0.080 ± 0.020	50	1.6	2.6	50	6.8 ± 1.8	3.3 ± 0.4	7.7 ± 1.5
7.2	407	0.250 ± 0.080	50	5.0	1.9	30			
	485	0.080 ± 0.025	80	1.0	4.1	135	6.1 ± 1.2	3.0 ± 0.4	6.9 ± 1.3
7.6	407	0.140 ± 0.030	50	2.8	3.3	90			
	431	0.075 ± 0.033	80	0.9	3.6	105	5.1 ± 1.3	3.1 ± 0.5	5.8 ± 1.4
8.0	366	0.140 ± 0.040	50	2.8	3.1	75			
	405	0.095 ± 0.045	80	1.2	3.9	120	3.7 ± 1.0	3.5 ± 0.3	4.2 ± 1.0
				Amino-Terminal Domain: Poly[d(A-T)]					
5.6	485	0.090 ± 0.015	20	4.5	1.5	20	16.7 ± 4.1	0.3 ± 1.2	18.9 ± 4.4
5.9	370	0.150 ± 0.050	50	3.0	2.1	40	6.8 ± 2.3	2.2 ± 0.9	7.7 ± 2.4
6.7	216	0.350 ± 0.130	50	11.2	1.6	25			
	296	0.075 ± 0.025	50	1.5	2.0	30	5.2 ± 0.8	2.1 ± 0.5	5.9 ± 0.9
7.2	240	0.085 ± 0.025	50	1.7	3.2	80	4.4 ± 2.3	2.2 ± 1.3	5.0 ± 2.4
8.0	158	0.145 ± 0.040	80	1.8	3.6	100			
	180	0.105 ± 0.030	80	1.3	3.7	110	3.6 ± 0.8	2.3 ± 0.6	4.1 ± 0.9
				Amino-Terminal Domain: Random DNA					
6.7	35	0.500	1						
	65	0.030	1						
	83	0.010	1						
	103	0.005	1				4.3 ± 0.4	−0.6 ± 0.5	4.9 ± 0.4

[a] The parameters of the last three columns of the table describe the dependence of the observed binding affinities on the NaCl concentration at constant pH. Each set of these parameters refers therefore to a single pH condition (several NaCl concentrations). [b] Determined by isotherm curve fitting. [c] Determined by linear weighted regression of the data plotted according to the method of Schwartz and Watanabe. [d] Determined as $(1/\Psi)\partial \log K\omega/\partial \log [NaCl]$ (assuming no anion release).

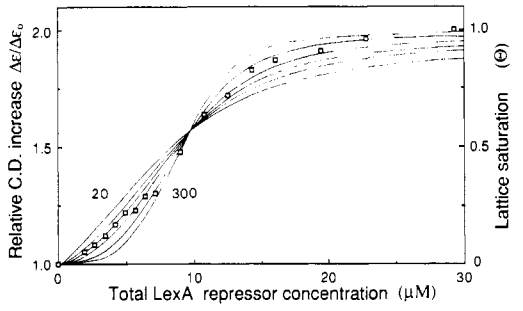

FIGURE 4: Comparison between a family of theoretical isotherms and an experimental association of LexA repressor to poly[d(A-T)] (□; 407 mM NaCl, pH 7.6). Theoretical plots were generated for a constant overall binding constant ($K\omega = 1.4 \times 10^5$ M^{-1}) by using a binding-site size of $n = 8$ base pairs and the following values for the cooperativity parameter ω: 20, 35, 50, 75, 150, and 300.

used for the determination of intrinsic binding constants and cooperativity parameters from the binding isotherms: (i) fitting of theoretical isotherms to the experimental data (eq 3) and (ii) determination of K and ω according to the method of Schwartz and Watanabe (eq 4).

(i) Isotherm Curve Fitting. As an example Figure 4 shows a comparison between a set of theoretical isotherms and an experimental association curve of LexA with poly[d(A-T)] (407 mM NaCl, pH 7.6). These theoretical curves were calculated with a constant value of $K\omega$ determined at half-saturation as described above ($K\omega = 1.40 \times 10^5$ M^{-1}). K and ω were varied reciprocally, giving rise to steeper binding isotherms with increasing ω values. The theoretical curves shown in Figure 4 cross each other somewhat above the midpoint of the titration curve ($\Theta = 0.5$) as expected if the cooperativity is not very strong [see, for example, Figure 2 in Kowalczykowski et al. (1986)]. For this reason the $K\omega$ values determined at half-saturation had to be corrected according to eq 1 (see Theoretical Background).

Visual inspection of the data in Figure 4 shows that the cooperativity parameter ω should be rather close to 50. In general, we focused on a reasonable fit of the experimental data in the first half of the binding isotherms since for $\Theta > 0.5$ the degree of lattice saturation gets very sensitive to the correct choice of the final ellipticity $\Delta\epsilon_{max}$. Additionally, the theoretical isotherms are calculated with the assumption of an infinite polynucleotide lattice leading also to deviations from the experimental values for a high degree of saturation (Kowalczykowski et al., 1986). The error for the determination of ω is nevertheless quite high and may be estimated to be of −50% and +100% since the fitting procedure loses in resolution with increasing ω, leading to an asymmetric error range. The curves in Figures 1 and 2 correspond to the best fits through the data points according to this procedure. In most cases the best fit is achieved for both LexA and its amino-terminal domain with $\omega = 50$ with a few exceptions of either higher or lower apparent cooperativity (see Table I). The intrinsic binding constants (derived from the knowledge of $K\omega$ and ω) are also shown in Table I. In the case of the data in Figure 4 the intrinsic binding constant is 2.8×10^3 M^{-1}.

The curves drawn through the data points of Figure 3 for the essentially noncooperative interaction of the amino-terminal domain with random DNA are theoretical best fit binding curves calculated from eq 2 which were adjusted to the experimental data points by varying K. Both the experimental values and the theoretical curves show that full lattice saturation ($\Theta = 1$) is essentially impossible to achieve under equilibrium binding conditions.

(ii) Linearization of the Binding Data. Figures 5 and 6 show another representation of the binding data (according

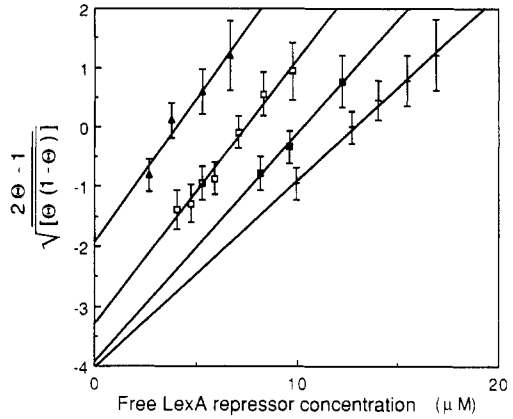

FIGURE 5: Binding data obtained for the interaction of the LexA repressor with poly[d(A-T)] plotted according to the method of Schwartz and Watanabe. Binding conditions were 10 mM sodium phosphate, pH 7.2, 407 mM NaCl (▲), 485 mM NaCl (+); pH 7.6, 407 mM NaCl (□); and pH 8.0, 407 mM NaCl (■).

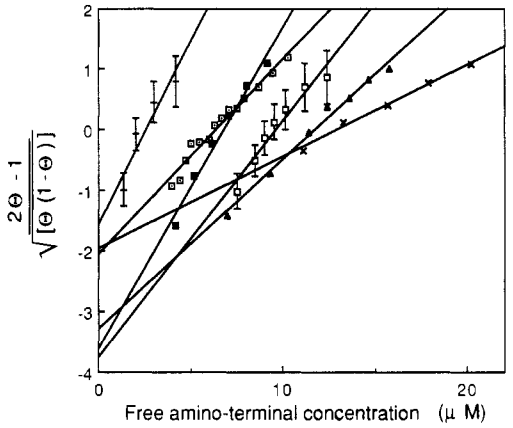

FIGURE 6: Binding data obtained for the amino-terminal domain of the LexA repressor with poly[d(A-T)] plotted according to the method of Schwartz and Watanabe $[(2\Theta - 1)/[\Theta(1 - \Theta)]^{1/2}]$ versus the free protein concentration) within the saturation range 20–80% of the polynucleotide lattice. Binding conditions were 10 mM sodium phosphate, pH 5.9, 360 mM NaCl (⊡); pH 6.7, 204 mM NaCl (+), 295 mM NaCl (×); pH 7.2, 230 mM NaCl (▲); pH 8.0, 136 mM NaCl (■) and 160 mM NaCl (□). For two experiments the experimental error on $(2\Theta - 1)/[\Theta(1 - \Theta)]^{1/2}$ is shown. The straight lines are the linear least-squares fits of the data.

to eq 4) for the entire repressor and the amino-terminal domain, respectively. The data points shown in these figures correspond to a range of fractional saturation of $20\% < \Theta < 80\%$. As predicted from eq 5, a plot of $(2\Theta - 1)/[\Theta(1 - \Theta)]^{1/2}$ versus L_{free} is in all cases a straight line with slope $K\omega\ (\omega/n)1^{1/2}$ and intersection with the ordinate $-(\omega/n)^{1/2}$. Table I shows that both types of analysis led to essentially the same $K\omega$ values as expected and that the differences observed for K and ω are reasonable in view of the precision of the measurements and the fact that isotherm fitting was mainly achieved for $0 < \Theta < 50\%$, whereas the linearized data set covered the range $20\% < \Theta < 80\%$. The average value of the different cooperativity parameters obtained under widely varying solvent conditions is 52 by curve fitting and 68 by the Watanabe procedure in the case of LexA and, respectively, 54 and 58 for the amino-terminal domain. These averages cooperativity values are interesting for a comparison of the two types of analysis and the two protein species. However, ω might change with the salt concentration and/or the pH value. Our data do not allow us to answer this question rigorously. Due to the strong dependence of $K\omega$ on both pH and salt concentration (see below), it is essentially impossible to hold one of these parameters constant and to vary the other one over a fairly broad range.

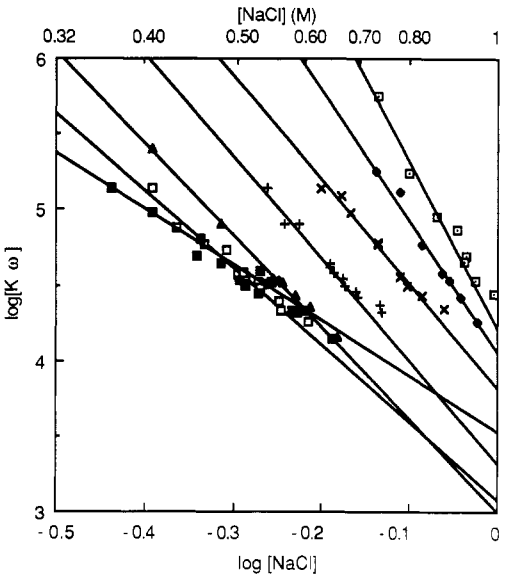

FIGURE 7: Dependence of the overall binding constant (log $K\omega$) of the LexA repressor for poly[d(A-T)] on the monovalent salt (log [NaCl]) concentration at different pH values: pH 5.6 (⊡), pH 5.9 (♦), pH 6.2 (×), pH 6.7 (+), pH 7.2 (▲), pH 7.6 (□), and pH 8.0 (■). Straight lines represent weighted linear least-squares fits of the experimental data points. The slopes and intercepts of these straight lines with the 1 M NaCl axis are listed in Table I.

What we can conclude is that "low pH/high salt" conditions led essentially to the same ω as "high pH/low salt" conditions. This means that either ω is independent of both parameters—what we believe to be most likely—or the dependency from the two parameters is opposite and is canceled out.

Salting-Back Experiments. A more detailed study of the relationship between $K\omega$ and the salt concentration of the solution was done by additional experiments according to the salting-back procedure (Kowalczykowski et al., 1984). These experiments consist in a stepwise dissociation of a preformed complex by addition of increasing amounts of a highly concentrated salt solution. As in the case of the association isotherms, $K\omega$ will arise directly from $L_{free\ 1/2}$ at half-dissociation of the complex. Additionally, K and $K\omega$ may be calculated for any fractional saturation Θ if ω is known. Since the salt concentration changes during these experiments, one has to assume that ω is independent of the salt concentration. In the case of LexA and its amino-terminal domain this assumption seems to be reasonable in that $K\omega$ values determined from $L_{free\ 1/2}$ and those from salting-back experiments gave very similar results. Figures 7 and 8 show the dependence of log $K\omega$ on log $[Na^+]$, respectively, for LexA and its amino-terminal domain at different pH values. In all cases the data can be reasonably fitted with straight lines by using a linear least-squares procedure. It is immediately obvious from these figures that both the slope of these lines and the overall binding constant increase quite dramatically with decreasing pH (see below). The data in Figures 7 and 8 allow a comparison of the overall binding constants of LexA and of the amino-terminal domain and a comparison between the affinities for poly[d(A-T)], random DNA, and operator DNA.

LexA Interacts More Strongly with Poly[d(A-T)] than Its Isolated DNA Binding Domain. The fact that LexA interacts more strongly with poly[d(A-T)] than the isolated amino-terminal domain is qualitatively obvious from the observation that under identical pH conditions higher salt concentrations are necessary in the case of LexA to establish equilibrium binding conditions. Therefore, a comparison of the binding

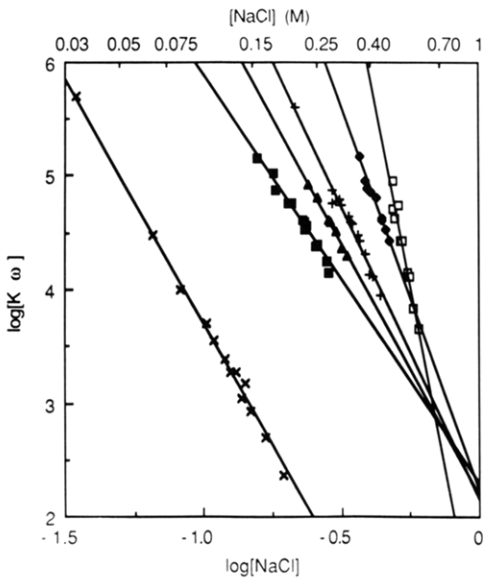

FIGURE 8: Dependence of the overall binding constant of the amino-terminal domain of the LexA repressor for poly[d(A-T)] (log $K\omega$) and pBR345 DNA (log K) on the monovalent salt concentration (log [NaCl)] at different pH values. Poly[d(A-T)]: pH 5.6 (□), pH 5.9 (♦), pH 6.7 (+), pH 7.2 (▲), and pH 8.0 (■). pBR345 DNA: pH 6.7 (×). Straight lines represent weighted linear least-squares fits of the experimental data points. The slopes and intercepts of these straight lines with the 1 M NaCl axis are listed in Table I.

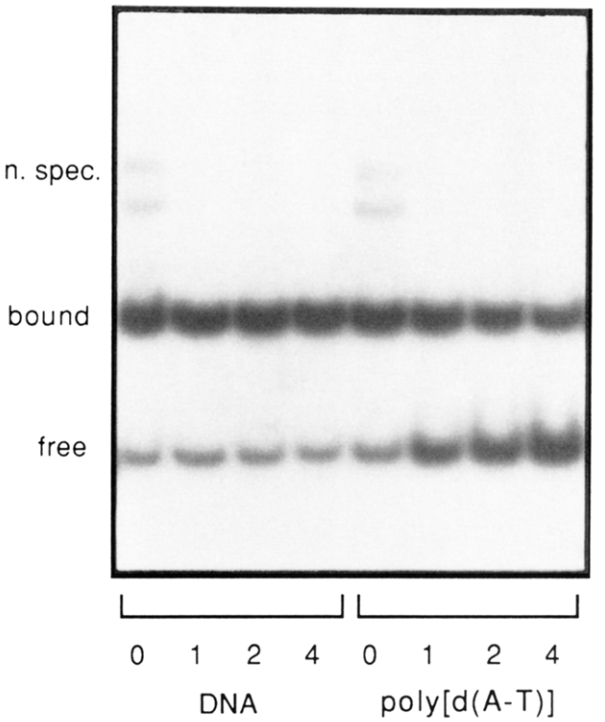

FIGURE 9: Competition of pBR345 DNA and poly[d(A-T)] with the specific interaction between the entire LexA repressor and the *recA* operator as monitored by gel retardation as described earlier (Schnarr et al., 1985). Lanes labeled 1, 2, and 4 contain increasing amounts of either pBR345 or poly[d(A-T)]: 0.14, 0.28, or 0.56 mM (in base pairs). A low ionic strength binding buffer was used for these experiments (10 mM Tris, pH 7.4).

affinities of the two proteins necessitates an extrapolation of the binding data to salt concentrations outside the experimental binding range for at least one of the protein species. LexA binds 20-fold more strongly at pH 8.0/300 mM NaCl, 30-fold more strongly at pH 7.2/400 mM, and 50-fold more strongly at pH 6.7/500 mM. These salt concentrations have been chosen between the highest and lowest salt concentrations useful for binding studies with, respectively, the amino-terminal domain and LexA to minimize errors from extrapolation. As shown previously for the specific interaction of the amino-terminal domain with the uvrA operator (Bertrand-Burggraf et al., 1987) and the *recA* operator (Hurstel et al., 1988), the amino-terminal domain preserves also a considerable binding affinity for poly[d(A-T)] despite the lack of the carboxy-terminal dimerization domain. The specific DNA binding studies have shown an about 14-fold stronger binding of LexA at pH 7.9, in fairly good agreement with a factor of 20 in the case of poly[d(A-T)] at pH 8.0.

LexA Interacts Much More Strongly with Poly[d(A-T)] than with Random DNA. Figure 8 allows further a comparison between the binding of the amino-terminal domain to poly[d(A-T)] and to random DNA. The slopes of both log–log plots for the binding data obtained at pH 6.7 are very similar, suggesting similar or identical electrostatic contributions to the binding energy of the two complexes. The overall binding constant $K\omega$ of the amino-terminal domain for the interaction with poly[d(A-T)] is, however, about 2000-fold greater than the binding constant K for random DNA.

This great difference in binding affinity is due to two different factors of similar importance. First, the binding to poly[d(A-T)] is cooperative with an average cooperativity factor of about 50–60 in the case of the amino-terminal domain, and, second, the intrinsic binding constant K for poly[d(A-T)] is thus greater by a factor of about 30–40 than that for random DNA.

As mentioned above, equilibrium CD measurements with random DNA could not be done for the entire repressor because of precipitation of the complex at intermediate ionic strength (about 30–150 mM NaCl). At higher ionic strength the complex with DNA is too weak to allow for a precise determination of the binding constant, whereas interaction with poly[d(A-T)] is observed under these conditions up to about 700 mM NaCl. The preference of the entire LexA repressor for poly[d(A-T)] is, however, obvious from Figure 9, where a specific complex of LexA with the recA operator has been challenged with poly[d(A-T)] and random DNA. Essentially no competition is observed with DNA, whereas the same amounts of poly[d(A-T)] compete successfully with the recA operator for LexA binding.

The data in Figure 7 may be used further to compare the binding of LexA to poly[d(A-T)] with that to the different SOS operators by extrapolation of $K\omega$ to lower salt concentrations. The association constants measured for the different operators vary from 5×10^9 M^{-1} for the umuDC operator to 3×10^7 M^{-1} for the uvrA operator at pH 7.0/200 mM KCl [for a compilation of these values see Bertrand-Burggraf et al. (1987)]. Assuming that NaCl and KCl are equivalent for protein–DNA interactions an extrapolation of the data in Figure 7 to 200 mM yields an overall association constant $K\omega$ of about 4×10^7 M^{-1}; that is, in the cooperative binding mode poly[d(A-T)] binds LexA as well as a weak SOS operator like uvrA.

pH Dependence of LexA Binding. A vertical cut through the log–log plots at a given salt concentration in Figures 7 and 8 allows a determination of the pH dependence of the overall binding constants. The salt concentrations used for this extrapolation should be close to the center of the data points shown respectively in Figures 7 (LexA) and 8 (amino-terminal domain) to minimize errors from extrapolation. Figure 10 shows such extrapolations at 600 mM NaCl in the case of the entire LexA repressor and at 350 mM NaCl in the case of the isolated amino-terminal domain. In both cases the overall

Table II: Equilibrium Parameters in Buffers Containing $MgCl_2$ Only[a]

pH	[$MgCl_2$] (mM)	$K\omega$ ($\times 10^{-6}$ M^{-1})	ω^b	K ($\times 10^{-3}$ M^{-1})	$(\omega/n)^{1/2}$	ω^c	$-(\partial K\omega/\partial \log [MgCl_2])$	log ($K\omega$) (at 1 M $MgCl_2$)	Z^d	a^e
				LexA Repressor: Poly[d(A-T)]						
5.6	189	0.120 ± 0.025	100	1.2	3.2	80				
	208	0.063 ± 0.020	50	1.0	3.0	70	5.3 ± 1.2	1.2 ± 0.7	13.9	−1.2
6.2	155	0.090 ± 0.020	100	0.9	3.4	90				
	164	0.050 ± 0.020	150	0.3	5.2	200	4.7 ● 1.2	1.1 ± 0.9	5.4	2.1
7.2	116	0.030 ± 0.010	50	0.6	2.0	30	2.8 ± 1.7	1.9 ± 1.5	8.0	−1.0

[a] The parameters of the last three columns of the table describe the dependence of the observed binding affinities on the NaCl concentration at constant pH. Each set of these parameters refers therefore to a single pH condition (several NaCl concentrations). [b] Determined by isotherm curve fitting. [c] Determined by linear weighted regression of the data plotted according to the method of Schwartz and Watanabe. [d] Determined independently from a (the number of anions released from the protein) as the difference of the slopes obtained in the presence of NaCl and $MgCl_2$ only, divided by $\Psi - \Phi$. [e] Number of released anions determined as $a = -\partial[\log K\omega]/\partial[\log [NaCl]] - \Psi Z$ (see text).

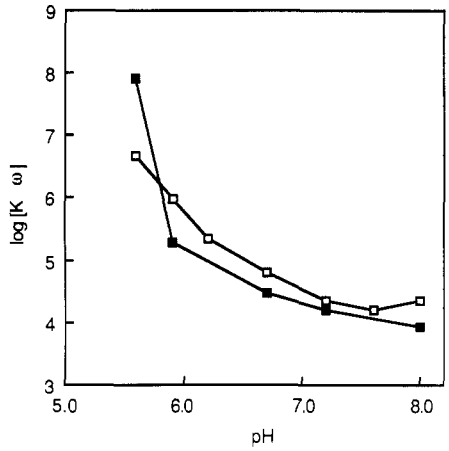

FIGURE 10: pH dependence of the overall binding constant for poly[d(A-T)] of the LexA repressor at 600 mM NaCl (□) and of its amino-terminal domain at 350 mM NaCl (■) as determined from the data in Figures 7 and 8.

binding constant $K\omega$ increases strikingly if the pH value becomes slightly acidic, whereas between pH 7 and pH 8 only minor changes are observed.

If the protonation of a functional group of LexA would be a prerequisite for efficient binding to DNA, the binding constant would be expected to decrease linearly in this moderately alkaline pH range as observed, for example, in the case of the lac repressor headpiece (Schnarr et al., 1983). This is obviously not the case for LexA, suggesting that term B of eq 5 may not explain this rather unusual pH behavior. The "titration curve model" (Lohman et al., 1980) seems also not suitable to explain this log $K\omega$ versus pH curve, because in this case the binding constants are expected to show a sigmoidal titration curve behavior as a function of pH. Especially below pH 6 no further changes of $K\omega$ would be expected if the titrable groups are histidines or the amino-terminal α-NH_2 group.

The interpretation of this unusual pH dependence of log $K\omega$ is further complicated by the observation that the slopes of the log $K\omega$ versus log [NaCl] plots for both LexA and its amino-terminal domain (Figures 7 and 8) increase markedly with decreasing pH values. The corresponding $\partial(\log K\omega)/\partial(\log [NaCl])$ values range from about 4 at pH 8 up to more than 10 at pH 5.6 (see Table I). According to eq 6 these values are potentially linked to the number of cations released from the DNA and to the number of anions released from the protein upon binding.

To address the question if anion release (or uptake) from the protein plays a major role, we have done additional measurements of log $K\omega$ as a function of the divalent cation Mg^{2+}. Table II shows that at least at pH 5.6 and pH 7.2 the corresponding $\partial(\log K\omega)/\partial(\log[MgCl_2])$ values are about half those found in the case of NaCl. According to eqs 6 and 7 this means that only very few or no anions are released from the protein. A combination of these equations allows a precise determination of the parameter a that is found to be about −1 at pH 5.6 and 7.2 and about +2 at pH 6.2 (see Table II). Taking into account the rather large experimental error in this type of measurement, we would argue that neither anion release nor anion uptake by LexA plays a major role upon DNA binding. Assuming thus that no anion release or uptake takes place, the slopes of Figures 7 and 8 may be converted directly into the number Z of cations released from the DNA by multiplication with $\Psi = 0.88$, giving rise to more than 12 cations released at pH 5.6 and only 4 at pH 8 upon binding of both LexA and its isolated DNA binding domain. Earlier measurements on LexA at pH 6.5 only (Schnarr & Daune, 1984) led to a Z value of 7 ± 1, in good agreement with the values reported in Table I.

In terms of the titration curve model this difference in the numbers of cations released at pH 5.6 and pH 8 would mean that LexA should contain at least eight titrable side chains forming salt bridges with the DNA backbone if the side chains are protonated. In view of the chemical composition of the protein (only two histidines are found within the amino-terminal domain) this explanation seems highly unlikely. Several other factors might contribute to this unusual pH dependence of the overall binding constant and the apparent number of released counterions.

First, one may argue that protonation of poly[d(A-T)] at acidic pH values might be involved in this phenomenon. Using an intrinsic pK value of 4.4 for dAMP (Fasman, 1975), one would expect that about 6% of the adenine bases might be protonated at pH 5.6, the highest pH value examined in our study. If LexA would bind preferentially to a segment containing protonated adenine bases, the pK value would be raised, leading potentially to a higher degree of protonation. The presence of positive charges close to the DNA helix axis might be energetically favorable for DNA binding, since LexA contains two adjacent negatively charged side chains (Glu-44 and -45) within its putative recognition helix (Lamerichs et al., 1989). However, one should keep in mind that adenine protonation occurs at the N1 position, interfering thus with one of the Watson–Crick hydrogen bonds leading potentially to a local deformation of the double helix.

Second, the two glutamic acid side chains mentioned above will also start to titrate around pH 5.6, since with an intrinsic pK value of 4.25 (Fasman, 1975) one would expect that about 4% of each of these glutamic acids would be protonated and thus be neutralized. Again, if the neutralization of these negative charges would be favorable for LexA binding, the pK value would be increased.

Third, low pH values might favor the dimerization of the protein, but since essentially the same pH behavior is observed

for both LexA and the amino-terminal domain, it is unlikely that this effect would be linked to an increased dimerization via the carboxy-terminal domain. We have nevertheless checked this possibility by equilibrium ultracentrifugation at pH 5.6/600 mM NaCl using otherwise the same procedure as described earlier (Schnarr et al., 1985). The dimerization constant of LexA under these conditions is very similar to that determined earlier at pH 7.2/150 mM NaCl, that is, 4×10^4 M^{-1} instead of 2×10^4 M^{-1}.

Fourth, the more efficient DNA binding observed at moderately acidic pH values might also be linked to a pH-induced conformational change within the DNA binding domain. However, CD measurements in the peptide absorption range did not reveal any change in the pH range used for the binding studies, excluding at least a major conformational change in this pH range.

Discussion

The interaction with poly[d(A-T)] is cooperative for both LexA and the isolated DNA binding domain, whereas no cooperativity is detected upon interaction of the DNA binding domain with random DNA. We are not aware of a similar matrix-dependent cooperativity for other DNA binding proteins. We have to assume that cooperativity may be due either to direct contacts between adjacent protein molecules or to a modification of the DNA structure adjacent to a bound protein despite the fact that the direct contact model is actually favored by most researchers working in this field.

If protein–protein contacts would be at the origin of the observed cooperativity, it should arise mostly from contacts between adjacent amino-terminal domains, and at least two arguments might explain why poly[d(A-T)] is favored with respect to random DNA: (i) Since poly[d(A-T)] is homologous to the consensus sequence, LexA may interact with this polymer in a specific binding mode, leading to a positioning of adjacent amino-terminal domains suitable for protein–protein contacts that could not be formed in a purely nonspecific binding mode with random DNA. (ii) The formation of these protein–protein contacts requires a deformation of the DNA matrix (torsion, bending) requiring less energy in the case of poly[d(A-T)] than in the case of random DNA.

If cooperativity would not be due to protein–protein contacts but due to a conformational change of adjacent DNA segments, we would naturally expect that this effect takes place in a similar way for LexA and its DNA binding domain. To explain the preference for poly[d(A-T)], we may argue that the "transmission" to adjacent matrix segments upon binding of a first ligand is facilitated in the case of poly[d(A-T)] with respect to random DNA. At present we are unable to decide which mechanism is operational in the case of LexA.

On the average the cooperativity parameter ω for both LexA and its amino-terminal domain was found to range from 50 to 70 depending slightly on the procedure used for the determination of ω. This ω value corresponds to an additional free energy $-RT \ln \omega$ of about 2.4 kcal/mol. A similar value has been determined for the interaction of the phage λ repressor upon cooperative binding to OR1 and OR2 (Johnson et al., 1979); however, in this case the cooperativity is mediated by the carboxy-terminal domain. As in the case of LexA the carboxy-terminal domain of phage λ repressor seems not to be directly involved in DNA binding.

The overall association constant $K\omega$ for the binding of the amino-terminal domain to poly[d(A-T)] is about 2000-fold stronger than its binding constant to random DNA. Qualitatively, this preference for poly[d(A-T)] has been confirmed for the entire repressor by using band-shift competition experiments. Taking into account that this preference is in part due to cooperativity in the case of poly[d(A-T)], the intrinsic binding constant K for poly[d(A-T)] is about 30–40-fold stronger than that found for random DNA, suggesting that poly[d(A-T)] constitutes in fact a contiguous pseudo-operator retaining some binding specificity as suggested from its homology with the consensus sequence.

We have shown further that the overall association constants for the interaction of both LexA and its amino-terminal domain with poly[d(A-T)] and random DNA depend strongly on both salt concentration and pH and that the steepness of the salt dependence is much more pronounced at slightly acidic pH values than at neutral or slightly alkaline pH. This behavior is not easily understood in terms of the current theories on the electrostatic contribution to protein–DNA interactions based on polyelectrolyte theory. The same holds true for the dependence of the overall binding constant on pH that shows no tendency to level off at acidic pH values as expected both for the titration curve and the "protonation requirement" models. A subtle pH-induced conformational change of the amino-terminal domain might be at the origin of this unusual behavior. Other possibilities that might contribute to this behavior are the presence of two negatively charged titrable side chains (Glu-44 and -45) within the putative DNA binding helix across amino acids 41–54 (Lamerichs et al., 1989) and the onset of adenine protonation at the N1 position (see above).

Finally, a comparison of the overall binding constant $K\omega$ of the entire LexA repressor for poly[d(A-T)] with that of its isolated amino-terminal domain reveals that LexA binds poly[d(A-T)] only 20–50-fold more strongly under a wide variety of salt and pH conditions. This result tends to demonstrate further that the additional energy due to the dimerization of LexA via the carboxy-terminal domain should be rather weak. The loss in binding free energy corresponds to about 1.8–2.3 kcal/mol per monomer of amino-terminal domain as compared to the binding of the entire repressor. The dimerization of LexA mainly via the carboxy-terminal domain is expected to contribute about 6.0 kcal/mol for each formed dimer (Schnarr et al., 1985, 1988). Assuming an equal partition of half of this energy on each monomer, that is, 3.0 kcal/mol, one may argue that the energy balance is fairly respected within the limits of experimental precision for both the determination of the dimerization and the DNA binding constants.

Acknowledgments

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Registry No. d(A-T), 26966-61-0; Mg, 7439-95-4.

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CORRECTION

A Comparative Monomolecular Film Study of 1,2-Di-*O*-palmitoyl-3-*O*-(α- and β-D-glucopyranosyl)-*sn*-glycerols, by B. Asgharian, D. A. Cadenhead,* D. A. Mannock, R. N. A. H. Lewis, and R. N. McElhaney, Volume 28, Number 17, August 22, 1989, pages 7102–7106.

Page 7105. In Table II, the T_m and T_h values for di-16:0-α-GlcDG and di-16:0-β-GlcDG are inverted. The data should read as follows.

lipid sample	T_m (°C)	ΔH_{cal} (kcal/mol)	T_h (°C)
di-16:0-α-GlcDG	57.2	9.5[c]	79.1[c]
di-16:0-β-GlcDG	61.0	9.0[d]	75.0