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Quantitative Analysis of DNA Binding by the *Escherichia coli* Arginine Repressor

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Allosteric activation of the hexameric arginine repressor (ArgR) for specific operator DNA binding appears to involve alteration in its quaternary structure. Current models for activation include subunit assembly and/or domain rearrangements in response to binding of the coeffector L-arginine. To investigate the molecular basis for ArgR operator interactions, we have carried out a series of quantitative analyses of ArgR subunit assembly and of the affinity, stoichiometry, cooperativity, and L-arginine- and DNA sequence-dependence of ArgR-DNA binding. The results indicate that subunit assembly plays no role in activation, although communication among subunits of the ArgR hexamer is required for specific DNA binding. The data suggest that DNA is also an allosteric effector of ArgR.

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Introduction

The arginine repressor of *Escherichia coli*, ArgR, is the master regulator of a set of genes known as the arg regulon.[1] ArgR transduces the signal from intracellular L-arginine (L-Arg) to repress transcription of a group of genes involved in arginine biosynthesis (reviewed by Maas[2]). ArgR is also required as a cofactor in the resolution of plasmid ColE1 multimers during replication.[3] ArgR homologs in *Bacillus subtilis* (AhrC) and *Salmonella typhimurium* have been shown to activate transcription of certain genes involved in arginine catabolism.[4,5] The ArgR polypeptide chain of 156 residues assembles into a hexamer of total molecular mass approximately 100 kDa.[6] Binding of L-Arg to the ArgR hexamer allosterically activates the protein for binding to DNA operator sequences upstream of each of the regulated genes, and in the cer locus for recombination. Thus, ArgR is a protein with multiple biochemical and physiological functions.

The mechanism by which binding of the corepressor L-Arg to ArgR allosterically activates the protein for DNA binding is presently unknown. The location of L-Arg binding sites at the interface between two trimeric layers in the ArgR hexamer, as determined from X-ray crystallographic analysis, suggested that L-Arg may stabilize the hexameric state.[7,8] The hexamer appears to be the active form of ArgR for DNA binding;[9–11] thus, hexamer stabilization could provide a link between L-Arg binding and DNA binding. On the other hand, trimers of ArgR have been reported to bind operator DNA,[12,13] with DNA binding apparently mediating their assembly into hexamers.[14,15] Structural comparison of apo and holo forms of ArgR also suggested that domain adjustments induced by L-Arg binding may alter the disposition of the DNA-binding surfaces of the protein.[8]

Operator-region DNAs from regulon members show a clear consensus site for ArgR binding, comprising a pair of half-sites in palindromic arrangement, 5′TGNAT(A/T)$_4$ATNCA3′, repeated with fixed spacing to form a tandem pair of palindromes (Figure 1). Footprinting and stoichiometry analyses[9–11] showed that one hexamer contacts natural operators over a span of nearly 40 bp containing the two palindromes. Contacts are made

1 half site (←)

```
            ←
      CTAGAGATTATTCAG
      TCTAATAAGTCAGCT
```

1 palindrome = pair of half sites (→ ←)

```
           →              ←
    CTAGGACAGTGAATAATTATTCACTCAG
    CGTGTCACTTATTAATAAGTGAGTCAGCT
```

tandem palindromes (→← →←)

```
       →           ←            →           ←
  AGTGAATAATTATTCAGTATAAGTGAATAATTATTCAGT
  TCACTTATTAATAAGTCATATTCACTTATTAATAAGTCA
```

control sequence

```
      TGCGTAATGCTATAATATTACAACATTTCGC
      CGCATTACGATATTATAATGTTGTAAAGCGT
```
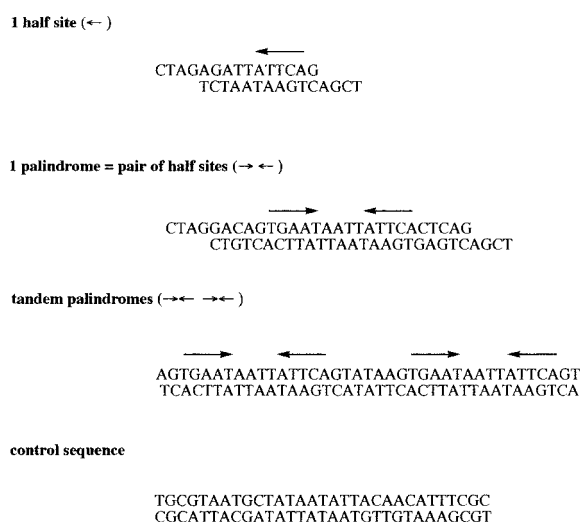
Figure 1. DNA oligonucleotides used in this work. The arrows indicate the conserved half-site sequence 5′ TGNAT 3′ derived from comparison of natural *arg* operators.[2] As described in the text, in some cases DNAs were end-filled prior to use by extension of underhanging 3′ ends with the large fragment of DNA polymerase I; in other cases DNAs were cloned into the *Sal*I and *Xba*I sites of plasmid pBend3 and excised on restriction fragments.

exclusively on one "face" of the DNA helix, and are facilitated by bending of the DNA by about 80° at a locus between the pair of palindromes as mapped by circular permutation. Detailed modeling using the protein structure showed that available high-resolution DNA chemical footprinting data could be accounted for by the binding of only four DNA-binding domains to typical operators containing tandem palindromes.[16] This result suggests that only four of the six equivalent subunits of hexameric ArgR are engaged in operator binding, consistent with the organization of Arg operators. Although the inference is consistent with other lines of indirect evidence, available results provide no direct information on whether there is a one-to-one correspondence between DNA half-sites and protein subunits, or whether all six subunits are potentially active in binding.

To provide basic information about DNA binding that can be used to constrain models for allosteric activation in the ArgR system, we have carried out a series of quantitative analyses of its DNA and subunit interactions. Measurements of affinity, stoichiometry, cooperativity, and L-Arg dependence suggest that communication among subunits is required for activation. The results also imply that DNA is an allosteric effector of specific binding, and that subunit assembly plays no role in activation.

Results

ArgR subunit assembly

The subunit assembly state of ArgR was determined by analytical ultracentrifugation using equilibrium sedimentation as a function of protein concentration in the presence and absence of L-Arg. Solution conditions were 20 mM Tris-HCl (pH 7.6), 10 mM MgCl$_2$, 0.2 M NaCl, 0.2 mM dithiothreitol, 4 °C. Radial distribution data were collected at several rotor speeds and several loading concentrations for both apo and holo ArgR. Experiments using short columns of solution (ca 2 mm) indicate that both apo and holo ArgR form stable hexamers in the concentration range of 1 to 10 µM total protein monomers (Table 1). This conclusion is supported by the average molecular masses obtained from global fitting to data collected at several rotor speeds, as well as by the lack of dependence of molecular mass on either rotor speed or loading concentration.

To better quantify hexamer stability, we used longer columns of solution (approximately 10 mm) to collect additional data for 1 µM apo ArgR at two rotor speeds (7000 and 10,000 rpm). The higher rotor speed was selected to deplete the meniscus in order to obtain better baseline values. Such an experiment, while time-consuming, offers the most sensitive approach to detecting lower molecular mass species in the ultracentrifuge when protein absorbance is limiting as it is here (see Materials and Methods). Global analysis of these data (Table 1 and Figure 2) is consistent with the 2 mm experiments, and yields an apparent molecular mass for the protein of 102,300 ± 1700.

An upper limit of the dissociation constant for a hexamer-trimer equilibrium was estimated by fitting various K_d values to the radial distribution data (not shown). The fit with $K_d = 0.25$ µM yielded clearly non-random residuals. The residuals improved steadily as the K_d value was reduced to 2.5 nM, suggesting that the stability of ArgR hexamers is at least as strong as 2.5 nM under the conditions used. Our results disagree with the earlier findings by Holtham *et al.*,[15] who reported dissociation of the ArgR hexamer with K_d in the range of 10 to 100 µM. Those authors report, however, that background noise from the solvent limited the evaluation of molecular mass as a function of concentration and restricted the use of global analysis procedures. Treatment of multicomponent systems depends critically on these types of analyses. We found that substituting DTT for β-mercaptoethanol reduced background noise, permitting global analysis of our data.

Sedimentation velocity experiments were performed to measure the sedimentation coefficient. At an ArgR monomer concentration of 185 µM, the uncorrected S-value determined in this measurement was 4.3 S, in agreement with the previous report.[15] The same S-value was found for both apo and holo ArgR within error (estimated at ca 3%).

Table 1. Sedimentation equilibrium analysis of ArgR

	Speed (rpm)			
Sample	8000	10,000	12,000	Global[a]
Apo ArgR, 10 μM	91,547	104,778	104,696	104,000(±5500)
Apo ArgR, 6 μM	109,544	108,558	101,901	104,100(±3500)
Apo ArgR, 2 μM	96,231	100,258	98,368	98,800(±6000)
Apo ArgR, 1 μM[b]	108,600(±9600)[c]	98,100(±4100)		102,300(±1700)
Holo ArgR, 10 μM	105,681	106,257	104,284	104,800(±5400)
Holo ArgR, 6 μM	108,064	107,243	106,010	106,400(±3400)
Holo ArgR, 2 μM	94,752	95,163	96,560	96,100(±9400)

Values are the best-fit molecular mass obtained from analysis of the data using a single-species model. Calculated monomer molecular mass = 16,994.5 Da; hexamer molecular mass = 101,967 Da.

[a] Values obtained by simultaneously fitting data from each set of three speeds.

[b] Long column experiment (see the text).

[c] Collected at 7000 rpm.

Sedimentation equilibrium experiments at this protein concentration show no difference in molecular mass between apo and holo ArgR. The S-value was converted to $s^{\circ}_{20,w}$ using the program Sednterp,[17] yielding an $s^{\circ}_{20,w}$ value for ArgR of 7.1 S. Shape and hydration information about ArgR was extracted by comparing the frictional coefficient calculated from the experimentally determined value of $s^{\circ}_{20,w}$ to the frictional coefficient calculated for an anhydrous sphere of equivalent mass. The ratio of the two frictional coefficients typically deviates from 1 by about 10% due to hydration and by larger amounts due to non-spherical shape;[18] for a spherical protein of
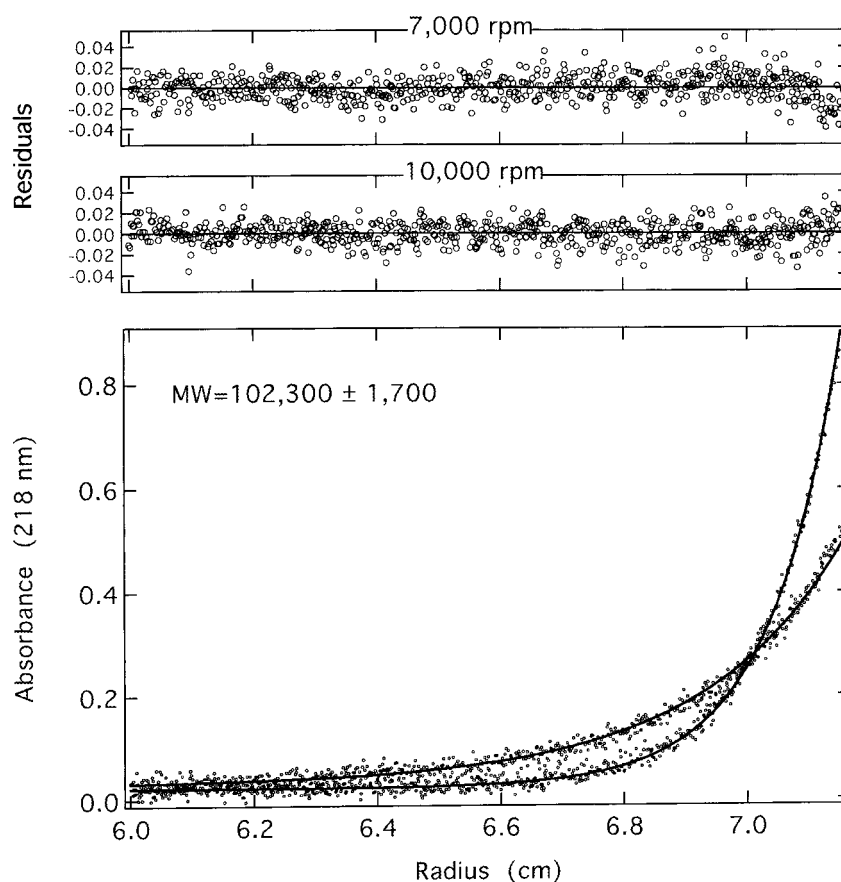

Figure 2. Sedimentation equilibrium analysis of apo ArgR. Samples were run at a loading concentration of 1 μM at two rotor speeds using a double-sector cell as described in the text and Materials and Methods. Open circles are radial distribution data (lower) or residuals (upper) from global fitting of the data from both speeds simultaneously to a single-species model (continuous lines) with molecular mass as an adjustable parameter. The best-fit molecular mass and error of the fit are shown.

this size with typical hydration the expected value of the ratio is approximately 1.13. For the ArgR hexamer with $s^\circ_{20,w}$ 7.1, the calculated ratio of frictional coefficients is 1.016, which would indicate that ArgR is highly spherical and anhydrous. However, the sedimentation equilibrium data indicate that approximately 10 % of the total protein is present in the form of higher-order multimers at these concentrations (not shown). Inclusion of this fraction yields a frictional coefficient ratio of 1.12, fully consistent with that expected for a hydrated sphere. Taken together, the data indicate that structural changes accompanying L-Arg binding are too small to be detected as a change in hydrodynamic size. Given the magnitude of errors in the measured sedimentation values, the maximum difference between apo and holo ArgR that is consistent with the data is no more than about 1 Å in Stokes' radius.

DNA design and affinity estimation

A set of DNA operator constructs was devised to meet the competing requirements for comparative quantitative analysis of affinity, stoichiometry, and cooperativity on DNA molecules with various half-site arrangements (Figure 1). In addition to native operators with tandem palindromes, one member of the regulon, argG, has an isolated third palindrome near its operator region.[9] As well, a single homologous palindrome is found in the cer locus where ArgR acts during resolution of ColE1 multimers.[3] Thus, binding of ArgR to both single and tandem palindromes is of interest for understanding its DNA-binding functions. For measurements of stoichiometry, oligonucleotides were designed bearing one or two palindromes, with the most conserved nucleotides chosen for the variable positions, as shown (Figure 1). A DNA oligonucleotide containing only a single half-site was also designed, as well as a control DNA lacking arg operator sequences. For the rigorous measurement of affinity and cooperativity and the bending analysis described in the following sections, each oligonucleotide was also cloned into plasmid pBend3[19] and excised on restriction fragments of 120-140 bp.

In order to establish the conditions required for stoichiometry determination with the oligonucleotides, preliminary measurements of their affinity were carried out by gel retardation using protein-excess titrations at fixed DNA concentration (ca 0.7 nM). For each DNA oligomer, the protein concentration at which half the free duplex DNA is depleted from its characteristic position in the gel, estimated by scanning the autoradiograms, is taken as the preliminary estimate of K_d. However, at the DNA concentrations used in these experiments, both the single and tandem palindromic DNA oligomers are present initially as two species with different mobilities (e.g. see Figure 3 lane 1). Gel-shift experiments, conducted in the absence of ArgR by titrating each labeled single strand with the unlabeled opposite strand and annealing prior
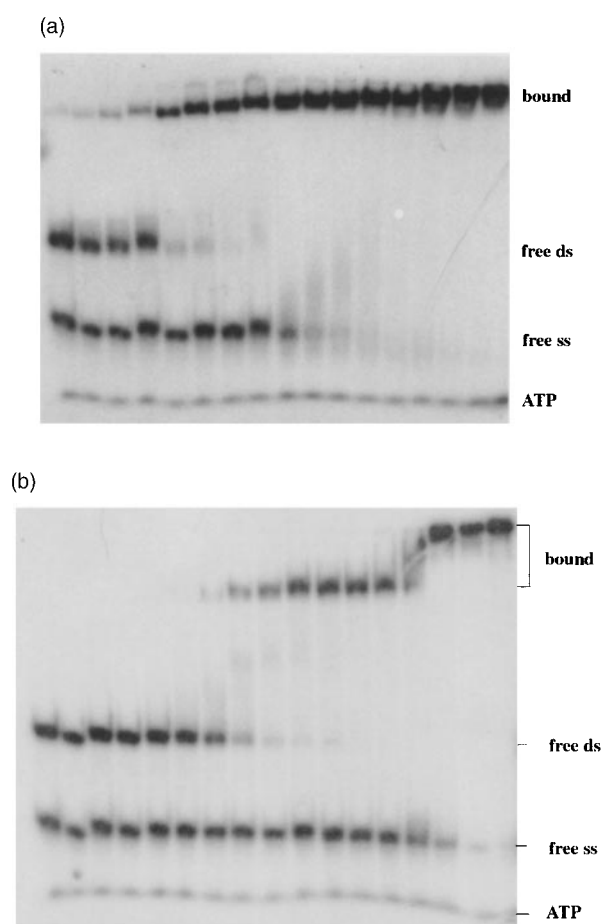

Figure 3. Estimation of ArgR affinity for the tandem palindrome oligonucleotide using gel-shift assay. The autoradiograms of the gels are shown. Radiolabeled DNA was at 0.7 nM; final protein concentrations in lanes from left to right are 0 ((b) only), 1.2, 2.4, 4.8, 9.6, 18, 36, 72, 144, 288, 576 nM, 1.2, 2.4, 4.8, 9.6, 18, 36 μM (hexamer). Identity of species indicated on the right was confirmed in control experiments (not shown): from bottom to top, unincorporated ATP from the kinase reaction; unbound single (ss) and double-stranded (ds) oligonucleotide; and bound forms. (a) Holo ArgR. Binding reactions carried out with 10 mM L-Arg in the binding buffer were resolved on gels containing 5 mM L-Arg with 1 mM L-Arg recirculating in the running buffer. (b) Apo ArgR. Binding reactions carried out and resolved on gels in the absence of L-Arg.

to electrophoresis (data not shown), demonstrate that the species with faster mobility represent the individual single-stranded oligomers, presumably in the form of stable hairpins. For duplexes bearing a symmetric tandem palindrome, the hairpin forms are expected to present a single palindrome in the duplex region. For duplexes bearing a single palindrome, the hairpins present a single half-site. The presence of single-stranded forms complicates quantitative analysis of binding affinity using oligonucleotides, and precludes analysis of binding at lower DNA concentrations; thus, the K_d estimates

Table 2. DNA binding by ArgR

No. of palindromes	Oligonucleotides			Restriction fragments					
	Length (bp)[a]	−L-Arg K_d	+L-Arg K_d	Length (bp)	−L-Arg		+L-Arg		Bend angle (deg.)
					K_d	ΔpL	K_d	ΔpL	
3	n.a.	n.a.	n.a.	270	50 nM	1.9	0.6 nM	1.9	n.d.
2	39	200 nM	7 nM	120	50 nM	1.9	0.8 nM	1.9	85
1	32	1.8 μM	100 nM	140	0.6 μM	1.9	16 nM	1.4	35
1/2	19	25 μM	2.5 μM	130	1.6 μM	1.7	0.1 μM	1.0	10
0[b]	31	12 μM	2.4 μM	120	1.6 μM	1.6	0.2 μM	1.1	10

A discussion of errors is given in Materials and Methods. n.a., not applicable; n.d., not determined.
[a] Duplex length after end-filling.
[b] Control DNA lacking *arg* operator sequences.

derived here must be regarded as upper limits. These limiting values of K_d are adequate to set the conditions for stoichiometry experiments using the oligomer duplexes, but for the precise measurements of affinity and cooperativity at very low DNA concentrations presented in later sections, the cloned DNAs corresponding to each operator configuration were used.

The affinity estimates for the binding of each DNA oligonucleotide to apo and holo ArgR are collected in Table 2. As expected from previous work with natural operators,[9–11] the apparent affinity increases with the number of half-sites. Typical gels showing ArgR binding to the tandem palindromic DNA oligomer in the presence and absence of L-Arg are shown in Figure 3. In the presence of L-Arg (Figure 3(a)), complex formation is detectable as a single new species with slow mobility at ArgR concentrations as low as ∼1 nM (hexamer). Half-depletion of the free DNA duplex occurs near 10 nM ArgR, slightly weaker than the affinity values previously reported for natural tandem palindromes on restriction fragments.[9–11] At ArgR concentrations of ca 300 nM, the free hairpin forms of the tandem palindrome also become depleted, and the bound forms undergo further reduction in mobility. The apparent affinity of the hairpin is similar to the affinity of the duplex bearing a single palindrome (cf. Table 2). As the ArgR concentration is increased to the micromolar range, the mobility of the bound forms decreases further until at the highest ArgR concentrations tested the bound forms barely enter the gel. The bound forms with decreasing mobility seen when protein is in great excess over DNA presumably represent complexes with additional ArgR hexamers bound. Essentially identical results, both the qualitative appearance of the complexes and the quantitative response to ArgR titration, were found for DNA molecules with only 2 bp spacing between tandem palindromes (data not shown), such as that found in the control region for ArgR autoregulation.

The absence of L-Arg (Figure 3(b)) causes differences in both mobility and affinity. Binding of the duplex DNA is first detected as a single complex with slow mobility at ArgR concentrations of ca 50 nM. The mobility of this complex appears to be faster than that of the high-affinity complex formed in the presence of L-Arg, but when mobility of each complex is normalized to that of the free duplex or hairpin in the same gel, there is little difference in relative mobilities of the two complexes. This is because the mobilities of all species are substantially faster in the absence of L-Arg. The presence of L-Arg increases affinity of the various oligonucleotides by about ten- to 30-fold (Table 2).

Stoichiometry

In a so-called stoichiometric titration, the concentration of one component is held fixed at a level substantially higher than its dissociation constant, while the concentration of the other component is incremented. Under these conditions the binding isotherm shows a sharp breakpoint and a well-defined plateau, which define the molar ratio of interacting components.[20] A stoichiometric titration can be conducted with either one or the other of the interacting components held at fixed concentration, but for multivalent binding partners the stoichiometries that can be achieved, and the molecular identities of the complexes that result, can be different depending on the ratio of components (Figure 4(a)). A previous stoichiometric titration with excess protein was carried out for ArgR by Charlier et al.[9] using a restriction fragment bearing a natural tandem palindrome with sub-nanomolar K_d. The results showed a molar ratio of 1:1, consistent with the ratio determined using a double-label protocol.[11] However, for the single palindrome and half-site DNA molecules with weaker binding and potentially higher molar ratios, the amounts of plasmid DNA required for stoichiometric titration are prohibitive, and long restriction fragments may interfere with forming multivalent complexes. Therefore oligonucleotides were used in the present experiments.

Both the fixed-protein and fixed-DNA types of stoichiometric titration were carried out for holo-ArgR using the synthetic DNA molecules bearing one, two, or four half-sites. To establish optimal conditions for each stoichiometry determination, the estimated binding constant for each DNA oligomer (Table 2) was used to calculate theoretical
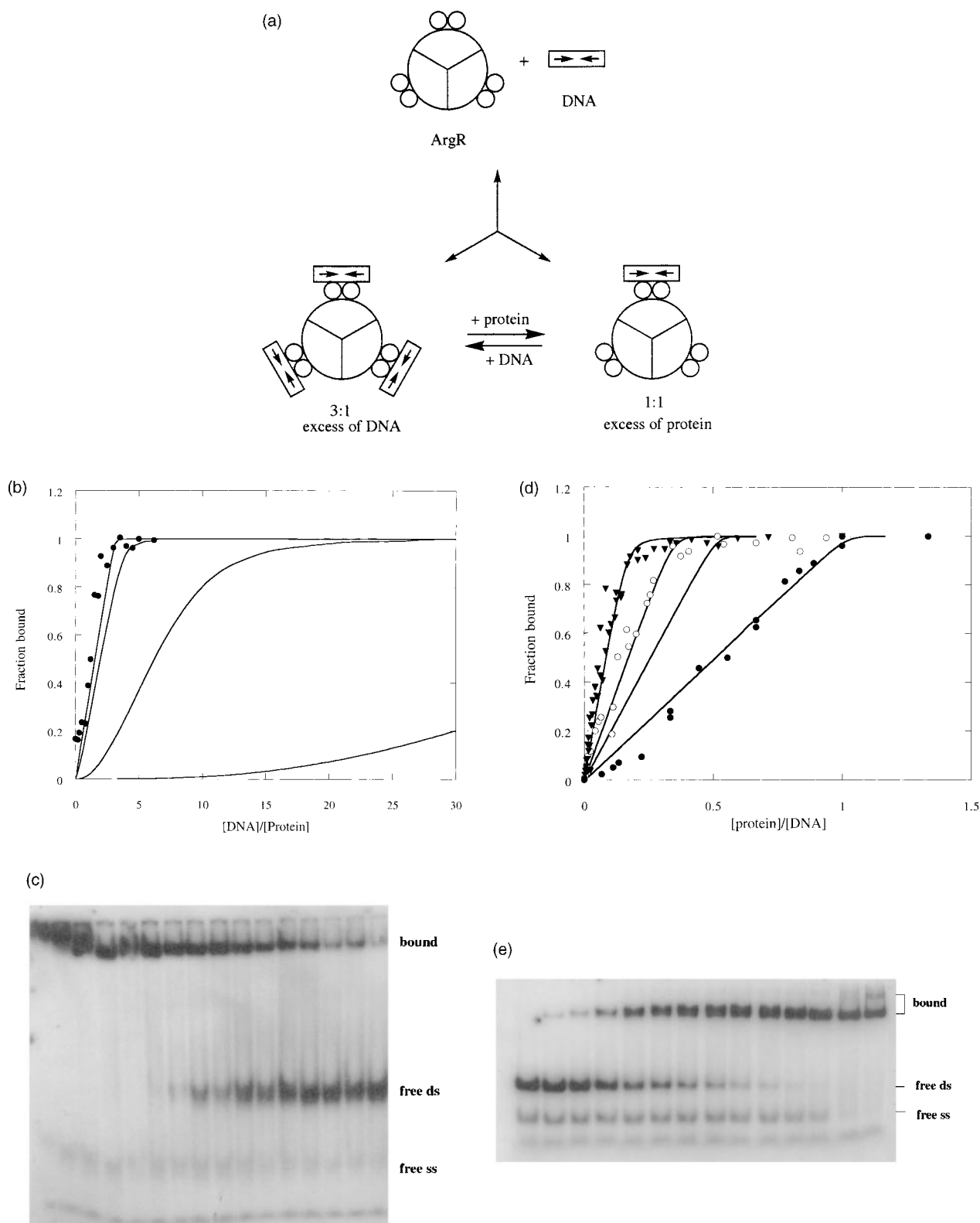
Figure 4 (*legend opposite*)

binding isotherms under different scenarios. Figure 4(b) shows a group of theoretical binding isotherms corresponding to the left reaction shown in Figure 4(a), and Figure 4(d) shows theoretical curves for the reaction on the right of (a). Each curve in Figure 4(b) was calculated with equation (1) (see Materials and Methods) for a different fixed protein concentration assuming formation of

a 3:1 DNA:hexamer complex with K_d ca 100 nM, similar to the affinity found for holo ArgR binding to the single palindromic DNA oligonucleotide. As the fixed protein concentration is raised from 10 nM to 10 µM, the binding isotherms become progressively steeper. A sharp breakpoint at a 3:1 ratio of DNA to protein is observed only when protein concentrations reach ca 10 µM; higher protein concentrations do not shift the curves further. These theoretical curves indicate that for accurate determination of the molar ratio with the single palindrome, a protein concentration of at least 10 µM must be used, and the DNA concentration should span at least a tenfold range above and below this. Molar ratios were determined experimentally by measuring each binding isotherm and fitting to it a series of theoretical isotherms calculated for different scenarios (see Figure legends).

Stoichiometric titrations carried out with fixed holo ArgR concentrations and variable DNA concentration show that the limiting molar ratio of DNA to protein at DNA excess depends on the number of half-sites present on the DNA. A typical gel is shown in Figure 4(c) for titration of ArgR with the single palindromic DNA. Binding data derived from this gel and others by densitometry are superimposed on the theoretical curves of Figure 4(b). The results show that the limiting value of molar ratio at DNA excess is three single palindromic DNA molecules per hexamer. This result implies that each palindrome is likely to be engaged by a pair of ArgR subunits as depicted in Figure 4(a). It also indicates that, although natural operators may engage only four subunits of the ArgR hexamer, all six subunits are active for DNA binding, and can be occupied simultaneously. For the tandem palindromic DNA oligonucleotide, the limiting value of the molar ratio achieved at the breakpoint is one DNA molecule per hexamer (not

shown), in agreement with the stoichiometry found previously with natural operators.[9,11] For the DNA oligonucleotide bearing only one half-site, the limiting value of molar ratio at DNA excess could not be determined with confidence in this experimental configuration because the very low affinity required the use of prohibitively large amounts of materials.

Reversed stoichiometric titrations were also carried out with each DNA oligonucleotide at a fixed concentration and variable ArgR concentrations. Titration of 1.5 µM tandem palindromic DNA with ArgR is shown in Figure 4(e). A single complex containing all the input duplex DNA is observed at protein concentrations up to about 2.5 µM. Quantification of the bound fraction of DNA in this complex shows that saturation is reached when the molar ratio is one ArgR hexamer per DNA molecule (Figure 4(d)), in agreement with the original determinations of stoichiometry on natural operator fragments containing tandem palindromes.[9,11] Similar stoichiometric titrations were carried out with fixed concentrations of the single palindrome and single half-site DNA molecules. The concentrations of protein and DNA were chosen to enable determination of the maximum number of each DNA molecule able to occupy all the subunits of the ArgR hexamer. The results show that three single palindromes or six half-site DNAs can be bound to ArgR (Figure 4(d)). Thus, consistent with results presented above, all subunits of the ArgR hexamer are active for DNA binding, and can be occupied simultaneously. As well, the results indicate a one-to-one correspondence between protein subunits and DNA half-sites.

At the highest protein concentrations used in Figure 4(e), where the ratio of ArgR hexamers to tandem palindrome DNA molecules is 2:1, a

Figure 4. Stoichiometries of the complexes of holo ArgR and DNA. (a) Cartoon of the binding equilibrium between a single palindrome DNA and an ArgR hexamer, showing that the molecular identity of states depends on the molar ratios of components. The ArgR hexamer is represented by a tripartite pie representing the two trimeric layers of the C-terminal hexamerization domain, with three pairs of peripheral circles representing the DNA-binding domains. The assumption that one DNA palindrome interacts with one pair of ArgR DNA-binding domains as depicted is validated in the present work. In the left reaction the limiting molar ratio is three DNA molecules per ArgR hexamer at DNA excess, while in the right reaction the limiting value is one DNA molecule per hexamer at protein excess. (b) Binding isotherms for titration of ArgR at fixed concentration with the single palindrome DNA as in the left reaction pathway of (a). Theoretical binding isotherms (continuous lines) were calculated using $K_d = 100$ nM for different fixed ArgR concentrations (from right to left at fraction bound = 0.2, ArgR = 10 nM, 100 nM, 1 µM, and 10 µM (hexamer). Experimental data (filled circles) derived from titrations of 10 µM ArgR with the single palindrome oligonucleotide using gel-shift assays like that in (c) are shown superimposed on the theoretical curves. (c) Typical autoradiogram showing results of gel-shift assay used to derive some of the experimental data plotted in (b). Final protein concentration, 10 µM; final DNA concentrations in lanes 1 to 16 are 0.65 nM; 1, 3, 5, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 45, 50 µM. (d) Binding isotherms for titration of DNAs at fixed concentration with ArgR as in the reaction pathway shown on the right of (a). Theoretical binding isotherms (continuous lines) were calculated for hexamer:DNA ratios of (left to right) 1:6, 1:3, 1:2, or 1:1 with each DNA as described in the text. Experimental data from stoichiometric titrations of each DNA are superimposed on the theoretical curves: tandem palindrome (filled circles), best-fit breakpoint 1.0; single palindrome (open circles), best-fit breakpoint 0.35; single half-site (inverted triangles), best-fit breakpoint 0.16. e. Typical autoradiogram showing results of gel-shift assay used to derive some of the experimental data plotted in panel (d). Final DNA concentration, 1.5 µM tandem palindrome; final protein concentrations in lanes 1 to 14 are 0, 0.17, 0.33, 0.50, 0.67, 0.83, 1.0,1.2, 1.3, 1.5, 1.7, 2.0, 2.5, 3.3 µM (hexamer).

second complex is observed with a mobility slower than that of the 1:1 complex. Only one species of slow mobility type is observed, and its mobility is quite distinct and only slightly slower than that of the 1:1 complex under our gel conditions. These features suggest that this second bound species represents a discrete form rather than an indefinite assembly, which might be expected to remain at the well as for the complexes formed at very high protein excesses in Figure 3. Although the appearance of the second bound species is correlated with depletion of the hairpin form of the DNA, binding experiments with the isolated hairpin DNA show that bound hairpin DNA comigrates with bound tandem palindrome duplex DNA (data not shown). This second bound species is also unlikely to represent a complex in which each hexamer has both duplex and hairpin DNA molecules bound, since it forms when ArgR is in excess over DNA. This band could represent a 2:1 ArgR:DNA species resulting from the addition of a second ArgR hexamer to the 1:1 complex at high protein concentrations. Such a complex would be consistent with the ability of the hexamer to bind three single palindromes. Attempts to determine the stoichiometry of this second bound species directly in titrations carried out at even higher fixed DNA concentrations were complicated by the poor resolution of bands in this part of the gels, as well as limitations of materials, but the molar ratios found at the highest concentrations were between one and two ArgR hexamers per DNA molecule (not shown), consistent with the tentative assignment of this band as a 2:1 hexamer:DNA complex.

Affinity and cooperativity

The stoichiometry of high-affinity ArgR binding to the tandem palindrome is one hexamer per DNA molecule over a wide range of component concentrations (this work),[9,11] and the highest affinities are in the nanomolar range. Thus, measurements of binding cooperativity on this DNA under 1:1 binding conditions can be used to evaluate the model of ArgR trimer-hexamer assembly coupled to DNA binding.[15] If hexamer dissociation occurs at nanomolar ArgR concentrations then DNA binding might be coupled to hexamer reassembly. This effect would be manifested as a DNA-binding isotherm that displays cooperativity with respect to ArgR concentration. The most critical test of the coupled assembly model is provided by the tandem palindrome because it binds at the lowest protein concentration, where hexamer dissociation should be most favored. Cooperativity can be assessed from the breadth of the binding isotherm on a semilogarithmic plot[20,21] in the limit of low DNA concentration. In the absence of cooperativity, the increase in ligand concentration required to raise the bound fraction from 10% bound to 90% bound, called the ligand concentration interval, ΔpL, is 1.91 log units, corresponding to an 81-fold increase in ligand concentration. Positive coopera-
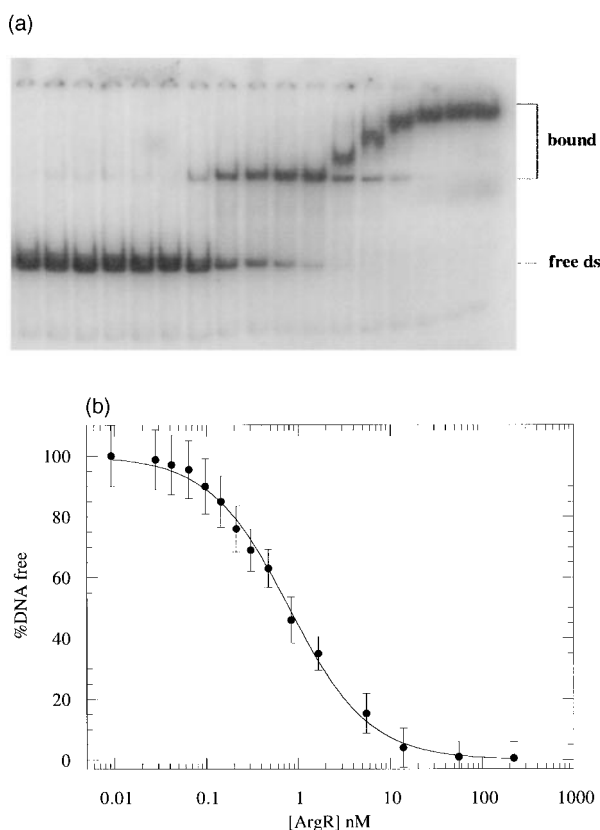

Figure 5. Affinity of holo ArgR for the tandem palindrome. (a) Gel-shift assay. The autoradiogram of a typical gel is shown. Radiolabeled *argF* operator DNA was at <10 pM; final protein concentrations in lanes 1 to 17 are 0.04, 0.06, 0.10, 0.14, 0.22, 0.30, 0.47, 0.83, 1.7, 5.5, 14, 56, 220, 330, 500, 670, 830 nM (hexamer). Other details are as in Figure 3(a). (b) Binding isotherm. DNA bound fraction determined by densitometry (circles) is plotted *versus* ArgR concentration. Error bars represent one standard deviation derived from triplicate determinations for each point. The continuous line is the theoretical binding isotherm calculated for $K_d = 0.8$ nM (hexamer).

tivity narrows the ligand concentration interval, while negative cooperativity broadens it. Although ArgR-DNA binding affinities have been reported,[9,10] cooperativities have not been analyzed quantitatively. In order to achieve the low DNA concentrations required for this type of titration, while avoiding duplex dissociation, restriction fragments were used representing each of the operator configurations of interest in this work.

Figure 5(a) shows a typical gel and Figure 5(b) the corresponding isotherm measured in the presence of 0.5 mM L-Arg for ArgR binding to the tandem palindromes of the native *argF* operator, on a DNA restriction fragment of approximately 120 bp. The complex formed in the nanomolar range of protein concentrations has an affinity of ca 0.8 nM (hexamer), and corresponds to the complex characterized previously with a stoichiometry

of one hexamer per DNA molecule and similar affinity.[11] The ligand concentration interval of 1.9 log units shows that formation of this complex is non-cooperative with respect to ArgR concentration. This result indicates that no other protein concentration-dependent process is linked to the binding of the hexamer to this DNA. Thus, even though DNA binding occurs in the nanomolar ArgR concentration range, the breadth of the isotherm demonstrates that assembly of ArgR hexamers is not coupled to DNA binding.

Essentially identical results were obtained with a restriction fragment bearing the cloned tandem palindrome depicted in Figure 1,[22] as well as with a 275 bp restriction fragment containing the three palindromes of the argG operator (Table 2). At higher protein concentrations, higher-order complexes with gradually decreasing mobilities are observed in Figure 5(a). The weak affinities of these complexes and their variable mobilities precluded direct determination of their molar ratios, but these features suggest that they may contain indefinite stoichiometries.

Binding isotherms for both apo and holo ArgR binding to restriction fragments bearing each of the other cloned operator constructs (Figure 1) were determined as for the tandem palindrome. The affinity and ligand concentration interval for each DNA are summarized in Table 2. As had been observed above with the oligonucleotides, affinity increases with the number of half-sites. The affinity is increased in the presence of L-Arg by about 50-fold for DNAs containing one, two, or three palindromes, but only by about tenfold for non-specific or single half-site DNA molecules. This result implies that L-Arg is an effector of DNA specificity. As well, apo ArgR binding to most of these DNA molecules is non-cooperative, indicating that even in the absence of L-Arg at nanomolar protein concentrations, hexamer assembly is not linked to DNA binding. Finally, the restriction-fragment affinity data show that, for each type of operator construct, the measured affinities were slightly underestimated in the oligonucleotide titrations, presumably due to the observed dissociation of the duplexes at high dilutions.

The ligand concentration interval is less than 1.9 log units for several of the entries in Table 2. A narrowed ligand concentration interval was correlated with the formation of higher-order complexes in the gels (not shown), similar to those observed at high ArgR concentrations in Figure 5(a). The stoichiometries of these complexes are not known. In Figure 5(a) the higher-order complexes form only after all the free DNA becomes bound in the highest affinity (1:1) complex, whereas in the other cases both types of complexes begin to form simultaneously at approximately 200 nM ArgR[22] (data not shown). In this range of ArgR concentration, ultracentrifugation analysis indicates that ArgR in the absence of DNA exists exclusively in the hexameric state. Thus, although depletion of the free DNA is steeper than predicted for non-cooperative

formation of a 1:1 complex, this result is not due to hexamer assembly equilibria coupled to DNA binding.

Binding of trimers to DNA

The ability of ArgR trimers to bind to DNA was assessed using the subunit exchange assay described by Van Duyne *et al.*[7] These authors showed that mixed multimers could be formed when intact ArgR is combined with truncated ArgR-C, a fragment of ArgR bearing only the C-
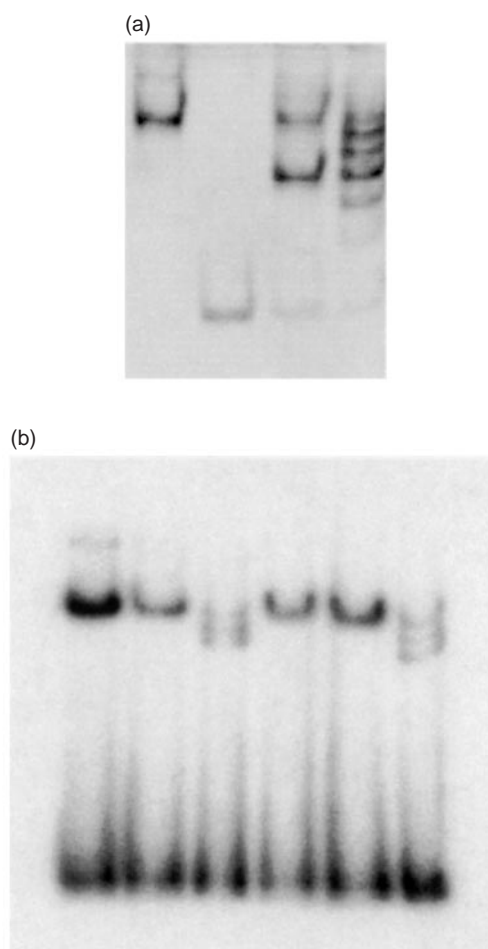
Figure 6. Binding of ArgR multimers to DNA. The native gel system described by Van Duyne *et al.*[7] was used to resolve mixed multimeric species of ArgR and ArgR-C as described in the text. (a) Coomassie-stained gel of proteins. Lane 1, ArgR; lane 2, ArgR-C; lane 3, ArgR and ArgR-C mixed under native conditions to form 3:3 hexamer (middle band); lane 4, ArgR and ArgR-C mixed under denaturing conditions to form mixed hexamers (from top to bottom: 6:0, 5:1, 4:2, 3:3, 2:4, 1:5, 0:6 ArgR:ArgR-C). (b) Autoradiogram of radio-labeled DNAs. Oligonucleotides were added to protein samples prepared as in (a). Left three lanes, tandem palindrome DNA; right three lanes, single palindrome DNA. Protein samples in lanes 1, ArgR; lanes 2, 3:3 hexamers; lanes 3, mixed hexamers. The free DNA has nearly migrated off the bottom of the gel.

terminal domain, which itself can form hexamers. Under native conditions the major species formed from this mixture is a 3:3 hexamer comprised of one trimer of intact ArgR subunits and one trimer of truncated ArgR-C subunits. Under denaturing conditions all possible combinations of subunit types can form (mixed hexamers). All the different multimer types can be resolved by native gel electrophoresis. We replicated the results of Van Duyne *et al.*[7] (Figure 6(a)) and then used arg operator DNA oligonucleotides to ask which multimeric holo-ArgR species are active in DNA binding (Figure 6(b)). The total concentrations of ArgR and DNA used in these experiments were high enough to permit detectable binding for interactions with K_d values as weak as ca 0.1 μM. The results for both the single and tandem palindromic arg operator DNA molecules demonstrate that both these DNA molecules can bind to the intact ArgR hexamer, as expected, as well as to the 5:1 and 4:2 multimeric species (top three bands in the stained gel, visible also on the autoradiogram). However, no binding of either DNA was detected to the 3:3 hexamer, nor to any of the other multimeric species containing fewer than four intact ArgR subunits (bottom four bands, visible in the stained gel but not in the autoradiogram), even when the autoradiographic films were grossly overexposed (not shown). Thus, even though all subunits of the intact ArgR hexamer are active for DNA binding and there is a one-to-one correspondence between ArgR subunits and DNA half-sites, a single ArgR trimer present in a 3:3 hexamer is unable to bind palindromic DNAs. This result is consistent with models derived from NMR[16] and X-ray[8] data, which suggest that each trimer of the ArgR hexamer contributes one subunit in binding to each DNA palindrome.

Bending

Previous bending measurements[10,11] on natural operators by the circular permutation method[23] indicated a large (70-90°) bend apparently centered between the pair of palindromes. To determine whether bending can occur within each palindrome or half-site, each cloned operator construct (Figure 1) was excised from pBend on a series of restriction fragments designed to shift the operator position with respect to the DNA ends. Binding was carried out under conditions chosen to yield a bound fraction of approximately 50% of a 1:1 hexamer:DNA complex. Bound and free DNA molecules were resolved by gel electrophoresis, and migration of each complex was normalized to that of the corresponding free DNA molecules in the same gel, none of which displayed any dependence of mobility on operator position[22] (data not shown). For each complex the bending center and angle were calculated from the relative mobilities as described by Thompson & Landy.[23] The bending angles derived from the analysis are presented in Table 2. Bending of the tandem palindromic

DNA fragment confirms a bending angle of approximately 85° apparently centered between palindromes as determined previously with argF. The single palindromic DNA fragment is also bent, by an amount that is roughly half as large as that determined for the tandem palindrome, and the bend center is localized approximately to the center of the palindrome. This result indicates that the 85° bend of the tandem palindrome is not centered between palindromes but rather that bending within each palindrome results in an apparent bend center between them. Circular permutation with only the tandem palindrome cannot distinguish these two configurations. The bending locus is also consistent with the observation that DNase I hypersensitive sites develop near the central end of each palindrome upon binding to natural tandem palindromes.[9–11] Bending of the half-site DNA was zero within the error of measurement. The difference in bending between the single half-site and the single palindrome, taken together with the stoichiometries of ArgR binding determined here, implies that bending of one palindrome requires the cooperation of two ArgR subunits.

Discussion

The results presented here provide constraints that can be used to evaluate mechanisms for ArgR allosteric activation. A number of studies have suggested that ArgR trimers can bind to DNA, and some studies have suggested that trimers assemble into hexamers on arg operators. Our results rule out this mechanism of activation, suggest alternative interpretations of the results that led to those models, and imply new features of allosteric activation for ArgR.

The sedimentation velocity results presented here show that no major conformational change accompanies the binding of L-Arg, indicating that structural rearrangements upon binding are relatively subtle in solution, consistent with observations on crystals.[7,8] In most cases studied here, high-affinity DNA binding is not cooperative whether L-Arg is present or not. This result, in addition to excluding the DNA-dependent hexamer assembly model, also rules out a requirement for L-Arg to stabilize the hexameric DNA-binding form of ArgR. Furthermore, the apo and holo ArgR affinities presented here for operator and non-operator DNA molecules indicate that L-Arg is an effector of DNA specificity and not only of affinity, as the presence of L-Arg differentially enhances operator affinity compared to non-operator affinity. The fact that the DNA affinity enhancement exerted by L-Arg depends on DNA identity implies that DNA is itself an allosteric effector for ArgR. The present finding that one DNA palindrome is bent upon binding to ArgR but each half-site is not bent demonstrates that binding of the palindrome enforces cooperation of

two ArgR subunits, again implying that DNA is an allosteric effector. Thus, even though crystallographic data show that the ArgR hexamer is organized as a pair of trimers and that all six subunits are equivalent in the absence of DNA, and, as shown here, each subunit of the hexamer can bind an individual DNA half-site, the effective minimal functional unit of ArgR in DNA binding is one pair of subunits. The idea that DNA can act as an allosteric effector has been suggested for a number of transcription factors (for recent examples, see, e.g. Lukens *et al.*[24] and Scully *et al.*[25]), although the detailed mechanisms of DNA-mediated activation have been elucidated in very few cases, and are likely to involve a wide range of molecular features.

How can these results be reconciled with earlier findings? The trimer model first emerged from studies by Burke *et al.*[12] who created the double mutant ArgRNV in which Asp residues 128 and 129 in the L-Arg binding site were changed to Asn and Val, respectively. This mutant displayed a loss of L-Arg dependence in its binding to operator DNA molecules, and formed two kinds of complexes, with fast and slow gel mobilities and similar affinities, on operators containing tandem palindromes. These two complexes were proposed to result from the binding of ArgRNV trimers and hexamers, respectively. The DNA bending angle was similar in the two complexes of ArgRNV, but less than that found for wild-type ArgR bound in a 1:1 complex to the tandem palindrome. These workers later observed a similar pair of complexes with wild-type ArgR also,[13] although in the conditions used the wild-type protein displayed extremely weak and L-Arg-independent operator binding, and had similar affinities for binding to tandem and single DNA palindromes. The wild-type ArgR results showed that the affinity of the presumed trimers was equal to or greater than that of the presumed hexamers, and that the presence of two complete palindromes was required in order to form both the fast and slow-moving complexes.

Other evidence adduced in support of the existence of ArgR trimers and their activity in DNA binding was presented by Miller *et al.*,[14] who demonstrated that the ArgR homolog from *B. subtilis*, AhrC, displayed DNA-binding affinity that increased with DNA concentration. They later extended this observation to *E. coli* ArgR,[15] which they found could also form two kinds of complexes ascribed to trimers and hexamers. To account for the DNA concentration dependence, they postulated that ArgR trimers bound DNA in a first step, and that the DNA-bound form mediated the assembly of hexamers in a subsequent step. The theoretical curves shown in Figure 4(b) provide an alternative explanation for the DNA-concentration dependence of ArgR apparent affinity. One feature of this family of curves that is relevant for quantitative analysis of K_d is that the midpoint of the isotherm (the half-saturation point) progressively shifts to the left as the curves become steeper with increasing concentration of the fixed component until the stoichiometric limit is reached. This behavior is observed despite the fact that all the curves shown in Figure 4(b) are calculated with equation (1) using a single value of K_d. A consequence of this behavior is that the half-saturation point cannot be used as a measure of binding affinity, K_d, because the midpoint is not independent of the concentration of the fixed component in this range of concentrations.

The absence of cooperativity in high-affinity DNA binding reported here establishes that hexamer assembly is not linked to DNA binding, and the results with mixed multimers on native gels suggest that ArgR trimers are unlikely to bind to DNA. The DNA affinity and stoichiometry measurements presented here also indicate that at high concentrations, ArgR may be able to form a two-hexamer complex on the tandem palindromic DNA. These results permit an interpretation of earlier findings that does not invoke the existence of DNA-binding trimers of ArgR. The faster-moving gel species observed in previous work could correspond to a single hexamer bound in a 1:1 complex with tandem palindromes, while the slower species may contain two bound hexamers, each interacting with one palindrome. This model would explain why all four half-sites were required in order to permit observation of both types of complexes in the previous work.

The implications of this model merit consideration even if we have been unsuccessful so far in testing it directly. If the proposal is correct, it suggests that the molecular phenotype of the mutant ArgRNV[12] might be described as a defect in the ability to discriminate between single and tandem palindromic operators. Under some solution conditions (e.g. see Chen *et al.*[13]) the wild-type protein can also lose this ability. These findings would then imply that normal operator binding relies on communication among four subunits of the ArgR hexamer, and that subunit communication can be lost by mutation or by altering solution conditions. A defect in subunit communication due to mutation or choice of conditions may be either a consequence or a cause of alterations in other properties, such as the slightly lower bending angle found for the ArgRNV mutant in both of its observed DNA complexes.[12] The similarity of the bending angles measured for the two types of complexes with ArgRNV could be related to the finding presented here (for wild-type ArgR) that the center of bending is within each palindrome rather than between the two palindromes. Bending in the recombination complex is likely to occur only within one palindrome, consistent with the bending results presented here and with modeling of ArgR-DNA interactions in the synaptic complex based on the recently solved structure of pepA.[26] The ArgRNV mutant is competent in recombination[12] despite its apparent

defect of inter-subunit communication, implying that the subunit communication requirements are different for recombination and regulation. The fact that hexamers of ArgRNV are required for recombination despite their defect in subunit communication suggests that hexamers serve a structural role in recombination that is distinct from their role in DNA operator recognition. A more general inference is that inter-subunit communication may play fundamentally different roles in operator recognition and in recombination, and that the role of DNA in enforcing subunit communication may also be fundamentally different in recombination and in operator recognition.

The results and analysis presented here have a number of hopeful implications for future studies of ArgR that may elucidate its allosteric activation mechanisms in greater detail. The molecular mechanism of ArgR binding to DNA appears to be less complicated than previously thought. The possibility that ArgRNV is an allosteric mutant also suggests that other mutants may be found or made that will further clarify the activation mechanisms of ArgR. The quantitative features of DNA binding established here can be evaluated in ArgR mutants to give new clues about activation mechanisms.

Materials and Methods

Materials

Phage T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) were obtained from New England Biolabs. DNase I, RNase, shrimp alkaline phosphatase and phage T4 DNA ligase were from USB. [γ-^{32}P]ATP (6000 Ci/mmol) was from NEN (DuPont).

Proteins and DNAs

ArgR was purified as described by Lim *et al.*[6] For ultracentrifugation studies the protein was further purified by chromatography on Sephadex A25 developed with a salt gradient. Purification of ArgR-C followed the method of Van Duyne *et al.*[7] Oligonucleotides were synthesized and purified in the DNA synthesis facility in the Molecular Biology Department at Princeton University, and were used without further purification. The single palindrome and single half-site oligomers were cloned into plasmid pBend3[19] using the *Sal*I and *Xba*I sites. *Bgl*II digestion of the resulting clones yielded the 140 and 130 bp DNA fragments for quantitative binding studies. A 275 bp DNA fragment containing the three palindromes of the argG operator was obtained by *Eco*RI and *Bam*HI digestion of plasmid pG275.[9] Plasmid pGT101[11] was digested with *Eco*RV and *Bam*HI providing a 120 bp DNA fragment containing the tandem palindromes of the argF operator. A control DNA fragment of 120 bp containing the trpEDCBA promoter/operator of *Serratia marcescens* was prepared by *Eco*RI and *Bam*HI digestion of plasmid pRK9.[27] All restriction fragments were purified from 6 % (w/v) non-denaturing polyacrylamide gels stained by ethidium bromide, and the fragments were isolated and purified by the crush-and-soak method. DNAs were dephosphorylated using shrimp alkaline phosphatase if necessary, 5' end-labeled with T4 polynucleotide kinase, annealed and end-filled if necessary, and used without further purification.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were carried out at 4 °C using a Beckman Optima XL-A analytical ultracentrifuge equipped with an An60 Ti rotor and using either six-channel or two-channel, 12 mm path length, charcoal-filled Epon centerpieces and quartz windows. Detection wavelengths were chosen to yield adequate absorbances for each sample at protein concentrations of 1 to 10 μM (monomer). The relatively weak extinction coefficient of ArgR in the aromatic region of the spectrum, due to the presence of only three Tyr residues per polypeptide chain and no Trp, dictated the use of much lower detection wavelengths at most protein concentrations. Using the method of Gill & von Hippel[28] we determined ε276 for native ArgR as 4290($\pm$40) M^{-1} cm^{-1} (monomer), virtually indistinguishable from the value calculated for the denatured state (4350 M^{-1} cm^{-1}). The buffer conditions for ultracentrifugation and extinction coefficient determination were 20 mM Tris-HCl (pH 7.6), 10 mM MgCl$_2$, 0.2 M NaCl, 0.2 mM DTT. Equilibrium was assumed when successive data scans resulted in difference plots with zero slope. Data were collected at several rotor speeds and represent the average of 20 scans using a scan step-size of 0.001 cm. Data were analyzed using the HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut (Storrs, CT, USA). Figure 2 was composed using IGOR Pro version 3.16 (WaveMetrics Inc., Lake Oswego, OR, USA) running an algorithm generously provided by James Lear. Sedimentation velocity experiments were run at 40,000 rpm and 4 °C using 185 μM ArgR ($\pm$L-Arg) (5 mM) in the two-channel centerpieces. Data were collected at a wavelength of 275 nm and were analyzed using the DCDT program developed by Walter Stafford.[29] Partial specific volume (0.738 ml g^{-1}) and solution density (1.00877 g ml^{-1}) were calculated using the Sednterp program.[17] The default value for concentration dependence (2.7 mg ml^{-1} protein) is used by the Sednterp program in extrapolating the S-value to infinite dilution to yield $s^{\circ}_{20,w}$.

Gel retardation

Gel-retardation experiments were performed as described by Tian *et al.*[11] DNAs or protein were diluted into ice-cold binding buffer (20 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM CaCl$_2$, 10 mM β-mercaptoethanol, 100 mM KCl, 10 % glycerol and 5 mM L-Arg as indicated) at the specified concentration. Serial dilutions of ArgR or DNA made in binding buffer were added and incubation was continued for 30 minutes on ice. The reaction mixtures were loaded onto gels running at 250 V at 4 °C in 0.5 × TBE buffer (1× is 90 mM Tris, 90 mM boric acid, 1 mM EDTA) with addition of 1 mM L-Arg as indicated. Gels of between 6 and 10 % (w/v) total acrylamide contained 5 mM Arg as indicated. Electrophoresis was continued at 100 V until the free DNA had migrated approximately two-thirds the length of the gel. The bound fraction was determined at each concentration from disappearance of the free DNA using densitometry and NIH Image software.

The largest source of error in determining K_d values by this method is the size of the increments in protein concentration used in the titrations. In general for the

affinity data reported here, range-finding experiments using relatively large increments were first used to establish the approximate half-saturation point as a preliminary estimate of K_d. Titrations with narrower concentration increments were then used over a concentration range within a factor of 10 above and below the estimated K_d. Within this range, twofold increments of protein concentration were generally used. Thus, each K_d value may vary by as much as twofold from the value reported. The magnitude of errors from this source could be reduced by further reduction in the increment size, but errors from other sources such as pipetting approach this magnitude, as verified by determining errors from multiple independent replicates of each titration. These errors were neglected in quantitative analysis of the data.

Theoretical calculations

Theoretical calculations were based on the equilibrium binding reaction:

$$m\text{ArgR} + n\text{DNA} \Leftrightarrow \text{ArgR}_m(\text{DNA})_n$$

described by the dissociation binding constant K_d:

$$K_d = \frac{[\text{ArgR}]^m[\text{DNA}]^n}{[\text{ArgR}_m(\text{DNA})_n]} \tag{1}$$

For chosen values of K_d, m, and n, a standard graphical calculator was employed to solve the equation and generate theoretical curves of bound fraction *versus* concentration of the variable component. Determination of the best-fit breakpoint of stoichiometric isotherms used the residual sum of squares method.[30]

Bending

Bending studies were conducted similarly as per Tian *et al.*[11] Fragments of equal length but with target DNA sequences at different positions were obtained by restriction enzyme digestions of pBend3 clones using *Bgl*II, *Xho*I, *Puv*II, *Ssp*I, or *Bam*HI. Electrophoresis was carried out at 400 V for six hours using long 8 % (w/v) acrylamide gels. The bending angle was calculated from its empirical relationship to relative electrophoretic mobility as described by Thompson & Landy,[23] and its locus was mapped by extrapolation according to the method described by those authors. The main source of error in determining the bending angle is the precision of measuring the migration distances. From numerous independent replicates we estimated the propagated error from this source to be approximately $\pm 10°$. We did not propagate these errors into the linear extrapolation used to estimate the locus of bending.

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