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# Heterodimers Reveal That Two Arabinose Molecules Are Required for the Normal Arabinose Response of AraC

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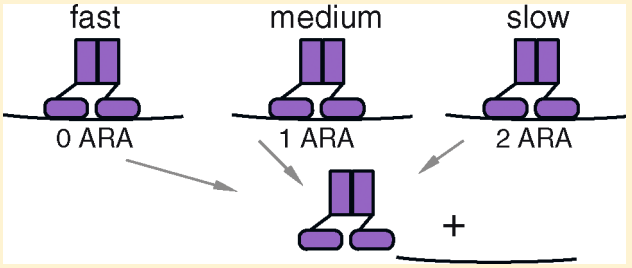
**ABSTRACT:** AraC protein, which regulates expression of the L-arabinose operon in *Escherichia coli*, is a dimer whose DNA binding affinity for pairs of DNA half-sites is controlled by arabinose. Here we have addressed the question of whether the arabinose response of AraC requires the binding of one or two molecules of arabinose. This was accomplished by measuring the DNA dissociation rates of wild-type AraC and heterodimeric AraC constructs in which one subunit is capable of binding arabinose and the other subunit does not bind arabinose. Solutions consisting entirely of heterodimers were formed by spontaneous subunit exchange between two different homodimers, with heterodimers being trapped by the formation of an intersubunit disulfide bond between cysteine residues strategically positioned within the dimerization interface. We found that the normal arabinose response of AraC requires the binding of two arabinose molecules. These results provide additional constraints on mechanistic models for the action of AraC.




The dimeric AraC protein, which regulates expression of the *araBAD* operon genes in *Escherichia coli* in response to the presence of arabinose,[1−3] is a member of a very large family of bacterial transcription regulatory proteins.[4] A few members of this family function as monomers, consisting of just a homologue of the DNA binding domain of AraC, e.g., MarA,[5] or contain a partially characterized domain, e.g., Rns.[6] Most, however, like AraC, also possess an additional regulatory domain that likely dimerizes the protein as shown experimentally for the MelR,[7] UreR,[8] XylS,[9] RhaR,[10] and ToxT[11] proteins. In these and many other AraC homologues, the additional domain contains a region with the sequence characteristics of a coiled coil as is found in the AraC dimerization interface.

Solubility and stability problems have limited the acquisition of biochemical and biophysical information about most of the AraC family members. This has been less true for AraC itself however, where many experimental data suggest that the protein utilizes a conceptually simple and appealing mechanism called the light switch. In the absence of arabinose, the 20-amino acid N-terminal arms of the dimerization domains of AraC are postulated to hold the C-terminal DNA binding domains in positions and orientations that favor binding of the protein to the $I_1$ and $O_2$ DNA half-sites[12] that are separated by 210 bp[13] (Figure 1). The resulting DNA loop represses expression from the *ara* $p_{BAD}$ promoter by preventing AraC from occupying the $I_2$ half-site whose occupancy is required for induction,[14] and also by interfering with the access of RNA polymerase to the adjacent $p_{BAD}$ promoter.[15] Induction ensues when arabinose binds to AraC, the arms relocate over the bound arabinose, and the presumably less constrained DNA binding domains can occupy the adjacent direct repeat half-sites, $I_1$ and $I_2$. This same mechanism explains the arabinose-induced increase in affinity for binding to direct repeat half-sites and the arabinose-induced decrease in the rate of dissociation from such DNA. It is unknown, however, whether the binding of one or two molecules of arabinose is required to generate these changes in DNA binding.

Despite a wide variety of experimental support consistent with the light switch mechanism of AraC as outlined above,[12−14,16−20] a number of significant questions remain. Some of these questions would be answered if the crystal structures of AraC in its repressing and inducing states were known. Despite efforts extending over several decades, no X-ray diffracting crystals of full-length AraC in the absence or presence of arabinose have been obtained. The crystal structures of the dimerization domain in the presence and absence of arabinose[21,22] and the solution structure of the DNA binding domain[23] are known. These domain structures and the requirements of binding to $I_1$ and $O_2$ in the absence of arabinose and to $I_1$ and $I_2$ in the presence of arabinose are satisfied by a family of domain arrangements for the full-length protein.[24]

Another unknown concerns the functional relevance of arabinose-induced structural changes in the arm. In the X-ray crystallographic structures, the N-terminal arms on the dimerization domain dramatically change structure in response to arabinose while the remainder of each dimerization domain shifts structure only slightly. The rmsd between the nonarm portion of an apo and holo dimerization domain is only 0.442 Å.[21,22] It is not definitively known whether arm restructuring is the sole controlling element or whether the subtle structural

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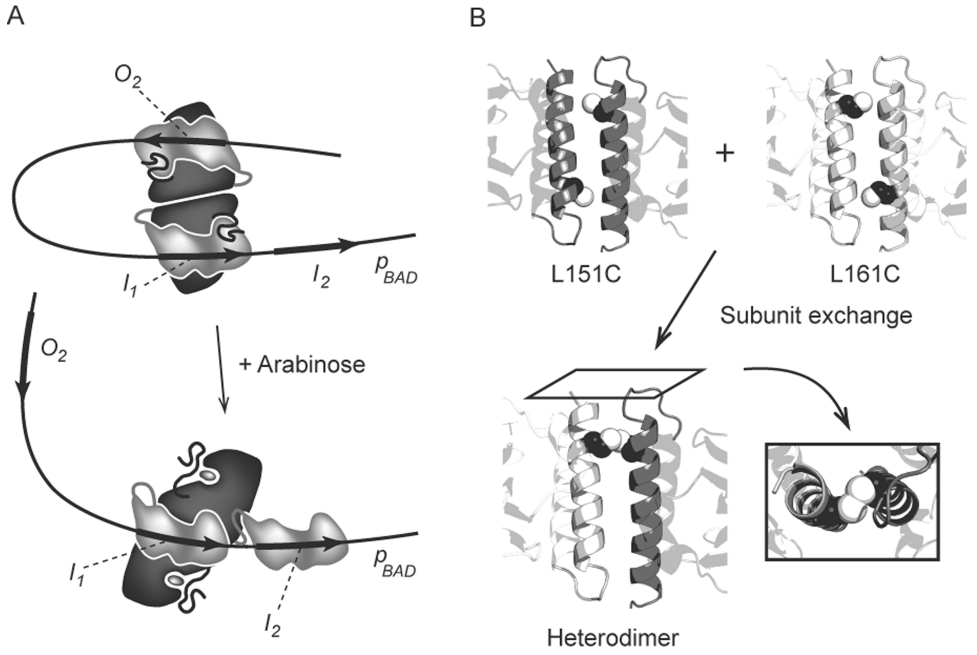


**Figure 1.** (A) Cartoon of the light switch mechanism of AraC regulation of $p_{BAD}$. In the absence of arabinose, the AraC DNA binding domains are in a configuration that favors binding to distal half-sites $I_1$ and $O_2$, a looped complex that represses transcription of $p_{BAD}$. When arabinose binds, the protein restructures to favor binding to adjacent DNA half-sites $I_1$ and $I_2$ in a complex that allows transcription. (B) Cartoon of the subunit exchange setup. The two central helices at the dimer interface that show the location of residues mutated to cysteine for the formation of disulfide trap are highlighted.

changes within the dimerization domain play an important role in determining the positional freedom of the DNA binding domains or possibly in communicating between the subunits.

Finally, the possibility raised by the light switch mechanism that the arms of AraC directly communicate with the DNA binding domains is weakened by the failure to find mutations in the DNA binding domains indicative of direct arm−DNA binding domain interactions.[16,25] In summary, the deduction of the geometric and mechanistic details of the mechanism by which AraC responds to arabinose and, very likely large numbers of the >40000-member AraC family responds to their inducers will be significantly aided by determining whether the two subunits of AraC function independently in controlling DNA binding.

## MATERIALS AND METHODS

**Proteins.** The AraC gene from plasmid pWR03[16] was subcloned between the *Nco*I and *Sac*I sites of the multicloning region of a pET-24d vector (Novagen) and verified by DNA sequencing. BL21(DE3) cells containing the vector were grown at 37 °C to a cell density corresponding to an $A_{550}$ of ∼0.8−1, shifted to 25 °C, grown for 30 min prior to induction with 0.4 mM IPTG, and then grown for 5 h or overnight. After being harvested, cells were resuspended in 3 volumes of 300 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 5 mM arabinose, and 5% (v/v) glycerol (pH 7), lysed by three passes through an Avestin EmulsiFlex C3 cell disruptor, diluted in half with 20 mM sodium phosphate, 5 mM arabinose, and 0.1 mM $NaN_3$ (pH 7), and centrifuged at 14000 rpm for 20 min in a Sorvall SS-34 rotor. The supernatant was chromatographed on a 5 mL HiTrap-Heparin HP column (GE Healthcare) equilibrated in 20 mM sodium phosphate, 5 mM arabinose, and 0.1 mM $NaN_3$ (pH 7) and eluted with a linear salt gradient from 0 to 1 M NaCl. AraC elutes at a NaCl concentration of ∼0.5 M. Peak fractions were pooled, diluted 4-fold with 20 mM imidazole, 5 mM arabinose, and 0.1 mM $NaN_3$ (pH 6.5), and passed over the HiTrap-Q HP column equilibrated in the same buffer. The flow through from the HiTrap-Q HP column, which contains most of the AraC, was reapplied to a HiTrap-Heparin HP column and eluted as described above. The resultant AraC was more than 95% pure as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Purification of AraC dimerization domain has been previously described.[22] Briefly, the overexpressed protein was bound to and eluted from Ni-NTA agarose beads (Qiagen); the hexahistidine tag was removed by trypsin proteolysis, and the final purification was achieved by chromatography on a HiTrap-Q HP column.

Protein concentrations were determined spectrophotometrically using the absorbance at 280 nm. The extinction coefficients of 47900 $M^{-1}$ $cm^{-1}$ for AraC and 39420 $M^{-1}$ $cm^{-1}$ for the dimerization domain were calculated from the amino acid composition with Sednterp.[26]

**Mutagenesis.** Expression plasmids containing either the AraC gene or the AraC dimerization domain gene, residues 1−184 with a hexahistidine tag, were mutagenized using a standard QuikChange mutagenesis protocol (Stratagene) and verified by DNA sequencing. Five dimerization domain constructs were generated with X to Cys amino acid substitutions in the dimer interface as part of the initial screen for trapped heterodimer formation. In addition, a mutation that eliminates arabinose binding, H80A, was introduced into the arabinose binding pocket for some of these constructs.

For full-length AraC, the two mutations introduced into the dimer interface for disulfide trapping were L151C and L161C. In addition, three DNA binding domain substitutions were made to remove cysteine residues, C205N, C268R, and C280S,


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and the H80A mutation in the arabinose binding pocket was made to eliminate arabinose binding. In some experiments, a C183S substitution was made in addition to the other cysteine substitutions in the DNA binding domains.

**Cell Growth, Induction, and Repression Measurements.** Both wild-type AraC and the various mutated AraC constructs used here were able to support growth on minimal arabinose medium when AraC was produced in trans by leakage synthesis alone from the pET-24 AraC expression vectors transformed into the AraC$^-$ strain, SH321 ($F^-$ Δ*araC-leu1022* Δ*lac74 galK*$^-$ *str*$^r$).[27] Leakage synthesis from the pET-24 vector, however, provides insufficient AraC to normally regulate the chromosomal *ara* $p_C$ promoter. Thus, the regulatory ability of the various AraC constructs was further assessed by measuring both repression of $p_C$ and induction of $p_{BAD}$ only after subcloning the constructs into the pSE380-based vector, pWR03.[16] Repression was assessed as the level of β-galactosidase activity produced under control of $p_C$ in the AraC$^-$, *pC-lacZ* fusion strain, SH10 ($F^-$ *araC-lacZ fusion, leu*$^-$ *str*$^r$ Δ*lac, araD*$^-$).[16] Induction was quantified as the amount of arabinose isomerase, the *araA* gene product,[28] produced by cells grown on glycerol or glycerol with arabinose in minimal M10 medium.[29] For these measurements, the SH321 strain was used.

**Heterodimer Formation.** Purified samples of the two different proteins containing appropriate cysteine modifications were first reduced by addition of 1 mM DTT and incubated for 1−2 h at 5 °C. The samples were then exhaustively dialyzed four times against 100−200 volumes of 100 mM NaCl, 50 mM sodium phosphate, and 0.1 mM $NaN_3$ (pH 7) to remove DTT. Following dialysis, samples were mixed in equimolar amounts, 10 μM each, and incubated at room temperature for a time sufficient for the completion of the subunit exchange. Exchange as indicated by the formation of disulfide-linked dimers was monitored by SDS−PAGE under nonreducing conditions. The half-time for the formation of covalent heterodimers of the dimerization domain or of full-length AraC was between 10 and 20 min, in the absence and presence of arabinose.

For the measurement of the kinetics of exchange, iodoacetamide was added to a final concentration of 1 mM to stop the reaction at each time point by alkylating all remaining cysteine residues. Initial attempts to form heterodimers of full-length AraC revealed spurious and confounding disulfide formation. Measurements of the thiol reactivity in wild-type AraC using DTNB revealed that some of its cysteine residues were highly reactive with half-times ranging from a few seconds to a few minutes. To minimize the undesired disulfide problem, we mutated three of the four cysteine residues in the DNA binding domain to residue types found in AraC homologues.[4] Initially, we did not alter C183 because this residue is highly conserved, but subsequent studies revealed that a C183S substitution retained activity close to the wild-type value. We did not alter C66, the only cysteine in the wild-type dimerization domain, because this residue is not DTNB reactive in the native state.

**L-Arabinose Binding.** Binding of arabinose was monitored using an intrinsic tryptophan fluorescence assay as previously described.[22]

**DNA Dissociation Kinetics.** DNA binding properties of the various AraC constructs were characterized by measurement of the dissociation kinetics using the electrophoretic mobility shift assay.[30] AraC samples were first equilibrated with 5′-Cy5-labeled $I_1$−$I_1$ DNA. The sequence of the labeled $I_1$−$I_1$ DNA was 5′-/5Cy5/GCCATAGCATTTTTATCCATAAGATTAGCATTTTTATCCATACCTC (the two $I_1$ half-sites that are separated by four bases are underlined). Dissociation kinetic measurements were initiated by addition of a 10-fold molar excess of unlabeled $I_1$−$I_1$ DNA, and at successive time intervals, samples were removed and immediately loaded onto a running polyacrylamide gel. Samples were subjected to electrophoresis at ∼9 V/cm for 3−10 min and at 5 V/cm for an additional 30−45 min. Following electrophoresis, gels were visualized using a Typhoon 9410 Variable Mode Imager, and the data were analyzed using ImageQuant TL.

**Thiol Reactivity.** The free thiol reactivity was monitored using Ellman's reagent, 5,5′-dithiobisnitrobenzoic acid (DTNB).[31,32] Protein samples, dialyzed to remove all reducing agent, were diluted to a concentration of 5−10 μM into 100 mM NaCl and 50 mM sodium phosphate (pH 7). For samples assayed in the presence of sugar, the sugars were diluted from a 1 M stock to a final concentration of 10 mM prior to the initiation of kinetic measurements. DTNB was added to a concentration of 1 mM, and the absorbance at 412 nm was followed as a function of time until it had reached a stable plateau or for up to 2 h. The data were fit to single- or multiple-exponential decay models. When multiple exponentials were used, their number equaled the number of reactive cysteine residues present, and a single-amplitude parameter was applied to each because the contribution to the signal from each cysteine should be the same.

## RESULTS

**Trapping Heterodimers.** The binding of arabinose by AraC decreases its rate of dissociation from adjacent DNA half-sites by a factor of ∼30.[30,33] To determine whether this shift can be achieved by the binding of one molecule of arabinose, or whether two are required, we measured the arabinose response of heterodimers of the protein in which only one subunit can bind arabinose. The necessary heterodimers were formed by mixing solutions containing equimolar amounts of AraC capable of binding arabinose with AraC incapable of binding arabinose, allowing spontaneous subunit exchange under nonreducing conditions, and selectively trapping the heterodimers by disulfide bond formation. The disulfide traps were made by introducing a cysteine residue into each of the two types of AraC such that a disulfide bond could form between the two cysteine residues only in a heterodimer (Figure 1B).

The principle dimerization interface in AraC is an antiparallel α-helical coiled coil. Locations for the introduction of the cysteine residues to be used in the disulfide trap were chosen by analysis of the dimerization interface reported in the X-ray crystallographic structure of the dimerization domain.[21,22] Pairs of residues were chosen on opposite chains such that their γ-carbons were separated by 3.5−4 Å, and each individual residue is separated by a considerably greater distance than this from the sequentially equivalent residue on the opposite chain. Suitable pairs appeared to be L151 and L161, Y147 and M164, and Y147 and E165. All of these mutations were initially generated in the dimerization domain and screened for suitable heterodimer formation and trapping. Each of the three candidate pairs proved to be satisfactory. The L151C:L161C mutation pair was chosen for the subsequent work with full-length AraC.

AraC constructs containing the various mutation combinations repressed $p_C$ between 70 and 100% as well as wild-type AraC and induced *ara* $p_{BAD}$ between 40 and 90% as well as


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wild-type AraC in vivo (Table 1). All constructs, when expressed in an AraC deletion strain, support growth on

**Table 1. Activity Measurements**

| | $p_{BAD}$ induction | | | $p_C$ repression | |
|---|---|---|---|---|---|
| | no. of arabinose isomerase units per cell[a] | | | Miller units[b] | |
| | uninduced | induced | % of wild type | value | % of wild type[c] |
| AraC (wild type) | 7 | 1700 | 100 | 2 | 100 |
| Δ3cys + L151C[d] | 4 | 1100 | 65 | 2 | 100 |
| Δ3cys + L161C | 5 | 1600 | 94 | 2 | 100 |
| Δ4cys + L151C[d] | 5 | 700 | 41 | 14 | 90 |
| Δ4cys + L161C | 5 | 1100 | 65 | 35 | 72 |

[a]Uncertainties in these values are approximately 25% of the values.[28] [b]All values are in comparison to the unrepressed value of SH10 cells used in these assays, 120 Miller units. [c]Defined as 100 × (unrepressed value − sample value)/(unrepressed value − wild-type value). [d]Δ3cys implies that the construct also contains C205N, C268R, and C280S mutations, and Δ4cys implies that the construct also contains C183S, C205N, C268R, and C280S mutations.

minimal medium with arabinose as the sole carbon source. As shown below, the arabinose and DNA binding properties of the proteins containing these mutations were similar to those of wild-type AraC protein.

**Mutants That Do Not Bind Arabinose.** In addition to the requirement of forming heterodimers of AraC, our experimental approach also requires the use of AraC mutants that do not bind arabinose but still fold, dimerize, and possess their normal activity in the absence of arabinose. Mutants with alterations in residue H80 are good candidates because the imidazolium side chain of this residue extends into the arabinose binding pocket and the AraC mutants H80A and H80R were previously found to be uninducible but to repress normally.[16] Indeed, purified H80R AraC protein does not bind arabinose.[34] To minimize any adverse biochemical properties caused by the large side chain of the arginine substitution and to overcome the low yields of purified H80R AraC, we used the H80A mutation for these experiments. As expected and required for these experiments, H80A AraC protein does not detectably bind arabinose (Table 2).

**Table 2. Arabinose Binding Affinities of AraC Mutants**

| sample[a] | $K_d$ (μM)[b] |
|---|---|
| wild-type AraC | 345 ± 23 |
| L151C:L161C | 353 ± 23 |
| H80A,L151C:L161C | 437 ± 47 |
| L151C:L161C,H80A | 434 ± 60 |
| H80A,L151C:L161C,H80A | no binding detected |

[a]The dimer interface mutants also contained the C205N, C268R, and C280S mutations. [b]Errors are the standard errors of the fit parameter.

Heterodimers constructed as described above in which only one subunit contains the H80A mutation were found to retain the ability to bind arabinose, presumably binding to only one subunit (Figure 2). Consistent with the design objective that these heterodimers bind only one molecule of arabinose, their change in average emission wavelength is only approximately half of that seen with constructs in which both subunits can bind arabinose. The heterodimer in which both subunits

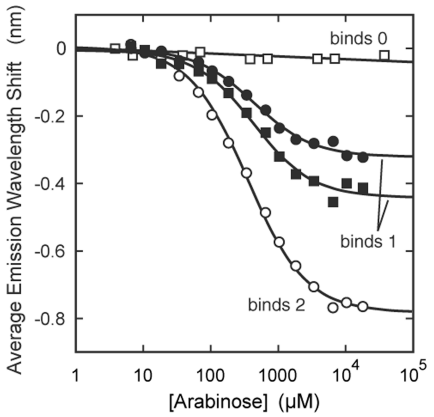


**Figure 2.** Arabinose binding of AraC heterodimer constructs. A plot of the shift in the average emission wavelength vs arabinose concentration is shown. All samples were heterodimers of AraC: (○) samples in which both subunits can bind arabinose, (■ and ●) two configurations in which only one subunit can bind arabinose, and (□) samples in which neither subunit can bind arabinose. The lines are the best fits to a simple binding equation except for the no binding case, which shows a straight line through the data. The maximal number of bound arabinose ligands is indicated in the figure. The protein concentration was ~0.5 μM, and the temperature was held at 20 °C. The buffer consisted of 75 mM KCl, 15 mM Tris, and 1 mM EDTA (pH 8).

contain H80A does not bind arabinose. The dissociation constants derived from analysis of the data obtained for the various constructs are summarized in Table 2.

**DNA Dissociation Properties of Heterodimers of AraC.** Using the techniques described above, heterodimers were made of full-length AraC containing various combinations of the dimer interface substitutions for trapping heterodimers (L151C and L161C) and the H80A substitution for preventing arabinose binding. All constructs also contained the three cysteine substitutions (C205N, C268R, and C280S) in the DBD to prevent spurious disulfide formation. Heterodimers were characterized by measuring the arabinose dependence of their DNA dissociation kinetics from direct repeat $I_1$−$I_1$ DNA.

Both subunits of the L151C:L161C heterodimer lacking the arabinose binding pocket mutations can indeed bind arabinose, and this heterodimer displays the normal wild-type AraC arabinose response in its dissociation from $I_1$−$I_1$ DNA (Figure 3). It dissociates with a half-time of ~200 min in the presence of arabinose. In the absence of arabinose, the dissociation was rapid (<5 min). The two heterodimers in which only one subunit can bind arabinose, H80A,L151C:L161C and L151C:H80A,L161C, displayed a substantially different response, with dissociation half-times of ~20 min in the presence of arabinose (Figure 3). For these also, in the absence of arabinose, the dissociation was rapid. In contrast, neither subunit of the H80A,L151C:H80A,L161C heterodimer can bind arabinose, and as expected, dissociation of this heterodimer from DNA was very rapid and not responsive to arabinose.

The kinetics of dissociation from DNA in the presence of arabinose of the L151:L161 heterodimeric protein, the two heterodimers that can bind a single arabinose molecule, and the heterodimer in which neither subunit can bind arabinose are shown together in Figure 4. Data on the parental construct (C205N, C268R, and C280S) are also included for comparison. Because the parental construct data set is essentially


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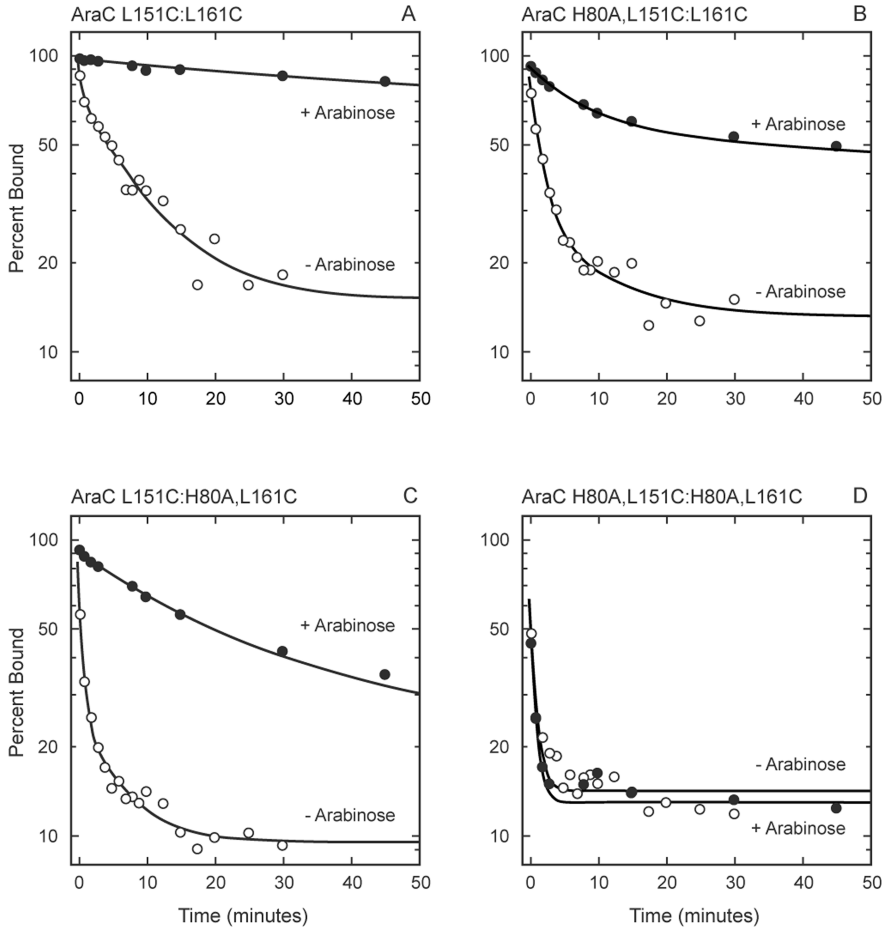


**Figure 3.** Kinetics of dissociation of AraC heterodimers from DNA. The percent of $I_1$–$I_1$ DNA bound by AraC heterodimers vs time is presented in each panel with and without 10 mM arabinose: (A) L151C:L161C, (B) H80A,L151C:L161C, (C) L151C:H80A,L161C, and (D) H80A,L151C:H80A,L161C with (○) 0 mM arabinose and (●) 10 mM arabinose. The lines are fits to exponential decay equations. All protein constructs also contain the C205N, C268R, and C280S substitutions.

indistinguishable from the L151:L161 heterodimeric protein data set, the parental data has been offset in the figure by +5 units along the *y*-axis for the sake of clarity. The combined results show that the binding of a single molecule of arabinose to AraC is insufficient to convert the protein to its normal inducing state.

## DISCUSSION

**Forming Heterodimers.** One way to address the question of whether the normal, wild-type, response to arabinose in the DNA binding of AraC represents the binding of one or two molecules of arabinose is to measure the properties of a heterodimeric AraC construct in which only one subunit is capable of binding arabinose. Several methods might be used to generate such a protein. First, the heterodimer might be directly encoded by joining two appropriate coding regions head to tail, as was done in studies of TetR.[35] This is not sensible in the case of AraC because of the direct involvement of the N-terminal arm of AraC in the protein's regulation. Another method of obtaining a solution consisting entirely of heterodimers is to redesign the dimer interface of a protein such that mixtures of two suitably altered protein subunits can form only the desired heterodimers. While this approach has been successful with pure parallel coiled coils[36] and the parallel coiled coil of CRP,[37] the more extensive dimer interface of AraC and its antiparallel coiled coil structure makes this approach less promising. A third approach could use spontaneous subunit exchange to produce the desired heterodimers. Ordinarily, when using such an approach, homodimers of the input species will remain. Further, continued subunit exchange would foil attempts to separate heterodimers from homodimers. Nonetheless, this third approach in combination with a small redesign of the dimer interface can be made to work by trapping heterodimers as they form,[38] thereby preventing their dissociation and allowing heterodimer formation to go to completion. This latter approach was used in the experiments described here. From equimolar mixtures of two input homodimers of AraC, solutions consisting entirely of heterodimers were formed and studied.

**Properties of the Mutants Required To Form the Heterodimers.** All of the proteins used in this study retain close to wild-type activity except for those carrying mutations, e.g., H80A, chosen to inactivate a specific function. The full-length AraC constructs were able to support growth on minimal medium with arabinose as the sole carbon source. All constructs exhibited close to wild-type repression activity at $p_C$ and close to wild-type induction activity at $p_{BAD}$ in vivo and were able to bind DNA in vitro, thus showing that the DBDs are properly folded. It is worth noting that the activity measurements required subcloning of all constructs from expression vector pET-24d into a pSE380-based vector, pWR03,[16] which has a considerably higher basal level of


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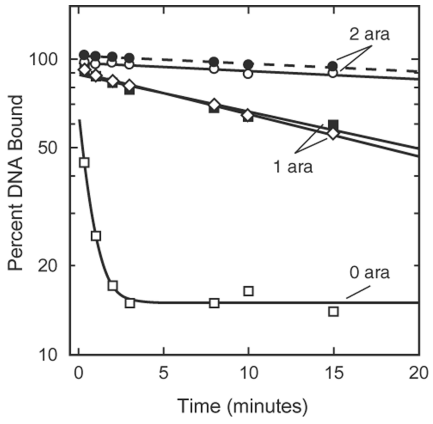


**Figure 4.** Composite of the kinetics of dissociation of AraC constructs from DNA in the presence of arabinose. The percent of $I_1-I_1$ DNA bound by five AraC constructs vs time is presented. All protein constructs contain the C205N, C268R, and C280S substitutions, and all kinetic data were obtained in the presence of 10 mM arabinose at room temperature: (●) parental strain with no additional mutations (the data have been offset by +5% along the ordinate for the sake of clarity), (○) L151C:L161C heterodimer, (■) H80A,L151C:L161C, (◇) L151C:H80A,L161C, and (□) H80A,L151C:H80A,L161C. Each line is a fit of a data set to a single-exponential decay equation. The constructs that can bind two, one, and zero arabinose molecules are indicated as 2 ara, 1 ara, and 0 ara, respectively.

expression. Leakage synthesis from pET-24d, which can yield sufficient AraC for some genetic purposes,[39] produced insufficient AraC for normal activity in our experiments. The insufficiency was exacerbated for AraC containing the dimer interface substitutions, possibly because of weakened dimerization affinity. Any weakening of the dimerization, however, becomes less important once the covalent cross-link is formed.

The Cys residues incorporated into the dimer interface for the disulfide trap do not interfere with normal DNA binding properties for either the various homodimers or the heterodimers. The L151C:L161C heterodimer protein, in which both subunits can bind arabinose, has DNA dissociation properties in the presence and absence of arabinose nearly identical to those of the homodimer lacking any substitutions in the dimer interface. Thus, the dimer interface mutations do not significantly alter the communication between the DNA binding domains and dimerization domains that is essential for the regulatory transition. This was expected because the considerable data leading to the light switch mechanism all indicate that the N-terminal arms and not the dimerization interface are what communicates the information about the status of arabinose binding to the DBDs.[12] Beyond the ability to bind DNA and respond to arabinose binding, the dimer interface substitutions only slightly alter the arabinose binding affinities, a change that appears to be physiologically insignificant.

**Arabinose Requirement of the AraC Regulatory Response.** The objective of these experiments was to determine whether the binding of a single molecule of arabinose to AraC is sufficient to fully alter the protein's DNA dissociation rate to that observed for wild-type AraC when it presumably binds two molecules. We were able to answer this question by constructing AraC heterodimers that behave like wild-type AraC dimers in the absence of arabinose, but in which only one subunit is capable of binding the sugar. Our results show that when only one subunit has bound arabinose, the protein dissociates from DNA at a rate intermediate between the rates at which apo-AraC, designated $ara_0$-AraC, and holo-AraC, designated $ara_2$-AraC, dissociate from DNA. This intermediate rate suggests that only one subunit of AraC has been transformed and that, normally, both are transformed.

Ideally, similar heterodimer experiments would also be performed in vivo, but these are not feasible for several reasons. The intracellular environment is reducing, and heterodimers could not be trapped by disulfide formation. Even if trapping were possible, when physiological levels of the two types of AraC are synthesized from two genes, translation of the two types of messengers will rarely be concurrent but would be separated by many minutes, and thus within a cell, significant quantities of homodimers would be present at least part of the time.

The fact that for dissociation from DNA, AraC capable of binding only one molecule of arabinose behaves in a manner intermediate between those of AraC incapable of binding arabinose and AraC capable of binding two molecules of arabinose indicates that in this case, the subunits of AraC respond independently rather than highly cooperatively to arabinose. As described in the introductory section, the arabinose-induced effect on DNA binding can be explained as resulting from an arabinose-induced increase in the degree of freedom of the AraC DNA binding domains to be oriented and positioned to bind to direct repeat DNA half-sites.[12−14,16−22] The results of the work described here indicate that normally, both DNA binding domains are allowed this increased degree of freedom and that the increased degree of freedom of both is required for the normal response.

## ■ CONCLUSIONS

Normally, the presence of arabinose strongly decreases the rate of dissociation of AraC protein from DNA. Our results show that when only one subunit AraC is capable of binding arabinose, the effect of arabinose is much attenuated, implying that the normal arabinose response involves the binding of two arabinose molecules to the dimeric AraC. Such behavior is consistent with an arabinose response mechanism in which each subunit binds and responds to arabinose in a manner independent of the other subunit.

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