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Strengthened Arm-Dimerization Domain Interactions in AraC*

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Constitutive mutations were sought and found in the N-terminal arm of the *Escherichia coli* regulatory protein of the arabinose operon, AraC protein. A new mutation, N16D, was of particular interest. Asn-16 is not seen in the crystal structure of the AraC dimerization domain determined in the absence of arabinose, because the N-terminal arm 18 residues are disordered, but in the presence of arabinose, residues 7–18 fold over the arabinose and make many interactions with it. In this state Asn-16 lies near two positively charged amino acids, Lys-43 and Arg-99. We propose that the introduction of the negatively charged aspartic residue at position 16 creates a charge-charge interaction network among Asp-16, Lys-43, and Arg-99 that holds the arm to the dimerization domain even in the absence of arabinose. This frees the DNA-binding domains and allows them to bind *cis* to I_1-I_2 half-sites and activate transcription. Mutating the two positively charged residues to alanines individually and collectively decreased or eliminated the constitutivity created by the N16D mutation.

The regulatory protein of the L-arabinose operon in *Escherichia coli* is thought to function by what is called the light switch mechanism (1, 2). In the absence of arabinose, the two DNA-binding domains of the dimeric AraC molecule are held not only by the eight amino acid peptide linkers between the dimerization domain and the DNA-binding domain but also by the N-terminal arms from the dimerization domains that bind to the DNA-binding domains. Even though each of these two connections may be flexible (3), their combination restricts the orientations freely available to the DNA-binding domains. This restriction makes it energetically disadvantageous for the two domains to contact the adjacent I_1 and I_2 half-sites but rather easy for the two domains to contact the nonadjacent half-sites I_1 and O_2 and form a DNA loop, represented in Fig. 1 (4). The situation changes upon the binding of arabinose. The binding of arabinose in the dimerization domains favors binding of the arms to the dimerization domains rather than the DNA-binding domains. This relieves the constraints on the DNA-binding domains and allows them to bind to the adjacent direct repeat I_1 and I_2 half-sites. This mode of DNA binding allows AraC to activate RNA polymerase for transcription from p_{BAD} (5–9).

According to the light switch mechanism, the binding of arabinose to the dimerization domain is normally required for the N-terminal arms to prefer to bind to the dimerization domains rather than the DNA-binding domains (1, 2). This model predicts the existence of two kinds of mutations in AraC that would allow it to activate transcription in the absence of arabinose. In the first type, the interactions between the arms and DNA-binding domains are weakened by mutations in either the arm or domain. In the second type, the arms are kept from the DNA-binding domains as a result of strengthened interactions between the arms and the dimerization domains that lead the arm to bind to the dimerization domain even in the absence of arabinose. Because either type of mutation frees the DNA-binding domains in the absence of arabinose, AraC activates the p_{BAD} promoter in the absence of arabinose, that is, it becomes constitutive. Although a sizeable number of constitutive mutations in AraC have been isolated (10–12), no mutations of the second type have been identified. Although it is quite possible that among the known constitutive mutations, some do have the effect of strengthening the arm-dimerization domain interaction, none stands out as obviously being of this type as judged from simple inspection of the structure of the dimerization domain that has been determined in the presence of arabinose (13). We have therefore sought additional constitutive mutations in AraC in hopes of finding mutations possessing a clear mechanistic basis and that would either support or refute the light switch mechanism.

EXPERIMENTAL PROCEDURES

Isolation of Constitutive Mutations—For mutagenesis, plasmid pWR03 (2) containing the entire AraC coding region was transformed into mutator cells (*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10:Tet*^{r}) (*E. coli* XL1-Red competent cells; Stratagene, La Jolla, CA) as described by Stratagene. The mutagenized plasmids were harvested via resin-based mini-prep (Wizard Plus DNA Miniprep purification system; Promega) and transformed into SH321 (*F*$^-$ Δ*araC-leu1022* Δ*lac74 galK*$^-$ *Str*^{r} *thi1*) (14). Constitutive mutant candidates were selected based on their abilities to grow on minimal arabinose-fucose plates containing 0.1% arabinose and 0.2% fucose. Colonies that grew up in 2 days were further tested by *in vivo* arabinose isomerase assay.

Arabinose Isomerase Assay—Cells were grown in M10 minimal salts medium plus 0.4% glycerol, 10 μg/ml thiamine, 0.02% L-leucine, and 0.2% casamino acids (15). When cells reached an A_{600} of ~0.8, they were harvested and assayed for their arabinose isomerase activities as described (15). When induction by arabinose was necessary, 0.2% arabinose was added to the culture 45 min before the assay.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the Stratagene QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following reagents were mixed together: 125 ng each of the two primers, 50 ng of parental plasmid template, 1 μl of 10 mM dNTP mix, 5 μl of 10× reaction buffer, 1 μl of *Pfu* DNA polymerase (2.5 units/μl; Stratagene, La Jolla, CA), and dH_2O to a final volume of 50 μl. All the other steps were as described by the Stratagene manual.

RESULTS

Isolation of Ara Constitutive Mutants—Induction of the arabinose operon is inhibited by the L-arabinose analogue, D-fucose (10, 12), but mutants can be isolated that are resistant to this inhibition by selecting for growth on medium where the only carbon sources are arabinose and fucose. Such mutants are

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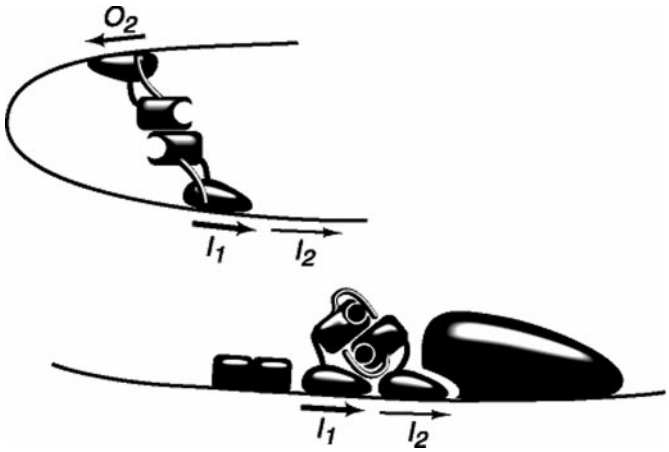

FIG. 1. **The light switch model of the response of AraC to arabinose.**

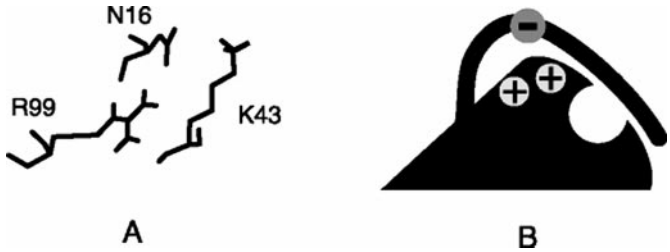

FIG. 2. *A*, liquorice model of the three residues Asn-16, Lys-43, and Arg-99 from the x-ray crystal structure of AraC dimerization domains in the presence of arabinose. *B*, a schematic drawing illustrating the charged triad in the context of the arm and the dimerization domain of AraC.

frequently found to be constitutive (10, 16). To isolate constitutive mutants, we therefore passed a plasmid coding for the AraC gene through a mutator *E. coli* strain XL1-Red, which is deficient in three of the primary DNA repair pathways. After accumulating mutations during propagation in the mutator cells, the plasmid was transformed into an AraC$^-$ strain.

Constitutive mutants, present at a frequency of ~0.2% among the transformants, were selected on the basis of their abilities to grow on minimal arabinose-fucose plates. The arabinose isomerase activities of the candidates were then assayed after growth in minimal medium in the absence of arabinose, and those with isomerase levels at least 3-fold above the wild type basal level were sequenced. Four different mutations were found, all lying in the N-terminal arm of AraC. They were L9K, S14P, N16D, and L19P. Of these, L19P was isolated twice, and N16D was isolated three times; L9K and S14P were isolated only once.

Constitutive mutations at positions 9, 14, and 19 have been isolated before (1, 12). The mutation N16D was new, however. Arabinose isomerase assay showed that, in the absence of arabinose, the N16D mutant activates the p_{BAD} promoter 67%, as well as wild type AraC induces in the presence of arabinose. By comparison, wild type AraC induces the p_{BAD} promoter less than 1% of the full induction level in the absence of arabinose.

In the structure of the AraC dimerization domain determined in the absence of arabinose, residues 1–18 are not seen, because the arms are disordered. In the structure determined in the presence of arabinose, however, the arms starting from residue 7 are visible and are seen to fold over the bound sugar. Asn-16 is close to two positively charged amino acids, Lys-43 and Arg-99 (Fig. 2). The closest distances between the side chains of the three residues are as follows: Asn-16—Lys-43, 3.5 Å; Asn-16—Arg-99, 2.0 Å; and Lys-43—Arg-99, 5.6 Å. It is very likely that changing residue 16 from asparagine to negatively charged aspartic acid creates a charge-charge interaction network between it and Lys-43 and Arg-99. We expect that, with the additional binding energy provided by this interaction, the arms are held to the dimerization domains most of the time even in the absence of arabinose, and this leads the protein to behave as though arabinose were present.

Effects of Alanine Substitutions Lys-43 and Arg-99—The hypothesis that in the N16D mutant the arms are held to dimerization domains through charge-charge interactions was tested by mutating the two positively charged amino acids Lys-43 and Arg-99 to alanines individually and collectively. If the electrostatic interactions increasing the strength of the binding of the arm to the core of the dimerization domain cause AraC to behave constitutively, then the phenotypes of the double or triple mutants should change accordingly. Alanine substitutions of Lys-43 and Arg-99 were generated by site-directed mutagenesis. The abilities of the mutants to activate the p_{BAD} promoter were examined by the arabinose isomerase assay both in the absence and in the presence of arabinose. Table I shows that changing either Lys-43 or Arg-99 to alanine in N16D AraC resulted in a partial loss of the constitutivity and that when both were changed to alanines, AraC no longer displayed any constitutive behavior. The triple mutant, N16D,K43A,R99A, behaved almost the same as the wild type AraC. As shown in Table I, the alanine substitutions did not affect the activation ability of either the wild type AraC or N16D in the presence of arabinose. The results are consistent with the idea that mutation N16D creates stronger arm-dimerization domain interactions and that these make AraC constitutive.

TABLE I
Activating abilities of AraC on p_{BAD} promoter

Activation by N16D AraC and the alanine substitution mutants was measured from the p_{BAD} promoter with the arabinose isomerase assay.

AraC protein	Activity	
	Minus Arabinose	Plus Arabinose
WT	40	4690
N16D	2740	4590
N16D, K43A	610	4650
N16D, R99A	260	3490
N16D, K43A, R99A	90	2150
K43A	20	5140
R99A	30	5240
K43A, R99A	70	4320

DISCUSSION

We searched for and found constitutive mutations in AraC protein. One of the mutations found, N16D, was not only new, but its mechanism of action can be understood and is consistent with the light switch mechanism for AraC action. In the three-dimensional structure of the AraC dimerization domain, Asn-16 lies very close to the two positively charged residues Lys-43 and Arg-99. Presumably then, a salt-bridge triad arises among Asp-16, Lys-43, and Arg-99, and the extra energy provided by this electrostatic network holds the arms to the dimerization domain even in the absence of arabinose.

Studies by both x-ray crystallography and NMR show that in almost all cases, the structural changes generated by changing the charge of a surface-exposed amino acid residue are confined to the immediate vicinity of the mutation (17–19). We expect this to be true of N16D, as well, although our attempts to crystallize the dimerization domain bearing the N16D mutation yielded only a crystal form with a space group that is unsuitable for structural determination. Under similar conditions wild type AraC dimerization domain also prefers to form these same unsuitable crystals but also occasionally forms

crystallographically useful crystals (13).

We think it likely that the salt bridges formed in the N16D mutant significantly stabilize the arm-domain interactions. Although some report that single salt bridges on the surfaces of protein contribute little stability (17–21), it is worth pointing out that some of the cited studies involved engineered ion pairs that may not have been in configurations necessary for optimal salt bridges. Moreover, when salt bridges involve three charged amino acids rather than two, cooperativity ought to add to the stabilizing effects (17). For example, on the surface of barnase are two exposed salt bridges between the C-terminal Arg-110 and aspartic residues at positions 8 and 12 in the first α helix of the protein. The contribution to the stability of the protein by the salt bridge between Asp-12 and Arg-110 is 1.25 kcal/mol, whereas that of the salt bridge between Asp-8 and Arg-110 is 0.98 kcal/mol. The energy of each is reduced by 0.77 kcal/mol when the other is absent, indicating that the two salt bridges are coupled.

Each of the two residues, Lys-43 and Arg-99, contributes to the constitutivity of the N16D mutant as shown by the fact that changing either of the two positively charged residues to alanine substantially reduced the level of constitutivity. Thus, a triad similar to the one found in barnase could form, and coupling between the two salt bridges would take place. The resulting stabilizing force is large enough for the arms to prefer binding to the core of the dimerization domain rather than to the DNA-binding domain or not binding to either domain.

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