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Altered DNA contacts made by a mutant AraC protein

Alan Brunelle, William Hendrickson* and Robert Schleif

Department of Biochemistry, Brandeis University, Waltham, MA 02254, USA

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ABSTRACT

Mutant AraC proteins were selected for their ability to induce but not to repress, or their ability to repress but not to induce the araBAD operon. One such unusual mutant is able to bind to the araI site with an affinity only two to three-fold weaker than the wild type AraC protein, but the mutant protein was shown, both in crude extracts and when purified, to contact only two of the three major groove regions of the DNA that are contacted by the wild type protein.

INTRODUCTION

Structures of a number of regulatory proteins determined by X-ray diffraction and studies of mutants have indicated that one motif for DNA sequence recognition utilizes a helix-turn-helix structure that binds in the major groove of the DNA (1-4). The AraC protein positively and negatively regulates expression of the arabinose operons in Escherichia coli (5-8). Since a dimer of this regulatory protein appears to contact three adjacent major groove regions of the DNA helix (9), the protein likely utilizes an alternative structure or, at the minimum, two helix-turn-helix structures per subunit for its sequence recognition capabilities. In studying this question we found a mutant that fails to make contact with one of the three major groove regions. Since the mutant protein binds DNA with nearly normal affinity, these results suggest caution in the interpretation of binding affinities of mutant proteins.

MATERIALS AND METHODS

Mutant selections

To select mutants in the AraC gene that were defective in their abilities to induce araBAD, hydroxylamine mutagenized AraC

overproducer plasmid (10) was transformed into a strain that is sensitive to the presence of arabinose by virtue of its being $araD^-$. If a mutant araC gene that prevented induction of the resident araBAD genes were introduced into these cells, they would become resistant to the presence of arabinose. Therefore, after transforming the araD cells with the mutagenized araC plasmid, amp^r colonies resistant to arabinose were selected on MacConkey plates containing 1% arabinose (11). The ability of the AraC protein encoded by any selected plasmid to repress araC was screened by transforming the plasmid into a Pc-lacZ fusion strain (12) and plating on MacConkey-lactose plates. This screening procedure works well with this high-level overproducer plasmid because repression seems to be complete as seen in this assay, that is, the wild type colonies are white. Other low level overproducer AraC plasmids yield wild type colonies that are pink on these plates. To eliminate totally defective mutants or those with increased nonspecific binding by AraC protein, only mutants defective in one, but not both, functions were screened with the gel binding assay.

Gel electrophoresis binding assay

180 base-pair DNA fragments containing the araI site and 290 base-pair fragments containing the $araO_1$ site were end-labeled with ^{32}P (13). After incubating both DNA fragments together with the indicated lysate at 25° for 10 min. in 20 µl binding buffer (10mM Tris-acetate, pH 7.4, 50mM KCl, 50mM arabinose or fucose, 1mM EDTA, 5% glycerol, 50µg/ml BSA, 1mM DTE, 0.05% NP40,), protein-DNA complexes were electrophoresed at 20° for 1 hour in 6% acrylamide-0.1% bisacrylamide gels in buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. Gels were dried and DNA was visualized by autoradiography.

Lysate preparation

5 ml cultures were grown to approximately 8×10^8 cells/ml in YT broth, cells were then pelleted, washed with Tris-HCl, pH 8.0, 1mM EDTA and resuspended in 0.5 ml cold lysate buffer (0.1 M potassium phosphate, pH 7.4, 50 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM DTE, 160 µg/ml phenylmethylsulfonate, 0.1 mM $ZnCl_2$). The suspension was sonicated to reduce the absorbance at 550 nm by greater than 90% (3 x 6 sec. pulses with a

microprobe) and the cell debris removed by centrifugation in a microfuge for 10 min. at 4°. The glycerol concentration was increased to 35% and aliquots were stored at -70°. About 50% of the binding activity is lost in the first few weeks of storage if the sample is stored at -20° but at least 50% of the initial activity is retained for at least 6 months if the sample is stored at -70°.

DNA contact determination

The binding interference assay was carried out as described by Hendrickson and Schleif (9) with the exception that cell lysates were the source of the AraC protein for most experiments. DNA fragments were treated so that each fragment was randomly modified, on average, once at either the N7 position of guanine in the major groove, the N3 position of adenine in the minor groove or on one of the backbone phosphates. Guanines and adenines were modified with dimethyl sulfate and phosphates were modified with ethylnitrosourea. The modified DNA was incubated in binding buffer containing arabinose, as described in Methods for the gel binding assay, with sufficient lysate to bind all of the DNA. A large excess of unlabeled *ara* DNA was then added to bind free protein and to allow any protein, which may have been weakly bound to critically modified *ara* sites, to dissociate. The gel electrophoresis assay was used as a preparative technique to

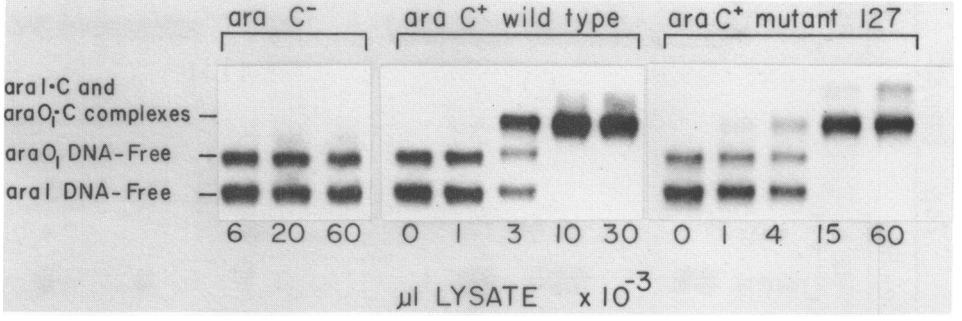
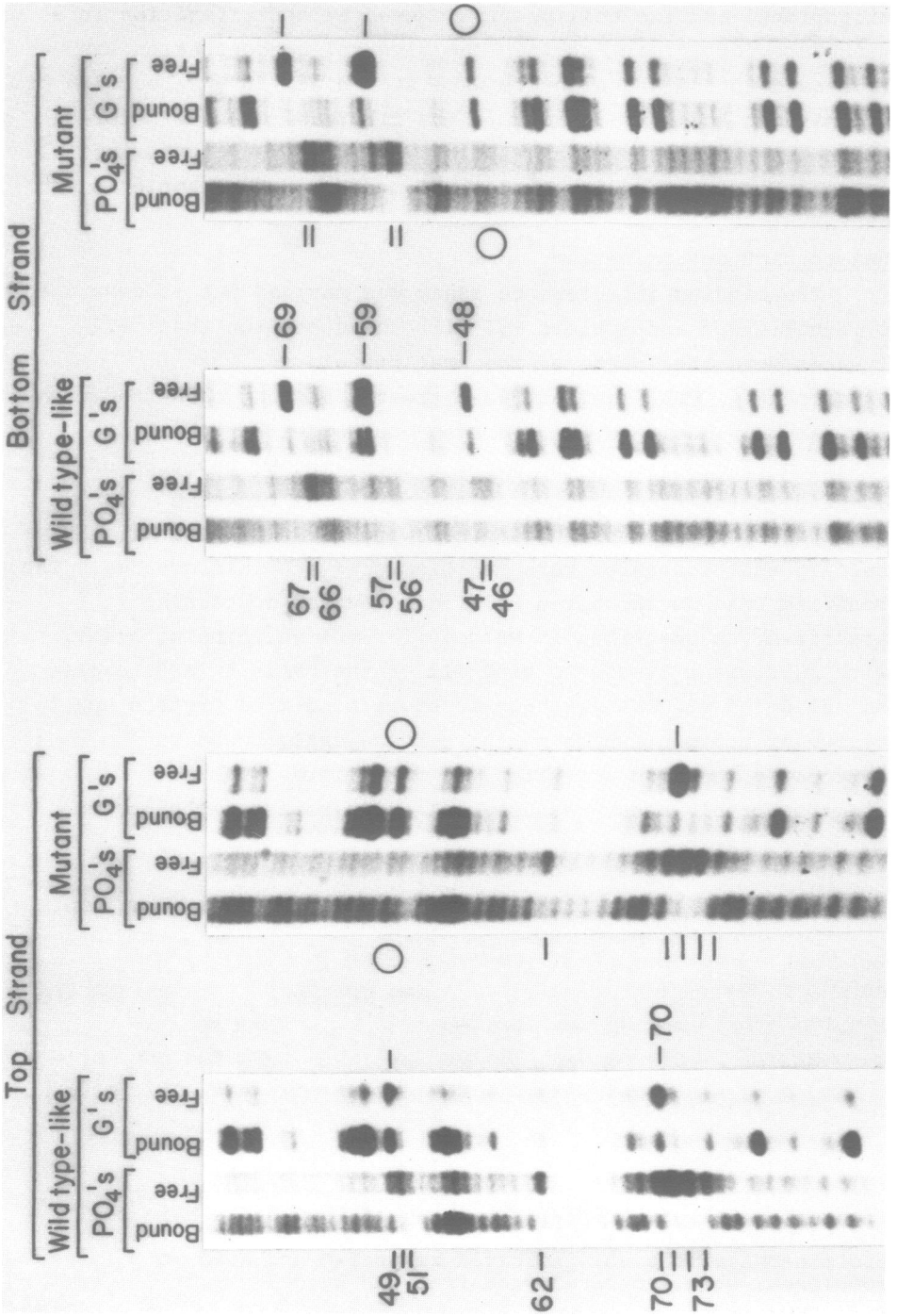


Figure 1. Gel electrophoresis assay of AraC protein in cell lysate binding to *ara* DNA.
Protein-DNA complexes, indicated as araI·C and $araO_1$·C complexes, were separated from free DNA by gel electrophoresis.


Bottom Strand
Mutant
PO4's
G's
Free
Bound
Wild type-like
−69
−59
−48
67
66
57
56
47
46
Top Strand
−70
49
51
62
70
73

separate araC·DNA complexes, presumably modified only at noncrucial DNA sites, from free DNA that was modified at positions that interfere with protein binding. The protein·DNA complexes and free DNA were recovered from the gels by running the DNA bands onto Schleicher and Schuell NA45 paper and then eluting the DNA from the paper. The DNA samples were cleaved as described before (9) at the modified bases so that their positions could be determined from the lengths of the resulting DNA fragments run on a sequencing gel. A G-track was used as sequencing size markers to determine the positions marked in figure 2.

RESULTS

For convenience in assaying and purifying mutant AraC proteins and for sequencing mutants, we used a plasmid that overproduces AraC protein about 1000-fold (14). Mutant AraC proteins defective in their ability to induce P_{BAD} but still able to repress P_C were studied further in the work described here. From their *in vivo* properties, the mutant proteins (see mutant selections in methods) were expected to be able to bind $araO_1$, the operator for P_C. The mutant proteins' inability to induce P_{BAD} could derive from (1) a lack of binding to *araI*, the induction site for P_{BAD}, (2) the inability of arabinose to convert the protein to its inducing state, or (3) from the inability of AraC protein in the inducing state to interact properly with RNA polymerase.

The gel electrophoresis binding assay can conveniently monitor DNA binding abilities of proteins (13,15-17). This

Figure 2. Guanine and phosphate contacts of mutant AraC127 and mutant AraC25 proteins at *araI*. DNA fragments that contain the *araI* site were ^{32}P-end-labeled at the 5'-position of either the upper strand (*Bst* EII site) or the lower strand (*Hind* III site). PO_4's: gel lanes with pre-ethylated DNA phosphates; G's: lanes with pre-methylated DNA guanines. Bound lanes: DNA isolated from AraC·DNA complexes. Free lanes: DNA isolated from the free DNA band. The numbers indicate the distance of the apparent contacts from the P_{BAD} transcription start site. Open circles are placed where contacts are lost with mutant AraC127. Locations of bands using mutant AraC25 lysate are identical to those using purified wild type AraC protein (9) and are shown here for comparison to the data for mutant AraC127.

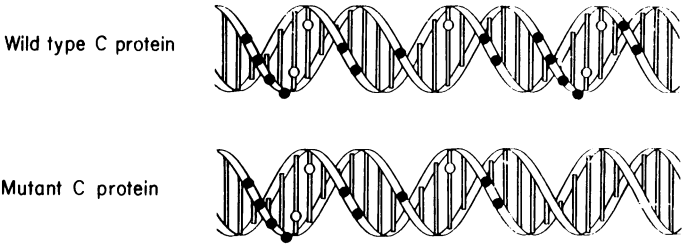


Figure 3: Diagram of the araI site showing contacts by wild type AraC protein and mutant AraC127. Protein contacts to guanines (open circles) and to phosphates (closed circles) are shown. Note that the binding of the wild type protein extends across three major grooves of the DNA at the araI site and the complete loss of contacts in the third major groove for AraC127.

assay was adapted to work with crude lysates (Fig. 1) and was used to measure binding of AraC protein to either the $araO_1$ or araI sites. After screening nearly one hundred mutants, AB127 was identified as an interesting candidate because it is able to bind the araI site even though it is unable to induce P_{BAD}.

To examine the mutant protein AB127 more carefully, the concentration of active AraC protein in lysates was first quantitated by titrating against a known amount of ara DNA at a concentration well above the equilibrium constant. The equilibrium binding constants of the protein for the araI and $araO_1$ sites were then determined in both the presence of arabinose (inducer) and fucose (an anti-inducer). The mutant protein binds to both sites with only a three-fold reduction in affinity as compared to the wild type protein. While association rates have not been determined for the mutant protein, all of the reduction in affinity can be accounted for in the three-fold increase in dissociation rate. The mutant protein's dissociation constant shows the same response to the presence of arabinose or fucose as does the wild type protein (13), thereby showing the mutant protein binds arabinose. DNA sequencing revealed that the mutation in this protein is a G to A change at position 849 (with respect to P_C), which changes amino acid 228 from asp to asn.

Since a failure to bind the araI site or arabinose was not the explanation for the mutant's properties, we examined the DNA

contacts made by the protein. Methylation of guanines or ethylation of phosphates can prevent the subsequent binding of a protein if the modifications are at sites of contact by the protein. DNA molecules were randomly modified. Then those altered so that binding by AraC protein was not possible were separated with the gel electrophoresis assay from those in which binding is possible. The sites of modification in these two subpopulations were then determined by cleaving the DNA at the modified sites and displaying the products on sequencing gels (Fig.2). Crude lysates were the protein source for determining the DNA contacts.

The wild type AraC protein displayed the same contacts as have been determined with pure AraC protein, that is all contacts are on one "face" of the DNA and extend across three major groove regions. AraC protein has been shown to bind the araI site as a dimer (9), therefore each monomer likely makes contacts in more than one major groove (Fig.2). (Figure 2 actually shows contacts made by another mutant, AB25, which has the same contacts as does wild type AraC protein.) For the mutant protein AB127, guanine contacts and the adjacent phosphate contacts on each side of the one major groove region are absent (fig.2). DNAse footprinting also showed an absence of protection in this same region of the araI site (data not shown). Subsequent examination of the contacts made by the purified mutant protein yielded the same results.

DISCUSSION

How could the mutant protein bind with an affinity similar to that of wild type if it has lost one third of its DNA contacts? If a single contact between protein and DNA provides on the order of 3 kcal/mole of binding energy, then the loss of one third of the DNA contacts between the mutant AB127 and and DNA would be expected to have a most drastic effect upon the protein affinity for the araI site.

One rationalization for the discrepancy between expectations and reality is that an appreciable fraction of the binding energy derived from the contacts made by wild type AraC protein to all three major groove regions is consumed in forcing

the protein and/or DNA into an altered and strained conformation. Such an altered conformation could be required for normal induction of the araBAD promoter. In the case of the mutant protein, the conformational change would not occur. This would have two effects. First, the araBAD promoter would not be inducible, and second, less of the mutant's binding energy would be consumed in the conformational change, leaving more available to contribute to the measured affinity.

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*Present address: Department of Microbiology, Hershey Medical Center, Hershey, PA, USA

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