In Vitro Recognition of Specific DNA Targets by AlcR, a Zinc Binuclear Cluster Activator Different from the Other Proteins of This Class*

(Received for publication, December 3, 1996, and in revised form, April 11, 1997)

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AlcR is the transactivator mediating transcriptional induction of the alc gene cluster in Aspergillus nidulans. The AlcR DNA-binding domain consists of a zinc binuclear cluster different from the other members of the Zn$_2$Cys$_6$ family by several features. In particular, it is able to bind to symmetric and asymmetric sites with the same affinity, with both sites being functional in A. nidulans. Here, we show that unlike the other proteins of the Zn$_2$Cys$_6$ binuclear cluster family, AlcR binds most probably as a monomer to its cognate targets. Two molecules of the AlcR protein can simultaneously bind in a noncooperative manner to inverted repeats. The consensus core has been determined precisely (5'-CCGCA-3'), and the AlcR-binding site in the aldA promoter has been localized. The sequence downstream of the zinc cluster is necessary for high affinity binding. Furthermore, our data show that the use of the carrier protein glutathione S-transferase in AlcR binding experiments introduces an important bias in the recognition of DNA sites due to its tertiary dimeric structure.

The Aspergillus nidulans activator AlcR is a member of the DNA-binding protein family whose DNA-binding domain contains a highly conserved zinc binuclear cluster (1, 2). The proteins in this class, such as GAL4 (3), PPR1 (4), and HAP1 (5) in Saccharomyces cerevisiae and UaY (6), PrnA (7), NirA (8), and AlcR (1, 2) in A. nidulans, are transcriptional activators that control a wide variety of metabolic pathways.

Among the Zn$_2$Cys$_6$ zinc binuclear cluster proteins, some have been characterized both biochemically and structurally by analysis of their three-dimensional conformations. Most of them, such as GAL4, PPR1, and UaY, bind to symmetric DNA sites as dimers through their coiled-coil dimerization element. HAP1 recognizes asymmetric sites (9), and it has been shown recently that the Zn$_2$Cys$_6$ zinc cluster is responsible for asymmetric binding, with the coiled-coil region stabilizing the complex (10). However, other proteins of this family, e.g. ARG2 and MAL63 (11), have been suggested to function as monomers.

* This work was supported in part by CNRS and the Université Paris-Sud and by European Communities Grant BIO2-CT93-0147. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) G168007 (alcA), G168009 (alcR), and G168011 (aldA).
† Supported by a grant from the Ministère de l’Education Nationale, de l’Enseignement Supérieur, et de la Recherche.
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AlcR, the specific transactivator of the alc cluster involved in ethanol utilization and in other related carbon metabolic pathways (12, 13), appears to be different from the other members of the Zn$_2$Cys$_6$ family. It contains in its DNA-binding domain, between the third and fourth cysteines, an unusual extended sequence of 16 residues instead of the six to eight usually found, and no predicted dimerization regions were found downstream of the zinc cluster. Furthermore, unlike the other members of this family, AlcR appears to bind with the same affinity to both symmetric and asymmetric sites containing the consensus motif 5'-CCGCA-3' (14, 15). Both types of targets localized in the alcR and alcA promoters have been shown to be functional in vivo (15, 16).

The AlcR-binding sites have been determined previously using a bacterial expression system with a glutathione S-transferase (GST)$^1$ fusion protein (GST-AlcR-(7–60)) (14, 15). Bacterially expressed fusion proteins are widely used to identify specific DNA sequences that are recognized by regulators. Among them, GST presents several advantages since it provides high yields of protein that can be easily purified to homogeneity. Furthermore, cleavage by thrombin releases the DNA-binding protein (17). The GST fusion protein system was successfully used to determine the DNA-binding sites for a number of proteins such as SWI5 in yeast (18), N-Myc in mouse (19), and T/E1A in human (20). In A. nidulans, binding sites for the CreA repressor (16) and NirA (21) and UaY (6) activators have been localized using GST fusion proteins. The binding sites have been shown to be functional in vivo, as for AlcR-binding sites.

In this report, we have compared the binding specificities of the GST-AlcR-(1–60) fusion protein and a longer AlcR-(1–197) protein, tagged at its carboxyl terminus with six histidine residues. We demonstrate that the use of GST introduces an important bias in the recognition of DNA sites as the result of its quaternary dimeric structure. It prevented the identification of an AlcR-binding site in the aldA promoter that is now established. It hindered the important observation that the AlcR protein binds preferably as a monomer to DNA, unlike the other proteins of the Zn$_2$Cys$_6$ binuclear cluster family. Two molecules of the AlcR protein can simultaneously bind in a noncooperative manner to symmetric sites, whereas only one molecule occupies a direct repeat site. Finally, we show also that the sequence downstream of the zinc cluster is necessary for high affinity binding.

EXPERIMENTAL PROCEDURES

Expression and Purification of AlcR Proteins—To construct the GST-AlcR fusion expression vector, a DNA fragment encompassing the AlcR DNA-binding domain (amino acids 1–60) was amplified by polymerase

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* The abbreviations used are: GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay.
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chain reaction and cloned in frame with the GST gene in the pGEX-2T plasmid (Pharmacia Biotech Inc.). The protein was expressed from the tac promoter and purified as described earlier (17, 14). The protein obtained was 80–90% pure as judged by SDS gel electrophoresis using Coomassie Blue staining.

The AlcR peptide was separated from GST by cleavage with thrombin (1 unit/mg of fusion protein; Sigma) in the presence of 2.5 mM CaCl2 for 10 min at 30 °C. The mixture was loaded onto a Resource S high pressure liquid chromatography column (6 ml; Pharmacia Biotech Inc.) pre-equilibrated in 10 mM phosphate (pH 7.2) and 0.1 M NaCl. AlcR was eluted using an increasing gradient of 1 M NaH2PO4, 10 mM phosphate (pH 7.2), and 0.1 M NaCl. The eluted AlcR peptide was then passed through a p-aminobenzamidine column (Sigma) to remove contaminating thrombin, and pH was adjusted to 6.0. A plasmid expressing AlcR(1–197) tagged with His6 at its C terminus was constructed by cloning the Ncol-BamHI fragment into the pET-22b vector (QIAGEN Inc.). The Ncol site was introduced into the ATG codon during polymerase chain reaction amplification. Escherichia coli BL21(DE3) cells bearing the expression plasmid were grown at 37 °C to A600 = 0.6. After 3 h of induction with 1 mM isopropyl-β-D-thiogalactopyranoside in the presence of 20 μM ZnCl2, the cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate buffer (pH 7.9) containing 0.3 M NaCl, 5 mM β-mercaptoethanol, and 20 μM ZnCl2. After sonication, the AlcR protein was partially purified using a NTA-nitrilotriacetic acid-agarose column. The eluted protein was monitored by SDS-polyacrylamide gel electrophoresis following the procedure of Maxam and Gilbert (22).

For EMSAs, end-labeled DNA probes were partially methylated by Dimethyl sulfate following the procedure of Maxam and Gilbert (22). The chemically modified probes (103 counts/s) were incubated with 1000 ng of partially purified AlcR(1–197) as described above. Bound and unbound DNAs were sliced from a preparative EMSA gel and electroeluted onto DEAE NA45 membrane in 1 × TBE buffer. Recovered DNAs were purified by phenol/chloroform extraction, cleaved by 1 M dimethyl phosphate following the procedure of Maxam and Gilbert (22), and subjected to electrophoresis on a 16% polyacrylamide gel containing 8 μm urea.

RESULTS

Three Different Bacterially Expressed AlcR Proteins—Previous studies have shown that the GST-AlcR(7–60) fusion protein binds to inverted and direct repeat sites with the same consensus core (5′-CCCGC-3′) in the alcR and alcA promoters. Both types of sites have been shown to be functional in vivo (15, 16, 2). The interactions between the AlcR fusion proteins and guanines within the motif appeared to be different in both types of target (14, 15). Since GST (the fusion protein carrier) is known to be dimeric in solution (23, 24) and hence could introduce a bias in the DNA binding specificity, we decided to perform in parallel the same binding experiments with another bacterially synthesized AlcR protein consisting of six histidine residues fused to the C terminus of the truncated AlcR protein (residues 1–197) and purified on a nickel column as described under “Experimental Procedures.” Such chimeric proteins are also widely used. For example, NMR studies of the Fru repressor from E. coli showed that the extra histidine residues have no influence on the protein conformation and its activity (25).

The three AlcR proteins utilized in this DNA binding study are depicted in Fig. 1 (A and B). As shown in Fig. 1B, the GST-AlcR(1–60) protein migrated on SDS gels according to its predicted size (34 kDa), whereas the AlcR(1–197) (~25 kDa) and AlcR(1–60) (~7 kDa) proteins exhibited aberrant electrophoretic mobility. These results remained unexplained. In the case of the AlcR(1–60) peptide, the molecular mass deter-
minded by mass spectrometry appeared to be 7.1 kDa. The GST-AlcR(1–60) protein contains only the AlcR DNA-binding domain, including the amino terminus (amino acids 1–6), which was deleted in our previous studies. Cleavage by thrombin resulted in the isolated AlcR(1–60) peptide, which was purified (see “Experimental Procedures”). The His-tagged AlcR(1–197) protein (Fig. 1C) comprises additional domains equivalent to those present in other proteins of the zinc cluster family, the so-called linker and dimerization regions. Therefore, questions may also be addressed to the role in AlcR binding of these two regions described as essential elements for the binding specificity of proteins of this Zn2Cys6 class.

Two Molecules of AlcR Bind to Inverted Repeat Sites—Two chimeric proteins, His-tagged AlcR(1–197) and GST-AlcR(1–60), were initially tested by gel retardation assays with a wild-type inverted repeat probe (probe b in the alcA promoter) (Fig. 2). Upon an increase in the AlcR(1–197) protein concentration, a second complex of higher molecular mass was formed (Fig. 3A). Competition experiments showed that both AlcR(1–197) complexes (I and II) are specific (data not shown). The apparent Kd for complex I was estimated as 4 × 10^{-8} M and that for complex II as 2 × 10^{-8} M, which is not significantly different. As shown in Fig. 3A, the mobility of the fast migrating complex (complex I) corresponded to that of the complex obtained with a single copy site (probe 1/2b), indicating the DNA interaction of one AlcR molecule. Therefore, the slow migrating complex (complex II) contains two AlcR molecules bound noncooperatively to a palindromic sequence.

With the AlcR(1–60) peptide containing only the zinc binuclear cluster, at very high protein concentration (1000 ng), two complexes were observed, indicating the binding of two AlcR molecules (Fig. 3B). The low affinity binding observed with the AlcR DNA-binding domain alone supports the idea that the sequence downstream of the zinc cluster (amino acids 61–197) contributes significantly to high affinity binding. This is indicative of either an increase in stability or thermostability of the complex and/or additional contacts between AlcR and DNA. Interestingly, it was observed previously that the binding of the AlcR(7–60) peptide is unstable, and DNA binding activity was restored by changing the conditions of gel band shift experiments as described in Ref. 14 and under “Experimental Procedures.”

With GST-AlcR(1–60), only one complex was obtained whatever the protein concentration (Fig. 3C). The apparent Kd for complex was estimated as 10^{-9} M, indicating a high affinity binding of GST-AlcR(1–60). Therefore, the presence of the GST moiety enhances GST-AlcR(1–60) affinity for its specific target by 10-fold. The presence of only one complex is not surprising since the GST protein naturally occurs as a dimer, and thus, one single complex contains two AlcR molecules. Therefore, the dimeric structure of the GST protein prevents AlcR binding to single sites.

AlcR Binds DNA Probably as a Monomer—The simplest interpretation of these experiments is that AlcR is able to bind DNA as a monomer. To test this hypothesis, transcription-translation assays in the reticulocyte lysate system were performed using two plasmid constructions encompassing alcR encoding His-tagged proteins of different length: AlcR-(1–163)-encoding proteins of different length: AlcR-(1–163)-

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4. R. Cerdan and E. Guittet, personal communication.

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**Fig. 2. Localization of the specific DNA targets in the *alcR*, *alcA*, and *aldA* promoters. Arrows indicate the orientation of the AlcR consensus motifs marked in *boldface*. The start of translation is indicated as position +1 tsp, transcription start point.**
The promoter (14) with a symmetric change of the last base pair A to T instead of A in the fifth position of the consensus core (5'-AGCGGCTCCGCT-3') (Fig. 2 and Table I). Previous gel shift experiments with GST-AlcR(7–60) and overlapping restriction fragments in the aldA promoter failed to demonstrate any retardation (1). Therefore, it was important to test this potential AlcR target with the AlcR-(1–197) protein in parallel with the GST-AlcR-(1–60) protein. This sequence is similar to the functional inverted repeat target in the aldR promoter (14) with a symmetric change of the last base pair A to T (probe aldA (B1)).

As shown in Fig. 5, nucleotide change prevented completely the binding of the GST-AlcR-(1–60) fusion protein to the probe. In contrast, the His-tagged AlcR-(1–197) protein was able to form two complexes with a slightly lower affinity as compared with the inverted repeat sequence present in the aldA promoter. A similar pattern of binding was observed when the last A was replaced by C. Taken together, these results imply that there is no strong preference in the fifth position of the consensus motif for tight binding. Therefore, the presence of the GST moiety hinders the identification of an AlcR palindromic target in the aldA promoter.

**Specificity of AlcR Recognition for Direct Repeats**—Previous footprinting and gel retardation experiments have shown that the direct repeat sequence in the aldR promoter (probe A) (Table I and Fig. 2) may be occupied by the GST-AlcR-(7–60) fusion protein only if the adjacent inverted repeat target (probe B) is bound by the fusion protein (14). As shown in Fig. 6A, no binding to the aldR direct repeat target was observed with the GST-AlcR-(1–60) fusion protein, even at high protein concentration. Conversely, the His-tagged AlcR-(1–197) protein formed a single complex with a lower affinity compared with the aldA direct repeat target (probe c) (see below). It is evident that one AlcR molecule is able to bind to the natural direct repeat in the aldR promoter, whereas the GST-AlcR fusion protein does not.

The direct repeat target (probe c) (Table I and Fig. 2) in the aldA promoter was recognized with high affinity by both AlcR proteins (apparent $K_d = 3.1 \times 10^{-8}$ M for the fusion and His-tagged proteins, respectively). Similarly, a single complex was observed, indicating the binding of one AlcR-(1–197) molecule and of one dimeric GST-AlcR-(1–60) molecule, respectively (Fig. 6B). Therefore, it was important to address the question whether the two sites could be occupied randomly, or if AlcR-(1–197) occupies preferentially one site. In addition, it was interesting to determine if the different pattern of binding specificity observed with GST-AlcR-(1–60) and His-tagged AlcR-(1–197) could be attributed to differences in the interacting guanines in the sites.

As illustrated in Fig. 7, interactions by methylation interference showed that AlcR-(1–197) made strong contacts with the two central guanines (positions -183 and -185) in the bottom strand within the 3′-site (with G at position -186 being also protected, however less), whereas the central G (position -200) in the top strand did not interfere. These results differ from those obtained with GST-AlcR-(1–60) or with GST-AlcR-(7–60) (15) (note that the affinity was too low to perform footprint experiments with AlcR-(1–60)): (i) both sites were protected instead of one; and (ii) all the guanines in the consensus motifs interfered with the complex formation, and furthermore, the methylation of the two upstream guanines in the motifs (positions -204 and -191) interfered (although differently) with the GST-AlcR-(1–60) protein. These experiments show clearly that in addition to the altered DNA specificity with GST-AlcR-(1–60) for the direct repeat probe c target, there is also a change in the interaction between the GST-AlcR fusion protein and DNA.

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**Fig. 4. Test for AlcR heterodimer formation.** A, autoradiography of SDS-polyacrylamide gel electrophoresis analysis of [35S]Met-labeled AlcR polypeptides expressed in the reticulocyte transcription-translation system. Two types of proteins of different length as indicated above the gel were expressed either separately or simultaneously. B, EMSAs with probe b in the presence of AlcR proteins produced by the in vitro reticulocyte lysate system. 0.1 pmol of labeled DNA and 5–10 μl of transcription-translation product were used in each reaction.

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| Probe (gene) | Sequence of the top strand 5′ to 3′ | Size |
|--------------|------------------------------------|------|
| b (aldA)     | GATGCATGGGGAAACCGGAGGAGGGCG       | 25-mer |
| 1/2b         | GATGCATGGGGAAATTACCGGAGGGCG       | 25-mer |
| c (aldA)     | GGTACGTGCGGAGGATCTCCGAGCGGAGA     | 28-mer |
| A (aldR)     | GATGGCGATGGGGAAATTCGGGAGGGCG      | 32-mer |
| B1 (aldA)    | GCCTAACAAGAGGGGTCCGGGTGACCC       | 28-mer |

*Consensus motifs are indicated in boldface.

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F. Lenouvel and B. Felenbok, unpublished results.

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**TABLE I**

Oligonucleotides used in this study

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**DISCUSSION**

Analyses of eukaryotic site-specific DNA-binding proteins are generally carried out either with bacterially synthesized chimeric proteins using fusion proteins such as glutathione S-transferase and β-galactosidase or with chimeric proteins consisting of additional histidine residues. We show here the limit of the approach based on the utilization of a heterologous protein linked to a DNA-binding domain for defining a specific target. Until now, to our knowledge, it had not yet been described that such an approach would introduce a serious bias related to the quaternary structure of the heterologous carrier protein. For example, the GST protein is a dimer in solution (23, 24), and β-galactosidase is a tetramer (27, 28). Therefore, these proteins fused to a DNA-binding domain impose a conformation resulting from their quaternary structure for selecting the DNA targets. This was clearly demonstrated by comparing the binding activities of the GST-AlcR-(1–60) and His-tagged AlcR-(1–197) proteins.

As a first approach, the localization of AlcR-specific binding sites was performed with the GST-AlcR fusion and His-tagged AlcR proteins for the palindromic target in the **aldA** promoter. Gel mobility shift assays were performed with a constant amount of labeled oligonucleotides (20 fmol; probe B1) (see Fig. 1 and Table I) and increasing amounts of GST-AlcR-(1–60) and AlcR-(1–197) proteins as indicated at the top of each gel. AlcR palindromic targets in the **aldA** promoter (probe B1) (see Table I) are represented at the bottom. Arrows indicate the orientation of the motif, with the modification of the last bases indicated above.

**FIG. 5.** Binding properties of the GST-AlcR fusion and His-tagged AlcR proteins for the palindromic target in the **aldA** promoter. Gel mobility shift assays were performed with a constant amount of labeled oligonucleotides (20 fmol; probe B1) (see Fig. 1 and Table I) and increasing amounts of GST-AlcR-(1–60) and AlcR-(1–197) proteins as indicated at the top of each gel. AlcR palindromic targets in the **aldA** promoter (probe B1) (see Table I) are represented at the bottom. Arrows indicate the orientation of the motif, with the modification of the last bases indicated above.

**FIG. 6.** Binding properties of GST-AlcR fusion and His-tagged AlcR proteins for direct repeat targets in the **alcR** and **alcA** promoters. Gel mobility shift assays were performed with a constant amount of labeled oligonucleotides (20 fmol; probes A and c) and increasing amounts of GST-AlcR-(1–60) and AlcR-(1–197) proteins as indicated at the top of each gel. AlcR direct repeat targets in the **alcR** (probe A) and **alcA** (probe c) promoters are represented at the bottom of the gels (see Fig. 2 and Table I). Arrows indicate the orientation of the consensus motif, with the modification of the last bases indicated. The sequence of the spacer is shown. A, binding of both proteins to the direct repeat target (probe A) in the **alcR** promoter; B, binding of both proteins to the direct repeat target (probe c) in the **alcA** promoter.

**FIG. 7.** Methylation interference footprinting pattern of the direct repeat target (probe c) in the **alcA** promoter with the His-tagged AlcR-(1–197) proteins. Methylation interference by dimethyl sulfate was performed as described under “Experimental Procedures.” The double-strand oligonucleotide encompassing the target (probe c) in the **alcA** promoter (see Fig. 2 and Table I) was labeled on the bottom or top strand and incubated with 1000 ng of estimated His-tagged AlcR protein (lane +). Constant amounts of labeled oligonucleotides were run in lanes P and +. Lanes P show the dimethyl sulfate cleavage pattern of the top and bottom strands, respectively, of unbound probe c. Full protected guanines by the His-tagged AlcR-(1–197) protein are indicated by closed circles; open circles represent weaker protection in the nucleotide sequence of the target (probe c).
deleted (GST-AlcR-(7–60)) unexpectedly resulted in a change in binding specificity. Therefore, this study was performed with a longer AlcR protein (AlcR-(1–197)) containing the amino terminus and the region downstream of the zinc cluster that includes the so-called linker and dimerization regions found in most proteins of the ZmCys6 class. Comparisons with GST-AlcR-(1–60) binding specificity showed that AlcR-(1–197) recognizes the same motif (5′-CCGCA-3′) organized in direct and inverted repeat sequences. In agreement with these results, physiological studies on A. nidulans have shown by deletion and site-directed mutagenesis that both types of targets are functional in the promoters of the alcR (16) and alcA (15) genes.

However, the use of the GST-AlcR-(1–60) fusion protein in previous studies has prevented the identification of important features that place AlcR in an original and unique position in the zinc binuclear cluster family. The most significant result is probably that AlcR is able to bind as a monomer to its targets. That could explain its unusual specificity for both inverted and direct repeats. In agreement with this result, the region downstream of the zinc cluster, which is organized in heptad repeats in the other zinc cluster proteins (GAL4 (3), PPR1 (4), and HAP1 (5)), is not present in AlcR. In fact, in AlcR, two downstream regions could be similar to leucine zippers. They contain four leucine or hydrophobic amino acids (able to replace leucine) every seven residues. However, proline residues are also present (12) (see Fig. 1C), which are known to impair α-helical structures (29). The second and strong argument is that no functional dimerization elements were detected in these reading of the manuscript and to Dr. M. Blight for its English version.

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*I. Nikolaev, F. Lenouvel, and B. Felenbok, submitted for publication.
**M. Mathieu and B. Felenbok, unpublished results.