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# The *Aspergillus nidulans* transcription factor AlcR forms a stable complex with its half-site DNA: a NMR study

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**Abstract** **The *Aspergillus nidulans* transcription factor AlcR is shown by NMR and gel retardation assay to form a stable complex with oligonucleotide sequences comprising the consensus half-site 5′-TGCGG-3′. Apparent μM dissociation constants are evaluated by both methods. The measured lifetime of the complex is 74±7 ms at 20°C with the following DNA sequence: 5′-C1G2T3G4C5G6G7A8T9C10-3′. The major chemical shift variations upon binding involve both the two adjacent GC pairs (G6 and G7) and, clearly, the AT pairs at both ends of the consensus sequence (T3 and A8), suggesting additional contacts of the protein with the DNA. This extensive and strong interaction with the half-site is another example of the variability in contacts of the fungal DNA-binding proteins containing $Zn_2Cys_6$ domains with their consensus sites. It is the first demonstration that a binuclear cluster protein can bind to DNA as a monomer with strong affinity.**



*Key words:* Transcription factor; AlcR; Protein–DNA complex; Exchange rate; NMR

## 1. Introduction

AlcR is a transcription factor from *Aspergillus nidulans* required for the transcriptional activation of the genes encoding the ethanol metabolizing enzymes and of other clustered *alc* genes which functions are unknown [1–3]. The AlcR protein contains at its N-terminus a DNA-binding domain which includes a Cys-$X_2$-Cys-$X_6$-Cys-$X_{16}$-Cys-$X_2$-Cys-$X_6$-Cys motif complexing two $Zn^{2+}$ atoms [4,5]. This $Zn_2Cys_6$ motif is conserved among many fungal transcription factors [4,6]. Contrary to the other known zinc binuclear cluster proteins, AlcR contains between the third and the fourth cysteine an unusual extended sequence of 16 residues instead of 6/9 usually found. Most fungal DNA-binding proteins containing $Zn_2Cys_6$ domains, recognize DNA sites with 5′-CGG-3′ triplet in inverted [7,8] or direct [9] orientation and with a spacing characteristic for each protein. However, AlcR differs also from these proteins since it binds to both symmetrical DNA sequences and direct repeats sharing the consensus half-site, 5′-TGCGG-3′. Each of these specific DNA targets are present in the promoters of the inducible genes such as *alcR* [10], *alcA* [11], *alcS* and *alcM* [3,12]. These targets are functional and are defined as UAS*alc* which are responsible for the transcriptional activation by AlcR of the *alcA* gene [11] and of the *alcR* gene itself [13].

In order to approach these singularities of AlcR at the atomic level, we initiated a NMR study of the interaction of an AlcR peptide comprising the Zn-cluster with an oligonucleotide bearing the 5′-TGCGG-3′ quintet. This peptide, containing the first 60 amino acids of the AlcR protein, was previously used for the identification of AlcR-specific binding sites as a fusion protein GST-AlcR(1–60) [10,11]. In this work, we demonstrate the ability of the AlcR DNA-binding domain to bind to a specific half-site DNA using gel retardation assay and NMR spectroscopy. We have determined the lifetime of the complex and identified the possible contacts between the protein and DNA, both aspects being discussed.

## 2. Materials and methods

### 2.1. DNA-binding test

The GST-AlcR(1–60) fusion protein was constructed by cloning the AlcR DNA-binding domain (corresponding to amino acids 1–60) into pGEX-2T (Pharmacia) 3′ of the glutathione-S-transferase. The linker region of the protein is GST-GlySer-AlcR and three residues were added at the C-terminus. Protein expression in LC137 *Escherichia coli* strain and purification were as described [10,14]. The AlcR peptide was separated from the GST by a cleavage with thrombin (Sigma) (1 U/mg of fusion protein) in presence of $CaCl_2$ 2.5 mM for 10 min at 30°C. The mix was loaded onto a HPLC column resource S (6 ml, Pharmacia) pre-equilibrated in buffer A (10 mM phosphate pH 7.2, 0.1 M NaCl). AlcR was eluted using an increasing gradient of $NH_4Cl$ 1 M, phosphate 10 mM pH 7.2, NaCl 0.1 M. The eluted AlcR peptide was then passed through a *p*-aminobenzamidine column (Sigma) to remove contaminating thrombin and the pH adjusted to 6.0.

Electrophoretic mobility shift assays (EMSAs) were performed with a MiniProteanII apparatus from Bio-Rad. The DNA-binding shift assay was as described by Kulmburg et al. [10] modified as follows: 8% polyacrylamide (30/0.36) gels were run in TBE 0.25X (24.5 mM Tris pH 8.0, 16.15 mM boric acid, 0.62 mM EDTA pH 8.0) at 4°C and 18V/cm for 30 min. A double-stranded oligonucleotide (25-mer) (see legend of Fig. 1) containing a specific half-site for AlcR was end-labelled with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP following recommendations of the manufacturer (Biolabs). The resulting gel was scanned with a phosphoimager and the apparent equilibrium dissociation constant calculated (±20%) as the concentration of protein necessary to complex 50% of the DNA probe.

### 2.2. Sample preparation for NMR study

The sequence of the oligonucleotide corresponds to target b [11] of the *alcA* promoter. To limit the size of the oligonucleotide studied, we used a 10-mer oligonucleotide with the following sequence:

5′d-(C1 G2 T3 G4 C5 G6 G7 A8 T9 C10)-3′
3′d-(G20 C19 A18 C17 G16 C15 C14 T13 A12 G11)-5′

The two strands of DNA decamer were synthesised by phosphoramidite chemistry [15]. The purification was carried out using anion ex-

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*Abbreviations:* AlcR, alcohol regulator of *Aspergillus nidulans*; NMR, nuclear magnetic resonance; $UAS_{alc}$, upstream activating sequence; GST, glutathione-S-transferase; TBE, Tris-boric acid-EDTA; HPLC, high-performance liquid chromatography; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; E-COSY, edited correlation spectroscopy; TSP, trimethylsilyl proponiate; TPPI, time proportional phase incrementation; FID, free induction decay


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change resin (Pharmacia QRH) under denaturing conditions (20 mM NaOH) pH 12.0 [16]. The duplex was prepared by mixing equimolar amounts of each strand. After the purification (see Section 2.1), the AlcR peptide was concentrated to 0.5 ml with a Centriprep 3 (Amicon) and the pH adjusted to 6.0. Finally, 0.05% sodium azide and 5% $D_2O$ were added to the sample. Protein and DNA concentrations were measured by fluorescence [17]. For the titration of the DNA with AlcR, the protein solution was lyophilised and added in aliquots to 0.3 mM duplex DNA in 0.400 ml of buffer (10 mM phosphate, 0.1 M NaCl, pH 6.0). The NMR sample contains a trace of TSP as an internal chemical shift reference.

*2.3. NMR spectroscopy*

Spectra were collected on a Bruker AMX-600 spectrometer. The titration was carried out at 20°C and monitored with 1D spectra. Because of the rapid exchange of imino and amino resonances of the free DNA with water, the water flip-back watergate version [18] was used. 2D exchange spectra [19] were taken midway through the titration (AlcR) : (DNA) ratio = 0.5. A series of 2D exchange spectra was acquired with different mixing times: 25, 50, 75, 100, 125, 150 and 175 ms. The spectra were acquired under the following experimental conditions: 96 summed scans, 2048 real t2 points with a spectral width of 12195.5 Hz, 128 t1 TPPI increments, and a relaxation delay of 2 s between scans. t1 FIDs were linearly predicted to 256 points. The spectra were apodized with appropriate shifted sine-bell functions in both dimensions and zero-filled to 1K along the t1 dimension. All experiments were processed using the GIFA NMR software [20].

*2.4. Determination of the AlcR–DNA complex lifetime*

The AlcR–DNA lifetime could be determined from the 2D exchange spectra taken at a (AlcR : DNA) ratio of 0.5, following the general procedure described by Jeener et al. [19]. Considering the following exchange between the free and bound DNA molecules,

$$\mathrm{AlcR} + \mathrm{DNA_{free}} \underset{k_{bf}}{\overset{k_{fb}}{\rightleftarrows}} \mathrm{AlcR{-}DNA_{bound}}$$

the rate constant $k_{fb}$ and $k_{bf}$ are related to the mole fractions $x_{f(free)}$ and $x_{b(bound)}$ of the species in chemical equilibrium through the following expressions: $k_{fb} = x_b k$, $k_{bf} = x_f k$.

The lifetime of the AlcR–DNA complex can be expressed as $\tau = 1/k$.

The exchange process can be described by the following expressions:

$$V_{bound} = x_b e^{-\sigma\tau_m}\left[\cosh(D\tau_m) - \frac{\delta}{D}\sinh(D\tau_m)\right]$$

$$V_{free} = x_f e^{\sigma\tau_m}\left[\cosh(D\tau_m) + \frac{\delta}{D}\sinh(D\tau_m)\right]$$

$$V_{exchange} = x_b x_f \frac{k}{D} e^{\sigma\tau_m}\sinh(D\tau_m)$$

$$\sigma = \tfrac{1}{2}(R_b + R_f);\ \delta = \tfrac{1}{2}(R_b - R_f);\ D = \sqrt{\delta^2 + x_b x_f k^2}$$

where $V_{bound}$, $V_{free}$ and $V_{exchange}$ represent the volumes of the bound, free and exchange peaks, $R_{b(bound)}$ and $R_{f(free)}$, the relaxation rate of the two forms and $\tau_m$, the mixing time.

A least-square fitting procedures was used to extract the $R_f$, $R_b$ and k parameters corresponding to the best fit of the theoretical decay–building curves to the measured data, using the MATLAB software.

## 3. Results

*3.1. Binding of AlcR(1–60) to a specific half-site*

Prior to the NMR experiments, we wanted to assess whether AlcR(1–60) was able to bind in vitro to a specific DNA half-site. A 25-mer oligonucleotide with the consensus core 5′-TGCGG-3′ and the flanking bases from the palindromic target of the *alcA* promoter was used in a gel shift assay. Increasing concentrations of the purified AlcR peptide were incubated with the labelled 25-mer oligonucleotide as depicted in Fig. 1. We clearly see that one complex is formed,

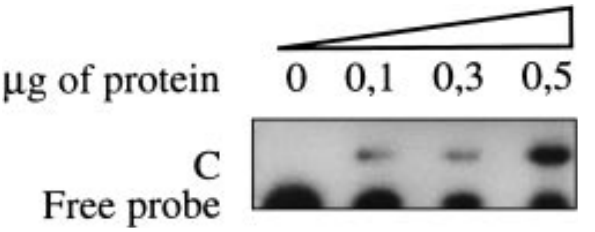


Fig. 1. DNA-binding ability of AlcR(1–60) to a specific half-site. Binding reactions were performed with 32 fmol of a 25-oligonucleotide of the following sequence: 5′-GCCCTCGTGCGGATCCG-TATGCATC-3′ and increasing concentrations of protein, 0.1, 0.3 and 0.5 µg of AlcR(1–60) corresponding to 13, 39 and 65 pmol, respectively.

indicating that one molecule of AlcR binds to its target as a monomer with an apparent dissociation constant of $10^{-6}$ M (±20%).

A binding was also observed with the 10-mer by gel retardation assay but at high concentration of protein, this result is already documented in previous studies in which the size of the oligonucleotide is one of the important parameters for AlcR–DNA complex stability in gel band shift experiments [5,10].

These encouraging data lead us to initiate a NMR study of the AlcR–DNA interaction.

*3.2. DNA chemical shifts analysis in the AlcR–DNA complex*

We have first assigned the free DNA using well-documented procedures [21–24] combining NOESY and TOCSY spectra in $H_2O$ and $D_2O$. The proximities extracted from NOESY experiments and coupling constants measured from an E-COSY spectrum were consistent with a right-handed B-DNA helix. The assignment of the proton resonances for the free DNA 10-mer are summarized in Table 1.

Chemical shifts of the DNA imino protons were monitored by titrating the DNA with increasing concentrations of the AlcR(1–60) peptide. The components of the AlcR–DNA complex were in slow exchange at the NMR chemical shift time scale as shown in Fig. 2 by the appearance of an extra set of resonances with increasing amounts of protein. The lifetime of the complex is, thus, longer than the inverse of the frequency difference between the free and bound state resonances. A similar titration of GAL4 with its half-site DNA has been recently published [25] but, contrary to the present case, the affinity constant was low and the lifetime of the complex was short since the complex was shown to be in fast equilibrium with the dissociated species at the chemical shift time scale. With AlcR, a 2D exchange experiment demonstrated that the imino protons were in intermediate exchange (at the T1 relaxation time scale) and allowed the straightforward assignment of the imino protons of the bound DNA from the assignment of the free DNA resonances. Most imino resonance chemical shifts change (Table 2) but the largest changes occur for the T3, G6, G7 and T13 imino resonances: 0.14, 0.36, −0.27 and 0.12 ppm, respectively. The largest chemical shift variations involve two adjacent GC pairs in the consensus sequence (G6 and G7). These imino protons of equivalent GC pairs also experienced the higher chemical shift variations in the GAL4 complex [25]. Furthermore, these GC pairs were shown to interact directly with GAL4 [26] and PPR1 [27] by crystallography studies of the protein–DNA complexes. It is striking

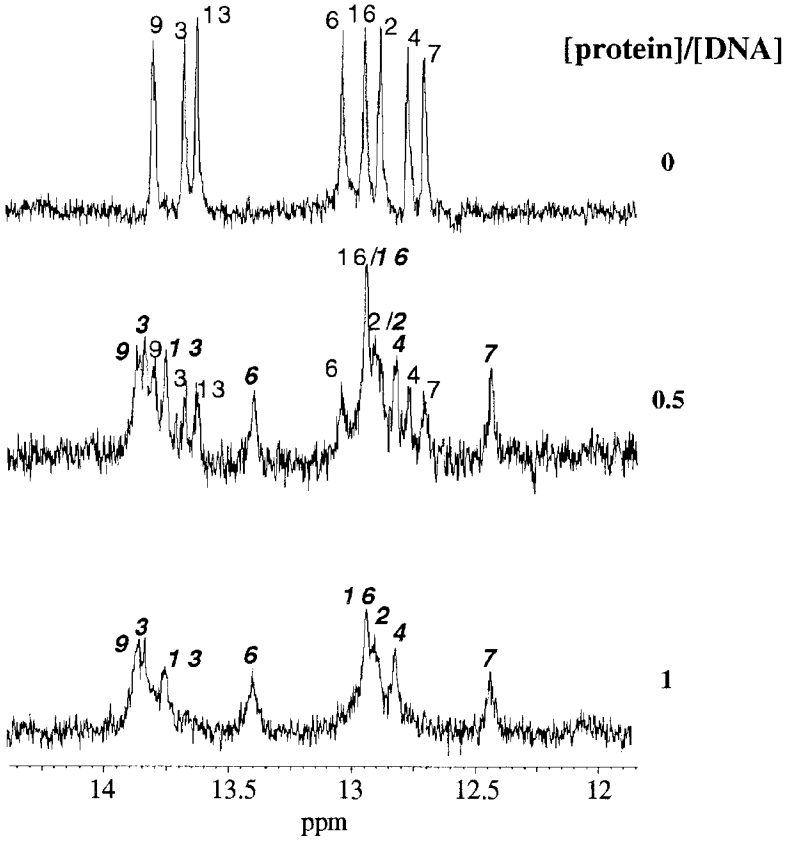


Fig. 2. Spectral changes in DNA imino resonances upon addition of AlcR protein. 1D $^1$H NMR spectra were shown between 12 and 14.25 ppm. Numbered resonances indicate the assignment of imino protons to each nucleotide of the DNA. In bold-italic are indicated the imino resonances of the bound form. Aliquots of AlcR(1–60) were added to the half-site DNA giving molar protein : DNA ratios. Buffer conditions are 10 mM phosphate, 0.1 M NaCl, pH 6.0 at 20°C.

to see that, beside the adjacent GC pairs of the consensus sequence, the largest chemical shift variation involves the nearby AT pair at both ends of the recognition site (T3 and T13, respectively). The large chemical shift variation of the imino proton at position 3 is specific for AlcR and was not described for the other proteins of the $C_6$ class. It belongs to the consensus DNA half-binding-site as established from gel band shift assays, footprinting experiments [10,11] and checked in vivo by the functionality of these binding sites [13]. Interestingly, this T3 could be changed only into an A without affecting the activity of the target (F. Lenouvel, I. Nikolaev and B. Felenbok, unpublished results). Taken to-

Table 1
$^1$H NMR chemical shifts[a] (ppm) for the free AlcR half-binding-site DNA at 20°C (pH 6.0)

| Position | Base | H8/H6 | H5 | H1′ | H2″ | H2′ | H3′ | H4′ | H5′-5″ | CH3 | NH | H4(1)C | H4(2)C | H2A | H6(1)A | H6(2)A |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C | 7.71 | 5.94 | 5.80 | 2.48 | 2.09 | 4.75 | 4.09 | 3.75 | | | 7.10 | 8.09 | | | |
| 2 | G | 8.03 | | 6.04 | 2.84 | 2.72 | 5.01 | 4.41 | 4.15/4.05 | | 12.91 | | | | | |
| 3 | T | 7.23 | | 5.84 | 2.50 | 2.13 | 5.09 | 4.91 | 4.35/4.23 | 1.51 | 13.71 | | | | | |
| 4 | G | 7.91 | | 5.88 | 2.72 | 2.64 | 5.01 | 4.39 | 4.39/4.13 | | 12.78 | | | | | |
| 5 | C | 7.25 | 5.32 | 5.64 | 2.30 | 1.85 | 4.83 | | | | | 6.44 | 8.36 | | | |
| 6 | G | 7.83 | | 5.54 | 2.74 | 2.66 | 4.99 | | | | 13.05 | | | | | |
| 7 | G | 7.77 | | 5.70 | 2.78 | 2.66 | 5.03 | 4.43 | 4.31/4.17 | | 12.71 | | | | | |
| 8 | A | 8.18 | | 6.28 | 2.94 | 2.62 | 5.01 | 4.47 | 4.25 | | | | | 7.89 | 6.02 | 7.63 |
| 9 | T | 7.19 | | 6.04 | 2.48 | 2.01 | 4.85 | | | 1.37 | 13.83 | | | | | |
| 10 | C | 7.59 | 5.66 | 6.28 | 2.28 | | 4.59 | | 4.21/4.03 | | | 7.05 | 8.24 | | | |
| 11 | G | 7.95 | | 5.70 | 2.80 | 2.64 | 4.89 | | | | 12.63 | | | 8.03 | 6.32 | 7.73 |
| 12 | A | 8.36 | | 6.38 | 3.04 | 2.78 | 5.09 | 4.53 | | | | | | | | |
| 13 | T | 7.23 | | 6.00 | 2.54 | 2.09 | | | | 1.37 | 13.65 | | | | | |
| 14 | C | 7.55 | 5.58 | 6.02 | 2.48 | 2.13 | 4.89 | 4.23 | 4.13 | | | 6.69 | 8.30 | | | |
| 15 | C | 7.45 | 5.60 | 5.63 | 2.40 | 2.07 | | | | | | 6.85 | 8.56 | | | |
| 16 | G | 7.91 | | 5.88 | 2.74 | 2.66 | | | | | 12.95 | | | | | |
| 17 | C | 7.37 | 5.44 | 5.56 | 2.38 | 2.03 | 5.02 | 4.17 | | | | 6.46 | 8.38 | | | |
| 18 | A | 8.30 | | 6.22 | 2.88 | 2.72 | 5.05 | 4.41 | 4.15 | | | | | 7.80 | | |
| 19 | C | 7.29 | 5.38 | 5.66 | 2.30 | 1.87 | | 4.25 | 4.13 | | | 6.79 | 8.38 | | | |
| 20 | G | 7.93 | | 6.16 | 2.60 | 2.36 | 4.69 | | | | | | | | | |

[a]Chemical shifts ±0.02 ppm were measured relative to TSP at 0 ppm.

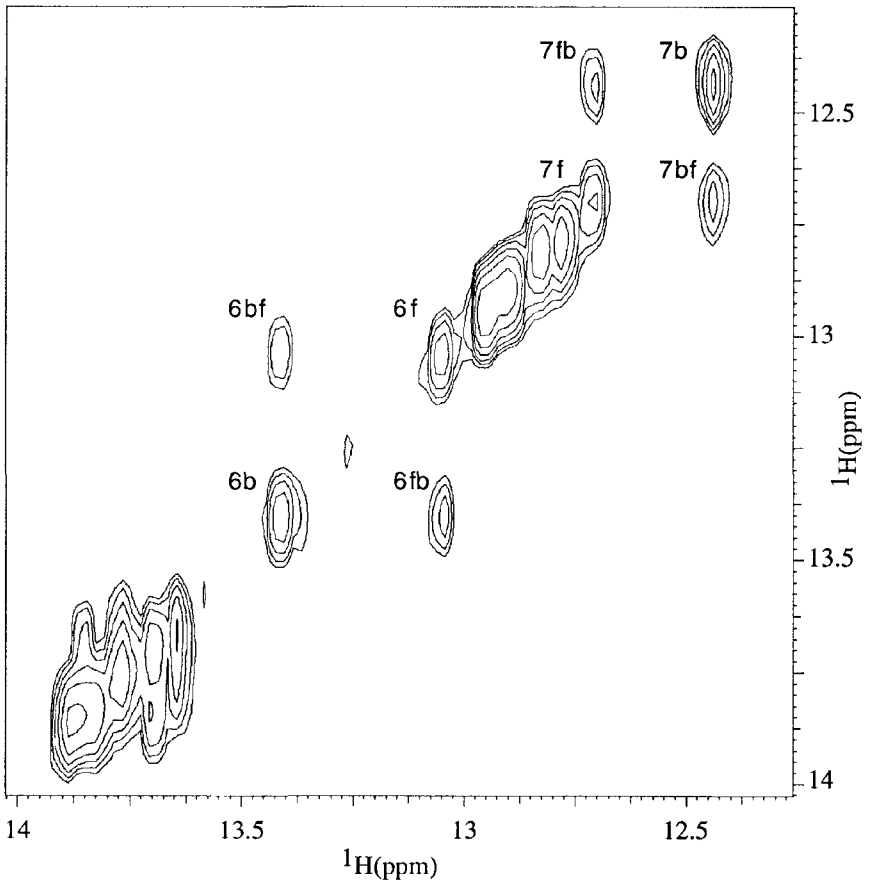


Fig. 3. 10-Mer imino region of the 2D exchange spectrum, pH 6 at 20°C. Auto (b, bound; f, free) and exchange (bf, fb) peaks of the guanine 6 and 7 are indicated. The mixing time was 100 ms.

gether, these molecular, physiological and structural data are in agreement and show that T or A at position 3 is essential for AlcR's contact. The other important chemical shift variation observed is outside the consensus core and involves the AT pair at position T13. These residues are part of the spacer region comprising two nucleotides, separating symmetrical sites found in several *alc* gene promoters, *alcR*, *alcA* [10,11], *alcM* and *alcS* [3,12]. All these spacer regions share the same consensus pair of nucleotides 5′-HT-3′ (H being a C, A or T), upstream the 5′-CCGCA-3′ sequence in which the second base is always a T (here T13). In methylation interference footprints, we have previously shown that the non-conserved base (H) was hypersensitive [11], indicating that it has no specific contacts with the AlcR peptide whereas the conservation of the T residue is in accordance with the suggestion that AlcR needs additional contacts with the DNA to get its optimal binding (I. Nikolaev, F. Lenouvel and B. Felenbok, unpublished results). Moreover, we have observed amino chemical shifts variations for cytosine 14, 15 and 17. The higher variations involve both, hydrogen-bounded/non-hydrogen-bounded amino protons of C15 with changes of 0.79 and 0.91 ppm, respectively. The hydrogen-bounded amino proton chemical shift of C14 changes by −0.32 ppm, the non-hydrogen-bounded amino proton in the bound state could not be detected. Finally, the amino protons of C17 change less significantly (0.10 and 0.02 ppm for the hydrogen-bounded and non-hydrogen-bounded, respectively). In the GAL4-half-site–DNA complex, the chemical shifts of the equivalent amino protons of C15 (C18) and C14 (C17) show also the largest variations. The crystal structure of the GAL4–DNA complex indicates that the C18 and C17 amino protons are directly involved in hydrogen bounds with the carbonyl of two lysine residues. Similar interactions are, thus, also expected with AlcR.

Table 2
Imino chemical shifts of the bound 10-mer (δ(NH)) and chemical shift variations between the bound and free forms (Δ(NH))

| Base | δ(NH) | Δ(NH) |
|---|---|---|
| G2 | 12.91 | 0.00 |
| T3 | 13.85 | 0.14 |
| G4 | 12.83 | 0.05 |
| G6 | 13.41 | 0.36 |
| G7 | 12.44 | −0.27 |
| T9 | 13.89 | 0.06 |
| T13 | 13.77 | 0.12 |
| G16 | 12.95 | 0.00 |

### 3.3. Lifetime of the complex

Exchange rates were measured from two different pairs of imino protons, those of guanines 6 and 7. These resonances are well-separated in the free and bound forms and do not overlap with others. The diagonal and cross-peak volumes of guanines 6 and 7 were measured at the seven different mixing times. Fig. 3 shows an extract of the 2D exchange spectrum at 100 ms. The exchange rate constant was derived from a least-

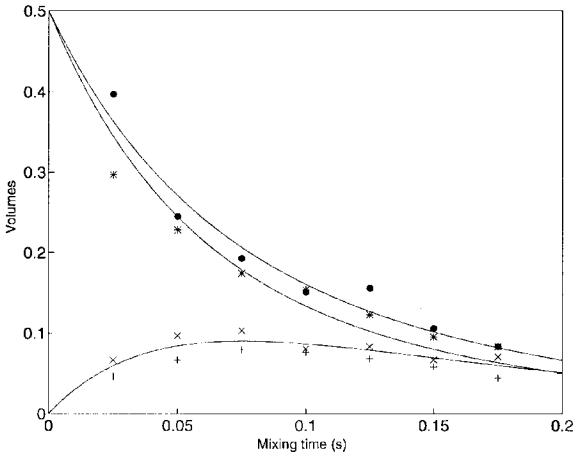


Fig. 4. Experimental data and fitted curves for the G7 imino resonances. Symbols are used to indicate experimental data points for the auto (●, ∗) and exchange (+, ×) peaks.

square fit of diagonal and cross-peak signal volumes against $\tau_m$ (Fig. 4). Two independent values of the exchange rate constant were calculated for the imino resonances of guanines 6 and 7 and found to be $13.8 \pm 1.4$ and $13.6 \pm 1.3$ $s^{-1}$, respectively, the experimental error in each volume measurement being estimated to $\approx 10\%$. Thus, the lifetime of the complex (1/k) is $74 \pm 7$ ms at 20°C, a value in agreement with a slow exchange at the NMR chemical shift time scale and a low dissociation constant. Assuming a diffusion-controlled reaction, this would lead to an equilibrium dissociation constant in the μM range, compatible with the value evaluated from the gel retardation assay. The fact that no free DNA is detectable by NMR at a (protein : DNA) ratio of 1.5 leads to a similar evaluation.

## 4. Discussion

Our results demonstrate the original binding of AlcR to its specific target as compared to the other zinc clusters of the $C_6$ family. First, we show that AlcR(1–60) binds to its half-site DNA with a strong affinity and with a long residence time in the complex as opposed to GAL4 [25]. The evaluated μM equilibrium dissociation constant is two orders of magnitude smaller than for GAL4, this is the first demonstration that a binuclear cluster can bind to DNA as a monomer with strong affinity.

One should clearly keep in mind that the present analysis was performed on the biologically significant Zn complex and not on the Cd complex. Mau et al. [28] have showed that some amide protons exchange 3–21× faster in Cd-GAL4 than in Zn-GAL4 and suggested that cadmium substitution is likely to cause a reduction of the global stability of the protein and of the recognition module. Rodgers et al. [29] showed that the Zn for Cd substitution results in relatively modest changes (2–3-fold decrease) in the affinity of a fragment of GAL4, comprising both the DNA-binding domain and an additional subdomain (GAL4(149*)), for its specific DNA sequence. However, the influence of the metal ion on the association constant of shorter fragments remains to be fully elucidated. We feel that the main reason for the stronger affinity and longer residence time in the complex observed, is more likely to be due to the specific interactions seen by NMR. The AlcR consensus site is longer than the other consensus sequences of the same family. The additional chemical shift variations, as compared to GAL4 [25], which were observed after complexation of the DNA site by AlcR demonstrate the more extensive interactions with the half-site. Several 3D structures of $Zn_2Cys_6$ DNA-binding domain, GAL4 [26,30–32], PPR1 [27], LAC9 [6] and, very recently, CYP1(HAP1) [33], and of two protein–DNA [26,27] complexes are known. These proteins bind to the same consensus site 5′-CGG-3′. Moreover, the specificity of interaction seems to be due to a fine tune between the linker which connects the zinc-cluster to a dimerization helix and the number of base pairs separating the two CGGs rather than direct interactions between the zinc cluster and the DNA. In our case, there is no evidence for such a dimerization element in close proximity that could play the same role. The binding domain itself would be responsible for the interaction specificity. This would then explain why AlcR is able to bind with a strong affinity to direct repeats as well as symmetrical sequences. This study provides a first insight into a comprehensive interaction of AlcR peptide with its consensus site. The 3D structure determinations of AlcR(1–60) and of its complex with several target DNA sequences are in progress in our laboratory using a $^{15}N$-labelled protein and will probably shed further light into the recognition mechanisms.

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