Chloride binding by the AML1/Runx1 transcription factor studied by NMR

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Abstract It is known that the DNA binding Runt domain of the AML1/Runx1 transcription factor coordinates Cl^- ions. In this paper we have determined Cl^- binding affinities of AML1 by ^{35}Cl nuclear magnetic resonance (NMR) linewidth analysis. The Runt domain binds Cl^- with a dissociation constant ($K_{d,Cl}$) of 34 mM. If CBFβ is added to form a 1:1 complex, the $K_{d,Cl}$ value increases to 56 mM. Homology modeling suggests that a high occupancy Cl^- binding site overlaps with the DNA binding surface. NMR data show that DNA displaces this Cl^- ion. Possible biological roles of Cl^- binding are discussed based on these findings.

Key words: AML1; Runt domain; ^{35}Cl; Nuclear magnetic resonance spectroscopy; Anion binding to protein

1. Introduction

Runx1, also termed AML1, CBFa2 or PEBP2αB, is together with runx2 and runx3 the mammalian members of the Runt family of heterodimeric transcription factors. All runx proteins share the Runt domain [1], a 128 amino acids long evolutionary conserved segment that is responsible for both DNA binding and heterodimerization with CBFβ. CBFβ acts to stabilize the DNA-bound state of the Runt protein without contacting DNA [2]. Both nuclear magnetic resonance (NMR) [3] and X-ray crystallography ([4]; Bäckström et al., unpublished) structures of the Runt domain have been determined and they reveal that the fold is Ig like. The Runt domain shares structural homology with the DNA binding domains of the NFκB, NFAT, p53 and STAT families, all of which contain the Ig fold. Runt proteins are involved in a diverse set of biological functions and human diseases. Biological functions in mammals that are dependent on Runt proteins are for instance osteogenesis [5] and definitive hematopoiesis [6,7]. The inherited human diseases cleidocranial dysplasia and familial platelet disorder with propensity to acute myeloid leukemia and many cases of acute lymphatic leukemia and acute myeloid leukemia have been linked to Runt proteins [8,9]. There exists experimental evidence that CBFβ is required for the function of at least one Runt protein in vivo [10].

We have found that amino acids 46–185 of AML1 with a C72S, C81S double mutation ($Runt_{185}$) coordinate a Cl^- ion with high occupancy to one of the putative DNA binding loops (Fig. 1) (Bäckström et al. unpublished). Also Cl^- ions with lower occupancies were found. The high occupancy Cl^- is coordinated by the N^{ε} and $N^{\eta 2}$ nitrogens of R139, the mainchain nitrogen of V170 and the oxygen atoms of one or two water molecules R139 and V170 are strictly conserved throughout species in Runt proteins. Given that Cl^- binds to the Runt domain in a region that presumably is involved in DNA binding we decided to investigate this feature in detail with NMR spectroscopy. In this paper we have characterized the Cl^- binding to $Runt_{185}$ in its free state and when complexed with DNA or amino acids 1–141 of CBFβ ($CBF\beta_{141}$) with NMR ^{35}Cl line-broadening measurements. We also address the question whether Cl^- regulates the DNA binding of Runt proteins.

2. Materials and methods

2.1. Protein purification and NMR sample preparation

$Runt_{185}$ was purified following the method outlined in [11]. Expression of $CBF\beta_{141}$ was made following the same protocol as for $Runt_{185}$ except that a plasmid carrying the *dnaY* gene [12] was co-transformed into the *Escherichia coli* BL21(DE3) cells. Recombinant $CBF\beta_{141}$ is produced at high levels and is completely soluble. Purification of $CBF\beta_{141}$ was accomplished using Ni/nitrilotriacetic acid agarose, DEAE Sepharose Cl-6B (Pharmacia Biotech) and Superdex 75 gel filtration (Pharmacia Biotech) columns. NMR samples were prepared by an additional gel filtration in the appropriate NMR buffer.

2.2. NMR spectroscopy

NMR spectra were acquired at 600 MHz proton frequency (58.8 MHz ^{35}Cl frequency) on a Bruker AVANCE spectrometer. The spectrometer was equipped with three rf-channels, pulsed field gradients and a 5 mm broad band (^1H/BB) probe. One-dimensional ^{35}Cl spectra were acquired with 512 data points, 340–5000 scans, a spectral width of 1200 Hz and a relaxation delay of 50 ms. NMR spectra were analyzed with XWIN-NMR.

2.3. Line-broadening assay

The desired quantity in the line-broadening assay is the ^{35}Cl excess transverse relaxation rate ($R_{2,e}$). $R_{2,e}$ is defined as the observed R_2 minus the R_2 of free Cl^-, where R_2 is equal to π multiplied with

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Abbreviations: $CBF\beta_{141}$, amino acids 1–141 of CBFβ; FWHH, full width at half height of an NMR signal; $K_{a,Cl}$, Cl^- association constant of a protein; $K_{d,Cl}$, Cl^- dissociation constant of a protein; NMR, nuclear magnetic resonance; $\Sigma_j P_j R_{2,j}$, low affinity binding sites in the Cl^- binding model; $R_{2,e}$, ^{35}Cl excess transverse relaxation rate; $R_{2,i}$, ^{35}Cl intrinsic transverse relaxation rate at chloride binding site i; $Runt_{185}$, amino acids 46–185 of AML1 with a C72S, C81S double mutation

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the full width at half height of the NMR signal (FWHH). Titration experiments were done by adding aliquots from 2 or 4 M NaCl stock solutions to samples containing the desired protein, DNA or complexes thereof. At each experimental point a one-dimensional ^{35}Cl spectra was acquired and the FWHH was measured. Linear and non-linear curve fitting was accomplished with the program Origin.

3. Results

3.1. Model of a $Runt_{185}$/DNA complex

Structural homologs of $Runt_{185}$ were identified with the DALI server [13]. One strong structural homolog is STAT-1. The X-ray structure of STAT-1 has been solved in complex with an 18 bp duplex DNA [14]. Superposition of the C^{α} trace of 55 amino acids around the A-B and E-F strands of $Runt_{185}$ (nomenclature according to [3]) and the corresponding residues in STAT-1 rendered an RMSD of 2.28 Å. Fig. 1 shows $Runt_{185}$ superimposed on STAT-1. Only the DNA of STAT-1 is shown. In the model, R80 of Runt appears to interact with the major groove while R139 and R142 interact with the minor groove of the DNA. These three residues are completely conserved in Runt domain containing proteins and their importance in DNA binding and disease have been shown by mutant analysis [15,16]. These facts support the model. The Cl^- ion coordinated by R139 and V170 completely overlaps with a DNA phosphate group in our model (Fig. 1).

3.2. Cl^- binding model and justification of assumptions

It is possible to determine the Cl^- dissociation constant for a protein ($K_{d,Cl}$) with ^{35}Cl line-broadening measurements. A simplified model for Cl^- binding can be used provided that the following assumptions can be justified: (i) the exchange process is fast on the NMR timescale, (ii) the difference in chemical shift between free and bound Cl^- is small, (iii) the amount of free Cl^-can be approximated with the total amount, and (iv) the lineshapes of the broadened lines are Lorentzian. $R_{2,e}$ may under these circumstances be expressed by a two site model [17] (Eq. 1) which contains both strong and weak Cl^- binding sites:

$$R_{2,e} = \frac{n_i C_{pr} K_{a,Cl} R_{2,i}}{1 + K_{a,Cl} C_{Cl}} + \sum_j P_j R_{2,j} \quad (1)$$

where n_i is the number of strong Cl^- binding sites (n_i is equal to unity in $Runt_{185}$ as deduced from the crystal structure), C_{pr} is the total protein or protein-complex concentration, $K_{a,Cl}$ is the association constant for Cl^- binding, $R_{2,i}$ is the ^{35}Cl intrinsic relaxation rate at site i, C_{Cl} is the total concentration of Cl^-. $\Sigma_j P_j R_{2,j}$ corresponds to any low affinity binding sites and gives rise to an apparent constant contribution to $R_{2,e}$. It is known that a plot of $\ln(R_{2,e})$ versus T^{-1} is first order with a positive slope for exchange processes in the fast regime [18]. Fig. 2 shows this plot for a $Runt_{185}$ sample. The experimental data could be fitted to a straight line with positive slope, hence we conclude that the exchange process is fast. We could not detect any change in ^{35}Cl resonance frequency during the

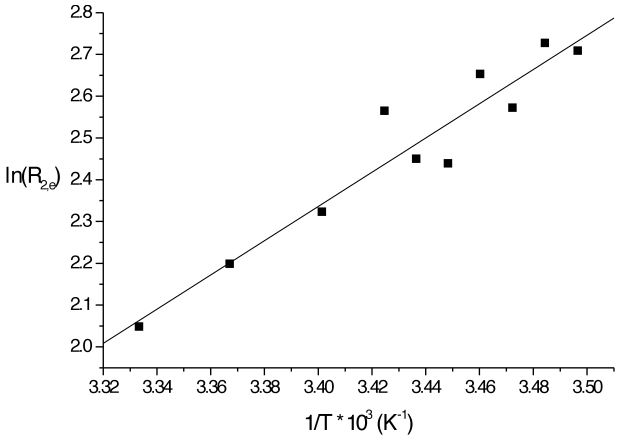

Fig. 2. Natural logarithm of $R_{2,e}$ plotted against the inverse absolute temperature. The sample contained 28 μM $Runt_{185}$ with 10 mM NaCl and 3 mM sodium phosphate at pH 6.8. The relaxation rate was fitted to a straight line using the least-squares method.

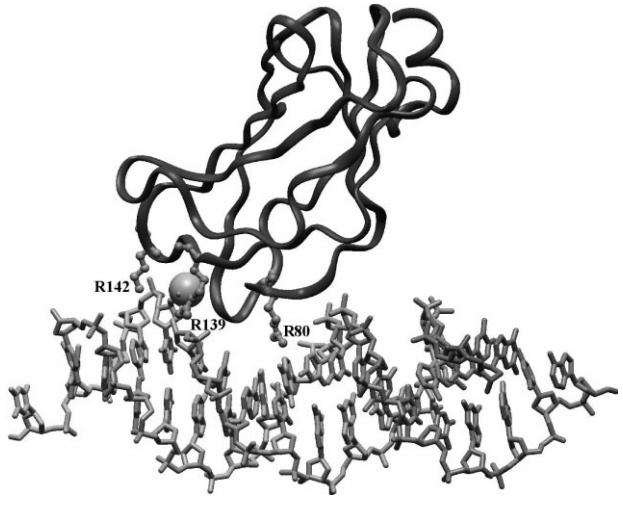

Fig. 1. Model of a $Runt_{185}$/DNA complex. The side chains of R80, R139 and R142 are shown in a ball and stick representation. The Cl^- ion coordinated by V170, R139 and one or two water molecules is represented as a gray sphere.

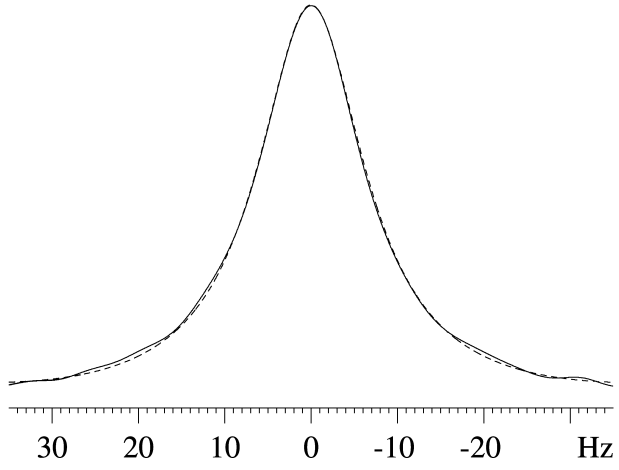

Fig. 3. Fit of a Lorentzian lineshape with a FWHH of 14.7 Hz (dotted line) to the observed ^{35}Cl resonance in one of the samples in a titration of NaCl to $Runt_{185}$ (solid line). The sample contained 31 μM $Runt_{185}$, 24 mM NaCl with 3 mM sodium phosphate at pH 6.8 and the spectra was acquired at 291 K.

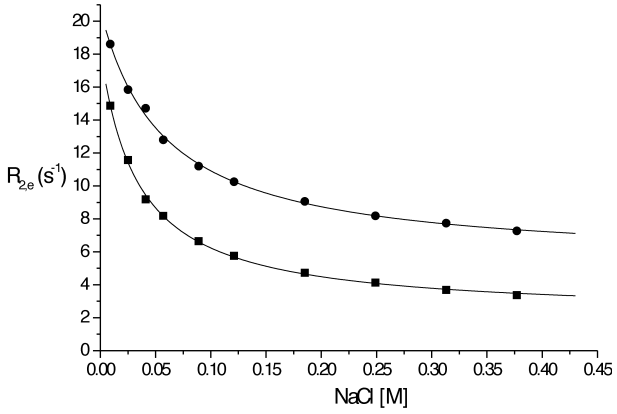

Fig. 4. $R_{2,e}$ from two binding experiments plotted as a function of the NaCl concentration. Squares correspond to 31 μM $Runt_{185}$. Circles correspond to a 29 μM 1:1 $Runt_{185}$/$CBF\beta_{141}$ complex. Both samples contained 3 mM sodium phosphate at pH 6.80. NMR data were acquired at 291 K. Solid lines are $R_{2,e}$ values calculated with the fitted parameters (Table 1 and Eq. 1).

titrations within our experimental accuracy showing that the chemical shift difference between free and bound Cl^- is small. The total amounts of Cl^- (mM range) in the experiments are much larger than the protein concentrations (μM range). The fraction bound Cl^- is thereby low and free Cl^- concentration can be approximated with the total concentration. All NMR spectra in our study have resonances with Lorentzian lineshapes (Fig. 3) which is a prerequisite for fast exchange. To determine if the $\Sigma_j P_j R_{2,j}$ parameter gives a statistically improved fit we carried out F tests [19]. In these tests we compared fits of $R_{2,e}$ to models with and without $\Sigma_j P_j R_{2,j}$. It turns out that the model including $\Sigma_j P_j R_{2,j}$ was significantly improved at the 99% level in all titrations. Our conclusion following the reasoning above is that Eq. 1 is valid for analysis of $R_{2,e}$.

3.3. Cl^- binding by $Runt_{185}$

The experimental results and the curve fitted to Eq. 1 of a NaCl titration to $Runt_{185}$ is shown in Fig. 4. All fitted parameters and the χ^2 value are summarized in Table 1. The quality of the fit is good based on the low χ^2 value. The dissociation constant for Cl^- binding to $Runt_{185}$ is in the order of 34 mM. $R_{2,i}$ is about 18 000 (s^{-1}). Low affinity binding contributes with a constant offset to the binding curve of 2.2 (s^{-1}).

3.4. Cl^- binding by a $Runt_{185}$/DNA complex

It is apparent from our $Runt_{185}$/DNA model (Fig. 1) that there exist steric interference between the Cl^- ion bound to free $Runt_{185}$ and a DNA phosphate atom in the complex. If the model is valid, then $Runt_{185}$ should be disabled to bind Cl^- in the DNA bound state. To investigate this relationship

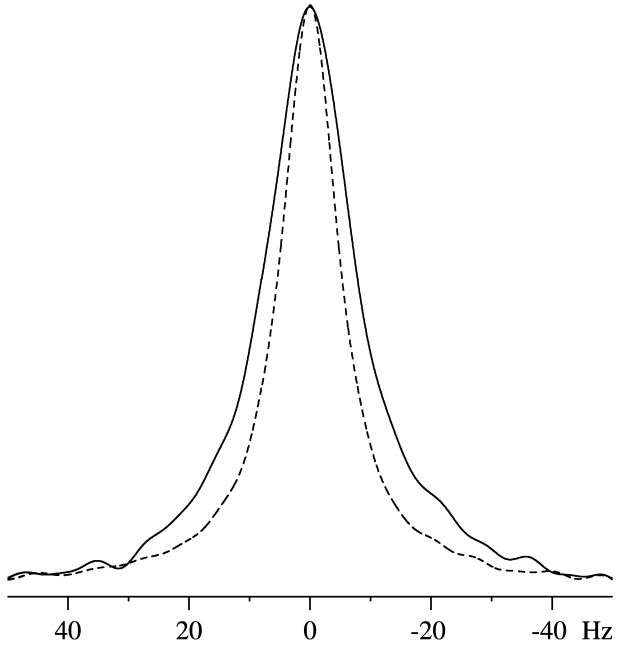

Fig. 5. ^{35}Cl NMR signals acquired at 291 K showing the decrease in linewidth upon DNA binding by $Runt_{185}$. The solid line corresponds to a sample containing 20 μM $Runt_{185}$ and the dashed line corresponds to a 1:1.5 $Runt_{185}$/DNA mixture containing 20 μM protein and 30 μM DNA. Both samples contain NaCl at 10 mM.

we acquired four ^{35}Cl spectra each containing 10 mM NaCl; the first on a reference NaCl sample; the second on 20 μM free $Runt_{185}$; the third after addition of a 14 bp DNA oligomer [11] to a concentration of 30 μM; and a fourth spectrum was acquired on a sample containing 30 μM of the free DNA. The reference ^{35}Cl FWHH was measured to 11 Hz. Free $Runt_{185}$ has a ^{35}Cl FWHH of 17 Hz. Addition of DNA to $Runt_{185}$ induced a decrease in the ^{35}Cl FWHH to 11 Hz (Fig. 5). Also the sample containing free DNA had a FWHH of 11 Hz.

3.5. Cl^- binding by a $Runt_{185}$/$CBF\beta_{141}$ complex

To examine whether CBFβ can modulate the Cl^- binding by Runt we measured $R_{2,e}$ of a 1:1 $Runt_{185}$/$CBF\beta_{141}$ complex as a function of NaCl concentration. Fig. 4 shows the data and the fit of Eq. 1 which is also summarized in Table 1. The $K_{d,Cl}$ of the complex is 56 mM, somewhat higher than the $K_{d,Cl}$ for free $Runt_{185}$. The value of $R_{2,i}$ has increased to 30 000 s^{-1} and the contribution from the low affinity sites has increased to 5.3 s^{-1}. In a separate experiment, we measured the NaCl dependence of $R_{2,e}$ of a sample containing 60 μM $CBF\beta_{141}$ The only effect seen in this experiment was a constant offset in $R_{2,e}$ of 6.4 s^{-1}.

Table 1
Results of non-linear fitting of $R_{2,e}$ to the binding parameters in Eq. 1

	High affinity sites			Low affinity sites	
	$K_{a,Cl}$ (M^{-1})	$K_{d,Cl}$ (mM)	$R_{2,i}$ (s^{-1})	$\Sigma_j P_j R_{2,j}$ (s^{-1})	χ^{2a}
$Runt_{185}$	29	34	18 000	2.2	0.12
$Runt_{185}$/$CBF\beta_{141}$	18	56	30 000	5.3	0.31

[a]The χ^2 values are defined as $\chi^2 = \sigma_i(x_i - x_f)^2$ where x_i are measured values and x_f are fitted values.

4. Discussion

In this paper we have determined $K_{d,Cl}$ values of the Runt domain of the AML1 transcription factor. Cl^- binding by $Runt_{185}$ can be described with a two site model containing low and high affinity binding sites. We have obtained a $K_{d,Cl}$ of 34 mM for uncomplexed $Runt_{185}$. In contrast to Runt, $CBF\beta_{141}$ binds Cl^- unspecifically as it only contains low affinity sites judged from the NaCl concentration independent $R_{2,e}$ value of 6.4 s^{-1}. Addition of DNA to $Runt_{185}$ completely abolished the Cl^- binding (Fig. 5). DNA and Cl^- evidently compete for binding sites in the same region of the protein surface.

One Cl^- ion bound with high occupancy was detected in the crystal structure of $Runt_{185}$ (Bäckström et al., unpublished) Our $Runt_{185}$/DNA model is consistent with overlapping binding sites for DNA and the high occupancy ion. The fact that association with DNA removes high affinity Cl^- suggests that the high affinity binding in solution is associated with high occupancy in the crystal structure, as expected. The low affinity binding class may then be associated with the lower-occupancy sites as well as with other, even weaker, binding sites not detected in the crystal structure.

One interesting question is whether $CBF\beta_{141}$ affects the strength of the Cl^- binding by $Runt_{185}$. NMR measurements on a $Runt_{185}$/$CBF\beta_{141}$ complex reveal a $K_{d,Cl}$ value of 56 mM. As $CBF\beta_{141}$ only binds Cl^- weakly we draw the conclusion that the only strong site in the complex is the $Runt_{185}$ site. Cl^- binding by $Runt_{185}$ is obviously about 1.6 times weaker in the heterodimeric complex compared to the free state. $R_{2,i}$ increases from 18 000 s^{-1} in free $Runt_{185}$ to 30 000 s^{-1} in the heterodimeric state. The magnitudes of the $R_{2,i}$ values are on the order of expected numbers for a protein-bound Cl^- [18].

From a functional point of view it is necessary to consider the relevance of $K_{d,Cl}$ values in the 30–60 mM range. Band3, an abundant integral membrane protein in human erythrocyte membranes that exchanges interior HCO_3^- for Cl^- ions from outside the membrane, contains Cl^- binding sites with $K_{d,Cl}$ values ranging from 65 to 80 mM [20]. Cytoplasmic and nuclear Cl^- concentrations in human cells are approximately equal [21] and span from about 15 to 60 mM [22,23]. The magnitude of these intracellular Cl^- concentrations and $K_{d,Cl}$ values indicates that the strength of Cl^- binding by AML1 might have functional implications. The possible role of Cl^- binding by Runt is unknown. We speculate that Cl^- might regulate DNA binding of the Runt domain in a negative or positive way. Cl^- might act as a competitive inhibitor to DNA or force the sidechain of R139 to adopt a conformation that is optimal for DNA binding. Another possibility is that Cl^- might affect the negative influences that N- and C-terminal sequences exert on the DNA binding of the Runt domain [11,24]. All scenarios could be consistent with Cl^- being excluded from the DNA bound state. However, our data do not allow distinction between these or any other possible functions for Cl^- binding. Whatever the function of Cl^-, we believe that all Runt domain containing proteins bind Cl^- considering that the binding residues R139 and V170 are strictly conserved.

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