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Identification of the metal coordinating residues in the DNA binding domain of the glucocorticoid receptor by ^{113}Cd-^{1}H heteronuclear NMR spectroscopy

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Two-dimensional ^{1}H-^{113}Cd HSQC and relay HSQC experiments were performed on the ^{113}Cd substituted DNA binding domain of the rat glucocorticoid receptor The results of these experiments combined with sequence-specific assignments allowed the identification of all coordinating cysteines It was found that C495 and not C500 is the fourth coordinating cysteine in the second zinc-finger A signal at ~2 ppm previously assigned to a ε-CH_3 of a methionine residue coordinating to a third, weakly bound, cadmium ion, was identified as the C443 β proton ligating to the metal ion in the first zinc-finger No indications were found for the presence of a previously suggested third metal ion binding site

Glucocorticoid receptor DNA binding domain, ^{113}Cd NMR, Metal coordination, Zinc-finger

1 INTRODUCTION

The glucocorticoid receptor (GR) is a 87 kDa DNA-binding protein mediating the physiological response of glucocorticoid hormones [1,2]. It is a member of the steroid/thyroid superfamily of ligand inducible nuclear transcription factors [3] In the presence of hormone it regulates gene expression by binding to specific DNA sequences The GR consists of several functionally distinct domains [4] The DNA binding domain is the most highly conserved of these segments and consists of 70 residues containing 9 cysteines conserved in the steroid/thyroid superfamily (Fig. 1). The cysteines coordinate two zinc atoms which are indispensable for protein folding and DNA binding [5] similar to the 'zinc-fingers' found in the gene-regulatory proteins of a wide range of organisms [6] Protein fragments spanning 70 residues encompassing the DNA binding domain have been expressed in *E coli* and bind specifically to the same DNA sequences as the intact receptor [5,7] EXAFS studies and UV/visible spectroscopy have shown that each zinc atom is coordinated tetrahedrally by four cysteines, leaving one conserved cysteine free [5] However, it has not been unambiguously established which cysteine residues are involved in metal binding. Severne et al [8] found that C500 could be substituted by serine or alanine without affecting the function of the receptor while mutation of C495 completely inactivated the protein. In contrast, Schena et al [9] concluded from random mutagenesis studies that either substitution of C495 by a tyrosine or of C500 by an arginine led to loss of receptor function In order to resolve this matter we performed ^{113}Cd-^{1}H HSQC and ^{113}Cd-^{1}H HSQC-NOE NMR studies [10,11] on a ^{113}Cd substituted 83 residue protein fragment containing the DNA binding domain C440-R510 of the rat glucocorticoid receptor (GR-DBD) (Fig 1) ^{113}Cd NMR has been widely used in the study of metalloproteins [12,13] and has recently been applied to several types of ^{113}Cd substituted zinc-finger proteins [14–21] The ^{113}Cd-^{1}H heteronuclear coupling constant is used to identify the metal binding ligands while the ^{113}Cd chemical shifts provide information about the coordination state of the metal. ^{113}Cd NMR studies on the DNA binding domain of the glucocorticoid receptor were previously performed [19] However, the absence of a sequential assignment of the protein at the time hampered the interpretation of their results. The coordination scheme derived from the ^{1}H-

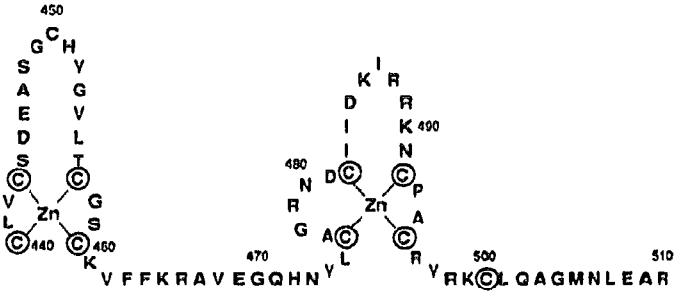

Fig 1. Sequence of the GR-DBD The conserved cysteines have been circled

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^{113}Cd NMR experiments reported here is based on the resonance assignment published earlier [22] and agrees with that suggested by the solution structure of the glucocorticoid receptor [23] and the estrogen receptor [24]

2 MATERIALS AND METHODS

The ^{113}Cd substituted glucocorticoid receptor DNA binding domain was prepared from the native zinc form [5,19] either by removal of the zinc at low pH followed by an increase of the pH and addition of $^{113}CdCl_2$ or by dialysis of the zinc form against a buffer containing 10 μM $^{113}CdCl_2$ Cadmium incorporation was checked by atomic absorption spectroscopy on a Varian Spectra AA-10 spectrometer which showed less than 5% residual zinc DNA-binding activity was assessed by gel mobility shift experiments NMR samples were prepared by dialysis and concentration with an Amicon flow cell against D_2O containing 300 mM NaCl, 0 1 mM NaN_3 and 1 mM DTE to prevent the oxidation of the cysteines and were typically 2–4 mM in protein concentration The pH of the samples was 6 5–7 0 Heteronuclear NMR experiments were performed at 305 K on a Bruker AMX 500 operating at 110 9 MHz for ^{113}Cd with a 5 mm broadband probe For the HSQC experiments a standard pulse sequence with an extended phase cycle on the first 180° pulse on cadmium was used [11] The delay Δ was set to 8 ms In the HSQC-NOE spectrum [11] a 90°(^{1}H)-τ_m- 90°(^{1}H) was added prior to data acquisition with the mixing time τ_m set at 125 ms In the case of the HSQC-HOHAHA experiment [11] an MLEV-17 pulse train of 20 ms was used before collection of the FIDs 36 t_1 increments of 1024 scans were obtained for the HSQC experiment which took 16 h to record 34 t_1 increments of 4608 scans were collected for the HSQC-NOE resulting in a total measuring time of 60 h For the HSQC-HOHAHA experiment, 34 t_1 increments of 1280 scans were collected This measurement took 20 h The sweep width was 2000 Hz for ^{113}Cd and 6250 Hz for ^{1}H The ^{113}Cd chemical shifts are reported relative to 1 M $CdSO_4$ The residual HDO resonance was suppressed by presaturation Spectra were processed using the 'TRITON' NMR software package developed at the Department of Chemistry, Utrecht University

3 RESULTS AND DISCUSSION

Fig 2 shows the ^{1}H detected ^{113}Cd-^{1}H HSQC spectrum [10,11] of the ^{113}Cd substituted glucocorticoid receptor DNA binding domain displaying the correlation between the two ^{113}Cd resonances in ω_1 and the ^{1}H chemical shifts belonging to the β protons of the coordinating cysteines in ω_2 The ^{113}Cd chemical shifts of 712 and 716 ppm relative to 1 M $CdSO_4$ are in good agreement with the values of 704 and 710 ppm relative to 1 M $Cd(ClO_4)_2$ reported by Pan et al [19], the slight difference probably being caused by the use of different

Table I

^{1}H NMR chemical shift of the cysteines in Zn(II) and Cd(II) GR-DBD at pH 6 5[a]

	Cd(II)			Zn(II)		
	$C^\alpha H$	$C^\beta H(1)$	$C^\beta(2)$	$C^\alpha H$	$C^\beta H(1)$	$C^\beta H(2)$
First finger						
C440	4 00	3 22	2 75	4 02	3 21	2 73
C443	5 20	2 84	2 10	5 05	2 80	1 96
C457	5 27	3 68	2 96	5 14	3 50	2 79
C460	4 06	3 17	3 17	4 07	3 05	3 05
Second finger						
C476	4 45	2 84	2 84	4 24	2 81	2 79
C482	4 02	3 00	2 61	3 91	2 97	2 51
C492	4 95	2 96	2 79	4 86	2 90	2 70
C495	4 23	3 00	2 90	4 13	2 94	2 73
C500	4 02	3 04	3 04	3 94	3 06	2 97

[a]The chemical shift of Cd(II) GR-DBD was obtained at 305 K while the chemical shift of Zn(II) GR-DBD was taken at 300 K [22]

reference compounds The chemical shifts are typical for cadmium(II) coordinated by four sulphur ligands [12]. The assignments of the cysteine residues in the ^{113}Cd substituted protein (Table I) were made by comparison of the chemical shifts and NOE patterns of the cadmium substituted protein with those found in the native zinc form [22] Identification of the β protons of the cysteine residues allowed us to assign the ^{113}Cd resonance at 712 ppm to the metal ion in the first metal binding site while the resonance at 716 ppm originates from the second site.

Similar to the DNA-binding domain of GAL4 [15], no large shifts in either cysteine β or α protons were noted upon substitution of Zn(II) by Cd(II) (Table I), indicating that substitution of Zn(II) by Cd(II) does not result in major conformational changes. A downfield shift, however, occurred at the T456 α proton which shifted from its already extremely low value of 6 25 ppm to 6 40 ppm. This indicates that the shift to low field of this proton is caused by its proximity to the metal ion The proton resonance at about 2 ppm was previously attributed to the ε-CH_3 group of methionine-505 on the basis of its chemical shift [19] and coordination of the methionine thioether sulfur to a *third* metal ion was

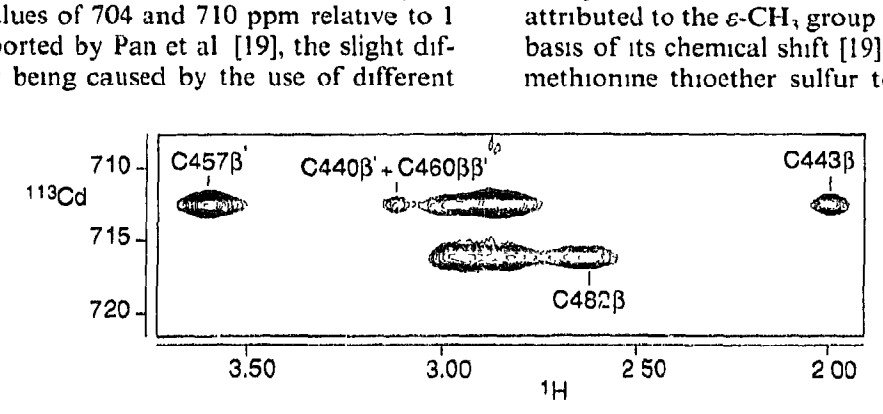

Fig 2 ^{113}Cd-^{1}H HSQC spectrum of the ^{113}Cd substituted GR-DBD showing the correlation between the frequencies of the cadmium ions and the β protons of the coordinating cysteines

invoked to explain its presence in a 1D HMQC spectrum This resulted in a complicated coordination scheme for the third metal ion which involved C450, C500 and M505 [19] Combination of our results with the assignments of the cysteine β proton resonances obtained earlier [22] suggest that this resonance should be assigned to C443 coordinating to the metal ion in the first zinc-finger We found no indications for the presence of a third metal binding site Inspection of the solution structure of the GR-DBD [23] reveals that the sulfur–sulfur distances for both C450–C500 and C450–M505 of about 10 Å, indicating that these residues could not coordinate to the same metal ion unless the protein underwent major conformational changes

Because of the overlap of the cysteine β protons (see also Table I and Fig 2), the HSQC spectrum alone is not sufficient to discriminate between C495 and C500 as coordinating ligands in the second zinc-finger Since the α protons of the cysteines show a larger chemical shift dispersion, relay HSQC spectra were recorded In this case, the HSQC-NOE experiment gave a much better signal to noise ratio than the HSQC-HOHAHA experiment, even when one takes into account the fact that for the HSQC-NOE experiment about three times more scans were acquired Fig 3A and B show cross-section parallel to f_2 at the f_1 frequency of the cadmium ion in the second zinc-finger of an HSQC-NOE spectrum. The experiment yields both intra- and inter-residue relay cross-peaks which may complicate the analysis However, careful comparison of the HSQC-NOE spectrum with homonuclear 2D NOE and HOHAHA spectra readily allowed both distinction between intra-residue and inter-residue cross-peaks In this way all the coordinating cysteines could be identified (Fig 3) The intensity of the cross-peaks to the α protons is variable because it depends on both the distances between the α proton and the two β protons and the heteronuclear ^{113}Cd-$\beta\beta'$ proton coupling constants In the case of C443 and C457 analysis of homonuclear 2D NOE and P E. COSY spectra shows that the conformation of the χ angle in these residues is *gg* (unpublished results), i.e short $\alpha\ \beta$ and $\alpha\ \beta'$ proton distances In addition, the coupling between one of the β protons and ^{113}Cd is large in both C443 and C457, as can be concluded from the intensity of the β cross-peaks in Figs 2 and 3A, resulting in strong α proton resonances for these residues (Fig 3A) The conformation of the χ angle is *gt* for all other cysteines (one short and one longer $\alpha\ \beta$ proton distance) resulting in reduced intensity for the α cross-peaks belonging to these residues as compared to C443 and C457 The broad signal at ~2 1 ppm in Fig 3B is probably due to residual intensity from the C443β peak coordinating to the first metal ion and does not indicate coordination of this cysteine to both Cd ions The spectra show that C495 is the fourth coordinating cysteine in the second zinc-finger establishing a coordination scheme as depicted in Fig 1 On the basis of our data, it is not possible to exclude unequivocally some role for C500 in metal coordination because its α and β proton resonances overlap with the resonances of the remaining cysteines (see also Table I) It is unlikely, however, that C500 acts as a *ninth* metal ligand since Freedman et al [5] showed that the GR-DBD contains two free cysteines (i e C500 and the non-conserved C450) not involved in metal binding In the three-dimensional structure of the GR-DBD, C500 is located close to the first metal binding site [23] A dynamic process in which C500 plays 'musical chairs' with the other four cysteines in the first metal binding site to coordinate the metal as suggested by Hard (personal communication) cannot be excluded on the basis of our results.

Schena et al [9] found that substitution of C500 by

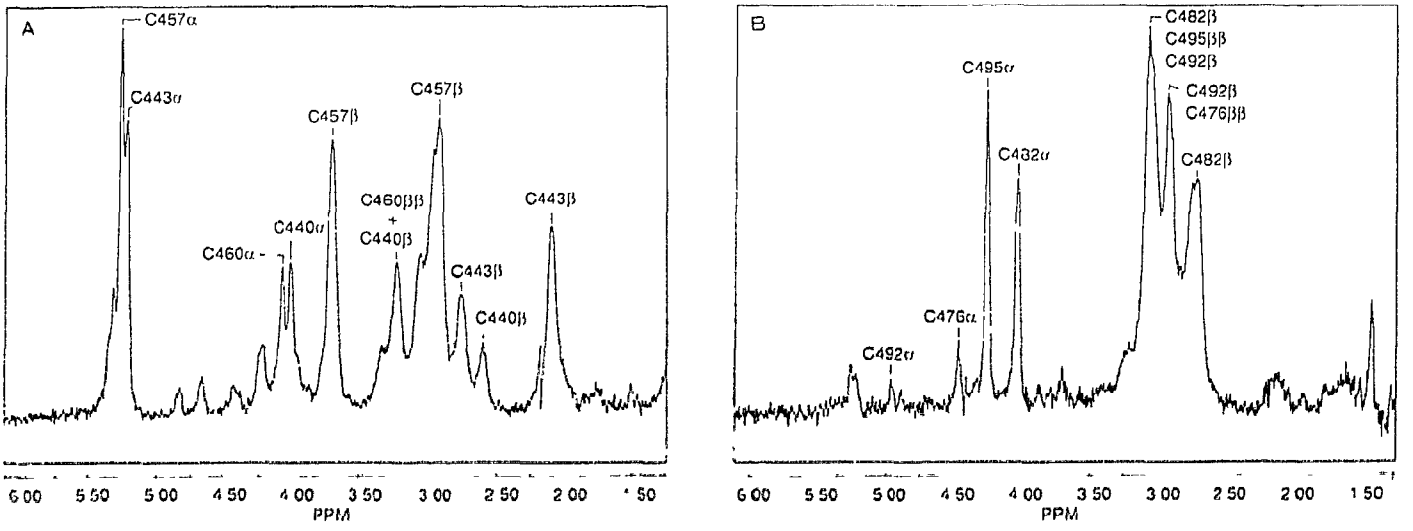

Fig 3 Cross-section from an HSQC-NOE experiment taken parallel to f_2 at the frequency of the metal ion in the first (A) and second (B) zinc-finger The NOE mixing time was 125 ms

an arginine resulted in a defective receptor, in contrast, Severne et al. [9] showed that C500 could be mutated to a serine residue without affecting receptor function This discrepancy may be explained by the different steric requirements of arginine and serine residues whereas serine is very similar in size to cysteine, arginine is a much larger amino acid and in addition has a positive charge. In the structure of the GR-DBD, C500 is part of an α helix [23] Mutation of C500 to an arginine residue may disrupt this helix preventing correct folding of the receptor, while serine substitution does not interfere with the folding process.

In conclusion, our data are consistent with the presence of two metal binding sites in GR-DBD. The resonance previously attributed to the M505 ε-CH_3 coordinating a third cadmium ion originates from the C443 β proton. No indications were found for a third, weaker binding, coordination site Relay HSQC experiments were used to establish the identity of the coordinating cysteines. It was found that C495 coordinates to the metal ion in the second zinc-finger. This is in agreement with the coordination scheme proposed by us [23] and others [24] previously based on proton NMR data.

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