

## DFT Calculation of Nitrogen Chemical Shifts in the Active Site of Vitamin D Receptor\*

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The complexed vitamin D receptor (VDR) is responsible for calcium homeostasis. Tryptophan is of special importance for the receptor's functions, as it appears just once in the VDR sequence and occupies the center of the ligand binding pocket. DFT calculations of nitrogen chemical shifts for Trp-NH<sub>SC</sub> moiety, presented in this work for liganded and free receptor, agree with NMR studies on the VDR specifically labeled with [UL]<sup>15</sup>N<sub>2</sub> Trp. Our calculations confirm orientation of the C(7)=C(8) vitamin D bond under the tryptophan ring. We suggest that interactions with water molecules are responsible for observed deshielding of indole Trp-nitrogen in unliganded VDR.

**Key words:** nuclear receptors, vitamin D receptor, protein ligand interactions, DFT calculations of nitrogen chemical shifts

The vitamin D receptor is a member of a nuclear receptor (NR) superfamily comprising receptors for the steroid, retinoid and thyroid hormones [1]. During the last ten years, most of the steroid receptors were crystallized in both apo (unliganded) and holo (liganded) forms [2–6]. These crystallographic data allowed for the determination of active sites in the ligand binding pockets (LBP) of the NR family. However, comparison of biological potency of wild type VDR and its mutants revealed that some contact sites found in solid state do not exist *in vivo* [7]. It can therefore be anticipated that knowledge of detailed architecture of a LBP in a medium close to the physiological *milieu* is necessary to understand its interaction with a ligand. LBP-VDR is too large (37.4 kDa) for full conformational identification by NMR techniques [8]. Nevertheless, employment of edited NMR techniques developed for proteins with molecular mass exceeding 15 kDa and selective isotope labeling permits partial determination of its structure. There is no doubt that tryptophan is one of the most important amino acids in the VDR sequence. Recently, NMR studies verified that in aqueous solutions, just like in the crystal state, the position of tryptophan in ligand-VDR complexes is preserved, even in the case of vitamin D analogs, which drastically differ (Table 1) in their biological activities [9–10]. Comparison of

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$^1\text{H}[^{15}\text{N}]$  HSQC spectra of apo and holo VDR revealed that the Trp282  $\text{N}^{\text{H}}$  signal shifts downfield by 4 ppm (from 125.8 to 129.8 ppm) when the ligand is removed [9]. It is worth mentioning that the structure of the free vitamin D receptor is still unknown, as only crystals of liganded VDR mutants have been obtained [5–6]. Probably the presence of a highly mobile domain (165–215 in human VDR) hampers crystallization efforts of the full length LBD-VDR. Recently, this 50 amino acid nonhomologous receptor fragment, excised in crystallized protein mutants, was modeled by SICHO (Side-CHain Only), while full length LBD-VDR was created by combined homology and lattice modeling [11].

**Table 1.** Literature data<sup>a</sup> for biological activities.

Compound (number)	VDR binding ratio <sup>b</sup>	Amount (pmol)	ICA	BCM
none (control)		0	5.5 ± 0.2	5.1 ± 0.2
1 $\alpha$ ,25-(OH) $_2$ D $_3$ (1)	1	260	6.2 ± 0.4	7.2 ± 0.5
2MD (3)	0.77	260	4.6 ± 0.7	14.4 ± 0.6
none (control)		0	2.3 ± 0.4	3.9 ± 0.1
1 $\alpha$ ,25-(OH) $_2$ D $_3$ (1)	1	260	5.6 ± 0.6	6.1 ± 0.2
2AM20R (4)	0.22	260	5.3 ± 0.6	5.8 ± 0.3
none (control)		0	–	9.3 ± 0.4
1 $\alpha$ ,25-(OH) $_2$ D $_3$ (1)	1	0.2 <sup>c</sup>	–	10.6 ± 0.1
2MbisP (5)	0.11	70 <sup>c</sup>	–	9.5 ± 0.1

<sup>a</sup>Literature data are taken from [10] and for compound 5 from [9]. ICA and BCM denote intestinal calcium absorption and bone calcium mobilization, respectively. <sup>b</sup>The binding capability of analogs is expressed as fraction of hormone activity. <sup>c</sup>Dose level in  $\mu\text{g}/\text{kg}$ .

In this work we calculate chemical shifts of Trp and its nearest (3.5 Å) neighbors (Ser 271, Phe 275 and Gln 313) in the free and liganded vitamin D receptor. Our aim is to search for such VDR structure in which the tryptophan residue is deshielded in apo and shielded in holo form.

## RESULTS AND DISCUSSION

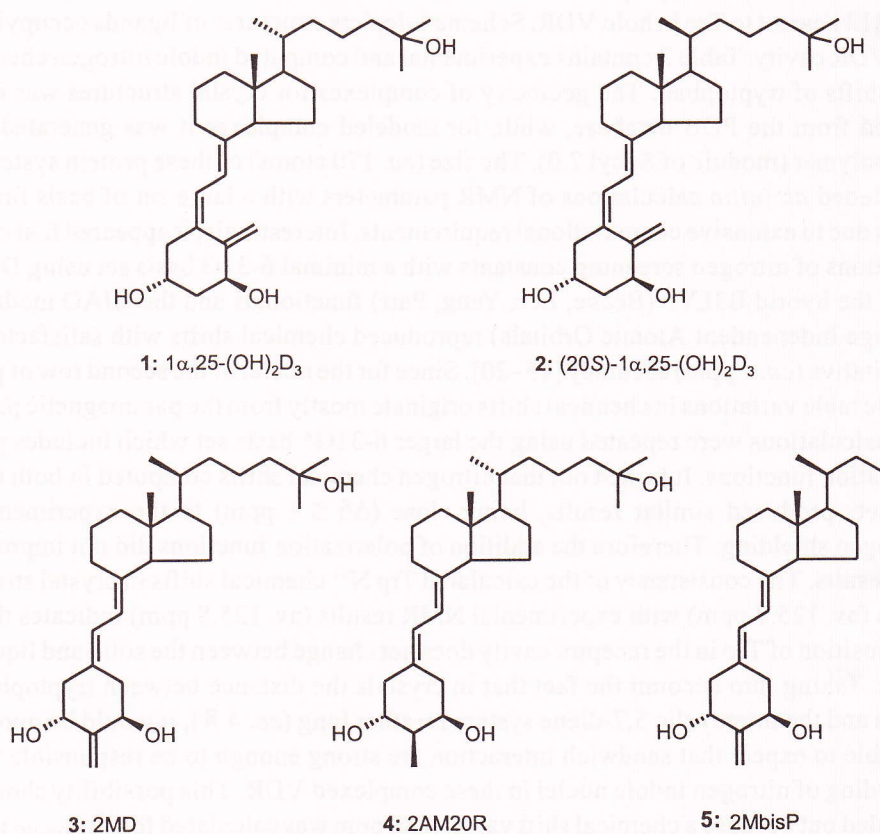
Since VDR crystallizes only in the presence of ligands [5–6], structural comparison of its two forms can only be done through molecular modeling. Among the amino acids residing in the ligand binding pocket (LBP), tryptophan (Trp) is without any doubt of crucial importance. Its natural mutation to arginine leads to severe hereditary vitamin D-resistant rickets [12], while the replacement of Trp with phenylalanine, serine or alanine decreases binding receptor capabilities between 100 (Phe) and 1000 (Ser, Ala) times [13–14]. Several studies attempted to explain the role

of Trp in ligand binding. X-ray analyses of structures of liganded VDR revealed that the indole group of tryptophan is always stacked with the vitamin D C(6)–C(7)=C(8) fragment, regardless of the biological potency of the ligand [6,10]. Since some contact sites found in solid state do not exist *in vivo*, a question arose whether this unique amino acid also conserves its orientation with respect to the anchored ligands in aqueous buffers, a *milieu* closer to physiological conditions. Recently published studies on the VDR specifically labeled with [UL]  $^{15}\text{N}_2$  Trp confirmed that it is the case [9]. Even though the analyzed ligands differed markedly in their biological activity (Table 1), Trp  $\text{H}^{\text{e1}}$  and Trp  $\text{N}^{\text{e1}}$  chemical shifts in the complexes were virtually identical, with nitrogen nuclei in holo and apo VDR clustering around 125.8 and 129.8 ppm, respectively (Table 2). These results indicate that the interaction between the ligands and Trp282 is not responsible for variations in calcemic activity observed in vitamin D analogs. Rather, it appears that the indole ring of the tryptophan residue acts as a common binding site for the intercylic 5,7-diene moiety of vitamin D compounds anchored in the VDR binding pocket. In this work we have focused our attention on DFT calculations of tryptophan chemical shifts and the amino acids: Ser271, Phe275, Gln313 nearest to Trp in holo VDR. Scheme 1 depicts structures of ligands occupying the VDR cavity. Table 2 contains experimental and computed indole nitrogen chemical shifts of tryptophan. The geometry of complexes for crystal structures was obtained from the PDB database, while for modeled complexes it was generated in Biopolymer (module of Sybyl 7.0). The size (*ca.* 170 atoms) of these protein systems precluded *ab initio* calculations of NMR parameters with a large set of basis functions due to extensive computational requirements. Interestingly, it appeared that calculations of nitrogen screening constants with a minimal 6-31G basis set using DFT with the hybrid B3LYP (Becke, Lee, Yang, Parr) functionals and the GIAO method (Gauge Independent Atomic Orbitals) reproduced chemical shifts with satisfactory qualitative (*ca.* 1 ppm) accuracy [15–20]. Since for the nuclei of the second row of periodic table variations in chemical shifts originate mostly from the paramagnetic part, the calculations were repeated using the larger 6-31G\* basis set which includes polarization functions. It turned out that nitrogen chemical shifts computed in both basis sets produced similar results, being close ( $\Delta\delta \leq 1$  ppm) to the experimental nitrogen shielding. Therefore the addition of polarization functions did not improve the results. The consistency of the calculated Trp  $\text{N}^{\text{e1}}$  chemical shifts in crystal structures (av. 125.7 ppm) with experimental NMR results (av. 125.8 ppm) indicates that the position of Trp in the receptor cavity does not change between the solid and liquid state. Taking into account the fact that in crystals the distance between tryptophan rings and the intercylic 5,7-diene system is rather long (*ca.* 4 Å), it would be unreasonable to expect that sandwich interaction are strong enough to be responsible for shielding of nitrogen indole nuclei in these complexed VDR. This possibility should be ruled out because a chemical shift value of 80 ppm was calculated for  $^{15}\text{N}_{\text{Trp-SC}}$  in a simulated complex in which this distance was shortened to 2 Å. In docking experiments of C-2 modified vitamins to full-length LBD-VDR, it was observed that the C(5)=C(6)–C(7)=C(8) diene moiety can shift along the Trp aromatic rings [21].

**Table 2.** Experimental<sup>a</sup> and calculated<sup>b,c</sup> nitrogen chemical shifts of tryptophan indole moiety (in ppm) in liganded VDR.

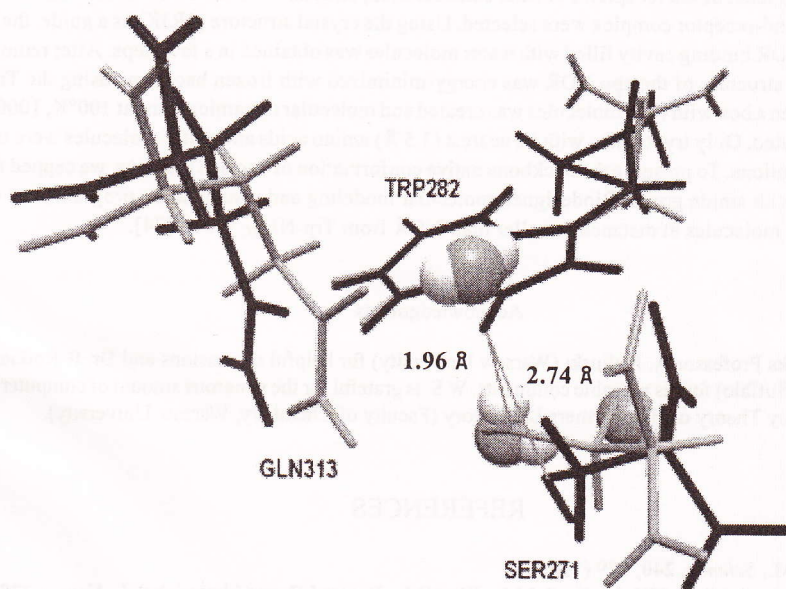
Ligand	PDB Code	Res. (Å)	<sup>15</sup> N <sup>ε1</sup> exp.	<sup>15</sup> N <sup>ε1</sup> calcd. <sup>b</sup>	<sup>15</sup> N <sup>ε1</sup> calcd. <sup>c</sup>
1 $\alpha$ ,25-(OH) <sub>2</sub> D <sub>3</sub>	1DB1	2.2	–	123.7	123.0
1 $\alpha$ ,25-(OH) <sub>2</sub> D <sub>3</sub>	1RK3	2.2	125.6	126.0	125.0
20( <i>S</i> )-1 $\alpha$ ,25-(OH) <sub>2</sub> D <sub>3</sub>	MC1288	1.5	–	124.6	123.9
2MD	1RJK	1.99	125.9	126.9	126.4
2AM20R	1RKH	2.28	–	125.0	124.4
2MbisP	1RKG	1.90	125.8	125.3	124.6

<sup>a</sup>Experimental data are taken from [9]. <sup>b</sup>Computing of the nitrogen shielding of Trp N<sup>ε1</sup> and reference NH<sub>3</sub> was performed with a 6-31G basis set using DFT with the hybrid B3LYP functionals and the GIAO method [15–20,22]. Geometry of NH<sub>3</sub> was optimized with aug-cc-pVDZ basis set using DFT B3LYP method. When the nitrogen shielding of Trp N<sup>ε1</sup> was calculated using the GIAO/B3LYP/6-31G approach and referred to NH<sub>3</sub> taken from Gaussian 03 software package chemical shifts (in ppm) for: 1DB1, 1RK3, MC1288, 1RJK, 1RKH, 1RKG denoted 125.2, 127.5, 126.0, 128.3, 126.4 and 126.7, respectively. <sup>c</sup>Computing of the nitrogen shielding of Trp N<sup>ε1</sup> and reference NH<sub>3</sub> were performed with expanded 6-31G\* basis set.



**Scheme 1.** Chemical structure of (1) 1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>3</sub> and (2) its 20-epi analogue, (3) 2MD: (20*S*)-2-methylene-19-nor-1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>3</sub>, (4) 2AM20R: 2 $\alpha$ -methyl-19-nor-1 $\alpha$ ,25-(OH)<sub>2</sub>D<sub>3</sub> and (5) 2MbisP: (20*S*)-2methylene-19-nor-1 $\alpha$ -(OH)-bishomopregnacalciferol.

However, in DFT calculations of screening constants the positioning of the indole nitrogen under the C(5)=C(6) bond causes chemical shift inconsistent with experimental results, excluding such ligand orientation from consideration. Natural mutation of Trp to Phe decreases the binding capabilities of the VDR receptor by two orders of magnitude. This suggests that Trp interacts with the ligand and with the nearest amino acids through the pirol moiety rather than through the phenyl ring. In all crystal structures the Trp NH group acts as donor of hydrogen bonds to Ser271 and Gln313 (Figure 1). In the Trp282Phe VDR mutant such interactions are erased and the active site is disturbed. To understand the deshielding of Trp indole nitrogen in an empty VDR binding pocket, molecular interactions between the receptor and water were studied. In crystals of holo VDR vitamins fill the binding pocket so tightly that there is no room for water around the Trp active site. Of course, when the receptor cavity is empty, water can freely interact with tryptophan. Using the crystal structure of holo VDR as a starting point, the lowest energy conformation of the unliganded receptor was found in an energy minimization procedure (Tripos force field) while keeping the backbone frozen. Next, water was added and molecular dynamics performed on the whole protein. Finally, Trp and its closest neighbors (three amino acids and water molecules distanced up to 3.5 Å) were retained for analysis of the computed screening constants. The resulting chemical shift of indole nitrogen nuclei was calculated at 135.4 ppm (vs exp. 129.0 ppm). It is therefore likely that interactions with water could be responsible for the observed downfield shift of Trp N<sup>ε1</sup> in unliganded form.



**Figure 1.** Superimposition of two VDR active sites at Trp282: the crystal structure (with ligand removed) is depicted in gray; the computational structure, after energy minimization and molecular dynamic with water is depicted in black. Two amino acids (Ser 271 and Gln313) and three water molecules forming hydrogen bonds with tryptophan indole NH group are included.

## CONCLUSIONS

*Ab initio* quantum mechanical calculations using GIAO/B3LYP method reproduce nitrogen chemical shifts of  $N_{\text{Trp-SC}}$  moiety in a 170 atom protein system with the accuracy of *ca.* 1 ppm. Since discrepancies in nuclear screening constants calculated with the minimal 6-31G and the larger 6-31G\* basis set do not exceed 1 ppm, the minimal set of basis functions is sufficient for calculations of nitrogen shieldings in the vitamin D-VDR complexes. Computation of nitrogen chemical shifts in a large protein system of known crystal structure and subsequent comparison with chemical shifts detected by  $^{15}\text{NMR}$  in aqueous buffers could constitute a useful tool for detecting differences in the structure of active protein sites in the solid and liquid state.

## COMPUTATIONAL DETAILS

The calculations were carried out using the GAUSSIAN 03 software package [22]. The nitrogen shielding calculations were performed with 6-31G and 6-31G\* basis sets of wavefunctions using DFT with the hybrid B3LYP functionals and the GIAO method (Gauge Independent Atomic Orbitals) [15–20]. The atom coordinates required for calculation of Trp chemical shifts in liganded and the free receptor were taken from PDB database (Table 2) and from computational models, respectively [23]. Docking simulations were performed by FlexX software from the Biopolymer module, which uses a generic algorithm for the search of conformational space of the ligand with respect to the receptor's binding site. Several simulations of 100,000 steps each were performed with various initial positions of the ligand with respect to the binding pocket of the receptor. For final consideration, structures of the lowest conformational energy of the ligand-receptor complex were selected. Using the crystal structure (1RJK) as a guide, the best model of the VDR binding cavity filled with water molecules was obtained in a few steps. After removing the ligand, the structure of the apo VDR was energy-minimized with frozen backbone using the Tripos force field. Then a box with  $\text{H}_2\text{O}$  molecules was created and molecular dynamics (runs at 300°K, 10000 iterations) executed. Only tryptophan with its nearest (3.5 Å) amino acids and water molecules were taken for DFT calculations. To preserve the backbone native conformation of excised residues, we capped them at C terminal with amide group. Biodesigner, molecular modeling and visualization program, was used for finding the molecules at distances smaller than 3.5 Å from Trp-NH<sub>SC</sub> group [24].

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