The use of a ditopic Gd(III) paramagnetic probe for investigating α-bungarotoxin surface accessibility
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Andrea Berninia, Ottavia Spiga, Vincenzo Venditti, Filippo Prisch, Mauro Botta, Gianluca Croce, Angela Pui-Ling Tong, Wing-Tak Wong, Neri Niccolai

Department of Chemistry, The University of Hong Kong, Pokfulam Road, HKSAR, China
Dipartimento di Biotecnologie, Università di Siena, I-53100 Siena, Italy
Dipartimento di Scienze e Innovazione Tecnologica, Università del Piemonte Orientale “Amedeo Avogadro”, I-15121 Alessandria, Italy
Department of Life Sciences, Imperial College London, London SW72AZ, UK
National Institutes of Health, Bethesda, MD, USA
Dipartimento di Biotecnologie, Università di Siena, I-53100 Siena, Italy
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ABSTRACT

Protein surface accessibility is a critical parameter which drives all intermolecular interaction processes. In this respect a big deal of information has been derived by analyzing paramagnetic perturbation profiles obtained from NMR protein spectra, particularly in the case that the effects due to different soluble paramagnets can be compared. Here Gd₂L₇, a neutral ditopic paramagnetic NMR probe, has been characterized in protein systems.

1. Introduction

The use of proteins as molecular devices in bionanotechnology requires a detailed knowledge of the mechanisms of their interactions with the surrounding molecular environment. It is apparent, indeed, that protein surface accessibility controls all the biological processes determined by protein–protein, protein–ligand and protein–nucleic acid interactions. Thus, mapping protein surface accessibility can provide a solid experimental basis to predict surface hot spots and, hence, to design bionanodevices with suitable mutants.

Nuclear magnetic resonance (NMR) studies on the effects induced by soluble paramagnetic probes, such as aminoxyl spin-labels [1–3], gadolinium complexes [4,5] and molecular oxygen [6], on protein and RNA NMR signals have provided a wealth of information about the complex dynamics contributing to surface accessibility. The fact that paramagnetic probes might be involved in biased approaches toward specific amino acid side chains or structural determinants has been considered and, consequently, the use of more than one probe has been suggested to enhance the resolution of this kind of studies [7,8].

Some interference to a purely random approach of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) to the protein surface, due to some hydrogen bonding between the N-oxyl moiety of the probe and protein backbone amide groups, has been suggested by comparing the paramagnetic profiles induced by different probes [7]. Relaxometric techniques have yielded no evidence of strong interactions of GadTPA-BMA (gadolinium(III) diethylenetriamine-N,N,N″,N‴-pentaacetate-bis(methylamide)) with specific molecular sites, even in the crowded molecular environments typical of biological fluids [9], suggesting that the latter paramagnet is well suited for accurate mapping of surface accessibility [4].

To overcome risks from toxicity [10,11] and reduced MRI (magnetic resonance imaging) contrasting activity at high magnetic fields [12] of conventional paramagnetic probes, engineering of a large variety of new paramagnets is required. NMR investigations on protein surface accessibility can take advantage of the latter MRI needs.
broadening the repertoire of neutral soluble paramagnets to study the interaction between proteins and small molecules.

In the present study a ditopic neutral complex of Gd(III) with L7 macrocycle (see Fig. 1 and caption for structure and full name), henceforth called GdL7, was used for investigating the role of size and hydrophobicity of paramagnetic probes in defining protein surface accessibility. Paramagnetic attenuations induced by GdL7 on well resolved CoH signals of 1H–13C heteronuclear single quantum coherence (HSQC) spectra of α-bungarotoxin, α-BTX, have been analyzed. This small neurotoxin, having well characterized structural and dynamic features [13,14], represents a suitable model system to study results in very good agreement with the recent crystallographic structure of the α1 subunit of the mouse nicotinic acetylcholine receptor[16]. Indeed, the protein moiety most accessible to GdDTPA-BMA results also to be the binding site with the α1 subunit of the mouse nicotinic acetylcholine receptor. Moreover, in the protein–receptor interface no water molecules are present, consistent with the lack of high water density sites in the α-BTX binding region predicted by MD simulations [15].

In the case that GdL7 would confirm the accessibility pattern toward the α-BTX surface which has been already derived from GdDTPA-BMA paramagnetic perturbations, the ditopic probe should be included in the list of molecular tools for understanding the dynamics of protein surface accessibility and, hence, for accurate mapping of protein surface hot spots.

2. Experimental

2.1. Sample preparation and NMR measurements

α-BTX was obtained from Sigma and used without any further manipulation. GdL7 was prepared as previously described [1]. NMR measurements, run at 303 K and pH 6.0 to reproduce the experimental conditions of the original structural study of α-BTX, were obtained with a Bruker Avance DRX 600 spectrometer.

The 1H–13C HSQC diamagnetic and paramagnetic spectra were obtained with 160 increments and 192 scans over 2048 data points, with an inter-scan delay of 5.0 s and an INEPT (Insensitive nuclei enhanced by polarization transfer) delay of 1.725 ms for a 2-day experiment time. Spectral widths of 6000 Hz and 12,000 Hz were used for proton and carbon dimensions, respectively. Data processing was performed with the NMRPipe software [17]. Data were zero-filled to a final matrix of 2048 × 512 points prior to apodization with Gaussian (line broadening of −6.0 Hz and Gaussian broadening of 0.1) and cosine-squared bell functions for the direct and indirect dimensions, respectively. The H chemical shifts were referenced on trimethylsilylpropionic-2,2,3,3-d4 acid sodium salt (TSP) at 0 ppm. NMR samples were prepared by dissolving the protein in H2O/D2O (95:5) to obtain a 1.0 mM solution of the protein. The paramagnetic NMR samples contained 0.5 mM GdL7, an optimal probe concentration to observe sizeable signal attenuations.

To compare cross peak volumes, Vp, obtained in different set of experiments and measured with a confidence higher than 90%, their autoscaled values Ṽp were used according to the relation [18]:

\[ Ṽp = \frac{Vp}{(1/n) \sum Ṽp} \]

Paramagnetic attenuations, Ap, were calculated from the autoscaled diamagnetic and paramagnetic peak volumes, respectively tp and t̃p, according to the relation [18]:

\[ A_p = 2 - \frac{t̃p}{tp} \]

In the case of glycines, signal intensities of both CoH1 and CoH2 correlations were measured and averaged Ap are reported.

Data analysis was performed with Sparky software (http://www.cgl.ucsf.edu/home/sparky).

2.2. Relaxivity measurements

The proton 1/T1 NMRD (nuclear magnetic resonance dispersion) profiles were measured on a Stelar fast field-cycling FFC-2000 (Mede, PV, Italy) relaxometer on about 0.25–1.0 mmol gadolinium solutions in non-deuterated water. The relaxometer operates under computer control with an absolute uncertainty in 1/T1 of ±1 %. The NMRD profiles were measured in the range of magnetic fields from 0.00024 to 1.6 T (corresponding to 0.01–70 MHz proton Larmor frequencies). Additional experimental data (300 and 600 MHz) were acquired on high resolution Bruker Avance NMR spectrometers and compared with those calculated from the fitting of the NMRD profiles. Longitudinal relaxation rates, 1/T1, were obtained by the inversion recovery method on a 0.5 mM aqueous solution of the complex. A capillary containing d8-benzene was inserted into the 5-mm tube to lock the field.

Variable-temperature 17O NMR measurements were recorded on a JEOL EX-90 (2.1 T) spectrometer equipped with a 5 mm probe. Solutions containing 2.0% of the 17O isotope (Cambridge Isotope) were used. The observed transverse relaxation rates were calculated from the signal width at half-height. Other details of the instrumentation, experimental methods, and data analysis have been previously reported [19].

**Fig. 1.** The 2D structural representation of GdL7(H2O)2 (A) and of GdDTPA-BMA(H2O) (B). L7 is 4,7,10,23,26,29-hexakis-(carboxymethyl)-2,12,21,31-tetraoxo-1,4,7,10,13,20,23,26,29,32-decaazatrcyclo[14.20]-p-xylene and DTPA-BMA is diethylentriamine-N,N,N′,N′′,N″-pentaacetate-bis(methylamide).
2.3. Depth index calculation

The 3D atom depths, reported as depth indexes \(D_i\)'s, were calculated on the basis of the minimized average NMR structure of \(\alpha\)-BTX (PDB code: 1IK8) [20].

\[ D_i = \frac{2V_{i,r}}{V_{0,r}} \]

where \(V_{i,r}\) is the exposed volume of a sphere of radius \(r\) (sampling radius) centered on atom \(i\) and \(V_{0,r}\) is the total volume of the same sphere. The sampling radius was set to 8 Å, as the smallest value which yields \(V_{i,r} = 0\) only for the most buried protein atom. To account for the involvement of water molecules in the protein surface accessibility, a probe radius of 1.4 Å, the water molecular radius, has been used for the computation of protein surface and, hence, the protein volume to be considered for calculating \(V_{i,r}\)'s. For each C–H correlation, the averaged carbon–hydrogen \(D_{i,\text{H}}\) value is reported.

2.4. Molecular dynamics simulation

A 16 ns MD simulation run in explicit water was performed starting from the \(\alpha\)-BTX NMR minimized average structure (PDB code: 1IK8), using the GROMOS (Groningen Molecular Simulation package) force field [22] and the GROMACS package [23]. Details on the MD run are supplied elsewhere [15]. All the analyses of the MD trajectory were carried out using standard GROMACS tools.

2.5. \(\text{Gd}_2\text{L}_7\) structural characterization

Single crystal diffraction data were collected on a Bruker SMART 1000 CCD diffractometer, using graphite monochromatic Mo K\(\alpha\) (\(\lambda = 0.71073\) Å) radiation. Data reduction and absorption correction were performed using SAINT (Bruker, 1998) and CrystalStructure (Rigaku/MSC, 2006). The structure was solved and refined by full-matrix least squares using SHELX-97 [24]. Hydrogen atoms were generated in calculated position using SHELX-97. The obtained structure is reported in Fig. 2A. CCDC ID: 844433 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

2.6. Structure prediction of GdDTPA-BMA

On the basis of the crystal structure of Gd(DTPA-BEA) [25], the structure of the homologous complex GdDTPA-BMA was modeled by using MOPAC [26] and reported in Fig. 2B.

3. Results

3.1. \(\text{Gd}_2\text{L}_7\) structural characterization

The X-ray structure of gadolinium complex was solved by single crystal X-ray diffraction analysis. Crystallographic data and details of data collection and refinement are given in Table 1. The asymmetric unit contains two crystallographically independent Gd(III) ions linked together through two phenyl groups to construct a centrosymmetric dinuclear secondary building unit of \([\text{Gd}_2\text{C}_4\text{H}_8\text{N}_9\text{O}_{18.5}\text{H}_2\text{O}]\). Each gadolinium ion is nine-coordinated and its coordination cage is constituted by three nitrogen and five oxygen atoms from the ligand and one from a water molecule. The average Gd–N, Gd–O\(_\text{ligand}\) and Gd–O\(_\text{water}\) distances are 2.68(3), 2.39(4) and 2.41(2) Å respectively, building a distorted tricapped trigonal prism. The two coordinated water molecules are located, opposite to the chelating ligands, in the molecular region which offers large surface accessibility. The Gd–Gd distance of 12.88(1) Å of the dinuclear unit is attributable to the phenyl chains which show an average length of 9.30(2) Å and form Gd–Ph\(_\text{plane}\)–Gd angles of 148.2(2)° and 135.4(2)°. In the \(\text{Gd}_2\text{L}_7\) structure, the two phenyl rings are perpendicularly close to each other and the C–N–C–C torsion angles reveal that their chains assume a twisted conformations. In the asymmetric unit the Gd complex is surrounded by different crystalline water molecules, which refill a large part of the volume cell, generating an extended hydrogen bonding network with the oxygen atoms of ligand carbohydrate groups and of coordinated water molecules. The crystal packing of the system is mainly driven by hydrogen bonding forces between crystalline water and the Gd complex. In fact a very restricted number of short contact (\(\sim\)sum of vdW radii) between the molecular units is recorded during the packing examination.

3.2. Molecular dynamics simulation of \(\alpha\)-BTX

In order to correlate local molecular flexibility, surface hydration and protein surface accessibility, a 16 ns MD simulation was performed on \(\alpha\)-BTX. As shown in Fig. 3A, residues 30–37 belonging to the so called \(\alpha\)-BTX finger II and to the protein carboxy terminus, residues 67–74, are very flexible protein moieties, as inferred by their high root mean square fluctuation (rmsf) values. In Fig. S3 of Supplementary materials, \(\alpha\)-BTX structure is given, highlighting the locations of protein fingers and of active site residues. It should be noted that both finger II and carboxy terminus are primarily involved in the binding with the acetylcholine receptor [16], and that in the latter \(\alpha\)-BTX moieties no MD defined high water density sites were found [15]. This finding supports the already suggested correlation between the extent of protein backbone flexibility and absence of structured water molecules [27].

3.3. Characterization of relaxation properties of \(\text{Gd}_2\text{L}_7\) and GdDTPA-BMA

Relaxation properties of \(\text{Gd}_2\text{L}_7\) and GdDTPA-BMA have been analyzed in detail by measuring their proton relaxivity, \(r_{1p}\), at 298 K and \(pH = 7.2\). Thus, \(r_{1p}\) values of 2.9 and 7.2 mM\(^{-1}\) s\(^{-1}\) were obtained at 600 MHz for GdDTPA-BMA and \(\text{Gd}_2\text{L}_7\), respectively. The fact that \(\text{Gd}_2\text{L}_7\) relaxivity is more than twice higher than that of GdDTPA-BMA is due not only to the presence of two paramagnetic centers, but also to the compact and large molecular structure causing slower rotational dynamics of the ditopic paramagnet in solution (see Fig. 2). The aqueous solutions of \(\text{Gd}_2\text{L}_7\) and GdDTPA-BMA have been also investigated by \(^1\)H and \(^19\)O NMR relaxometric techniques in order to

![Fig. 2](image-url)
Table 1
Crystallographic data and details of data collection and refinement.

<table>
<thead>
<tr>
<th>Crystallography Structure</th>
<th>Data Collection and Refinement Details</th>
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</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C44 H97 Gd2 N10 O36.50</td>
</tr>
<tr>
<td>Formula weight</td>
<td>1664.82</td>
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<tr>
<td>Temperature</td>
<td>299(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 A</td>
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<tr>
<td>Crystal system</td>
<td>Monoclinic</td>
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<tr>
<td>Space group</td>
<td>P21/c</td>
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<tr>
<td>Unit cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>17.560(1) Å</td>
</tr>
<tr>
<td>b</td>
<td>22.669(1) Å</td>
</tr>
<tr>
<td>c</td>
<td>20.837(1) Å</td>
</tr>
<tr>
<td>Volume</td>
<td>8164.2(7) Å</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
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<tr>
<td>Density (calculated)</td>
<td>1.354 mg/m³</td>
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<tr>
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<td>F(000)</td>
<td>3404</td>
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<tr>
<td>Crystal size (mm)</td>
<td>0.22 × 0.16 × 0.07 mm</td>
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<td>Theta range for data collection</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>-20 &lt;= k &lt;= 26</td>
</tr>
<tr>
<td></td>
<td>-26 &lt;= l &lt;= 26</td>
</tr>
<tr>
<td>Reflections collected</td>
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</tr>
<tr>
<td>Independent reflections</td>
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</tr>
<tr>
<td>Compleness to theta</td>
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<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F²2</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
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</tr>
<tr>
<td>Goodness-of-fit on F²</td>
<td>1.099</td>
</tr>
<tr>
<td>Final R indices</td>
<td>R1 = 0.0639, wR2 = 0.1869</td>
</tr>
<tr>
<td></td>
<td>R1 = 0.1264, wR2 = 0.2132</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>1.041 and -0.547 e.A⁻³</td>
</tr>
</tbody>
</table>

The mean residence lifetime τₐ at 298 K for Gd₂L₇ (3.0 μs) is sensibly longer than that measured for GdDTPA-BMA (2.2 μs) [29] and one of the longest reported so far for a neutral Ga-complex.

The knowledge of τₐ allows the evaluation of the other relaxation parameters by the measurement and analysis of the magnetic field dependence of the proton relaxivity on a fast-field cycling relaxometer over an extended frequency range (0.01–70 MHz). This was done for Gd₂L₇ at 298 K and pH = 6.2. The relaxivity is constant from 0.01 to ca. 2 MHz, showing a dispersion around 6 MHz and remaining nearly constant at high fields (10–100 MHz). The shape and amplitude of the curve obtained are consistent with a dimeric species where each Gd center is coordinated by a single water molecule (q = 1) and it is also clear that the relaxivity at low fields is partially limited by the long τₐ value, as for GdDTPA-BMA. The best-fit parameters were obtained from the analysis of the data in terms of the Solomon–Bloembergen–Morgan equations for the inner-sphere relaxation mechanism and Freed’s equation for the outer sphere component [30].

The relevant results are the sensibly longer rotational correlation time, τ₀, and distance of closest approach of the outer sphere water molecules, a, as compared to GdDTPA-BMA, whereas...
their electronic relaxation times are rather similar. Furthermore, using the best fit values of $\Delta^2$ and $\tau_v$ (Table 2) it may be calculated for $T_{1e}$ a value of ca. 480 ns at 600 MHz. Then, it is clear that at the measuring field the electronic relaxation does not influence the relaxation properties of the Gd(III) complexes.

It must be underlined here, that the interplay of inner- and outer-sphere contributions is anyways controlled, in a complex way, by structural features and hydration dynamics of the protein.

Since the obtained $T_{1e}$ values are strictly dependent on the exchange rate of the water molecule coordinated to the Gd(III) ion, the optimal GdL7 concentration to achieve $\alpha$-BTX NMR signal attenuations similar to the ones previously obtained in presence of 2.0 mM GdDTPA-BMA [15] was empirically evaluated by adding increasing amount of a GdL7 solution to the protein sample. Thus, a final GdL7 concentration of 0.5 mM was chosen for the present investigation.

3.4. Paramagnetic attenuation analysis of $\alpha$-BTX NMR signals

In 0.5 mM and 2.0 mM paramagnetic solutions respectively of GdL7 and GdDTPA-BMA, $\alpha$-BTX $^1H$ protein signals appear similarly broadened even though the water signal is much wider in the second case. GdL7 and GdDTPA-BMA relaxivities, as well as their interaction with water molecules, account for this finding. This preliminary observation indicates that GdL7 is a very suitable paramagnetic probe for quantitative volume determinations of peptides lying close to the solvent surface.

In Fig. 4 the fingerprint region of $\alpha$-BTX $^1H-^13C$ HSQC spectra, recorded in the absence and in the presence of GdL7, is shown. Peak volumes of 45 well resolved CoH correlations could be quantified with enough accuracy to make speculations on paramagnetic signal attenuations, $A_{Gd\alpha}$. Thus, in the case of the highly perturbed R30 CoH, $A_{Gd\alpha}$ has not been reported due to severe spectral overlapping, see Fig. 4. A comparative analysis of $A_{Gd\alpha}$ with the ones previously measured for $\alpha$-BTX CoH signals in the presence of GdDTPA-BMA [15], $A_{Gd\alpha}$ is shown in Fig. 3B. $A_{Gd\alpha}$’s and $A_{Gd\alpha}$’s are, in general very similar, being their average difference $\Delta = 0.15$ and with a $\sigma_\alpha = 0.11$. To discuss the extent of paramagnetic attenuations, the standard deviation of $A_{Gd\alpha}$’s, $\sigma_\alpha$, has been calculated. Since a $\sigma_\alpha$ value equal to 0.36 was found, strong paramagnetic effects have been assessed only for those CoH correlations whose $A_{Gd\alpha}$ values were larger than 1.36, as in the case of P10, A31, S34, A36, G37 and V40. It is interesting to note that the improved quality of the $^1H-^13C$ HSQC spectrum obtained in the presence of GdL7, comparing the one obtained in the presence of GdDTPA-BMA, allows an accurate peak volume determination for six more correlations, including the one of V40 CoH, very attenuated and not determined in our previous investigation [15].

Only few CoH correlations exhibit $A_{Gd\alpha}$ values below 0.64, i.e. smaller than the average $A_{Gd\alpha}$ by a factor of $\alpha$. The small size of the neurotoxin and the long distance perturbation effect of the paramagnetic probe [31] account for this finding.

4. Discussion

It has been already shown that the structural interpretation of perturbations induced by soluble and neutral paramagnetic probes is not straightforward [15]. The use of atom depths, rather than accessible surface areas, ASA, has been proposed as a step forward to discuss the extent of paramagnetic relaxation effects, PRE, in terms of protein structure [15]. Atom depth, indeed, reflects more properly than ASA the through-space character of those dipolar interactions between electronic and nuclear spins generating the observed paramagnetic perturbations. Backbone nuclei can have ASA = 0 being, at the same time, near to the protein surface and, hence, closely approachable by the paramagnetic probe. On the basis of the used reference structure, average depth indexes, $D_{ia}$, of each $\alpha$-BTX CoH group have been calculated (see Fig. 3C) where the lower is the $D_{ia}$ value the deeper is the $i$ backbone methyne. The comparative analysis of $D_{ia}$, rmsf and $\alpha$ values, suggested in Fig. 3, points out that high flexibility and close surface proximity of $\alpha$-BTX backbone are often needed to observe strong paramagnetic attenuations, as in the case of P10, A31, S34, R36. The large probe accessibility to the inner residue G37, belonging to the active site moiety of the protein finger II together with the four latter ones, shows how the lack of a bulky side chain favors a close approach between $\alpha$-BTX backbone atoms and GdL7. This finding is consistent with other previous paramagnetic surveys of protein surface accessibility with Gd-paramagnets [15]. However, the strong paramagnetic attenuation exhibited also by the buried V40 suggests how the static average NMR structure, used as reference for our analysis, cannot be fully satisfactory for the interpretation of dynamic results such as PRE.

Data shown in Fig. 3 indicate also that backbone flexibility and surface exposure are not the only requirements for efficient paramagnetic

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**Table 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GdL7</th>
<th>GdDTPA-BMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta^2$ (s$^{-2}$ 10$^{-19}$)</td>
<td>5.0 ± 0.3</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>$\tau_v$ (ps)</td>
<td>19 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>$E_v$ (kJ mol$^{-1}$)</td>
<td>6.0 ± 0.9</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>$\tau_m$ (µs)</td>
<td>3.0 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>$\Delta H^\circ$ (kJ mol$^{-1}$)</td>
<td>48.0 ± 0.4</td>
<td>47.6 ± 1.1</td>
</tr>
<tr>
<td>$\Delta H$ (10$^7$ rad s$^{-1}$)</td>
<td>$-4.1$ ± 0.3</td>
<td>$-3.8$ ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ Fixed during the least-squares procedure.

---

**Fig. 4.** The overlapped $^1H-^13C$ HSQC spectra of the fingerprint region of $\alpha$-bungarotoxin recorded in the absence (green, lighter line) and in the presence of 0.5 mM GdL7 (red, darker line). Labels are given for the most attenuated signals (signals other than CoH are labeled accordingly).
perturbations. In fact, several surface exposed backbone CH groups belonging to flexible protein moieties are characterized by average or even low $\alpha_i$ values. This is the case of $\alpha12$, $\alpha13$, $\alpha14$, $\alpha17$, $\alpha18$ finger I residues, confirming that protein surface hot spots, delineated by the soluble paramagnetic probes, cannot be simply related to the protein structure and dynamics.

It has been already suggested that protein hydration has to be carefully considered for more accurate structure–PRE correlations [15]. In fact, systematic analyses of hydration site distribution on protein surfaces from MD simulations [32,33] and multiple solvent crystal structure determinations [34] have now established the close correlation between hydration pattern and contact sites of small molecules.

Accordingly, the presence of high density water molecules in specific $\alpha$-BTX surface regions, as predicted by MD simulations, explains the observed hindered approaches of $\text{Gd}_2\text{L}_7$ and $\text{GdDTPA-BMA}$ to finger I and III, defined in Fig. S3 of Supplementary material.

The reduced probe accessibility of finger III residues can be partially ascribed also to the involvement of this protein moiety in the formation of dimeric species, observed both in the crystal state [35] and in solution [36]. It is interesting to note that protein backbone flexibility together with the distribution of hydration sites [15] and the approach of the used gadolinium complexes to the $\alpha$-BTX surface, identify two protein sides. In side A, i) reduced backbone flexibility, ii) presence of many high density hydration sites, and iii) protein surface scarcely perturbed by $\text{Gd}_2\text{L}_7$ and $\text{GdDTPA-BMA}$ can be noticed. A complementary trend of the above mentioned features can be observed in the $\alpha$-BTX side B, the one which incorporates residue involved in the acetylcholine receptor binding.

The high resolution structure of the complex between $\alpha$-BTX and the extracellular domain of the $\alpha_1$ subunit of acetylcholine receptor, $\alpha_{211}$, has been recently resolved [16] and it can be used as a reference for discussing the extent of $\text{Gd}_2\text{L}_7$ induced perturbations to the $\alpha$-BTX backbone CH groups. On the basis of the Protein Data Bank [37] structure the $\alpha$-BTX/$\alpha_{211}$ complex, PDB code 2QC1, it is apparent that the highest $\alpha_i$ values are all grouped in the toxin side B where the binding with $\alpha_{211}$ occurs. Conversely, all the surface exposed $\alpha$-BTX methynes which experience only limited paramagnetic attenuations are located in a wide region which is almost opposite to the protein binding site (see Fig. 5).

The strong similarity of $A_{\text{Gd}}$ and $A_{\text{Gd}2}$ values cannot be attributed to a mere coincidence of specific protein–probe interactions, but rather to the similarity of the effective paramagnetic relaxation mechanisms, equally modulated by $\alpha$-BTX surface dynamics. Here, as already observed for other protein systems [8], $\alpha$-BTX active site appears as the protein moiety which is the most exposed to the paramagnetic perturbation. The absence of nearby hydration sites can explain the preferential $\alpha$-BTX accessibility of the receptor binding region. Protection of $\alpha$-BTX active site from potential protein–protein interaction can be also inferred by the enhanced accessibility of the used paramagnetic probes.

It can be concluded that paramagnetic perturbations, induced by soluble and neutral Gd complexes on suitable NMR signals, can be generally confirmed as a powerful source of information to delineate protein surface dynamics. The ditopic probe $\text{Gd}_2\text{L}_7$, whose scarce effectiveness as MRI contrast agent (unpublished results) can be attributed to its exceedingly long rate of water exchange, seems to be, for the same reason, very suitable for protein accessibility investigations. Indeed, $\text{Gd}_2\text{L}_7$ induced paramagnetic perturbations can be analyzed in spectra where water suppression can be achieved better than in the presence of $\text{GdDTPA-BMA}$. This finding suggests that efficient hot spots detectors should be found among those Gd(III) based paramagnetic probes, where $\text{H}_2\text{O}$ cannot exchange from the inner ion coordination sphere to the bulk solvent ($q = 0$ systems). Thus, a general remark can be made: design of gadolinium paramagnets can take advantage of the complementary needs required by MRI contrast agents and protein surface hot spots detectors.

**Abbreviations**

$\alpha$-BTX $\alpha$-Bungarotoxin

DTPA-BMA diethylenetriamine-N,N,N′,N″,N‴-pentacacetate-bis(methylamide)

HSQC heteronuclear single quantum coherence

INEPt insensitive nuclei enhanced by polarization transfer

MD molecular dynamics

MRI magnetic resonance imaging

NMR nuclear magnetic resonance

NMRD nuclear magnetic resonance dispersion

rmsf root mean square fluctuation

TSP trimethylsilylpropionic-2,2,3,3-d$_4$ acid

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at doi:10.1016/j.inorgbio.2012.03.004.

**References**


![Fig. 5. Solvent accessible surface representation of the crystal structure (PDB code: 2QC1) of the extracellular domain of $\alpha_1$ subunit of the murine acetylcholine receptor (yellow) bound to $\alpha$-bungarotoxin (gray). The toxin surface is colored according to paramagnetic attenuations observed for backbone methyne signals and to the color bar.](image-url)