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# High-pressure NMR study of the complex of a GTPase Rap1A with its effector RalGDS

## A conformational switch in RalGDS revealed from non-linear pressure shifts

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**Abstract Unusually large non-linear $^{1}H$ and $^{15}N$ nuclear magnetic resonance chemical shifts against pressure have been detected for individual amide groups of the Ras-binding domain of Ral guanine dissociation stimulator (GDS). The non-linear response is largest in the region of the protein remote from the Rap1A-binding site, which increases by about two-fold by the complex formation with its effector protein Rap1A. The unusual non-linearity is explained by the increasing population of another conformer (N′), lying energetically above the basic native conformer (N), at higher pressure. It is considered likely that the conformational change from N to N′ in the Ras-binding domain of RalGDS works as a switch to transmit the effector signal further to molecules of different RalGDS-dependent signaling pathways.** 

*Key words:* High-pressure NMR; Chemical shift; RalGDS; Rap1A; Signal transduction

### 1. Introduction

Signal transductions in living organisms are mediated by molecular interactions involving protein molecules. For enzymes, induced-fit models have been introduced in explaining enzyme–substrate or effector interaction. Basically, all protein–protein or protein–ligand interactions will involve some kind of conformational change on the protein side. However, this picture has rarely been supplemented by direct experiments. The utility of high-pressure nuclear magnetic resonance (NMR) to study protein–protein interactions was previously demonstrated by Urbauer et al. who used J-coupling and hydrogen exchange rate for the system of apocalmodulin and the calmodulin-binding domain of neuromodulin [1]. In our present work, analysis of chemical shift behavior against pressure is mainly used to probe the conformational change of a protein–protein complex.

NMR chemical shift carries a wealth of information regarding the environment and the local electronic structure in the vicinity of the nucleus under study [2]. Thanks to some pioneering works in the field [3–7] and to super conducting magnets now available at very high fields, chemical shifts have become particularly sensitive parameters for local conformational changes in biopolymers. In the high-pressure NMR studies carried out at 17.6 T, $^{1}H$ and $^{15}N$ chemical shifts have been successfully used to report site-specific structural changes of protein structures under pressure [8–19]. In particular, amide $^{1}H$ and $^{15}N$ chemical shifts sensitively report structural changes of a polypeptide backbone [9–11,13–17]. More specifically, $^{1}H$ chemical shifts are shown to be sensitive to NH$\cdots$O=C distances [9,14,15], whereas $^{15}N$ shifts reflect torsion angle variations ($\phi$, $\psi$, $\chi_1$) of the polypeptide backbone [11,18]. For most previously studied proteins such as lysozyme [8], BPTI [9,11,12], gurmarin [10], (1–36) helix of bacteriorhodopsin [17], protein G [14] and melittin [15], the $^{1}H$ and $^{15}N$ chemical shifts revealed surprising linearity with pressure in the pressure range between 1 bar and 2000 bar. The linear chemical shift change with pressure is understood as resulting from a linear change in averaged internuclear distances and torsion angles, and is considered to arise from a small shift of population within the basic native ensemble [11].

Recently, amide $^{1}H$ and $^{15}N$ chemical shifts of a binding domain of a signal transducing protein, the Ras-binding domain (RBD) of the guanine nucleotide dissociation stimulator (RalGDS) for GTPase Ral, has been found to show marked non-linear behavior with pressure, besides local unfolding at relatively low pressure [20]. Similar non-linearity has been found in β-lactoglobulin [16]. The marked non-linearity cannot be explained by a shift of population within the basic native ensemble (N), but only by the involvement of a conformational ensemble (N′) different from that of N. The latter must have a smaller partial molar volume than N so that its population increases with pressure and gives non-linear shift [16]. Thus, the non-linear behavior of pressure shift is a novel probe for an alternative conformer N′, which lies energetically close to N, but is not usually recognizable because of its small

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relative population at 1 bar. Such an alternative conformation could be crucially important in protein function, particularly for signal transduction function mediated by protein–protein interaction. In the present work, the presence of a low-lying excited state conformer is examined in $^{15}$N-labeled RalGDS-RBD and its complex with a small GTPase, Rap1A, one of its effector proteins in its signal transduction pathway. Rap1A is a member of the Ras superfamily and has almost the same three-dimensional structure as Ras [21]. Pressure-induced $^{1}$H and $^{15}$N chemical shifts of individual amide groups are analyzed quantitatively in terms of linear and non-linear contributions with respect to pressure.

## 2. Materials and methods

### 2.1. Sample preparation

$^{15}$N-uniformly labeled RBD of human RalGDS (amino acids 11–97) was prepared as described earlier [22]. The $^{15}$N-enriched RalGDS-RBD was measured in its free form and in the complex with non-labeled Rap1A.$Mg^{2+}$.GppNHp. The GTP-analog GppNHp was used in place of GTP to prevent hydrolysis of the phosphodiester bond. The sample of the complex contained 1.2 mM RalGDS-RBD and 1.5 mM Rap1A.$Mg^{2+}$.GppNHp in 15 mM Tris–HCl, 150 mM NaCl and 10 mM dithioerythritol (DTE), pH 7.3, in 95% $^{1}H_2O$/5% $^{2}H_2O$. The presence of DTE prevents RalGDS-RBD and its complex from forming intermolecular or intramolecular disulfide bridges [23].

### 2.2. High pressure NMR spectroscopy

The on-line cell high-pressure NMR method [7] was used to study pressure-induced conformational changes. Two-dimensional $^{15}$N/$^{1}$H HSQC NMR measurements [24] at 750 MHz were performed on free $^{15}$N-labeled RalGDS-RBD [20] and on its complex with non-labeled Rap1A in an aqueous environment (95% $^{1}H_2O$/5% $^{2}H_2O$). The spectra were recorded at 297 K in the range of 30–2000 bar at 500 bar intervals. The spectral widths for $^{1}$H and $^{15}$N were 10 000 and 2400 Hz, respectively. 256 complex points were sampled in the $t_1$ domain and 2048 complex points in the $t_2$ domain with hypercomplex phase sensitive detection in $t_1$ [25]. Water suppression was accomplished with the WATERGATE technique incorporating the 3–9–19 pulse sequence [26]. At all pressures, chemical shifts were measured relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as an internal reference. The $^{15}$N chemical shifts were indirectly referenced to DSS [27]. Data were processed with the XWIN-NMR package (Bruker) and nmrPipe [28] running on a Silicon Graphics O2 workstation.

### 2.3. Analysis of pressure-induced shifts

When the shift is markedly non-linear, the amount of shift at 2 kbar [8–12] does not have a proper meaning. Hence, the analysis in terms of linear and non-linear components is considered more appropriate. We fitted the variation of experimental $^{1}$H and $^{15}$N chemical shifts of individual residues against pressure to the following equation to the second order in pressure [16]:

$$\delta_i = a_i + b_i(p-p_0) + c_i(p-p_0)^2 \quad (1)$$

where $p$ is the pressure (bar), $\delta_i$ is the chemical shift (ppm) for $i$-th residue, $c_i$ (ppm/bar$^2$) and $b_i$ (ppm/bar) are the second-order (non-linear) coefficient and the first-order (linear) coefficient (ppm/bar), respectively. $p_0$ is the atmospheric pressure (1 bar) and $a_i$ (ppm) is the chemical shift at this pressure. The linear coefficient represents the pressure response of mostly the basic native structure, whereas the non-linear coefficient represents the pressure response of the protein at elevated pressures.

## 3. Results

Fig. 1a and b show the superposition of the $^{15}$N/$^{1}$H HSQC spectra of $^{15}$N-uniformly labeled RalGDS-RBD at 30 bar (blue), at 1000 bar (green) and at 2000 bar (red) for the free form (residues 11–97) and the complex with Rap1A, respectively. Nearly all the cross-peaks in the spectrum shift continuously and reversibly with pressure. All the cross-peaks maintain their full intensities up to 1500 bar, and at 2000 bar the cross-peak intensities of only several residues, namely 32, 67, 79, 80, 89, decrease without new appearing cross-peaks. The observation indicates that the protein maintains its folded structure up to 2000 bar, in contrast to free RalGDS-RBD, for which an unfolding intermediate with local melting is found above ~500 bar [20]. Although pressure is generally known to cause dissociation of multimeric proteins [29], apparently no such event takes place either, because of the strong binding between RalGDS-RBD and Rap1A.$Mg^{2+}$ (dissociation constant = 0.01 mM at pH 7.4 at 310 K) [30]. The $^{1}$H and $^{15}$N resonance assignments of the free RBD of RalGDS-RBD(1–97) comprising residues 1–97 had been accomplished [21], complete $^{1}$H, $^{15}$N and $^{13}$C resonance assignments of the N-terminal truncated domain RalGDS-RBD (11–97) at 1 bar being given in the free form and the complex with Rap1A.$Mg^{2+}$.GppNHp (Maurer, T., Hermann, C., Horn, G. and Kalbitzer, H.R., unpublished results). Based on these, the assignments at other pressures were straightforward from the continuous pressure dependence of chemical shifts.

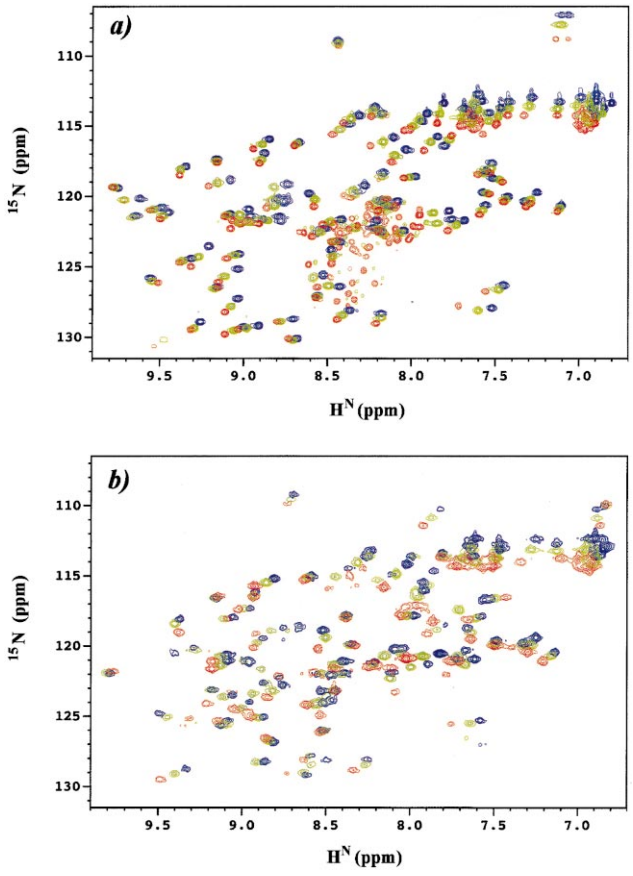


Fig. 1. $^{15}$N/$^{1}$H HSQC spectra of $^{15}$N-uniformly labeled RalGDS-RBD (residues 11–97) measured at 30 bar (blue), 1000 bar (green) and 2000 bar (red). a: Free RalGDS-RBD; b: RalGDS-RBD complexed with non-labeled Rap1A.$Mg^{2+}$.GppNHp.

We found distinct non-linearity with pressure in many of their cross-peaks of RalGDS-RBD in the free and complex forms (Fig. 2, $^{1}$H (a,b) and $^{15}$N (c,d)). The linear and non-linear coefficients $b_i$ and $c_i$ for individual amides are deter-

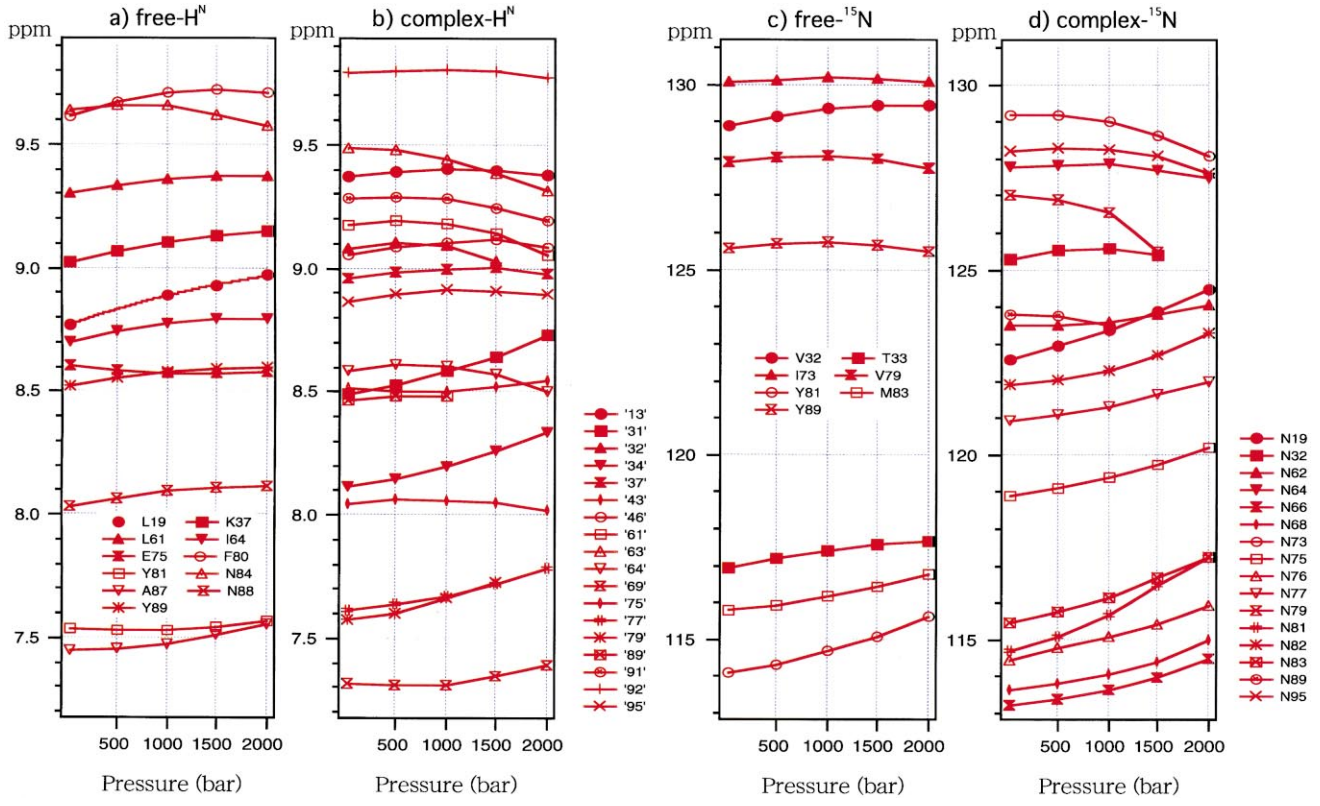


Fig. 2. Plot of amide $^{1}H$ and $^{15}N$ chemical shifts for selected residues of RalGDS-RBD against pressure. a: Amide protons of free RalGDS-RBD; b: amide protons of RalGDS-RBD complexed with Rap1A.$Mg^{2+}$.GppNHp; c: amide nitrogens of free RalGDS-RBD; and d: amide nitrogens of RalGDS-RBD complexed with Rap1A.$Mg^{2+}$.GppNHp.

mined based on Eq. 1. We found that the average absolute values of $b_i$ are similar for the free ($2.80\times10^{-4}$ ppm/bar for $^{15}N$; $0.50\times10^{-4}$ ppm/bar for $^{1}H$) and the complex forms ($1.72\times10^{-4}$ ppm/bar for $^{15}N$; $0.46\times10^{-4}$ ppm/bar for $^{1}H$). On the other hand, the non-linear coefficients $c_i$ (ppm/bar$^2$), plotted against the amino acid sequence in the free and complex forms (Fig. 3), show distinctly larger values in the complex than in the free form. The average absolute values of $c_i$ for the complex are $1.05\times10^{-7}$ ppm/bar$^2$ for $^{15}N$ and $1.76\times10^{-8}$ ppm/bar$^2$ for $^{1}H$, while the average absolute values of $c_i$ for the free RalGDS-RBD are $4.90\times10^{-8}$ ppm/bar$^2$ for $^{15}N$ and $1.01\times10^{-8}$ ppm/bar$^2$ for $^{1}H$. The locations of the amide groups with large non-linear shifts are shown by red, magenta, pink and blue (in the decreasing order) on the structure of RalGDS-RBD complexed with Rap1A (Fig. 4). The astonishing feature in Fig. 4 is that the largest non-linearity is found in regions remote (opposite) from the Rap1A-binding site of RalGDS-RBD.

## 4. Discussion

Previously, we showed that the RBD of free RalGDS starts to denature at relatively low pressures, giving at 2000 bar and at 297 K nearly an equal fraction of the protein in a native state (N), an intermediate state (I) and an unfolded state [20]. As Fig. 1 depicts, up to 2000 bar, there is no indication of denaturation or the formation of an unfolding intermediate I in RalGDS-RBD complexed with Rap1A. Clearly, the binding of the small GTPase Rap1A has greatly stabilized the native structure of RalGDS-RBD against pressure denaturation. Stabilization of a native protein structure against thermal or chemical denaturation upon binding with a ligand or a macromolecule has often been observed. The result in Fig. 1 shows that this is also the case for pressure denaturation.

The HSQC spectra of the free and complex forms of RalGDS-RBD showed distinctly non-linear chemical shift changes (Fig. 2). However, when analyzed based on Eq. 1, the averaged absolute values of the linear coefficients $b_i$ are almost the same for free RalGDS-RBD ($2.80\times10^{-4}$ ppm/bar for $^{15}N$; $0.50\times10^{-4}$ ppm/bar for $^{1}H$) and RalGDS-RBD complexed with Rap1A ($1.72\times10^{-4}$ ppm/bar for $^{15}N$; $0.46\times10^{-4}$ ppm/bar for $^{1}H$)), and even for BPTI ($2.79\times10^{-4}$ ppm/bar for $^{15}N$; $0.43\times10^{-4}$ ppm/bar for $^{1}H$) [10]. The linear coefficient represents the fluctuation of a protein structure extrapolated to 1 bar, namely the fluctuation of the dominant basic native conformer N, monitored at individual amide sites [16]. Therefore, the above observation indicates that the conformational fluctuation is comparable among the three proteins within the basic native conformer.

In contrast, large differences are found among these protein systems in the average absolute values of non-linear coefficients $c_i$: $4.90\times10^{-8}$ ppm/bar$^2$ for $^{15}N$ and $1.01\times10^{-8}$ ppm/bar$^2$ for $^{1}H$ for the free RalGDS-RBD and $1.05\times10^{-7}$ ppm/bar$^2$ for $^{15}N$ and $1.76\times10^{-8}$ ppm/bar$^2$ for $^{1}H$ for the complex, while they are negligibly small for BPTI. The large $c_i$ values for the free and complexed forms of RalGDS-RBD suggest the presence of N′ conformers that lie energetically close to N. In a simple case, the free energy difference

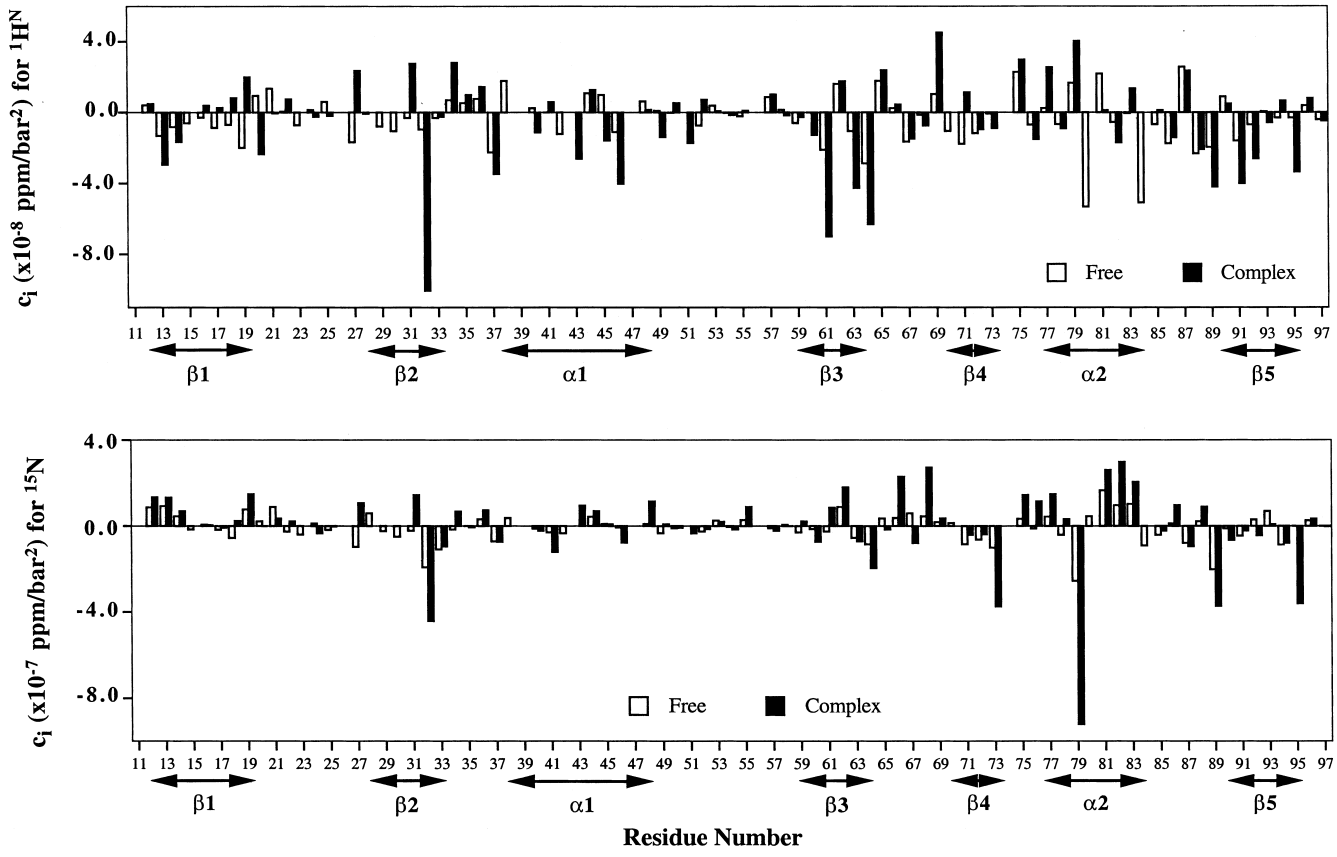


Fig. 3. Top panel: Plot of the second order coefficients ($c_i$) (Eq. 1) of the pressure-induced $^1$H chemical shifts for individual amide groups of RalGDS-RBD. Open column: Free RalGDS-RBD; filled column: RalGDS-RBD complexed with Rap1A.$Mg^{2+}$.GppNHp. Bottom panel: The same for the $^{15}$N chemical shifts.

$\Delta G = G_{N'} - G_N$ is given by

$$\Delta G = G_{N'} - G_N = \Delta G_0 + (p - p_0)\Delta V \quad (2)$$

where $\Delta V$ is the difference in partial molar volume between N and N′. $\Delta V$ is generally negative, because the higher energy state N′ usually has a more open and hydrated conformation than N. Thus, the free energy difference is expected to decrease and the fraction of N′ to increase with increasing pressure, resulting in a non-linear pressure shift. It is not possible to evaluate how much fraction of N′ coexists with N for RalGDS-RBD at 1 bar from the present data alone. However, the full reversibility of the pressure shifts and the non-linearity at very low pressures ($\sim$500 bar) indicate that some fraction of N′ is present at all pressures including 1 bar. In dihydrofolate reductase, an N′ conformer is detected at $\sim$10% at 1 bar, in which case the conformational transition is slow ($< 20$ s$^{-1}$) so that two separate signals are observed for N and N′ [18]. In the present case, the conformational transition between N and N′ is rapid in the NMR time scale so that the

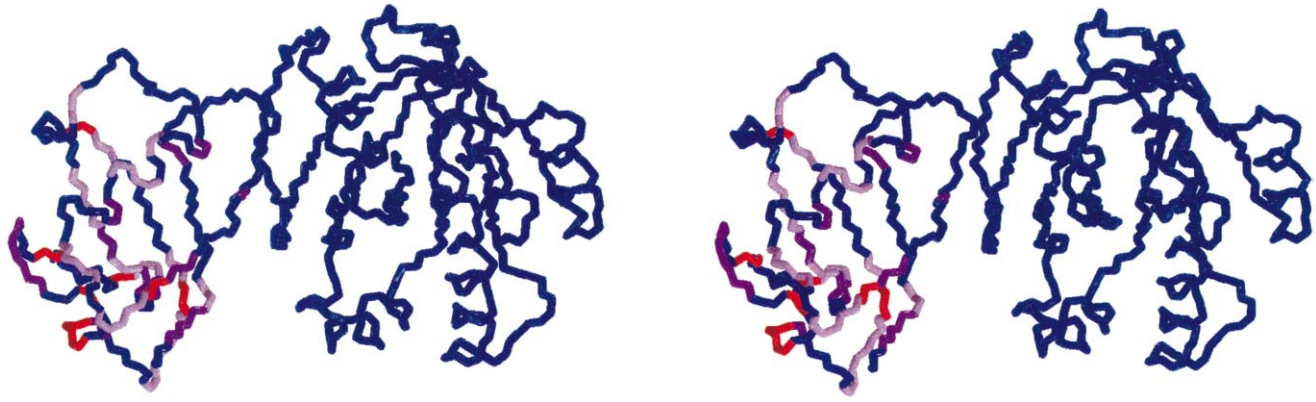

Fig. 4. Stereo view showing regions showing large non-linearity coefficient $c_i$ (Eq. 1) in the $^{15}$N pressure shifts of RalGDS-RBD complexed with Rap1A.$Mg^{2+}$.GppNHp. In the increasing absolute values of $c_i$ (ppm/bar$^2$), blue $<$ 5.0e-08 (plus unassigned peaks), 5.0e-08≦pink $<$ 1.0e-07, 1.0e-07≦magenta $<$ 2.5e-07, 2.5e-07≦red.

signals of N and N′ merge into one, suggesting that the rate of conversion is higher than $\sim 10^3$ $s^{-1}$.

The structure of N′ for the RalGDS-RBD–Rap1A complex cannot be shown with definite atomic coordinates. However, the region of conformational change of RalGDS-RBD in the transition from N to N′ can be depicted by mapping the non-linear shift coefficient $c_i$ on the three-dimensional structure of the complex (Fig. 4). A remarkable feature in Fig. 4 is that an unusually large conformational change takes place in a region of RalGDS-RBD remote from the Rap1A-binding site. It is likely that the conformational change from N to N′ in the RBD of RalGDS works as a switch to transmit the effector signal further to molecules of different RalGDS-dependent signaling pathways [31].

The present example shows that non-linear pressure shift is a sensitive measure of conformational fluctuation outside the range of the basic native conformer. Such a fluctuation can be slow (in the time scale of μs–ms) and is hard to detect site-specifically by most conventional techniques, but easy by high-pressure NMR. The general feature of this phenomenon will be discussed in more detail elsewhere [32].

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