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Conformation of 3′CMP bound to RNase A using TrNOESY

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Abstract

The conditions for accurately determining distance constraints from TrNOESY data on a small ligand (3′CMP) bound to a small protein (RNase A, <14 kDa) are described. For small proteins, normal TrNOESY conditions of 10:1 ligand:protein or greater can lead to inaccurate structures for the ligand-bound conformation due to the contribution of the free ligand to the TrNOESY signals. By using two ligand:protein ratios (2:1 and 5:1), which give the same distance constraints, a conformation of 3′CMP bound to RNase A was determined (glycosidic torsion angle, $\chi = -166°$; pseudorotational phase angle, $0° \leqslant P \leqslant 36°$). Ligand–protein NOESY cross peaks were also observed and used to dock 3′CMP into the binding pocket of the apo-protein (7rsa). After energy minimization, the conformation of the 3′CMP:RNase A complex was similar to the X-ray structure (1rpf) except that a C3′-endo conformation for the ribose ring (rather than C2′-exo conformation) was found in the TrNOESY structure.

Keywords: Tansferred nuclear Overhauser effect spectroscopy; Nuclear Overhauser effect; Pseudorotational phase angle; 3′CMP; RNase A

Transferred nuclear Overhauser effect spectroscopy (TrNOESY)[1] is extensively used in determining the structure of a ligand bound to a protein [1–6]. The observed nuclear Overhauser effect (NOE) is the population weighted average of the bound and free NOE under fast chemical exchange conditions. For a high molecular weight protein, the observed NOE arises essentially from the ligand in the bound state even if the free ligand is in excess [7–10]. This approach works well for proteins that exceed 25 kDa [7,11–13], but few structures of ligands bound to proteins less than 25 kDa have been solved using this approach [8,14–16]. Theoretical calculation [17] shows that the NOE from small ligands can contribute substantially to the observed signal when the protein is less than 20 kDa. Although direct nuclear magnetic resonance (NMR) methods can be employed for ligand:protein complexes less than 25 kDa, these approaches become more difficult when the ligand is not tightly bound to the protein and/or if few inter-proton constraints are present between ligand and protein. In this study, we explore the usefulness and limitations of TrNOESY experiments in generating a ligand structure when bound to a protein under 20 kDa.

We have chosen the cytidine 3′-monophosphate:ribonuclease A (3′CMP:RNase A) system, since it involves a small ligand bound weakly to a small protein. RNase A is a 13,686 Da protein containing 124 residues in a single polypeptide chain [18]. The X-ray structure of RNase A was first determined by Kartha et al. [19]. The enzyme consists of a long four-stranded anti-parallel β-sheet and three short α-helixes. The X-ray structures of RNase A with different pyrimidine nucleotide inhibitors were solved to help understand the catalytic mechanism [20,21], which involves His-12, His-112 and Lys-41. The ^{1}H chemical shifts of RNase A in aqueous solution were assigned [22], and the solution structure of the apo-protein was determined by NMR

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[1] *Abbreviation used:* RNase, ribonuclease A; 3′CMP cytidine 3′-monophosphate; TrNOESY, transferred nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy.

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spectroscopy [23]. The NMR structure of apo-RNase A is very similar to the reported X-ray structures.

Simple pyrimidine nucleotides are competitive inhibitors of RNase A, and their binding constants as a function of pH are known [24–31]. Under the conditions in this study, the 3′CMP:RNase A complex has a dissociation constant of about 50 μM, which is well suited for TrNOESY experiments. We show here that by maintaining a low ligand:protein ratio, the free ligand contribution to the NOE can be minimized and an accurate three-dimensional (3D) structure for the ligand bound to the protein can be obtained. In addition, because we observed protein:ligand NOEs at this low protein–ligand ratios in addition to the TrNOESY, these NOE constraints were used to dock the ligand into the apo-structure (either NMR or X-ray) to achieve a holo-protein complex that is essentially in agreement with the X-ray crystal structure of 3′CMP bound to RNase A [20].

Materials and methods

Materials

3′CMP (lot # 36H7822) and RNase A (lot # 104H7110) were purchased from Sigma Chemical Co. and used without further purification. D_2O (99.83%) was purchased from Cambridge Isotope Lab. Acetic acid-*d*4 (99.8%) was purchased from Aldrich Chemical Co.

Preparation of protein sample

RNase A was dissolved in sodium acetate buffer solution (0.1 M, pH 5.5), and the concentration was determined spectrophotometrically by using $\varepsilon_{278} = 9800\ M^{-1}\,cm^{-1}$ [1]. The concentration of 3′CMP was measured by using $\varepsilon_{260} = 7600\ M^{-1}\,cm^{-1}$ [24]. The pH was not corrected for isotope effects.

NMR measurements

Standard ^1H NMR experiments [32,33] were run on a Bruker DRX-400 MHz spectrometer at 300 K without spinning. Presaturation of HDO was used for all ^1H experiments. The 2D nuclear Overhauser effect spectroscopy (NOESY) spectra were collected with a 4160 Hz spectral width at 60, 90, 130, 160, 190 and 220 ms mixing times. Data was processed with a Gaussian window function in the t2 dimension and with a 60° cosine-square function and zero filled to yield a 2048×1024 matrix. T1 noise was reduced by dividing the first data point by 2 before the second FT [34]. Baseline correction using a filter function was applied to all spectra [35].

To identify spin-diffusion effects, a transferred rotating frame NOE correlated spectroscopy (TrROESY) experiment was run under the same condition as the TrNOESY experiment at a single mixing time (210 ms). The 2D rotating frame NOE correlated spectroscopy (ROESY) spectra were acquired using spin-lock times of 60, 90, 120, 150, and 180 ms [5]. Coherent magnetization transfer that occurs by J-coupling pathways, was eliminated by using a $180°(x)180°(-x)$ spin-lock sequence [36]. The experimental conditions were the same as the TrNOESY experiments. For the chemical shift assignment of protons on the ribose, the 2D TOCSY spectrum was run with a spin-lock time of 28 ms [35].

Molecular modeling

Simulated annealing and energy minimization of 3′CMP were conducted on a Silicon Graphics Octane workstation in Sybyl 6.4 (Tripos). Simulated annealing without distance constraints was carried out for 20 cycles to generate a family of different ribose ring conformations of 3′CMP. The molecules were heated to 1000 K and equilibrated for 2 ps, which was followed by exponential cooling to 300 K and annealing for 5 ps. To increase the number of conformations about the glycosidic bond of 3′CMP, we changed the χ value of the starting structures to ensure that different *anti* and *syn* conformations were present for each ribose ring conformation of 3′CMP. Forty starting conformations of 3′CMP were used to generate the final structure. Energy minimization was done for at least 400 iterations or until the energy converged by using Powell minimization (*in vacuo*). Two different force fields, Amber [37] and MMFF94 [38], were used with a distance-dependent dielectric constant of 4, a non-bonding cutoff of 8 Å and Kollman and MMFF94 charges. NMR distances were used with a constraint range of ±5% of each value. A penalty energy of 200 kcal/(mol Å^2) was applied for distances outside this range. The final structures were ordered according to energy. Structures with NOE violations (energies that were greater than 20 kcal/mol above the low-energy structure) were excluded. The final NMR derived 3′CMP structure was then used to provide a docked structure of the ligand–protein complex. The NOE contacts found in the 150 ms NOESY spectrum using a 2:1 3′CMP:RNase A were assigned as strong (1.8–2.5 Å), medium (1.8–3.0 Å), or weak (1.8–5.0 Å). The RNaseA: 3′CMP structure was determined by docking the TrNOESY structure of the ligand into the binding pocket of the X-ray structure of either the holo-(1rpf) or apo-protein (7rsa). The complex was energy minimized with the 3′CMP TrNOESY structure using the NMR-derived constraints. Protein residues within 5 Å of 3′CMP were allowed to minimize.

Theoretical details

RNase A was titrated with 3′CMP, and the 1D ^1H spectra were analyzed to determine a dissociation constant, K_d, of 3′CMP bound to RNase A. The binding of the ligand (L) to a single site on the protein (P) is a two-state second-order exchange [39]:

$$L + P \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

K_d is given by

$$K_d = k_{off}/k_{on} = ([L]_0 - [PL])([P]_0 - [PL])/[PL] \quad (1)$$

where $[L]_0$ and $[P]_0$ are the total concentration of the ligand and protein, respectively. For fast exchange, a single chemical shift is observed (δ_{obs}), which is the weighted average of the chemical shifts of the bound (δ_{PL}) and free states (δ_L) [40]:

$$\delta_{obs} = \delta_L[L]/[L]_0 + \delta_{PL}[PL]/[L]_0 \quad (2)$$

Eqs. (1) and (2) are rearranged to express δ_{obs} as a function of total ligand and protein concentrations, K_d and the chemical shift difference of the ligand in its bound and free states:

$$\delta_{obs} - \delta_L = (\delta_{PL} - \delta_L)\Big\{\big([L]_0 + [P]_0 + K_d\big) - \big([L]_0 + [P]_0 + K_d\big)^2 - 4\big([L]_0[P]_0\big)^{1/2}\Big\}\Big/2[L]_0 \quad (3)$$

The data were fitted as a plot of $\delta_{obs} - \delta_L$ *vs* $[L]_0$ using nonlinear regression.

The volumes of the NOE cross peaks and diagonal peaks were determined with the same scaling factor for all spectra. To extend the linear region of the NOE build-up curve, the percentage NOE intensity (I) was taken to be the ratio of the NOE cross peak volume divided by the volume of the diagonal peak of H1′ at the same mixing time [10,41]. The NOE build-up curves were fitted to a second degree polynomial equation to determine the initial NOE build-up rate. The relation between NOE intensity (I) and mixing time (τ_m) is:

$$I(\tau_m) = 1 - \exp(-\mathbf{R}\tau_m) = \mathbf{R}\tau_m - (1/2)\mathbf{R}^2\tau_m^2 + (1/6)\mathbf{R}^3\tau_m^3 - \cdots \quad (4)$$

where $\mathbf{R}$ is the relaxation matrix, which has elements, R_{ij}, that describe the initial build-up rate of the NOE cross peak between H_i and H_j.