Protein Conformation

Design of a Conformation-Sensitive Xenon-Binding Cavity in the Ribose-Binding Protein**

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NMR spectroscopy with laser-polarized ^{129}Xe has been used to investigate biomolecular systems, for example, detecting protein cavities in myoglobin, lipoxygenase, lysozyme, and a lipid-transfer protein, through interaction-induced chemical shift changes.[1–4] With the maltose-binding protein (MBP) from *E. coli*[2] and the chemotaxis protein Y[3] the chemical shift of interacting ^{129}Xe was found to respond to the protein conformation because of cavities that change size or shape in different conformers,[3,4] altering the shift of bound ^{129}Xe or the xenon binding affinity. Xenon probing requires no protein derivatization, and exploits simple spectra with no background signals and high sensitivity through optical polarization.[2,3]

It has been estimated that about half of the natural proteins contain cavities of sufficient size to bind xenon,[5] but only a few can be expected to bind xenon in a conformation-sensitive manner. Our studies of the conformation-sensitive xenon binding in MBP[4] led us to attempt to engineer a cavity for conformation-sensitive xenon binding into the ribose-binding protein (RBP), which lacks an intrinsic site of this type.

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Periplasmic transport proteins, including MBP and RBP, close around their ligands in a "clam-shell"-like manner as they bind.[6] The ^{129}Xe chemical shift in RBP solutions responds only slightly to this process (Figure 1). Xenon in protein solutions exchanges rapidly among all available sites of interaction giving a single observed chemical shift (δ_{obs}), which is an average over all site-specific chemical shifts (δ_i) weighted by their respective binding constants (K_i) [Eq. (1)].[3,7,8]

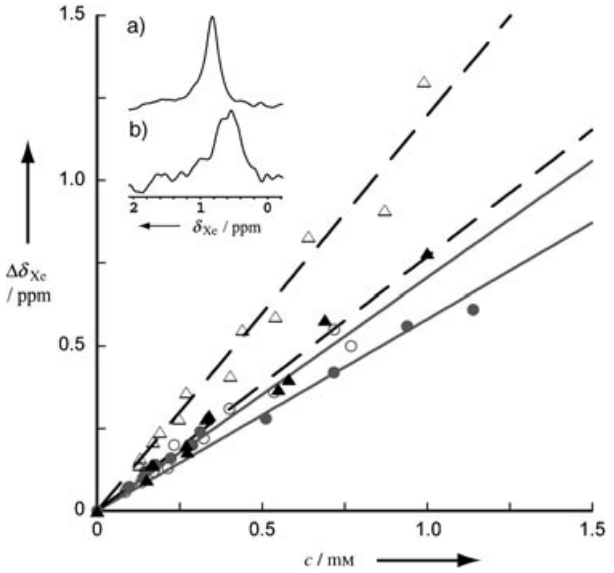

Figure 1. Change in ^{129}Xe chemical shift ($\Delta\delta_{Xe}$) with protein concentration (c) for the open (○, solid gray line) and closed (●, solid gray line) conformations of wild-type RBP and for the open (△, black dashed line) and closed (▲, black dashed line) conformations of L19A RBP. $\Delta\delta_{Xe}$ is the difference between the ^{129}Xe chemical shifts of each titration point and the buffer. Insets: a) ^{129}Xe NMR spectrum for 0.64 mM L19A RBP in the open conformation at a chemical shift of 0.83 ppm. b) ^{129}Xe NMR spectrum for 0.69 mM L19A RBP in the closed conformation at a chemical shift of 0.58 ppm. The ^{129}Xe chemical shift and linewidth are sensitive to the conformation of L19A RBP but not to that of wild-type RBP. The source of linewidth sensitivity is under current investigation.

$$\delta_{obs} = (\sum \delta_i K_i)\, c(\text{protein}) \qquad (1)$$

The concentration-normalized chemical shift ($\Sigma\delta_i K_i$) values (slopes of the lines) for the open and closed conformations of RBP are (0.7 ± 0.1) and (0.6 ± 0.1) ppm mM^{-1}, respectively, whereas for MBP these are (1.2 ± 0.1) and (2.1 ± 0.1) ppm mM^{-1}.[2] The low sensitivity of the ^{129}Xe shift to the RBP conformation indicates that wild-type RBP does not contain a conformation-sensitive xenon-binding cavity.[3,4] A search of the RBP structures with the program VOIDOO, which identifies internal voids, found a cavity located away from the ligand-binding cleft (Figure 2). Its size, about 50 Å^3, is the same in the open and closed conformations.

Mutating a buried, nonpolar side chain, such as Leu, Ile, or Phe, to a smaller nonpolar side chain, such as Ala, can create a cavity of sufficient size to bind xenon.[4,5,9] Bulky

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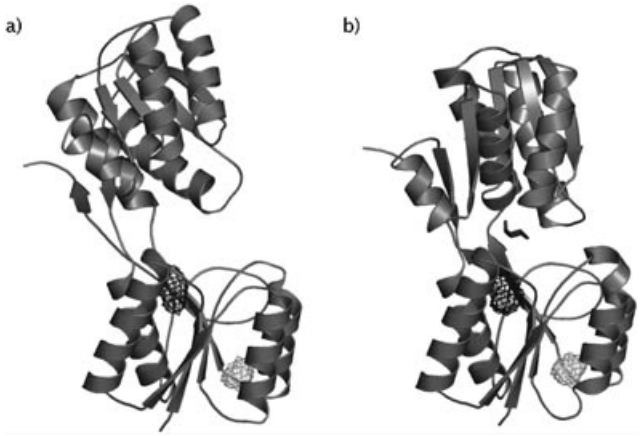

Figure 2. Cartoon backbone depictions of a) the open, or ligand-free, b) the closed, or ligand-bound, conformation of RBP.[6,11] Shown in light gray mesh is the surface of the cavity present in wild-type RBP that does not bind xenon; it is located away from the binding site and is the same size in both conformations. The black mesh shows the cavity created by the L19A mutation. Its increased size in the closed conformation is apparent as well as its proximity to bound ribose (with the ring shown by black sticks) and the loops that connect the two domains.

nonpolar side chains in regions that change structure between the two conformers were inspected and six potential sites for mutation were selected for initial screening: L19, L88, F187, F214, I233, and I240. The effect of a change to alanine at each site was evaluated by deleting atoms in the PDB file and calculating the size of the resulting cavity. Two changes produced cavities that differed significantly in size (~30 Å^3) between the two conformations, and L19A RBP was selected to test experimentally. The cavity sizes estimated by VOIDOO for the open and closed conformations of L19A RBP are 74 and 102 Å^3, respectively. Smaller cavities can result if residues surrounding a cavity created by mutation relax.[9,10] This was not considered a significant problem in the design process because xenon has been shown to bind to small flexible cavities, increasing the cavity volume by restoring the lining residues to their original positions.[9]

Figure 2 shows that the cavity of L19A RBP is directly below the ribose-binding cleft near loops that serve as a hinge during ligand binding. Of the residues that line the cavity, F15, F16, N64, and D89 make direct contact with ribose in the closed conformation.[11] The proximity of the cavity to functionally critical residues could affect the conformation change and decrease affinity for ribose. The affinities of wild-type and L19A RBP for ribose were measured using isothermal titration calorimetry (ITC) which gave K_d values of (0.11 ± 0.01) μM for wild-type (in agreement with previous work[12]) and (0.30 ± 0.05) μM for L19A RBP. The concentration-normalized ^{129}Xe shifts for open ((1.2 ± 0.1) ppm mM^{-1}) and closed L19A RBP ((0.8 ± 0.1) ppm mM^{-1}) were determined (Figure 1). The mutation increases the ^{129}Xe shift of the open conformation by 0.5 ppm mM^{-1} and of the closed conformation by 0.2 ppm mM^{-1}, enhancing the discrimination of open and closed conformations.

To verify that xenon binds in the designed cavity, ^{1}H-^{15}N correlation spectra (HSQC) were obtained in the presence and absence of xenon as done previously for MBP and other proteins.[4,13] Resonance signals that shift upon xenon addition are primarily from amide protons that line the cavity.[4,13] ^{1}H-^{15}N HSQC spectra of open and closed wild-type RBP were collected in the presence and absence of 90 mM xenon. The overlayed spectra of wild-type RBP show that 90 mM xenon does not shift signals in either wild-type conformer, indicating that xenon does not bind to the wild-type cavity.[14] In the L19A RBP spectra a subset of the signals shift relative to their wild-type counterparts, reflecting perturbations from introducing the L19A mutation (Figure 3). For both open and

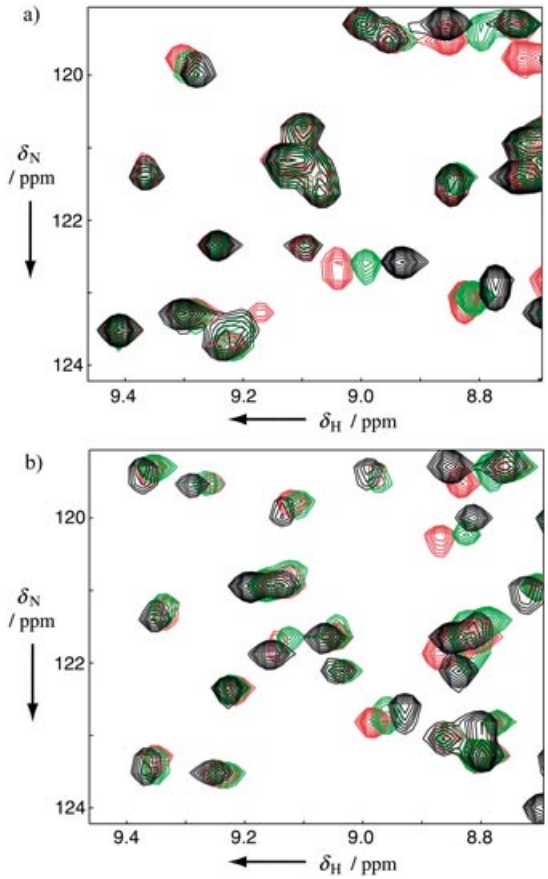

Figure 3. Representative portions of the ^{1}H-^{15}N HSQC spectra for the open (a) and closed (b) conformations of wild-type RBP in the absence of xenon (black), L19A RBP in the absence of xenon (red), and L19A RBP in the presence of 93 (a) or 72 mM (b) xenon (green).

closed L19A RBP specific resonance signals shift with increasing xenon concentration, indicating specific binding.[4] Every signal that shifts with increasing xenon concentration also shifts upon introduction of the L19A mutation, indicating that residues affected by xenon binding are associated with the introduced cavity, verifying creation of a specific xenon-binding site. Several signals affected by xenon binding shift toward the wild-type signals, suggesting that xenon restores these residues to their original environments, as in T4 lysozyme.[9]

The xenon binding affinity was measured by following the change in amide shift with xenon concentration (Figure 4).[4] Five resonance signals that shifted at least ten Hertz in each conformation were fitted as previously described[4] and averaged to give values of (70 ± 30) and $(40 \pm 20)\,\text{M}^{-1}$ for the open and closed conformations, respectively, yielding affinities similar to those for MBP.[4]

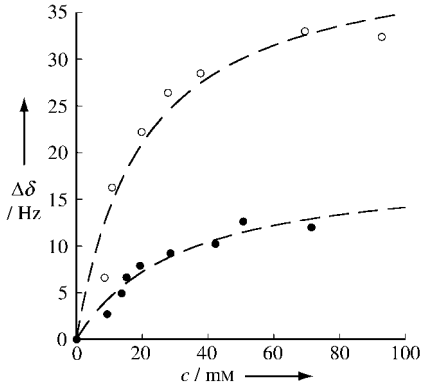

Figure 4. The total change in amide ^{1}H chemical shift ($\Delta\delta$) versus xenon concentration (c) for the same resonance signal in the open (○) and closed (●) conformations of L19A RBP. Similar binding curves and fits were obtained for four other resonance signals. The different xenon binding affinity of the open and closed conformations reflects the conformation-sensitive nature of the xenon-binding cavity created by the L19A mutation.

The limiting xenon chemical shifts relative to the shift in buffer, (δ_i) [Eq. (1)], in the xenon-binding cavity were determined by comparing titrations in which the cavity was available and blocked,[4] giving $\delta_{\text{open}} = (7 \pm 3)$ and $\delta_{\text{closed}} = (5 \pm 3)$ ppm. Cavity-induced downfield shifts have been shown to be inversely proportional to cavity volume in proteins, zeolites, and clathrates.[4,15,16] The shift values for the cavity of L19A RBP indicate that it is smaller in the open conformation, as predicted by the design process.

Cavity-creating mutations in T4 lysozyme were shown to decrease the Gibbs energy of unfolding in proportion to the size of the cavity.[11] Urea-induced unfolding of open wild-type and L19A RBP was followed by circular dichroism, and analyzed to give ΔG_u values of (5.8 ± 0.1) and (3.3 ± 0.1) kcal mol^{-1}, respectively. Combining this with ribose affinity measurements from ITC and the calibration from T4 lysozyme[11] gives estimates of 25 Å^3 (open) and 54 Å^3 (closed) for the cavities in RBP, a change of about 30 Å^3 as modeled.[14]

Analysis of the L19A–^{129}Xe interaction shows that the conformational responsiveness arises from both a higher xenon affinity and a larger shift induced by the cavity in the open conformation contributing to the averaged limiting ^{129}Xe chemical shift (δ_{obs}) [Eq. (1)]. The higher xenon binding affinity of the smaller cavity likely arises from increased van-der-Waals contact in the smaller cavity.[9]

We were able to introduce a conformation-sensitive xenon-binding cavity into the ribose-binding protein with a straightforward design process, demonstrating the feasibility of engineering ^{129}Xe NMR reporter sites into proteins of interest for assaying ligand binding or conformation changes. The idea of designed sites for xenon can be further generalized[17] and is being applied to biological problems.

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