# Closed Form of Liganded Glutamine-Binding Protein by Rotational-Echo Double-Resonance NMR†

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ABSTRACT: Rotational-echo double-resonance NMR has been used to determine internuclear distances in the complex of glutamine-binding protein and its ligand, L-glutamine. The distances between the ligand and Tyr185 are consistent with the results of molecular dynamics simulations constrained by three REDOR-determined distances to His156. This model is also consistent with six other REDOR-determined internuclear distances, most of which agree with values from the first report of an X-ray structure of the complex of glutamine-binding protein and L-glutamine.

We recently reported the determination of several internuclear distances in the complex between glutamine-binding protein (GlnBP)[1] and its ligand, L-glutamine (Hing et al., 1994), using stable-isotope labeling and rotational-echo double-resonance (REDOR) NMR. GlnBP is a 25 kDa protein that is an essential component of the glutamine transport system in *Escherichia coli* (Ames, 1986). The REDOR-determined distances were also used as constraints in molecular dynamics calculations which led to a proposed structure for the complex. The modeling was limited by the fact that (i) REDOR distances were measured from the ligand to residues in only one of the two GlnBP domains and (ii) the simulations were not sufficiently long to observe a stable closed structure. In this paper, we describe a new type of REDOR measurement which includes the determinations of distances between L-glutamine and residues in both domains. We also compare these distances to those from an extended molecular dynamics simulation, as well as to distances from the first report of an X-ray crystallograpic analysis of the complex (Hsiao et al., 1996).

## MATERIALS AND METHODS

*Solid-State NMR Samples and Spectrometer.* Isotopically labeled protein was prepared following the procedure described previously by Hing et al. (1994). GLnBP labeled by [*ring*-4-$^{13}$C]Trp was complexed to L-[*amine*-$^{15}$N]glutamine in a solution that was 2.7 mg/ml protein, 1% poly(ethylene glycol) 8000, 20 mM sucrose, 2 mM 4-morpholinepropanesulfonic acid, and 1 mM dithiothreitol (Studelska et al., 1996). The solution containing the ternary complex was frozen at −20 °C and then cooled with liquid nitrogen before lyophilization. Two other complexes were prepared that contained buffer but no cryo- or lyoprotectants. [*m*-$^{19}$F]Tyr-[*uniform*-$^{15}$N]GlnBP was complexed as described before (Hing et al., 1994) to (i) L-[5-$^{13}$C]glutamine and (ii) unlabeled L-glutamine. Powdered, lyophilized protein complex (100−200 mg) was packed into 7.5 mm outside diameter zirconia rotors fitted with Kel-F spacers and drive cap. Cross-polarization, magic-angle spinning spectra were obtained at 4.7 T using a four-channel probe with a single 9 mm diameter solenoidal coil which permits $^{1}$H, $^{19}$F, $^{13}$C, and $^{15}$N detection or dephasing (at 200, 188, 50, and 20 MHz, respectively). Fluorine incorporation into GlnBP was measured by direct $^{19}$F NMR detection, calibrated by comparisons to spectra of materials with known fluorine content. REDOR experiments began after a 2.0 ms matched spin-lock cross-polarization transfer from protons at 50 kHz, followed by proton decoupling at 100 kHz. The sequence repetition time for most experiments was 2 s. There was no indication of large-amplitude molecular motion either from slow cross-polarization transfer rates or from unusually fast spin−lattice relaxation rates. The magic-angle stators were obtained from Chemagnetics (Fort Collins, CO). A controlled spinning speed of 5000 Hz was used for REDOR experiments.

*REDOR.* REDOR provides a direct measure of heteronuclear dipolar coupling between isolated pairs of labeled nuclei (Gullion & Schaefer, 1989). In a solid with an I−S-labeled spin pair, for example, the S-spin rotational echoes that form each rotor period following a proton to S-spin cross-polarization transfer can be prevented from reaching full intensity by insertion of two I-spin $\pi$ pulses per rotor cycle, one in the middle of the rotor period and the other at the completion of the rotor period (for the GlnBP experiments discussed here, I is either $^{19}$F or $^{15}$N and S is $^{13}$C). Both I- and S-spin pulses were applied using an XY8 phase-cycling scheme to suppress offset effects and compensate for pulse imperfections (Gullion et al., 1990). The REDOR difference (the difference between an S-spin NMR spectrum obtained under these conditions and one obtained with no I-spin $\pi$ pulses) has a strong dependence on the dipolar coupling and hence the internuclear distance. Measurements of carbon−

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[1] Abbreviations: GlnBP, glutamine-binding protein; REDOR, rotational-echo double resonance; NMR, nuclear magnetic resonance.

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Table 1: Comparison of Observed and Calculated Internuclear Distances for Liganded Glutamine-Binding Protein

| dephasing nucleus | observing nucleus | REDOR[a] (Å) | model[b] (Å) | X-ray[c] (Å) |
|---|---|---|---|---|
| $C_\delta$ of L-Gln | $N_{\epsilon 2}$ of His156 | $4.3 \pm 0.2$[d] | 4.4 | 3.7 |
| $C_{\epsilon 1}$ of His156 | N of L-Gln | $6.2 \pm 0.2$[d] | 6.0 | 5.4 |
| $C_{\epsilon 1}$ of His156 | $N_\epsilon$ of L-Gln | >7[d] | 6.9 | 5.2 |
| $C_\delta$ of L-Gln | $N_\xi$ of Lys115 | $4.4 \pm 0.2$ | 5.6 | 3.5 |
| $C_\delta$ of L-Gln | $N_{\delta 1}$ of His156 | >6.5 | 5.9 | 5.2 |
| $F_{\epsilon 1}$ or $F_{\epsilon 2}$ of Tyr185 | $N_{\epsilon 2}$ of His156 | $8.1 \pm 0.5$ | 8.2 | 8.7 |
| $F_{\epsilon 1}$ or $F_{\epsilon 2}$ of Tyr185 | $N_{\delta 1}$ of His156 | $8.6 \pm 0.5$ | 9.5 | 8.6 |
| $F_{\epsilon 1}$ or $F_{\epsilon 2}$ of Tyr185 | $C_\delta$ of L-Gln | $9 \pm 1$[b] | 7.3 | 7.0 |
| N of L-Gln | $C_\xi$ of Tyr185 | $7 \pm 1$[b] | 6.6 | 4.4 |

[a] From Hing et al. (1994) unless otherwise noted. [b] This work. [c] Hsiao et al. (1996). [d] REDOR constraint for simulation.

nitrogen and carbon−fluorine internuclear distances of 6 and 12 Å, respectively, are possible (Gullion & Schaefer, 1989; Hing et al., 1994). The dephasing of magnetization in REDOR arises from a local dipolar field gradient and involves no polarization transfer. REDOR has no dependence on I or S chemical-shift tensors and does not require resolution of an I−S coupling in the chemical-shift dimension.

*Molecular Modeling of the GlnBP Complex.* The molecular modeling was done in three parts: (i) separate energy minimizations of L-glutamine and GlnBP, (ii) docking of the energy-minimized L-glutamine to the open form of GlnBP, and (iii) molecular dynamics simulation of the GlnBP-Gln complex.

The minimum-energy conformation of glutamine was determined by *Quanta 4.0 Grid Scan.* The four backbone torsion angles ($\psi$, $\chi_1$, $\chi_2$, and $\chi_3$) of L-glutamine were varied from 60 through 300° in 120° increments, each with 100 steps of energy minimization using a conjugate-gradient method. The most stable conformation had the following torsion angles: $\psi = 60.3°$, $\chi_1 = 65.3°$, $\chi_2 = 67.9°$, and $\chi_3 = -154.2°$. Next, the crystal structure of GlnBP (Hsiao, 1994) was minimized using 100 steps of a steepest-descent method, followed by 200 steps of the conjugate-gradient method. This procedure yielded an rms energy gradient of less than 0.1 kcal mol$^{-1}$ Å$^{-1}$ for the open GlnBP structure.

The energy-minimized L-glutamine was placed in the cleft of the energy-minimized GlnBP, imposing three REDOR distance constraints between glutamine and His156 (Table 1). The van der Waals contacts between glutamine and GlnBP were eliminated by constrained-energy minimization using 100 steps of the conjugate-gradient method. The force constant for each of the three constraints was 50 kcal mol$^{-1}$ Å$^{-1}$. The complex was then equilibrated for 20 ps, during which the velocity was either scaled or assigned if the temperature deviated from 300 K by more than ±5 K. After equilibration, the simulation was continued for 300 ps, during which time the REDOR constraints to His156 were maintained. In the simulation, Newton's equations of motion were integrated numerically, using the Verlet algorithm (Verlet, 1967), with a time step of 1 fs under a constant NVE condition. The united-atom model was employed for both minimizations and simulations. Electrostatic energies were treated with a distance-dependent dielectric constant of 4, truncated at 8 Å by a switching function. Water molecules were not included in the binding site. A separate 100 ps simulation was performed for GlnBP in the absence of glutamine ligand.

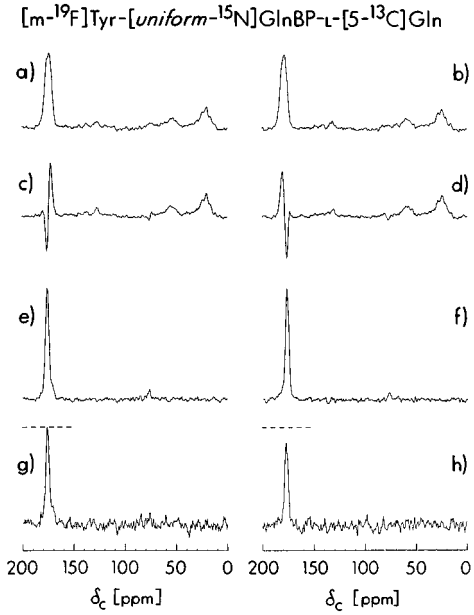


FIGURE 1: REDOR $^{13}$C NMR spectra of [*m*-$^{19}$F]Tyr-[*uniform*-$^{15}$N]-GlnBP-L-[5-$^{13}$C]Gln after evolution for 120 rotor cycles with magic-angle spinning at 5 kHz. Full-echo spectra (no $^{19}$F dephasing pulses) are shown in parts a and b. DANTE inversion of a part of the carbonyl-carbon peak near 180 ppm is shown in part c and near 175 ppm in part d. The results of substracting part c from part a and part d from part b are shown in parts e and f, respectively. These are $S_o$ spectra. The results of double subtractions of echo spectra with and without $^{19}$F dephasing pulses, with and without the DANTE inversion, are shown in parts g and h, respectively, at twice vertical gain. These are $\Delta S$ spectra.

All the computations were carried out on a Silicon Graphics Power Challenge computer. The 300 ps simulation of the GlnBP complex with 2112 atoms required 8 days of (single) CPU time using an R8000 processor.

## RESULTS

*REDOR for [$^{19}$F]Tyr-Labeled GlnBP.* The dominant peak in the $^{13}$C NMR spectrum of [*m*-$^{19}$F]Tyr-[*uniform*-$^{15}$N]-GlnBP-L-[5-$^{13}$C]Gln (Figure 1) is due to the 277 carbonyl carbons at natural abundance in GlnBP (3 intensity units) and the single $^{13}$C carbonyl label in bound glutamine (1 intensity unit). The resonance for the latter is not resolved but appears at low field, exclusively in the left-hand half of the broad carbonyl-carbon peak. This means that 40% of the left-hand half of the carbonyl-carbon peak is due to glutamine label and 60% is due to protein, while all of the right-hand half of the peak is due to protein. Using rotor-synchronized DANTE selection (Bork & Schaefer, 1988), the REDOR $\Delta S/S_o$ of the left-hand half of the line is 0.44, while that of the right-hand half is 0.38 (Figure 1). Thus, $0.4(\Delta S/S_o)_{label} + (0.6)(0.38) = 0.44$, which leads to $(\Delta S/S_o)_{label} = 0.53$. Fifty-three percent dephasing in 120 rotor cycles with 5 kHz magic-angle spinning corresponds to a $^{13}$C−$^{19}$F dipolar coupling of 35 Hz and, assuming only a single nearest-neighbor fluorine, most likely Tyr185 (Hing et al., 1994), an internuclear separation of $9 \pm 1$ Å. In a separate REDOR experiment performed on the same protein complexed to natural-abundance glutamine, $\Delta S/S_o$ for the entire carbonyl-carbon line was 0.36 (spectra not shown), consistent with the results of Figure 1.

*REDOR for [$^{13}$C]Tyr-Labeled GlnBP.* The aromatic-carbon region of the REDOR $^{13}$C NMR spectra of [*ring*-4-

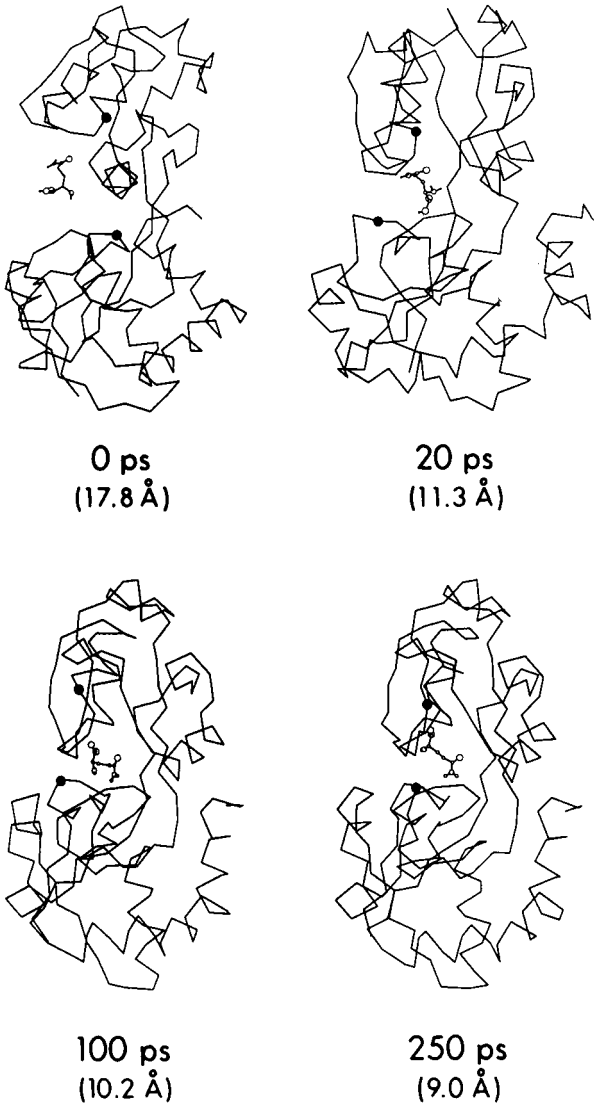


FIGURE 2: Backbone representation of the open (top left) and closed (bottom right) forms of glutamine-binding protein. L-Glutamine is shown in a ball-and-stick format. The open form is based on the crystal coordinates of the unliganded protein (Hsiao, 1994). Snapshots of the time evolution from the open to the closed form are based on a REDOR-constrained molecular dynamics simulation for the liganded protein complex. Positions of the peptide nitrogens of Glu74 (top) and Ser120 (bottom) are indicated by solid circles.

$^{13}$C]Tyr-GlnBP-L-[*amine*-$^{15}$N]Gln has peaks at 155 and 158 ppm of approximately equal intensity arising primarily from the ten labeled tyrosine residues (spectra not shown). The 3 ppm shift is associated with variations in the electronic structure of the oxygenated ring carbon and has been observed in other proteins with tyrosine $C_4$ labels (McDowell et al., 1996). A $\Delta S/S_o$ of 2% was observed for the 155 ppm peak after 96 rotor cycles of $^{15}$N dephasing. Assuming proximity of the $^{15}$N amine label to a single, nearest-neighbor Tyr $^{13}$C label, the 2% dephasing translates (Gullion & Schaefer, 1989) to a $^{15}$N−$^{13}$C distance of 7 ± 1 Å (Table 1).

Molecular Dynamics Simulation. Closing of the cleft of the GlnBp−Gln complex as a function of time is shown in Figure 2. The two domains start to approach one another after only 20 ps when the substrate is inside the cleft. The closing process is essentially completed after 100 ps; the cleft remains closed for the duration of the simulation. In the absence of glutamine, the GlnBP cleft does not close after 100 ps. The arrangement of L-glutamine to the three residues in the binding site for which REDOR measurements were made is shown in Figure 3. Closure of the cleft involves rotation about a single hinge region, a process which has been observed for other periplasmic binding proteins (Oh et al., 1993).

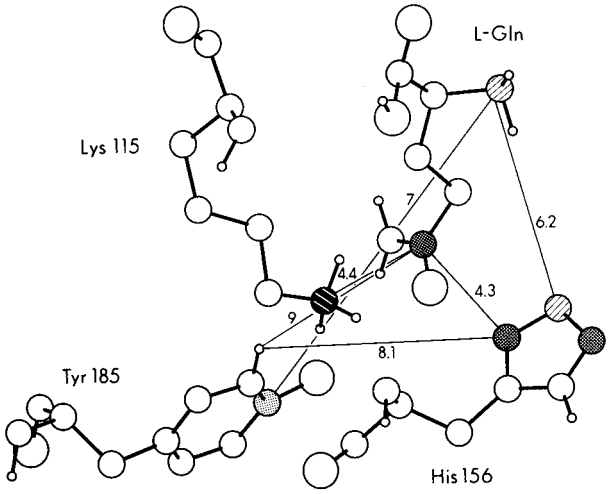


FIGURE 3: Ball-and-stick representation of the binding site of glutamine-binding protein showing REDOR-determined distances (in angstroms) between L-glutamine and residues Lys115, His156, and Tyr185. Labeled atoms (see Table 1) are highlighted.

## DISCUSSION

Even though the molecular dynamics simulation was constrained only by REDOR distances measured between L-glutamine and His156, the REDOR-measured distances to Lys115 (smaller domain) and Tyr185 (larger domain) are in good agreement with all those obtained from the model, as well as with most of those reported by crystallographic analysis (Table 1). The most important exception is the distance from the amine nitrogen of the ligand and the oxygenated aromatic carbon of Tyr185. The 4.4 Å distance of the X-ray structure is hard to reconcile with the observed 2% [$^{13}$C]Tyr REDOR dephasing by amine-$^{15}$N-labeled L-glutamine. At the moment, we have no explanation for this discrepency.

Agreement between the REDOR-constrained molecular dynamics model and the X-ray structure for hydrogen-bond formation is only fair (Hsiao et al., 1996). In particular, Asp10, Arg75, and Asp157 appear to be misplaced by the modeling (not shown). The mechanism for closure of the GlnBP cleft seems to be the hydrogen-bond bridges that L-glutamine makes as the ligand forms an electrostatic shield between the two domains. These kinds of interactions are certain to be dependent on the details of the interaction potentials used in the simulation. Thus, without experimental distances between L-glutamine and at least some of the residues forming hydrogen bonds as constraints, it is not surprising that the molelcular dynamics simulations do not define the positions of Asp10, Arg75, and Asp157. On the other hand, the model and X-ray structure disagree on the placement of Tyr185 (Table 1), for which there is also a discrepency between experimental REDOR and X-ray distances (Table 1). Perhaps future refinements of both REDOR-constrained simulations and X-ray structures will improve the overall agreement.

## REFERENCES

Ames, G. F.-L. (1986) *Annu. Rev. Biochem. 55*, 397−425.
Bork, V., & Schaefer, J. (1988) *J. Magn. Reson. 78*, 348−354.
Gullion, T., & Schaefer, J. (1989) *Adv. Magn. Reson. 13*, 57−83.
Gullion, T., Baker, D. B., & Conradi, M. S. (1990) *J. Magn. Reson. 89*, 479−484.
Hing, A. W., Tjandra, N., Cottam, P. F., Schaefer, J., & Ho, C. (1994) *Biochemistry 33*, 8651−8661.
Hsiao, C.-D., Sun, Y.-J., Rose, J., Cottam, P. F., Ho, C., & Wang, B.-C. (1994) *J. Mol. Biol. 240*, 87−91.
Hsiao, C.-D., Sun, Y.-J., Rose, J., & Wang, B.-C. (1996) *J. Mol. Biol. 262*, 225−242.
McDowell, L. M., Lee, M., McKay, R. A., Anderson, K. S., & Schaefer, J. (1996) *Biochemistry 35*, 3328−3334.
Oh, B.-H., Pandit, J., Kang, C.-H., Nikaido, K., Gokcen, S., Ames, G. F.-L., & Kim, S.-H. (1993) *J. Biol. Chem. 268*, 11348−11355.
Studelska, D. R., Klug, C. A., Beusen, D. D., McDowell, L. M., & Schaefer, J. (1996) *J. Am. Chem. Soc. 118*, 5476−5477.
Verlet, L. (1967) *Phys. Rev. 159*, 98−103.

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