Investigation of Slow Dynamics in Proteins: NMR Pulse Sequence Development and Application in Triosephosphate Isomerase

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Abstract

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The dynamics of proteins on the millisecond time scale are on the same time scale as typical catalytic turnover rates, and can sometimes be closely related to enzymes’ functions. Solid state NMR, equipped with magic angle spinning, is a very good technique to detect such millisecond dynamics, because it is suitable for many protein systems such as membrane proteins, and the anisotropic interactions recoupled in the solid state NMR can supply valuable geometric information regarding the dynamics. In this thesis, I mainly focus on the developing new dynamics detection pulse sequences based on previous Centerband-Only Detection of Exchange (CODEX) experiment and applying CODEX experiments to an enzyme system, triosephosphate isomerase (TIM), for studying the function of the millisecond dynamics in catalysis.

Two newly developed pulse sequences, Dipolar CODEX and R-CODEX use the $^{13}$C-$^{15}$N (Dipolar CODEX) and $^1$H-$^{13}$C or $^1$H-$^{15}$N (R-CODEX) dipolar couplings to detect dynamics. Compared with the chemical shift anisotropy used in the CODEX experiment, the dipolar coupling has a more direct relationship with the molecular geometry and could be better for extracting geometric information regarding reorientations. A special characteristic of the R-CODEX sequence is that the use of an R-type dipolar recoupling sequence can suppress the effect of $^1$H-$^1$H homonuclear couplings. This approach paves the way to detect both the correlation time and reorientational angle of the dynamics in fully protonated samples. These two pulse sequences are tested by detecting the $\pi$ flip motion of urea and methylsulfone imidazole. The R-CODEX experiment is compared with two other millisecond dynamics detection methods: 2D-exchange experiments and line-shape analysis, using the example of in crystalline L-phenylalanine hydrochloride. The millisecond ring flip motion of the aromatic ring in L-phenylalanine hydrochloride is characterized in detail for the first time. The comparison between these three methods
shows that the R-CODEX experiment does not require a chemical shift change in the process of the motion and that it can detect the dynamics even if there is the peak overlap in the spectra.

Triosephosphate isomerase (TIM) is a well-known highly efficient enzyme. Its loop motion (loop 6) has been extensively studied and been proven to be correlated with product release and be a rate-limiting step for the catalysis. Another highly conserved loop near the active site, loop 7 also has large changes in dihedral angles during ligand binding. Its motion is suspected to be correlated with loop 6 based on mutant experiments and solution NMR studies. However, the core sequence of loop 7, YGGS, is missing in the solution NMR spectrum. We assigned the GG pair in loop 7 (G209-G210) using $^{1,13}$C, $^{15}$N glycine labeling and solid state NMR experiments, and detected the loop 7’s motion using $^{1,13}$C glycine labeling and CODEX experiments. We found that loop 7’s motional rate ($300\pm100\text{ s}^{-1}$) at -10°C agrees well with previously detected motional rates of loop 6 extrapolated from higher temperatures using an Arrhenius plot. This suggests that the motion of loop 6 probably correlates with loop 7. At the same time, the line-shape analysis for another GG pair (G232-G233), which forms hydrogen bonds with the ligand, indicates a ligand release rate ($400\pm100\text{ s}^{-1}$) similar to loop 7’s rate, supporting the hypothesis that the ligand release is also probably correlated with the motion of loop 7 and loop 6.
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Chapter 1  Introduction
1.1 Protein function and slow motions
Heraclitus taught that everything in the world is in motion. This is also true for proteins, which are subject to various motions ranging from single bond vibrations on the femtosecond time scale to rearrangements of subunits over the course of several hours [1-3]. The idea that such dynamics play important roles in protein function has been widely accepted and is supported by both theoretical and experimental work [2-9]. The dynamics in proteins, including bond vibrations and librations, side chain rotations, collective “breathing” of the protein core, loop motions and the rearrangements of the subunits may be closely related to biological functions such as enzyme catalysis, signal transduction, protein transport and antigen recognition.

The goal of this thesis is to focus on protein dynamics that have correlation times longer than a millisecond. To my knowledge, this kind of slow dynamics can be related to several different processes and functions of proteins. The best-studied is enzyme catalysis. Although millisecond motions are too slow to directly influence the transition state, which has a lifetime of a femtosecond, or the chemical reaction coordinate, the slow conformational motion can prepare the correct electrostatic environment for catalytic reaction and facilitate substrate binding and product release [1]. This is supported by the fact that most of the detected millisecond dynamics are around the active pocket [10-12], and mutations that perturb dynamical processes are associated with significant loss of function even if the structure of the protein stays the same [13-15]. Meanwhile, the detected enzyme dynamics have frequency rates corresponding to the turnover rates, suggesting that millisecond dynamics may limit the overall catalytic reaction [10, 12, 16, 17]. Another important function of dynamics is in protein folding. The slow dynamics that carry out the interconversion between states separated by a high energy barrier in the energy landscape will affect the main folding direction [18, 19], and the slow large conformational changes that occur in chaperon proteins are also important to induce the rearrangement of the client to the right conformation [20]. Furthermore, slow dynamics processes are also involved in many molecular machines [21] such as those involved in protein synthesis, signal transduction [22] and muscle contraction [23]. Therefore, detecting these slow dynamics processes and understanding their mechanisms are critical to revealing the enigma of many biological functions.

1.2 Advantages of solid state NMR experiments in detection of slow dynamics
The essence of dynamics detection is to find and detect a physical parameter that can distinguish between different motional states and that can be studied to produce a quantitative dependence on the correlation time and reorientational geometry of the dynamics. Technological advances in physics and engineering have enabled the application of many techniques, such as fluorescence [24] and NMR methods [25], to
detecting protein dynamics. Compared with other methods, NMR, and especially solid state NMR, holds many advantages. The first advantage is that NMR does not require an extra label as an indicator. Aside from deuterium enrichment experiment, common NMR labeling schemes for example, replacing $^{12}$C and $^{14}$N with $^{13}$C and $^{15}$N, have a very small kinetic isotope effects (KIE) [26], and hence the NMR method can minimally disturb protein function. Additionally, NMR can detect the dynamics of multiple sites with atomic resolution. This is very helpful to study the correlation of motions in different parts of proteins. In the solid-state NMR, an additional advantage is that anisotropic interactions like the dipolar or quadrupolar coupling are only sensitive to an orientational change rather than an environmental change. For example, substrate binding to the active site will not be detected in the solid-state NMR methods based on the dipolar coupling if the target residue in the active site does not have a spatial motion. This can provide a good method for studying the relationship between protein dynamics and protein function. In the next section, these anisotropic interactions will be introduced.

1.3 The anisotropic interaction in solid state NMR

1.3.1 Chemical shift anisotropy

In the NMR experiment, the magnetic moments of nuclei precess about the main magnetic field at their respective Larmor frequencies. However, in reaction to the main magnetic field, the electrons that surround the nucleus also generate a secondary field that shifts the resonant frequencies of the nuclei from the Larmor frequency to a new frequency. This frequency shift induced by the shielding interaction from the surrounding electrons is the chemical shift, which is sensitive to environmental effects on electron structure and forms the basis of the modern NMR experiment.

Because atomic electron structure does not have spherical symmetry (except for the s-orbital), chemical shielding is expressed by a second order tensor. To describe the chemical shielding more conveniently, the principal axis frame (PAF) in which the chemical shielding tensor operates is diagonalized are used (Figure 1.1).

$$
\begin{pmatrix}
\sigma_{xx} & 0 & 0 \\
0 & \sigma_{yy} & 0 \\
0 & 0 & \sigma_{zz}
\end{pmatrix}
$$

Figure 1.1 The chemical shielding tensor in the principal axis frame (PAF). $\sigma_{xx}$, $\sigma_{yy}$ and $\sigma_{zz}$ are the principal values along the principal axis $x^{PAF}$, $y^{PAF}$ and $z^{PAF}$.

The chemical shielding Hamiltonian for a certain orientation can be easily obtained by transforming the shielding tensor in the principal axis frame to the lab frame, and the spin with this orientation will have a
specific resonance frequency that differs from the Larmor frequency (Figure 1.2). In a powder sample, the spins with various orientations relative to the main magnetic field have different resonance frequencies and form a continuous and broad spectrum called “powder pattern” (Figure 1.2). The shape of the “powder pattern” is determined by the principal values and orientational distribution (a non-uniform distribution can change the powder pattern) [27]. Therefore, the chemical shift anisotropy can easily distinguish different orientations by their different resonance frequencies. The powder pattern change caused by the dynamics can also manifest information about the reorientational geometry.

![Diagram of principal axis frame and Lab frame transformation](image)

Figure 1.2 The effect of the chemical shielding on the resonance frequency of the spin. The spin with principal value (0 ppm, 0 ppm, 60 ppm) and the Euler angle (0°, 30°, 0°) is exemplified on the top. The bottom blue figure is the static spectrum of the powder sample in the solid state NMR.

### 1.3.2 Dipolar coupling

Similar to the interaction between pairs of bar magnets, the nuclear spins that possess magnetic moments interact with each other through the magnetic field (Figure 1.3). This through-space interaction, which does not involve the electron clouds, is called the dipolar interaction. The Hamiltonian of the dipolar interaction is [28]
\[
H_{jk}^{DD} = d_{jk} \left( 3(\hat{I}_j \cdot e_{jk})(\hat{I}_k \cdot e_{jk}) - \hat{I}_j \cdot \hat{I}_k \right)
\]

\[
d_{jk} = -\frac{\mu_0 \gamma_j \gamma_k}{8\pi^2 r_{jk}^3}
\]

Equation 1.1

where \(\gamma_j\) and \(\gamma_k\) are the gyromagnetic ratios of the two spins, \(e_{ij}\) is the unit vector determined by the centers of the two spins and \(r_{jk}\) is the distance between two spins.

![Dipolar interaction diagram](image)

Figure 1.3 Dipolar interaction.

In high magnetic fields, Equation 1.1 can be simplified by the secular approximation to

\[
H_{jk}^{DD}(\Theta_{jk}) = \frac{1}{2} \left( 3\cos^2\Theta_{jk} - 1 \right) d_{jk} \left( 3\hat{I}_j \cdot \hat{I}_k - \hat{I}_k \cdot \hat{I}_j \right)
\]

Equation 1.2

where \(\Theta_{jk}\) is the angle between the vector \(e_{jk}\) and the main field direction. In Equation 1.1 and Equation 1.2, it is found that the magnitude of the dipolar coupling only depends on the type of the two nuclei, the distance between them and the direction of the dipolar coupling relative to the main field. Therefore, the dipolar coupling is a perfect interaction to detect the conformational reorientation (Figure 1.4).
1.3.3 The effect of the magic angle spinning on anisotropic interactions

Magic angle spinning (MAS) has been widely used in solid state NMR to obtain high resolution spectra since it was developed in 1959 [29, 30]. It is based on the idea that a sufficiently rapid mechanical rotation about an axis inclined at the magic angle (54°44’) relative to the direction of the main magnetic field can remove the most of the anisotropic interactions in solids (Figure 1.5). An intuitive understanding of this effect is that the fast magic angle spinning averages anisotropic interaction in all directions except the interaction component along the rotation axis, i.e. 54°44’ relative to the main field direction [31].

According to Equation 1.2, the anisotropic interaction along that orientation has zero value. As a result, magic angle spinning can remove the anisotropic interactions.

Figure 1.5 The effect of the MAS on the spectra for the $^{15}$N-$^1$H dipolar coupling interaction.
The anisotropic interaction under magic angle spinning can be treated more formally through frame transformations.

Principal axis frame

Rotation frame

\[
{R}(\alpha_p, \beta_p, \gamma_p) \quad \text{Lab frame}
\]

\[
{R}(0, \beta_R=54.7^\circ, \gamma_R=\omega_R t)
\]

Figure 1.6 Frame transformations of the dipolar coupling.

Take the heteronuclear dipolar coupling as an example. After the frame transformations, as shown in Figure 1.6, the resulting dipolar coupling is

\[
\omega_{D_0}(t) = -\frac{\sqrt{2}d}{2} \sin 2 \beta_p \cos (\gamma_p + \omega_R t) + \frac{d}{2} \sin^2 \beta_p \cos (2 \gamma_p + 2 \omega_R t)
\]

Equation 1.3

where \( d \) is the dipolar coupling constant. Equation 1.3 shows that the dipolar coupling interaction on the spin under the magic angle spinning is a periodic function, and the period is the period of the magic angle spinning. At the end of one rotor period, the magnetization dephased by the dipolar coupling will come back to the starting point (Figure 1.7) as if there was no action of the dipolar interaction. This kind of “rotor echo” is good to narrow the line-width (Figure 1.5), but not good for dynamics detection because the phase developed by the dipolar interaction cannot be accumulated to distinguish different orientations. Therefore, the specific pulse sequences are developed to prevent the “rotor echo” induced by the mechanical rotation.
One example of a pulse that can recouple the anisotropic interaction is to insert a π pulse in the middle of the rotor period (Figure 1.8) [32, 33]. In Figure 1.7, at the first half rotor period, the magnetization evolves under the dipolar coupling and has a maximum phase in the middle of the rotor period, but the rotor rotation makes the magnetization refocus in the second half rotor period. The π pulse in the middle of rotor can prevent the magnetization from refocusing in the second half rotor period. This method is easy to implement and has a large scaling factor for the recoupled anisotropic interaction. As a result, it is a very good choice for dynamics study. However, in the recoupling heteronuclear dipolar coupling case, the homonuclear dipolar coupling of one of the nuclei will affect the efficiency of the recoupling. Therefore, some special pulse programs that can recouple the heteronuclear dipolar coupling while removing the homonuclear dipolar coupling should be considered. These pulses are introduced in Chapter 2 and Chapter 3.

Figure 1.8 The pulses to recouple the chemical shift anisotropy and dipolar coupling. π pulses are inserted in the middle and at the end of the rotor periods.
1.4 Solid State NMR experiments to detect millisecond dynamics with magic angle spinning

As shown in last section, the strengths of all these anisotropic interactions, such as chemical shift anisotropy, dipolar interaction and quadrupole interaction, depend on its orientation with respect to the main magnetic field. A change of molecular orientation due to motion will induce a change in strengths of these nuclear spin interactions. Therefore, two principles can be used to detect millisecond dynamics: the first is exchange methods that detect the exchange of magnetizations with different anisotropic interaction strengths; the second is line-shape analysis to detect the extent that motions average anisotropic interactions, but the line-shape will have significant change only when motional rates are comparable to the anisotropic interaction strength. Therefore, for motions that have correlation times around or longer than a millisecond, their motional rates are often too slow compared to several kilohertz or even larger anisotropic interactions. Therefore, here we mainly focus on exchange methods.

The core concept of the magnetization transfer methods is that a specific interaction is detected before and after a mixing period, during which the magnetization is transferred along with the motion of the spin. Under the assumption that the motion is a first order jump motion (i.e. the transition rate is equal to the concentration times a rate constant), the amount of magnetization transferred- which is dependent on the dynamics- is a single-exponential function of the mixing time. Therefore, in theory magnetization transfer methods can detect any dynamics accurately. The lower limit of the the detectable motional rate is the life-time of the magnetization in the mixing time. Therefore, the magnetization transfer method is usually limited by $T_1$. The upper limit of the detectable motional rate is set by how well the interaction chosen to detect the position of the spin can distinguish between the different positions of the spins that reflect the states involved in the dynamic motion. In solid state NMR, anisotropic interactions such as the dipolar coupling and the chemical shift anisotropy (CSA) can be used for dynamics detection. The strengths of these interactions are usually several kilohertz and are strong enough to detect millisecond dynamics.

In terms of the method used to distinguish the different positions of the spin, magnetization transfer methods can be divided into 2D type and 1D type. The basic 2D exchange experiment (Figure 1.9) [34-36] first proposed in 1979 [36], which takes advantage of the isotropic chemical shift, is the most representative of the 2D magnetization methods. This method was generalized to static solid state NMR when the recoupled chemical shift anisotropy [37] and the quadrupole interaction [38] were used to detect motions that do not change the isotropic chemical shift of the spin. Along with the isotropic chemical shift, these anisotropic interaction also have an effect on the evolution of the magnetization during $t_1$ and $t_2$, generating the specific 2D powder patterns that reflects the rate and reorientational angle of the dynamics [27]. Because the signal-to-noise ratio and the resolution of the powder pattern is low, the
magic angle spinning technique was introduced into dynamics detection [39, 40]. The off-diagonal peaks resulting from the spin exchange at the low magic angle spinning speed can be used to extract the dynamics correlation time.

![Diagram of 2D exchange experiment]

Figure 1.9 The basic 2D exchange experiment [36].

While the 2D exchange experiments open the door to detecting very slow dynamics, a series of 2D spectra with different mixing times is required to obtain the exchange rate. Therefore, several 1D exchange experiments were developed to solve this problem as reviewed by Luz et al. [41]. The principle of 1D exchange experiments is to excite the different orientations of the spins to different extents, then observe how the molecular reorientation changes the magnetization distribution amongst the different orientations. Using this principle, the selective inversion experiment was developed to detect the exchange of two spins with different chemical shifts [42]. The spins at one chemical shift were inverted by a selective pulse, and then the magnetization recovery due to the molecular exchange between the inverted and unperturbed spins was recorded as a T₁ inversion-recovery experiment [43]. Williams et al. [44] also applied this principle to the quadrupole interaction in static solid-state NMR. A certain frequency in the deuterium powder pattern was selectively inverted, and its recovery rate was detected as the molecular motion rate. This experiment implies that with the orientational dependence of anisotropic interactions, solid state NMR experiments can detect the exchange of spins with the same isotropic chemical shift. Moreover, due to the more direct relationship between the anisotropic interactions and molecular orientation, the solid state NMR method can detect the molecular reorientation more directly and strictly. Another kind of solid state experiment, ODESSA (One Dimensional Exchange Spectroscopy by Sideband Alternation) [45] (Figure 1.10) and its variants [46-48] cleverly used the interference of the mechanical rotor spinning with the magnetization evolution under the chemical shift anisotropy (CSA) to invert the even numbered spinning side bands before the mixing time. This overcame the difficulty of using a selective pulse, especially in cases where the anisotropic interaction is not very strong (i.e. the powder pattern is not wide enough to just invert one part of it), but this kind of experiment still highly depends on the spinning side band and cannot be applied in modern fast-spinning magic angle spinning experiments.
At the beginning of the new millennium, the CODEX (Center-band Only Detection of Exchange) experiment [32] (Figure 1.10) was developed to detect millisecond dynamics. For this experiment, magic angle spinning frequency can be increased to moderate magic angle spinning frequencies (10~20 kHz), which are enough to effectively narrower peaks (Figure 1.5) and obtain a high resolution spectrum. At these magic angle spinning frequencies, there is usually only one center-band that contains contributions from all the orientations. Therefore, a new concept was implemented; the CODEX experiment creates a scaling factor for the spins that change their conformation before and after the mixing time, by recoupling the chemical shift anisotropy (CSA) before and after the mixing time. Therefore, with increased mixing time, the populations of the exchanging spins increases as well and the intensity of the center peak decreases.

**Inversion Experiment:**

**ODESSA:**

**CODEX:**

Figure 1.10 Simulations of a 1D inversion exchange experiment, an ODSSA experiment and a CODEX experiment. All the simulations are on a model of two-site jump of two $^{13}\text{C}$ with 10 kHz chemical shift anisotropy (CSA) and the main principal axes of the two exchanging $^{13}\text{C}$ make a flip angle of 60°.
After the original CODEX experiment [32, 49] (Figure 1.11 (b)), which used a REDOR [33] type pulse to recouple the chemical shift anisotropy of the $^{13}$C or $^{15}$N, a series of new CODEX-type experiments have been developed. Reichert et al. developed S-CODEX [50] (Figure 1.11 (c)) and CONTRA [51] (Figure 1.11 (d)), which moved the π pulse away from center of the rotor periods, to scale the recoupled chemical shift anisotropy down and detect the reorientational angle in the situation of large chemical shift anisotropies where the signal reaches its asymptotic value with very short dephasing and recoupling times. Besides the chemical shift anisotropy, dipolar coupling is also used to detect the dynamics because both the strength and the orientation of the dipolar coupling relates in a simple and direct way to the molecular structure. Schaefer et al. used CODEX with and without $^{13}$C–$^{19}$F dipolar cancelation and demonstrated the presence of a large amplitude motion of a fluorinated aromatic ring [52]. Recently, Krushelnitsky et al. [53] (Figure 1.11 (e)) and our group [54] (Figure 1.11 (f)) developed the Dipolar CODEX, which uses REDOR [33] pulse for dipolar coupling recoupling. However, in the case of $^1$H-$^{13}$C or $^1$H-$^{15}$N, the $^1$H’s homonuclear coupling is a large obstacle for the dynamics detection, and thus a highly deuterated sample is required [53] in the Dipolar CODEX experiment. The R-CODEX (Figure 1.11 (g)) is designed to solve this $^1$H’s homonuclear coupling problem. The symmetry-based R- type pulse R181$^7$ [55] can recouple the $^1$H-X dipolar coupling while effectively suppressing the $^1$H homonuclear coupling. Meanwhile, the R-type pulse sequence can detect the reorientation angle for the strong $^1$H-$^{13}$C or $^1$H-$^{15}$N dipolar interaction.

CODEX experiments have been applied to detect dynamics in macromolecular materials [56-65] and some protein systems [66-69]. Several problems limit CODEX experiments’ application in proteins: the first is that CODEX experiments only have less than 50% signal intensity compared with normal cross-polarization experiments. Moreover, in complex protein samples, CODEX experiments are often required to be incorporated into 2D experiments to obtain enough resolution. More than 20-folds experimental time relative to usual 2D experiment is required. Furthermore, the skewed population ratio between exchanging states in proteins make the intensity decay of CODEX experiments very small, which requires much more experimental time. However, several methods described in this thesis can effectively decrease experimental times of CODEX experiments in proteins. We used specific labeling scheme to obtain separated peaks in 1D experiments, and the extremely skewed population ratio of two exchanging states is adjust to equal population by controlling the concentration of the ligand.
1.5 Protein dynamics in the crystalline sample

As for X-ray crystallography, solid state NMR experiments must sometimes address the question as to whether proteins have the same structure and dynamics in the microcrystalline sample as in the solution or native conditions. According to our experience in the small molecule crystal, the interactions between different molecules are strong and it is nearly impossible to have the large unsymmetric motion. However, there is a large fraction of water (40%-70%) in proteins’ crystal. This water has different forms in the crystal. The water just outside of the protein forms a hydration shell, which has interaction with proteins and less mobile than free water but much more mobile than water in ice. Some other water forms water channels that allow the ligand’s diffusion in most part of the proteins [70], which is proved by the ligand soaking experiments. Therefore, proteins and water in crystals of proteins are not like the strict crystal lattice as we expect for the small molecular, and there is space for the conformational dynamics and flexibility of proteins. Another concerned factor is the viscosity. It is a general method to add polymers into protein solution for crystallization, which increases the viscosity of solution and increases the
difficulties of ligand diffusion and proteins’ motions. However, this effect on proteins’ catalytic rate and dynamics depends on the polymer’s chemical properties, the concentration of the polymer, ligands’ property and the comparison between the activation energy of proteins’ dynamics and the hindrance potential caused by the solution’s viscosity. An indirect but effective method to estimate the effect of the crystallization on proteins’ dynamics is to check the biological activity either by the microspectrophotometer experiments which monitor the formation of the chromophoric intermediates, or by the activity assay experiments in the solution phase of a crystalline suspension [70-72]. Since the dynamics is related to proteins’ functions, the effect of the crystallization on proteins’ dynamics can be estimated from proteins’ catalysis efficiency in crystals. Taking triosephosphate isomerase (TIM) as an example. According to the concentration of the PEG for TIM crystallization, the resistance to the loop 6’s motion from the solution is estimated as 0.4 J/mol in Appendix IV, which can be omitted compared to the activation energy of loop motion (58 kJ/mol) [17, 73, 74]. Moreover, there are only two O...H-N hydrogen bonds formed between the residues on loop 6 in different TIM molecules, but these crystal contacts of the two TIM molecules cause a short distance of two carbonyl oxygens (Figure 1.13). Therefore, it is difficult to define the effect of these crystal contacts on the motion of loop 6. Furthermore, the enzyme activities of TIM in solution and in microcrystalline sample are similar according to the kinetic experiments[74]. Therefore we can conclude that this loop 6’s motion is probably not influenced by the crystallization. Meanwhile, it is worthwhile to note that cellular conditions are very crowd [75], this loosely packed and hydrated condition is more related to the real environments where enzymes conduct their functions.

Figure 1.12 The crystal contacts in the crystal structure of the unligated TIM (PDB: 1I45).
1.6 References

Chapter 2  The Principals of Designing CODEX
Type Experiments
2.1 Overview
The general theoretical principles and experimental details of designing the center-band-only detection of exchange (CODEX) type experiments are discussed in this chapter. In CODEX type experiments, molecular dynamics is investigated through the recoupled anisotropic interaction, which is relevant to the conformational change of molecules. The correlation time of the dynamics can be obtained by simply fitting the data with a single exponential decay curve, without potential complications introduced by adding other fitting parameters. The reorientational angle of the motion can be extracted by fitting the dephasing curve with respect to the corresponding simulations. In the analysis of the difference tensor (Equation 2.6) of the anisotropic interaction before and after mixing time, the dephasing curve depends on the sine function of the reorientational angle, and is especially sensitive to the small angle. Meanwhile, the length of the dephasing and refocusing times, along with the strength of the anisotropic interaction, determines both the signal intensity and the effective detection range of the correlation time. This chapter discusses several experimental details such as the coherence choice and rotor synchronization, to apply this CODEX principal to different kinds of anisotropic interactions.


2.2 Magnetization evolution in the CODEX type experiment
A general pulse sequence for a CODEX type experiment is shown in Figure 2.1. It mainly consists of short recoupling pulses during the dephasing and refocusing periods, spaced by an incremented mixing time during which the magnetization is transferred between the different conformations.

![Figure 2.1 The general pulse sequence for a CODEX type experiment](image)
The conformational exchange processes such as the reorientation of the chemical shift anisotropy (CSA) of a $^{13}$C spin is assumed to be described by a two-site jump model, as shown in Scheme 2.1. No exchange motion during the dephasing and refocusing periods is assumed in the following theoretical derivation (although for numerical simulations the exchange during those periods is included); the longitudinal relaxation rates ($T_1$) and transverse relaxation rates ($T_2$) of different exchanging conformations are assumed to be the same here (and the spin-spin relaxation times $T_2$ for both conformations are assumed to be the same (The situation that $T_1$s or $T_2$s for different conformations are different is discussed in the Appendix V). The normalized populations of two exchange sites are $P_A$ and $P_B$. The exchange rates $k_1$ and $k_{-1}$ satisfy $k_1 \times P_A = k_{-1} \times P_B$.

\[ P_A \rightarrow_{k_1} P_B \rightarrow_{k_{-1}} P_A \]

Scheme 2.1

After cross polarization, a transverse magnetization $I_{x,0}$ is created. During the dephasing time, the magnetization $I_{x,0}$ precesses under the recoupled CSA interaction according to

\[ I_x^A = \langle P_A e^{-t_{dephase}/T_2} \times (\cos \phi^A_{dephase} I_x + \sin \phi^A_{dephase} I_y) \rangle > \]
\[ I_x^B = \langle P_B e^{-t_{dephase}/T_2} \times (\cos \phi^B_{dephase} I_x + \sin \phi^B_{dephase} I_y) \rangle > \]

Equation 2.1

where $\langle >$ represents powder average over all molecular orientations in rotor, $I_x^A$, $I_x^B$ are the magnetizations separately belonging to two different conformations and $\phi^A_{dephase}$ and $\phi^B_{dephase}$ are the accumulated phases under the action of the recoupled CSA of two different conformations. A subsequent 90° pulse flips the in-phase magnetization to generate stored longitudinal magnetization as shown in Equation 2.2. The results of anti-phase part are the same with those of the in-phase part if one replaces the sine function with the cosine function. Any remaining transverse magnetization will either decay by transverse relaxation or be eliminated by phase cycling.

\[ I_z^A (t_{mix} = 0) = \langle P_A e^{-t_{dephase}/T_2} \times \cos \phi^A_{dephase} \rangle > \]
\[ I_z^B (t_{mix} = 0) = \langle P_B e^{-t_{dephase}/T_2} \times \cos \phi^B_{dephase} \rangle > \]

Equation 2.2
\( P_A \) and \( P_B \) are the populations of the two conformations as shown in Scheme 2.1. During the mixing time, the Zeeman magnetization is modulated by longitudinal relaxation (we assume longitudinal relaxation rates for different conformations are the same here and we will discuss the the exchange process between different conformations, which can be described by

\[
\frac{d}{dt}\begin{pmatrix}
I_z^A(t_{\text{mix}}) - P_A I_0^0 \\
I_z^B(t_{\text{mix}}) - P_B I_0^0
\end{pmatrix} = \begin{pmatrix}
-k_1 - 1/T_1 & k_{-1} \\
k_1 & -k_1 - 1/T_1
\end{pmatrix}\begin{pmatrix}
I_z^A(t_{\text{mix}}) - P_A I_0^0 \\
I_z^B(t_{\text{mix}}) - P_B I_0^0
\end{pmatrix}
\]

Equation 2.3

where \( I_0^0 \) is the equilibrium magnetization of \(^{13}\text{C}\). Solution of Equation 2.3 yields

\[
\begin{pmatrix}
I_z^A(t_{\text{mix}}) \\
I_z^B(t_{\text{mix}})
\end{pmatrix} = \begin{pmatrix}1 - \exp(-k_{t_{\text{mix}}} - 1/T_1) & k_{t_{\text{mix}}} \end{pmatrix} \begin{pmatrix}
P_A I_0^0 \\
P_B I_0^0
\end{pmatrix} + \begin{pmatrix}1 & k_{t_{\text{mix}}} \end{pmatrix}
\begin{pmatrix}
I_z^A(t_{\text{mix}} = 0) \\
I_z^B(t_{\text{mix}} = 0)
\end{pmatrix}
\]

Equation 2.4

in which “1” is the unit matrix. Recalling that \( I_0^0 \) does not depend on the phase of the cross polarization, the first part of Equation 2.4 can be eliminated by alternating the phases of cross polarization and receiver simultaneously. Then, Equation 2.4 can be simplified to

\[
\begin{pmatrix}
I_z^A(t_{\text{mix}}) \\
I_z^B(t_{\text{mix}})
\end{pmatrix} = e^{-t_{\text{phasing}}/T_1} e^{-t_{\text{mix}}/T_1} \begin{pmatrix}1 & k_{t_{\text{mix}}} \end{pmatrix} \begin{pmatrix}
P_A \times < \cos \phi_{\text{dephase}}^A > \\
P_B \times < \cos \phi_{\text{dephase}}^B >
\end{pmatrix} e^{(-k_{t_{\text{mix}}} - 1/T_1) t_{\text{mix}}}
\]

Equation 2.5

where

\[
\begin{align*}
a_{11} & = P_B e^{-k_{e\times t_{\text{mix}}}} + P_A \\
a_{12} & = P_A (1 - e^{-k_{e\times t_{\text{mix}}}}) \\
a_{21} & = P_B (1 - e^{-k_{e\times t_{\text{mix}}}}) \\
a_{22} & = P_A e^{-k_{e\times t_{\text{mix}}}} + P_B \\
k_{e} & = k_1 + k_{-1}
\end{align*}
\]

Equation 2.6
Before the refocusing period, the 90° pulse flips the longitudinal magnetization back to the transverse plane, and the magnetization evolves under the same recoupling condition as the dephasing period as shown in Equation 2.7. After the z-filter, the signal intensities for two different conformations are

\[
I^A_x(t_{\text{mix}}) = e^{-t_{\text{dephase}}/T_2} e^{-t_{\text{recoupling}}/T_2} \left[ P_A \cos \phi^A_{\text{dephase}} \cos \phi^A_{\text{refocus}} \left( P_B e^{-k_\omega t_{\text{mix}}} + P_A \right) 
+ P_B \cos \phi^B_{\text{dephase}} \cos \phi^B_{\text{refocus}} P_A (1 - e^{-k_\omega t_{\text{mix}}}) \right] 
\]

\[
I^B_x(t_{\text{mix}}) = e^{-t_{\text{dephase}}/T_2} e^{-t_{\text{recoupling}}/T_2} \left[ P_A \cos \phi^A_{\text{dephase}} \cos \phi^A_{\text{refocus}} \left( P_B e^{-k_\omega t_{\text{mix}}} + P_A \right) 
+ P_B \cos \phi^B_{\text{dephase}} \cos \phi^B_{\text{refocus}} P_A (1 - e^{-k_\omega t_{\text{mix}}}) \right] 
\]

Equation 2.7

The final intensity is

\[
I_{\text{in-phase}}^{\text{mix}}(t_{\text{mix}}) = I^A_x(t_{\text{mix}}) + I^B_x(t_{\text{mix}}) 
= e^{-t_{\text{dephase}}/T_2} e^{-t_{\text{recoupling}}/T_2} \left[ P_A \cos \phi^A_{\text{dephase}} \cos \phi^A_{\text{refocus}} \left( P_B e^{-k_\omega t_{\text{mix}}} + P_A \right) 
+ P_B \cos \phi^B_{\text{dephase}} \cos \phi^B_{\text{refocus}} P_A (1 - e^{-k_\omega t_{\text{mix}}}) \right] 
+ e^{-t_{\text{dephase}}/T_2} e^{-t_{\text{recoupling}}/T_2} \left[ P_A \cos \phi^A_{\text{dephase}} \cos \phi^A_{\text{refocus}} \left( P_B e^{-k_\omega t_{\text{mix}}} + P_A \right) 
+ P_B \cos \phi^B_{\text{dephase}} \cos \phi^B_{\text{refocus}} P_A (1 - e^{-k_\omega t_{\text{mix}}}) \right] 
\]

Equation 2.8

Because \( \phi^A_{\text{dephase}} = \phi^A_{\text{refocus}} = \phi^A \), \( \phi^B_{\text{dephase}} = \phi^B_{\text{refocus}} = \phi^B \), \( t_{\text{dephase}} = t_{\text{recoupling}} = t_r \), and \( t_{\text{total}} = t_{\text{mix}} + t_z \), Equation 2.8 can be further simplified to

\[
I_{\text{in-phase}}^{\text{mix}}(t_{\text{mix}}) = e^{-2t_r/T_2} e^{-t_{\text{mix}}/T_2} \left[ P_A \cos \phi^A \left( P_B e^{-k_\omega t_{\text{mix}}} + P_A \right) 
+ P_B \cos \phi^B \cos \phi^A (1 - e^{-k_\omega t_{\text{mix}}}) \right] 
\]

Equation 2.9

Equation 2.9 results from the in-phase component of Equation 2.1. If we change the phase of the 90° pulse before the mixing time, the anti-phase component in the Equation 2.1 can be selected, and has a similar form as Equation 2.9:

\[
I_{\text{anti-phase}}^{\text{mix}}(t_{\text{mix}}) = e^{-2t_r/T_2} e^{-t_{\text{mix}}/T_2} \left[ P_A P_B (\cos \phi^A - \cos \phi^B) e^{-k_\omega t_{\text{mix}}} + (P_A \cos \phi^A + P_B \cos \phi^B)^2 \right] 
\]

Equation 2.10

The combination of the anti-phase and in-phase signal will have the simple form:
2.3 The detection of the exchange rate and population ratio

Equation 2.11 shows that aside from the mixing time dependant decay caused by spin-spin and spin-lattice relaxation, the final intensity of the spectra has only a single exponential decay of the mixing time at the rate of $k_{ex}$. The signal loss due to the spin-spin relaxation is a constant in the $k_{ex}$ detection experiment because the dephasing and refocusing times are fixed for all the experiments. For the cases that recouple the dipolar coupling and store the in-phase component or the cases that recouple chemical shift anisotropy, the effect of spin-lattice relaxation can be eliminated by keeping the total time of the mixing and z-filter time constant so that the signal losses from spin-lattice relaxation are the same for all the experiments. For the case that recouple dipolar coupling and store the anti-phase component, a second exponential index should be used to deal with the $T_1$ relaxation. After correcting for these relaxation effects, the exchange rate can be easily obtained by fitting the signal intensity to a single exponential decay function with respect to mixing times.

Meanwhile, the population ratio between the two exchanging states is found in the ratio between the long mixing time intensity and the zero mixing time intensity as shown in Equation 2.12. When the dephasing and refocusing time is long enough, $\cos(\varphi^A - \varphi^B)$ approaches to zero due to the powder average of different orientations of powder samples. Therefore, the populations of the two exchanging conformation can be easily obtained.

\[
\frac{I(t_{mix} = \infty)}{I(t_{mix} = 0)} = 1 - 2P_AP_B\left[1 - < \cos(\varphi^A - \varphi^B) > \right] \approx 1 - 2P_A(1 - P_A)
\]

Equation 2.12

This value is always equal or larger than 1/2. If the motional model is such that the molecule can equally access to $M$ orientational states, the ratio between the long mixing time intensity and the zero mixing time intensity becomes [1, 2]
\[
\frac{I(t_{\text{mix}} = \infty)}{I(t_{\text{mix}} = 0)} = \frac{1}{M}
\]

Equation 2.13

and this ratio is always smaller than 1/2. Therefore, the CODEX experiment can distinguish between these two different motional models and give more information about protein dynamics.

2.4 The detection of motional geometry

Besides the correlation time and population ratio, the CODEX experiment can detect the reorientation angle of the motion [1, 2]. In Equation 2.14, the normalized signal intensity highly depends on the difference of the recoupled anisotropic interactions under the two exchanging conformations. This interaction difference contains the reorientational angle’s information. By gradually increasing the dephasing time, a dephasing curve can be obtained to extract the reorientational angle by fitting the experimental results to the corresponding simulations.

\[
\frac{I(t_{\text{mix}} = 0) - I(t_{\text{mix}} = \infty)}{I(t_{\text{mix}} = 0)} = 2 P_x P_y \left[ 1 - \cos(\phi_x^A - \phi_x^B) \right] >
\]

Equation 2.14

To understand the relationship between the dephasing curve and the flip angle $\beta_f$, the exchange of two dipolar couplings is assumed as shown in Figure 2.1.

Figure 2.2 The exchanging of two dipolar coupling interactions discussed with tensors $\Lambda^A$ and $\Lambda^B$. The principal frames of two dipolar couplings are labeled with principle axes $x^A, y^A, z^A$ and $x^B, y^B, z^B$. The principal value of the uniaxial dipolar couplings are along the $z^A$ and $z^B$ directions. The flip angle between the two dipolar couplings is $\beta_f$. The molecular frame is set so that the Y axis is perpendicular to the plane of $z^A$ and $z^B$, and the X, Z axis make a 45° angle with the rotation axis.
A convenient way [1] to calculate \( \omega^A - \omega^B \) is to calculate the difference tensor between \( \Lambda^A \) and \( \Lambda^B \) in the lab frame as Equation 2.15.

\[
\Delta \Lambda = \Lambda_{\omega^A}^A - \Lambda_{\omega^B}^A = R^{-1}(180^\circ, 45^\circ + \frac{\beta}{2}, 0^\circ) \Lambda^A R(180^\circ, 45^\circ + \frac{\beta}{2}, 0^\circ) - R^{-1}(0^\circ, 45^\circ - \frac{\beta}{2}, 0^\circ) \Lambda^A R(0^\circ, 45^\circ - \frac{\beta}{2}, 0^\circ)
\]

\[
= R^{-1}(180^\circ, 45^\circ + \frac{\beta}{2}, 0^\circ) \begin{pmatrix}
-d/2 & 0 & 0 \\
0 & -d/2 & 0 \\
0 & 0 & d
\end{pmatrix} R(180^\circ, 45^\circ + \frac{\beta}{2}, 0^\circ) - \\
R^{-1}(0^\circ, 45^\circ - \frac{\beta}{2}, 0^\circ) \begin{pmatrix}
-d/2 & 0 & 0 \\
0 & -d/2 & 0 \\
0 & 0 & d
\end{pmatrix} R(0^\circ, 45^\circ - \frac{\beta}{2}, 0^\circ)
\]

\[
= \begin{pmatrix}
\frac{3d}{2} \sin \beta_r & 0 & 0 \\
0 & 0 & 0 \\
0 & 0 & -\frac{3d}{2} \sin \beta_r
\end{pmatrix}
\]

Equation 2.15

where \( d \) is the dipolar coupling constant and \( \beta_r \) is the flip angle defined in Figure 2.2. Then, \( \omega^A - \omega^B \) under the magic angle spinning can be easily obtained as

\[
\omega^A(t) - \omega^B(t) = C_1 \cos(\gamma_p + \omega_r t) + S_1 \sin(\gamma_p + \omega_r t) + C_2 \cos(2\gamma_p + 2\omega_r t) + S_2 \sin(2\gamma_p + 2\omega_r t)
\]

with

\[
C_1 = -\frac{3\sqrt{2}}{4} d \times \sin \beta_r \times \sin 2\beta_p (1 - \frac{1}{3} \eta \cos 2\alpha_p)
\]

\[
S_1 = -\frac{\sqrt{2}}{2} d \times \sin \beta_r \times \sin \beta_p \sin 2\alpha_p
\]

\[
C_2 = \frac{1}{4} d \times \sin \beta_r \times \left[3 \sin^2 \beta_p + (1 + \cos^2 \beta_p) \cos 2\alpha_p\right]
\]

\[
S_2 = -\frac{1}{2} d \times \sin \beta_r \times \cos \beta_p \sin 2\alpha_p
\]

Equation 2.16

where \( t \) is the dephasing (refocusing) time, \( \alpha_p, \beta_p, \gamma_p \) are the Euler angles that transform the molecular frame in Figure 2.2 to the rotor frame and \( \beta_r \) is the flip angle defined in Figure 2.2.
From Equation 2.14 and Figure 2.15, the dephasing curve is determined by the sine function of the flip angle. Therefore, as shown in Figure 2.3, the flip angle can be obtained by fitting the dephasing curve. It is obvious that the CODEX type experiment is more sensitive to the small flip angle, due to the property of the sine function.

![Figure 2.3](image)

Figure 2.3 The simulations of CODEX dephasing curves with different flip angles. The principal value of chemical shift anisotropy (CSA) is 2 kHz, which has the direction perpendicular to the jump axis. The value of $k_{eq}$ is 0.5 kHz. No relaxation is added in the simulations. The mixing time for $I_0$ is equal to 0 while the mixing time for $I_t$ is equal to 100 ms.

2.5 The signal-to-noise in CODEX type experiments

The signal-to-noise of the CODEX experiment is very important in practical applications and especially in applications to large biological systems. According to Equation 2.11,

$$I(t_{mix} = 0) = \frac{1}{2} e^{-t_{phase+refocus}/T_2} e^{-t_{total}/T_1}$$

$$I(t_{mix} = 0) - I(t_{mix} = \infty) = e^{-t_{phase+refocus}/T_2} e^{-t_{total}/T_1} P_A P_B \left[ 1 - \cos(\omega^A - \omega^B) t_r \right]$$

Equation 2.17

In Equation 2.17, the CODEX experiment essentially loses half of the signal intensity relative to the standard cross polarization experiment due to the selection of in-phase or anti-phase coherence. Also, the final signal intensity depends on the spin-lattice relaxation during the mixing and z-filter time, and spin-spin relaxation during the dephasing and refocusing part. Because the mixing time and z-filter time are determined by the correlation time of the dynamics and cannot be changed, methods to increase the signal-to-noise focus on shortening the dephasing and refocusing times $t_r$. In Equation 2.17 and Equation 2.16, the decay amplitude $I(t_{mix} = 0) - I(t_{mix} = \infty)$ is determined by $t_r$ and $d \times sin \beta_r$. For example, by using a stronger anisotropic interaction (larger $d$) one can obtain enough decay at a short dephasing time $t_r$. 
2.6 Timescales at which CODEX is sensitive

The CODEX type experiment is very efficient for detecting dynamics with the correlation times ranging from milliseconds to seconds. However, spin-lattice relaxation and the magnetization exchange due to dipolar spin diffusion set a detection limit for the longest correlation time. Basically speaking, the magnetization transfer caused by spin diffusion cannot be distinguished from the magnetization transfer caused by the chemical exchange. As a result, the motional rate should be much faster than the spin diffusion rate. The correlation time of $^{13}$C spin diffusion can be as short as 100 ms in sparsely labeled proteins [3, 4], while the natural abundance small molecules have the $^{13}$C spin diffusion of around 1 s [5]. However, fast magic angle spinning up to 60 kHz can largely reduce or quench the $^{13}$C spin diffusion even in the fully labeled protein [6, 7]. One method to solve this spin diffusion problem is using $^{15}$N labeling scheme. The dipolar coupling between the possible closest amide $^{15}$N is only 55 Hz (for α-helix), which has a negligible spin diffusion within 500 ms [3].

The lower limit for the detectable correlation times depends on the effect of the dynamics during the dephasing and refocusing parts. In the derivation above, we have assumed that there were no reorientation events occurring during the dephasing and refocusing periods. This is of course correct only when the dephasing and refocusing periods are much shorter than the correlation time of dynamics. During the dephasing and refocusing periods, the dynamics will interfere with the evolution of the magnetization under the recoupled anisotropic interaction. The amplitude of the initial signal becomes smaller [8, 9] even when the mixing time is zero as shown in Figure 2.4. Meanwhile the amplitude of the decay decreases with increasing the motional rate as shown in Figure 2.4 (left figure), although the time constant for the motion is still faithfully represented in this experiment. This decrease of the decay amplitude from the motion’s averaging effect makes the reorientation angle detected by CODEX type experiments become artefactually smaller. However, stronger anisotropic interaction can be used to shorten the dephasing and refocusing times and decrease the dynamics’ effect as shown in Figure 2.4 (right figure). Additionally, the dephasing curves for determining the flip angle would also have large effects due to the dynamics during the dephasing as shown in the Figure 2.5, and hence the dynamics effect should be considered in the fitting process to extract flip angle. Thus CODEX type experiments cannot accurately study motions which have a rate much faster than the coupling constant of the anisotropic interaction.
2.7 Implementation of the CODEX type experiments

2.7.1 The choice of anisotropic interaction
As discussed in the above, a strong anisotropic interaction is preferred in order to decrease the signal loss in the dephasing and refocusing times and to broaden the detection range of the correlation time. Table
2.1 lists the usual anisotropic interactions in proteins. Considering the scaling factor for chemical shift recoupled by the \( \pi \) pulse in the middle of the rotor period is around 0.4 and the scaling factor for the recoupled dipolar coupling is about 0.3-0.5 \([10-12]\), the amide nitrogen and carbonyl carbon’s CSA, \(^{13}\text{C}-^{1}\text{H}\) and \(^{15}\text{N}-^{1}\text{H}\) dipolar couplings are good candidates for shortening the dephasing time.

<table>
<thead>
<tr>
<th>Type of anisotropic interaction</th>
<th>Principal Value</th>
<th>Typical dephasing time for (30^\circ) flip motion</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{13}\text{C} ) CSA of C(\alpha)</td>
<td>2 kHz (~15 ppm ([13]))</td>
<td>0.8 ms (8 Tr)</td>
</tr>
<tr>
<td>(^{13}\text{C} ) CSA of C'</td>
<td>10 kHz (~70 ppm ([13]))</td>
<td>0.2 ms (2 Tr)</td>
</tr>
<tr>
<td>(^{15}\text{N} ) CSA of amide N</td>
<td>6 kHz (~100 ppm ([14]))</td>
<td>0.4 ms (4 Tr)</td>
</tr>
<tr>
<td>(^{13}\text{C} - ^{1}\text{H} ) dipolar coupling</td>
<td>21.5 kHz (1.12 Å ([15]))</td>
<td>0.1 ms (1 Tr)</td>
</tr>
<tr>
<td>(^{15}\text{N} - ^{1}\text{H} ) dipolar coupling</td>
<td>10.8 kHz (1.04 Å ([16]))</td>
<td>0.2 ms (2 Tr)</td>
</tr>
<tr>
<td>(^{13}\text{C}’ - ^{15}\text{N} ) dipolar coupling</td>
<td>1.3 kHz (1.34 Å ([14]))</td>
<td>1.5 ms (15 Tr)</td>
</tr>
</tbody>
</table>

Table 2.1 Typical principal values of usual anisotropic interactions in proteins. In the dephasing and recoupling time, the REDOR pulse sequence is used for recoupling the dipolar interaction while a series of \( \pi \) pulses are used to recouple chemical shift anisotropy (CSA) as shown in the origin CODEX paper \([1]\). The magic angle spinning frequency is set as 10 kHz (i.e. one rotor period 1 Tr is equal to 100 \(\mu\)s). The exchanging rate is 0.5 kHz while the flip angle is \(30^\circ\).

The second consideration is the geometry of the motion relative to the principal axis of the anisotropic interactions. CODEX type experiments are not sensitive to the motions in which the rotation axis is along the most shielded principal axis, for example, the rotation around the C’-N will not generate signal decay in the CODEX experiment using \(^{13}\text{C} - ^{15}\text{N} \) dipolar coupling. However, this motion will result in a large signal decay for the CODEX experiments using C’ or \(^{15}\text{N} \)’s CSA as detecting interactions, because their most shielded component, \(\delta_{33}\), is perpendicular to the rotation axis (Figure 2.6). Therefore, detecting dynamics using several different kinds of anisotropic interactions at the same time can characterize the dynamics more thoroughly.

![Figure 2.6 Orientations of the chemical shift tensors of \(^{13}\text{C} \) and \(^{15}\text{N} \). \([14, 17, 18]\)](image-url)
Certainly, in practical applications, other factors such as spin diffusion and spectral resolution will also limit the choice of the anisotropic interaction.

2.7.2 The choice of the in-phase vs. anti-phase component

After the dephasing period, the phase of the 90° storage pulse determines whether the in-phase or anti-phase component of the evolved magnetization is stored, as shown in Section 2.2. In fact, Figure 2.7 shows that the in-phase, anti-phase or a combination of them will have similar results when the dephasing and refocusing times are long enough compared to the inverse of the recoupled anisotropic interaction, but when the dephasing and refocusing times are relatively short, the anti-phase component is better for characterizing the correlation time of the dynamics because the amplitude of the decay during the mixing time is considerably large.

![Figure 2.7 Simulations of the CODEX experiments with different principal values of CSA and different dephasing (refocusing) times. The simulations use a two-site jump model with equal populations and 0.2 kHz exchange rate. The CSA is uniaxial with various principal values. The reorientation angle between the two positions of the unique axis of the CSA (jump angle) is 120°. The sample rotation frequency is 10 kHz. Two dephasing times, 0.2 ms (2 Tr, a-c) and 0.5 ms (5 Tr, d-e), are simulated.](image)

Furthermore, relaxation also affects the choice of in-phase and anti-phase component. In Section 2.2, the relaxation rates for in-phase and anti-phase components are assumed to be the same. However, this is only true when the recoupled anisotropic interaction is the CSA. In the case of the dipolar coupling, especially dipolar couplings of 1H-13C or 1H-15N spin pairs, the anti-phase component decays fast during the mixing time due to the fast 1H spin-lattice relaxation as shown in Section 3.2.1 and cannot be used for CODEX...
type experiments. Moreover, the spin-lattice’s effect on the experiments is eliminated by keeping the total time of the mixing and z-filter periods the same. However, this method is based on the fact that $T_1$ is the same for the selected coherence in the mixing time and final detectable coherence, which is not true for the anti-phase component. Therefore, the CODEX type experiment which takes advantage of the anti-phase component should consider $T_1$ effect in the decay curve fit.

### 2.7.3 Rotor synchronization

In magic angle spinning solid-state NMR, rotor synchronization of the mixing time is required for most of the 1D or 2D longitudinal magnetization exchange experiments [1, 19]. Due to the spatial dependence of the anisotropic interaction, the rotation of the sample modulates the spin Hamiltonian during the rotor period, resulting in varying strengths of the anisotropic interaction with respect to the rotor position. The recoupled anisotropic interaction generated by the recoupling pulse during the refocusing part depends on the starting time point of the pulse sequence, and hence on the length of the mixing time. Therefore, the dephasing time, the refocusing time and the mixing time should be rotor-synchronized to make the recoupled Hamiltonians the same in the dephasing and refocusing periods. This rotor synchronization requirement can be easily implemented as shown in Figure 2.8.

![Figure 2.8 The rotor synchronized CODEX pulse sequence. The solid lines are 90° pulses while the hollow rectangles are 180° pulses. After cross polarization, the $^{13}$C’s magnetization of is flipped immediately to the z-direction to wait for the rotor trigger. In both Varian and Bruker, the pulse program can be postponed until it receives a signal from the magic angle spinning controller. The first z-filter time (just after CP) is used to insert the 2nd rotor trigger signal, and its length can be an arbitrary short length.](image)

However, this requirement for the rotor synchronization during the dephasing and refocusing times means that the increment of the dephasing and refocusing times must be an integral multiple of rotor periods. This affects the detection of the flip angle because only a few data points can be obtained before the dephasing curve rises to a plateau as shown in the Figure 2.9.
Figure 2.9 The dephasing curve of the 60° flip jump of the 10 kHz chemical shift anisotropy. If the magic angle spinning frequency is 10 kHz (rotor period is 0.1 ms), only one data point can be observed in the most meaningful rising part of the dephasing curve.

One solution to this problem is to scale the anisotropic interaction by moving the position of the π pulse while keeping the dephasing and refocusing times the same [7, 20]. In Figure 2.10, when T_π is equal to zero, there is no CSA recoupled during the dephasing and refocusing parts. By increasing the T_π, the recoupled CSA becomes stronger, and then the dephasing curve gradually increases.

Figure 2.10 The dephasing curve of CSA generated by moving the position of the π pulse. The magic angle spinning frequency is 10 kHz. The system that is simulated has a 60° jump of a 10 kHz chemical shift anisotropy. The dephasing and refocusing times are 2 rotor periods (0.2 ms) for all the simulations. The first and third π pulses in the dephasing and refocusing periods move together as shown in the top figure. The π pulses in the center of dephasing and refocusing periods are used to refocus the isotropic chemical shift.
Another method to obtain the full dephasing curve of a strong anisotropic interaction is to decouple the anisotropic interaction during part of dephasing time. This method is usually used to detect the dipolar coupling of $^1\text{H}-^{13}\text{C}$ or $^1\text{H}-^{15}\text{N}$ [12, 21, 22], where rotor synchronization is also necessary. The principal is that the sum of the recoupling pulse and decoupling pulse are kept the same and rotor synchronized, but the recoupling pulse is gradually increased from zero. Figure 2.11 is an example of this scheme where the new developed R-CODEX is used to extract the flip angle of the $^{13}\text{C}-^1\text{H}$ dipolar coupling (more discussion is on the chapter 3).

Figure 2.11 The dephasing curve of the $^{13}\text{C}-^1\text{H}$ dipolar coupling by R-CODEX (more discussion about this pulse sequence is in Chapter 3). $RR$ is the sub-cycle of the recoupling pulse of $^{13}\text{C}-^1\text{H}$ dipolar coupling, $R18^\gamma_1$ [15]. The magic angle spinning frequency is 11 kHz (i.e. one rotor period is 0.909 ms and one $RR$ sub-cycle is 0.101 ms). The simulation system is the 60° flip jump of the $^{13}\text{C}-^1\text{H}$ dipolar coupling (23.33 kHz). The dephasing and refocusing times are kept the same for all the simulations.

However, the requirement of the rotor synchronization about the mixing time can be weakened by the $\gamma$-encoded recoupling scheme [23] like symmetry-based pulse sequences, which has a very useful phase-time relationships [24, 25] (Equation 2.18).

$$\exp\{-i\mu\phi\} = \exp\{im\omega_r(t_2 - t_1)\}, \text{ i.e. } \phi = \frac{m\omega_r(t_2 - t_1)}{\mu}$$

Equation 2.18
where the rank number $m$ and $\mu$ separately represent the transformation property of the recoupling anisotropic interaction with respect to rotations of the molecular framework and rotations of the nuclear spin polarizations [26]; $\omega_r$ is the magic angle spinning frequency; $t_1$ and $t_2$ is the starting time point of the dephasing and refocusing periods; and $\phi$ is the phase shift of the recoupling pulse in the refocusing period relative to the recoupling pulse in the dephasing part.

Figure 2.13 and Figure 2.14 show how the phase-time relationship in Equation 2.18 can break the limitation of the rotor synchronization of the mixing time in the R-CODEX experiment (Figure 2.12).

Figure 2.12 The R-CODEX using a windowed R-type sequence (R-type pulse $R_{185}^3$ [15, 25, 27]) to refocus the CSA of the $^{13}\text{C}$ for detecting dynamics.

Figure 2.13 Simulations for exchange of two carbons with the same CSA principal value (50 ppm) and different CSA orientations (flip angle 40°). In the simulation, the MAS frequency is 11.111 kHz, the dephasing time is 720 $\mu$s (8 rotor periods), and $k_{ex}$=0.4 kHz. Left figure: The simulations without phase-adjustment in the refocusing pulse. The signal intensities change irregularly. When the mixing times are not rotor synchronized, the intensities fluctuate a lot during the rotor period. Right figure: The overall phase of the refocusing pulses is adjusted according to Equation 2.18. The intensities have smooth change even there are non-rotor-synchronized data.
Figure 2.14 Simulations for a static $^{13}$C with 50 ppm chemical shift anisotropy. The magic angle spinning frequency is 11.111 kHz (i.e. one rotor period is 90 μs). The dephasing (refocusing) time is 720 μs (8 rotor periods). The black squares represent the results without the phase-adjust in the recoupling pulse during the refocusing part, while the red spheres represent the results with the phase-adjust according to Equation 2.18.

2.8 References


Chapter 3 The Development of New Versions of CODEX
3.1 Overview

As discussed in the previous chapter, CODEX type experiments detect dynamics using the fact that molecular conformational reorientation before and after the mixing time prevents the magnetization dephased using orientational dependant interaction from refocusing. As a result, all the anisotropic interactions such as chemical shift anisotropies and heteronuclear dipolar couplings, which have different strengths at different conformations, can be used in the CODEX type experiments. In 1999, Schmidt-Rohr et al. developed the original CODEX experiment to recouple chemical shift anisotropy (CSA) to detect the dynamics, and it has been applied to explore the dynamics in macromolecular materials [1-10] and some protein systems [11-13]. However, there are still several advantages to developing CODEX type experiments using different anisotropic interactions, such as the dipolar coupling. Firstly, dipolar couplings such as $^1$H-$^{13}$C and $^1$H-$^{15}$N are usually stronger than the CSA in current commercial available magnetism, use of a stronger coupling can decrease signal loss and increase detection range of the correlation time as discussed in Chapter 2. Moreover, knowledge of the principle values and orientations of the anisotropic interaction tensor is necessary to measure the reorientational angle of the molecular dynamics. The principle values and directions of CSA tensors depend strongly on the environment of the functional group [14, 15]. Although it is possible to compute the CSA values [14, 15], or measure them for the limiting structure in favorable cases [16-19], this might not always be practical especially for high energy or relatively poorly populated states in a conformational exchange process. However, the principal values of the dipolar coupling are much less depends on the chemical environment and their tensors’ orientation relate in a simple and direct way to the molecular structure. In addition, the CODEX type experiments are sensitive to the motion about the axis, perpendicular to the principal axis with the maximum principal values. Therefore, the combination of several CODEX experiments with anisotropic interactions, which have different principal axes, can characterize the reorientation of the molecular dynamics better. In this chapter, two new CODEX experiments, Dipolar CODEX and R-CODEX are introduced. The Dipolar CODEX experiment is used in the case of weak dipolar coupling ($^{13}$C-$^{15}$N) where both nuclei are unaffected by homonuclear dipolar coupling. On the other hand, the R-CODEX takes advantage of strong dipolar coupling ($^1$H-$^{13}$C or $^1$H-$^{15}$N) in a dense proton environment.

This chapter is partially based on our papers: “Characterization of slow conformational dynamics in solids: Dipolar CODEX” published in Journal of Biomolecular NMR, 2009, 45: 227-232 and “Investigation of Slow Molecular Dynamics using R-CODEX” published in Journal of Magnetic Resonance 2012 with permission of the journals, and when we developed the Dipolar CODEX, Krushelnitsky et al. also developed similar pulse program published in Journal of the American Chemical Society 131:12097-12099.
3.2 Dipolar CODEX

3.2.1 Dynamics detection using $^{15}\text{N-}^{13}\text{C}$ dipolar coupling
The Dipolar CODEX experiment is designed to detect the dynamics using the weak dipolar coupling such as the dipolar coupling between $^{13}\text{C}$. $^{15}\text{N}$. Several changes are made to better characterize the dynamics for this weak dipolar coupling. First, only the anti-phase component of the magnetization is stored after the dephasing time for better performance when the anisotropic interaction is weak as shown in Chapter 2. Furthermore, the recoupling pulses during the dephasing and refocusing times should have high efficiency to recouple the weak dipolar coupling. The REDOR (rotational echo double resonance) [20] is used here with higher scaling factor ($\sqrt{2}/\pi \approx 0.45$) [21], compared with other pulse sequences like SPI-R$^3$ [22] ($1/2\sqrt{2} \approx 0.35$) and symmetry-based R or C type pulse [23] ($\sim 0.2$).

![Diagram of Dipolar CODEX pulse program](image)

Figure 3.1 The Dipolar CODEX pulse program: 90° pulses are denoted using filled black lines. 180° pulses are denoted using hollow square symbols. CP: adiabatic cross polarization. DD: dipolar decoupling. (The detail information is in the experimental section) Tr: one rotor period. A REDOR (rotational echo double resonance) pulse program element was used for dephasing and refocusing the $^{13}\text{C}$. $^{15}\text{N}$ dipolar coupling. The basic phase cycles ($\phi$) are in the Appendix II.

With the above considerations, the pulse sequence of the Dipolar CODEX is shown as Figure 3.1. After the cross polarization, $^{13}\text{C}$ spins develop an anti-phase coherence ($C_yN_z$) with a phase that depends on the structure and crystallite orientation, under the influence of the recoupled $^{13}\text{C}$. $^{15}\text{N}$ dipolar coupling interactions. The two conformers involved in the conformational exchange process therefore develop different phases, here denoted $\phi_A$ and $\phi_B$. The subsequent 90-degree pulse on $^{13}\text{C}$ converts $C_yN_z$ to $C_zN_x$ for longitudinal storage. A relatively long mixing time from milliseconds to seconds is introduced during which the conformational exchange process redistributes the magnetization between two exchange sites, i.e. a site that has developed a phase angle $\phi_A$ during the dephasing period will develop a phase angle $\phi_B$ during the refocusing period. After the z-filter, the final intensity can therefore be represented by
Equation 3.1

\[ I_{x_{\text{anti-phase}}}^{\text{anti-phase}}(t_{\text{mix}}) = e^{-2i/T_2} e^{-t_{\text{mix}}/T_1} e^{-t_{\text{filter}}/T_1} \left[ P_A P_B (\sin \varphi_A - \sin \varphi_B)^2 e^{-t_{\text{mix}}/T_1} + (P_A \sin \varphi_A + P_B \sin \varphi_B)^2 \right] \]

where \( t_1 \) is the dephasing time, \( t_{\text{mix}} \) is the mixing time, \( t_{\text{filter}} \) is the z-filter time and \( P_A \) and \( P_B \) are the population ratios of the population ratios of the two exchanging conformations.

The effect of the spin-lattice relaxation in Dipolar CODEX experiment is different from that in original CODEX, only the anti-phase component \((C_Nz)\) is chosen to be stored in the mixing time as the coherence of \(C_Nz\). As shown in Section 2.7.2, the anti-phase component has the same performance with the combination of the in-phase and anti-phase components as in the original CODEX, but the coherence in the mixing time \((C_Nz)\) is different from the coherence in the z-filter \((C_z)\). As a result, the signal loss due to spin-relaxation is not a constant even when the total time of the mixing and z-filter periods is the same.

According to the derivation in Appendix I following reference [24], the relaxation rate of the \(C_Nz\) coherence is significant only when the motion rates of the \(^{13}\text{C}-^{15}\text{N}\) bond or the chemical shift anisotropic tensor of \(^{13}\text{C}\) and \(^{15}\text{N}\) are comparable with the Larmor frequencies of the \(^{13}\text{C}\) or \(^{15}\text{N}\). However, this relaxation will still affect the result in detecting the slow dynamics. Meanwhile, the spin flip will change the direction of the dipolar coupling (Figure 3.2) and then the dephase angle in Equation 3.1, resulting a decay without motions. The rate of this process is the rate that spins jump between spin-up and spin-down states, which actually can be described by \(T_1\). Therefore, a double exponential decay function, with one index for the relaxation of \(C_Nz\) coherence and spin flip effect, the other for the correlation time of dynamics, should be used here to fit the decay curve. The function of the z-filter here is just to remove the unfavorable coherence, rather than eliminating the effect of the spin-lattice relaxation.

Figure 3.2 The effect of the spin flip on the Dipolar CODEX experiment. In a) and b), the spin flip changes the direction of the dipolar coupling and cause the decay that does not result from motions. In c), the spin flips happen in both spins and will not change the direction of the dipolar coupling.
The reorientational angle can be extracted from the amplitude of the decay, but the difference between $\phi_A$ and $\phi_B$ which determines the magnitude of exponential decay are complicated functions of dephasing time and especially the reorientational angle. Therefore, the long mixing time experiments ($S$) can extract the flip angle by investigating how the signal intensity changes with the function of dephasing times. However, as shown in the above equation, the spin-spin relaxation is also the function of the dephasing time. Hence a reference spectra ($S_{ref}$) is taken with a very short mixing time like one rotor period. As shown in Equation 3.2, the normalized signal $S/S_{ref}$ removes the effect of the spin-spin relaxation. The spin-lattice relaxation, however, cannot be removed, but it can be obtained by fitting the decay curve when detecting the correlation time of dynamics and can be incorporated into the simulations.

$$\frac{S}{S_{ref}} = \left[ (P_A^2 + P_B^2) + 2P_A P_B \times \frac{<\sin \theta \sin \phi_2>}{<\sin^2 \phi_1>} \right] e^{-\left(t_{mix\_long} - t_{mix\_short}\right)/T_1}$$

Equation 3.2

where $t_{mix\_long}$ is the mixing time of $S$ while $t_{mix\_short}$ is the mixing time of $S_{ref}$.

If the spin-lattice relaxation is much longer than the correlation of the dynamics, Equation 3.2 can be simplified as shown in Equation 3.3 to remove the effects of spurious signals from other immobile sites.

$$\frac{S_{ref} - S}{S_{ref}} = 2P_A P_B \times \left( 1 - \frac{<\sin \theta \sin \phi_2>}{<\sin^2 \phi_1>} \right)$$

Equation 3.3

### 3.2.2 Motions of crystalline urea

The structure of urea has been well studied by X-ray and neutron diffraction [25-27]. In the crystal structure, urea is a planar molecule. Two equivalent ureas with space group $P4_2_1$ are packed in one unit cell with the carbonyl of each urea pointing in opposite directions and the two ureas’ planes perpendicular to each other (Figure 3.2). As seen in the projection down $c$, the crystal structure is quite open with the molecules and the hydrogen bonds confined to the two planes which intersect and leave hollow tunnels with a square cross section about $4\AA \times 4\AA$ along the $c$ axis. This big space supplies great freedom for the motion around the $C=O$ bond ($c$ axis), i.e. the motion that brings the NH$_2$ groups into the hollow tunnels. Meanwhile, from the viewpoint of molecular symmetry, the NH$_2$ group is also allowed to reorient about the C-N bond. Previously, both of these motions were modeled to fit the change in the atomic anisotropic thermal parameters of the neutron diffraction structure [28]. In NMR experiments, the whole-body motion
around C=O bond was also shown based on the \(^{14}\text{N}\) line-width and relaxation time in pulsed NQR experiments [29], the angular dependence of the second moment of proton [30] and the orientational dependence of the quadrupolar resonance splitting of the deuterons in urea-d\(_4\) [31]. The energy barrier for this whole-body motion is suggested to be about 10 kcal/mol. However the rate constants for the motion were not measured. Williams et al. [32] detected the motion rates of the urea for the first time by the T\(_1\) anisotropy experiments and DANTE selective inversion-recovery experiments in a wide temperature range from 30 °C to 120 °C. According to those experimental data and extrapolations based on the Arrhenius equation (Figure 3.3), the correlation times of the motions cover the time range from seconds to milliseconds. Therefore, crystalline urea, with a very simple structure and wide range of motion rates, is a very good model compound for the slow dynamics studies.

Figure 3.3 The neutron diffraction structure of the urea. The coordinates are from the Cambridge Structural Database (Entry No.: UREAXX08).

Figure 3.4 A summary of urea’s motions. The figure at right shows the motional model for urea. The figure at left is the Arrhenius plot of the whole body motion and the cis-trans isomerization. The units for the abscissa are 1000/K, and the units for k are s\(^{-1}\).
3.2.3 Experimental

$^{13}$C (99%), $^{15}$N (98%) isotopically enriched urea (Cambridge Isotope Laboratories Inc.) was recrystallized from water and crushed into powder form.

The pulse sequence for the Dipolar CODEX experiment is shown in Figure 3.1. Experiments were performed on a Varian Infinity 400 MHz triple resonance instrument, using a T3 triple resonance, MAS probe in a $^1$H/$^{13}$C/$^{15}$N configuration with 4 mm rotor. The magic angle spinning frequencies ranged from 6 to 9 kHz ($\pm$3 Hz). The temperature ranged from -10°C to 10°C ($\pm$0.1°C). Typical RF $\pi$ pulse’s lengths were 4.9 $\mu$s for $^{13}$C, 5.6 $\mu$s for $^{15}$N, and 6.2 $\mu$s for $^1$H. The carbon’s initial signal was enhanced by the adiabatic passage Hartmann-Hahn cross polarization. The constant $^1$H RF field had an amplitude of 45 kHz for all the experiments. The $^{13}$C channel’s tangential RF field followed Equation 3.4[33]. The $\omega_{HH}$ ranged from 36-39 kHz according to different spinning frequencies. The tangential parameters $\Delta$ and $\beta$ were 24 kHz and 13 kHz. The contact time was 1.3 ms. 60 kHz CW $^1$H decoupling was applied during dephasing and refocusing parts and about 60 kHz TPPM $^1$H decoupling was applied during acquisition.

$$
\omega^C(t) = \omega_{HH} + \beta \times \tan\left[\left(\frac{2t}{\tau} - 1\right) \times \arctan\left(\frac{\Delta}{\beta}\right)\right]
$$

Equation 3.4

A reference spectrum was taken by with mixing time of one rotor period. Since $T_1$ is long enough (more than 100 s) to be ignored, the normalized signal $(S_{ref} - S)/S_{ref}$ was used to fit the flip angle.

3.2.4 The investigation of the whole-body motion of urea using Dipolar CODEX

Figure 3.4(a) shows the mixing time dependence of the carbon peak intensity, which is clearly an exponential decay. The normalized signals $(S_{ref} - S)/S_{ref}$ are fit using a single exponential decay function to obtain the exchange rate describing the whole-body rotation of urea (Figure 3.4(b)). Figure 3.5 shows that the logarithm of the exchange rates thus extracted (Table 3.1) gives a straight line when plotted against inverse temperature, indicating the Arrhenius behavior, consistent with previous studies [32].
Figure 3.5 a) The carbon’s center peak intensity decays with increasing the mixing time. Measurements are made with at 268 K, MAS frequency of 6 kHz, and dephasing time of 4 rotor periods. b) The normalized center peak intensities in different temperatures are fit to a single exponential function. The error bar is the spectrum noise divided by the reference intensity.

![Figure 3.5](image)

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<td>3.2±0.5</td>
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Table 3.1 Exchange rates obtained from single exponential decay fits of the peak intensities are given for different temperatures.

Figure 3.6 Arrhenius plot of the natural log of the rate constant for urea whole body reorientation as a function of inverse temperature. The data at left top (triangle) are the prior literature data [32] taken at relatively high temperatures (30-100 °C). The data at the bottom right (square) are based on our Dipolar CODEX experiments.

![Figure 3.6](image)
The geometric information is another point of interest in dynamics research. As described in Chapter 2, the hop angle can be obtained by systematically varying the dephasing and refocusing times, while fixing the mixing time (Figure 3.6 (a)). However, the simulations presented in Figure 3.6 (b) show that the Dipolar CODEX experiments actually have poor ability to distinguish different $^{15}$N-$^{13}$C-$^{15}$N hop angles for the urea model, in which there are two nitrogen connected with carbon simultaneously.

Figure 3.7 The simulation of Dipolar CODEX data for different hop angles. Two different motional models are simulated: the jump motion of a single $^{13}$C-$^{15}$N bond (a) and the flip motion of the urea model, i.e. two nitrogens simultaneously connected with the same carbon (b). In both simulations, the $^{13}$C-$^{15}$N bond length used is 1.335 Å (based on X-ray data in the reference [27]); the MAS frequency is 8 kHz; the exchange rate is 500 s$^{-1}$; the mixing time is 15 ms for S experiments and 0.125 ms for $S_{\text{ref}}$ experiments; The field strengths for 90° and 180° pulse on $^{13}$C and $^{15}$N channels are 100 kHz in the simulations.

This insensitivity to the angle results from the faster magnetization dephasing rate of the urea’s carbon during the dephasing time, due to the two $^{15}$N neighbors (Equation 3.5). At the same time, the unit of increase for the dephasing time must be 2 rotor periods (Figure 3.1). Therefore, too few data points can be taken for the initial change (0-0.5 ms) of the dephasing curve, which is the most valuable region for distinguishing flip angles as shown in Figure 3.6 (b).

\[
C_x \rightarrow C_x N^A_x + C_x N^B_x \rightarrow \left[ C_x \cos \phi \cos \phi^B - 4C_x N^A_x N^B_x \sin \phi \sin \phi^B \right] + \left[ 2C_x N^B_x \cos \phi \sin \phi^B + 2C_x N^A_x \sin \phi \cos \phi^B \right]
\]

Equation 3.5

The principle of Scaled-CODEX [34] can be used to solve this problem. As shown in Figure 3.6, the $\pi$ pulse prevents the averaging of the dipolar coupling by mechanical magic angle spinning. The dephasing amplitude of the magnetization ($\phi$) depends on the position of the $\pi$ pulse, i.e. the length of $t_d$. Therefore, gradually increasing the length of $t_d$ (Figure 3.7) can better characterize the initial change of the dephasing curve (Figure 3.8). However, in practice the actual signal intensities of the first few time points
are very small (Figure 3.8 (a) and (b)) due to the choice of the anti-phase coherence. Therefore, $t_d$ should start at 20 $\mu s$ or 30 $\mu s$, even if the normalized intensities (Figure 3.8 (c)) of the first few time points ($t_d < 20 \mu s$) are large and have significant differences between different flip angles.

Without $\pi$ pulse, \[ \varphi = \int_0^{T_d} \omega(t)dt = \int_0^{t_d} \omega(t)dt + \int_{t_d}^{T_d} \omega(t)dt = 0 \]

With $\pi$ pulse at $t_d$, \[ \varphi = \left( -\int_0^{t_d} \omega(t)dt + \int_{t_d}^{T_d} \omega(t)dt \right) = -2 \times \int_0^{t_d} \omega(t)dt \]

Equation 3.6

Figure 3.8 The pulse sequence used for the simulations in Figure 3.8.

Figure 3.9 The simulations of Dipolar CODEX experiments for different hop angles based on the model for urea’s whole body $\pi$ flip motion as described in Figure 3.3. The simulations follow the pulse sequence in Figure 3.7, with the magic angle spinning frequency of 8 kHz and the exchanging rate of 0.5 kHz. The mixing time in (a) is 15 ms ($S$), while the mixing time in (b) is 0.125 ms ($S_{ref}$). The y-axis in (a) and (b) is the signal intensities relative to the initial carbon’s magnetization at the beginning of the simulation.
3.3 R-CODEX

3.3.1 Dynamics detection using $^1$H-X dipolar couplings

The R-CODEX experiment is designed to characterize the motion of the dipolar coupling axis of a $^1$H-X pair (X is $^{13}$C or $^{15}$N). Due to the strong dipolar coupling constant of $^1$H-X, the scaling factor is not as important as for the situation of $^{13}$C-$^{15}$N. In most protein samples, the $^1$H-X bond of interest is located in a dense $^1$H network, which can result in a severe signal loss during the dephasing and refocusing times. Therefore, the recoupling pulse used must have the ability to suppress the strong $^1$H-$^1$H homonuclear coupling. Compared to other recoupling schemes with the ability to suppress the $^1$H-$^1$H homonuclear coupling (like T-MREV [37]), the R187 (and the similar R185) can be applied for a wide range of magic angle sample spinning frequencies (~10 kHz-20 kHz) while efficiently suppressing the $^1$H homonuclear coupling [36]. This aspect lends it to practical use in protein studies. Meanwhile, the scaling factor for R187 (0.3 [35, 36]) is only a little smaller than the scaling factor of T-MREV (0.4~0.5 according to the different number of symmetry pulse in one rotor periods [37, 38]). Furthermore, R187 can be used to characterize the reorientational angle more accurately with better digitizing the dephasing. In addition, the use of R187 pulse sequence can specifically detect the motion of the directly connected $^1$H-X bond with little interference of the motion of the remote $^1$H. All these advantages will be discussed in light of experimental results, numerical simulations and theoretical considerations.

![Diagram of R-CODEX pulse sequences](image)

Figure 3.10 The R-CODEX pulse sequences used in these studies to detect conformational exchange rates (a) and reorientation angles (b). 90° pulses are denoted using black lines, and 180° pulses are represented by hollow rectangles. The 180° pulse in the middle of dephasing and refocusing periods refocuses the dephasing due to the isotropic chemical shift. CP: cross polarization. Tr: Rotor period. *R18$: R18_7 with 180° phase shift to prevent averaging of the recoupled dipolar coupling by 180° pulse. The mixing time...
should be an integral number of rotor periods [39]. The dephasing and refocusing periods should be even multiples of the rotor periods to reduce net evolution of other anisotropic interactions. In (b), the mixing time was fixed while the number of $RR$ blocks (denoted by one hollow (R) and one solid ($\bar{R}$) square) of the R18$^1$ pulse was increased systematically. All phases represented by a-g are given in the Appendix II.

### 3.3.2 Motions of imidazolium methylsulfonate

Prior studies of the imidazolium’s motion were motivated by the importance of its proton conductivity both in biological systems and fuel cells. One of the mechanisms proposed for proton transfer is called the Grotthuss process [40], which involves a $\pi$ flip of the whole imidazolium plane. However, studies of pure imidazole by the 1D/2D $^{15}$N exchange experiments have not observed significant reorientations even at 100°C [41, 42]. Recently, Goward [43] et al. reported a new imidazolium material crystallized from the methylsulfonic acid. The reorientation of the imidazolium plane with a correlation time between milliseconds and seconds (Figure 3.10) has been proven by line-shape analysis, dipolar coupling constant detection and CODEX experiments [43, 44]. Considering its simple $^1$H-X bond motion and high signal to noise, imidazolium methyl sulfonate is a very good model compound to test the R-CODEX experiments.

![Figure 3.11 The reorientational model of the imidazolium methyl sulfonate](image)

### 3.3.3 Experimental

$^{15}$N (98%) isotopically enriched imidazole (Cambridge Isotope Laboratories Inc.) was mixed with equimolar methyl sulfonate acid (diluted to 15%, to minimize the release of heat) and crystallized at room temperature. It was crushed into powder form and center-packed in a 4 mm rotor.

All experiments were performed on a Chemagnetics Infinity 300 MHz double resonance instrument, using an APEX double resonance MAS probe in $^1$H/$^13$C or $^1$H/$^{15}$N configuration with a 4 mm rotor. The sample temperature was maintained at 20 °C ($\pm0.1$ °C). For the $^{13}$C R-CODEX experiment, the $^8R18^1$ element (180° phase shift relative to R18$^1$) in Figure 1 (a) was replaced with 100 kHz CW decoupling.
because one rotor period was sufficient to fully dephase the $^{13}$C magnetization. In the angle detection experiment (Fig 1b), the total time of dephasing (refocusing) element was 2 rotor periods. The CW decoupling time was decreased incrementally with increasing the number of R187$^1$ blocks. The magic angle spinning frequency was 11 kHz ($\pm$3 Hz). The $^1$H field strength for the R187$^1$ element was 99 kHz, and the $^{13}$C field strength for all the 90° and 180° pulses was 100 kHz. The initial $^{13}$C signal was enhanced using adiabatic passage through the Hartmann-Hahn condition [45]. The constant $^1$H RF field during cross polarization had an amplitude of 52 kHz. The $^{13}$C tangential RF field followed Equation 3.7, where $\omega_{HH}^{\text{tang}}$ was 41 kHz and the tangential parameters $\Delta$ and $\beta$ were 12 kHz and 7 kHz, and the contact time was 3 ms. 60 kHz TPPM $^1$H decoupling was applied during acquisition.

$$\omega_C(t) = \omega_{HH}^{\text{tang}} + \beta \times \tan \left[ \left( \frac{2t}{\tau} - 1 \right) \arctan \left( \frac{\Delta}{\beta} \right) \right]$$

Equation 3.7

For the $^{15}$N R-CODEX experiment, the magic angle spinning frequency was 8.889 kHz, and the dephasing and refocusing time was 4 rotor periods (450 μs). The $^1$H field strength for the R187$^1$ element was 80 kHz, and the $^{15}$N field strength for all 90° and 180° pulses was 40 kHz. The initial $^{15}$N signal was enhanced using a flat Hartmann-Hahn cross polarization element, with a $^1$H RF field that had an amplitude of 40 kHz and a $^{15}$N RF field amplitude of 32 kHz. The contact time was 3 ms. 60 kHz TPPM $^1$H decoupling was applied during acquisition.

The $^{13}$C chemical shift was referenced to the $^{13}$C adamantane’s methylene peak at 40.26 ppm [46]. All the $^{15}$N chemical shifts are calibrated by the 0 ppm frequency calculated from $^{13}$C’s 0 ppm frequency, which was referenced from the $^{13}$C adamantane’s methylene peak at 40.26 ppm.

3.3.4 Investigation of the $\pi$ flip motion of imidazolium methyl sulfonate using R-CODEX

Spectra from R-CODEX experiments with different mixing times are shown in Figure 3.11 (a) and Figure 3.12 (a). The peak intensities for carbon and nitrogen lines generally decay markedly with increasing mixing time as expected (peak near 122 ppm in Figure 3.11 (a) and the peak in Figure 3.12 (a)). The peak intensity of the immobile carbon in the rotational axis has only a small amplitude decay with increasing mixing time (peak near 139 ppm in Figure 3.11 (a)), which can be attributed to the contribution of the motion of the dipolar vector connecting the immobile carbon with remote protons (i.e. the imidic $^1$H directly bonded to $^{15}$N). As shown in Figure 3.11 (b) and Figure 3.12 (b), the signal decay fits well to a single exponential function, and the extracted exchange rates are comparable to previous results from
line-shape analysis [43]. Analogous results from the $^{13}\text{C}$ and $^{15}\text{N}$ R-CODEX experiments indicate that the $^{13}\text{C}$-$^1\text{H}$ and $^{15}\text{N}$-$^1\text{H}$ motions are concerted (or at least have the same time constant) as expected. Moreover, the plateau values in Figure 3.11 (b) and Figure 3.12 (b) are 0.5, consistent with the two site jump model proposed with equal populations. The peak intensities of both carbon and nitrogen with the shortest mixing time are about 30% and 20% relative to cross polarization spectra. This signal loss results from the intrinsic 50% loss of CODEX type experiments as shown in Chapter 2 and the relaxation during the dephasing and refocusing periods.

Figure 3.12 $^{13}\text{C}$-$^1\text{H}$ R-CODEX spectra with different mixing times (black: 0.09 ms, blue: 1.36 ms, green: 2.73 ms, magenta: 4.55 ms, red: 27.27 ms). The peak near 139 ppm is the apical carbon in the imidazolium ring. The peak near 122 ppm is due to the two basal carbons. The two mobile carbons’ peaks are merged and unresolved. (b) Peak intensity vs. mixing time for apical and basal carbons. The black lines are single exponential fits with an exchange rate $k_{\text{ex}}$ of 0.32$\pm$0.03 kHz.

Figure 3.13 $^{15}\text{N}$-$^1\text{H}$ R-CODEX spectra with different mixing times (black: 0.09 ms, blue: 1.12 ms, green: 2.81 ms, magenta: 6.19 ms, red: 28.12 ms). The two $^{15}\text{N}$ peaks are merged and unresolved. (b) Peak intensities (black squares) are plotted vs. mixing time. The black line is a single exponential fit with exchange rate $k_{\text{ex}}$ of 0.27$\pm$0.02 kHz.
The reorientation angle of the basal $^1$H-$^{13}$C bond was characterized as shown in Figure 3.13. The intensities of both mobile and immobile carbons decay, but the intensities of mobile carbon decay much more in the long mixing time as compared with that in the short mixing time. The build-up curve of the normalized intensities $(S_{\text{short}} - S_{\text{long}})/S_{\text{short}}$ is fit using the corresponding simulations to obtain the reorientational angle as 82° with a 95% confidence interval [70°, 90°] (Figure 3.14). This agrees well with the reorientation angle of 81° determined from the neutron diffraction [47] (Cambridge Structural Database Entry: IMZMAL11). The relatively wide confidence interval partly results from limited S/N (the measurements were performed using natural abundance $^{13}$C materials). Also, the build-up curve depends on a sine function of reorientation angle (derived in Chapter 2.4), and thus is relatively insensitive at large angles as shown in Figure 3.13 (c). Meanwhile, the increment unit of the R187, pulse in the dephasing and refocusing times is an incomplete cycle, which also will introduce small errors [48].

Figure 3.14 $^{13}$C-$^1$H R-CODEX spectra with different R187, recoupling times during the dephasing (and refocusing) period. (black: 0 μs, blue: 20.2 μs, green: 40.4 μs, magenta: 60.6 μs, red: 80.8 μs). The mixing times are 0.18 ms in (a) and 27 ms in (b). In (c), the normalized intensities change as the R187, recoupling times are simultaneously increased (i.e. both the dephasing and refocusing time). Squares are experimental data for the immobile apical $^{13}$C while triangles are experimental data for the mobile $^{13}$C. The single lines are simulations of $^{13}$C-$^1$H bond exchanging with different reorientation angles, which vary from 10° to 90°. The exchange rate in the simulation is 0.3 kHz.

Figure 3.15 RMSD plot in the reorientational angle fitting
As shown in Chapter 2.4 and Figure 3.15, the use of an accurate dipolar coupling is important for determining the angle accurately. Two aspects should be considered to obtain the $^1$H-X dipolar coupling constant for the simulations. The first is whether the bond length is stable enough that a general value can be applied for all the cases. The hydrogen bonds like N-H···O and C$^\alpha$-H···O [49] will affect the C$^\alpha$-H and N-H$^N$ lengths. However, previous experiments [50] and theoretical calculations [51] show that short hydrogen bonds (N···O < 2.7 Å) can have a significant effect (>0.01 Å) on X-H bond lengths. Another factor to affect the bond length is the microsecond dynamics, which will average the dipolar coupling and decrease the dipolar coupling constant, but the averaged dipolar coupling still can detect the second-millisecond dynamics. In solid-state NMR, many studies have been done to study this dynamical effect on the H-X bond length [52-57]. They show that except for several residues with microsecond dynamics, the H-X bond lengths of most residues in the same protein are almost the same (± 0.1 Å). The second consideration for the $^1$H-X dipolar coupling constant is whether there is difference for the bond length detected by the NMR methods and neutron diffraction. Due to much higher time resolution in the data collection, the neutron diffraction data are not affected by the microsecond diffusion dynamics, and hence it is not proper to calculate the dipolar coupling constant directly from the neutron diffraction data. Meanwhile, considering the difference of the scaling factor between the experiment and theory calculation, a model compound should be used to extract the recoupled dipolar coupling constant by the 2D DIPSHIFT [58] experiment, in which the heteronuclear dipolar coupling is reintroduced during the first dimension using the same dipolar coupling recoupling sequence. In the current simulations, the dipolar coupling was assumed to be 23.3±0.5 kHz, the value extracted from the 2D experiment on the imidazolium ring of histidine-hydrochloride (Figure 3.16). This dipolar coupling constant (corresponding to 1.09±0.01 Å) is the same as the previous R187$_1$ experiment [35], but is 0.02 Å longer than the neutron diffraction result [59] presumably due to the vibrational averaging effects [60, 61].

**Figure 3.16** The dephasing curves of R-CODEX experiments for the jump of a single $^{13}$C-$^1$H bond. In (a), different flip angles are simulated with fixed $^{13}$C-$^1$H bond of 1.105 Å. In (b), the bond length is varied while the flip angle is fixed as 60°. Comparing (a) and (b), we can conclude that the little change on the bond length will cause a big change on the flip angle fitting.
3.3.5 The effect of vicinal protons

In most protein samples, the $^{1}$H-$^{15}$N (or $^{1}$H-$^{13}$C) bond of interest is located in a dense $^{1}$H network. Besides effectively suppressing the $^{1}$H homonuclear dipolar couplings during the dephasing and recoupling time, R-CODEX experiments can minimize the effects from the motions of additional nearby $^{1}$Hs. The single quantum dipolar spin operators generated in this R-CODEX experiment corresponding to various neighboring spin pairs ($H' C_z$SITE1$+H C_z$SITE1$ $ compared to $H' C_z$SITE2$+H C_z$SITE2$ $) do not commute, so the weak dipolar interaction between a $^{13}$C and a remote $^{1}$H is truncated by the directly bonded $^{1}$H-$^{13}$C coupling. Therefore, in a R-CODEX the artefactual decay due to the motion of the remote $^{1}$H has a very small amplitude (Figure 3.17).
Figure 3.18 The effect of motion of a remote \(^1\text{H}\) on \(^{13}\text{C}-^{1}\text{H}\) R CODEX and Dipolar CODEX (which uses a REDOR element to recouple the \(^1\text{H}-{^{13}\text{C}}\) dipolar interaction). The simulation system contains the apical carbon, the \(^1\text{H}\) bonded to it and the imidic \(^1\text{H}\) bonded to \(^{15}\text{N}\) (depicted as red in the right figure). We assume the imidazolium ring has a 180° flip with \(k_{ex}\) of 0.5 kHz. The left figure shows the remote \(^1\text{H}\)s (imidic \(^1\text{H}\) bonded to \(^{15}\text{N}\)) have a substantial effect for dipolar CODEX and a modest one for R-CODEX.

3.4 References


Chapter 4  A comparison of solid state NMR dynamics detection methods applied to L-phenylalanine
4.1 Overview

NMR is a powerful technique for probing dynamical properties of proteins that have correlation times in the second to millisecond range [1]. Generally speaking, millisecond dynamics can have three effects on the NMR experiment: first, in the hindrance of magnetization refocus under the echo or spin lock pulse; second, in the line-widths of the peaks, and third, in the magnetization transfer along with the spatial reorientation of the spins. The CPMG and $R_{1p}$ experiments, which capitalize on the first effect, have been widely applied in solution NMR, and applications of the $R_{1p}$ experiment in solid state NMR are rapidly developing. The second and third effects have a long history of use in both solution and solid state NMR, and recent experimental developments are based on the third effect. In this chapter, three methods for dynamics analysis based on the second and third effects, namely 2D exchange experiments and CODEX type experiments (based on the magnetization transfer) and line shape analysis (based on the line widths) are compared in terms of application to protein experiments. A natural abundance amino acid, L-phenylalanine hydrochloride, is used as a model for motions in solid proteins. The millisecond $\pi$ flip motions of its aromatic ring are characterized in detail for the first time.

This chapter is partially based on our paper: “Detection of Slow Dynamics by Solid-State NMR: Application to L-Phenylalanine Hydrochloride” Concepts in Magnetic Resonance, 2012, submitted, with permission of the journal.

4.2 The motion of phenylalanine

The aromatic ring in phenylalanine has $C_2$ symmetry (Figure 4.4). Therefore, the motion of the aromatic ring along the symmetric axis- the $C^\alpha-C^\gamma$ bond is predictable, and has been detected in single phenylalanine amino acids [2-5], peptides [6-9] and proteins [4, 10-13]. These experiments show that the dynamics of the aromatic ring are highly dependent on the environment. Even in the crystal of single L-phenylalanine molecule, the motion of the aromatic ring is complex and depends on the different crystallization conditions. In the work of Frey et al. [3], the aromatic ring’s motion in three crystal forms of L-phenylalanine were investigated. In L-phenylalanine crystallized from hydrochloride acid (Figure 4.1), the millisecond $\pi$ flip rotation of the aromatic ring was observed at room temperature; in the L-phenylalanine crystallized from a mixture of ethanol and water, no motion was detected; in the L-phenylalanine crystallized from water, two states of the molecule were observed, a immobile state and one with a $10^9\text{ s}^{-1}$ aromatic ring motion. The possible reason for these different rates is that the huge bulk of the rotating aromatic ring may result in a high rate dependence on the crowdedness in the environment.
Despite the fact that the spatial hindrance for the rotation of the aromatic ring is relatively large, the millisecond or slower dynamics are only observed in a few examples, such as the L-phenylalanine hydrochloride crystal [3], the cyclic penta-peptides [8] and the phenylalanines in the BPTI [11]. In all of these examples, the extra line-broadening in $^{13}\text{C}$ or $^1\text{H}$ spectra were used as proof of the millisecond or slower dynamics, but only a range of rates was estimated for the aromatic ring motion. Therefore, one of the goals of this chapter is to detect this millisecond motion more accurately, providing insight into the slow motion of the bulky group.

4.3 Experimental

L-phenylalanine was purchased from Sigma-Aldrich. The L-phenylalanine hydrochloride was crystallized by dissolving L-phenylalanine into 37% hydrochloride solution above 90 °C. The saturated L-phenylalanine solution was slowly cooled to room temperature and needle crystals were obtained in two days. The powder diffraction data in Figure 4.2 show that our L-phenylalanine hydrochloride has the same crystalline form as the structure stored in the Cambridge Structure Database [14] (Entry: PHALNC01). These needle crystals were crushed into powder forms and center-packed in the rotor for NMR experiments.
All NMR experiments in this chapter were performed on a Varian Infinity 300 MHz double resonance instrument, using an APEX double resonance probe in a $^{1}\text{H}/^{13}\text{C}$ configuration with 4 mm rotor. The line-shape analysis experiments were done using 70 kHz TPPM [15] decoupling during acquisition. The magic angle spinning frequency was 11 kHz for the experiments from -40 to 0 °C, and 8 kHz for the experiments from 20 to 60 °C. The initial $^{13}\text{C}$ magnetization was enhanced by adiabatic passage Hartmann-Hahn cross polarization [16] with a contact time of 2.5 ms. The constant $^{1}\text{H}$ RF field during cross polarization had an amplitude of 50 kHz, and the $^{13}\text{C}$ channel’s tangential RF field followed Equation 4.1 [16]. The $\omega^{\text{HH}}_c$ in Equation 4.1 for carbon ranged from 39-42 kHz for spinning frequencies 11-8 kHz. The tangential parameter $\Delta$ was 10 kHz and $\beta$ was 8 kHz. Typically every 1D experiment requires about 2 hours.

$$\omega^c(t) = \omega^{\text{HH}} + \beta \times \tan\left(\frac{2t}{\tau} - 1\right) \times \arctan\left(\frac{\Delta}{\beta}\right)$$

Equation 4.1

For the 2D exchange experiments, the magic angle spinning rotation frequency was 11 kHz, and the temperature was -25 °C. The initial $^{13}\text{C}$ magnetization was enhanced by 2.5 ms adiabatic passage Hartmann-Hahn cross polarization with the same parameters as the CP experiment described above. The first dimension was acquired with the TPPI method [17], and 256 points were taken with a 50 μs dwell time. The mixing time varied from 0.1 ms to 80 ms. The mixing time varied from 0.1 ms to 80 ms, and typically a 2D spectrum requires about 13 hours.

All R-CODEX experiments used a 11 kHz magic angle spinning rotation frequency, and 50 kHz TPPM decoupling [18] during the acquisition. The initial $^{13}\text{C}$ magnetization was enhanced by adiabatic passage Hartmann-Hahn cross polarization with the same parameters as the low temperature line-shape experiments. The dephasing and refocusing times were composed of a R187 pulse [19] of duration one rotor period, followed by 100 kHz CW decoupling of duration one rotor period. The R187 pulse contained nine ($\pi/70^a$)$($π$)_{290}^a$ blocks with the constant $^{1}\text{H}$ RF field strength (99 kHz). The mixing times were varied in order to sample the exchange rates for different temperatures. Typically a R-CODEX spectrum for one mixing time requires about 2 hours.

All simulations were performed using Spinevolution 3.4.1 with ChemExLib [20]. All spectra were processed using NMRPipe [21].
4.4 Results and Discussion

4.4.1 2D exchange experiments

2D exchange experiments can not only assign the two exchanging spins using the cross peak, but can also detect the exchange rate. In Figure 4.3, the cross peaks with the frequency as peak $\delta_1$ and $\delta_2$, $\varepsilon_1$ and $\varepsilon_2$ indicate that the aromatic groups exchange in pairs. Combined with 1D spectra, these exchange experiments prove that the ring undergoes a $\pi$ flip motion about the $C^\beta-C^\gamma$ bond. The rate of the flip motion can be determined by recording the intensities of the cross peaks between $\delta_1$ and $\delta_2$ with different mixing times (Figure 4.4). The experimental fit gives a rate of $130\pm20$ Hz. The cross peak intensities are normalized using the corresponding diagonal peaks to remove the effect of $T_1$ during the mixing time and any instability in environmental factors (e.g. temperature effects on the amplifiers). The cross peak between $\varepsilon_1$ and $\varepsilon_2$ are not used for the fitting because the intense diagonal peaks may affect their intensity, especially when the mixing time is short and the intensities are already weak.

![Figure 4.3 The assignment of the aromatic region of L-phenylalanine hydrochloride. The 2D exchange spectrum of ring carbons in crystalline L-phenylalanine hydrochloride was recorded at -25 °C with mixing time of 30 ms (left bottom) and 1D CP spectrum was recorded at the same temperature (left top). The motional model for L-phenylalanine hydrochloride is shown schematically on the right.](image)

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</table>

Table 4.1 $^{13}$C chemical shifts for the aromatic ring region of L-phenylalanine hydrochloride. $\delta_1/\delta_2$ vs. $\varepsilon_1/\varepsilon_2$ were identified based on solution NMR assignments[22].
Figure 4.4 2D exchange experiments on L-phenylalanine. The normalized intensities of the two cross peaks (red triangles and black squares) between $\delta_1$ and $\delta_2$ with increasing the mixing time. The black and red line are the fit to the equation $I_{\text{cross}} / I_{\text{diagonal}} = a \times (1 - e^{-k_{\text{ex}} t}) / (1 + e^{-k_{\text{ex}} t})$, where $I_{\text{diagonal}}$ is the diagonal peak intensity at 135.1 ppm, $I_{\text{cross}}/I_{\text{diagonal}}$ is the normalized cross peak intensity, and $a$ is the parameter that corrects for possible deviation caused by the overlap of diagonal peaks. The best fits for both cross peaks’ intensities yield $k_{\text{ex}}=130\pm/\pm20$ s$^{-1}$ and $a=0.97\pm/\pm0.05$.

4.4.2 1D exchange experiments

In addition to the 2D exchange experiment, the CODEX type 1D magnetization transfer method, R-CODEX [23] was also used to detect the motion of the phenylalanine. This method uses the $^1\text{H}-^{13}\text{C}$ dipolar coupling rather than chemical shift differences to sense motion. Due to the anisotropic property of the $^1\text{H}-^{13}\text{C}$ dipolar coupling, any motion that appreciably alters the direction of $^1\text{H}-^{13}\text{C}$ bond can be characterized. In R-CODEX spectra (Figure 4.5a), the intensities of the peaks involved in dynamical processes decay with increased mixing time ($\delta_1$, $\delta_2$, $\epsilon_1$ and $\epsilon_2$). The peaks for the static spins, $\gamma$ and $\zeta$, have negligible decay. (The limited decay that is observed can likely be attributed to the contribution from the distant protons and the peak overlap between the static and mobile peaks.) Therefore, even if the mobile spin overlaps with the static spin, R-CODEX experiments can still yield accurate motional rates. The exponential decay rates for all the mobile spins involved in the same dynamic process are in agreement within error, as expected (Figure 4.5b). Therefore, in the congested spectral region with spins having a concerted motion, full assignment is not always necessary. Moreover, the strongest peak (“M” in Figure 4.5a), which also contains the same dynamic information, can be selected to optimize the signal to noise ratio of the measurement. Following the same method, the motion rates at -15 °C and 0 °C are also detected (Figure 4.6). In Figure 4.7, the activation energy (57 kJ/mol) is obtained using an Arrhenius plot. This high energy barrier is typical for other rings that undergo a flipping motion.[7] The intensities at long mixing times are approximately half of the initial peak intensity, indicating that the population ratio for the two exchanging conformations is 1:1, which is of course expected for the 180° flip model for the aromatic ring.
Figure 4.5 $^{13}$C R-CODEX experiments at -25 °C. (a) $^{13}$C R-CODEX spectra emphasizing the NMR lines for the mobile ring sites of crystalline L-phenylalanine hydrochloride at -25 °C; mixing times as indicated. The peak positions selected correspond to assignments listed in Table 4.1. The position “M” indicates maximum intensity (not a particular peak position). The inserted smaller spectra display the whole aromatic region. (b) Single exponential decay fits for all peak intensities. The best fit rates are $170\pm40$ s$^{-1}$ ($\delta_1$), $160\pm20$ s$^{-1}$ ($\delta_2$), $160\pm30$ s$^{-1}$ ($\epsilon_1$), $150\pm10$ s$^{-1}$ ($\epsilon_2$), and $160\pm20$ s$^{-1}$ (M). All the peaks’ intensities were scaled by their intensities at the shortest mixing time.

Figure 4.6 The single exponential decay fits of the time dependent intensities of the strongest peak in the mobile region of R-CODEX spectra at -15 °C (a) and 0 °C (b). The rates ($k_{ex}$) obtained from the best fits (shown in the black lines) are $480\pm60$ s$^{-1}$ for data at -15 °C and $1900\pm200$ s$^{-1}$ for data at 0 °C.

Figure 4.7 The Arrhenius plot of the $\pi$ flip motion of the aromatic ring in L-phenylalanine hydrochloride. The unit for the abscissa is 1000/K, and the scale for $k_{ex}$ is $10^{3}$ s$^{-1}$. The activation energies extracted from linear regressions (black line) are 57 kJ/mol.
4.4.3 Line-shape analysis

The chemical shift difference for the $\delta_1$ and $\delta_2$ is 270 Hz while the chemical shift difference between $\varepsilon_1$ and $\varepsilon_2$ is 128 Hz. Therefore, the line-shape analysis, which is comparably sensitive to the motion rate as the chemical shift difference, can be used to characterize millisecond dynamics in this case. Figure 4.8 shows the comparison between the $^{13}$C spectra of the aromatic ring at different temperatures and the best simulations. The immobile carbon $\zeta$ is a good reference in the simulation to provide the information like the carbon line-widths in the absence of motion. The two exchange pairs, which have unique chemical shift differences (3.6 ppm vs. 1.7 ppm), are sensitive to somewhat different motion rates and make the results more accurate. The detected motion rates and the activation energy (Figure 4.9) agrees well with those detected by R-CODEX experiments, suggesting that the millisecond motion of the $^1$H-$^{13}$C bonds detected by R-CODEX experiment is the $\pi$ flip motion (Table 4.2).

Figure 4.8 $^{13}$C spectra of the aromatic ring of the L-phenylalanine hydrochloride crystal at different temperatures (left) and the corresponding best fit spectra (right). In the simulation, the six carbons in the aromatic ring were included with the isotropic chemical shifts determined in the Table 1. The $T_2$ values used in the simulations were estimated from the line-width of the $\zeta$ carbon, i.e. 6.4 ms for the spectrum recorded at 0 °C and 8 ms for spectra recorded at -40, -25 and -15 °C.
Figure 4.9 The Arrhenius plot comparing the line-shape analysis method (black triangle) and R-CODEX method (red solid circles). The unit for the abscissa is 1000/K, and the scale for $k_{ex}$ is $10^3$ s$^{-1}$. The activation energies extracted from linear regressions are $55+/-3$ kJ/mol (line-shape analysis) and $57+/-3$ kJ/mol (R-CODEX).

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<thead>
<tr>
<th>T (°C)</th>
<th>line-shape</th>
<th>Rcodex</th>
<th>2D exchange</th>
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<tr>
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<td>200+/-60 s$^{-1}$</td>
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<tr>
<td>-25</td>
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Table 4.2 The rate constants, $k_{ex}$, for aromatic ring flip motion in the L-phenylalanine hydrochloride crystalline as determined by three different experimental methods.

4.4.4 Comparison of 2D exchange, R-CODEX and 1D line-shape analysis experiments
The above experiments show that the 2D exchange experiment, R-CODEX experiment and 1D line-shape analysis experiment are all very powerful methods to characterize motion on the atomic scale, and have different advantages and disadvantages. The 2D exchange experiment and 1D line-shape analysis experiment use isotropic chemical shift to detect the motion while the R-CODEX experiment uses the dipolar coupling to detect the motion. Therefore, a chemical shift difference is a prerequisite for the 2D exchange experiment and 1D line-shape analysis experiment. Meanwhile, the difference in the detection interaction means that the detected process may be different. Any changes in either the environment or the spin itself that affect the chemical shift will be reflected in the 2D exchange experiment and 1D line-shape analysis experiment. However, the R-CODEX experiment is only sensitive to real motion of the $^1$H-$^{13}$C bond. Moreover, R-CODEX is robust even when the spectrum is crowded, but 2D exchange experiment and 1D line-shape analysis experiment require that the target peaks are separated from other peaks.
Another consideration in practical application is the experimental time. The 1D line-shape experiment needs the least time; even in our natural abundance sample, only one hour was needed for a spectrum with a good signal-to-noise ratio. Another advantage of the 1D line-shape experiment is that it can be used in the presence of the spin diffusion. Therefore, in the exploration of a new protein, the fully labeled sample used for the assignment can be prepared to quickly use in a 1D line-shape experiment. The R-CODEX experiment requires much more time. Several 1D experiments (at least 5) with different mixing times are required to detect the motional rate, and the intensity of the R-CODEX experiment is only ~30% relative to the 1D line-shape experiment, therefore one day is usually needed for a decent spectra. The 2D exchange experiment takes the longest, requiring several 2D spectra with different mixing times that usually take 3-4 days.

4.5 Conclusions

The millisecond motion of the L-phenylalanine hydrochloride is characterized in detail. This is likely to be a useful and well behaved model system for motion studies in general. This system was characterized using line-shape analysis, 2D exchange and R-CODEX experiments. The results from these three methods are consistent within experimental uncertainty (Table 4.2) and suggest a π flip motion. The line-shape analysis required the least experimental time, but the detection range for dynamics was limited by the chemical shift difference between two exchanging spins, and the need for fitting parameters such as the assumed T₂ in absence of dynamics and the chemical shifts for two exchanging spins. The overlap between the peak of interest and other peaks will also complicate the fitting and limit available information significantly. The 2D exchange experiment can extract much useful dynamical information in addition to motional rate (e.g. the population ratio for the reorientational conformations), but a significant amount of experimental time is required. 2D exchange experiments also require substantial chemical shift differences to separate the cross peaks from the strong diagonal peaks. 1D exchange experiments like R-CODEX have the advantage that they do not depend on the chemical shift difference and require less experimental time.

4.6 References

Chapter 5  Investigations of the Slow Loop Motion in Triosephosphate Isomerase (TIM) Using CODEX Experiments
5.1 Objectives

Triosephosphate isomerase (TIM) is a highly efficient enzyme that catalyzes the isomerization of dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde 3-phosphate (D-G3P), which is essential for the energy production in glycolysis. It is a catalytically perfect enzyme that catalyzes the reaction when almost every time the enzyme meets its substrate, and its specificity constant, $k_{cat}/K_m$ is on the order of $10^8$ M$^{-1}$s$^{-1}$. This kind of enzyme requires a hydrophilic active pocket to bind its substrate at just below the rate of the diffusion [1]. However, TIM requires a hydrophobic environment to stabilize the transition state [2, 3] and suppress the side reaction [2, 4]. The solution of this conflict is the conformational dynamics. Crystallographic studies have shown that a loop (loop 6) will be open for the ligand access; and then will close to create a hydrophobic environment; at the end, the loop will be open again to release the product [5-7]. Elegant kinetics and dynamics studies [8-13] have been done for the motion of this loop 6, and it has been proven that in the biologically significant direction (DHAP as substrate), the motion of loop 6 is probably correlated with product release and is likely a rate limiting step of the reaction. Furthermore, X-ray crystallography experiments show that both the open and closed conformations exist in ligated [14] or unligated TIM [15], but the population ratio between the open and closed is very different and skewed, i.e. most of unligated TIMs have the open conformation and most of ligated TIMs have the closed conformation. The hydrogen bonds formed between ligand and loop 6 may stabilize the closed conformation and cause this large conformational change.

According to the X-ray crystallography structures, another active site loop (loop 7), which has a highly conserved sequence, YGGS, in nearly all the TIM sequences and undergoes a large dihedral angle changes from the open to the closed conformation, can also stabilize the closed conformation. Two more hydrogen bonds are formed between loop 7 and loop 6 after the conformation changed from the open conformation to the close conformation. Therefore, loop 7 is very important to understand the mechanism of TIM’s dynamics. However, only a few studies on loop 7 have been reported, leaving many intriguing questions open like whether the motions of loop 6 and loop 7 are correlated. In this chapter, loop 7 and especially the highly conserved sequence (Y208-G209-G210-S211) is studied by solid state NMR techniques. More chemical shifts for G209 and G210 are unambiguously assigned to reflect structural information. Moreover, the CODEX experiment will be applied to reveal the dynamical properties of loop 7.
5.2 Introduction

Triosephosphate isomerase (TIM) is a ~52 kDa dimeric enzyme (26 kDa per subunit) which catalyzes the reversible interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP), the two products of the hexose ring when it is split in the glycolytic pathway. This interconversion process directs DHAP down the same "pay-off" pathway as GAP, doubling the energy produce per glucose and simplifying the regulation (Figure 5.1) [16]. Moreover, this interconversion keeps the balance of GAP and DHAP. The accumulation of DHAP disrupts the regulation of normal cell functions, leading to neuromuscular disorder and chronic hemolytic anemia [17].

![Figure 5.1 The function of Triosephosphate isomerase (TIM) in glycolysis. TIM interconverts DHAP and GAP and conducts a net flux from DHAP to GAP, producing two extra ATP for one molecule glucose.](image)

In addition to its important function in metabolism, TIM is an important model system for enzyme studies. The TIM barrel, a conserved protein fold consisting of eight α-helix and parallel β-strands, is named after TIM. This structural motif is by far the most commonly observed protein fold, constituting approximately 10% of all enzymes and including five of the six primary classes of enzymes [18]. The active site of most TIM barrel proteins are situated in the lower loop region created by the eight loops that connect the C-terminal end of the β-strands with adjacent α-helix [19]. Many TIM barrel enzymes also share a conserved phosphate binding pocket, with the phosphate moiety often found in the substrate and cofactors [18].

Meanwhile, in terms of catalysis, TIM is a very efficient enzyme. In the biologically significant (endergonic) direction (from DHAP to D-GAP), the active site of TIM can catalyze the initial proton abstraction from DHAP approximately $10^9$ times faster than the solution catalysis by a simple base [2].
According to the free energy profile calculated by from the rate constants (Figure 5.2), the free energy barrier is depressed from 26 kcal/mol [20] for the simple base catalysis to 13.5 kcal/mol for TIM catalysis [21-23]. Therefore, TIM is a representative protein to explore the high efficiency of enzymes generally.

Figure 5.2 The free energy profile of a simple base catalyst [20] (black) and TIM catalysis [21] (red).

5.2.1 TIM conformational changes
To efficiently coordinate different processes during the catalysis, conformational changes are necessary. Structural studies from more than 100 X-ray crystal structures of TIM and TIM-ligand complexes [2] have highlighted two important loop motions (Figure 5.3) for substrate binding and catalysis: the large displacement of the loop 6 (the tip moves ~7Å) and the large dihedral angle flip of the loop 7 (Table 5.1). Through these loop motions, TIM can form a tight phosphate binding pocket for the ligand (Figure 5.4), which can stabilize the transition state by ~12 kcal/mol [24].

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Table 5.1 Dihedral angle changes in loop 7 after G3P binds to TIM. The dihedral angles of apo TIM are extracted from the PDB file 1I45 and the dihedral angles of ligated TIM are extracted from the PDB file 1NEY.
Figure 5.3 The motions of loop 6 and loop 7 after ligand binding. The apo TIM coordinates (PDB file 1I45, orange) and ligated TIM coordinates (PDB file 1NEY, green) are superimposed in both figures. The right figure is a zoom-in view of the GGS part of the loop 7 (the peptide with orange carbons is apo form while the peptide with green carbons is the ligated form).

Figure 5.4 Hydrogen bonds between ligand and protein in the open and closed conformations. The X-ray structure of DHAP ligated TIM (PDB: 1NEY, green) is superimposed with X-ray structure of unligated TIM (PDB: 1I45, blue).
Figure 5.5 The functions of loop 6 and loop 7 motions shown in the superposition of the unligate TIM (PDB: 1YPI, blue) and the transition state analogue PGH ligated TIM (PDB: 7TIM, green). In the figure at left, the motions of the loop 6 and loop 7 sequesters the ligand from solution and creates a perfect environment for the catalytic reaction. In the figure at right, the rotation of Gly209 in loop 7 leaves the space to make Glu165 correctly positioned for the reaction.

Moreover, closing loop 6 is very important for efficient TIM catalysis. According to the proposed reaction mechanisms, either the classical mechanism [25, 26] or the criss-cross mechanism [27, 28], the first intermediate enediol:TIM is generated after the initial proton abstraction by Glu165 (Figure 5.6), which has a much lower energy barrier compared with a simple base catalysis (Figure 5.2). This high catalytic efficiency requires that the substrate and the base (the carboxylate moiety in the side chain of Glu165) have similar pKa values [29]. However, the pKa of the α proton of aldehydes or ketones in substrate is expected to be around 16-20 in solution [2, 30] while the pKa of the Glu165 side chain carboxylate is about 4 [31]. It is proposed that the closure of the loop 6 sequestered ligand from solution and increases the pKa of the carboxylate in Glu165 to match the pKa of the substrate [2, 3, 32]. At the same time, the side-reaction, elimination of the enediolate phosphate (Figure 5.7), which is faster than DHAP: D-GAP isomerization by at least a factor of 100 in solution, is suppressed in TIM by a factor of 10^5 to 10^8 in TIM as compared to in solution [33, 34]. In the mutation experiment [35] in which four tip residues in loop 6 (170-Ile-Gly-Thr-Gly-173) was deleted, the mutant TIM’s catalysis activity decreased 10^5 fold, and it no longer prevented the elimination reaction of enediolate phosphate. Meanwhile, the isotopic partitioning studies in the same mutant TIM showed that the main reason for the decrease in activity is the much higher energy barrier to enolize substrates bound to TIM [35]. Furthermore, systematic mutation studies on the hinge of loop 6 [36-38] found that for all mutations with an activity between 10%-70% of WT activity, the reduced k_cat/K_m relative to the wide-type mainly resulted from slower Michaelis complex formation between the substrate and TIM and slower product release rate. One interesting observation of this experiment was that the rate of phosphate elimination to form the by-product, methylglyoxal, was not affected by the mutations. Moreover, there is another TIM-
barrel protein: methylglyoxal synthase that has similar active sites’ structures with TIM but efficiently catalyzes the side reaction of TIM (i.e. the conversion of DHAP to methylglyoxal). After comparing the X-ray structures of transition state analogue 2-phosphoglycolated (2PG)-ligated TIM and 2PG-ligated methylglyoxal synthase [39], we found in methylglyoxal synthase, there are no loops to sequester the substrate from solution and no residues that can constrain the phosphoryl oxygen in the C2 plane of 2-PG and prevent the elimination of phosphate moiety. These results may suggest that the structure of active site in TIM, especially the structure of loop 6 is very important to stabilize the intermediate to facilitate the reaction and prevent the side reaction while the dynamics of loop 6 is very important to control the reaction rate.

Figure 5.6 The proton abstraction process by Glu165.
Figure 5.7 The phosphate elimination reaction which happens easily in solution, but it is prevented in TIM by the tight hydrogen bonding of the phosphate group with the TIM’s loops.

Meanwhile, loop 7, especially the highly conserved YGGS part (114 out of 133 TIM sequences in SWISSPROT database [40]) also plays a very important role in the TIM catalysis. In the closed conformation, loop 7 supplies hydrogen bonds to stabilize both the ligand and the closed form of the loop 6 (Figure 5.8) [41]. Moreover, the motion of loop 7 enables an about 3 Å shift of the active site base Glu165 (Figure 5.5) into its active position. Mutations in loop 7 have pronounced effects on the catalysis efficiency. The replacement of chicken TIM’s loop 7 (208-YGGS) with the corresponding loop 7 from an archaeal homologue (208-TGAG) caused a 102-fold loss of $k_{cat}$ and 4-fold increase of $K_m$ [13] and the mutation Y208F also caused a 600-fold decrease of $k_{cat}$ and 4-fold increase of $K_m$ [41]. From these kinetics data, we found these mutations on loop 7 mainly affect $k_{cat}$. Combined with the observation that $K_i$ of PGA increased ~200-fold for Y208F mutant TIM and the fact that the phenol hydroxyl group of Y208 formed a hydrogen bond with the closed form of loop 6, the first guess was that after the mutations loop 6 could not close perfectly and ligands were exposed to solution. However, this hypothesis was rejected by the fact that the substrate decomposition rate for the mutant TIMs was nearly the same as wide-type TIM [13, 41]. The viscosity experiments and isotopic experiments found that the rate-limiting step for the Y208F mutant TIM was enolization rather than the product off step for the wide-type TIM, and the energy barrier to produce enediol intermediate in the mutant TIM was much higher than that in wide-type TIM. This suggested that the mutations perhaps slightly distorted the perfect catalytic environment in wide-type TIM and the reaction intermediate was less well stabilized.
Figure 5.8 The hydrogen bonds formed between loop 7 (Tyr208-Ser211) to loop 6 (Pro166-Ala176) and the ligand in the DHAP-ligated TIM (PDB: 1NEY). The residues of loop 6 are depicted in orange and DHAP is depicted in green.

Due to the importance of the loop closure for the catalysis, questions will be asked like what drives the loop motion and whether the ligand is dispensable for the loop motion. The experiments in X-ray crystallography provide insight to these questions. Subunit b of a unligated rabbit TIM structure (PDB entry: 1R2R, resolution: 1.5 Å) [15] was observed with a closed conformation, and the projected ligand atom position was occupied by several water molecules that formed hydrogen bonds with residues in active sites. At the same time, in several TIM structures such as a human TIM (PDB entry: 1HTI) [42], only one subunit has the close conformation with the ligand bound while the other subunit is in the open conformation without ligand bound. Meanwhile, the two subunits in a yeast TIM-DHAP complex (PDB entry: 1NF0) [14] exhibit both the closed conformation with ligand and the open conformation without the ligand. Most explanations point to the crystal contacts to rationalize these abnormal structures.

However in the example of rabbit TIM above (i.e. apo TIM with closed loop 6, PDB entry: 1R2R), Aparicio et al. [15] found that the closed loop could potentially adopt the open conformation without any steric clashes. Moreover, loop 6 is open in another structure (PDB entry: 1R2S [15]) grown in similar conditions and with nearly the same crystal form. These experiments support that both the closed and open form conformations exist simultaneously whenever the ligand exists or not, but the ligand could adjust the population ratio of these two conformations. Under the particular situation, a higher crystal growth intake rate may generate the crystal with the lower population conformation. One interesting TIM category is P. falciparum TIM. Unlike most of other TIMs, the P. falciparum TIM has many crystal structures that have an open conformation for loop 6 even when ligands are present in the active site as shown in Figure 5.9. One possible reason is that the highly conserved S96 in other TIM sequences is changed to F96 in P. falciparum TIM. As shown in Figure 5.10, the bulk side chain of F96 would cause the steric clash for the closed form of loop 6 and may shift the population ratio between open and closed
conformations. From these special structures of ligated P. falciparum TIM, a two step binding process can be expected [41]: the ligand first forms the Michaelis complex with TIM, and then the TIM has a conformation change including the closure of loop 6 and large dihedral angle changes in loop 7 to push the ligand to the right position for the reaction.

Figure 5.9 The superposition of subunit b in the crystal structure 1LZO (green) [43] with the apo TIM structure [44] (PDB: 1YDV, pink, resolution: 2.2 Å), the 3PG (3-phosphoglycerate) ligated TIM structure [43] (PDB: 1M7O, yellow, resolution: 2.4 Å) and G3P ligated TIM [45] (PDB: 1M7P, blue, resolution: 2.4 Å).

Figure 5.10 The superposition of subunit b in the crystal structure 1LZO (green) and another PGA-ligated P. falciparum TIM structure, 1LYX (cyan, resolution: 1.9 Å) [43].

5.2.2 The dynamics of loop 6 and loop 7
According to last section, we know that the conformational changes of loop 6 and loop 7 are very important for the catalytic reaction in TIM. The first intriguing question is how fast loop 6 and loop 7 change their conformations between open and closed states. The motional rate detection for the conformational change of loop 6 was initiated by Williams et al. [8]. They selectively incorporated indole ring labeled deuterio-tryptophan into the N-terminal hinges of loop 6 at position 168, and applied $^2$H solid
echo experiment in the static solid state NMR to study the motion of loop 6. During the echo sequence, the molecular motion interferes with the coherence evolution and prevented complete refocusing of magnetization, resulting in a variety of characteristic line shapes. The motional rate, excursion angle and population ratio between open and closed conformations were obtained through fitting the $^2$H echo spectra with different dephasing times. They observed the loop motion rate approximately matched TIM’s catalytic turnover rate; the motional rate of loop ($2-3\times10^4$ s$^{-1}$) in the unligated sample, G3P (substrate analogue) ligated and PGA (transition state analogue) ligated TIM were nearly the same. However, the observations that the loop motional rate did not depend on the ligand seemed not to agree with the kinetic experiments. Take G3P and DHAP as examples. In the conversion of DHAP to GAP, the loop motion appeared to be the rate limiting step according to the isotope data, and hence the loop motional rate should comparable to the turn-over rate ($7.5 \pm 0.2 \times 10^2$ s$^{-1}$) [46]. In the reverse reaction, the proton transfer was the rate-limiting step and loop motion should be faster than the turn-over rate ($8.7 \pm 0.3 \times 10^3$ s$^{-1}$) [46]. Therefore, the loop rate depended on the ligands. It is perhaps because the extremely skewed population distribution between open and closed conformations made the spectra change due to dynamics very small. Meanwhile, in the $^2$H spectra fitting, both population ratio and motional rate were involved in the fitting (the excursion angles were obtained from crystal structures), so the uncertainty of the population ratio broaden the error bar of the fitted motional rate. Rozovsky et al. [10] improved this experiment by preparing the sample with half open conformation and half close conformation through controlling the concentration of G3P. This method largely enhanced the effect of dynamics on the $^2$H spectra, and the pre-known population ratio can decrease the number of fitting parameters in the fit of $^2$H spectra. Moreover, a kinetics model was proposed in this paper (Figure 5.11) based on the fact that the opening of loop 6 was precondition of the ligand binding. In the dynamics detection by $^2$H experiment, this model was further simplified to a two-site jump model (Figure 5.12) because the frequency changes of $^2$H reporter in loop 6 were only related to the conformational change of the $^2$H reporter group relative to main magnetic field. It is important to note that in this simplified model, $k_1$ is not related to $k_{close}$, but related to $k_{open}$, and the population ratio between open conformation ($P_{open}$) and closed conformation ($P_{closed}$) (Equation 5.1). With this model, loop 6’s opening rates at different temperatures were detected and an activation energy of 14 kcal/mol was obtained from an Arrhenius equation. The advantage of this $^2$H experiment in static solid-state NMR experiment is that the $^2$H spectral changes can clearly reflect the conformational change (the second step in Figure 5.11) and are not related to the ligand binding (the first step in Figure 5.11). However, this experiment is limited by the spectral sensitivity due to a broad linewidth of hundreds kilohertz, and hence only one reporter can be used. Moreover, the sample condition in the solid-state NMR (microcrystalline form) is different from the physiological condition. Therefore,
extra kinetic experiments were conducted to prove that TIM still has similar activity in the microcrystalline state as that in solution state [8].

\[ \text{TIM}^{\text{open}} + \text{ligand} \xrightarrow{k_{\text{on}}/k_{\text{off}}} \text{TIM}^{\text{open}}/\text{ligand} \xrightarrow{k_{\text{close}}/k_{\text{open}}} \text{TIM}^{\text{close}}/\text{ligand} \]

Figure 5.11 Kinetic model of ligand binding and conformational change in TIM.

\[ (\text{TIM}^{\text{open}} + \text{TIM}^{\text{open}}/\text{ligand}) \xrightarrow{k_1/k_{\text{open}}} \text{TIM}^{\text{close}}/\text{ligand} \]

Figure 5.12 Kinetic model for the motional rate detection by $^2$H lineshape experiments.

\[ k_1 = k_{\text{open}} \times P_{\text{closed}} / P_{\text{open}} \]

Equation 5.1

Solution NMR chemical exchange experiments were also applied to detect the motion of loop 6. 5’-fluoro-troptophan was incorporated into the same position as for the $^2$H experiments [9]. Due to the environmental sensitivity of fluorine, two separated lines were observed in the $^{19}$F spectra for the half G3P ligated sample at 0 °C. They resulted from the open and closed conformations rather than the ligated and unligated states because the fluorine was 8 Å away from the active site and it pointed away from the ligand binding site. Its solvent exposure changes in the conformational changes. Therefore, the model in Figure 5.12 was used to interpret the peak coalescence at higher temperatures (25 °C and 30 °C). The loop open rates at different temperatures can be estimated from the peak broadening and coalescence phenomena. These $^{19}$F experiments provided very clear proof of loop 6’s motion, but concerns about structural and functional perturbation caused by $^{19}$F substitute limited $^{19}$F labeling at more sites. With the improvement of solution NMR techniques, TROSY experiments made it possible to record global $^{13}$C, $^{15}$N spectra with narrow line-widths for large proteins like TIM (52 kDa for dimer). Massi et al. [11] detected the contribution of milliseconds-microseconds loop motions (Rex) on the $^{15}$N’s transverse relaxation using TROSY Hahn spin-echo pulse sequences [47] applied to the G3P fully ligated yeast TIM. $R_{\text{ex}}$ of backbone $^{15}$N at multiple residues on loop 6 were detected at different temperatures and their activation energies were extracted using the Arrhenius equation were nearly the same, proving that milliseconds-microseconds motions of residues on loop 6 was correlated. In these solution NMR experiments, a two site exchange model was assumed and $R_{\text{ex}}$ depended on the square of the chemical shift difference between two states. It was very difficult to define these two states as open and closed conformations because the ligand binding also could induce chemical shift changes. However, in the model shown in Figure 5.11, $k_{\text{on}} \gg k_{\text{off}}$ and $k_{\text{close}} \gg k_{\text{open}}$ [48], so the population of TIM$^{\text{open}}/\text{ligand}$ state
was very low. The model of Figure 5.12 still can be used whatever the chemical shift differences were caused by the loop motion or ligand binding. Moreover, with TROSY detected R₂ method [49] on backbone ₁⁵N₅, the milliseconds-microseconds motion of loop 6 was also detected for chicken TIM samples which were unligated and fully ligated with a transition state analogue PGA [12]. There were no significant increases for the transverse relaxation rates of the residues on loop 6 compared with the transverse relaxation rates of other residues. This result may indicate the loop 6’s motion in the presence of transition state analogue PGA was much slower than loop 6’s motion in the presence of substrate analogue G3P.

Besides NMR methods, laser induced temperature-jump experiments [48] were also used to study the motion of loop 6. The fast temperature jump induced by laser heating was used to disturb the equilibrium between different states, and the relaxation process was observed by the change of fluorescent intensities of the tryptophan 168 at the N-hinge of loop 6 in the G3P ligated TIM. This T-jump method was very sensitive and the relaxation process was not only related to $k_{\text{open}}$, but also related to $k_{\text{close}}$. Moreover, with the help of equations for dissociation constant $K_d$, $K_d = (k_{\text{off}} / k_{\text{on}})(k_{\text{open}} / k_{\text{close}})$, information about the ligand binding process in Figure 5.11 can be obtained. Therefore, $k_{\text{open}}$, $k_{\text{close}}$, $K_d$ and $k_{\text{on}} / k_{\text{off}}$ were determined at different temperatures and the activation energies of loop close and loop open was extracted as 13.8 kcal/mol and 14.1 kcal/mol, similar to the results in ²H experiments [10]. At the same time, enthalpy differences among the three states in model of Figure 5.11 were determined for the first time, proving that the ligand binding (the first step in Figure 5.11) contributes most of the entropy to the whole process. However, this method was limited by the heat diffusive cooling process, the observable time window is from 20 ns to 1 ms. Therefore, the presumably very slow loop motion for PGA ligated TIM could not be detected.
Figure 5.13 Summary of $k_{open}$ detected by different methods in the W90Y, W157F mutants of yeast TIM. Measurements from $^2$H spectra in solid-state NMR [10] are labeled in solid squares with deuteron labeled indole ring of W168, those from $^{19}$F solution NMR [9] are labeled in hollow squares with 5'-fluorotroptophan at W168, those from TROSY-Hahn spin echo experiments [11] are labeled in solid triangles with fully $^{15}$N and 85% deuteron labeled TIM and those from temperature jump experiments [48] are labeled in hollow triangles.

The opening rates detected by different methods are summarized in Figure 5.13. The motional rates at different temperatures fit well in the Arrhenius plot with the activation energy of about 14 kcal/mol. An intriguing question is what is the biological function for this loop motion with such a high energy barrier. Albery et al. [21] have shown that the rate-limiting step in the isomerization of DHAP to D-GAP is the product release. As discussed above, loop 6’s motion rate is comparable to the catalytic turn-over rate in the isomerization of DHAP to D-GAP. Moreover, in $^{31}$P chemical exchange experiments corresponding to the phosphor moiety of the bound G3P and free G3P [9], the product releasing rate was comparable to the opening rate of loop 6. Therefore, the opening motion of loop 6 is probably the rate-limiting step of the isomerization of DHAP to D-GAP. The mutant experiments on chicken TIM [12] also built strong correlation between the loop motion and catalytic function. In this experiment, two hinges of loop 6 (Figure 5.14) were mutated to P166G167G168/G174G175G176. The motional rate of loop 6 detected by TROSY-detected R$_2$ experiments was one order of magnitude slower than that of WT TIM while the turnover rate $k_{cat}$ reduced $10^3$-fold than WT TIM.
The milliseconds-microseconds motion of the loop 6 has been proved to be closely related to the catalytic function. Another interesting question is whether loop 6 has other dynamics in different time-scales such as nanosecond motions. Williams et al. [8] observed relatively short $T_1$ (~0.2 s) for $^2$H incorporated into the indole ring at W168 and long $T_1$ (>10 s) for $^2$H incorporated to $\alpha$-position at W168. Due to the lack of nanosecond overall tumbling that drove effective spin-lattice relaxation process in solution, these $T_1$ in static solid-state NMR experiments result from local motions of the deuterated group at the time scale of nanosecond. The relatively short $T_1$ in the indole ring fitted a model of 5°-7° libration with the rate of $10^8$-$10^9$ s$^{-1}$ (the large angle libration can be excluded because it could cause pounced line-shape narrowing which was not observed). The long $T_1$ at $\alpha$-position suggested the lack of substantial motion (>10°) for the backbone at nanosecond time scale. This backbone result was in agreement with the TROSY detected $T_1$ experiments [12], in which all backbone $^{15}$N $T_1$ on loop 6 were similar to most of other residues in TIM. This backbone rigid-body motion at nanosecond time scale favors the closing of the loop because if the residues on the loop 6 were very flexible, extra energy is required to compensate the entropy loss after the binding [12, 50].

While the loop 6 has been studied extensively, the studies for loop 7’s dynamical property are relatively fewer. By TROSY detected $R_2$ experiments in unligated chicken TIM, Kempf et al. [12] observed that the Val 212 on the loop 7 had much faster transverse relaxation ($R_2 = 150$ s$^{-1}$) than the average transverse relaxation of the protein ($R_2 = 41$ s$^{-1}$), indicating a microseconds-milliseconds motion of loop 7. Massi et al. [11] also characterized the motion of loop 7 by detecting the dynamics’ contribution to the transverse relaxation $R_{ex}$ in fully G3P ligated yeast TIM. They detected a significant value of $^{15}$N $R_{ex}$ for loop 7 residue Asn 213, indicating a microseconds-milliseconds motion. Moreover, they observed the activation energies for motions of loop 6 (30+/−10kJ/mol) and loop 7 (27+/−25kJ/mol) were similar, suggesting that loop 7 and loop 6 have correlated motions. The sequence analysis and mutation experiments also supported this correlated motion conclusion. The loop 6’s N-terminal hinge sequence PXW (X=I,L,V) accompanied loop 7’s YGGS sequence in 114 out of 133 TIM sequences, and for those few deviations, both of them were not conserved [40]. The mutation of loop 7’s YGGS sequence to TGAG [13] caused a
large increase of the motion rate for Val167 in loop 6 (9000 vs. 18000 s\(^{-1}\)) and a large decrease of the motional activation energy (6 vs. 58 kJ/mol). One explanation was that the mutation broke the connection between loop 6 and loop 7, and loop 6 could move more freely. Moreover, this mutation resulted in a 100-fold decrease of enzymatic activity. The isotope exchange experiments involving protonated GAP in 100% D\(_2\)O showed that more deuterated product was generated for the mutant TIM than for WT TIM, suggesting an imperfect loop closure during the catalysis. These experiments proved the interaction between loop 6 and loop 7 was very important for catalysis. However, one possible problem in these solution NMR experiments was that all these relaxation measurements depended on a chemical shift difference. The origin of these chemical shift differences may be the conformational change of loop 6 and loop 7 as expected, but they could instead be effects of ligand binding either. It is not very clear whether the detected process is the loop motion process or the ligand binding process. Therefore, a method that can specifically detect the loop motion should be introduced.

Figure 5.15 The contacts between loop 6 and loop 7 in the unligated state (blue, PDB entry: 1I45) and the ligated state (orange, PDB entry: 1NEY). Two hydrogen bonds between G173-Ser211 and between Tyr208-Ala176 stabilize the closed conformation [41, 51]. An interesting motion can be observed related to Pro166, Glu165 and Gly209. The “swung-out” position of the Proline ring leaves space for a flip of Gly209, which allows Glu165 to move to the right position for the catalysis. This provides a support for the prediction that the motions of loop 6 and loop 7 should be concerted.

5.2.3 Labeling scheme
CODEX experiments have the advantage that only one dimension is required for dynamics detection, and hence, ideally, one can use one dimension for the dynamics detection (by incrementing of the mixing time, using about 6-10 points) and two traditional (\(^{13}\)C-\(^{13}\)C or \(^{13}\)C-\(^{15}\)N) dimensions to resolve the peaks in a complex protein system. However, as shown in Chapter 2, CODEX type experiments have a relatively
high demand for acquisition time (signal to noise). Considering the relaxation during the dephasing and
developing, peak intensities even with short mixing times, are only expected to be about 20-30% of the
intensity of the direct cross polarization experiments, while peak intensities with long mixing times is
expected to be about 10-20%. Therefore, specific labeling that can identify a target residue in a one
dimensional spectrum is preferred for large protein systems like TIM. In loop 7 we are interested in both
Gly209 and Gly210 which have large dihedral angle changes (Figure 5.15). Moreover, TIM only has
three Gly-Gly pairs: Gly8-Gly9, Gly209-Gly210 and Gly232-Gly233 (Figure 5.17). As shown in Figure
5.18, Gly8-Gly9 are at the first beta-sheet and do not have conformational changes in the catalytic process,
so they provide very good reference peaks for dynamics detection. Gly232-Gly233 are on loop 8 and
form two critical hydrogen bonds with the bound ligand (Figure 5.4). Mutations on the 234-GGAS-237
sequence of loop 8 in trypanosomal TIM [52] cause the loss of catalytic ability and a two orders of
magnitude increase in the dissociation constant for the transition state analogue PGA, but X-ray structures
show that loop 6 and loop 7’s conformations are the same for the mutant TIM and WT TIM, so they are
very good indicators for ligand binding. Therefore, glycine has been chosen for selectively labeling for
dynamics investigations.

Selective labeling of protein by residue type, through growing the host E. coli on a defined medium
supplemented with certain labeled amino acids, has been used for a long time to simplify NMR spectra
[53-57]. Cross labeling and isotopic dilution as a result of scrambling through metabolic pathways are the
main concerns. The most effective approach is to use auxotroph strains, which have been modified to
have genetic lesions to limit the biosynthesis of certain amino acid of interest [58]. For glycine labeling
(Figure 5.16), legion of glyA (serine hydroxymethylase) [59] can prevent scrambling [60].

![Figure 5.16 The metabolism of glycine to form other amino acids. The data are from the KEGG (Kyoto
Encyclopedia of Genes and Genomes) database [61].](image-url)
We consider two labeling patterns: $^{13}$C glycine and $^{13}$C,$^{15}$N glycine. The carbonyl carbon is chosen due to the large chemical shift anisotropy (~70 ppm). Gly209 and Gly210 haven’t been assigned in solution NMR. A $^{13}$C,$^{15}$N glycine labeling pattern can be used to assign the carbonyl carbon. As shown in Figure 5.17, TIM only has three Gly-Gly pairs: Gly8-Gly9, Gly209-Gly210 and Gly232-Gly233. Using $^{13}$C - $^{15}$N correlation spectrum with short transferring times; the $^{13}$C and $^{15}$N chemical shifts of these three one-bond $^{13}$C'-$^{15}$N pairs can be assigned.

Figure 5.17 The sequence of yeast TIM, with the three GG pairs labeled in red.

Figure 5.18 The conformations of three G-G pairs in the apo (PDB entry: 1I45, green) and DHAP-ligated structure (PDB entry: 1NEY, purple). Th atoms of DHAP is depicted with large sphere.
5.2.4 The kinetic model
As discussed in Section 5.2.2, the ligated TIM has been observed with open conformation in some cases. Moreover, loop 6 should be open to allow ligand enter into the active site. Therefore, we can hypothesize that ligands first bind to the open conformation of TIM, and then the TIM’s loops undergo the large “jump-like” conformational changes forming the close form with the ligand. This process can be summarized in the model as shown in Figure 5.19.

\[
\text{TIM}^{\text{open}} + \text{ligand} \xrightarrow{k_{\text{on}}} \text{TIM}^{\text{open}}/\text{ligand} \xrightarrow{k_{\text{off}}} \text{TIM}^{\text{closed}}/\text{ligand}
\]

Figure 5.19 The kinetic model of ligand binding and the conformation change in TIM.

However, CODEX type experiments, detecting the conformation change process, can only distinguish two states, i.e. open conformation (TIM\textsuperscript{open} and TIM\textsuperscript{open}/ligand) and closed conformation (TIM\textsuperscript{closed}/ligand) as shown in Figure 5.20.

\[
(TIM^{\text{open}} + TIM^{\text{open}}/\text{ligand}) \xrightarrow{k_{\text{close}}/k_{\text{open}}} TIM^{\text{closed}}/\text{ligand}
\]

Figure 5.20 A simplified model for TIM’s conformational change

In this simplified two-site jump model, the open conformation is in equilibrium with the closed conformation, so

\[
k_{\text{close}}^* \times p_{\text{open}} = k_{\text{open}} \times p_{\text{closed}}
\]

Equation 5.2

where \(p_{\text{open}}/p_{\text{closed}}\) is the population ratio of TIM\textsuperscript{open} and TIM\textsuperscript{open}/ligand compared to the population of TIM\textsuperscript{closed}/ligand. The observed rate, i.e. the exponential decay rate of the motional atom can be expressed as

\[
k_{\text{ex}} = k_{\text{open}} + k_{\text{close}}^* = k_{\text{open}} + k_{\text{open}} \times p_{\text{closed}} \frac{p_{\text{closed}}}{p_{\text{open}}} = \frac{k_{\text{open}}}{1 - p_{\text{open}}}
\]

Equation 5.3

If the population fraction of the TIM\textsuperscript{open}/ligand is known, the opening rate of the loop can be determined with CODEX type experiments.
5.3 Experimental

5.3.1 Materials
All chemicals were purchased from Sigma-Aldrich with the exception of the isotopically labeled chemicals which were purchased from Cambridge Isotope Laboratory.

The modified pKK223-3 vector, containing *Saccharomyces cerevisiae* (baker’s yeast) Triosephosphate Isomerase gene, was a generous gift from Prof. Knowles of Harvard University and Prof. Sampson of SUNY, Stonybrook and used for expressing the wild type Triosephosphate Isomerase. The glycine auxotrophic E. coli strain JW2535-1 [60] was purchased from the E. coli Genetic Resources at Yale, the Coli Genetic Stock Center. The λDE3 Lysogenization Kit was purchased from Merck Company.

All recipes for the solutions and buffers are listed in the Appendix III.

5.3.2 Transformation of the Gly-auxotroph strain with the plasmid containing the TIM gene
The λDE3 prophage was integrated into the glycine auxotroph strain JW2535-1 using the λDE3 Lysogenization Kit, to supply the T7 RNA polymerase to express the TIM in the pET vector. Competent cells were prepared following the instructions from the QIAexpressionist™ (hand book from the QIAGEN company). A modified pKK223-3 vector, containing the TIM gene, was transformed into JW2535-1 competent cells using standard protocols (hand book from the QIAGEN company). The glycine auxotroph of the prepared cells was tested in the M9 plates.

Figure 5.21 The tests of the glycine auxotroph of the JW2535-1 strain with the pKK223-3 vector containing TIM gene. Except glycine and serine, the M9 plates had the same amino acids concentration as the M9 minimum media in section 5.3.3. The concentrations of glycine and serine were a) glycine 0 g/L, serine 2 g/L; b) glycine: 0 g/L, serine: 0 g/L; c) glycine 1 g/L, serine 0 g/L; d) glycine 1 g/L, serine 1 g/L.

These experiments proved that the metabolic pathways that can transfer other amino acids especially serine to glycine were cut off in our used glycine auxotroph strain. The positive result in c) was because other amino acids were transformed to serine.
5.3.2.1 Protein preparation

Cells were grown in 25g/L Luria broth (LB) solution at 37°C with shaking rate of 250 rpm. 50μg/L ampicillin was used to select the desire E. coli cells. Cells were harvested by centrifugation at 5000 g at 4 °C for 10 minutes when O.D. 600 of the LB media reached ~0.6. The pellet was resuspended in M9 wash buffer, and then spun down. The washed cells were gently resuspended in the following M9 minimum media with volume ratio of 4:1~5:1 between the LB and M9 minimum media. The following solutions are separately autoclaved or filter-sterilized and then combined to 1.5 L minimum medium:

1. 150 mL M9 buffer(10x) (autoclave)
   - 9 g Na₂HPO₄, 4.5gKH₂PO₄, 7.5gNaCl, 1.5g NH₄Cl
   - 3 mL 1 M MgSO₄
   - 1.5 mL 100 mM CaCl₂

2. 100 mL Carbon source (autoclave)
   - 4.5 g D-glucose

3. 1100 mL Amino acid solution (L-Form) (autoclave)
   - 1.5 g: Ala, Lys, Asp(aspartic acid), Glu(glutamic acid), Asn, Gln
   - 0.75 g Pro, Tyr, Ile, Phe, Val, Arg(arginine)
   - 0.75 g Ser
   - 0.3 g Thr

4. 150 mL amino acid solution 2: (autoclave)
   - 0.75 g Leu, Met,
   - 0.15 g Cys, Try, His

5. 30 mL solution C (filter sterilization)

6. 0.75 mL vitamin stock (filter sterilization)

7. 0.15 mg Thiamine (filter sterilization)

8. 1.5 gram 1-13C, 15N labeled glycine or 1.5 gram 1-13C labeled glycine (filter sterilization)

IPTG (final concentration to 0.4 mM) was added into the minimum media for induction after cells were equilibrated at 37 °C with good aeration for about an hour. About 10 hours after the induction, cells were harvested by centrifugation at 5000 g for 30 minutes. About 10~15 g of wet cell pellet was obtained from 1.5 liter minimum media. The pellet was then immediately resuspended in a resuspension buffer (~15 g wet cell pellet for 100 mL). Cells were lysed using a French press with 10,000 PSIG. 10,000 g centrifugation was used to remove the membranes, and then the ammonium sulfate was added to the
supernatant to 45% saturation (258 g/L). After equilibration for 2 hours, the solution was centrifuged for 30 minutes at 13,000× g to remove DNA. The supernatant was dialyzed extensively with Buffer A (described in the Appendix III). After that, protamine sulfate was added up to a final concentration of 1.5 mg/mL. After an hour’s equilibration, the solution was spun at 13,000 g for 20 minutes to remove nucleic acids that bind to protamine sulfate. The supernatant was dialyzed with Buffer B, and then passed through the Q sepharose fast flow anion exchange column. The eluted protein solution was then dialyzed with Buffer C and loaded onto a second Q sepharose fast flow anion exchange column. A linear gradient of 0-150 mM KCl (Buffer C, Buffer D) was applied to elute different proteins at different times. The desired protein solution determined by electrophoresis was purified again by phenyl sepharose fast flow anion exchange after dialyzed with Buffer E and a linear gradient of 1.4-0 M ammonium sulfate. The electrophoresis was used to verify the purify of the TIM solution (Figure 5.22). If TIM was not pure enough, another KCl gradient Q-sepharose column could be run to eliminate impurity. Finally, the pure TIM solution was dialyzed with buffer F and concentrated to about 150 mg/mL. Typically, 150 mg of pure TIM can be obtained from 1 liter minimum medium.

![Figure 5.22](image) The SDS-PAGE experiment to verify the purity of TIM after the phenyl-sepharose column. The third lane is the protein marker, and the last lane is the commercial TIM.

### 5.3.3 Activity assays

Activity of TIM is determined according to the reaction from GAP to DHAP [62]. As shown in Figure 5.23, in the presence of the α-Glycerol 3-phosphate dehydrogenase (GDH), the product of the TIM reaction (DHAP) reacts to consume the UV activity indicator β-nicotinamide adenine dinucleotide (NADH). Therefore, monitoring the NADH’s characteristic UV absorbance at 340 nm can measure the activity of the TIM. To accurately measure the TIM’s activity, the concentration of the TIM cannot be very high (<0.02 mg/mL).
The activity assay was conducted on the 0.75 ml scale in a quartz cuvette at 25°. The substrate concentration (10 mM) was chosen to have a large excess to $K_m$ (1.5 ± 0.1 mM for GAP [46]), the rate of the reaction catalyzed by GDH was much faster than that by TIM and the concentration of NADH was also much higher than its $K_m$ (2.7 μM [63]), so that the recorded initial reaction rate was close to the TIM’s maximum reaction rate. Therefore, the reaction solution contained 100 mM triethanolamine-HCl (TEA) at pH 7.6, 10 mM GAP, 0.3 mM NADH, 4 μmol/min GDH, and ~ 0.04 μmol/min TIM. 4800 units/mg is observed for our purified TIM.

### 5.3.4 Measurements of $K_d$ for the substrate analogue G3P

To detect the $K_d$ of the TIM, the indole ring in the tryptophan residues are used as the fluorescence probe to distinguish the open and close conformation [48]. Under the assumption that most of the ligated TIMs are in the close conformation while the most apo TIMs are in the open conformation [2], the characteristic fluorescence emission intensities of the tryptophan are investigated with different ligand concentrations (Figure 5.24). $K_d$ can be obtained by fitting the fluorescence intensity to the equation [64]:

$$
\Delta F = \frac{\Delta F_{\text{max}} \times [\text{ligand}]}{[\text{ligand}] + K_d}
$$

Equation 5.4
Figure 5.24 The emission spectra of TIM with different G3P concentrations. The emission intensity change with increasing ligand concentrations.

However, in the process of G3P titration, the added ligand solution dilutes the protein solution, and hence the $\Delta F$ in Equation 5.4 will continuously decay even the TIM has been saturated. A new modified fitting equation is derived below to calibrate this dilution effect.

In the ligand binding equilibrium $PL \xrightarrow{K_d} P + L$, the relationship between bound protein and $K_d$ can be derived as

$$K_d = \frac{[P][L]}{[PL]} \Rightarrow [PL] = [P]_0 \frac{[L]}{[L] + K_d},$$

where $[P]_0$ is the total protein concentration, $[PL]$ is the bound TIM’s concentration and $[L]$ is the concentration of the free G3P. Since the concentration of TIM is very low (~0.6 μM) relative to $K_d$ (~1 mM), $[L]$ can be thought as the total concentration of the G3P.

At the low concentration of TIM, we can define the initial fluorescence intensity of the protein $F_0$, the fluorescence intensity after adding the concentrated ligand solution $F_t$ as [65]

$$F_0 = k_{open} \times [P]_0^*, \quad F_t = k_{open} \times ([P]_0 - [PL]) + k_{close} \times [PL]$$

where $[P]_0^*$ is the total TIM concentration before diluted by the ligand solution, $k_{open}$ and $k_{close}$ are the open and close conformations’ emission coefficients containing the quantum efficiency, incident radiant power, molar absorptivity and the path length of the cell. Therefore,
\[ \Delta F = F_0 - F_i = k_{\text{open}} ([P]_0^* - [P]_0) + (k_{\text{open}} - k_{\text{close}}) \times [PL] \]
\[ = k_{\text{open}} ([P]_0^* - [P]_0) + (k_{\text{open}} - k_{\text{close}}) \times [P]_0 \times \frac{[L]}{[L] + K_d} \]
\[ \Rightarrow \Delta F = \frac{k_{\text{open}} [P]_0^*}{c} [L] + (k_{\text{open}} - k_{\text{close}}) \times \left(1 - \frac{[L]}{c}\right) \frac{[L]}{[L] + K_d} \]

Equation 5.5

where \( c \) is the G3P concentration in the concentrated G3P solution. In the Equation 5.5, three parameters \((k_{\text{open}} [P]_0^*/c, k_{\text{open}} - k_{\text{close}}, K_d)\) rather than two parameters in Equation 5.4 should be used to fit the data, but new equation accounts for the effect of dilution.

Figure 5.25 K\(_d\) detection by fitting the fluorescence data with different equations. When the G3P concentration is very high (>10 K\(_d\)), the fluorescent intensity should be constant according to Equation 5.4, but the dilution from adding G3P concentrated solution makes \( \Delta F \) become higher.

These steady-state fluorescence spectra were measured on a Perkin Elmer LS55 Luminescence spectrometer in Professor Ruben Gonzalez’s lab. About 80 units TIM (~0.6 \( \mu \)M) was dissolved in 1 mL buffer of 50 mM Tris and 30 mM NaCl. The TIM solution was held in a 2×10 mm quartz cuvette. In the titration, 200 mM G3P dissolved in a buffer of 50 mM Tris and 30 mM NaCl was added into the cuvette. The excitation wavelength was 290 nm.

With the method introduced above, the G3P’s K\(_d\) was investigated with different pH, PEG concentration and temperatures. It was found that small fluctuation of the pH and the presence of the PEG (Table 5.2) will not affect G3P (D form)’s K\(_d\). This PEG effect was smaller than the results obtained by Williams et al. [66](about 2-fold decrease of K\(_d\)). One possible reason is that the concentration of PEG they used was 30%, which was much higher than ours (10%). The D-G3P’s K\(_d\) at different temperatures were listed in
Table 5.3, which agreed with previous detection by Desamero et al. [48]. As shown in Table 5.3, temperature has large effect on $K_d$, and should be carefully considered in the NMR sample preparation.

<table>
<thead>
<tr>
<th>pH</th>
<th>6.51</th>
<th>6.77</th>
<th>7.09</th>
<th>6.77</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10%</td>
</tr>
<tr>
<td>$K_d$ (mM)</td>
<td>0.77+/-0.08</td>
<td>0.7+/-0.1</td>
<td>0.76+/-0.07</td>
<td>0.6+/-0.1</td>
</tr>
</tbody>
</table>

Table 5.2 The effect of pH and PEG concentration on the D-G3P’s $K_d$ at room temperature.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ºC</td>
<td>0.2+/-0.1 mM</td>
</tr>
<tr>
<td>22 ºC</td>
<td>0.7+/-0.1 mM</td>
</tr>
<tr>
<td>29 ºC</td>
<td>1.4+/-0.1 mM</td>
</tr>
</tbody>
</table>

Table 5.3 The effect of the temperature on the D-G3P’s $K_d$ at pH 6.8.

### 5.3.5 Solid state NMR sample conditions

For the apo TIM sample, the enzyme was concentrated to 100-200 mg/ml in the buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA and pH 6.8 at 4 ºC). The concentrated polyethylene glycol (PEG) (average molecular weight was ~4000) solution (40% w/v) in the same buffer was slowly added to the protein solution in a sealed pipet tip until the solution appeared cloudy. The final concentration of the polyethylene glycol was approximately 15%. The solution was then left in the 4 ºC fridge for several hours for crystallization.

For the G3P bound samples, the disodium salt of G3P (secondary pKa=6.45 [67]) was dissolved in the same buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA and pH 6.8 at 4 ºC) as concentrated TIM solution and added into the protein solution and equilibrated with enzyme for one hour. Then PEG solution was added into protein solution following the same procedure as unligated sample. The crystallization condition for G3P bound sample was the same as the apo sample.

After crystallization, the sample in the sealed pipet tip was first centrifuged down and quickly transferred into the rotor at 4 ºC. Typically, about 40 mg sample (about 40 μL) was center packed in a 4 mm rotor.

To control the percentage of the bound TIM, the concentration of the G3P was pre-calculated as shown below:
\[
K_d = \frac{[TIM]_{\text{unligated}}[G3P]_{\text{free}}}{[TIM / G3P]_{eq}} = \frac{([TIM]_0 - [TIM / G3P]_{eq})([G3P]_0 - [TIM / G3P]_{eq})}{[TIM / G3P]_{eq}}.
\]

\[
[G3P]_0 = \frac{K_d \times [TIM / G3P]_{eq} + [TIM / G3P]_{eq}}{[TIM]_0 - [TIM / G3P]_{eq} + [TIM / G3P]_{eq}}.
\]

Equation 5.6

where \(K_d\) is the dissociation constant, \([G3P]_0\) was the initial concentration of the G3P in the pipette tip before it bound to the TIM, \([TIM]_0\) was the total concentration of the TIM, \([TIM/G3P]_{eq}\) was the concentration of the bound TIM in equilibrium, \([TIM]_{\text{unligated}}\) and \([G3P]_{\text{free}}\) were concentrations of unligated TIM and free G3P in equilibrium. We can simplify Equation 5.6 to

\[
[G3P]_0 = \frac{K_d \times y + y \times [TIM]_0}{1 - y}.
\]

Equation 5.7

where \(y\) is the ratio between \([TIM/G3P]_{eq}\) and \([TIM]_0\).

One of the problems in the preparation of the G3P half ligated TIM is the temperature dependence of \(K_d\) as shown in Table 5.3. To solve this problem, the G3P concentration is calculated using the \(K_d\) at 4 °C, and the half ligated sample is prepared by conducting all the experiments like the addition of G3P, protein crystallization and the centrifugation at 4 °C. This ensures that in the protein crystals at 4 °C, the population ratio for the open and closed conformation is 1:1. Then in the solid state NMR sample (protein crystals), the active site concentration is as high as 29 mM (calculated from the apo TIM crystal structure 1NEY [14]), and the concentration of the bound G3P is about 15 mM. Therefore, even if the sample temperature changes during the NMR, the small change of \(K_d\) (Table 5.3) cannot induce a significant change on the population ratio between the apo and bound states. In our half ligated sample, there was 1.44 μmol TIM was precipitated from 300 μL concentrated TIM solution. To prepare the half-ligated sample, 0.78 μmol D-G3P was added into the solution. In equilibrium at 4 °C (\(K_d=0.2\) mM), 0.72 μmol D-G3P was bound to TIM while 0.06 μmol D-G3P was in solution.
5.3.6 NMR experiments

5.3.6.1 Pulse programs used in the experiments

![Diagram](image)

Figure 5.26 2D $^{13}$C-$^{13}$C correlation spectra were acquired using Proton Driven Spin Diffusion (PDS) [68] without $^1$H irradiation.

![Diagram](image)

Figure 5.27 2D $^{13}$C-$^{15}$N correlation spectra were acquired using the selective Double Cross Polarization (DCP) method [69, 70].

![Diagram](image)

Figure 5.28 The CODEX experiment used for measurements in glycine $^{1-13}$C labeled TIM [71]

5.3.6.2 Selectivity of Double Cross Polarization (DCP)

To increase the spectral resolution and exclude glycines that are not involved in the motion, a selectively $^{1-13}$C, $^{15}$N glycine labeling pattern was used as shown and discussed in Section 5.2.3. This labeling scheme ensures that the only one-bond $^{15}$N-$^{13}$C pairs are in a GG sequence pair. The DCP (Double Cross Polarization) [69, 70] experiment was used to specifically identify these adjacent $^{15}$N-$^{13}$C dipolar
couplings in the glycine-glycine pairs. The simulations in Figure 5.29 show that only the directly bound C’-N pairs have significant intensities in a DCP spectra.

Figure 5.29 Simulations of the magnetization transfer from $^{15}$N to $^{13}$C using DCP pulse sequence elements. The black data show the significant transfer for the neighboring C’ and N (Gly209’s C’ - Gly210’s N, 1.33 Å) while the far pairs, for example Gly171’s C’ - Gly173’s N (3.68 Å, red) and Gly210’s C’ – Gly210’s N (2.4 Å, blue) have very little transfer. In the simulations, all the coordinates are from PDB entry 1I45 [14], the power on $^{15}$N channel (35 kHz), $^{13}$C channel (25 kHz) and the shape on the $^{13}$C channel’s power (2.5 kHz) as the same as used in the experiments. In my experiment, 3 ms contact time was used, and cross peaks’ intensities of long $^{13}$C-$^{15}$N pairs (blue and red) are less than 6% of the cross peak intensity of the short $^{13}$C-$^{15}$N pair (black).

5.3.6.3 Spectroscopic set-up for CODEX experiment

As discussed in Chapter 2, rotor synchronization is very important for CODEX type experiments. Although modern solid state NMR instruments can keep the spinning very stable, extra effort is necessary to keep the spinning synchronized for CODEX type experiments. A trigger signal, which comes from the laser reflection intensity change caused by the black mark on the rotor end, can solve this problem. We make the dephasing and refocusing times start immediately after this trigger signal (the “Trigger” in Figure 5.28), and ensure that the rotor position at the beginning of the dephasing time is the same as the rotor position at the beginning of the refocusing time. One possible problem for this set-up is that the mixing time could be one rotor period longer than intended if the rotor rotates a little bit slower. Therefore, the mixing time is practically set a half rotor period shorter than the expected value, and then after the mixing time, the instrument will wait for the trigger signal to start the following pulses.

Another possible problem for dynamics detection is the stability of the instrument. Since CODEX type experiments sacrifice more than half of the signal for dynamics detection and the detection totally depends on the peak intensity, the instrument stability is very important for CODEX type experiments. Therefore, a special acquisition mode is set as illustrated by Figure 5.30. For each mixing time, only a
basic phase cycle is done for each block (32 scans, usually ~100 seconds), then the experiment with the following mixing time is taken. This acquisition mode can compensate partly for the effect of amplifier’s instability caused by the environment.

![Image](image_url)

**Figure 5.30 Acquisition mode for CODEX type experiments**

### 5.3.6.4 Sample heating and G3P decomposition

We found that during the course of our NMR experiment, the G3P decomposes [72] due to sample heating. In the solid state NMR experiment, sample heating is mainly from two sources: the rapid sample spinning and the high power $^1$H decoupling. The room temperature "drive” gas and the friction between the rotor and the gas stream can increase the sample temperature [73]. At the same time, the radiofrequency (RF) power dissipation in the a “lossy” (low dielectric and salty) protein sample will hugely increase the sample temperature, especially during the high power $^1$H decoupling [74]. According to the thesis of Yimin Xu [72], 7 ms of 70 kHz $^1$H decoupling can induce a 15 °C temperature jump. One method to protect the G3P from decomposition is to conduct the experiment at a low temperature and use relatively short and low power $^1$H decoupling, and a relatively long pulse delay to allow temperature equilibration before the next experiment. In our experiments, we were able to take advantage of the long distance between the carbonyl carbon and the nearest proton to make of $^1$H decoupling power during the acquisition as low as 45–55 kHz while obtaining high resolution data. Furthermore, we found G3P would decompose if the TIM sample was not pure enough. We could run an extra KCl gradient column as described in Section 5.3.3. 2D DCP spectra taken before and after the CODEX experiment were used to check whether the G3P decomposed (Figure 5.31).
Figure 5.31 The DCP spectra used to investigate G3P’s decomposition. The DCP spectrum shown in green was taken before the CODEX experiment. The DCP spectra shown in red and purple were taken after the CODEX experiment with impure and pure TIM samples.

5.3.6.5 NMR spectroscopy

All experiments were performed on a Chemagnetics Infinityplus 400 MHz double resonance instrument, using an T3 triple resonance MAS probe in $^1$H/$^{13}$C or $^1$H/$^{13}$C/$^{15}$N configuration with a 4 mm rotor. The VT temperature was maintained at -15 °C (±0.1 °C) except as specifically indicated. After the calibration and correlation for experimental heating, the sample temperature in rotor corresponds to -10 ± 3 °C according to the K$^{79}$Br temperature calibration [75] for the same probe with the same magic angle spinning frequency. The magic angle spinning frequency was 10 kHz for all the experiments. The typical $^{13}$C field strength for 90° and 180° pulses was 100 kHz, and $^{15}$N field strength for 90° and 180° pulses was 62.5 kHz. In the $^{13}$C-$^{13}$C correlation experiment and CODEX experiment, the initial $^{13}$C signal was enhanced using adiabatic passage through the Hartmann-Hahn condition [76]. The constant $^1$H RF field during cross polarization had an amplitude of 45 kHz. The $^{13}$C tangential RF field followed Equation 5.8 [77], where $\omega^{\text{HH}}$ was 35 kHz and the tangential parameters $\Delta$ and $\beta$ were 7 kHz and 5 kHz, and the contact time was 1.1 ms. In the $^{13}$C-$^{15}$N correlation experiment (Figure 5.28), the initial $^{15}$N signal was enhanced using adiabatic passage through the Hartmann-Hahn condition [76]. The constant $^1$H RF field during cross polarization had an amplitude of 45 kHz. The $^{15}$N tangential RF field followed Equation 5.8 [77], where $\omega^{\text{HH}}$ was 35 kHz and the tangential parameters $\Delta$ and $\beta$ were 6 kHz and 3 kHz, and the contact time was 1.3 ms. In the process of the DCP transfer, the $^{15}$N’s carrier frequency was set at 119.9 ppm, and a constant amplitude of 35 kHz was applied on the $^{15}$N channel. The $^{13}$C’s carrier frequency was set at
165.5 ppm, and its RF field follows Equation 5.8 [77], where \( \omega_{HH} \) was 25 kHz and the tangential parameters \( \Delta \) and \( \beta \) were 2.5 kHz and 1.5 kHz. The contact time was 3 ms and a \( \omega_{H}=90 \) kHz \(^1\)H CW (continuous wave) decoupling was applied during the whole DCP process.

\[
\omega^C(t) = \omega_{HH} + \beta \times \tan \left[ \frac{2t}{\tau} - 1 \right] \times \arctan \left( \frac{\Delta}{\beta} \right)
\]

Equation 5.8

For the \(^{13}\)C-\(^{13}\)C correlation spectra, the acquisition times for both dimensions were 18 ms. 50 kHz TPPM \(^1\)H decoupling was applied during the acquisition. The carrier frequency was 179.5 ppm, and the dwell time for the first dimension was 50 \( \mu \)s. The length of the mixing time was 350 ms. For the \(^{13}\)C-\(^{15}\)N correlation experiment, the acquisition time for the direct \(^{13}\)C dimension was 8~10 ms with 50 kHz TPPM \(^1\)H decoupling, and the acquisition time for the indirect \(^{15}\)N dimension was 5~6 ms with 60 kHz TPPM \(^1\)H decoupling. The carrier frequency of \(^{13}\)C was 165.5 ppm, the carrier frequency of \(^{15}\)N was 119.9 ppm, and the dwell time for the indirect dimension was 200 \( \mu \)s. In the CODEX experiments, the dephasing time and refocusing time were one rotor period, i.e 0.1 ms. 85 kHz \(^1\)H CW decoupling was used during the dephasing and refocusing times. The acquisition time was 7.7 ms with 50 kHz TPPM \(^1\)H decoupling.

All \(^{13}\)C chemical shifts were referenced to the DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) standard (0ppm) using the methylene \(^{13}\)C peak of adamantane at 40.48 ppm [78] and all \(^{15}\)N chemical shifts were indirectly calibrated using the \(^{13}\)C frequency and the gyromagnetic ratio of \(^{13}\)C to \(^{15}\)N as published in BMRB (Biological Magnetic Resonance Bank).

5.4 Results and Discussion

5.4.1 Assignment of Gly209 and Gly210 in TIM

Figure 5.32 and Figure 5.33 show that with our specific labeling patterns (1-\(^{13}\)C glycine and 1-\(^{13}\)C,\(^{15}\)N glycine) and chosen NMR experimental conditions, only three Gly-Gly pairs have strong cross peaks in \(^{13}\)C-\(^{13}\)C and \(^{13}\)C-\(^{15}\)N correlation spectra (Gly8-Gly9, Gly209-Gly210 and Gly232-Gly233). These glycine in the G-G pairs are numbered 1-6 as shown in Figure 5.32 and Figure 5.33. The chemical shifts of G1-G2 agree very well with previous solution and solid NMR assignments for the G8-G9 (Table 5.5). Therefore, G1-G2 are assigned to G8-G9. G3-G4 and G5-G6 are difficult to assign due to the lack of assignment in previous experiments for Gly209, Gly210, Gly232 and Gly233 pairs. However, previous assignments gave a clue that Gly233’s \(^{15}\)N was assigned to ~120 ppm and the Gly232’s C’ was assigned to ~176 ppm in both solution and solid state NMR (Table 5.7). Moreover, in our experiments, \(^{15}\)N chemical shift of G4 has a large change between G3P (substrate analogue) bound state and PGA
(transition state analogue) bound state (Figure 5.32 and Figure 5.33). This can only happen for $^{15}$N G210, which are in the active site (Figure 5.18) and readily affected by the electrostatic environment change from the substrate to transition state as discussed below. At the same time, $^{15}$N chemical shift of G6 differences between unligated state and G3P (substrate analogue) bound state, which can be explained by the hydrogen bond formed with the ligand (Figure 5.18). Therefore, G3 and G4 are assigned to G209 and G210; G5 and G6 are assigned to G232 and G233. All assignments are listed in Table 5.4, and they are compared with previous assignments in Table 5.5, Table 5.6 and Table 5.7. The assignments of the Gly8, Gly9 are consistent in all the data sheets. The Gly209, Gly210 and Gly232, Gly233 pairs are difficult to distinguish. Therefore, the most down field peak in the nitrogen dimension is assigned to the Gly232-Gly233 pair while the most high field peak in nitrogen dimension is assigned to Gly209-Gly210 pair.

![Figure 5.32 The PDSD DCP spectra for apo (red, left) and fG3P-ligated TIM (blue, right).](image-url)
Figure 5.33 The PDSD and DCP spectra for fully PGA ligated TIM.

<table>
<thead>
<tr>
<th></th>
<th>Apo C'</th>
<th>Apo N</th>
<th>G3P bound C'</th>
<th>G3P bound N</th>
<th>PGA bound C'</th>
<th>PGA bound N</th>
</tr>
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<td></td>
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<td></td>
<td>173.1 ppm</td>
<td></td>
</tr>
<tr>
<td>Gly9</td>
<td>171.4 ppm</td>
<td>112.9 ppm</td>
<td>171.5 ppm</td>
<td>112.8 ppm</td>
<td>171.3 ppm</td>
<td>112.6 ppm</td>
</tr>
<tr>
<td>Gly209</td>
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<td></td>
<td>175.0 ppm</td>
<td></td>
<td>168.8 ppm</td>
<td></td>
</tr>
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<td>172.4 ppm</td>
<td>104.6 ppm</td>
<td>175.1 ppm</td>
<td>104.5 ppm</td>
</tr>
<tr>
<td>Gly232</td>
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<td></td>
<td>176.2 ppm</td>
<td></td>
<td>176.7 ppm</td>
<td></td>
</tr>
<tr>
<td>Gly233</td>
<td>118.5 ppm</td>
<td></td>
<td>121.8 ppm</td>
<td></td>
<td>121.7 ppm</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4 Glycine-Glycine assignments from $^{13}$C-$^{13}$C PDSD spectra and $^{13}$C-$^{15}$N DCP spectra. The error bars for $^{13}$C are +/-0.3 ppm and for $^{15}$N are +/-0.5 ppm.
<table>
<thead>
<tr>
<th></th>
<th>Gly 8</th>
<th></th>
<th>Gly 9</th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td>C'</td>
<td>N</td>
<td>C'</td>
<td>N</td>
</tr>
<tr>
<td>Yeast TIM, SS-NMR¹</td>
<td>Apo</td>
<td>173.3 ppm</td>
<td>111.9 ppm</td>
<td>171.5 ppm</td>
</tr>
<tr>
<td>Yeast TIM, solution NMR²</td>
<td>Apo</td>
<td>173.3 ppm</td>
<td>111.8 ppm</td>
<td>171.8 ppm</td>
</tr>
<tr>
<td>Chicken TIM, solution NMR³</td>
<td>Apo</td>
<td>173.2 ppm</td>
<td>112.6 ppm</td>
<td>171.0 ppm</td>
</tr>
<tr>
<td>Yeast TIM, our assignment</td>
<td>Apo</td>
<td>173.1 ppm</td>
<td>112.9 ppm</td>
<td>171.4 ppm</td>
</tr>
<tr>
<td>Yeast TIM, SS-NMR⁴</td>
<td>G3P bound</td>
<td>173.1 ppm</td>
<td>112.9 ppm</td>
<td>171.4 ppm</td>
</tr>
<tr>
<td>Yeast TIM, our assignment</td>
<td>G3P bound</td>
<td>173.2 ppm</td>
<td>112.8 ppm</td>
<td>171.5 ppm</td>
</tr>
<tr>
<td>Chicken TIM, solution NMR⁵</td>
<td>PGA bound</td>
<td>173.2 ppm</td>
<td>112.3 ppm</td>
<td>171.4 ppm</td>
</tr>
<tr>
<td>Yeast TIM, our assignment</td>
<td>PGA bound</td>
<td>173.1 ppm</td>
<td>112.6 ppm</td>
<td>171.3 ppm</td>
</tr>
</tbody>
</table>

Table 5.5 The comparison between our assignments and previous assignments for Gly 8 and Gly 9.  
¹BMRB entry: 16565 [79]; ²BMRB entry: 7216, ³BMRB entry: 15064 [12]; ⁴BMRB entry: 16565 [79]; ⁵BMRB entry: 15065 [12]. The Gly 8 and Gly 9 in Yeast TIM are corresponding to the Gly 9 and Gly 10 in chicken TIM.

<table>
<thead>
<tr>
<th></th>
<th>Gly 209</th>
<th></th>
<th>Gly 210</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C'</td>
<td>N</td>
<td>C'</td>
<td>N</td>
</tr>
<tr>
<td>Yeast TIM, SS-NMR¹</td>
<td>Apo</td>
<td>170.1 ppm</td>
<td>113.4 ppm</td>
<td>175.9 ppm</td>
</tr>
<tr>
<td>Yeast TIM, solution NMR²</td>
<td>Apo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken TIM, solution NMR³</td>
<td>Apo</td>
<td></td>
<td>117.6 ppm</td>
<td></td>
</tr>
<tr>
<td>Yeast TIM, our assignment</td>
<td>Apo</td>
<td>175.4 ppm</td>
<td>104.2 ppm</td>
<td>172.2 ppm</td>
</tr>
<tr>
<td>Yeast TIM, SS-NMR⁴</td>
<td>G3P bound</td>
<td>170.0 ppm</td>
<td></td>
<td>122.2 ppm</td>
</tr>
<tr>
<td>Yeast TIM, our assignment</td>
<td>G3P bound</td>
<td>175.0 ppm</td>
<td>104.6 ppm</td>
<td>172.4 ppm</td>
</tr>
<tr>
<td>Chicken TIM, solution NMR⁵</td>
<td>PGA bound</td>
<td>116.5 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast TIM, our assignment</td>
<td>PGA bound</td>
<td>168.8 ppm</td>
<td>104.5 ppm</td>
<td>175.1 ppm</td>
</tr>
</tbody>
</table>

Table 5.6 The comparison between our assignments and previous assignments for Gly209 and Gly210.  
¹BMRB entry: 16565 [79]; ²BMRB entry: 7216, ³BMRB entry: 15064 [12]; ⁴BMRB entry: 16565 [79]; ⁵BMRB entry: 15065 [12].
Table 5.7 The comparison between our assignments and previous assignments for Gly232 and Gly233.
1BMRB entry: 16565 [79]; 2BMRB entry: 7216, 3BMRB entry: 15064 [12]; 4BMRB entry: 16565 [79]; 5BMRB entry: 15065 [12].

The $^{15}$N chemical shift of Gly233 has a large change between unligated TIM and G3P bound TIM (118.5 ppm vs. 121.8 ppm), but there is nearly no change between G3P (substrate analogue [80]) bound TIM and PGA (transition state analogue [6, 81]) bound TIM (121.8 ppm vs. 121.7 ppm). It has been well-known that $^{15}$N chemical shifts can be influenced by various conformational factors, including hydrogen bonding, torsion angles and conformations of neighboring residues [82-85]. Figure 5.34 describes the environment of the Gly232-Gly233 and their interaction with the ligand in the apo and substrate bound conformation. There is no orientational change of Gly232-Gly233 between the open and closed and the same for their adjacent residues (the mobile loops 6 and 7 are more than 6 Å away from G232 or G233). Therefore, the most possible reason for the large $^{15}$N chemical shift change of the Gly233 between apo and G3P bound states is the hydrogen bond formed with the ligand, and this $^{15}$N chemical shift is a good indicator of the property of the ligand binding.
In Gly209-Gly210 pair, it is interesting that chemical shifts of both Gly209 and Gly210’s carbonyl carbons are nearly the same between unligated and G3P (substrate analogue [80]) bound states (175.4-172.3 ppm vs. 175.0-172.2 ppm), but so different between G3P bound and PGA (transition state analogue [6, 81]) bound sample (175.0-172.2 ppm vs. 168.8-175.1 ppm). However, according to the X-ray structure (Figure 5.3), the large dihedral angle change of the Gly209-Gly210 (loop 7) happens in the binding of the substrate; If the conformational changes induce the observed chemical shift changes, this chemical shift difference should be observed between an unligated state and a ligated state, rather than between the G3P bound and PGA bound states. Meanwhile, the X-ray structures of G3P bound and PGA bound TIM are almost the same (Figure 5.35) accept a little displacement of the ligand and Glu 165.
Figure 5.35 The crystal structure comparison between G3P-ligated TIM (PDB: 6TIM, green, resolution: 2.2 Å) and the PGA-ligated TIM (PDB: 2YPI, grey, resolution: 2.5 Å). The two structures are superimposed according to all the backbone atoms. The residues in the range of 6 Å around Gly209 or Gly210 are shown. The major differences in the ligand and Glu165 or Pro166 are highlighted.

A reasonable explanation is that the electrostatic environment in the PGA bound state is different than in the G3P bound state. $^{31}$P and $^{13}$C NMR experiments on the labeled ligand showed that the bound PGA is fully ionized with one negative charge on the carboxylate and two negative charges on the phosphate moiety [86]. Meanwhile, it has been suggested that a proton is taken up by the active site (probably Glu165) upon PGA binding (based on pH titration experiments) [6, 87]. However, the bound G3P is a monoanion or a dianion forms and the binding of G3P does not require the enzyme to uptake a proton [87, 88]. This proton taken up by Glu165 in the PGA bound state can form a hydrogen bond with the ligand (Figure 5.35) and agrees with the hypothesis postulated by Pauling [89]: the transition state has a more tightly binding with the enzyme. Therefore, the chemical shift changes of both Gly209 and Gly210s’ carbonyl carbons between the G3P bound state and the PGA bound state probably reflect this important electrostatic potential change in the active site for stabilize the transition state.
5.4.2 The detection of TIM’s dynamics by CODEX experiment

5.4.2.1 Dynamics of the ligand release

As shown in the assignment section, the $^{15}$N chemical shift of Gly233 is a good marker for ligand binding. Therefore, the ligand release process can be investigated by studying the line-shape of the half-ligated TIM sample. The DCP spectrum of the half G3P-ligated sample was taken and superimposed with the DCP spectra of the unligated and fully G3P-ligated samples. In the slice of the $^{15}$N dimension of the Gly232C’-Gly233N’s cross peaks (right figure of Figure 5.37), it is clear that the half ligated peak is in the middle of the unligated and fully ligated peaks, showing that there are approximately equal populations between the unligated and ligated states, and the line-width of the half-ligated peaks was broaden by the dynamics. The fitting of this half ligated peak (Figure 5.38) obtain the exchange rate for the ligand binding and releasing is around 400 s$^{-1}$ (the error bar is from 150 s$^{-1}$ to 1000 s$^{-1}$).

Figure 5.36 Chemical structures of ligands DHAP, the proposed transition state [6], the substrate analogue G3P and the transition state analogue PGA.
Figure 5.37 Superposition of the DCP spectra for the unligated TIM (red), G3P half-ligated TIM (green) and G3P fully-ligated TIM (blue). In the right figure, the slice of the $^{15}$N dimension is cut at the Gly232’s carbonyl frequency.

Figure 5.38 Line-shape fitting of a $^{15}$N dimension slice of the cross peak G232C$'$-G233N. The experimental data were green and the fittings with different chemical exchange rates are labeled in dotted lines. In the fitting, $T_2$ is set as 2.6 ms according to the line-width of the unligated sample. The best fitted rate is 400 s$^{-1}$.

5.4.2.2 The upper-limit time scale of the CODEX experiment

As shown in Chapter 2, the spin diffusion between $^{13}$Cs will induce spurious signals in CODEX experiments. The spin diffusion rate is determined by the dipolar coupling between spin diffusion pairs which falls off in strength with the third power of distance. Therefore, in our L-$^{13}$C glycine labeled TIM, the spin diffusion is very slow because the adjacent $^{13}$Cs are at least have a distance of 3.3 Å. To prevent the detection of spurious decay, the spin diffusion of the labeled TIM is estimated by 2D proton driven
spin diffusion (PDSD) experiments. Figure 5.39 shows that there are nearly no cross peaks resulting from the spin diffusion when the mixing times are shorter than 100 ms. Therefore, in CODEX experiments, the decay points mixing times shorter than the 100 ms can be ascribed to the dynamics rather than the spin diffusion.

Figure 5.39 Investigation of spin diffusion in the apo glycine-1-^{13}C labeled TIM. The ^{13}C-^{13}C 2D correlation experiments through spin diffusion are recorded with different mixing times labeled in the slices. The 2D experiment on the top is the ^{13}C-^{13}C 2D experiment with 350 ms mixing time. The slices cut at Gly9 (red) and Gly210 (blue)’s frequency are displayed to illustrated the spin diffusion rate.

Figure 5.39 Investigation of spin diffusion in the apo glycine-1-^{13}C labeled TIM. The ^{13}C-^{13}C 2D correlation experiments through spin diffusion are recorded with different mixing times labeled in the slices. The 2D experiment on the top is the ^{13}C-^{13}C 2D experiment with 350 ms mixing time. The slices cut at Gly9 (red) and Gly210 (blue)’s frequency are displayed to illustrated the spin diffusion rate.
5.4.2.3 CODEX experiments on G3P half-ligated 1-13C glycine labeled TIM

CODEX experiments [71] were carried out on 1-13C glycine labeled TIM at -10 °C, which was half-ligated G3P, an analogue of the substrate. Due to the low resolution of the carbonyl carbon, the target residues, Gly209 and Gly210, cannot be separated from other C’ peaks. However, one of the advantages of the CODEX is that only the peaks belonging to the mobile atoms displaying intensity decay. Therefore, the intensity decays in the unresolved peaks can be attributed to the mobile glycine and fitted to obtain the motional rates (Figure 5.40).

Figure 5.40 CODEX experiments on the half G3P ligated 1-13C glycine labeled TIM (left) and the fitting of the intensity decay of the two main bulk peaks using a single exponential decay function (right figure). The intensities in the right figure are chosen by the strongest intensities of the two unresolved peaks as indicated by the dash lines in the left figure.

Figure 5.41 Difference spectra of CODEX experiments on G3P-ligated TIM. The spectrum for the shortest mixing time (0.1 ms) was subtracted from all the spectra. The residues with a flip angle larger than 25° are labeled in the spectra. The * means the chemical shift under the G3P bound condition.
Difference spectra are introduced to identify the source of the intensity decay in Figure 5.41. All Gly residues that have more than 25° conformational change (Table 5.8) have been indicated. As shown using simulations in Figure 5.42, only Gly171, Gly209 and Gly210 with large flip angles are expected to significant contributions to the intensity decays. Therefore, the decay at the 173.2 ppm is mainly due to Gly210 in loop 7 while the decay at the 175.2 ppm can be ascribed to Gly171, Gly173 in the loop 6 and Gly209 in loop 7. The flip rate of the loop 7 can be obtained from fitting these decays (especially for the peak at 173.2 ppm). In Figure 5.43, our detected motion rate of loop 7 fits very well with previous detections of the loop 6’s open rates in Arrhenius plot, supporting that there is correlation between loop 6 and loop 7’s motions [12].

<table>
<thead>
<tr>
<th>Gly</th>
<th>apo (ppm)</th>
<th>G3P bound (ppm)</th>
<th>angle (°)</th>
<th>Gly</th>
<th>apo (ppm)</th>
<th>G3P bound (ppm)</th>
<th>angle (°)</th>
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<td>0.7</td>
<td>233</td>
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</table>

Table 5.8 The carbonyl carbon’s chemical shifts of all the glycine residues and the flip angle of the carbonyl bond between the apo (PDB entry: 1I45) and bound TIM (PDB entry: 1NEY). The chemical shifts are according to previous ssNMR assignments [79] and our assignments in Table 5.4.
Figure 5.42 Simulations of intensity decays for different flip angles in the CODEX experiment.

Figure 5.43 Arrhenius plot of detected motion rates. The loop 6’s motion rates were detected from $^2$H line-shape fit in solid state NMR [10] $^{19}$F line-shape fit in solution NMR [9] (black hollow squares), TROSY-detected $R_{ex}$ experiment (black solid triangles), Temperature-jump experiments [48] (black hollow triangles), and loop 7’s rate was detected in our CODEX experiment (red, 300+/−100 s$^{-1}$ are used here). The unit of the x-axis is 1000/K.

5.4.2.4 CODEX experiments on the PGA half ligated 1-$^{13}$C glycine labeled TIM
The CODEX experiments [71] are also carried out on 1-$^{13}$C glycine labeled TIM at 20 °C with PGA, the analogue of the transition state. Similar to the results in the Temperature-jump experiment [90], no motions has been detected in the millisecond range (Figure 5.44), proving that the TIM has tight binding with the transition state. This suggests the idea that the dynamics of loop 6 and loop 7 mediate the
reaction: in the unligated state, the TIM favors the open conformation and has a very high loop open rate to increase the ligands’ chance to access the active site; after the ligand binds, the closed conformation is favored and the open rate becomes slower to reserve the ligand and prepare for the reaction; when the reaction goes to the transition state, the ligand binds tighter and the loop open rate is very slow to prevent water exposure and stabilize the transition state; At the end, the loop open rate increases for product release.

Figure 5.44 CODEX experiments on the half PGA ligated 1-\textsuperscript{13}C glycine labeled TIM at 20 °C.

5.5 Conclusions

Using selective amino acid labeling, four important residues, G8, G9, Gly209, Gly210, Gly232 and Gly233 were assigned. In the analysis of their chemical shifts in the unligated state, the substrate analogue (G3P) bound state and transition state analogue (PGA) bound state, the active site’s electrostatic environments in the transition state was found to be very different to the substrate bound state. In the dynamics study, the nitrogen chemical shift of Gly233 was assigned as a marker for ligand binding and release. Through line-shape fitting, an exchange rate of about 400 s\textsuperscript{-1} was obtained. At the same time, the motion rate of the highly conserved loop 7’s large dihedral angle motion was directly detected to be 300+/−100 s\textsuperscript{-1} by the CODEX experiment. This motion rate is not only similar to that of ligand release rate as detected in the line-shape analysis experiment but also agrees well with an extrapolated values for loop 6’s motion rate, suggesting a concerted motion for the loop 6, loop 7 and the ligand release. Comparison between the motion rate with the release of the substrate analogue G3P and of the transition state analogue PGA shows that the transition state binds more stably to TIM.
5.6 References


[56] D.M. LeMaster, F.M. Richards, 1H-15N heteronuclear NMR studies of Escherichia coli thioredoxin in samples isotopically labeled by residue type, Biochemistry, 24 (1985) 7263-7268.

[57] L.P. McIntosh, F.W. Dahlquist, Biosynthetic incorporation of 15N and 13C for assignment and interpretation of nuclear magnetic resonance spectra of proteins, Quarterly Reviews of Biophysics, 23 (1990) 1-38.


Chapter 6  Conclusion
With the development of the X-ray crystallography, we see more and more beautiful protein structures that reveal exciting mechanisms. However, an indisputable fact is that many important proteins’ function is closely related to their motions. We cannot fully understand these proteins’ functions without studying their dynamics. Therefore, establishing the methods to study proteins’ dynamics is an import task.

Compared with other techniques such as fluorescence methods, NMR experiments can detect the dynamics with atomic resolution without an extra chemical functional group. Solution NMR experiments like CPMG and R₁ρ experiments [1, 2] are well established and detect many important millisecond dynamics of proteins. However, solution NMR is restricted to the study of soluble proteins of moderated molecular weight. Moreover, most solution NMR experiments for detecting motion are based on chemical shift differences, which do not have a quantitative relationship with the motional geometry. To obtain the conformational information regarding motions, anisotropic interactions should be used. In this regard, fast magic angle spinning solid state NMR is very good technique, which overcomes the broad lines caused by anisotropic interactions, and one can recouple the anisotropic interaction for dynamics detection. Based on the CODEX experiment [3], which uses chemical shift anisotropy to detect seconds-to-milliseconds dynamics, we developed two new experiments, Dipolar-CODEX and R-CODEX which utilize the dipolar coupling. The dipolar coupling has a simple relationship relative to the molecular geometry. Also, dynamics detection using more interactions supplies a chance to construct the whole conformational change without the knowledge from the X-ray structure. For example, considering the amide nitrogen in a protein, if we can detect reorientational of its N-αC bond, N-C’ bond, N-H bond and N chemical shift anisotropy, we can describe the motion around this nitrogen in detail.

With CODEX experiments, we directly detected the large dihedral angle motion of the highly conserved YGGS sequence in loop 7 of TIM, and proved that it is correlated with the motion of loop 6. This large conformational change of loop 7 supplies multiple hydrogen bonds with loop 6 and shifts the TIM conformation from the open conformation to the closed conformation. Meanwhile, as shown in X-ray structures [4], the conformational change of loop 7 will push the side chain of the key catalytic residue Glu165 to the right position. This implies that after the closing of loop 6, Glu165 has been prepared for the catalytic reaction. At the same time, we found that when TIM is bound to a transition state analogue (PGA), the loop motion rate decreased to prevent the water exposure of the transition state, which reveals that not only the conformational change conducted by the dynamics, but also the correlation time of the dynamics is very important for effective catalysis. Furthermore, line-shape analysis found that the rate of ligand release and loop motion are similar, indicating that this ligand release is involved in the loop opening.
CODEX type experiments have been shown to be successful for millisecond dynamics detection in proteins. In the future, combined with the special labeling schemes, this powerful solid state NMR method can be applied to large membrane proteins which have large-angle motion.

References

Appendix I  Calculation of the coherence $C_{zN}$’s relaxation rate caused by the motion of the $^{13}\text{C}-^{15}\text{N}$ dipolar coupling

In the system with $^{13}\text{C}-^{15}\text{N}$ bond, the Hamiltonian is the sum of the time-independent Hamiltonian from the main field $H_0$, and a stochastic Hamiltonian of $H_1(t)$. $H_1(t)$ can be the $^{13}\text{C}-^{15}\text{N}$ dipolar coupling or the chemical shift anisotropy of the $^{13}\text{C}$ and $^{15}\text{N}$, and $H_1(t)$ can be decomposed to the spatial part $F_k^q(t)$ and a tensor spin operator $A_{kp}^q$ as

$$H_1(t) = \sum_{q=-k}^{k} \sum_{p} (-1)^q F_k^q(t) A_{kp}^q$$

Equation 1

The Master equation can be written as

$$\frac{d\sigma(t)}{dt} = -i[H_0, \sigma(t)] - \hat{\Gamma}(\sigma(t) - \sigma_0)$$

$$\hat{\Gamma} = \frac{1}{2} \sum_{q=-k}^{k} \sum_{p} j^q(\omega_p^q)[A_{kp}^q, [A_{kp}^q, \cdot]]$$

$$j^q(\omega_p^q) = \text{Re}\left\{ \int_{-\infty}^{\infty} F_k^q(t) F_k^q(t + \tau) \exp\{-i\omega\tau\} d\tau \right\}$$

Equation 2

where $\hat{\Gamma}$ is the relaxation operator and $j(\omega)$ is the spectra density function. To calculate the relaxation constant, Equation 2 is expanded in terms of the eigenvector $B_r$ of the $H_0$ as

$$\frac{d b_j(t)}{dt} = \sum_{r} \{-i\Omega_{rs} b_j(t) - \Gamma_{rs} [b_j(t) - b_{jr}]\}$$

$$b_j(t) = \langle B_j | \sigma(t) \rangle / \langle B_j | B_j \rangle$$

$$\Omega_{rs} = \langle B_r | [H_0, B_s] \rangle / \langle B_r | B_s \rangle$$

$$\Gamma_{rs} = \langle B_r | \Gamma B_s \rangle / \langle B_r | B_s \rangle$$

$$\Gamma_{rs} = \frac{1}{2} \sum_{q=-k}^{k} \sum_{p} j^q(\omega_p^q) \left\{ \langle B_r | [A_{kp}^q, [A_{kp}^q, B_s]] \rangle / \langle B_r | B_s \rangle \right\}$$

Equation 3
From Equation 3, we can see that only the interactions that contain the tensor spin operator that makes $[A_{kp}^q, [A_{kp}^q, C_z N_z]] \neq 0$ can contribute to the relaxation. Only the $^{13}\text{C}-^{15}\text{N}$ dipolar coupling and the chemical shift anisotropies of $^{13}\text{C}$ and $^{15}\text{N}$ can do this. In the following, I will separately derive the relaxation constants due to dipolar coupling and CSA.

For the dipolar coupling contribution, according to the Table 5.3 at page 372 in the reference [1], the $[A_{kp}^q, [A_{kp}^q, B_z]]$ can be calculated as

\[
\begin{align*}
[A_{20}^0, [A_{20}^0, C_z N_z]] &= 0, \quad [A_{21}^0, [A_{21}^0, C_z N_z]] = 0, \quad [A_{22}^0, [A_{22}^0, C_z N_z]] = 0, \\
[A_{20}^1, [A_{20}^1, C_z N_z]] &= \frac{1}{8} C_z N_z, \quad [A_{20}^1, [A_{20}^1, C_z N_z]] = \frac{1}{8} C_z N_z, \quad [A_{22}^1, [A_{22}^1, C_z N_z]] = \frac{1}{8} C_z N_z, \\
[A_{21}^1, [A_{21}^1, C_z N_z]] &= \frac{1}{8} C_z N_z, \quad [A_{20}^2, [A_{20}^2, C_z N_z]] = 0, \quad [A_{20}^2, [A_{20}^2, C_z N_z]] = 0
\end{align*}
\]

Equation 4

Due to the secular approximation, $C_z N_z, C_z N_z$ do not cross-relaxation with other coherence, and the dipolar cross-relaxations between $C_z$ and $C_z N_z$ or $C_z$ and $C_z N_z$ do not occur. Therefore, the relaxation constant can be derived as

\[
\Gamma = \frac{1}{2} \times \frac{1}{8} \langle C_z N_z | C_z N_z \rangle \left( j^j(\alpha_0^0) + j^j(\alpha_0^1) + j^j(\alpha_1^0) + j^j(\alpha_1^1) \right)
\]

\[
= \frac{1}{8} \langle C_z N_z | C_z N_z \rangle \left( j^j(\alpha_0^0) + j^j(\alpha_0^1) \right)
\]

Equation 5

Therefore, generally speaking, the contribution of the dipolar relaxation is only significant when the $^{13}\text{C}$-$^{15}\text{N}$ bond’s motion rate comparable to the Larmor frequencies of the $^{13}\text{C}$ or $^{15}\text{N}$.

Meanwhile the chemical shift anisotropy will also contribute to the relaxation of $C_z N_z$ coherence. For $^{13}\text{C}$ CSA, following the table in Page 385 in the reference [1]

\[
\begin{align*}
[A_{20}^0, [A_{20}^0, C_z N_z]] &= 0, \quad [A_{20}^1, [A_{20}^1, C_z N_z]] = \frac{1}{8} C_z N_z, \quad [A_{20}^1, [A_{20}^1, C_z N_z]] = \frac{1}{8} C_z N_z, \\
[A_{20}^2, [A_{20}^2, C_z N_z]] &= 0, \quad [A_{20}^2, [A_{20}^2, C_z N_z]] = 0
\end{align*}
\]
Equation 6

The relaxation constant can be derived as

\[
\hat{\Gamma} = \frac{1}{2} \times \frac{1}{8} \left\{ \langle C_z N_z | C_z N_z \rangle j^1(\omega_0) + j^1(\omega_0^{-1}) \right\} \\
= \frac{1}{8} \langle C_z N_z | C_z N_z \rangle j^1(\omega_c)
\]

For \(^{15}\text{N}\) CSA, the result is similar to the \(^{13}\text{C}\) CSA,

\[
\hat{\Gamma} = \frac{1}{8} \langle C_z N_z | C_z N_z \rangle j^1(\omega_N)
\]

Therefore, generally speaking, the contribution of the CSA is only significant when the \(^{13}\text{C}-^{15}\text{N}\) bond’s motion rate comparable to the Larmor frequencies of the \(^{13}\text{C}\) or \(^{15}\text{N}\).

Reference:

Appendix II Phase cycling for the pulse sequences

1. Phase cycling for the Dipolar CODEX

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<th>$C_{90}$</th>
<th>$C_{90}$</th>
<th>$C_{90}$</th>
<th>$C_{90}$</th>
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2. The phase cycling for the R-CODEX

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Appendix III Buffer recipes for TIM preparation

1. M9 wash buffer

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<tr>
<th>Volume</th>
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<td>4.5g</td>
</tr>
<tr>
<td>NaCl</td>
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<tr>
<td>NH₄Cl</td>
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2. Metals 44 (4°C, filter sterile)

<table>
<thead>
<tr>
<th>Volume</th>
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<tbody>
<tr>
<td>K₂EDTA●2H₂O</td>
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</tr>
<tr>
<td>ZnCl₂</td>
<td>0.522g</td>
</tr>
<tr>
<td>FeCl₂●4H₂O</td>
<td>0.502g</td>
</tr>
<tr>
<td>MnCl₂●4H₂O</td>
<td>0.18g</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄●6H₂O</td>
<td>0.0185g</td>
</tr>
<tr>
<td>CuCl₂●2H₂O</td>
<td>0.0156g</td>
</tr>
<tr>
<td>Co(NO₃)₂●6H₂O</td>
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</tr>
<tr>
<td>Boric Acid</td>
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3. Solution C (4°C, filter sterile, could be kept until it’s not clear any more)

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<th>Volume</th>
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<tr>
<td>KOH</td>
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</tr>
<tr>
<td>Metals 44</td>
<td>50mL</td>
<td>25mL</td>
</tr>
<tr>
<td>Nitrilotriacetic Acid</td>
<td>10g</td>
<td>5g</td>
</tr>
<tr>
<td>MgCl₂●6H₂O</td>
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<tr>
<td>CaCl₂●2H₂O</td>
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Adjust pH with 5M KOH to 6.7.
4. **Vitamin stock** (4°C, filter sterile, then could be kept in fridge in dark)

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<thead>
<tr>
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<tr>
<td>4-Aminobenzoic Acid</td>
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</tr>
<tr>
<td>D-Biotin</td>
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5. **Resuspension buffer**

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<tr>
<td>50mM Tris-HCl</td>
<td>Trizma base 0.61g</td>
</tr>
<tr>
<td>150mM NaCl</td>
<td>NaCl 0.88g</td>
</tr>
<tr>
<td>20mM KH₂PO₄</td>
<td>KH₂PO₄ 0.27g</td>
</tr>
<tr>
<td>1mM DTT</td>
<td></td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>1 bottle</td>
</tr>
<tr>
<td>pH</td>
<td>7.8 at room temperature</td>
</tr>
</tbody>
</table>

6. **Buffer for purification**
   - Buffer A: 50 mM Trisma base, 300 mM NaCl, 1 mM EDTA, pH=7.8 at 4 °C
   - Buffer B: 50 mM Trisma base, 150 mM NaCl, 1 mM EDTA, pH=7.8 at 4 °C
   - Buffer C: 10 mM Trisma base, pH=7.8 at 4 °C
   - Buffer D: 10 mM Trisma base, 150 mM NaCl, 1 mM EDTA, pH=7.8 at 4 °C
   - Buffer E: 1.4 M ammonium sulfate, 50 mM Na₂HPO₄
   - Buffer F: 50 mM Trisma base, 50 mM NaCl, 1 mM EDTA, 0.02%(w/v) NaN₃, pH=6.8 at 4 °C
Appendix IV The effect of viscosity on the loop motion

To estimate the energy required to overcome the solution’s resistance in the solution, the motion of the loop 6 (Figure 1) is roughly simplified to the motion of a sphere in the solution (Figure 2).

Figure 1. The motion of loop 6 according to the unligated (PDB: 1I45) and ligated (PDB: 1NEY) X-ray structures.

Figure 2. The motional model extracted from Figure 1 to calculate the energy required for loop 6’s motion in the solution. In this model, the loop 6 is roughly simplified to a sphere. The hydrodynamics diameter of this sphere is set as 7.9 Å which is the diameter of the loop. The moving distance is set as 7.4 Å which is the moving distance of the loop 6’s tip.

According to the Stoke’s law derived by George Gabriel Stokes, the drag force exerted on the sphere is

\[ F_d = 6\pi\mu R v \]  

Equation 1

where \( \mu \) is the dynamics viscosity, \( R \) is the radius of the sphere, \( v \) is the sphere’s velocity.

Therefore, the energy required for the loop motion is
\[ E = F_d \times l = 6\pi \mu R v \times l \quad \text{Equation 2} \]

where \( l \) is the moving distance.

In our case, the dynamics viscosity of 10\% PEG solution is \( 12.7 \times 10^{-3} \) Pa/s \([1]\) and \( R = 7.9/2 \) Å = 3.95 Å. The moving velocity \( v \) can be estimated as the product of the moving distance 7.4 Å and the loop’s close rate \( 13 \times 10^3 \) s\(^{-1}\) \([2]\). Put all these number into Equation 2, we obtain the energy required to overcome the resistance from the solution is 0.4 J/mol.

Reference

Appendix V Effects of T₁ and T₂ on the CODEX experiments

As shown in Section 2.3 and 2.3, if we assume the spin-lattice relaxation time (T₁) and spin-spin relaxation time (T₂) are the same for different exchanging conformations, effects of T₁ and T₂ on CODEX experiments can be eliminated by normalizing data properly and doing reference experiments. Although this assumption seems correct for many situations, we should consider the situations that T₁ or T₂ changes with different conformations.

Longitudinal relaxation mainly has effects during the mixing time and z-filter time. Through simulations in Figure 1, we can observe that longitudinal relaxation has significant effect on the decay curve only when the difference of longitudinal relaxation rates is comparable or larger than the motional rate of dynamics. In Solid-State NMR, T₁ of ¹³C and ¹⁵N in uniformly ¹³C, ¹⁵N labeled proteins are usually sub-seconds at moderate magic angle spinning frequencies (10~20 kHz) [1-5]. The change of longitudinal relaxation rates will not often exceed tens of s⁻¹. Therefore, it is a good assumption that T₁ will not affect CODEX experiments to detect dynamics in millisecond time scale.

![Figure 1](image_url)

Figure 1. Simulations of CODEX decay curves with different longitudinal relaxation rates. The system assumed has a 60° jump of a uniaxial CSA interaction with a 10 kHz principal value. The jump rate kₑ is 1000 s⁻¹. The magic angle spinning frequency is 10 kHz.

Transverse relaxation mainly has effect in the dephasing and refocusing part. Equation 2.1 can be modified as
\[ I_x^A = P_A e^{-\text{dephase} / T_2^A} \times (\cos \phi_{\text{dephase}} I_x + \sin \phi_{\text{dephase}} I_y) > \\
= P_A e^{-\text{dephase} / T_2^A} \left[ < \cos \phi_{\text{dephase}} > I_x + < \sin \phi_{\text{dephase}} > I_y \right] \\
I_x^B = P_B e^{-\text{dephase} / T_2^B} \times (\cos \phi_{\text{dephase}} I_x + \sin \phi_{\text{dephase}} I_y) > \\
= P_B e^{-\text{dephase} / T_2^B} \left[ < e^{i \phi_{\text{dephase}} (1/T_2^A - 1/T_2^B)} > \cos \phi_{\text{dephase}} > I_x + < e^{i \phi_{\text{dephase}} (1/T_2^A - 1/T_2^B)} > \sin \phi_{\text{dephase}} > I_y \right] \\
\]

Equation 1

The term containing the relaxation rate difference, \( e^{i \phi_{\text{dephase}} (1/T_2^A - 1/T_2^B)} \) can be considered to modulate the dephasing process of one conformation, or in other words to make the observable anisotropic interaction larger or smaller. Therefore, this transverse relaxation rate difference will cause errors in the flip angle fitting process as shown in Figure 2. Due to the challenge to separate incoherent and coherent contributions to the transverse relaxation rates for dynamics detection [6], there were lack of T2 data in fully labeled proteins. However, based on line-widths of \(^{13}\)C and \(^{15}\)N peaks which are often tens to hundreds Hertz, we can estimate \(^{13}\)C and \(^{15}\)N’s T2 values in the millisecond range for fully labeled proteins. Therefore, the difference of transverse relaxation rates is often hundreds s\(^{-1}\), which will cause about 10° deviation as shown in Figure 2.

Figure 2. Simulations of CODEX decay curves with different transverse relaxation rates. The system assumed has a flip motion of a uniaxial CSA interaction with a 2 kHz principal value. The jump rate \( k_{\text{ex}} \) is 1000 s\(^{-1}\). The magic angle spinning frequency is 10 kHz. The simulation systems for the red rectangles, blue triangles and black circles have transverse relaxation rates as shown in the figure legend and a jump angle of 60°. The simulation systems for magenta and green dashed lines have the same T2 value (10 ms) and jump angles of 70° and 50° as shown in the figure. These two simulations depicted as dashed lines are used to see how much deviation can be caused by the difference of transverse relaxation rates.
References:


