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UNIVERSITY OF CALIFORNIA,
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NMR characterization of *Ciona intestinalis* $\beta\gamma$ -Crystallin: Implications for Calcium
Binding and $\beta\gamma$ -Crystallin Evolution

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Matthew D. Jimenez

Dissertation Committee:
Professor Rachel W. Martin, Chair
Professor Eric O. Potma
Professor Jennifer A. Prescher

DEDICATION

To my family who supported me throughout this PhD experience.

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2. **Jimenez, M. J.**; Mozafari, Mina; Hoang, T; Al-Botaif, M; Ko, J; Martin, R. W.; ”Backbone and side-chain NMR resonance assignments of *Ciona intestinalis* $\beta\gamma$ -crystallin.” Submitted July 13, 2026.

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7. “Investigating the Structural Change of Ci- $\beta\gamma$ -Crystallin at Various Calcium Concentrations” **Jimenez, M. J.**, Martin, R. W. Experimental NMR Conference, Asilomar, CA. (Poster, April, 2023)
8. “Towards determining the structure of native and aggregated states through the utilization of bicelles in Deuterium solid state NMR.” **Jimenez, M. J.**, Martin, R. W. Rocky Mountain Conference, Copper Mountain Resort, CA. (Poster, July, 2022)

ABSTRACT OF THE DISSERTATION

NMR characterization of *Ciona intestinalis* $\beta\gamma$ -Crystallin: Implications for Calcium Binding and $\beta\gamma$ -Crystallin Evolution

By

Matthew D. Jimenez

Doctor of Philosophy in Chemistry

University of California, Irvine, 2026

Professor Rachel W. Martin, Chair

$\beta\gamma$ -crystallins are a diverse family of proteins characterized by a conserved Greek-key fold and found across a wide range of organisms. Although vertebrate $\beta\gamma$ -crystallins are best known as structural proteins of the eye lens, members of this family are thought to have evolved from ancestral calcium-binding proteins. The *Ciona intestinalis* $\beta\gamma$ -crystallin (*Ci*- $\beta\gamma$) is particularly informative for understanding the evolution of this protein family because it retains calcium-binding properties while maintaining similar structural features to newer vertebrate lens $\beta\gamma$ -crystallins. Understanding how calcium binding influences the structure and dynamics of *Ci*- $\beta\gamma$ therefore provides insight into molecular properties that may have accompanied the evolution of the $\beta\gamma$ -crystallin family. This dissertation uses nuclear magnetic resonance (NMR) spectroscopy to characterize the structural, calcium-binding, and dynamic properties of *Ci*- $\beta\gamma$. Backbone and side-chain resonance assignments were established for the protein, providing the foundation for residue-specific characterization of its molecular properties. Calcium-dependent changes were investigated using ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectroscopy and chemical shift perturbation analysis, allowing the effects of calcium binding to be monitored throughout the protein. These studies demonstrate that calcium binding produces localized changes in the chemical environment of residues surrounding the calcium-binding sites while also influencing regions outside the

immediate coordination sphere. ^{15}N relaxation measurements were used to characterize the backbone dynamics of $Ci\text{-}\beta\gamma$ in both calcium-bound and calcium-free states. Longitudinal relaxation rates (R_1), transverse relaxation rates (R_2), and heteronuclear NOE measurements were used to evaluate residue-specific motions on picosecond-to-nanosecond timescales. ModelFree analysis was used to estimate generalized order parameters and identify regions exhibiting differences in backbone flexibility. Comparison of the apo and calcium-bound states reveals how calcium binding influences the dynamic behavior of the protein. Together, these NMR studies provide a comprehensive residue-specific characterization of the structure, calcium-dependent behavior, and dynamics of *Ciona intestinalis* $\beta\gamma$ -crystallin. The results demonstrate how calcium binding contributes to local structural organization and broader conformational dynamics, providing insight into the molecular characteristics of an evolutionarily relevant $\beta\gamma$ -crystallin and the evolution of calcium-binding proteins into the diverse $\beta\gamma$ -crystallin family.

Chapter 1

Biomolecular NMR spectroscopy: Methods for Protein Structure and Dynamics

1.1 Introduction to Biomolecular NMR

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique that provides information about the structure, dynamics, and interactions of molecules in solution. Since its development, NMR spectroscopy has become an essential tool for studying biological macromolecules. NMR has provides detailed structural and dynamic information under near physiological conditions making it an indispensable tool for investigating protein folding, ligand binding, molecular recognition, and conformational exchange. NMR enables proteins to be studied in solution, allowing both their structures and dynamic behavior to be characterized.

In structural biology, NMR spectroscopy complements techniques such as X-ray crystallogra-

phy and cryogenic electron microscopy (cryo-EM). While X-ray crystallography can provide high-resolution structures, it generally requires the formation of well-ordered crystals and often represents a single conformational state [2]. Cryo-EM has revolutionized the structural determination of larger macromolecular complexes but remains less suitable for many small proteins due to low signal-to-noise ratios and the lack of distinctive morphological features for particle alignment [3]. In contrast, solution-state NMR is effective for relatively small proteins and is applicable to more than one conformation [2]. There have been advances including transverse relaxation-optimized spectroscopy (TROSY), predeuteration, and selective isotope labeling strategies that have expanded the size range of proteins and protein complexes that can be investigated [4, 5]. These capabilities allowed biomolecular NMR to become an essential technique for studying protein folding, ligand binding, conformational exchange, and protein dynamics.

The fundamental principles of NMR arise from the magnetic properties of atomic nuclei. Nuclei possessing a nonzero nuclear spin behave as tiny magnetic dipoles. When placed within a strong external magnetic field, these nuclei adopt discrete energy states aligned either with or against the applied field. The energy difference between these spin states is proportional to the magnetic field strength, allowing transitions between them to be induced by radiofrequency (RF) pulses at the resonance Larmor frequency of the nucleus. Following excitation, the nuclei emit an oscillating signal known as the free induction decay (FID), which is converted into a frequency-domain spectrum through Fourier Transformation (FT) [6, 7]. The resonance frequency of each nucleus depends not only on the external magnetic field but also on its local environment. Electrons surrounding a nucleus partially shield it from the applied magnetic field, which produces slight variations in resonance frequency known as chemical shifts. These chemical shifts serve as a probe for protein structure and interactions due to them being highly sensitive to molecular structure, secondary structure, local conformation, and hydrogen bonding [8, 9].

Proteins contain hundreds to thousands of chemically distinct nuclei, which can result in spectral overlap in one dimensional NMR experiments. To overcome this limitation, multi-dimensional NMR experiments correlate the resonances of different nuclei through scalar (j) couplings or dipolar interactions, which allows the distribution of resonances across two or more frequency dimensions [10]. These experiments enable the sequential assignment of backbone and side-chain resonances which provide the foundation for structural determination, dynamic characterization, and interaction studies. This chapter introduces the biomolecular NMR techniques employed throughout this dissertation. The following sections describe the multidimensional experiments used for backbone and side-chain resonance assignment, methods for measuring residual dipolar couplings to obtain orientational restraints, and relaxation experiments used to characterize protein dynamics. Together, these methodologies establish the experimental framework for investigating the structural and dynamic properties of *Ciona intestinalis*- $\beta\gamma$ crystallin (*Ci*- $\beta\gamma$).

1.2 $\beta\gamma$ -Crystallins and Their Evolutionary Significance

Crystallins are a diverse group of proteins that are major structural components of the vertebrate eye lens. Their primary role in the lens is to maintain transparency and refractive properties; however, the evolutionary origins of crystallins are distinct from their modern structural role in the eye. Many crystallins are derived from proteins that originally performed other biological functions and were subsequently recruited as structural components of the lens. This evolutionary repurposing of pre-existing proteins makes crystallins an important system for investigating how changes in protein structure, stability, and function can contribute to the emergence of new biological roles [11, 12].

The $\beta\gamma$ -crystallin superfamily is informative for studying these evolutionary processes. Members of this superfamily share a characteristic $\beta\gamma$ -crystallin fold composed primarily of β -strands arranged into Greek-key motifs. Although $\beta\gamma$ -crystallin proteins are found as struc-

tural components of the vertebrate lens, related proteins also occur in organisms that do not possess a lens. The presence of the $\beta\gamma$ -crystallin fold outside the lens suggests that the structural framework associated with modern crystallins predates its recruitment as a structural lens protein. Studying non-lens members of the $\beta\gamma$ -crystallin superfamily can therefore provide insight into molecular properties that may have been present in earlier members of the crystallin lineage [13, 12].

An additional feature of several $\beta\gamma$ -crystallins is their ability to bind calcium. Calcium binding is of particular interest because interactions with metal ions can influence protein structure, stability, and dynamics. The conservation of calcium-binding sites among members of the $\beta\gamma$ -crystallin superfamily raises questions about the evolutionary significance of this property. In particular, determining how calcium binding affects the structure and dynamics of a $\beta\gamma$ -crystallin may provide insight into whether metal binding was associated with functional properties of proteins preceding their recruitment as lens crystallins. Changes in calcium-binding sites or their surrounding structural environments may also represent one mechanism through which members of the superfamily diversified during evolution [14, 13].

Ciona intestinalis- $\beta\gamma$ crystallin (*Ci*- $\beta\gamma$) provides a useful model for investigating the relationship between calcium binding, protein structure, and dynamics. *Ci*- $\beta\gamma$ is a small $\beta\gamma$ -crystallin protein containing two calcium-binding sites, allowing the effects of calcium binding to be investigated in a relatively simple protein system. Comparing the apo and calcium-bound states provides an opportunity to determine how calcium binding influences the molecular properties of the protein and whether these effects are localized to the calcium-binding sites or propagated throughout the $\beta\gamma$ -crystallin fold.

An important question is whether calcium binding primarily produces structural rearrangement or instead alters the dynamic behavior of an already folded protein. Protein function is influenced not only by its static three-dimensional structure but also by motions occurring over a range of timescales. Changes in protein dynamics may represent an important mecha-

nism through which ligand binding influences function without requiring large changes in the overall protein fold. Characterizing both the structure and dynamics of $Ci\text{-}\beta\gamma$ in the presence and absence of calcium can therefore provide a more complete picture of the molecular consequences of calcium binding.

Solution-state NMR spectroscopy is well suited to this problem because it provides residue-specific measurements of protein structure and dynamics in solution. Establishing comprehensive resonance assignments allows changes in individual residues to be monitored upon calcium binding, while measurements of chemical shifts, residual dipolar couplings, and nuclear relaxation provide complementary information about structure and molecular motion. These approaches provide the experimental framework for investigating how calcium binding influences the structure and dynamics of $Ci\text{-}\beta\gamma$ and how these properties may relate to the evolutionary history of the $\beta\gamma$ -crystallin lineage. For this chapter, I am going to discuss the NMR experiments used to solve the structure and probe $Ci\text{-}\beta\gamma$ dynamic properties.

1.3 Backbone Resonance Assignment

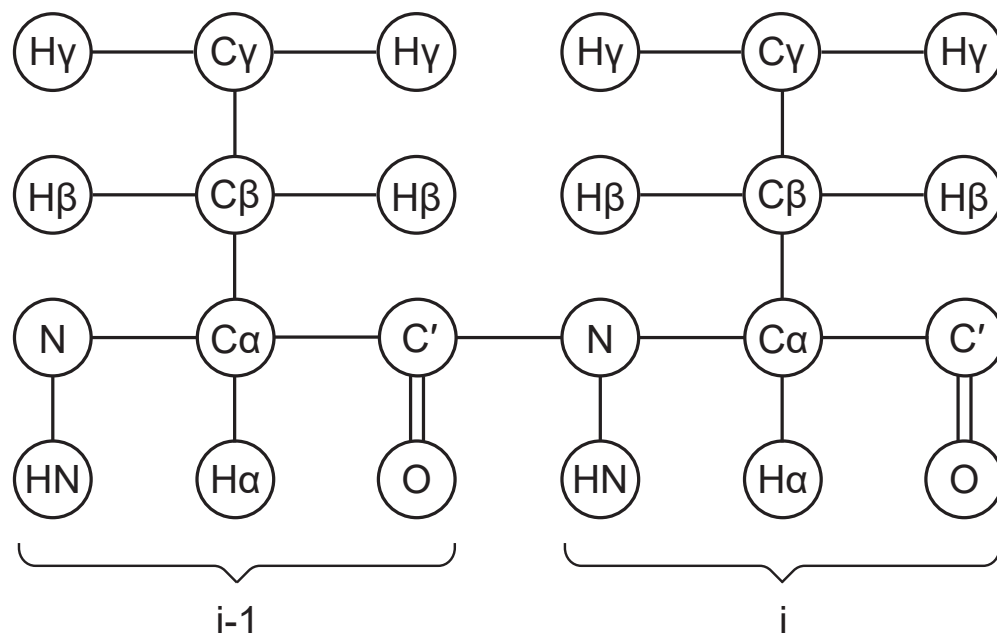


Figure 1.1: This figure depicts two amino acids within a protein backbone. Section i is one amino acid and section i-1 stands for the preceding residue. NMR experiments are used to assign any of the nuclei seen in i or i-1 sections.

NMR resonance assignments are essential for biomolecular NMR studies because they establish the relationship between observed resonances in an NMR spectrum and specific atoms within a protein sequence. Backbone assignments are composed of the amide, amide proton, carbonyl carbon, C_α and C_β found in the backbone. These assignments provide a foundation for structural and dynamic analyses such as chemical shift perturbation measurements, secondary structure prediction, residual dipolar coupling analysis, and relaxation experiments. Backbone assignments require a series of multidimensional triple-resonance NMR experiments. These experiments utilize scalar J-coupling interactions to establish correlations among backbone nuclei. These experiments transfer magnetization between ^1H , ^{13}C , and ^{15}N through the peptide backbone, which allows for the identification of neighboring amino acid residues 1.1. Complementary NMR experiments that provide intraresidue and

sequential correlations allow for the backbone to be fully assigned. The experiments used to assign the backbone include HNCO, HN(CO)CA, HNCA, HN(CA)CO, CBCA(CO)NH, and HNCACB 3D-experiments [15, 16, 17, 18]. These experiments provide complementary information regarding the connectivity of the backbone nuclei, which enable the sequential assignments of the residues throughout the protein sequence.

1.3.1 ^1H - ^{15}N Heteronuclear Single Quantum Coherence

A ^1H - ^{15}N HSQC experiment is used to obtain a 2D representation of the amide resonances within a protein by correlating each amide proton with its directly bonded ^{15}N nucleus (Figure 1.2). In this experiment, magnetization is initially excited on the proton and transferred to the directly bonded ^{15}N through scalar coupling. The magnetization is then transferred back to the proton for detection (Figure 1.3). This experiment should produce a single resonance for every residue except prolines. HSQCs are essential in NMR since amides are highly sensitive to the local chemical environment which means they can be used to monitor structural changes, ligand binding, and conformational exchanges [16]. The HSQCs parameters used to assign Ci - $\beta\gamma$ in both calcium-treated (holo) and EDTA-treated (apo) forms are on tables 1.1 and 1.2.

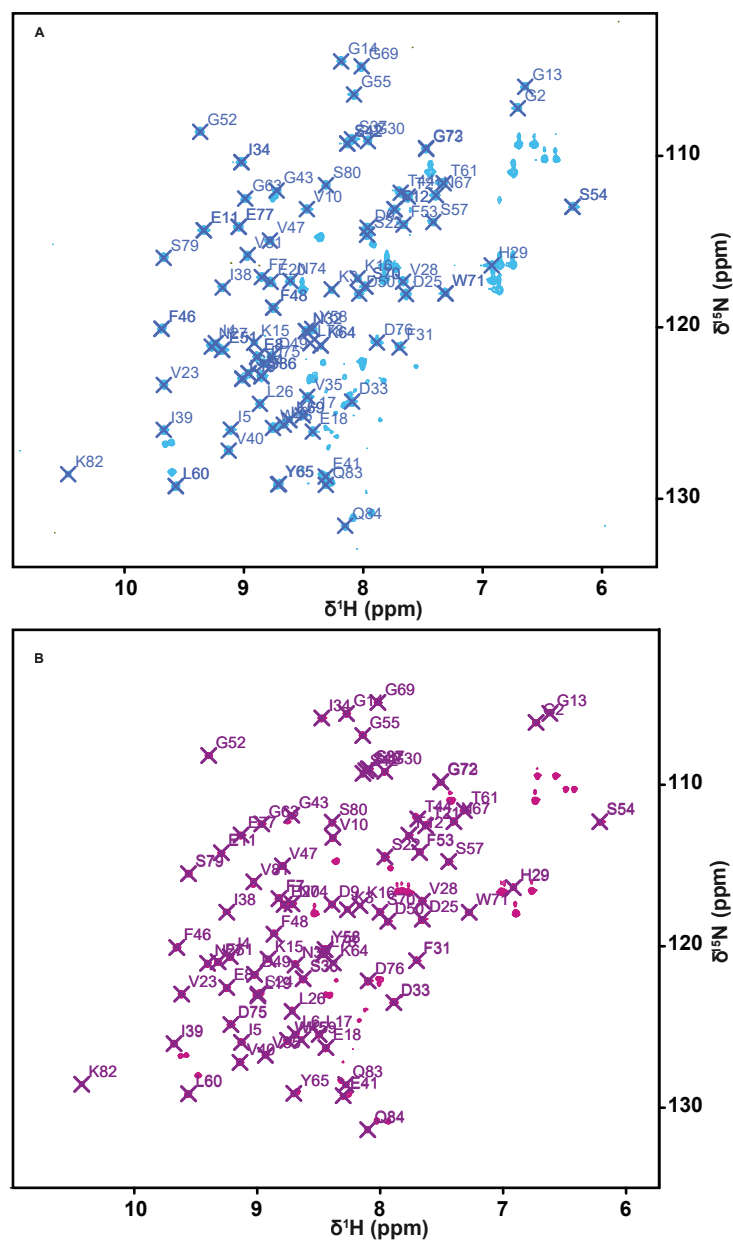


Figure 1.2: Two ^1H - ^{15}N HSQC spectra showing the NH backbone assignments of apo (A) and holo (B) form of *Ci*- $\beta\gamma$.

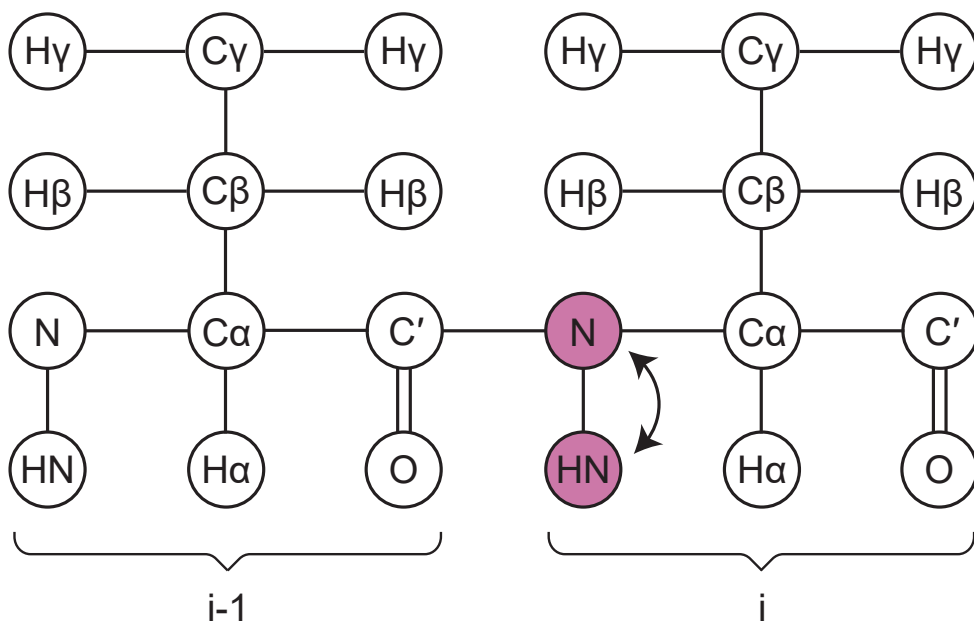


Figure 1.3: Magnetization transfer schematic of a ^1H - ^{15}N HSQC experiment.

Table 1.1: ^{15}N HSQC NMR parameters of apo form Ci - $\beta\gamma$ crystallin.

	^1H	^{15}N
SW (Hz)	12500.00	2836.66
Number of scans	2	
Number of points	2048	282
90 time (μ)	11.64	40.17
Decoupling power	13.19	312.52
Center frequency (ppm)	4.700	117.00

Table 1.2: ^{15}N HSQC NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin.

	^1H	^{15}N
SW (Hz)	12500.00	2836.66
Number of scans	2	
Number of points	1024	282
90 time (μ)	11.33	39.85
Decoupling power	13.19	13.19
Center frequency (ppm)	4.700	117.00

1.3.2 HNCO

The HNCO experiment provides a 3D correlation from the amide proton and nitrogen of the current residue (i) and the carbonyl carbon of the preceding residue (i-1) (Figure 1.4). In this experiment, magnetization is transferred from the amide proton to the directly bonded backbone nitrogen and then selectively to the carbonyl carbon of the preceding residue through scalar J-couplings. The magnetization is then transferred back to the proton for detection 1.5. The carbonyl dimension identifies the carbonyl carbon of the preceding residue (i-1), which provides sequential connectivity along the protein backbone and allows the carbonyl carbon resonance of residue (i-1) to be assigned [16, 17]. The HNCO parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.3 and 1.4.

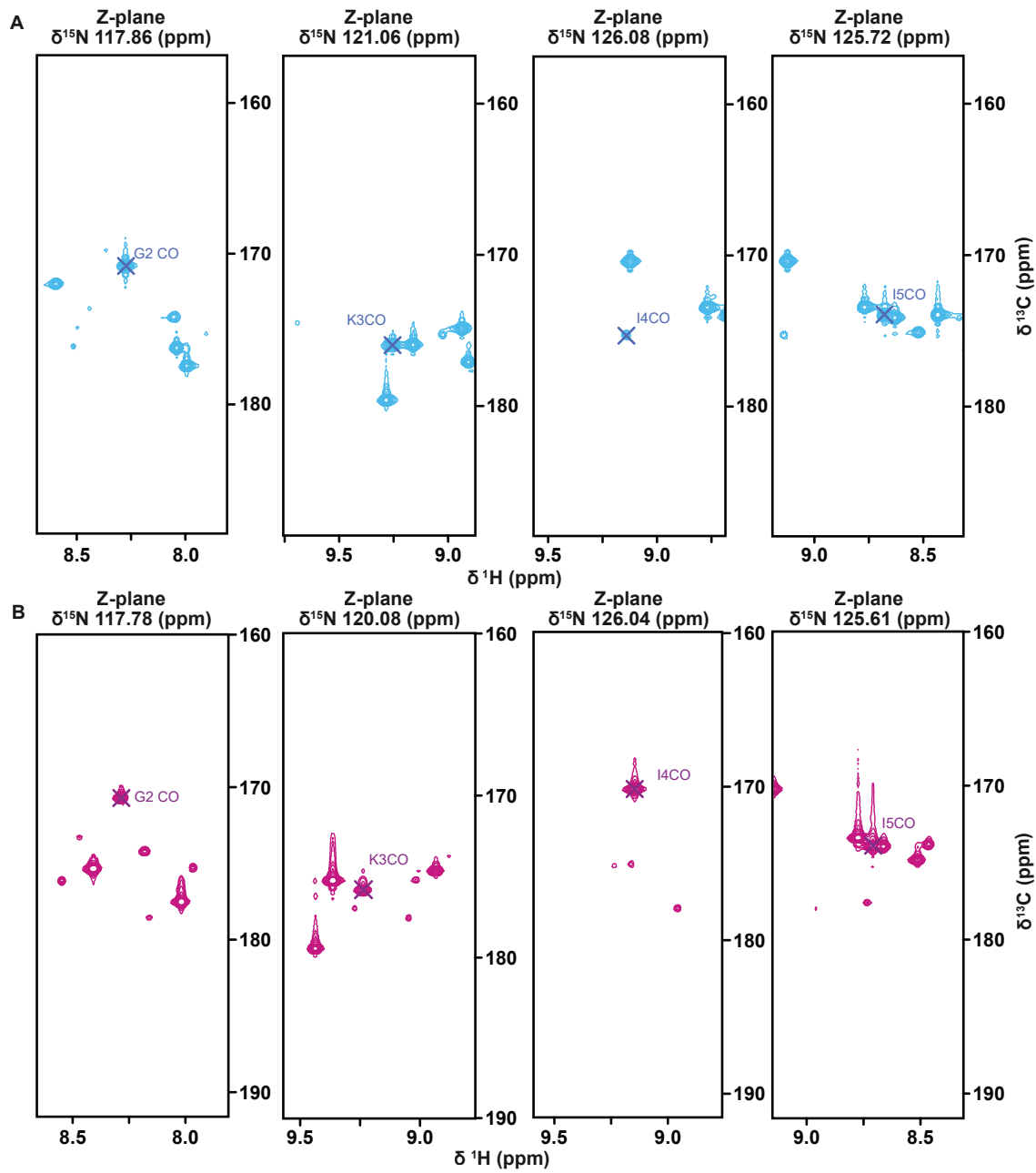


Figure 1.4: Four zoomed in 2D slices of the HNCO experiment showing the $(\text{CO})_{i-1}$ assignments for both apo (A) and holo (B) form of $\text{Ci-}\beta\gamma$.

Table 1.4: HNCO NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin [1].

	^1H	^{13}C	^{15}N
SW (Hz)	12999.68	4000.00	3600.04
Number of scans	4		
Number of points	2218	64	48
Transmitter offset (ppm)	-306.86		
90 time (μ)	8.60	13.25	43.00
Decoupling power	60	41	48
Center frequency (ppm)	4.79	174.03	118.78

1.3.3 HN(CA)CO

The HN(CA)CO experiment extends the HNCO experiment by introducing magnetization transfer through carbonyl carbon to the C_α carbon, which allows the observation of carbonyl correlations from both the current residue (i) and the preceding residue (i-1) (Figure 1.6). A HN(CA)CO experiment transfers magnetization from the amide proton to the directly bonded nitrogen. From there, the magnetization transfers from the nitrogen to the C_α carbon which then goes to the carbonyls in both i and i-1 residues. For detection, the magnetization is transferred back to the amide proton (Figure 1.7). The resulting correlations provide additional information about the sequential connectivity of the backbone and is used with the HNCO experiment to assign the carbonyl carbons [16, 19]. The HN(CA)CO parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.5 and 1.6.

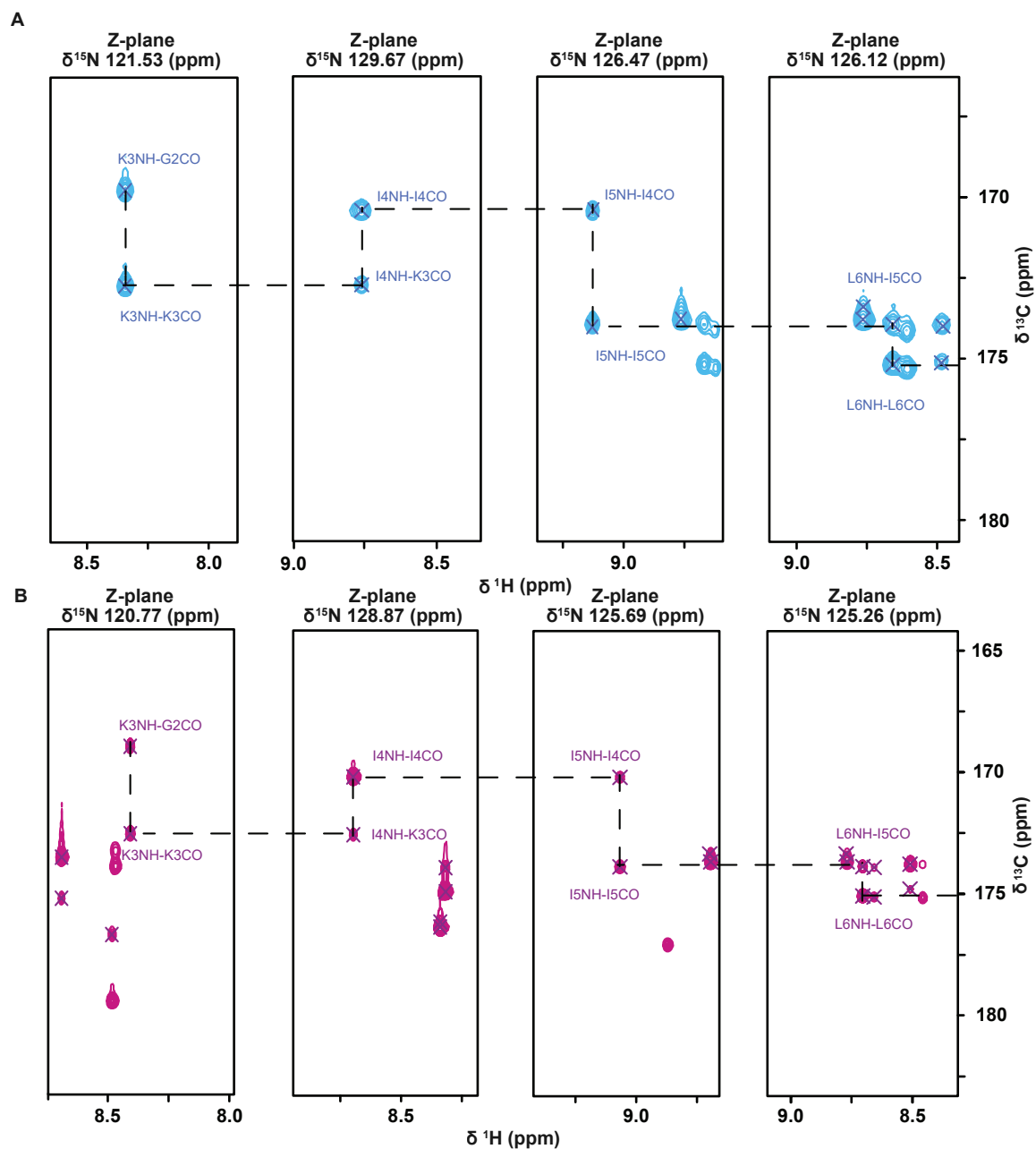


Figure 1.6: Four zoomed in 2D slices of the HN(CA)CO experiment showing the $(\text{CO})_i$ and $(\text{CO})_{i-1}$ residue assignments for both apo (A) and holo (B) form of $Ci-\beta\gamma$.

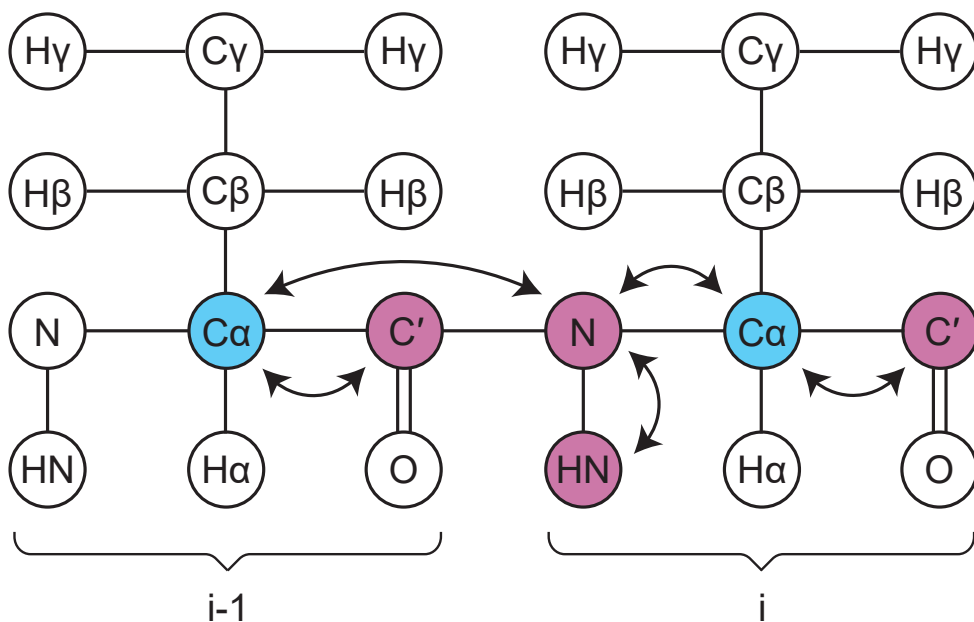


Figure 1.7: Magnetization transfer schematic of a HN(CA)CO experiment.

Table 1.5: HN(CA)CO NMR parameters of apo form *Ci-βγ* crystallin [1].

	¹ H	¹³ C	¹⁵ N
SW (Hz)	12999.68	4000.00	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-305.00		
90 time (μ)	10.00	12.40	45.50
Decoupling power	60	41	48
Center frequency (ppm)	4.79	56.15	120.18

Table 1.6: HN(CA)CO NMR parameters of holo form *Ci-βγ* crystallin [1].

	1H	13C	15N
SW (Hz)	12019.23	6033.64	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-310.76		
90 time (μ)	8.70	13.00	42.00
Decoupling power	60	41	48
Center frequency (ppm)	4.79	56.05	118.79

1.3.4 HNCA

The HNCA experiment provides correlations between the amide proton and nitrogen of the current residue (i) and the C_α resonances of both residue i and the preceding residue (i-1) (Figure 1.8). In a HNCA experiment, magnetization is transferred from the amide proton to the directly bonded nitrogen and then to the C_α carbons of both residues i and i-1. The magnetization is transferred back to the nitrogen and then to the amide proton for detection (Figure 1.9). The HNCA experiment is paired with the HN(CO)CA to identify the C_α resonances of the current and preceding residues [16, 18]. The HNCA parameters used to assign *Ci-βγ* in both forms are on tables 1.7 and 1.8.

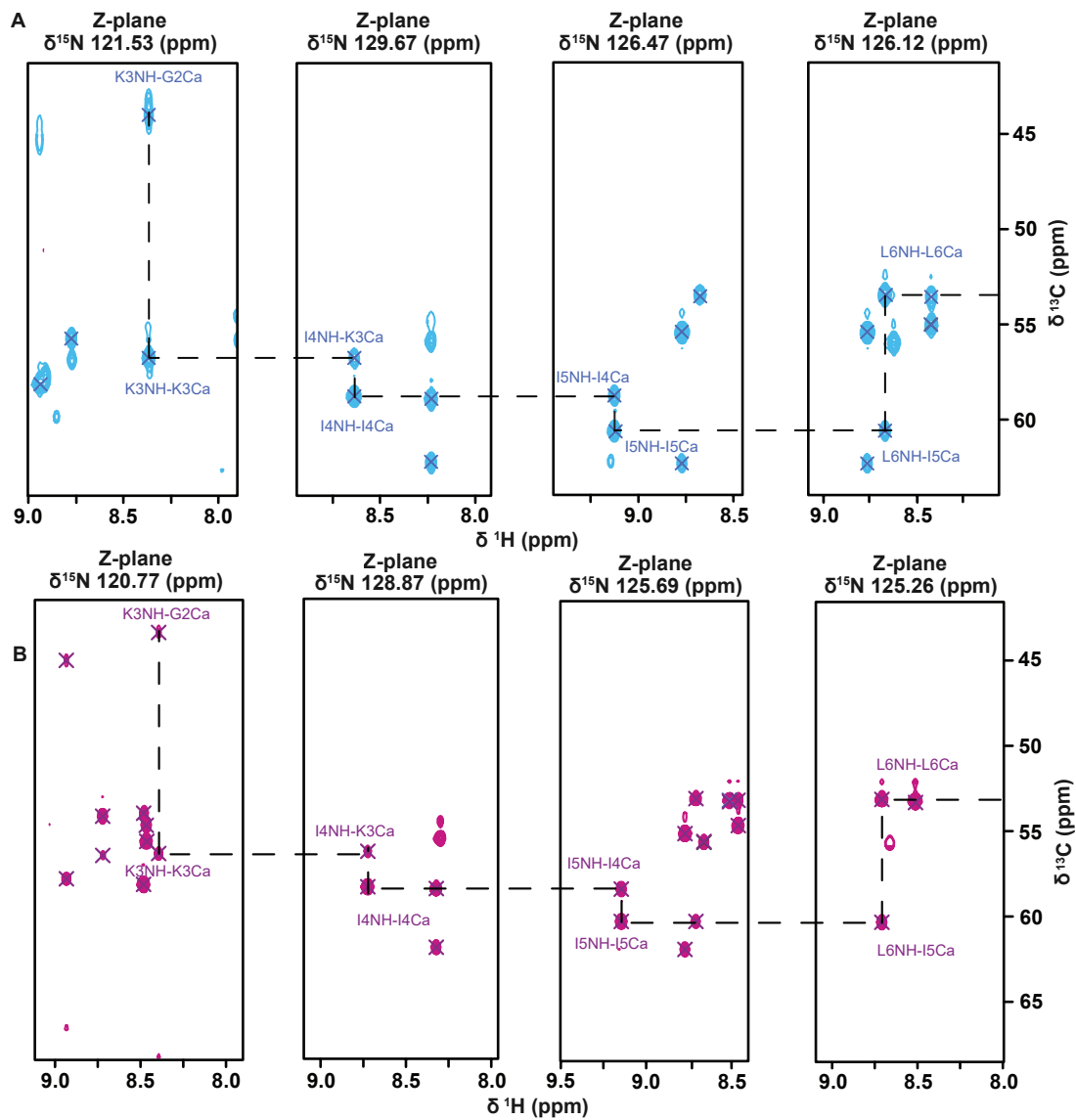


Figure 1.8: Four zoomed in 2D slices of a HNCA spectra showing the $(C_{\alpha})_i$ and $(C_{\alpha})_{i-1}$ residue assignments for both apo (A) and holo (B) form of $Ci-\beta\gamma$.

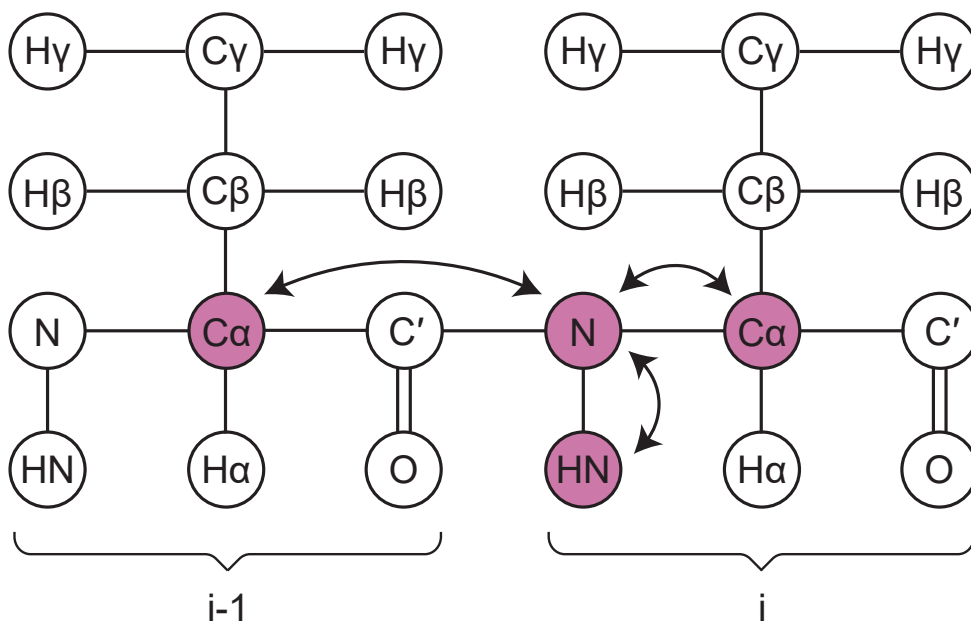


Figure 1.9: Magnetization transfer schematic of a HNCA experiment.

Table 1.7: HNCA NMR parameters of apo form *Ci-βγ* crystallin [1].

	^1H	^{13}C	^{15}N
SW (Hz)	12999.68	6034.29	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-305.00		
90 time (μ)	10.00	12.30	45.50
Decoupling power	60	41	48
Center frequency (ppm)	4.79	56.27	120.18

Table 1.8: HNCA NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin [1].

	^1H	^{13}C	^{15}N
SW (Hz)	12019.23	6033.64	3600.04
Number of scans	4		
Number of points	2048	64	48
Transmitter offset (ppm)	-306.90		
90 time (μ)	8.60	14.00	43.00
Decoupling power	60	41	48
Center frequency (ppm)	4.79	56.04	118.79

1.3.5 HN(CO)CA

The HN(CO)CA experiment extends on the HNCA experiment by correlating the amide proton and nitrogen of the current residue (i) with the preceding residue (Figure 1.10). In a HN(CO)CA experiment, magnetization is passed from the amide proton to the nitrogen in the backbone and then to the carbon on the carbonyl. From the carbonyl, magnetization is then transferred to C_α and then the magnetization is transferred back following the same process for detection (Figure 1.11). The HN(CO)CA experiment acts as a filter that identifies the preceding residues C_α signal which allows the identification of C_α for both i and i-1 in the HNCA experiment [16, 20]. The HN(CO)CA parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.9 and 1.10.

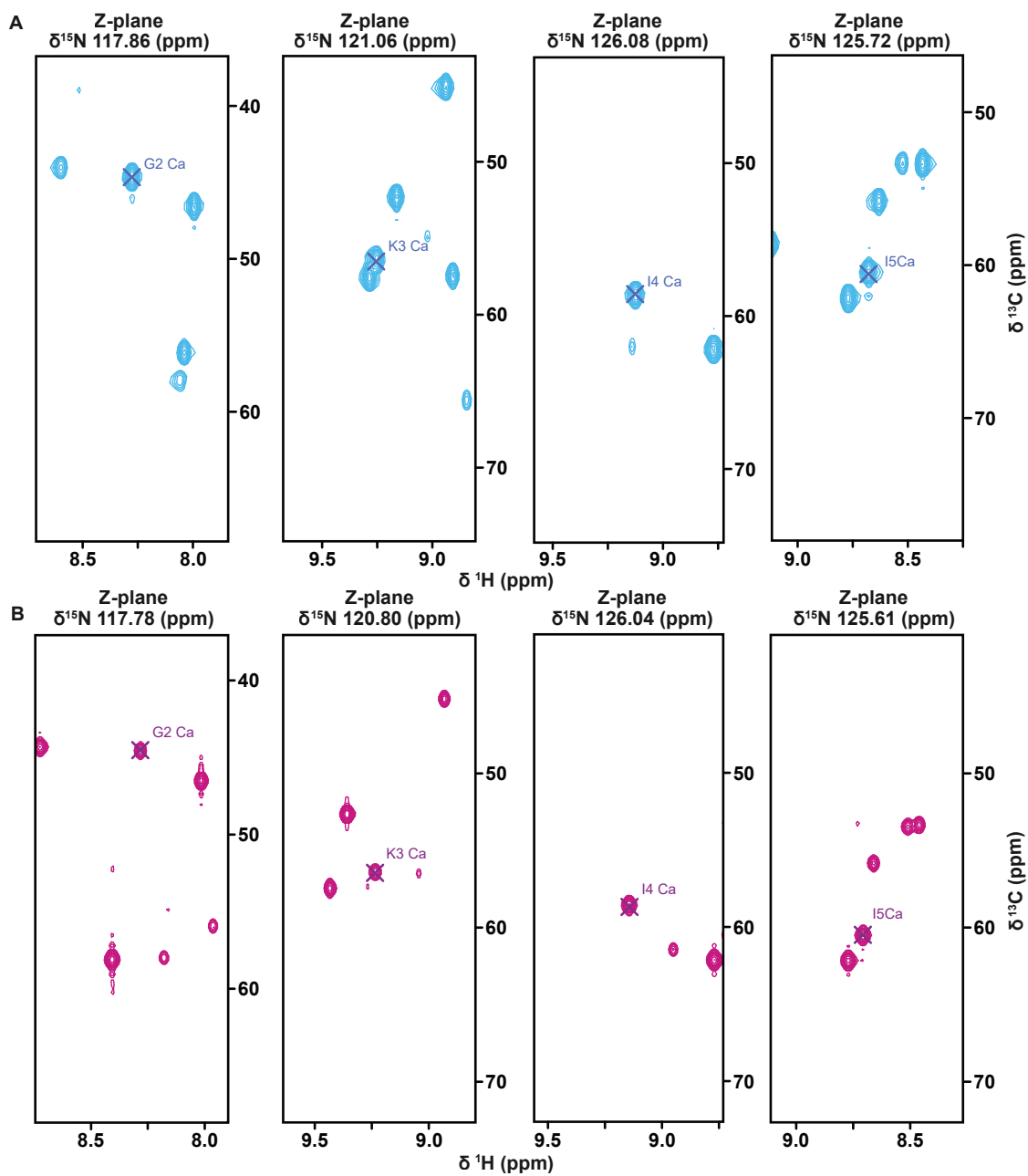


Figure 1.10: Four zoomed in 2D slices of the HN(CO)CA experiment showing the $(\text{C}_\alpha)_{i-1}$ residue assignments for both apo (A) and holo (B) form of $\text{Ci-}\beta\gamma$.

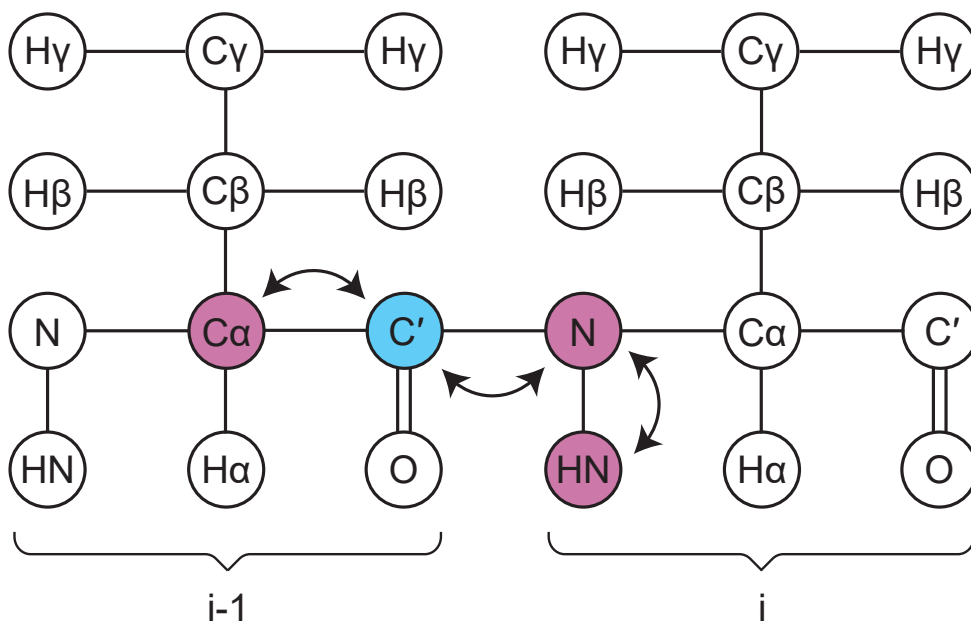


Figure 1.11: Magnetization transfer schematic of a HN(CO)CA experiment.

Table 1.9: HN(CO)CA NMR parameters of apo form Ci - $\beta\gamma$ crystallin [1].

	^1H	^{13}C	^{15}N
SW (Hz)	12999.68	10055.41	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-305.00		
90 time (μ)	10.00	12.40	45.50
Decoupling power	60	41	48
Center frequency (ppm)	4.79	56.15	120.18

Table 1.10: HN(CO)CA NMR parameters of holo form *Ci*- $\beta\gamma$ crystallin [1].

	1H	13C	15N
SW (Hz)	12019.23	6033.64	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-310.80		
90 time (μ)	8.70	13.00	42.00
Decoupling power	60	41	48
Center frequency (ppm)	4.79	56.05	118.79

1.3.6 CBCA(CO)NH

The CBCA(CO)NH experiment correlates amide resonances of the current residue (i) with C_α and C_β resonances of the preceding residues (i-1) (Figure 1.12). Magnetization is transferred from the H_α and H_β to their directly bonded C_α and C_β . Magnetization is then transferred from C_β to C_α where its then transferred to the carbonyl carbon. Once it reaches the carbonyl carbon, the magnetization is transferred to the nitrogen then to the amide proton for detection (Figure 1.13). The resulting correlations identifies the C_α and C_β of the preceding i-1 [16, 21]. The CBCA(CO)NH parameters used to assign *Ci*- $\beta\gamma$ in both forms are on tables 1.11 and 1.12.

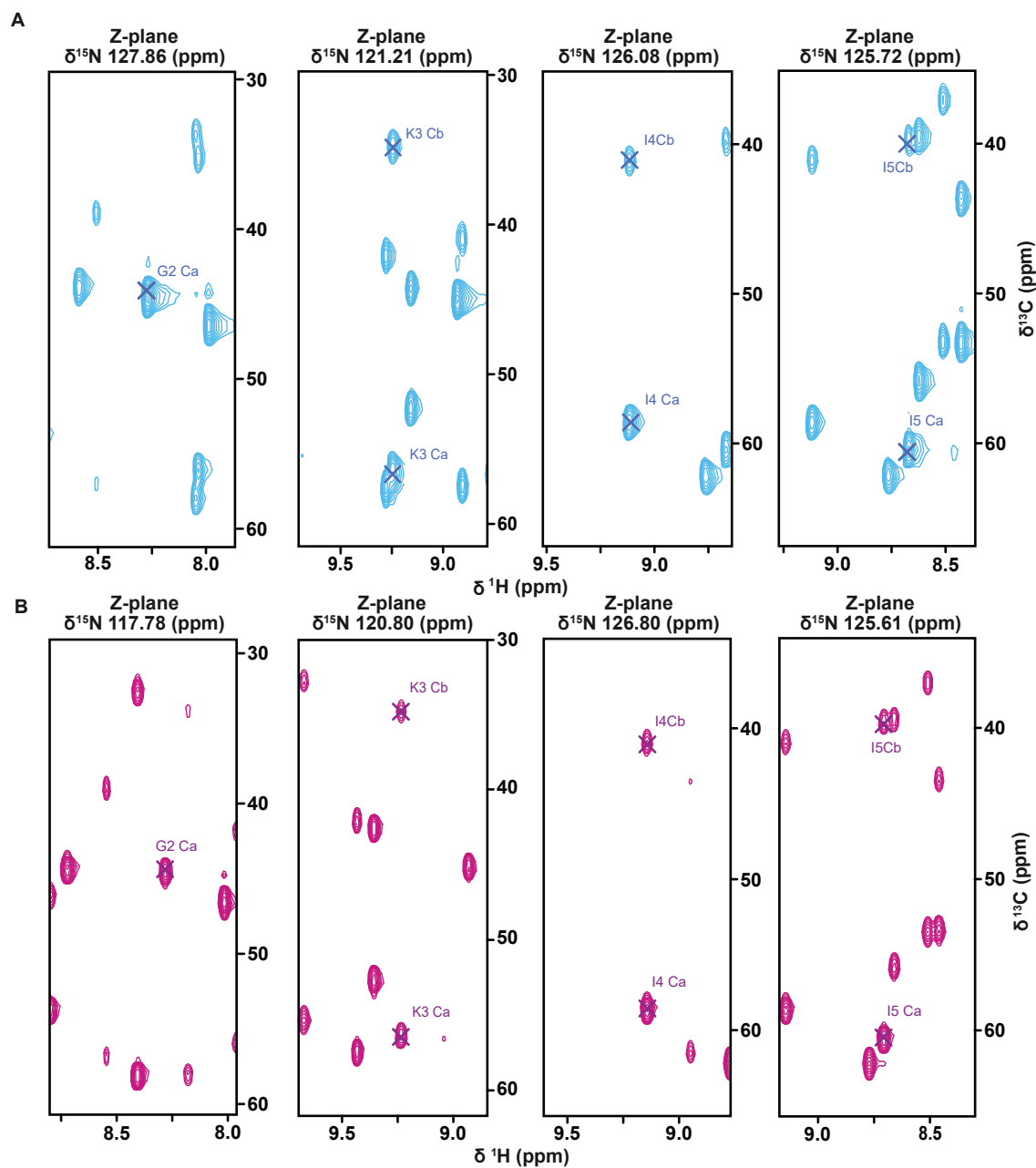


Figure 1.12: Four zoomed in 2D slices of the CBCA(CO)NH experiment showing the $(C_{\alpha})_{i-1}$ and $(C_{\beta})_{i-1}$ residue assignments for both apo (A) and holo (B) form of $Ci-\beta\gamma$.

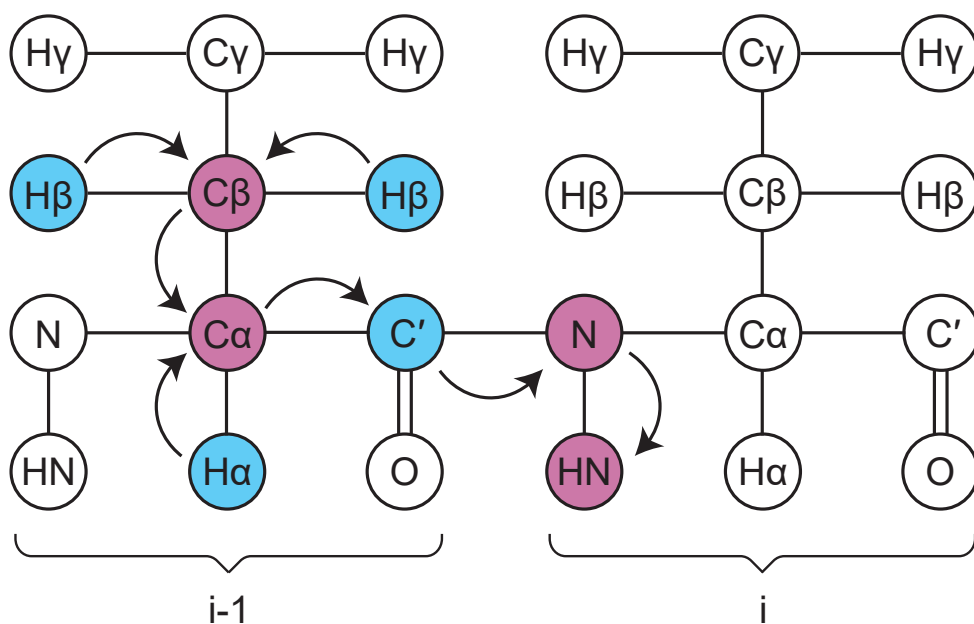


Figure 1.13: Magnetization transfer schematic of a CBCA(CO)NH experiment.

Table 1.11: CBCA(CO)NH NMR parameters of apo form *Ci-βγ* crystallin [1].

	¹ H	¹³ C	¹⁵ N
SW (Hz)	12999.68	16000.00	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-305.00		
90 time (μ)	10.00	12.40	45.50
Decoupling power	60	41	48
Center frequency (ppm)	4.79	46.16	120.18

Table 1.12: CBCA(CO)NH NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin [1].

	1H	13C	15N
SW (Hz)	12019.23	16000.00	3600.04
Number of scans	4		
Number of points	2048	64	36
Transmitter offset (ppm)	-304.90		
90 time (μ)	8.70	13.30	42.00
Decoupling power	60	41	48
Center frequency (ppm)	4.79	46.03	118.79

1.3.7 HNCACB

The HNCACB experiment correlates amide resonances with both C_α and C_β chemical shifts from the current (i) and preceding residues (i-1) (Figure 1.14). Magnetization is transferred from H_α and H_β to C_α and C_β then from C_β to C_α . From C_α , magnetization is transferred to the nitrogen and then to the amide proton for detection. Magnetization is transferred in both i and i-1 residue which allows for the identification of C_α and C_β for both i residue and i-1 residue (Figure 1.15). This experiment is paired with a CBCA(CO)NH experiment to identify C_α and C_β for the i residue [16, 21]. The HNCACB parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.13 and 1.14.

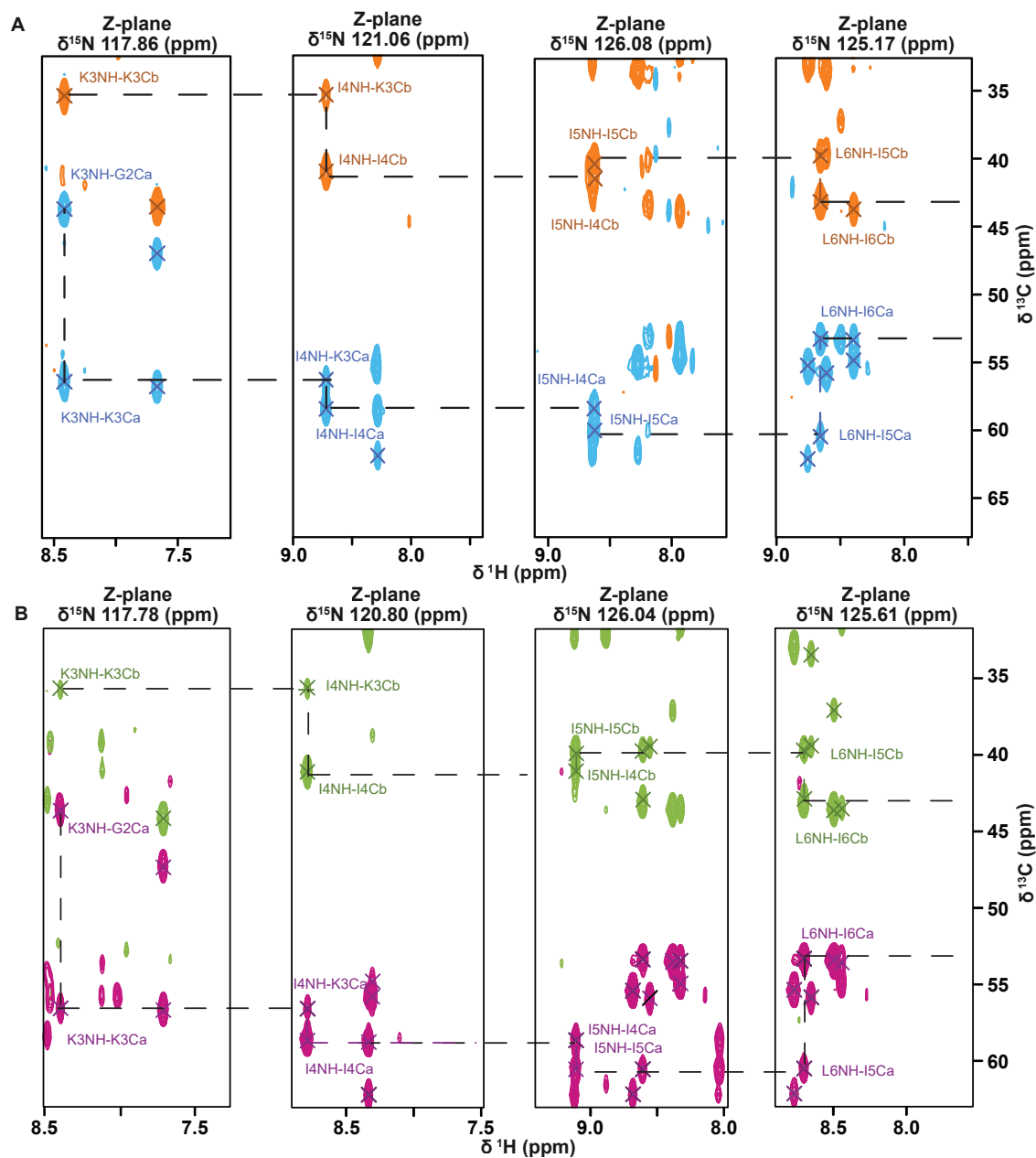


Figure 1.14: Four zoomed in 2D slices of the HNCACB spectra showing the $(C_\alpha)_i$, $(C_\alpha)_{i-1}$, $(C_\beta)_i$, and $(C_\beta)_{i-1}$ residue assignments for both apo (A) and holo (B) form of Ci - $\beta\gamma$.

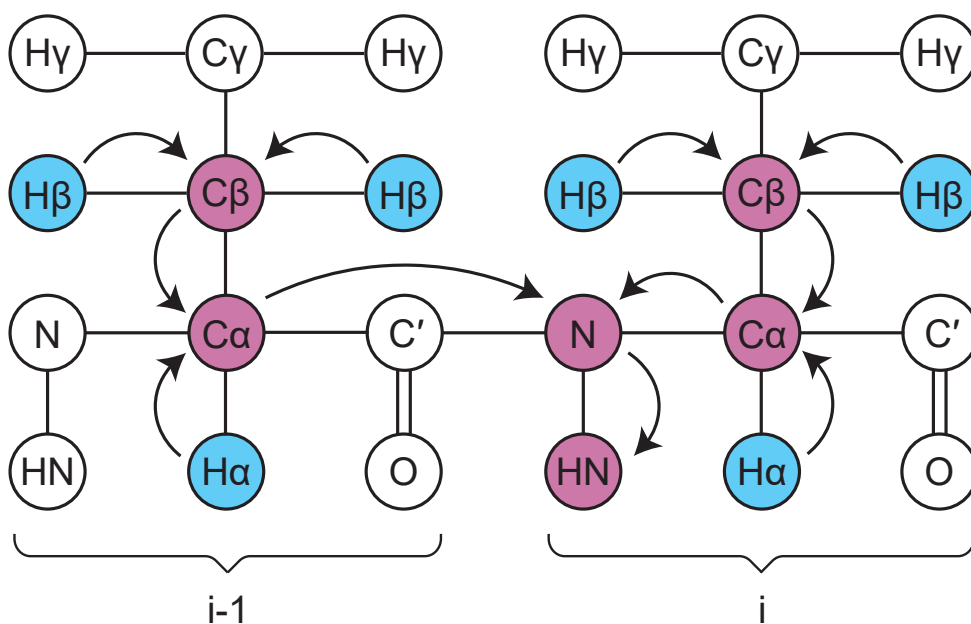


Figure 1.15: Magnetization transfer schematic of a HNCACB experiment.

Table 1.13: HNCACB NMR parameters of apo form *Ci*- $\beta\gamma$ crystallin [1].

	1H	^{13}C	^{15}N
SW (Hz)	12999.68	16000.00	3600.04
Number of scans	4		
Number of points	2048	48	36
Transmitter offset (ppm)	-305.00		
90 time (μ)	10.00	12.40	45.50
Decoupling power	60	41	48
Center frequency (ppm)	4.79	46.15	120.18

Table 1.14: HNCACB NMR parameters of holo form *Ci*- $\beta\gamma$ crystallin [1].

	¹ H	¹³ C	¹⁵ N
SW (Hz)	12019.23	16000.00	3600.04
Number of scans	4		
Number of points	2048	64	36
Transmitter offset (ppm)	-304.90		
90 time (μ)	8.70	13.30	42.00
Decoupling power	60	41	48
Center frequency (ppm)	4.79	46.03	118.79

1.3.8 Summary

These complementary 3D NMR experiments provide both sequential and intraresidue correlations that enable the assignment of backbone resonances throughout the protein. Once backbone assignments have been established, the resulting chemical shifts can serve as the foundation for secondary structure prediction, side-chain resonance assignment, ligand-binding studies, residual dipolar coupling measurements, and protein dynamics analyses.

1.4 Side-Chain Resonance Assignment

To complete the resonance assignments, the side-chain nuclei need to be identified. Side-chain chemical shifts are essential for characterizing residue-specific interactions, defining tertiary structure, identifying ligand-binding interfaces, and measuring side-chain dynamics. Multidimensional heteronuclear experiments are required to correlate aliphatic and side-chain nuclei with the previously assigned backbone resonances. A combination of ¹H-¹³C HSQC, HBHA(CO)NH, CCCONH, and hCCH-TOCSY experiments provides complementary information that should enable the assignments from the protein backbone into the side chains

[22, 23, 24].

1.4.1 ^1H - ^{13}C Heteronuclear Single Quantum Coherence

A ^1H - ^{13}C HSQC experiment provides a 2D representation of the protonated carbon resonances within a protein by correlating each proton with its directly bonded ^{13}C nucleus through J-coupling. In a ^1H - ^{13}C HSQC experiment, magnetization is transferred from the proton to the directly bonded carbon and then transferred back to the carbon for detection (Figure 1.17). A ^1H - ^{13}C HSQC identifies the CH, CH_2 , and CH_3 's groups within a sample (Figure 1.16). These correlations provide information for identifying and assigning side-chain resonances and can be used to characterize changes in the chemical environment of a protein [16]. The HSQC parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.15 and 1.16.

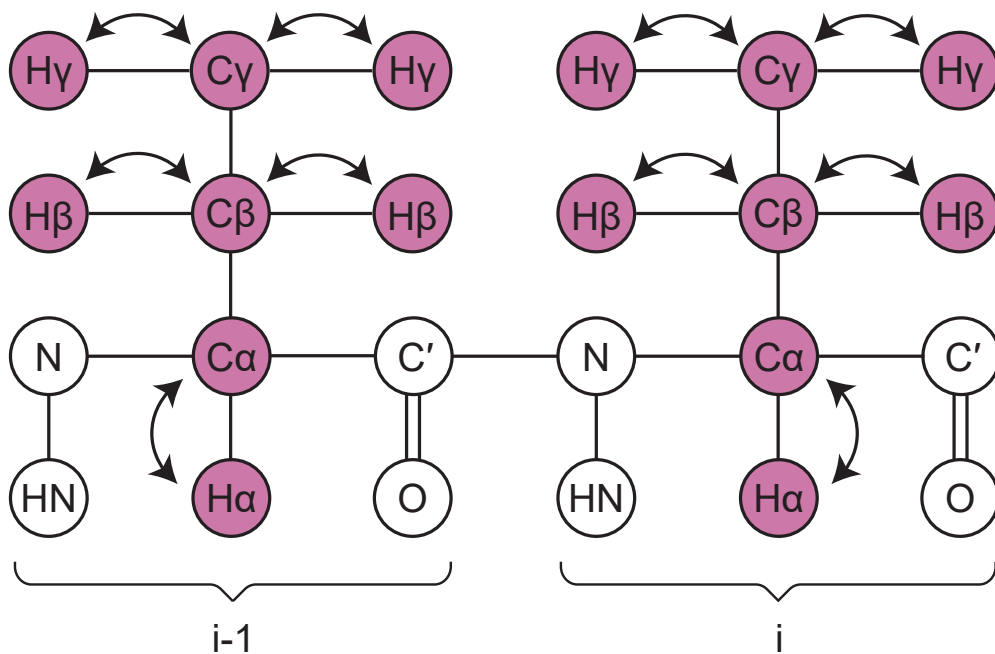


Figure 1.17: Magnetization transfer schematic of a ^1H - ^{13}C HSQC experiment.

Table 1.15: ^{13}C HSQC NMR parameters of apo form Ci - $\beta\gamma$ crystallin.

	^1H	^{13}C
SW (Hz)	10416.67	33183.54
Number of scans	2	
Number of points	1024	256
90 time (μ)	11.66	10.10
Decoupling power	13.19	312.52
Center frequency (ppm)	4.700	75.00

Table 1.16: ^{13}C HSQC NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin.

	^1H	^{13}C
SW (Hz)	10416.67	33183.54
Number of scans	2	
Number of points	1024	256
90 time (μ)	11.17	9.98
Decoupling power	13.19	312.52
Center frequency (ppm)	4.700	75.00

1.4.2 HBHACONH

The HBHA(CO)NH experiment correlates the amide proton and nitrogen of residue i with the H_α and H_β resonances of the preceding residue ($i-1$) (Figure 1.18). In a HBHACONH experiment, magnetization is transferred from H_α and H_β to their corresponding C_α and C_β . Magnetization is then transferred from C_β to C_α where its transferred to the carbonyl carbon. Once it reaches the carbonyl carbon, the magnetization is transferred to the backbone nitrogen then to directly attached proton for detection (Figure 1.19). The resulting correlations identify the H_α and H_β resonances of the preceding residues and provide sequential information for side-chain resonance assignments [16, 22]. The HSQC parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.19 and 1.18.

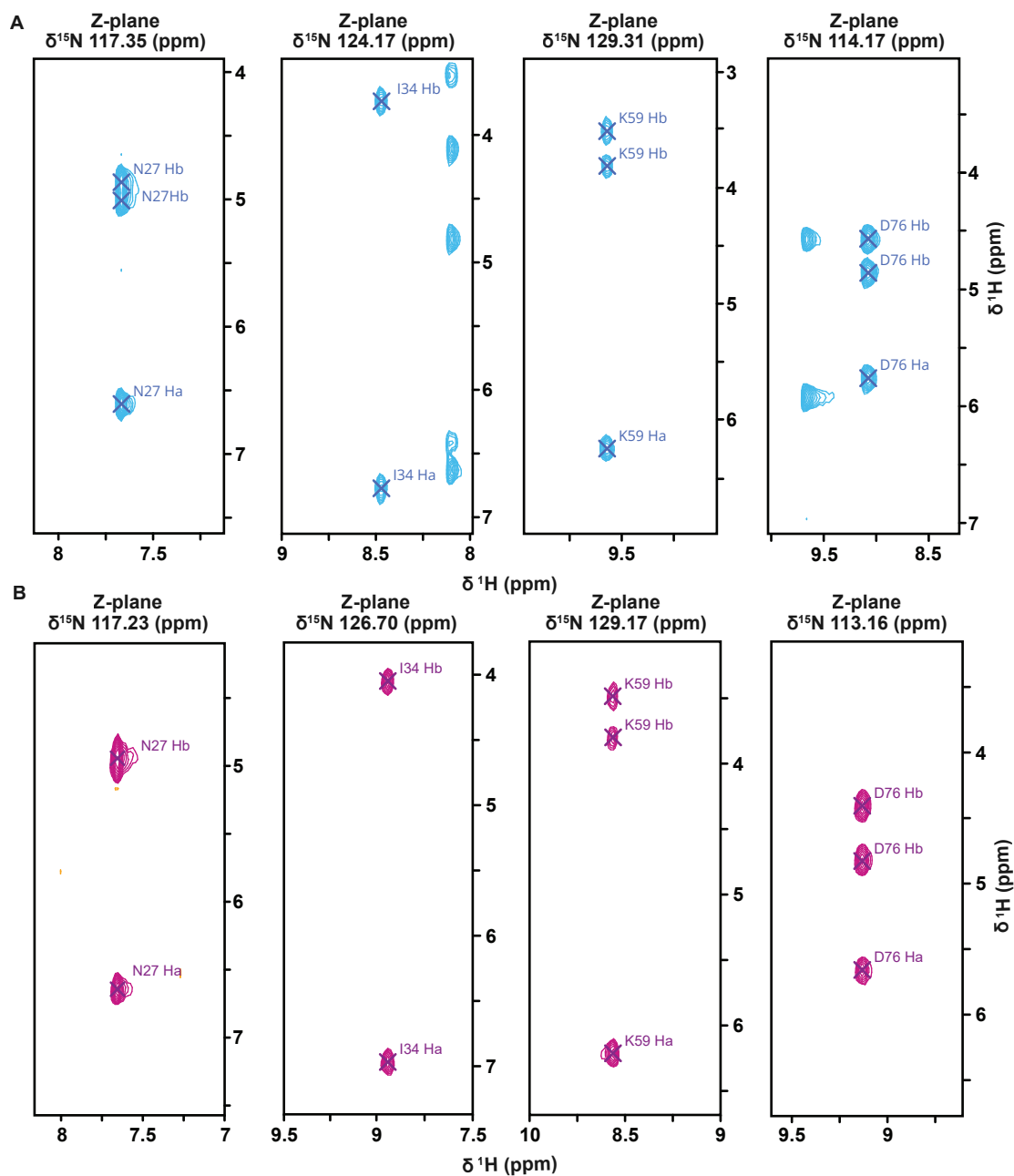


Figure 1.18: Four zoomed in 2D slices of the HBHACONH spectra showing the $\text{H}_{\alpha i-1}$ and $\text{H}_{\beta i-1}$ residue assignments for both apo (A) and holo (B) form of $\text{Ci-}\beta\gamma$.

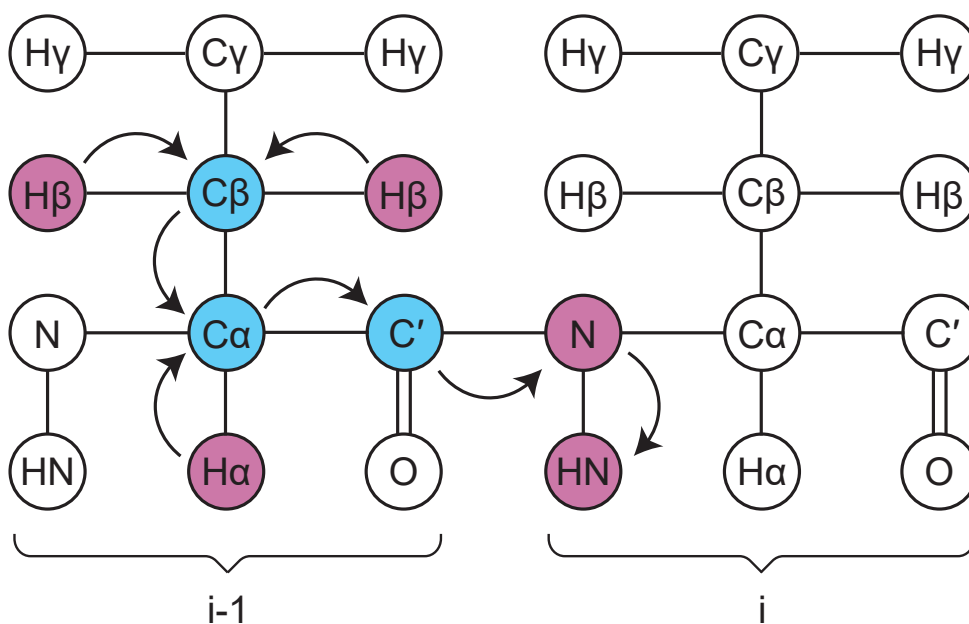


Figure 1.19: Magnetization transfer schematic of a HBHACONH experiment.

Table 1.17: HBHACONH NMR parameters of apo form Ci - $\beta\gamma$ crystallin.

	^1H	^{15}N	^1H
SW (Hz)	12500.00	2788.04	12500.00
Number of scans	16		
Number of points	2048	122	128
90 time (μ)	11.65	25.00	11.65
Decoupling power (W)	0.50		0.50
Center frequency (ppm)	4.69	119.80	4.69

Table 1.18: HBHACONH NMR parameters of holo form *Ci-βγ* crystallin.

	1H	15N	1H
SW (Hz)	12500.00	2836.66	12500.00
Number of scans	16		
Number of points	2048	124	128
90 time (μ)	11.17	25.00	11.17
Decoupling power (W)	0.71		0.71
Center frequency (ppm)	4.70	117.00	4.70

1.4.3 CCCONH

The CCCONH experiment correlates the amide proton and nitrogen of the current residue (i) with the aliphatic carbon resonances of the preceding residue (i-1) (Figure 1.20). Magnetization is transferred through the aliphatic carbon network of the preceding residue and then to the carbonyl carbone. The magnetization is then transferred to the backbone nitrogen and back to the amide proton for detection (Figure 1.21). These correlations allow the identification and assignment of the aliphatic carbon resonances within the same residue [16, 23]. The CCCONH parameters used to assign *Ci-βγ* in both forms are on tables 1.19 and 1.20.

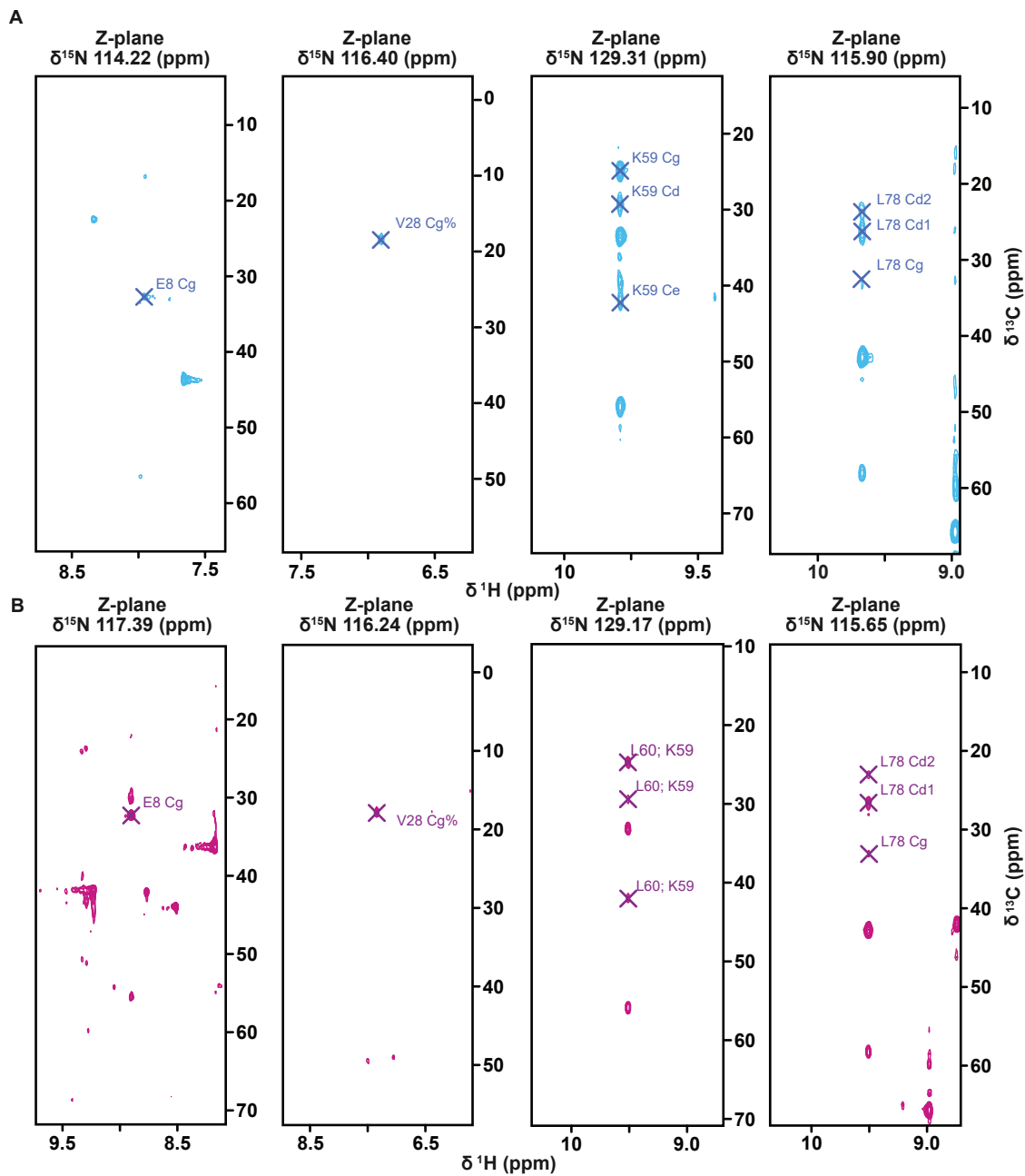


Figure 1.20: Four zoomed 2D slices of the CCONH experiment showing the $C_{\gamma i-1}$, $C_{\delta i-1}$, $C_{\epsilon i-1}$ residue assignments for both apo (A) and holo (B) form of $Ci-\beta\gamma$.

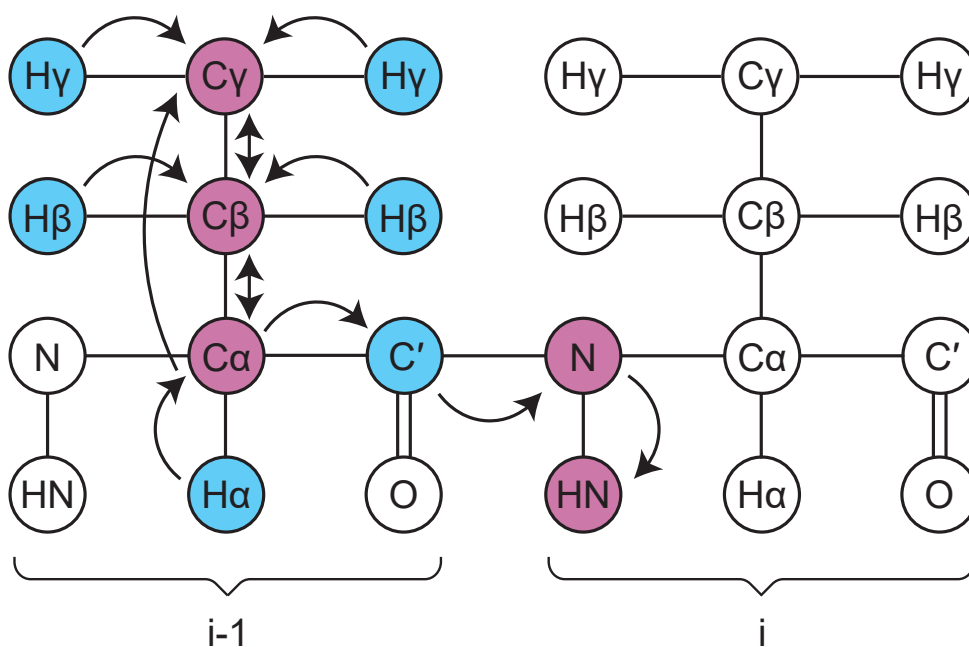


Figure 1.21: Magnetization transfer schematic of a CCONH experiment.

Table 1.19: CCONH NMR parameters of apo form Ci - $\beta\gamma$ crystallin.

	^1H	^{13}C	^{15}N
SW (Hz)	11363.64	16088.48	2836.66
Number of scans	16		
Number of points	2048	128	40
Mixing time (msec)	12		
90 time (μ)	9.00	10.00	38.00
Decoupling power	13.19	312.52	9.32
Center frequency (ppm)	4.70	43.00	117.00

Table 1.20: CCONH NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin.

	^1H	^{13}C	^{15}N
SW (Hz)	11363.64	16088.48	2836.66
Number of scans	16		
Number of points	2048	128	40
Mixing time (msec)	12		
90 time (μ)	9.00	10.00	38.00
Decoupling power	13.19	312.52	9.32
Center frequency (ppm)	4.700	43.00	117.00

1.4.4 HCCH-TOCSY

The HCCH-TOCSY experiment correlates protonated carbon resonances throughout an individual side-chain spin system which allows the observation of the CH , CH_2 , CH_3 groups. (Figure 1.22). Magnetization is transferred between the side-chain hydrogen nuclei to their attached carbon. Isotropic ^{13}C mixing then occurs and magnetization is transferred back to the side-chain hydrogen for detection (Figure 1.23). The resulting correlations allow protonated carbon resonances to be associated with the same amino acid and facilitate the assignment of side-chain resonances [16, 24]. The HCCH-TOCSY parameters used to assign $Ci\text{-}\beta\gamma$ in both forms are on tables 1.21 and 1.22.

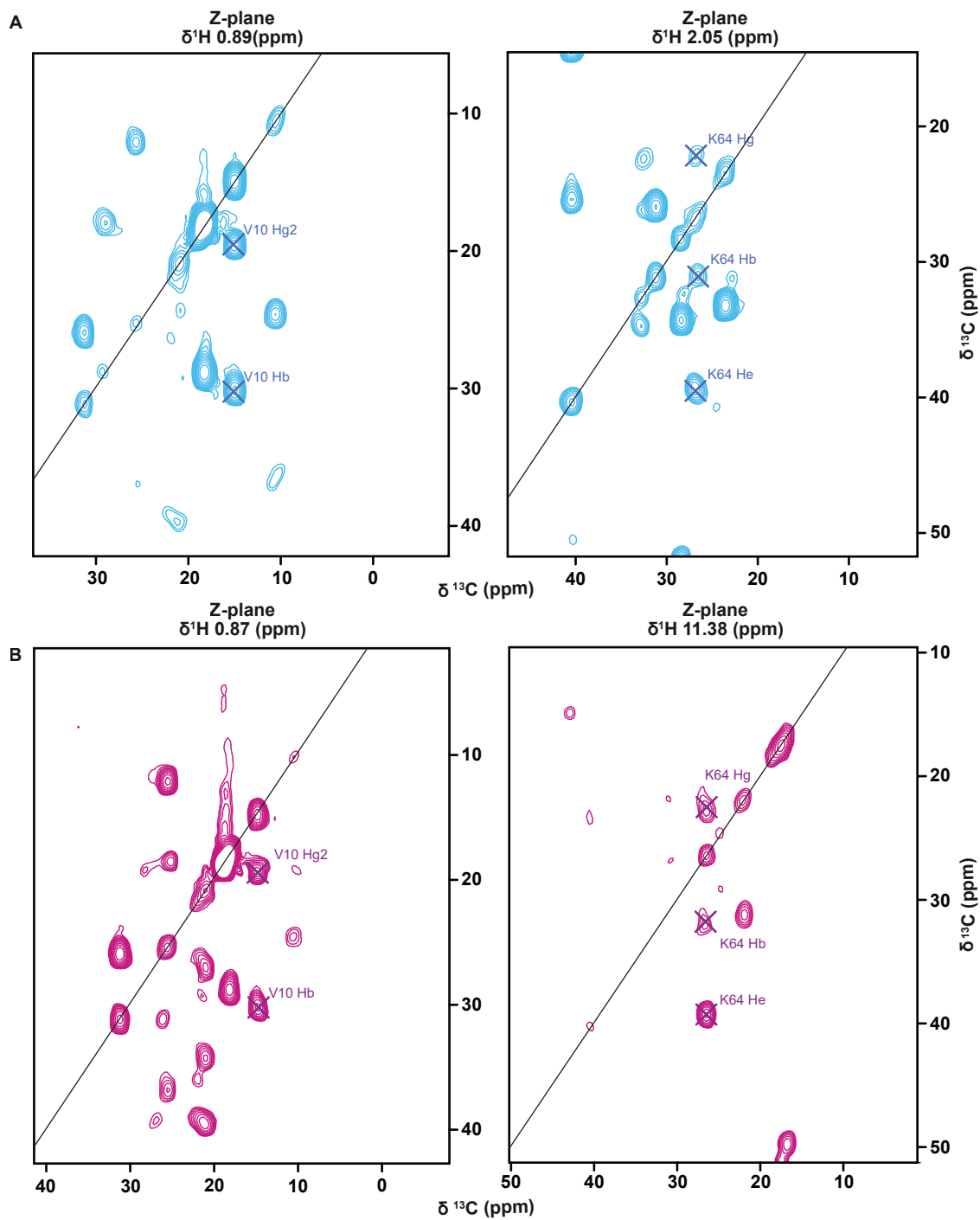


Figure 1.22: Two zoomed in HCCH-TOCSY spectra identifying the H_α , H_β , H_γ , H_δ , and H_ϵ residue assignments attached to the aliphatic carbons for both apo (A) and holo (B) form of *Ci-βγ*.

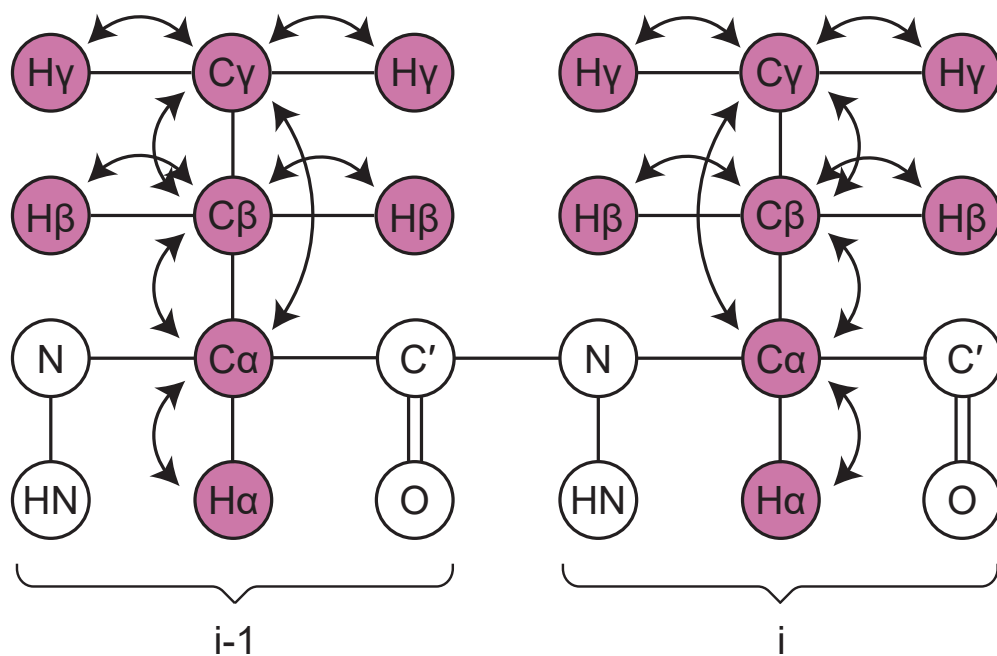


Figure 1.23: Magnetization transfer schematic of a hCCH-TOCSY experiment.

Table 1.21: HCCH-TOCSY NMR parameters of apo form Ci - $\beta\gamma$ crystallin.

	1H	^{13}C	^{13}C
SW (Hz)	11363.64	16088.48	16088.48
Number of scans	16		
Number of points	2048	64	128
Mixing Time (msec)	12		
90 time (μ)	11.82	10.18	10.18
Decoupling power (W)	13.19	312.52	312.52
Center frequency (ppm)	4.70	43.00	43.00

Table 1.22: HCCH-TOCSY NMR parameters of holo form *Ci*- $\beta\gamma$ crystallin.

	¹ H	¹³ C	¹³ C
SW (Hz)	11363.64	16088.48	16088.48
Number of scans	16		
Number of points	2048	64	128
Mixing Time (msec)	12		
90 time (μ)	11.33	10.12	10.12
Decoupling power (W)	13.19	312.52	312.52
Center frequency (ppm)	4.70	43.00	43.00

1.5 Residual Dipolar Couplings

Solution NMR experiments are often performed under isotropic conditions, where rapid molecular tumbling averages dipolar interactions to zero. Averaging out dipolar interactions removes orientational information that can be used to define molecular structure. Residual dipolar couplings (RDCs) are measured by weakly aligning proteins in solutions so that dipolar interactions are only partially averaged. These residual interactions provide long-range orientational restraints describing the average orientation of internuclear bond vectors relative to the molecular alignment tensor. RDCs are utilized to validate protein structure, refine structural models, characterize domain orientations, and investigate conformational dynamics [25].

1.5.1 In-Phase/Anti-Phase experiment

RDCs are measured from the splitting between the amide proton and nitrogen. In an isotropic solution, the splitting that is observed is only the scalar coupling. When the sample is weakly aligned with bicelles, the dipolar interactions is no longer average to zero

and the splitting now becomes a mix of scalar coupling and residual dipolar couplings. In a In-Phase/Anti-Phase (IPAP) NMR experiment, separate in-phase and anti-phase spectra are acquired. This allows the frequency difference between the two components of the amide J coupling doublet to be measured. Under weak alignment conditions, the observed splitting corresponds to the amide J coupling and residual dipolar coupling (Figure 1.24B). To get RDCs, another IPAP experiment will be ran under isotropic conditions so the scalar coupling can be removed [26] (Figure 1.24A). The IPAP parameters used to obtain RDCs of $C_{i-\beta\gamma}$ in both forms are on tables 1.23 and 1.24.

Table 1.23: IPAP NMR parameters of apo form *Ci*- $\beta\gamma$ crystallin.

	1H	15N
SW (Hz)	12500.00	3282.44
Number of scans	2	
Number of points	2048	328
Delay Time (msec)	1	
90 time (μ)	10.44	42.44
Decoupling power (W)	13.19	11.63
Center frequency (ppm)	4.70	121.20

Table 1.24: IPAP NMR parameters of holo form *Ci*- $\beta\gamma$ crystallin.

	1H	15N
SW (Hz)	12500.00	2358.48
Number of scans	2	
Number of points	2048	234
Delay Time (msec)	1	
90 time (μ)	10.65	40.81
Decoupling power (W)	13.19	11.4
Center frequency (ppm)	4.70	118.10

1.6 Protein Dynamics

Proteins are dynamic molecules that undergo motions over a wide range of timescales from rapid bond vibrations occurring on the picosecond timescale to large conformational changes occurring over milliseconds. These motions play important roles in protein folding, ligand recognition, enzyme catalysis, molecular recognition, and allosteric regulation [16]. NMR relaxation experiments allows the investigation of both protein structure and dynamics in

solution. NMR relaxation experiments provide residue-specific information about molecular motion by monitoring the return of nuclear magnetization to equilibrium through radio frequency excitation. Relaxation rates are influenced by fluctuations in local magnetic fields generated by molecular motion which makes them sensitive probes of protein dynamics. The measured relaxation parameters for backbone amide nuclei in this thesis are the heteronuclear nuclear overhauser effect (hetNOE), the longitudinal relaxation time (T1), and transverse relaxation time (T2). Together, these experiments characterize backbone flexibility over picosecond-to-nanosecond timescales and can identify residues undergoing slower conformational exchange [27, 28].

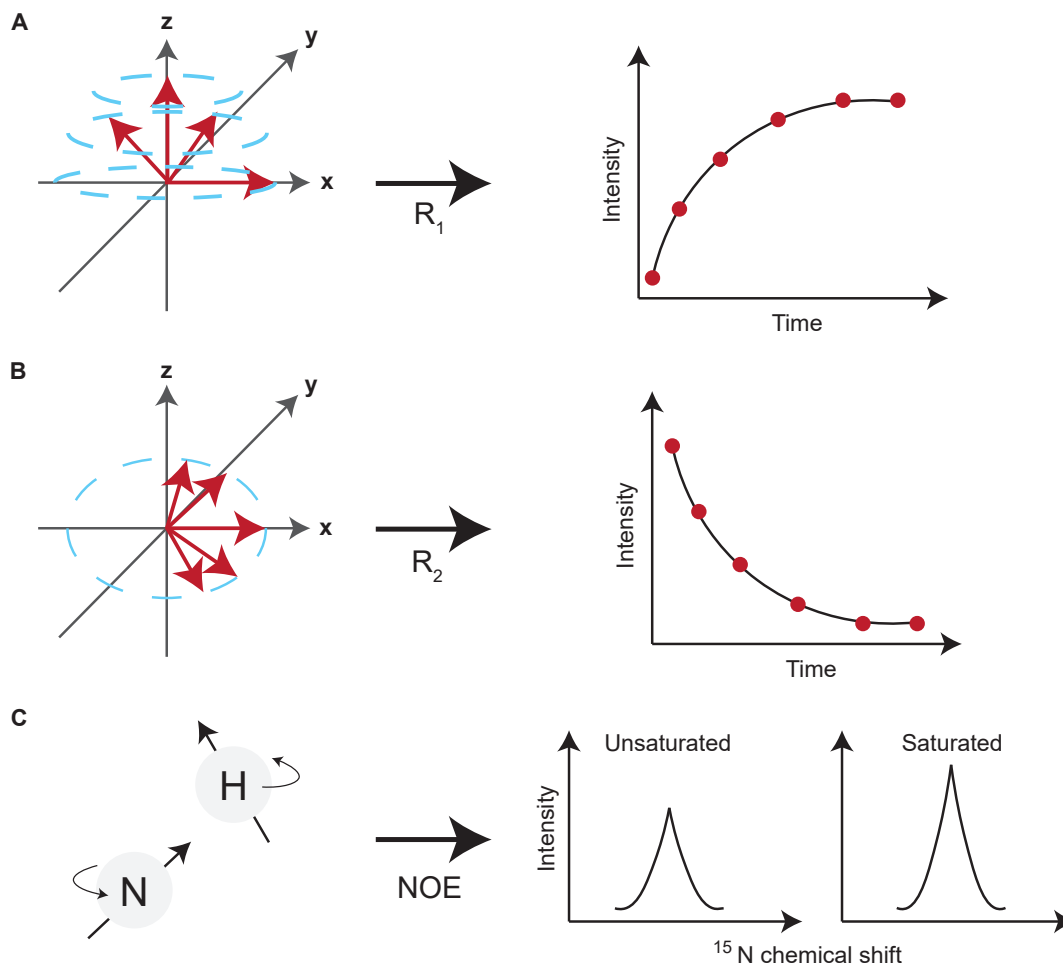


Figure 1.25: This figure demonstrates the NMR relaxation measurements used to characterize protein backbone dynamics. Panel A is the measurement of the longitudinal relaxation rate (R_1) by detecting the recovery of magnetization of the Z axis as a function of time. Panel B is the measurement of transverse relaxation rate (R_2) by detecting the recovery of magnetization in the xy plane as a function of time. Panel C is comparing the difference of intensity that occurs in an HetNOE experiment from a unsaturated and a saturated plane.

1.6.1 T1

T1 relaxation rates are influenced by overall molecular tumbling and fast internal motions occurring on the picosecond-to-nanosecond timescale. T1s measures the recovery of magnetization along the direction of the external magnetic field (the longitudinal plane). Residue specific T1 values are determined through measuring the recovery signal intensity at multiple

mixing time and fitting the data to an exponential recovery function (Figure 1.25B). These measurements provide information about overall rotational diffusion and contribute to the characterization of protein backbone dynamics [28, 29]. The T1 parameters used to obtain the translational relaxation rates of $Ci\text{-}\beta\gamma$ in both forms are on tables 1.25 and 1.26.

Table 1.25: T1 NMR parameters of apo form $Ci\text{-}\beta\gamma$ crystallin.

	1H	15N	
SW (Hz)	7812.50	1520.51	
Number of scans	8		
Number of points	2048	152	11
Delay Time (msec)	3		
90 time (μ)	8.93	31.33	
Decoupling power (W)	9.26	4.57	
Center frequency (ppm)	4.70	118.00	

Table 1.26: T1 NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin.

	1H	15N	
SW (Hz)	7812.50	1520.51	
Number of scans	8		
Number of points	2048	152	11
Delay Time (msec)	3		
90 time (μ)	9.23	31.00	
Decoupling power (W)	9.26	4.48	
Center frequency (ppm)	4.70	118.00	

1.6.2 T2

T2 relaxation rates are influenced by overall molecular tumbling, local backbone flexibility, and slower conformational exchange processes on the microsecond-to-millisecond timescale. T2 experiments measure the decay of magnetization in the transverse plane at multiple mixing times. Fitting this data in an exponential recovery function will obtain relaxation rates along the transverse plane for each nuclei (Figure 1.25A). These measurements measure molecular tumbling, local flexibility, and slower conformational exchange in the microsecond-to-millisecond timescale [28, 29]. The T2 parameters used to obtain the transverse relaxation rates of $Ci\text{-}\beta\gamma$ in both forms are on tables 1.27 and 1.28.

Table 1.27: T2 NMR parameters of apo form $Ci\text{-}\beta\gamma$ crystallin.

	1H	15N	
SW (Hz)	7812.50	1520.51	
Number of scans	4		
Number of points	2048	152	12
Delay Time (msec)	3		
90 time (μ)	8.94	30.83	
Decoupling power (W)	9.26	4.42	
Center frequency (ppm)	4.70	118.00	

Table 1.28: T2 NMR parameters of holo form $Ci\text{-}\beta\gamma$ crystallin.

	^1H	^{15}N	
SW (Hz)	7812.50	1520.51	
Number of scans	4		
Number of points	2048	152	12
Delay Time (msec)	3		
90 time (μ)	9.16	31.03	
Decoupling power (W)	9.26	4.48	
Center frequency (ppm)	4.70	118.00	

1.6.3 HetNOE

The hetNOE experiment measures the enhancement of the ^{15}N signal resulting from dipolar interactions with directly bonded amide protons. The experiment is performed by acquiring two $^1\text{H}\text{-}^{15}\text{N}$ HSQC spectra where one spectra contains information of the nuclei peak intensities once the amide protons are saturated and another spectra without proton saturation. The ratio of the peak intensities from the saturated and reference spectra yields the hetNOE values for each residue (Figure 1.25C). These measurements are sensitive to fast backbone dynamics on the picosecond-to-nanosecond timescale and they can be used to identify rigid, well-ordered regions and flexibility[28, 29]. The hetNOE parameters for both apo and holo form are found on tables 1.29 and 1.30.

Table 1.29: HetNOE NMR parameters of apo form *Ci*- $\beta\gamma$ crystallin.

	1H	15N	
SW (Hz)	7812.50	1520.51	
Number of scans	4		
Number of points	2048	152	2
Delay Time (msec)	5		
90 time (μ)	8.94	30.83	
Decoupling power (W)	9.26	4.42	
Center frequency (ppm)	4.70	118.00	

Table 1.30: HetNOE NMR parameters of holo form *Ci*- $\beta\gamma$ crystallin.

	1H	15N	
SW (Hz)	7812.50	1520.51	
Number of scans	4		
Number of points	2048	152	2
Delay Time (msec)	5		
90 time (μ)	9.16	31.02	
Decoupling power (W)	9.26	4.48	
Center frequency (ppm)	4.70	118.00	

1.6.4 Summary

T1, T2, and hetNOE measurements provide information regarding different aspects of molecular motion. T1s measure the overall molecular tumbling and fast internal motions within the picosecond-to-nanosecond timescale through longitudinal relaxation, T2s measure molecular tumbling, local flexibility, and slower conformational exchange in the microsecond-to-millisecond timescale through transverse relaxation, and hetNOEs measure fast backbone

motions indicating rigid regions or disordered regions through monitoring the change in intensity. When analyzed together, these relaxation parameters can be used to determine residue-specific order parameters (S^2), internal correlation times, and contributions from conformational exchange. With this information, one can map out the protein backbone dynamics that complements structural information obtained from resonance assignments and residual dipolar couplings. This is useful for distinguishing the proteins rigid regions from flexible loops and identify residues undergoing slower conformational changes[28, 29].

Chapter 2

Backbone and side-chain NMR resonance assignments of *Ciona* *intestinalis*- $\beta\gamma$ crystallin

2.1 Abstract

The structural $\beta\gamma$ -crystallin from the tunicate *Ciona intestinalis* serves as an evolutionary waypoint between ancestral metal cation-binding crystallins and the $\beta\gamma$ crystallins of the vertebrate eye lens. *Ci*- $\beta\gamma$ maintains the metal cation-binding sites of ancestral crystallins while also sharing common structural motifs with vertebrate lens crystallins. Due to its structure and strong interaction with Ca^{2+} , *Ci*- $\beta\gamma$ is more thermally and chemically stable than vertebrate lens crystallins, making it an ideal starting point for understanding $\beta\gamma$ crystallins' evolution for molecular stability. In this study, we present the side-chain and updated backbone resonance assignments of *Ci*- $\beta\gamma$ with TALOS-N predictions on the H_N , H_α , C_α , C_β and N chemical shifts. These assignments provide a foundation for future studies of metal binding, dynamics, and stability of proteins in the $\beta\gamma$ -crystallin family.

2.2 Biological Context

The $\beta\gamma$ -crystallins belong to an evolutionarily conserved protein superfamily found in organisms ranging from microbes to vertebrates. These proteins are characterized by a paired Greek key motif that assembles into a compact, stable β -sheet rich fold. In vertebrates, $\beta\gamma$ -crystallins function as structural proteins that maintain lens transparency and contribute to the refractive index gradient required for vision. However, homologous $\beta\gamma$ -crystallins are found throughout all domains of life, where they perform a variety of biological functions [30, 31, 32]. Their broad phylogenetic distribution suggests that the $\beta\gamma$ -crystallin fold evolved long before its specialization within the vertebrate eye lens [12]. Many non-lenticular members of the superfamily retain metal-ion binding motifs, indicating that cation binding likely represented an ancestral function of $\beta\gamma$ -crystallins prior to their recruitment as structural lens proteins.

Among extant chordates, the tunicate *Ciona intestinalis* occupies a position nearest the point in the tree of life where vertebrates diverge from the rest of the deuterostomes [33]. As a protein from an early-diverging chordate lineage, the *Ci*- $\beta\gamma$ crystallin (*Ci*- $\beta\gamma$) preserves characteristics of both ancestral and vertebrate crystallins. *Ci*- $\beta\gamma$ contains two calcium-binding sites and retains the double Greek key motif characteristic of ancestral $\beta\gamma$ -crystallin [34], while sharing similarities with vertebrate lens crystallins. Gene duplication of an ancestral $\beta\gamma$ -crystallin gave rise to the two-domain architecture observed in modern vertebrate lens crystallins, whereas metal-binding sites were largely lost during the evolution of the lens [35, 36, 37]. Therefore, *Ci*- $\beta\gamma$ serves as an evolutionary waypoint linking ancestral metal-binding $\beta\gamma$ -crystallins with their vertebrate descendants. Previous studies showed that calcium-bound *Ci*- $\beta\gamma$ is even more thermally and chemically stable than vertebrate lens crystallins,[38, 39], which may reflect an ancestral role of the $\beta\gamma$ -crystallin fold prior to its adaptation as a lens protein. In the tunicate, this protein is expressed in the sensory organ as well as the palps, suggesting dual functionality in this organism [34].

NMR resonance assignments are essential to characterize the biophysical basis of calcium binding, protein stability, and conformational dynamics in *Ci*- $\beta\gamma$ for both the apo and holo forms. Furthermore, backbone chemical shift assignments provide information regarding local secondary structure propensities and establish a framework for future investigations of metal-dependent structural changes, backbone dynamics, and intermolecular interactions. Here, we report the updated backbone and side-chain resonance assignments of *Ci*- $\beta\gamma$. HNCO, HN(CO)CA, HNCA, HN(CA)CO, CBCA(CO)NH, and HNCACB experiments were performed to assign the backbone. HN, N, C α , and C β chemical shifts were obtained for all non-proline residues for both the apo and holo forms. Unambiguous H α and H β assignments were obtained for 78/84 residues by performing a ^1H - ^{15}N HSQC and a 3D HBHACONH experiment. Extensive side-chain assignments, including C γ , C δ , and C ϵ , were also measured using a 3D CCONH experiment. Backbone assignments were used to predict secondary structure and calculate residue-specific order parameters with TALOS-N [40]. These assignments establish a foundation for future investigations of calcium-dependent structural stability, conformational, dynamics, and molecular evolution within the $\beta\gamma$ -crystallin lineage.

2.3 Methods and Experiments

2.3.1 Expression and purification of *Ci*- $\beta\gamma$ -Crystallin

An *Escherichia coli* codon-optimized plasmid containing the *Ci*- $\beta\gamma$ -crystallin gene was purchased from Blue Heron Biotechnology (Bothell, WA). The protein sequence is **MGKIILFEDV EFGGKKLELE TSVSDLNVHG FNDIVSSIIV ESGTWFVFDD EGFSGPSYKL TPGKYPNPGS WGGNDDELSS VKQQ**. Expression of *Ci*- $\beta\gamma$ for the side-chain NMR experiments was performed as follows: bacteria containing a pET-28 plasmid with the *Ci*- $\beta\gamma$ sequence and a kanamycin resistance gene were plated on a kanamycin (IBI scientific, Dubuque IA USA) Luria-Bertani (LB) agar plate at 37°C for 16 h. An overnight culture was inoculated with a colony from the *Ci*- $\beta\gamma$ plate and incubated at 37°C for 12-16 h. 500 mL of kanamycin LB media was inoculated with 5 mL of

overnight culture and incubated at 37°C until OD 1.1 was reached. The media was then spun down at 4,000 RPM for 30 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in $^{13}\text{C}/^{15}\text{N}$ labeled media and incubated at 37°C for one hour. It was then induced with 500 μL of 1 mM IPTG (Goldbio, St. Louis, MO USA) and incubated at 16°C for 48 hours. The media was then spun down and flash frozen for storage. To purify *Ci- $\beta\gamma$* , cell pellets were resuspended in 10 mM BisTris (Promega, Madison WI USA), 0.05% NaN_3 (Fisher Bioreagents, India), 10 mM CaCl_2 (Fisher chemical, Germany), pH 7 and lysed with sonication. The lysate was then purified via one anion exchange and one run of size exclusion chromatography (SEC). The low salt buffer utilized was 10 mM BisTris, 0.05% NaN_3 at pH 7 and the high salt buffer was 10 mM BisTris, 1 M NaCl , 0.05% NaN_3 at pH 7 for anion exchange. The buffer utilized for SEC was 10 mM HEPES (Fisher Bioreagents, Taiwan), 150 mM NaCl (Spectrum Chemical MFG Corp, New Brunswick NJ), and 0.05% NaN_3 at pH 7. Purity was checked through mass spectrometry and gel electrophoresis. Sample preparation for backbone chemical shift assignments was performed using a similar procedure as previously described [38].

2.3.2 NMR measurements and data analysis

Experiments for the backbone assignments were performed at 25°C on a Varian *Unity* INOVA system operating at 800 MHz equipped with a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ 5 mm tri-axis PFG triple-resonance probe. The protein concentrations were 1.7 mM in the presence of 10 mM Tris (pH 7.1), 0.05% NaN_3 and 10 mM EDTA or 10 mM CaCl_2 . HNCO , $\text{HN}(\text{CO})\text{CA}$, HNCA , $\text{HN}(\text{CA})\text{CO}$, $\text{CBCA}(\text{CO})\text{NH}$, and HNCACB experiments were acquired to assign the protein backbone. All the data for the backbone assignments were processed in NMRPipe and sparky. The assignments were performed in CCPNMR. Experiments for the side-chain assignments were performed at 25°C on a Bruker AVANCE NEO 800MHz spectrometer equipped with a BCU II chiller and a 5 mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ inverse triple resonance probe. The protein concentration for the side chain experiments was 0.5mM in 10mM HEPES (pH

7.1) 50mM NaCl, 0.05% NaN₃ with 10mM EDTA or 10mM CaCl₂. HBHA(CO)NH, ¹H-¹⁵N HSQC, ¹H-¹³C HSQC, CC(CO)NH, and a hCCH-TOCSY were acquired to assign the protein side-chains. All the data for the side-chain assignments were processed in TopSpin v 4.3 and the assignments were assigned on CCPNMR. The parameters for all NMR experiments can be found in chapter 1.

2.3.3 Secondary structure calculation

TALOS-N calculations were performed for both the apo and holo forms of *Ci*- $\beta\gamma$ using these backbone assignments. In the apo but not the holo form, chemical shift analysis indicates that Val 23, Ser 24, and Ser 80 are involved in β -sheet secondary structure. In contrast, Ser 37 is in a β -sheet secondary structure in holo form but not the apo form (Figure 2.1A). These results indicate that there are localized differences in secondary structure when calcium is bound. Residue Val 23 and Ser 24 exhibit reduced β -strand character and lower predicted RCI-S² values, suggesting an increase in local flexibility. Ser 37 exhibits β -strand character when calcium is bound with minimal differences in S², indicating that there is subtle rearrangement in the backbone upon calcium binding. Ser 80 shows minor differences in both β -strand character and S², demonstrating that calcium binding has little effect on its local rigidity (Figure 2.1B and C). Overall, calcium binding does not appear to induce major refolding of *Ci*- $\beta\gamma$, but instead redistributes local conformational populations and alters secondary structure in specific regions. The loss of β -strand character at Val 23, Ser 24, and Ser 80, with the increased β -strand character at Ser 37 likely reflects a subtle calcium-induced change in the backbone conformation and hydrogen-bonding interactions. Comparison of TALOS-N predictions with the crystal structure revealed similar secondary structure, although several residues near secondary structure boundaries showed different assignments, potentially reflecting differences in conformation and dynamics between a crystal structure and a molecule in solution.

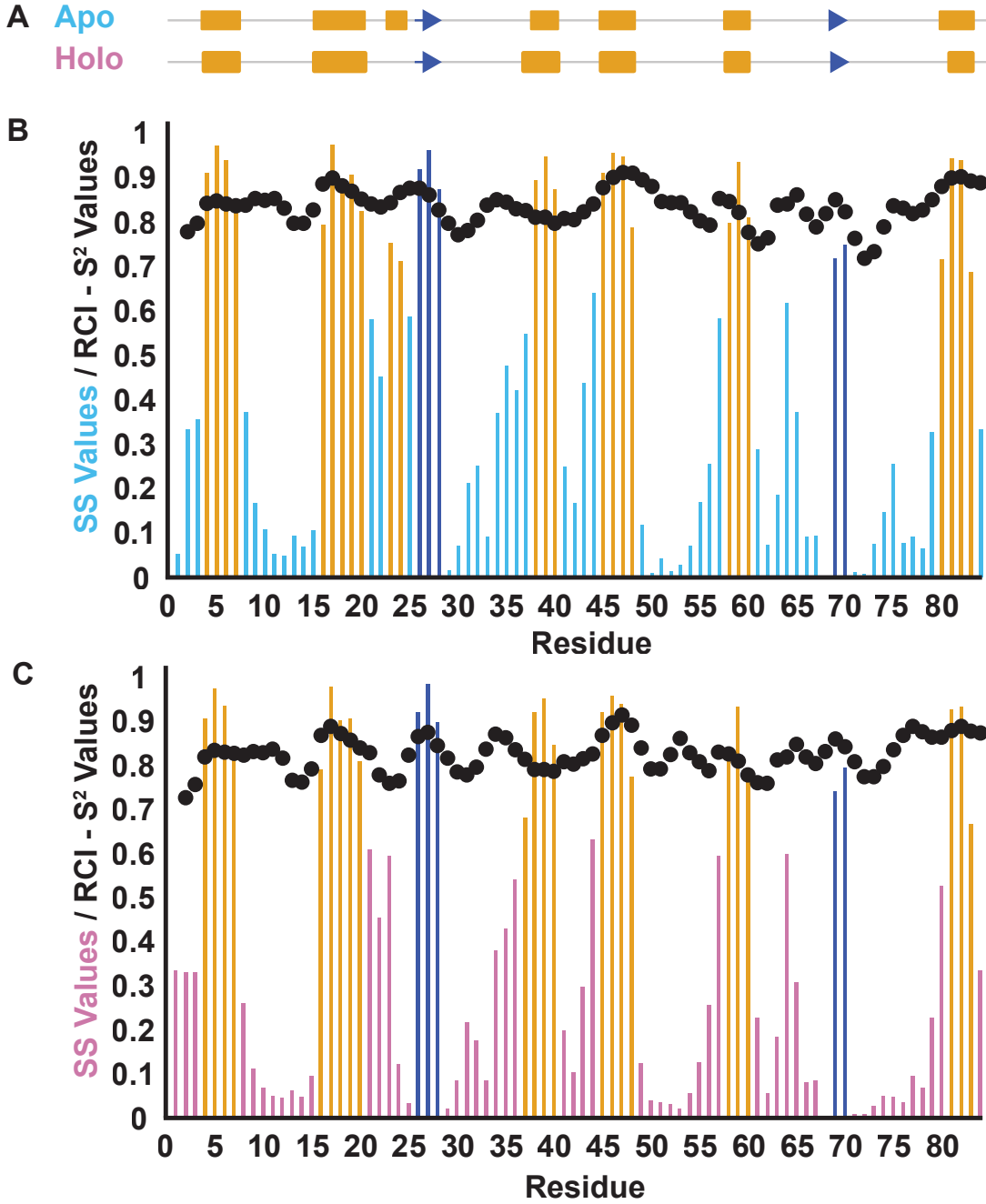


Figure 2.1: The *Ci*- $\beta\gamma$ secondary structure and RCI-S² TALOS-N predictions for both apo and holo form. **A** Predicted β -sheet secondary structure (orange) and α -helix secondary structure (blue) from the TALOS-N prediction. **B** RCI-S² and secondary structure prediction of the apo form of *Ci*- $\beta\gamma$. **C** RCI-S² and secondary structure prediction of the holo form. Both measurements show a difference in β sheet-secondary structure when Ca^{2+} is bound.

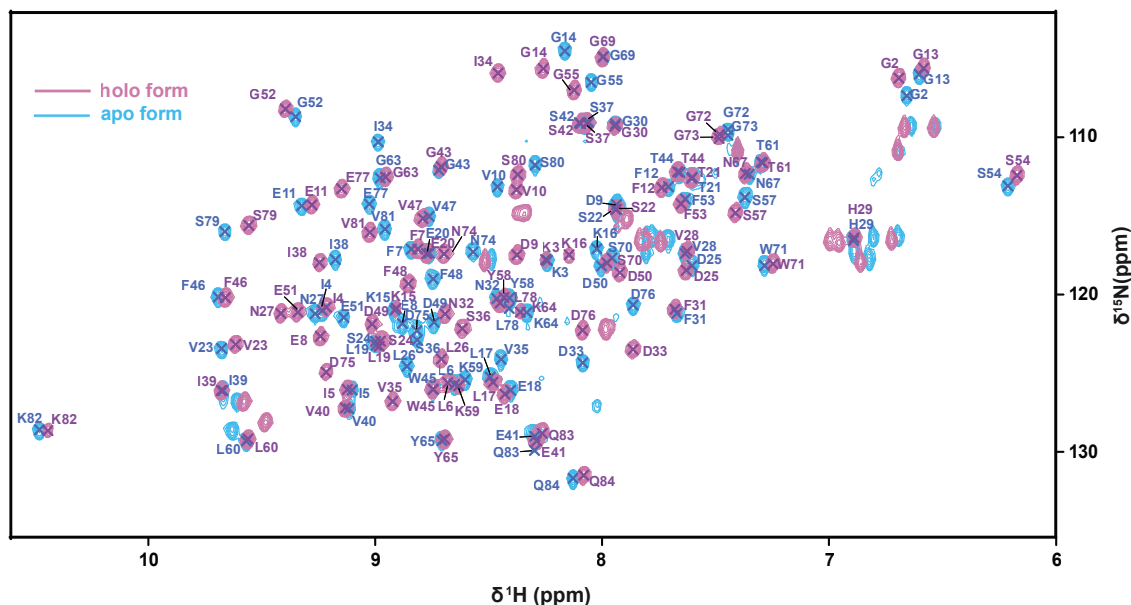


Figure 2.2: ^1H - ^{15}N HSQC of apo and holo form of *Ci*- $\beta\gamma$ at 298 K The cross peaks assignments are labeled with their respective one letter residue codes and sequence numbers.

2.4 Extent of assignment and data deposition

The overall backbone assignment completeness was 94.4% for the apo form and 95.2% for the holo form of *Ci*- $\beta\gamma$. The chemical shifts for HN, N, C α , C β were assigned for 80 out of 84 residues in both forms. The backbone assignments for both the apo and holo forms are labeled on overlaid ^1H - ^{15}N HSQC spectra (Figure 2.1). The four unassigned residues correspond to prolines 56, 62, 66, and 68, which lack backbone amide protons and therefore cannot be observed in standard amide-detected experiments. H α and H β resonances were assigned for 78 of 84 in the apo form and 79 of 84 in the holo form. In the apo form, the missing H α /H β assignments corresponded to the four residues preceding prolines, the C-terminal residue, and Glu 77. In the holo form, only the four residues preceding prolines and the one at the C-terminus were unassigned. Backbone chemical shift assignments were deposited in the BMRB under accession numbers 26860 (apo) and 26861 (holo).

Side-chain assignments were obtained for 96.5% of the C_γ , C_δ , and C_ϵ resonances for both the apo and holo forms. In the apo form, the unassigned nuclei were Ile 39 $C_\gamma 2$, Thr 61 $C_\gamma 2$,

and Gln 84 C_γ . In the holo form, the unassigned nuclei were Ile 34 C_δ 1, Thr 61 C_γ 2, and Gln 84 C_γ . The missing Ile 34 C_δ 1, Ile 39 C_γ 2, and Thr 61 C_γ 2 resonances can be attributed to spectral overlap or line broadening potentially arising from local dynamics. The Gln 84 C_γ resonance could not be assigned because it is the C-terminal residue and lacks the sequential connectivity required by the side-chain assignment experiments utilized during this study. Side-chain chemical shift assignments were deposited in the BMRB under accession numbers 53880 (apo) and 53882 (holo).

Overall, we report near complete backbone and side-chain resonance assignments for both the apo and holo form of *Ci*- $\beta\gamma$. The comparison of the apo and holo chemical shifts demonstrates that calcium binding does not produce large scale structural rearrangements, but instead results in localized changes in β -strand propensity and backbone rigidity. TALOS-N analysis indicates differences in secondary structure surrounding residues involved in calcium binding which supports a model in which calcium stabilizes specific regions of the protein while preserving the $\beta\gamma$ -crystallin fold. These assignments provide a comprehensive NMR framework for investigating the molecular basis of calcium-dependent stability, conformational dynamics, and metal binding in *Ci*- $\beta\gamma$. Due to *Ci*- $\beta\gamma$ evolutionary significance of being an evolutionary intermediate between ancestral $\beta\gamma$ -crystallin and vertebrate lens crystallins, this data has established an important foundation for future structural, biophysical, and evolutionary studies aimed at understanding how the $\beta\gamma$ -crystallin fold adapted from a calcium ion-binding protein to a specialized structural protein in the vertebrate lens.

Chapter 3

Structural basis of calcium-dependent stabilization in *Ciona intestinalis*

$\beta\gamma$ -crystallin

3.1 Abstract

The *Ciona intestinalis* $\beta\gamma$ -crystallin represents an evolutionary intermediate between ancestral calcium-binding proteins and the $\beta\gamma$ -crystallins found in the vertebrate lens. Previous studies have established that calcium binding substantially increases the thermal and chemical stability of *Ci*- $\beta\gamma$; however, the molecular basis for this stabilization remains unclear. In this study, solution-state NMR spectroscopy was used to characterize the residue-specific effects of calcium binding and to investigate changes in the protein hydrogen-bonding environment upon calcium binding. Calcium titration experiments monitored by ^1H - ^{15}N HSQC spectroscopy identified residues affected by calcium binding and revealed predominantly slow exchange behavior among residues within and surrounding the calcium-binding sites. Chemical shift perturbations were used to quantify the calcium-dependent response of residues

undergoing fast exchange. Variable-temperature ^1H - ^{15}N HSQC experiments were used to compare the hydrogen-bonding environments of the apo and holo forms of *Ci*- $\beta\gamma$. Calcium binding increased the number of residues exhibiting chemical shift behavior consistent with intramolecular hydrogen bonding, particularly among residues located near the calcium-binding sites. Previously determined chemical shift assignments were also used as inputs for CS-Rosetta structure calculations. The predicted structures were highly similar to the previously determined crystal structure, indicating that calcium binding does not require a major rearrangement of the overall $\beta\gamma$ -crystallin fold. Together, these results suggest that calcium-dependent stabilization of *Ci*- $\beta\gamma$ arises primarily from local structural changes that reinforce the protein while preserving its overall architecture.

3.2 Introduction

The $\beta\gamma$ -crystallins are a family of proteins characterized by a conserved double-Greek key fold and are best known for their structural and refractive roles in the vertebrate lens [30, 12, 31, 32]. Members of the $\beta\gamma$ -crystallins family are found across all three domains of life: bacteria, archaea, and eukaryotes. In bacteria and archaea, $\beta\gamma$ -crystallin domains are frequently associated with calcium binding [41, 42, 43]. These observations have led to the proposal that the vertebrate lens crystallins evolved from an ancestral calcium-binding protein and acquired specialized structural and refractive functions [44, 45, 46]. Understanding how these proteins retain ancestral structural features while acquiring new functional roles provides insight into the molecular mechanisms underlying the evolution of the $\beta\gamma$ -crystallins family.

The *Ciona intestinalis* $\beta\gamma$ -crystallin (*Ci*- $\beta\gamma$) is useful for investigating this transition because it retains characteristics from both ancestral and vertebrate $\beta\gamma$ -crystallins. The gene structure of *Ci*- $\beta\gamma$ contains separate exons for each Greek-key motif, which is also observed in vertebrate lens crystallins [35, 34]. In contrast, the related $\beta\gamma$ -crystallin from the sponge *Geodia cydonium* is encoded by a single exon as in the microbial crystallins [36]. This

difference in gene organization and the structural and functional characteristics of *Ci*- $\beta\gamma$ suggests that the protein occupies an intermediate position in the evolutionary transition from ancestral calcium-binding $\beta\gamma$ -crystallins to vertebrate lens crystallins.

In addition to sharing characteristics associated with vertebrate crystallins, *Ci*- $\beta\gamma$ retains the calcium-binding properties characteristic of ancestral $\beta\gamma$ -crystallins. Previous biochemical and biophysical studies demonstrated that *Ci*- $\beta\gamma$ contains two calcium-binding sites that bind with high affinity and undergoes stabilization upon calcium binding [38]. The calcium binding sites are located within the Greek key domains and are coordinated by conserved residues that contribute to the binding. Calcium binding produces a difference in the stability of the apo and holo forms of *Ci*- $\beta\gamma$. The apo form is stable to approximately 45°C, whereas the calcium-bound form remains folded to about 94°C [38]. Calcium-binding also increases resistance to chemical denaturation with the holo protein remaining folded at high concentrations of urea [38, 47]. The differences in both thermal and chemical stability between the apo and holo forms demonstrate the stabilizing effect that calcium binding has on *Ci*- $\beta\gamma$. The molecular mechanism underlying this stabilization remains unclear. More specifically, the mechanisms by which calcium binding at the individual sites influences the surrounding structure and whether these local effects are propagated throughout the protein to produce the observed increase in global stability remain unclear.

The previously determined backbone and side-chain chemical shift assignments described in Chapter 2 provide a residue-specific framework for investigating these calcium-dependent structural changes. In this study, ^1H - ^{15}N HSQC spectroscopy was used to monitor changes in individual residues during calcium titration and variable-temperature experiments. Calcium titration experiments were used to identify residues affected by calcium binding and characterize their exchange behavior. Chemical shift perturbations were used to quantify calcium-dependent changes for residues undergoing fast exchange, while residues exhibiting slow exchange were identified from the appearance of distinct apo and holo resonances.

Variable temperature NMR experiments were used to compare the hydrogen-bonding environments of residues in the apo and holo forms. The previously established chemical shift assignments were used as inputs for CS-Rosetta structure calculations to determine whether the local changes associated with calcium binding are accompanied by major changes in the overall protein fold. Together, these experiments provide a residue-specific view of how calcium binding alters the local structural network of *Ci*- $\beta\gamma$ and whether these changes account for its enhanced stability.

3.3 Materials and Methods

3.3.1 Expression and purification of *Ci*- $\beta\gamma$ -crystallin

An *Escherichia coli* codon-optimized plasmid containing the *Ci*- $\beta\gamma$ -crystallin gene was purchased from Blue Heron Biotechnology (Bothell, WA). The protein sequence is MGKIILFEDVEFGGKKLELETSVSDLNVHGFNDIVSSIIVESGTWVFVDD EGFSGPSYKLTPGKYP-NPGSWG GNDDELSSVKQQ.

Expression of *Ci*- $\beta\gamma$ for the calcium titration and variable-temperature experiments was performed using a pET-28 plasmid containing the *Ci*- $\beta\gamma$ sequence and a kanamycin resistance gene. Bacterial cells were plated on Luria-Bertani (LB) agar containing kanamycin (IBI Scientific, Dubuque, IA) and incubated at 37°C for 16 h. A single colony was used to inoculate an overnight culture, which was incubated at 37°C for 16 hours. A 500-mL culture of LB containing kanamycin was inoculated with 5 mL of the overnight culture and grown at 37°C until an optical density of 1.1 was reached. Protein expression was induced by addition of 500 μ L of 1 mM IPTG (GoldBio, St. Louis, MO), followed by incubation at 20°C for 16 hours. Cells were harvested by centrifugation and flash frozen until purification.

For purification, cell pellets were resuspended in 10 mM Tris, 0.05% NaN₃, and 10 mM CaCl₂ at pH 7 and lysed by sonication. The lysate was purified using two anion exchange

chromatography steps followed by size-exclusion chromatography (SEC). The low salt anion exchange buffer contained 10 mM Tris and 0.05% NaN₃ at pH 7, while the high-salt buffer contained 10 mM Tris, 1 M NaCl, and 0.05% NaN₃ at pH 7. The SEC buffer contained 10 mM HEPES, 150 mM NaCl, and 0.05% NaN₃ at pH 7. Protein purity was assessed using mass spectrometry and gel electrophoresis.

3.3.2 Calcium titration experiments

Calcium titration experiments were performed on a Bruker Neo NMR spectrometer operating at a ¹H Larmor frequency of 800 MHz. Samples were prepared in Shigemi NMR tubes. Calcium was added to *Ci-βγ* at protein to calcium molar ratios of 1:0, 1:0.75, 1:1, 1:2, 1:3, 1:4, and 1:6. The initial NMR sample contained 0.1 mM *Ci-βγ* in 10 mM HEPES, 0.05% NaN₃, and 10% D₂O at pH 7. The calcium titrant was prepared using the same buffer as the protein sample to minimize changes in sample composition during the titration.

¹H-¹⁵N HSQC spectra were collected at each calcium concentration. Chemical shift changes were monitored for individual assigned residues as calcium was added. Residues were classified according to their exchange behavior based on the appearance and movement of HSQC resonances during the titration.

3.3.3 Variable-temperature HSQC experiments

Variable-temperature ¹H-¹⁵N HSQC experiments were performed on the same Bruker Neo 800 MHz spectrometer. Separate samples were prepared for the apo and holo forms of *Ci-βγ*.

The apo sample contained 0.5 mM *Ci-βγ*, 3 mM EDTA, 150 mM NaCl, 0.05% NaN₃, and 10% D₂O at pH 7. HSQC spectra were collected in 5°C increments from 20 to 40°C. The holo sample contained 0.5 mM *Ci-βγ*, 3 mM Ca²⁺, 150 mM NaCl, 0.05% NaN₃, and 10% D₂O at pH 7. HSQC spectra were collected in 5°C increments from 25 to 70°C. Samples were allowed to equilibrate for several minutes at each temperature before data acquisition.

3.3.4 Chemical shift perturbation and exchange analysis

Chemical shift perturbations during the calcium titration were calculated from changes in the ^1H and ^{15}N dimensions according to

$$\Delta\delta_{\text{obs}} = \sqrt{\Delta\delta_H^2 + \frac{(\alpha\Delta\delta_N)^2}{2}}, \quad (3.1)$$

where $\alpha = 0.2$ was used to scale the nitrogen chemical shift dimension.

For residues exhibiting fast exchange, dissociation constants (K_d) were estimated by fitting the observed chemical shift perturbations to a two-site binding model:

$$\Delta\delta_{\text{obs}} = \Delta\delta_{\text{max}} \left\{ \frac{n[P]_t + [L]_t + K_d - \sqrt{(n[P]_t + [L]_t + K_d)^2 - 4n[P]_t[L]_t}}{2n[P]_t} \right\}, \quad (3.2)$$

where $[P]_t$ and $[L]_t$ represent the total protein and calcium concentrations, respectively, n is the number of calcium-binding sites, and $\Delta\delta_{\text{max}}$ is the maximum chemical shift perturbation. A value of $n = 2$ was used to account for the two calcium-binding sites in *Ci*- $\beta\gamma$. The parameters K_d and $\Delta\delta_{\text{max}}$ were optimized by minimizing the sum of squared differences between the experimental and calculated chemical shift perturbations [48].

3.3.5 CS-Rosetta structure calculations

Previously determined chemical shift assignments for the apo and holo forms of *Ci*- $\beta\gamma$ were used as inputs for structure prediction using CS-Rosetta [49]. TALOS+ [50] was used to evaluate the chemical shifts and identify potential referencing outliers before structure calculations. The previously determined crystal structure of *Ci*- $\beta\gamma$ (PDB: 2BV2) was excluded

from the CS-Rosetta calculations to minimize structural bias [51].

CS-Rosetta calculations were performed using a NMRbox virtual machine [52]. One thousand structures were generated for each form, and convergence was assessed by comparing the C α RMSD and all-atom energy of the generated structures. The ten lowest-energy structures were selected for structural comparison. RMSD values between the CS-Rosetta structures and the previously determined crystal structure were calculated using the MatchMaker tool in UCSF Chimera version 1.14 [53].

3.4 Results

3.4.1 Calcium binding alters the hydrogen-bonding environment of *Ci*- $\beta\gamma$

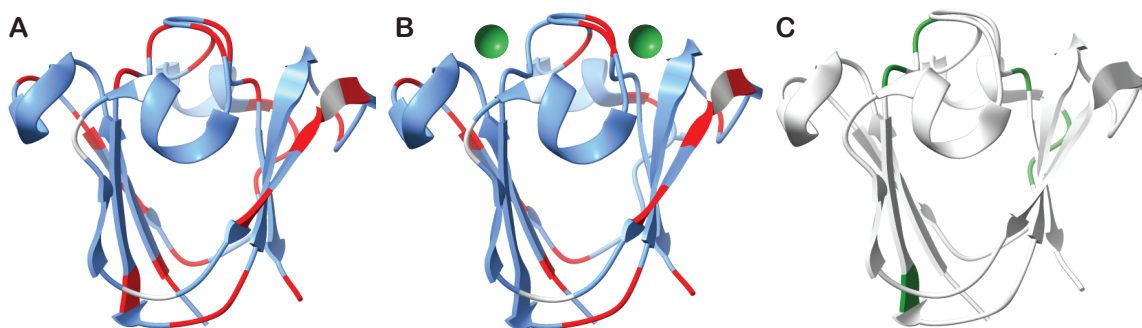


Figure 3.1: Part A is portraying which residues are interacting intramolecularly (blue) and interacting with its solvent (red) in the apo form of *Ci*- $\beta\gamma$. Part B is portraying which residues are interacting intramolecularly (blue) and interacting with its solvent (red) in the holo form of *Ci*- $\beta\gamma$. Part C are showing the residues that change from interacting with solvent to participating in intramolecular interactions upon addition of calcium are labeled in green. The -4.6 pbb threshold corresponds to -0.0046 ppm on the plotted scale.

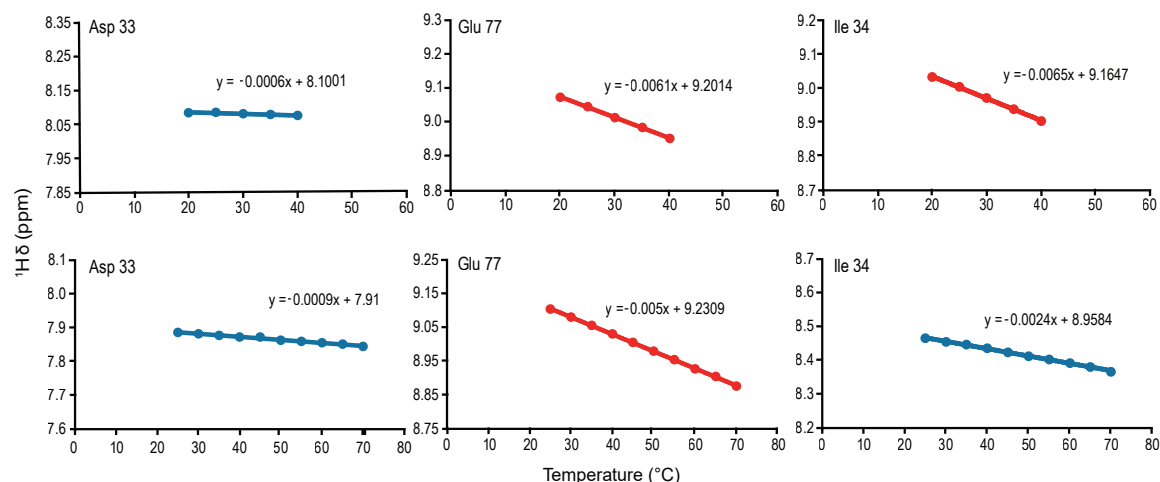


Figure 3.2: Representative chemical shift perturbations based on ^1H - ^{15}N HSQCs for selected residues of the apo (top) and holo (bottom) forms of *Ci*- $\beta\gamma$ crystallin. Fewer points are measured for the apo form because the protein is only stable up to 40 $^{\circ}\text{C}$ in the absence of calcium. Blue line represents intramolecular interactions and red line represents intermolecular interactions.

Variable-temperature ^1H - ^{15}N HSQC experiments were used to investigate changes in the local hydrogen-bonding environment of *Ci*- $\beta\gamma$ upon calcium binding. Spectra were collected from 20-40 $^{\circ}\text{C}$ for the apo form and from 25-70 $^{\circ}\text{C}$ for the holo form. The temperature dependence of the amide proton chemical shifts was evaluated for each residue. The temperature dependence of amide proton chemical shifts provides information about the local hydrogen bonding environment of each residue. Amide protons involved in hydrogen bonding to solvent exhibit larger temperature-dependent chemical shift changes, whereas residues involved in more stable intramolecular hydrogen bonds exhibit smaller changes. A threshold of -4.6 ppb/K was used to distinguish these two environments, following previously established criteria (Figure 3.2) [54].

In the apo form, 56 of the 84 residues exhibited temperature-dependent chemical shift behavior consistent with intramolecular hydrogen bonding. In the holo form, this number increased to 61 residues. Thus, calcium binding increased the number of residues exhibiting

chemical shift behavior consistent with intramolecular hydrogen bonding. Several residues near the calcium-binding sites exhibited changes in their temperature-dependent chemical shift behavior upon calcium binding. The two calcium-binding sites contain Glu 8, Ile 34, Ser 36, and Asp 76 at one site and Asp 33, Asp 49, Glu 77, and Ser 79 at the second site. In the apo form, Glu 8, Asp 33, Asp 49, and Asp 76 exhibited behavior consistent with intramolecular hydrogen bonding, whereas Ile 34, Ser 36, Glu 77, and Ser 79 exhibited behavior consistent with stronger solvent interactions. In the holo form, Glu 8, Asp 33, Ile 34, Ser 36, Asp 49, Asp 76, and Ser 79 exhibited behavior consistent with intramolecular hydrogen bonding, while Glu 77 remained in interacting intermolecularly. Although Glu 77 preferred to interact with the solvent in both states, its temperature dependence chemical shift perturbation (CSP) changed from -6.1 ppb/K in the apo form to -5.0 ppb/K in the holo form. This change indicates that calcium binding is still altering the local environment of Glu 77. Additional changes were observed for Val 23, Ser 24, and Glu 41, which changed from interacting with its solvent in the apo form to behavior consistent with intramolecular hydrogen bonding in the holo form. These residues are located outside the direct calcium-binding sites, indicating that the effects of calcium binding extend beyond the residues that directly coordinate the ion (Figure 3.1).

3.4.2 Calcium binding produces residue specific chemical shift changes

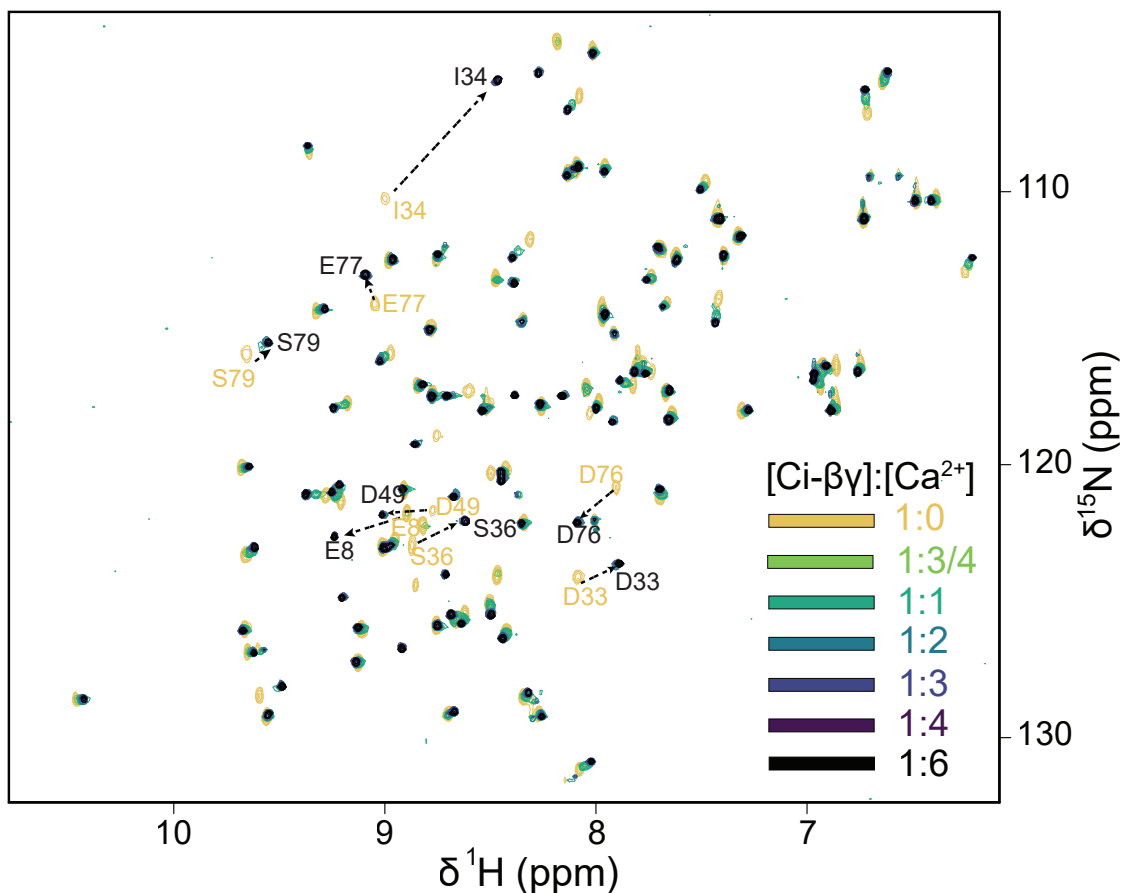


Figure 3.3: ^{15}N - ^1H HSQC of $Ci\text{-}\beta\gamma$ that portrays the chemical shift perturbations as Ca^{2+} was added in different ratios. The more shift occurs as more Ca^{2+} is binding to $Ci\text{-}\beta\gamma$.

^1H - ^{15}N HSQC experiments at various calcium titrations were collected to map the residue specific changes in $Ci\text{-}\beta\gamma$. Increasing concentrations of calcium produced chemical shift changes throughout the protein revealing that calcium binding alters the local environment of multiple residues (Figure 3.3). The largest chemical shift changes were concentrated around the two previously identified calcium-binding sites. Several residues surrounding these sites exhibited substantial chemical shift changes, such as, Asp 9, Val 10, Asn 32, Val 35, Ile 38, Phe 48, Asp 50, and Asp 75. Several residues outside the immediate vicinity of

the binding sites also exhibited changes during the titration, including Lys 3, Glu 41, and Gln 83. These observations indicate that the structural response to calcium binding extends beyond the residues directly involved in calcium binding.

3.4.3 Calcium-binding residues exhibit predominantly slow exchange

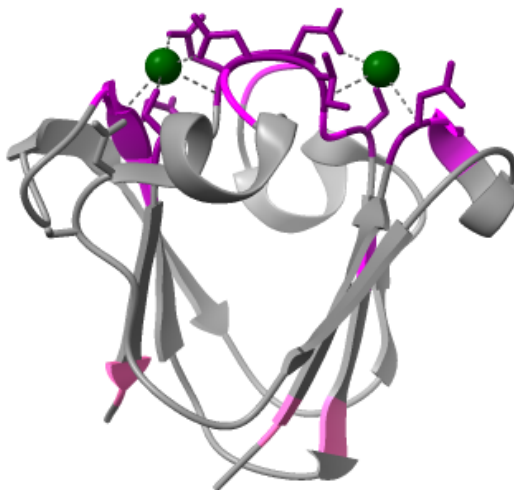


Figure 3.4: The slow exchange residues marked on the X-ray crystal structure.

The chemical shift changes observed during the calcium titration showed different exchange behaviors. In fast exchange, the apo and holo states interconvert rapidly relative to the NMR timescale which resulted in a single resonance that moves continuously as calcium is added. In slow exchange, the two states interconvert more slowly which resulted in separate resonances for the apo and holo forms. Seven of the eight residues that comprise the two calcium-binding sites exhibited slow exchange [48]. Ser 79 was the only calcium-binding residue that exhibited fast exchange. The predominance of slow exchange among the calcium-

binding residues is consistent with the high calcium affinity previously reported for *Ci- $\beta\gamma$* [38]. The slow-exchange residues identified throughout the titration are shown on the crystal structure in Figure 3.4. Chemical shift perturbations from residues exhibiting fast exchange were used to estimate dissociation constants. The resulting K_d values are provided in the Supporting Information. Since most of the residues directly involved in calcium binding exhibited slow exchange, quantitative affinity measurements were limited to the residues that could be analyzed within the fast-exchange regime. The predominance of slow exchange at the calcium-binding sites indicates that calcium binding produces distinct apo and holo chemical environments for most of the residues involved in calcium binding. Together with the chemical shift changes observed at surrounding residues, these results suggest that calcium binding also produces changes in the local structure of *Ci- $\beta\gamma$* .

3.4.4 CS-Rosetta structures show conservation of the overall fold

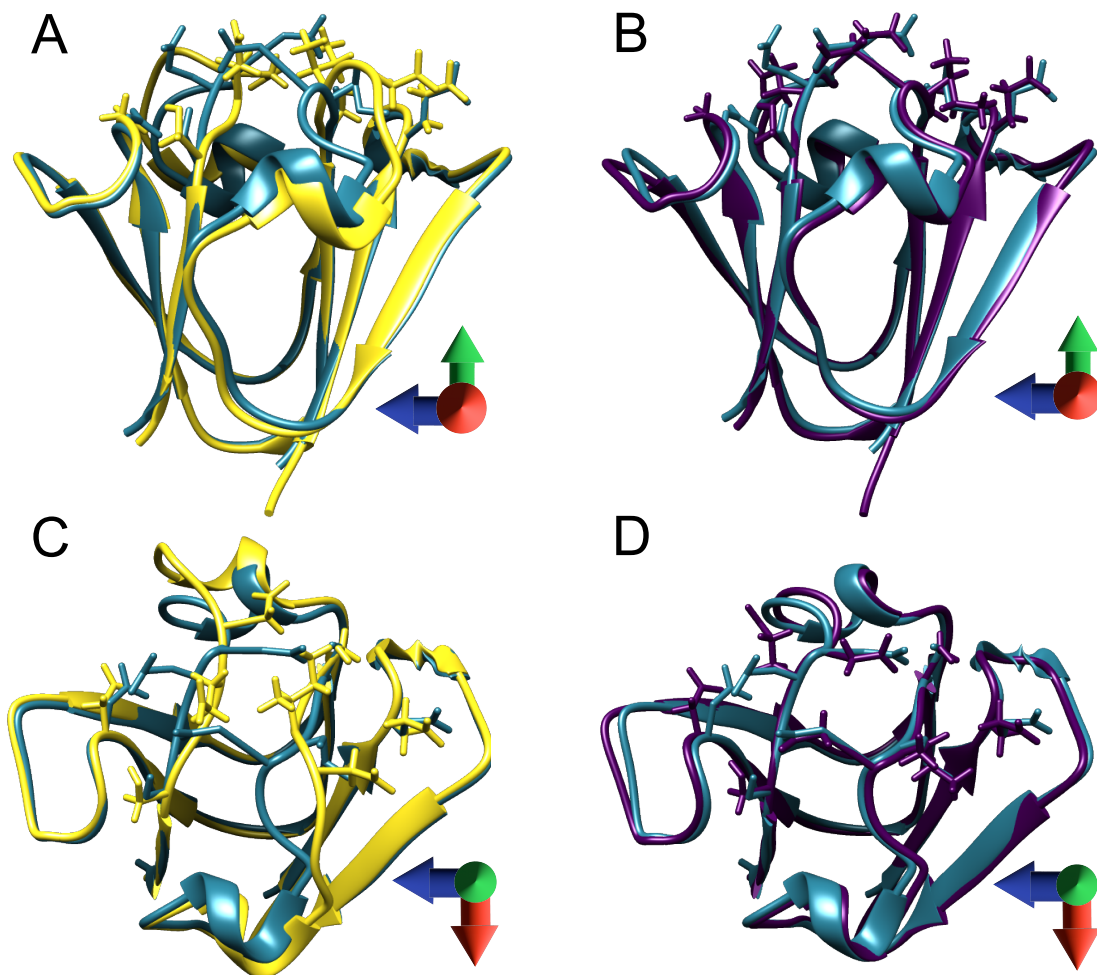


Figure 3.5: Comparison of the previously determined crystal structure of holo *Ci*- $\beta\gamma$ (blue) with the CS-Rosetta structures predicted for the apo (yellow) and holo (purple) forms. The overall $\beta\gamma$ -crystallin fold is conserved between the structures, while the largest structural differences are observed near the calcium-binding sites.

Previously determined chemical shift assignments for the apo and holo forms were used as inputs for CS-Rosetta to generate solution structures. The predicted structures converged after 1,000 models, providing a consistent structural model for both forms of *Ci*- $\beta\gamma$. The predicted holo structure showed close agreement with the previously determined crystal structure of the calcium-bound protein (PDB: 2BV2), with an RMSD of 0.539 Å (Table B.1). The predicted apo structure showed a larger RMSD of 1.439 Å relative to the crystal structure.

Despite this difference, the overall $\beta\gamma$ -crystallin fold was retained in both predicted structures. The positions of the eight calcium-binding residues were also compared between the predicted structures and the crystal structure (Table B.2). Most residues showed relatively small positional differences, particularly in the holo structure. The largest difference was observed for Asp 33 in the predicted apo structure, which differed by approximately 2.85 Å from its position in the crystal structure. The preservation of the overall fold despite the residue-specific changes observed during calcium titration and variable-temperature experiments suggests that calcium binding does not produce a major rearrangement of the *Ci*- $\beta\gamma$ structure. Instead, the structural differences between the apo and holo forms appear to be localized primarily around the calcium-binding sites and their surrounding residues.

Table 3.1: Full structure RMSD

Structure Comparison	RMSD (all 83 pairs)
2BV2 to Predicted EDTA	1.439 Å
2BV2 to Predicted Ca ²⁺	0.539 Å

Table 3.2: Distances between binding site residues

Binding Site Residue	Distance 2BV2 to Predicted EDTA (Å)	Distance 2BV2 to Predicted Ca ²⁺ (Å)
Glu 8	0.01911	0.096149
Ile 34	0.51782	0.3688
Ser 36	0.10621	0.26508
Asp 76	0.58027	0.23153
Asp 33	2.847	0.26496
Asp 49	0.10981	0.44077
Glu 77	0.70371	0.60459
Ser 79	0.32415	0.25065

3.5 Discussion

Previous biochemical studies established that calcium binding dramatically increases the thermal and chemical stability of *Ci-βγ* [38, 47]. The NMR experiments presented here provide a residue-specific view of the molecular changes associated with this stabilization. The calcium titration and variable-temperature experiments reveal that calcium binding produces changes in the local environments of residues within and surrounding the calcium-binding sites, while CS-Rosetta structure calculations indicate that these changes occur without major disruption of the overall protein fold.

3.5.1 Calcium binding reinforces the local structural environment

The variable-temperature experiments provide evidence that calcium binding alters the hydrogen-bonding environment of *Ci-βγ*. The number of residues exhibiting behavior consistent with intramolecular hydrogen bonding increased from 56 in the apo form to 61 in the holo form. Several residues that underwent this transition are located near the calcium-binding sites, including Ser 36, Asp 49, Asp 76, and Ser 79. These changes suggest that calcium binding does not simply stabilize the four residues that directly coordinate the ion. Instead, calcium binding alters the local environment of a broader network of residues. Val 23, Ser 24, and Glu 41 also transitioned toward behavior consistent with intramolecular hydrogen bonding despite not being direct calcium-binding residues. These observations suggest that calcium binding promotes local structural organization extending beyond the binding sites. The enhanced intramolecular hydrogen-bonding environment provides a possible molecular explanation for the increased thermal stability of the holo protein. By reducing the number of residues whose amide groups interact strongly with solvent and increasing the number exhibiting behavior consistent with intramolecular interactions, calcium binding may reduce local structural flexibility and stabilize the folded state.

3.5.2 Calcium-dependent stabilization extends beyond the binding sites

The calcium titration experiments provide additional evidence that the effects of calcium binding extend beyond the direct binding residues. The largest chemical shift changes occurred at the two known calcium-binding sites; however, residues surrounding these sites also exhibited substantial perturbations. The presence of slow exchange among residues such as Asp 9, Val 10, Asn 32, Val 35, Ile 38, Phe 48, Asp 50, and Asp 75 indicates that the structural response to calcium binding extends into the surrounding protein environment. The predominance of slow exchange among the calcium-binding residues is consistent with a stable calcium-bound state. Rather than rapidly averaging between apo and holo conformations, many residues populate chemically distinct states on the NMR timescale. This behavior is prominent near the calcium-binding sites and supports the presence of persistent calcium-dependent structural changes. The calcium titration therefore provides a residue-specific complement to the previously observed macroscopic stabilization of $Ci\text{-}\beta\gamma$. The biochemical measurements established that calcium binding increases thermal and chemical stability, while the NMR measurements demonstrate that this stabilization is accompanied by widespread changes in the local environments of residues surrounding the binding sites.

3.5.3 Calcium binding stabilizes $Ci\text{-}\beta\gamma$ without major rearrangement of the global fold

The CS-Rosetta structures provide an important structural context for interpreting the NMR data. The predicted holo structure agrees closely with the previously determined crystal structure, with an RMSD of 0.539 Å. The apo structure also retains the overall $\beta\gamma$ -crystallin architecture, although its RMSD relative to the holo crystal structure is larger at 1.439 Å. These results indicate that the substantial residue-specific chemical shift changes observed during calcium titration do not correspond to a major global rearrangement of the protein.

Instead, calcium binding appears to produce localized structural changes within a conserved global fold. The larger differences observed for selected calcium-binding residues in the apo structure are consistent with increased conformational freedom in the absence of the stabilizing calcium ion. Overall, the NMR and structural data support a model in which calcium binding stabilizes *Ci*- $\beta\gamma$ primarily through local structural reinforcement. Calcium coordinates residues within the two binding sites, alters the chemical environments of nearby residues and promotes an intramolecular hydrogen-bonding environment that is maintained within the overall $\beta\gamma$ -crystallin fold. This localized stabilization provides a molecular mechanism that is consistent with the dramatic increase in thermal stability observed upon calcium binding.

3.6 Conclusion

The NMR experiments presented in this chapter provide a residue-specific description of the molecular changes that accompany calcium binding to *Ci*- $\beta\gamma$ -crystallin. Calcium titration experiments identified extensive chemical shift perturbations within and surrounding the two calcium-binding sites, with the majority of calcium-binding residues exhibiting slow exchange. Variable-temperature HSQC experiments further demonstrated that calcium binding increases the number of residues exhibiting chemical shift behavior consistent with intramolecular hydrogen bonding. These changes extend beyond the direct calcium-binding residues, indicating that calcium binding influences a broader local structural network. CS-Rosetta structure calculations showed that these local changes occur without major disruption of the overall $\beta\gamma$ -crystallin fold. Together, the results support a model in which calcium binding stabilizes *Ci*- $\beta\gamma$ through localized structural reinforcement rather than a large-scale conformational rearrangement. The ability of calcium to strengthen local interactions while preserving the ancestral $\beta\gamma$ -crystallin architecture provides insight into how calcium-binding proteins can maintain both metal-dependent stability and a conserved structural framework.

Chapter 4

Calcium-dependent dynamics of *Ciona intestinalis*- $\beta\gamma$ crystallin

4.1 Abstract

The calcium-binding sites of *Ciona intestinalis*- $\beta\gamma$ (*Ci*- $\beta\gamma$) are important for understanding the connection between ancestral calcium-binding proteins and the $\beta\gamma$ -crystallin proteins found in vertebrate lenses. The influence of calcium binding on the backbone dynamics of *Ci*- $\beta\gamma$ was investigated using ^1H - ^{15}N relaxation measurements. Longitudinal relaxation rates (R_1), transverse relaxation rates (R_2), and ^1H - ^{15}N heteronuclear NOE measurements were acquired at 500 and 800 MHz for the calcium-bound (holo) and EDTA-treated (apo) states. These measurements were used to characterize backbone dynamics and identify regions exhibiting differences between the two states. Generalized order parameters (S^2) were obtained to further characterize the amplitude of fast backbone motions on the picosecond-to-nanosecond timescale. The relaxation measurements indicate that *Ci*- $\beta\gamma$ remains predominantly ordered in both apo and holo states. Calcium binding does not produce a uniform change in backbone dynamics throughout the protein. Instead, differences between the two

states are localized to specific regions, with the magnitude and direction of the changes depending on the residue and relaxation parameter examined. The relaxation measurements revealed more pronounced apo-to-holo differences at 800 MHz, particularly in R_2/R_1 at several residues. S^2 analysis showed that most residues exhibited high S^2 values in both states, consistent with a predominantly restricted backbone, while selected regions displayed lower order parameters. These results suggest that calcium binding is associated with localized changes in the dynamic behavior of Ci - $\beta\gamma$ rather than global rigidification of the protein. These findings provide a dynamic perspective on how calcium coordination may influence the molecular behavior of an ancestral $\beta\gamma$ -crystallin fold and provide insight into the evolutionary significance of calcium-binding sites that were subsequently lost in vertebrate lens crystallins.

4.2 Introduction

$\beta\gamma$ -crystallins comprise a diverse family of proteins characterized by a conserved Greek-key motif and are found across a wide range of organisms, including bacteria, invertebrates, and vertebrates [30, 12, 31, 32]. *Ciona intestinalis*- $\beta\gamma$ (Ci - $\beta\gamma$) represents an evolutionarily informative member of this protein family because it retains calcium-binding sites that were present in ancestral $\beta\gamma$ -crystallins. Over the course of evolution, the calcium-binding sites have been lost from vertebrate lens $\beta\gamma$ -crystallins while the overall $\beta\gamma$ -crystallin fold has been maintained [44, 45, 46]. Ci - $\beta\gamma$ therefore provides an opportunity to examine how calcium binding influences the molecular behavior of a $\beta\gamma$ -crystallin fold that retains these ancestral metal-binding features.

Ci - $\beta\gamma$ contains two calcium-binding sites. The first site comprises Glu 8, Ile 34, Ser 36, and Asp 76, while the second site comprises Asp 33, Asp 49, Glu 77, and Ser 79 [38, 47]. Calcium binding can influence protein behavior by altering local interactions and conformational sampling within and surrounding a binding site. Such effects may extend beyond residues

that directly coordinate the metal ion and may produce changes in protein dynamics without requiring a large-scale structural rearrangement [55, 56, 57]. Characterizing these changes can therefore provide insight into how calcium coordination influences the molecular behavior of Ci - $\beta\gamma$ and may help clarify the potential significance of calcium-binding sites in the evolutionary history of $\beta\gamma$ -crystallins.

NMR relaxation measurements provide residue-specific information about protein backbone dynamics and can be used to examine changes in molecular motion associated with ligand binding. Measurements of longitudinal relaxation (R_1), transverse relaxation (R_2), and ^1H - ^{15}N heteronuclear NOE (hetNOE) provide complementary information about the local environment and mobility of individual backbone amides. These measurements can be used to identify regions that exhibit differences in dynamic behavior between protein states. R_2/R_1 analysis characterizes molecular tumbling, rotational diffusion, and internal flexibility [27, 28, 29]. FastModelFree is a software that is used to automate the analysis of spin-relaxation data. FastModelFree gathers the R_1 , R_2 , and hetNOE to calculate the generalized order parameter (S^2) of each residue in the protein [58]. S^2 analysis describes the amplitude of fast internal motion of individual N-H bond vectors [59, 60]. Comparing these parameters between the calcium-bound and calcium-free states therefore provides a residue-specific approach for determining whether calcium binding alters backbone dynamics and whether such changes are localized to particular regions of the protein.

The effects of calcium binding on protein dynamics may not be uniform throughout a binding site or across the entire protein. Individual residues may respond differently depending on their local environment, interactions, and position within the $\beta\gamma$ -crystallin fold. It is therefore important to determine whether calcium coordination produces a coordinated change in the dynamics of the binding sites or instead produces heterogeneous, residue-specific changes. Such differences may distinguish a global change in protein rigidity from localized changes in conformational sampling.

In this study, backbone dynamics of $Ci\text{-}\beta\gamma$ were characterized in the calcium-bound (holo) and EDTA-treated (apo) states using $^1\text{H}\text{-}^{15}\text{N}$ relaxation measurements acquired at 500 and 800 MHz. R_1 , R_2 , and heteronuclear NOE measurements were used to compare residue-specific relaxation behavior between the two states, and FastModelFree analysis was used to evaluate the amplitude of fast-timescale backbone motion through generalized order parameters. This study therefore sought to determine whether calcium binding produces localized changes in backbone dynamics at the two calcium-binding sites or broader changes in the dynamic behavior of $Ci\text{-}\beta\gamma$. Understanding this dynamic response provides insight into the molecular behavior of an ancestral calcium-binding $\beta\gamma$ -crystallin fold and the potential significance of calcium-binding sites that were subsequently lost during the evolution of vertebrate lens crystallins.

4.3 Materials and Methods

4.3.1 Expression and purification of $Ci\text{-}\beta\gamma$ -crystallin

Expression and purification of $Ci\text{-}\beta\gamma$ is the same as Chapter 3.

4.3.2 NMR Experiments

T1, T2, and HetNOE experiments were ran to obtain R_1 , R_2 , and S^2 parameters. T1 and T2 correlate to $1/R_1$ and $1/R_2$. The T1, T2, HetNOE experiments follow the same parameters set from Chapter 1.

4.3.3 FastModelFree analysis

FastModelFree analysis was performed using CCPN [61] and NMRbox [52]. R_1 , R_2 , and $^1\text{H}\text{-}^{15}\text{N}$ heteronuclear NOE measurements were used to calculate generalized order parameters (S^2) for the calcium-bound and EDTA-treated states. The FastModelFree analysis was

performed independently for the 500 and 800 MHz datasets. The setup and input preparation for FastModelFree followed the procedure described by the Nebraska Center for Structural Biology FastModelFree documentation [58].

S^2 values were used to characterize the amplitude of fast internal motion of individual N-H bond vectors. Higher S^2 values indicate more restricted internal motion, whereas lower values indicate greater amplitudes of fast-timescale motion. Residues for which FastModelFree did not produce an S^2 value were not included in comparisons of order parameters.

4.4 Results

4.4.1 ^1H - ^{15}N Relaxation measurements of holo and apo *Ci*- $\beta\gamma$ -crystallin

Backbone dynamics of the protein were characterized in the apo and calcium-bound (holo) states using ^1H - ^{15}N heteronuclear NOE, R_2/R_1 , and S^2 measurements at 500 and 800 MHz. The protein consists of 84 residues and contains two proposed calcium-binding sites comprising Glu 8, Ile 34, Ser 36, and Asp 76 at one site and Asp 33, Asp 49, Glu 77, and Ser 79 at the second site. Changes between the apo and holo states were evaluated independently at 500 and 800 MHz, rather than through direct comparison of absolute relaxation values across field strengths, to account for the field dependence of the relaxation parameters.

4.4.2 Heteronuclear NOE measurements

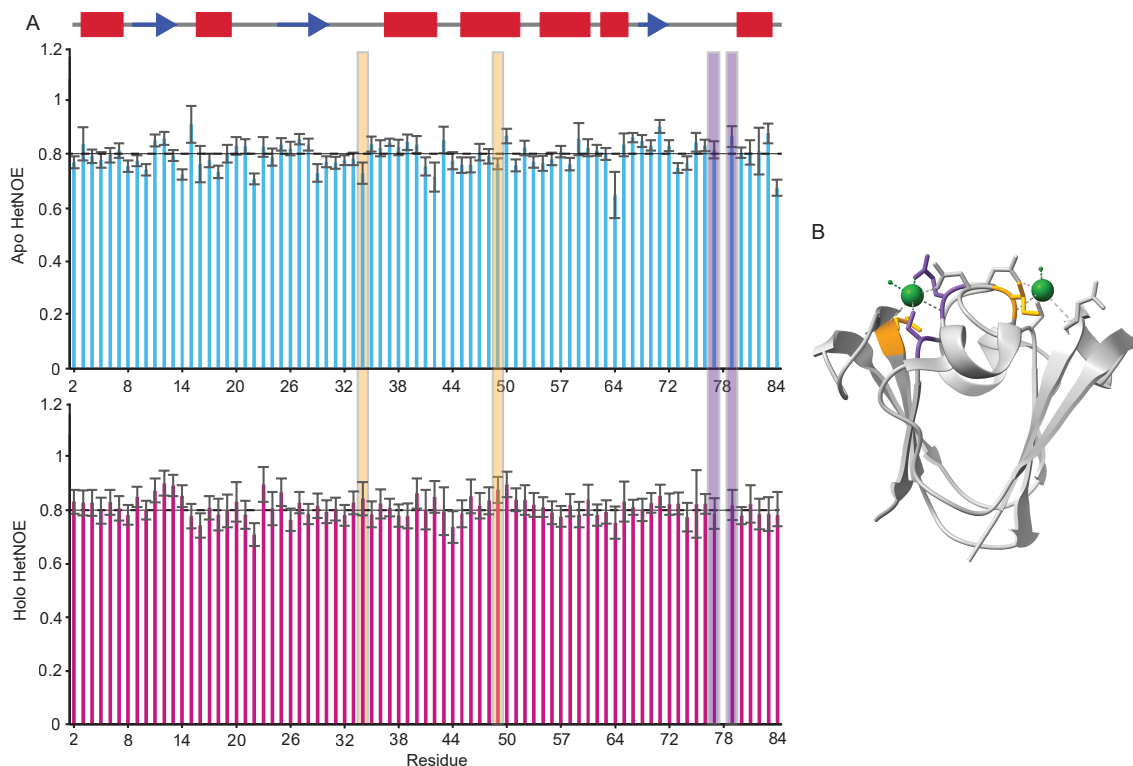


Figure 4.1: (A) HetNOE analysis of apo (top graph) and holo form (bottom graph) of *Ci-βγ*. The black dotted line is at 0.8 to show that the majority backbone is fairly restricted on a fast timescale. (B) A structure of *Ci-βγ* where the same changes in S^2 for both datasets were colored by residue as a increase (yellow) or decrease (purple).

The ^1H - ^{15}N heteronuclear NOE measurements were used to characterize fast-timescale backbone dynamics in the apo and holo states. At 500 MHz, the majority of holo NOE values were between 0.65 and 0.75, with higher values observed at Phe 53 (0.812) and Asp 75 (0.824). Lower values were observed at Lys 59 (0.583), Gln 83 (0.606), and Gln 84 (0.625). The apo state exhibited a broader distribution, with most NOE values between 0.60 and 0.75. Several residues displayed comparatively lower NOE values, including Val 40 (0.598), Ser 42 (0.610), Lys 59 (0.610), Lys 64 (0.465), Asn 74 (0.627), Ser 80 (0.606), Gln 83 (0.631), and Gln 84 (0.561). Thus, the 500 MHz measurements indicated generally positive heteronuclear NOE values in both states, consistent with a predominantly ordered backbone with localized regions of increased fast-timescale mobility.

At 800 MHz, the NOE values were higher than those observed at 500 MHz, with the majority of measurements between 0.75 and 0.90 in both states. In the holo state, higher NOE values were observed at Phe 12 (0.903), Gly 13 (0.895), Asp 49 (0.878), and Asp 50 (0.899), whereas lower values occurred at Ser 22 (0.711), Thr 44 (0.739), Lys 64 (0.755), and Asn 74 (0.775). The apo state exhibited a similar distribution, with higher values observed at Lys 15 (0.915), Trp 71 (0.908), and Gln 83 (0.882), and lower values at Ser 22 (0.711), Ser 42 (0.718), Lys 64 (0.651), and Gln 84 (0.679). Several residues, including Ser 22 and Lys 64, therefore remained among the residues with comparatively lower NOE values in both apo and holo states.

Changes in heteronuclear NOE values at the proposed calcium-binding residues were not uniform between the apo and holo states. At 800 MHz, the largest increases were observed at Ile 34 and Asp 49, where NOE values increased from 0.732 to 0.845 and from 0.768 to 0.878. Glu 77 and Ser 79 exhibited decreases, with NOE values changing from 0.820 to 0.789 and from 0.870 to 0.821. The remaining calcium-binding residues did not exhibit a consistent response upon calcium binding. These residue-specific changes indicate that calcium binding does not produce a uniform alteration in fast-timescale backbone dynamics at the proposed binding sites.

The heteronuclear NOE measurements indicate that the protein maintains predominantly positive NOE values in both the apo and holo states, consistent with a generally ordered backbone on the timescale probed by the experiment. Localized regions of lower NOE values were observed in both states, most notably around Lys 64 and, at 800 MHz, Ser 22. Calcium binding produced residue-specific changes in heteronuclear NOE values rather than a uniform increase or decrease across the sequence or within the calcium-binding sites. The field-dependent differences in the absolute NOE values further emphasize the importance of evaluating the apo-to-holo changes independently at each magnetic field strength.

4.4.3 FastModelFree analysis of backbone dynamics

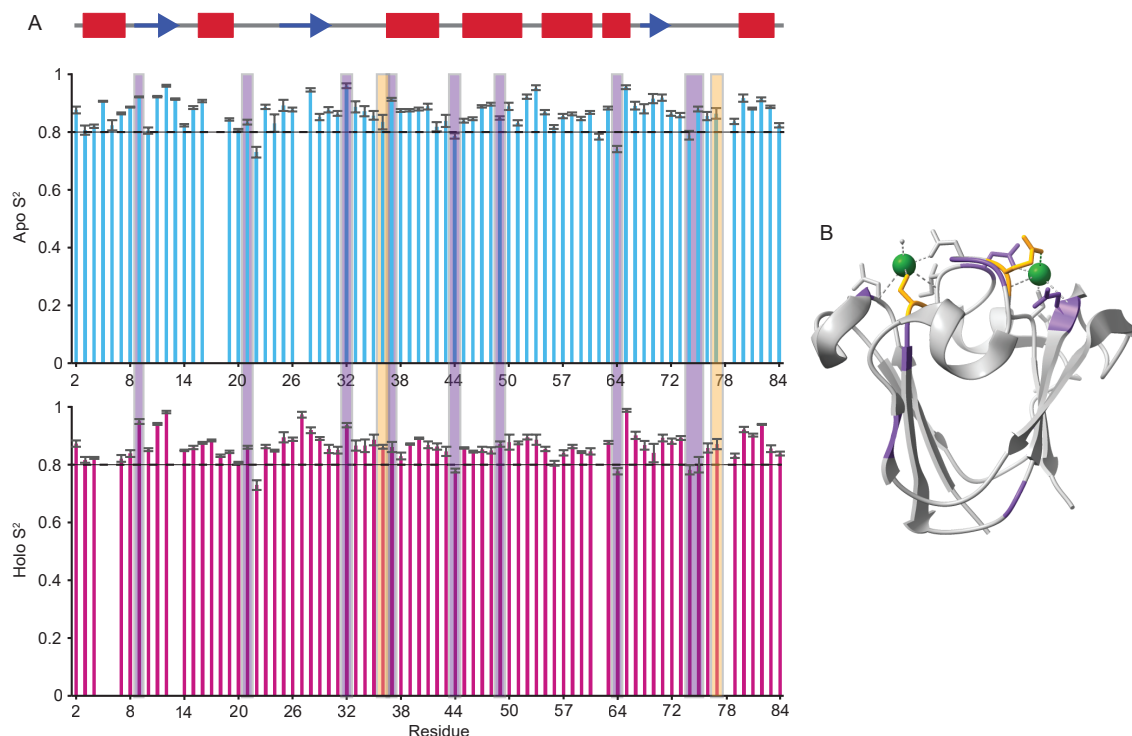


Figure 4.2: (A) FastModelFree analysis of apo (top graph) and holo form (bottom graph) of *Ci*- $\beta\gamma$. The black dotted line is at 0.8 to show that the majority backbone is relatively ordered. (A) A structure of *Ci*- $\beta\gamma$ where the same changes in S^2 for both datasets were colored by residue as a increase (yellow) or decrease (purple).

The S^2 values indicate that the backbone is generally well ordered in both the apo and holo states. At 500 MHz, most measurable S^2 values were approximately 0.8–0.9, with lower values observed at several localized positions, including residues Ser 22, Ser 36, Thr 44, Ser 57, Thr 61, Lys 64, and Asn 74. At 800 MHz, the majority of S^2 values similarly remained near 0.8–0.9. Several residues displayed comparatively lower S^2 values, most notably residues Ser 22, Thr 44, Lys 64, Asn 74, and Asp 75. Thus, neither state exhibited evidence for extensive fast-timescale backbone flexibility throughout the sequence.

The calcium-binding residues did not exhibit a uniform change in S^2 upon calcium binding. At 500 MHz, S^2 increased at Ile 34 and Ser 36 but decreased at Glu 8 and Asp 76, while Asp 33, Asp 49, Glu 77, and Ser 79 exhibited smaller changes. At 800 MHz, S^2 increased

slightly at Ser 36, Asp 49, and Glu 77, decreased at Glu 8 and Asp 33, and remained nearly unchanged at Asp 76. Overall, the changes in S^2 were relatively small and varied in direction between residues.

4.4.4 R_2/R_1 relaxation measurements

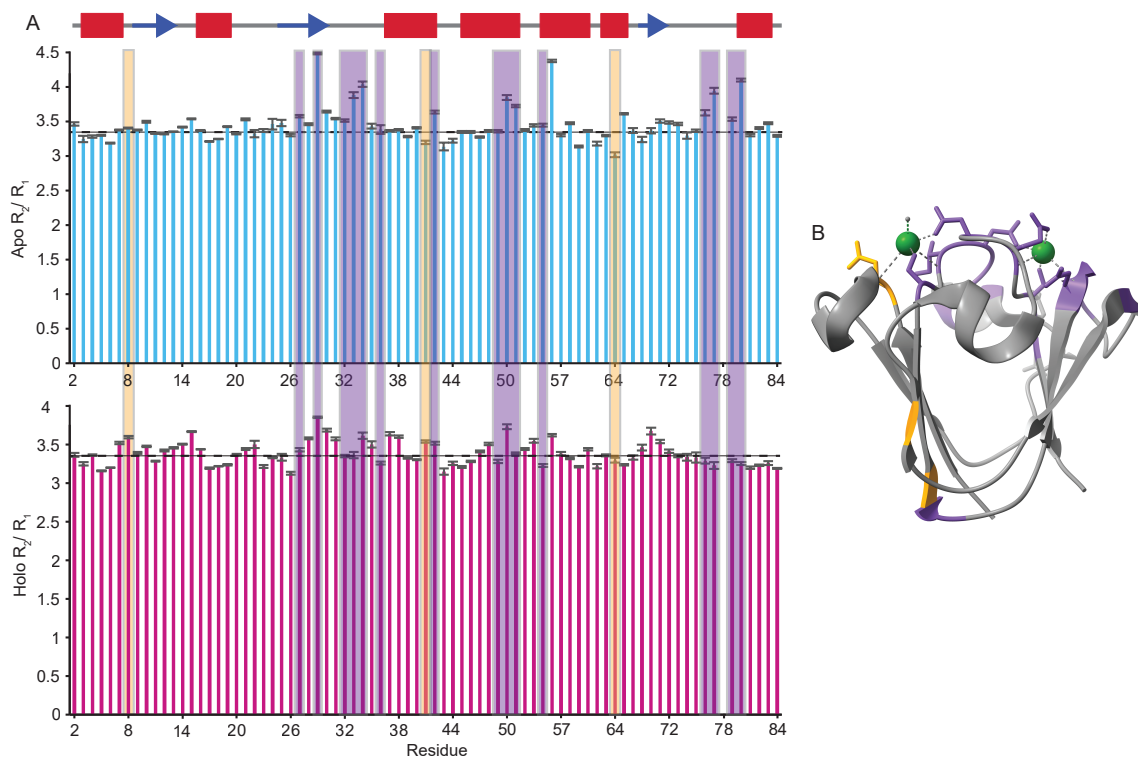


Figure 4.3: (A) R_2/R_1 relaxation measurements of apo (top graph) and holo form (bottom graph) of $Ci-\beta\gamma$. The black dotted line is at 3.44 (apo) and 3.40 (holo) to show that the majority backbone is relatively ordered. (B) A structure of $Ci-\beta\gamma$ where the same changes in S^2 for both datasets were colored by residue as a increase (yellow) or decrease (purple).

R_2/R_1 exhibited a more consistent response to calcium binding. At 500 MHz, all eight proposed calcium-binding residues exhibited decreases in R_2/R_1 upon calcium binding. At the first proposed binding site, R_2/R_1 decreased from 2.125 to 2.083 for Glu 8, from 2.319 to 2.177 for Ile 34, from 2.263 to 2.071 for Ser 36, and from 2.289 to 2.177 for Asp 76. At the second proposed binding site, R_2/R_1 decreased from 2.279 to 2.157 for Asp 33, from 2.121 to 2.111 for Asp 49, from 2.410 to 2.157 for Glu 77, and from 2.301 to 2.137 for Ser 79.

A similar trend was observed at 800 MHz. Seven of the eight calcium-binding residues exhibited decreases in R_2/R_1 upon calcium binding. At the first binding site, R_2/R_1 decreased from 4.031 to 3.628 for Ile 34, from 3.378 to 3.273 for Ser 36, and from 3.616 to 3.303 for Asp 76. At the second binding site, decreases were observed for Asp 33 (3.875 to 3.378), Asp 49 (3.353 to 3.296), Glu 77 (3.935 to 3.243), and Ser 79 (3.527 to 3.305). Glu 8 was the only proposed calcium-binding residue that exhibited an increase in R_2/R_1 at 800 MHz, increasing from 3.397 in the apo state to 3.610 in the holo state. Thus, seven of the eight calcium-binding residues exhibited a decrease in R_2/R_1 at both field strengths.

The decrease in R_2/R_1 was not restricted to the proposed calcium-binding residues. Several additional residues exhibited decreases at both 500 and 800 MHz, including Gly 27, Val 32, Leu 29, Val 42, Ala 50, Leu 51, Val 55, and Leu 80. Among these residues, Val 55 and Leu 80 exhibited large decreases at 800 MHz, with R_2/R_1 changing from 4.369 to 3.634 and from 4.094 to 3.265. Leu 29 also exhibited a decrease at 800 MHz, from 4.476 to 3.869. Fewer residues exhibited increases in R_2/R_1 at both field strengths. Some examples are Lys 41 and Lys 64, which increased from 2.033 to 2.053 and from 2.096 to 2.125 at 500 MHz, and from 3.191 to 3.555 and from 3.013 to 3.310 at 800 MHz. Overall, the relaxation data indicate that calcium binding produces a reproducible change in the relaxation behavior of residues associated with the proposed binding sites, while the high S^2 values and heterogeneous NOE response indicate that the protein remains predominantly well ordered in the holo state.

4.5 Discussion

4.5.1 Calcium binding modulates local dynamics within a predominantly ordered backbone

The combined relaxation data indicate that calcium binding alters the dynamic behavior of $Ci\text{-}\beta\gamma$ without producing a global increase in backbone rigidity. The heteronuclear NOE

and S^2 measurements indicate that the backbone remains well ordered in both the apo and holo states. Most S^2 values remained near 0.8-0.9, while the heteronuclear NOE values were predominantly positive at both magnetic field strengths. Although several localized regions exhibited lower values in both states, these regions were retained upon calcium binding, indicating that calcium binding does not substantially alter the overall fast-timescale flexibility of the backbone.

The high S^2 values throughout the majority of the sequence indicate that the apo protein already exhibits relatively restricted backbone motion. Calcium binding therefore does not appear to be required to establish the overall structural organization of the protein. It is most likely that the apo state contains a pre-existing structural framework capable of accommodating calcium. This is consistent with the absence of a systematic increase in S^2 at the proposed binding residues. Some residues become slightly more restricted following calcium binding, whereas others become slightly more mobile. Consequently, calcium binding appears to alter local motions rather than simply suppressing them.

The heteronuclear NOE measurements provide additional support for this interpretation. Although several binding residues exhibit changes in NOE upon calcium binding, the direction of these changes is not consistent across the two sites. A uniform increase in NOE would be expected if calcium binding produced a generalized reduction in fast-timescale backbone mobility. Instead, the mixed NOE response suggests that calcium binding changes the local dynamic environment of individual residues in different ways. This behavior is consistent with a binding mechanism in which formation of the metal-binding site requires small adjustments in local conformational dynamics rather than a global structural transition.

R_2/R_1 measurements show evidence of a calcium-dependent dynamic response. Seven of the eight proposed calcium-binding residues exhibited decreases in R_2/R_1 at both 500 and 800 MHz, whereas Glu 8 decreased at 500 MHz but increased at 800 MHz. The effect is also distributed across both proposed binding sites, suggesting that calcium binding influences

the broader dynamic environment of the metal-coordination regions rather than affecting only a single residue. Several non-binding residues, including Gly 27, Leu 29, Val 32, Val 42, Ala 50, Leu 51, Val 55, and Leu 80, also exhibited decreases in R_2/R_1 at both magnetic field strengths. The presence of consistent changes at residues outside the proposed calcium-binding sites suggests that the relaxation response to calcium binding extends beyond the directly coordinating residues. In particular, the large decreases observed at Val 55 and Leu 80 at 800 MHz indicate that residues distant in sequence from the proposed binding sites can also experience altered relaxation behavior. Lys 41 and Lys 64 also exhibited increases in R_2/R_1 at both field strengths, demonstrating that the response is not uniformly directional throughout the protein. These observations support a model in which calcium binding alters the dynamic environment of both the proposed binding sites and additional regions of the protein rather than simply rigidifying the residues that directly coordinate calcium.

The large R_2/R_1 decreases observed at Asp 33, Ile 34, and Glu 77 are notable because these residues occur within or immediately adjacent to the clustered binding-site regions. Asp 33, Ile 34, and Ser 36 form a contiguous sequence segment, while Asp 76, Glu 77, and Ser 79 form a second cluster near the C-terminus. This pattern suggests that the response to calcium binding may extend beyond individual coordinating side chains to neighboring backbone positions. Such behavior would be expected if calcium coordination reorganizes local packing, hydrogen-bonding interactions, or side-chain conformational preferences within the binding regions. R_2 is sensitive to both overall molecular tumbling and slower conformational exchange, and R_2/R_1 alone cannot uniquely distinguish among changes in these contributions. Therefore, the present data does not establish that calcium binding directly eliminates μ s-ms conformational exchange. Instead, the reproducible decrease in R_2/R_1 indicates that calcium binding changes the relaxation environment of the binding regions. This change may reflect alterations in local motions, conformational exchange, or other contributions to transverse relaxations, but R_2/R_1 cannot distinguish among these mechanisms. Complementary experiments specifically sensitive to chemical exchange would be required to make a definitive

assignment of μ s-ms exchange.

Another important observation is that the largest dynamic outliers are not restricted to the proposed calcium-binding residues. For example, residues such as Ser 22, Thr 44, and Lys 64 exhibit relatively low S^2 values, compared with the highly ordered regions of the protein. These positions may represent intrinsically more mobile portions of the backbone or structural regions with increased conformational freedom. Their presence in both apo and holo datasets argues against a model in which calcium binding simply converts the entire protein from a flexible to a rigid state. Instead, the protein appears to contain a combination of relatively rigid regions and localized dynamic regions, with calcium binding selectively perturbing the behavior of residues surrounding the metal-binding sites.

An overall model emerging from these observations is that calcium coordination stabilizes or reorganizes a pre-existing binding-site architecture rather than inducing extensive global folding. In the apo state, the protein is already relatively ordered, but the residues surrounding the calcium-binding sites retain a dynamic environment that can respond to metal coordination. Upon calcium binding, this local environment is reorganized, producing a pronounced and reproducible change in R_2/R_1 while leaving the overall S^2 distribution largely intact. The resulting holo state can therefore remain globally ordered while exhibiting altered local dynamics at the metal-binding regions.

4.5.2 Implications for the ancestral calcium-binding $\beta\gamma$ -crystallin fold

The dynamic behavior of *Ci*- $\beta\gamma$ provides a perspective on the functional significance of calcium-binding sites retained in this evolutionary intermediate. The persistence of a predominantly ordered backbone in both apo and holo states suggests that calcium coordination is not required to maintain the overall dynamic stability of the $\beta\gamma$ -crystallin fold. Instead, calcium binding is associated with localized changes superimposed on an existing dynamic

architecture. This behavior may be relevant to the evolutionary transition between ancestral calcium-binding proteins and vertebrate lens $\beta\gamma$ -crystallins. The calcium-binding sites retained by *Ci*- $\beta\gamma$ can influence local conformational dynamics without producing a global structural or dynamic transition. Loss of these sites during the evolution of vertebrate lens crystallins may therefore represent the removal of a localized regulatory feature rather than the loss of a requirement for maintaining the overall $\beta\gamma$ -crystallin fold. The present data cannot establish that the observed dynamic changes were the evolutionary reason for retention or loss of the calcium-binding sites. However, they demonstrate that calcium coordination is coupled to measurable, residue-specific changes in the dynamics of *Ci*- $\beta\gamma$. These findings provide a dynamic complement to structural and biochemical characterization of the protein and suggest that the ancestral calcium-binding sites may have influenced the local conformational behavior of the $\beta\gamma$ -crystallin fold.

4.6 Conclusion

The effects of calcium binding on the backbone dynamics of *Ciona intestinalis*- $\beta\gamma$ -crystallin were characterized using ^1H - ^{15}N relaxation measurements and FastModelFree analysis. Comparison of the calcium-bound (holo) and EDTA-treated (apo) states at 500 and 800 MHz demonstrated that *Ci*- $\beta\gamma$ remains predominantly ordered in both states. The high S^2 values and predominantly positive heteronuclear NOE measurements indicate that the protein remains globally well ordered in both states, with no evidence for widespread changes in fast-timescale backbone flexibility upon calcium binding.

The data indicate that calcium binding is associated with localized and heterogeneous changes in protein dynamics. Differences in R_2/R_1 , heteronuclear NOE, and S^2 values were observed at specific residues, with the magnitude and direction of these changes varying throughout the sequence. The 800 MHz R_2 measurements revealed pronounced differences in several regions, while heteronuclear NOE measurements showed that residues within the

calcium-binding sites could respond differently to calcium binding. These results demonstrate that the calcium-binding sites do not behave as dynamically uniform units and that calcium coordination does not simply rigidify the surrounding protein.

These findings suggest that calcium binding modulates the local dynamic environment of *Ci*- $\beta\gamma$ while preserving the overall dynamic architecture of the $\beta\gamma$ -crystallin fold. This provides a dynamic perspective on the ancestral calcium-binding sites retained in *Ci*- $\beta\gamma$ and suggests that their loss during the evolution of vertebrate lens crystallins may have removed a localized dynamic feature rather than a requirement for maintaining the overall protein fold. Although these measurements cannot establish the evolutionary reason for retention or loss of the calcium-binding sites, they demonstrate that calcium coordination is coupled to measurable, residue-specific changes in the dynamics of *Ci*- $\beta\gamma$.

4.7 Future Directions

Further investigation of the dynamic behavior of *Ci*- $\beta\gamma$ could provide a more detailed understanding of how calcium coordination influences the protein. In particular, additional ^1H - ^{15}N relaxation measurements at 700 MHz would provide an intermediate magnetic field strength between the 500 and 800 MHz datasets. Measurements at a third field strength would provide additional constraints for evaluating the field dependence of the relaxation parameters and determining whether the calcium-dependent changes observed in R_2/R_1 are reproducible across magnetic field strengths. The additional dataset could also provide further constraints for model-free analysis and help establish whether the observed changes in backbone dynamics are consistent across the different experimental conditions.

A second direction would be to directly investigate whether the residues undergoing a slower conformational exchange. The present R_2/R_1 measurements revealed reproducible calcium-dependent changes at several residues within and surrounding the proposed calcium-binding

sites. However, R_2/R_1 alone cannot distinguish changes arising from internal motions, molecular tumbling, or contributions from conformational exchange. ^{15}N CEST experiments could be used to determine whether residues exhibiting changes in relaxation behavior also undergo μs -ms timescale exchange between distinct conformational states. Comparing CEST profiles between the apo and holo states would allow changes in the populations and exchange behavior of these states to be evaluated directly. This would help determine whether the calcium-dependent changes observed in the present study reflect alterations in slower conformational exchange in addition to the changes in fast-timescale backbone dynamics characterized by S^2 and heteronuclear NOE measurements.

Together, relaxation measurements at an additional magnetic field strength and experiments targeting slower conformational exchange would provide a more complete description of the dynamic response of *Ci*- $\beta\gamma$ to calcium binding. These experiments would help distinguish the timescales affected by calcium coordination and further test the model, in which calcium binding alters localized dynamic behavior while preserving the predominantly ordered backbone of the protein. Defining how calcium coordination influences protein dynamics across multiple timescales will provide a more complete understanding of the molecular response of calcium binding and help place the dynamic behavior of the ancestral $\beta\gamma$ -crystallin fold in an evolutionary context.

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Appendix A

Supplementary information- Chapter 1

A.1 Residual Dipolar Coupling values

Table A.1: Apo form of $Ci-\beta\gamma$ residual dipolar coupling values

Sequence	Residue	$Ci-\beta\gamma$ with DLPC/DHPC bicelles	$Ci-\beta\gamma$ with DMPC/DHPC bicelles
2	GLY	7.963	-14.307
3	LYS	2.132	15.950
4	ILE	-0.320	-1.506
5	ILE	2.912	5.903
6	LEU	-6.263	-10.698
7	PHE	0.549	14.252
8	GLU	7.634	-13.593
9	ASP	-1.015	-20.811
10	VAL	7.913	11.117

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Table A.1 – continued from previous page

Sequence	Residue	$Ci-\beta\gamma$ with DLPC/DHPC bicelles	$Ci-\beta\gamma$ with DMPC/DHPC bicelles
11	GLU	6.167	15.547
12	PHE	4.024	-10.924
13	GLY	-0.637	-11.656
14	GLY	-11.210	-16.724
15	LYS	-4.256	-18.292
16	LYS	2.983	6.654
17	LEU	0.234	3.283
18	GLU	3.377	15.844
19	LEU	-1.896	-7.141
20	GLU	-0.962	20.685
21	THR	2.222	14.189
22	SER	-4.762	-1.980
23	VAL	7.579	-1.628
24	SER	3.166	-1.213
25	ASP	-2.204	-20.733
26	LEU	7.200	-1.165
27	ASN	-0.718	0.118
28	VAL	-1.402	27.995
29	HIS	2.972	3.032
30	GLY	-1.414	-13.220
31	PHE	-0.601	-8.705
32	ASN	10.104	30.545
33	ASP	6.030	31.490
34	ILE	-1.525	7.026

Continued on next page

Table A.1 – continued from previous page

Sequence	Residue	$Ci-\beta\gamma$ with DLPC/DHPC bicelles	$Ci-\beta\gamma$ with DMPC/DHPC bicelles
35	VAL	1.456	-13.514
36	SER	-1.023	-15.034
37	SER	-3.066	-11.322
38	ILE	-1.160	5.520
39	ILE	2.425	-2.957
40	VAL	0.536	-1.838
41	GLU	1.484	-4.188
42	SER	9.547	8.556
43	GLY	5.149	24.332
44	THR	1.580	-11.275
45	TRP	2.323	-10.553
46	PHE	4.780	-11.662
47	VAL	2.834	2.073
48	PHE	4.188	0.830
49	ASP	3.273	29.143
50	ASP	-3.297	-3.411
51	GLU	-8.707	1.172
52	GLY	7.086	-2.472
53	PHE	2.510	8.034
54	SER	3.368	22.735
55	GLY	6.139	8.640
56	PRO	n/a	n/a
57	SER	-0.314	3.877
58	TYR	1.768	8.178

Continued on next page

Table A.1 – continued from previous page

Sequence	Residue	$Ci-\beta\gamma$ with DLPC/DHPC bicelles	$Ci-\beta\gamma$ with DMPC/DHPC bicelles
59	LYS	-6.363	-10.452
60	LEU	8.416	-12.507
61	THR	-9.587	-20.857
62	PRO	n/a	n/a
63	Gly	0.595	16.251
64	LYS	0.501	-0.926
65	TYR	-1.220	0.398
66	PRO	n/a	n/a
67	ASN	9.175	-3.951
68	PRO	n/a	n/a
69	GLY	5.497	16.637
70	SER	-6.195	23.949
71	TRP	1.442	38.661
72	GLY	6.375	-1.661
73	GLY	6.253	1.396
74	ASN	7.736	-14.484
75	ASP	3.734	-1.869
76	ASP	2.589	-11.604
77	GLU	0.482	5.718
78	LEU	16.018	15.796
79	SER	-0.402	26.422
80	SER	-0.674	3.765
81	VAL	-0.831	-10.597
82	LYS	7.966	-8.298

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Table A.1 – continued from previous page

Sequence	Residue	$Ci-\beta\gamma$ with DLPC/DHPC bicelles	$Ci-\beta\gamma$ with DMPC/DHPC bicelles
83	GLN	1.734	2.393
84	GLN	1.333	-13.622

Appendix B

Supplementary information- Chapter 3

CS Rosetta calculations

The full structure comparison between the previously published crystal structure (2BV2) of the holo form and the CS-Rosetta predicted structure for the apo form, shown in Table B.1, yields an RMSD between 1-3 Å which typically indicates loop rearrangements or ligand binding events. Comparison of the crystal structure with the CS-Rosetta predicted structure for the apo form yielded an RMSD of less than 1 Å, indicating that the structures are nearly identical. Changes in the positions of binding site residues between the apo and holo forms and the calcium-bound crystal structure were assessed using the built-in Distance function in Chimera 1.14, detailed in Table B.2. Together these observations suggest subtle structural changes occur as a result of binding.

Table B.1: Full structure RMSD

Structure Comparison	RMSD (all 83 pairs)
2BV2 to Predicted EDTA	1.439 Å
2BV2 to Predicted Ca ²⁺	0.539 Å

Table B.2: Distances between binding site residues

Residue	Distance 2BV2 to Predicted EDTA (Å)	Distance 2BV2 to Predicted Ca ²⁺ (Å)
Gly 2	0.01911	0.096149
Lys 3	0.51782	0.3688
Ile 4	0.10621	0.26508
Asp 76	0.58027	0.23153
Asp 33	2.847	0.26496
Asp 49	0.10981	0.44077
Glu 77	0.70371	0.60459
Ser 79	0.32415	0.25065

Calcium titration binding affinity calculations

Table B.3: Fitted K_d and $\Delta\delta_{max}$

Sequence	Residue	K_d (mM)	fitted $\Delta\delta_{max}$ (Å)
2	GLY	0.0004	3.047
3	LYS	0	0.348
4	ILE	0.0019	1.049
5	ILE	0.0012	0.354
6	LEU	0.0849	1.015

Continued on next page

Table B.3 – continued from previous page

Sequence	Residue	K_d (mM)	fitted $\Delta\delta_{max}$ (Å)
7	PHE	0.0046	0.502
8	GLU	0	6.248
9	ASP	0	12.138
10	VAL	0.0028	1.995
11	GLU	0.0010	0.776
12	PHE	0.0004	0.514
13	GLY	0.0001	1.334
14	GLY	0	3.985
15	LYS	0.0034	0.150
16	LYS	0	2.102
17	LEU	0.1525	1.919
18	GLU	0.0002	0.845
19	LEU	0.0178	0.211
20	GLU	0.0012	0.179
21	THR	4.95E-06	0.544
22	SER	0.0008	0.607
23	VAL	0.0017	1.298
24	SER	0.0028	0.277
25	ASP	0.0005	0.595
26	LEU	0.0013	3.215
27	ASN	0.0004	1.641
28	VAL	0.0279	0.364
29	HIS	0.0004	0.306
30	GLY	0.0001	0.500

Continued on next page

Table B.3 – continued from previous page

Sequence	Residue	K_d (mM)	fitted $\Delta\delta_{max}$ (Å)
31	PHE	0	0.962
32	ASN	0.0022	4.848
33	ASP	0.0005	3.916
34	ILE	9.92E-05	18.469
35	VAL	0.0002	12.725
36	SER	0.0017	6.276
37	SER	0.0050	0.160
38	ILE	0	1.187
39	ILE	0.0005	0.227
40	VAL	0.0041	0.004
41	GLU	0	3.038
42	SER	0.0010	0.384
43	GLY	0.0014	0.613
44	THR	0	0.291
45	TRP	0.0015	0.130
46	PHE	0.0014	0.645
47	VAL	0.0011	0.209
48	PHE	0.0006	2.181
49	ASP	0.0011	4.543
50	ASP	0	2.150
51	GLU	0.0008	1.379
52	GLY	0.0001	0.857
53	PHE	5.39E-05	0.593
54	SER	0.0002	2.031

Continued on next page

Table B.3 – continued from previous page

Sequence	Residue	K_d (mM)	fitted $\Delta\delta_{max}$ (Å)
55	GLY	0.0005	1.994
56	PRO	n/a	n/a
57	SER	0.0008	3.035
58	TYR	0.0016	0.970
59	LYS	0.0002	1.344
60	LEU	4.72E-06	0.445
61	THR	0.0009	0.236
62	PRO	n/a	n/a
63	Gly	0.0055	0.404
64	LYS	0.0041	0.275
65	TYR	0.0001	0.506
66	PRO	n/a	n/a
67	ASN	1.70E-05	0.167
68	PRO	n/a	n/a
69	GLY	0.0009	0.176
70	SER	0.0008	0.730
71	TRP	0.0008	0.590
72	GLY	0.0005	1.128
73	GLY	0.0006	0.976
74	ASN	0.0022	2.382
75	ASP	0	10.616
76	ASP	0.0011	6.129
77	GLU	0.0004	3.937
78	LEU	0.0055	1.018

Continued on next page

Table B.3 – continued from previous page

Sequence	Residue	K_d (mM)	fitted $\Delta\delta_{max}$ (Å)
79	SER	0.0008	2.446
80	SER	0.0006	2.944
81	VAL	0.0007	1.276
82	LYS	0.0002	0.670
83	GLN	0	1.083
84	GLN	0.0013	1.355

Temperature ramp up HSQCs

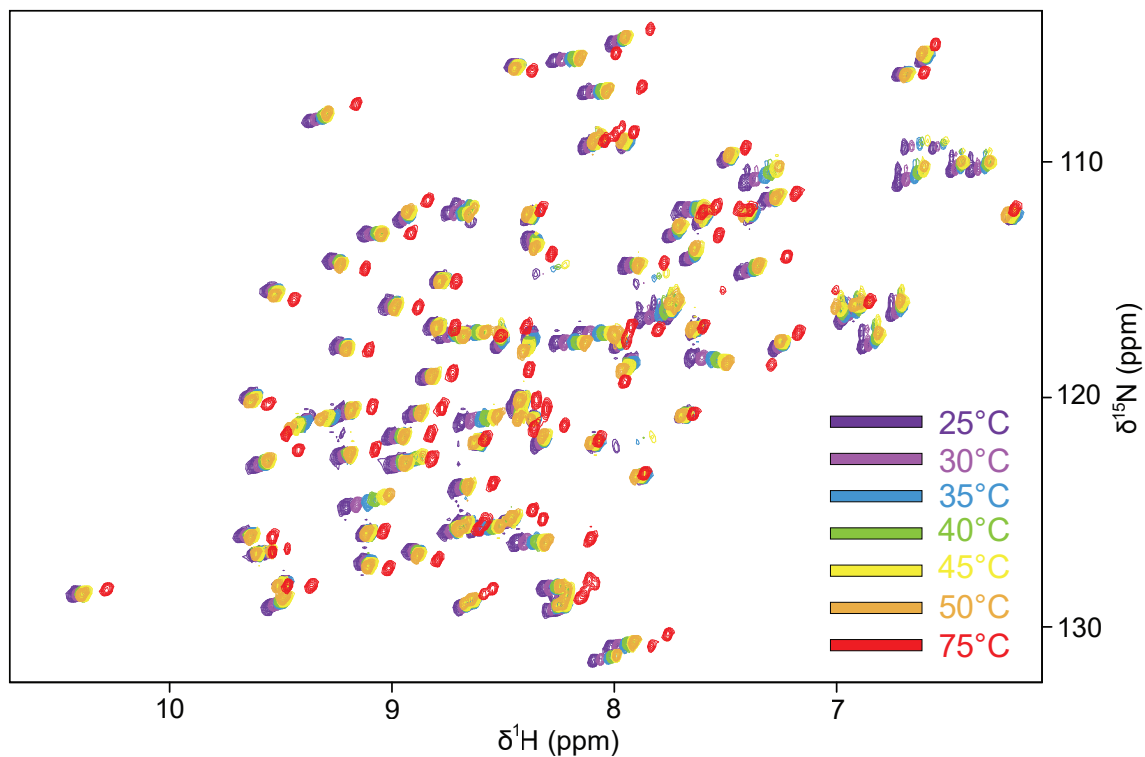


Figure B.1: ^{15}N - ^1H HSQC of *Ci*- $\beta\gamma$ -crystallin that portrays the chemical shift perturbations as temperature increased. The protein remains fully folded until 75 °C in the holo form.

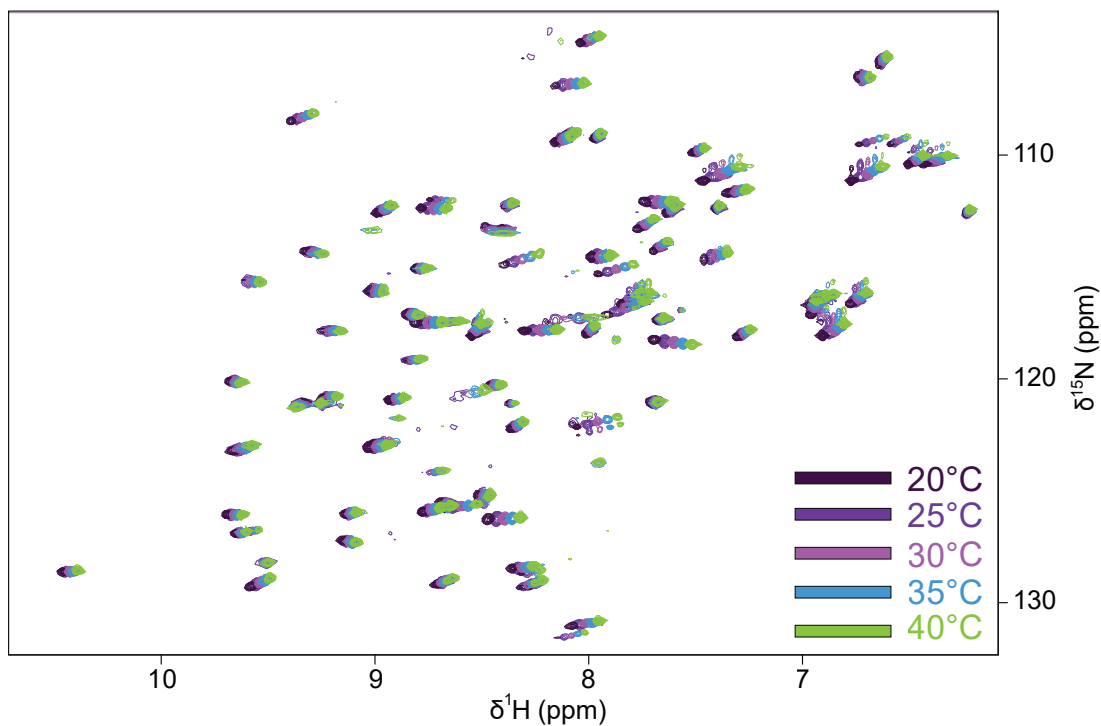


Figure B.2: ^{15}N - ^1H HSQC of *Ci*- $\beta\gamma$ -crystallin that portrays the chemical shift perturbations as temperature increased. The protein remains fully folded until 45 °C in the apo form where it starts to aggregate.

Temperature ramp up chemical shift perturbations

Table B.4: Chemical shift perturbations of *Ci*- $\beta\gamma$ in apo and holo form as a result of temperature ramp up

Sequence	Residue	Apo CSP	Holo CSP
2	GLY	-0.00254	-0.00287
3	LYS	-0.00730	-0.00714
4	ILE	-0.00327	-0.00318
5	ILE	-0.00226	-0.00239
6	LEU	-0.00191	-0.00241
7	PHE	-0.00232	-0.00271

Continued on next page

Table B.4 – continued from previous page

Sequence	Residue	Apo CSP	Holo CSP
8	GLU	-0.00261	-0.00400
9	ASP	0.000577	-0.000571
10	VAL	-0.00153	-0.00273
11	GLU	-0.00386	-0.00377
12	PHE	-0.00385	-0.00362
13	GLY	-0.00194	-0.00177
14	GLY	-0.00613	-0.00642
15	LYS	-0.00317	-0.00319
16	LYS	-0.00760	-0.00796
17	LEU	-0.00249	-0.00309
18	GLU	-0.00712	-0.00747
19	LEU	-0.00388	-0.00422
20	GLU	-0.00604	-0.00617
21	THR	-0.00182	-0.00215
22	SER	-0.00434	-0.00420
23	VAL	-0.00465	-0.00450
24	SER	-0.00687	-0.00425
25	ASP	-0.00854	-0.00808
26	LEU	-0.00374	-0.00395
27	ASN	0.00101	0.00141
28	VAL	-0.00147	-0.00133
29	HIS	-0.00151	-0.00199
30	GLY	-0.00127	-0.00147
31	PHE	-0.000761	-0.00152

Continued on next page

Table B.4 – continued from previous page

Sequence	Residue	Apo CSP	Holo CSP
32	ASN	-0.00659	-0.00862
33	ASP	-0.000576	-0.000907
34	ILE	-0.00648	-0.00216
35	VAL	-0.00452	-0.00288
36	SER	-0.0101	-0.00128
37	SER	-0.00105	-0.00310
38	ILE	-0.00323	-0.00348
39	ILE	-0.00348	-0.00329
40	VAL	-0.00289	-0.00310
41	GLU	-0.00464	-0.00375
42	SER	-0.00372	-0.00254
43	GLY	-0.00566	-0.00603
44	THR	-0.00595	-0.00595
45	TRP	-0.00381	-0.00377
46	PHE	-0.00242	-0.00227
47	VAL	-0.00293	-0.00221
48	PHE	-0.00233	-0.00317
49	ASP	-0.00393	-0.00452
50	ASP	-0.00354	-0.000517
51	GLU	-0.000904	0.000880
52	GLY	-0.00517	-0.00498
53	PHE	-0.00346	-0.00334
54	SER	-0.000858	-0.000742
55	GLY	-0.00663	-0.00613

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Table B.4 – continued from previous page

Sequence	Residue	Apo CSP	Holo CSP
56	PRO	n/a	n/a
57	SER	-0.00530	-0.00495
58	TYR	-0.00231	-0.00244
59	LYS	-0.00714	-0.00717
60	LEU	-0.00432	-0.00428
61	THR	-0.00421	-0.00340
62	PRO	n/a	n/a
63	Gly	-0.00319	-0.00301
64	LYS	-0.00159	-0.00205
65	TYR	-0.00333	-0.00301
66	PRO	n/a	n/a
67	ASN	-0.000579	-0.000544
68	PRO	n/a	n/a
69	GLY	-0.00425	-0.00411
70	SER	-0.00206	-0.00195
71	TRP	-0.00291	-0.00273
72	GLY	-0.00253	-0.00254
73	GLY	-0.00249	-0.00253
74	ASN	-0.00677	-0.00633
75	ASP	-0.00262	-0.00432
76	ASP	-0.00154	-0.00109
77	GLU	-0.00614	-0.00505
78	LEU	-0.00229	-0.00235
79	SER	-0.00523	-0.00281

Continued on next page

Table B.4 – continued from previous page

Sequence	Residue	Apo CSP	Holo CSP
80	SER	-0.00157	-0.00176
81	VAL	-0.00319	-0.00349
82	LYS	-0.00401	-0.00370
83	GLN	-0.00346	-0.00440
84	GLN	-0.00606	-0.00612

Appendix C

Supplementary information- Chapter 4

C.1 500 Mhz relaxation and ModelFree data of $Ci\text{-}\beta\gamma$ in apo and holo form

Table C.1: Apo 500 MHz relaxation and ModelFree data of $Ci\text{-}\beta\gamma$

Sequence	Residue	NOE	R ₁	R ₂	S ²
2	GLY	0.710 ± 0.023	2.59 ± 0.023	6.19 ± 0.387	0.851 ± 0.008
3	LYS	0.733 ± 0.065	2.42 ± 0.037	5.37 ± 0.062	0.796 ± 0.013
4	ILE	0.748 ± 0.025	2.56 ± 0.020	5.54 ± 0.040	0.852 ± 0.004
5	ILE	0.632 ± 0.026	2.73 ± 0.007	5.61 ± 0.070	0.878 ± 0.006
6	LEU	0.619 ± 0.025	2.70 ± 0.007	5.55 ± 0.038	0.863 ± 0.005
7	PHE	0.717 ± 0.024	2.68 ± 0.009	5.67 ± 0.068	0.880 ± 0.003
8	GLU	0.715 ± 0.016	2.69 ± 0.010	5.71 ± 0.047	0.884 ± 0.003

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Table C.1 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
9	ASP	0.619 ± 0.020	2.69 ± 0.008	5.56 ± 0.031	0.861 ± 0.004
10	VAL	0.717 ± 0.018	2.51 ± 0.010	5.37 ± 0.050	0.825 ± 0.003
11	GLU	0.693 ± 0.020	2.73 ± 0.008	5.95 ± 0.107	0.898 ± 0.002
12	PHE	0.746 ± 0.021	2.82 ± 0.015	5.84 ± 0.019	0.913 ± 0.003
13	GLY	0.711 ± 0.019	2.64 ± 0.011	5.32 ± 0.016	n/a
14	GLY	0.639 ± 0.016	2.54 ± 0.004	5.35 ± 0.023	0.823 ± 0.003
15	LYS	0.733 ± 0.049	2.53 ± 0.012	5.69 ± 0.070	0.830 ± 0.004
16	LYS	0.700 ± 0.069	2.61 ± 0.022	5.51 ± 0.044	0.858 ± 0.005
17	LEU	0.660 ± 0.024	2.67 ± 0.005	5.58 ± 0.087	0.877 ± 0.002
18	GLU	0.683 ± 0.025	2.58 ± 0.005	5.17 ± 0.056	n/a
19	LEU	0.734 ± 0.028	2.59 ± 0.007	5.50 ± 0.055	0.852 ± 0.002
20	GLU	0.712 ± 0.030	2.51 ± 0.010	5.35 ± 0.031	0.828 ± 0.003
21	THR	0.728 ± 0.025	2.53 ± 0.016	5.35 ± 0.052	0.831 ± 0.004
22	SER	0.706 ± 0.018	2.28 ± 0.037	5.18 ± 0.096	0.749 ± 0.012
23	VAL	0.693 ± 0.032	2.63 ± 0.023	5.73 ± 0.082	0.872 ± 0.006
24	SER	0.694 ± 0.026	2.54 ± 0.009	5.62 ± 0.096	0.836 ± 0.003
25	ASP	0.772 ± 0.023	2.65 ± 0.047	5.81 ± 0.074	0.892 ± 0.009
26	LEU	0.733 ± 0.020	2.66 ± 0.017	5.60 ± 0.046	0.874 ± 0.005
27	ASN	0.748 ± 0.015	2.76 ± 0.014	6.21 ± 0.125	0.910 ± 0.004
28	VAL	0.759 ± 0.018	2.68 ± 0.017	5.86 ± 0.025	n/a
29	HIS	0.713 ± 0.037	2.67 ± 0.017	6.28 ± 0.028	0.876 ± 0.006
30	GLY	0.730 ± 0.018	2.73 ± 0.015	5.67 ± 0.043	0.891 ± 0.004
31	PHE	0.684 ± 0.023	2.69 ± 0.012	5.88 ± 0.083	0.888 ± 0.003
32	ASN	0.719 ± 0.018	2.73 ± 0.013	6.03 ± 0.068	0.896 ± 0.004

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Table C.1 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
33	ASP	0.696 ± 0.021	2.70 ± 0.041	6.15 ± 0.060	0.886 ± 0.013
34	ILE	0.677 ± 0.035	2.69 ± 0.038	6.25 ± 0.083	0.885 ± 0.012
35	VAL	0.716 ± 0.024	2.65 ± 0.036	5.99 ± 0.116	0.871 ± 0.011
36	SER	0.714 ± 0.024	2.43 ± 0.058	5.50 ± 0.078	0.798 ± 0.019
37	SER	0.728 ± 0.011	2.69 ± 0.010	5.75 ± 0.107	0.885 ± 0.003
38	ILE	0.717 ± 0.027	2.66 ± 0.013	5.77 ± 0.066	0.878 ± 0.004
39	ILE	0.757 ± 0.029	2.61 ± 0.023	5.47 ± 0.029	0.853 ± 0.004
40	VAL	0.598 ± 0.026	2.66 ± 0.016	5.41 ± 0.036	0.806 ± 0.016
41	GLU	0.642 ± 0.030	2.60 ± 0.030	5.29 ± 0.052	0.836 ± 0.006
42	SER	0.610 ± 0.045	2.58 ± 0.013	5.74 ± 0.087	0.852 ± 0.004
43	GLY	0.649 ± 0.040	2.55 ± 0.057	5.65 ± 0.094	0.863 ± 0.012
44	THR	0.690 ± 0.028	2.39 ± 0.017	5.12 ± 0.079	0.787 ± 0.005
45	TRP	0.670 ± 0.028	2.63 ± 0.018	5.42 ± 0.058	0.858 ± 0.005
46	PHE	0.700 ± 0.027	2.59 ± 0.009	5.60 ± 0.089	0.850 ± 0.003
47	VAL	0.653 ± 0.027	2.67 ± 0.013	5.61 ± 0.057	0.875 ± 0.004
48	PHE	0.657 ± 0.025	2.70 ± 0.013	5.86 ± 0.081	0.889 ± 0.004
49	ASP	0.696 ± 0.019	2.60 ± 0.016	5.53 ± 0.024	0.859 ± 0.003
50	ASP	0.757 ± 0.023	2.58 ± 0.038	6.13 ± 0.073	n/a
51	GLU	0.734 ± 0.018	2.48 ± 0.013	5.81 ± 0.094	0.814 ± 0.004
52	GLY	0.699 ± 0.022	2.71 ± 0.011	5.82 ± 0.104	0.890 ± 0.003
53	PHE	0.732 ± 0.021	2.78 ± 0.013	6.03 ± 0.079	0.917 ± 0.004
54	SER	0.676 ± 0.023	2.54 ± 0.016	5.24 ± 0.027	0.823 ± 0.003
55	GLY	0.731 ± 0.020	2.46 ± 0.014	5.89 ± 0.071	0.808 ± 0.005
56	PRO	n/a	n/a	n/a	n/a

Continued on next page

Table C.1 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
57	SER	0.662 ± 0.023	2.53 ± 0.014	5.16 ± 0.025	0.761 ± 0.018
58	TYR	0.761 ± 0.026	2.60 ± 0.010	5.48 ± 0.030	0.855 ± 0.003
59	LYS	0.611 ± 0.056	2.53 ± 0.020	5.35 ± 0.092	0.831 ± 0.006
60	LEU	0.677 ± 0.026	2.63 ± 0.013	n/a	0.861 ± 0.004
61	THR	0.719 ± 0.024	2.38 ± 0.026	5.43 ± 0.071	0.781 ± 0.008
62	PRO	n/a	n/a	n/a	n/a
63	GLY	0.683 ± 0.019	2.69 ± 0.012	5.59 ± 0.047	0.881 ± 0.003
64	LYS	0.465 ± 0.084	2.11 ± 0.043	4.42 ± 0.067	0.666 ± 0.013
65	TYR	0.633 ± 0.030	2.82 ± 0.015	5.84 ± 0.017	0.901 ± 0.004
66	PRO	n/a	n/a	n/a	n/a
67	ASN	0.675 ± 0.013	2.69 ± 0.029	5.68 ± 0.055	0.880 ± 0.007
68	PRO	n/a	n/a	n/a	n/a
69	GLY	0.683 ± 0.017	2.62 ± 0.036	5.72 ± 0.088	0.874 ± 0.009
70	SER	0.735 ± 0.016	2.69 ± 0.032	5.93 ± 0.081	0.900 ± 0.008
71	TRP	0.722 ± 0.027	2.71 ± 0.025	5.89 ± 0.036	0.889 ± 0.009
72	GLY	0.703 ± 0.020	2.57 ± 0.015	5.34 ± 0.033	0.839 ± 0.003
73	GLY	0.703 ± 0.020	2.57 ± 0.015	5.34 ± 0.033	0.838 ± 0.004
74	ASN	0.627 ± 0.027	2.37 ± 0.036	5.10 ± 0.057	0.789 ± 0.007
75	ASP	0.746 ± 0.033	2.64 ± 0.032	5.54 ± 0.034	0.864 ± 0.005
76	ASP	0.670 ± 0.018	2.60 ± 0.032	5.95 ± 0.105	0.874 ± 0.009
77	GLU	0.665 ± 0.028	2.57 ± 0.044	6.20 ± 0.068	0.845 ± 0.015
78	LEU	0.077 ± 0.293	1.44 ± 0.111	5.00 ± 0.343	0.403 ± 0.044
79	SER	0.683 ± 0.033	2.60 ± 0.021	5.98 ± 0.107	0.854 ± 0.007
80	SER	0.606 ± 0.016	2.72 ± 0.021	6.33 ± 0.066	0.873 ± 0.008

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Table C.1 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
81	VAL	0.733 ± 0.039	2.68 ± 0.014	5.53 ± 0.044	0.873 ± 0.004
82	LYS	0.840 ± 0.079	2.70 ± 0.019	5.57 ± 0.062	0.883 ± 0.005
83	GLN	0.632 ± 0.035	2.71 ± 0.016	5.33 ± 0.037	0.729 ± 0.028
84	GLN	0.561 ± 0.024	2.52 ± 0.013	5.25 ± 0.031	0.801 ± 0.005

Table C.2: Holo 500 MHz relaxation and ModelFree data of *Ci*-βγ

Sequence	Residue	NOE	R ₁	R ₂	S ²
2	GLY	0.698 ± 0.058	2.60 ± 0.0218	5.46 ± 0.0338	0.848 ± 0.007
3	LYS	0.700 ± 0.0637	2.47 ± 0.0295	5.17 ± 0.0278	0.824 ± 0.004
4	ILE	0.653 ± 0.0636	2.59 ± 0.0108	5.36 ± 0.0164	0.844 ± 0.003
5	ILE	0.726 ± 0.0628	2.73 ± 0.00612	5.48 ± 0.0161	0.884 ± 0.002
6	LEU	0.646 ± 0.0548	2.71 ± 0.00628	5.44 ± 0.0184	0.860 ± 0.005
7	PHE	0.730 ± 0.0637	2.65 ± 0.0111	5.48 ± 0.0244	0.863 ± 0.004
8	GLU	0.715 ± 0.0414	2.70 ± 0.00644	5.62 ± 0.0240	0.878 ± 0.002
9	ASP	0.705 ± 0.0534	2.79 ± 0.00528	5.89 ± 0.0178	0.909 ± 0.002
10	VAL	0.725 ± 0.0458	2.58 ± 0.00765	5.51 ± 0.0236	0.841 ± 0.003
11	GLU	0.715 ± 0.0571	2.75 ± 0.00665	5.67 ± 0.0259	0.899 ± 0.002
12	PHE	0.704 ± 0.0516	2.86 ± 0.00568	5.90 ± 0.0208	0.930 ± 0.002
13	GLY	0.664 ± 0.0514	2.65 ± 0.00457	5.34 ± 0.0176	0.861 ± 0.001
14	GLY	0.690 ± 0.0469	2.58 ± 0.00458	5.44 ± 0.0215	0.839 ± 0.001
15	LYS	0.771 ± 0.0582	2.60 ± 0.00690	5.61 ± 0.0129	0.848 ± 0.002
16	LYS	0.675 ± 0.0688	2.59 ± 0.00696	5.36 ± 0.0145	0.844 ± 0.002
17	LEU	0.724 ± 0.0597	2.64 ± 0.00501	5.37 ± 0.0104	0.859 ± 0.001
18	GLU	0.699 ± 0.0697	2.55 ± 0.00791	5.12 ± 0.0166	0.825 ± 0.002

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Table C.2 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
19	LEU	0.705 ± 0.0638	2.58 ± 0.0110	5.30 ± 0.0144	0.846 ± 0.002
20	GLU	0.673 ± 0.0700	2.55 ± 0.0108	5.21 ± 0.0319	0.832 ± 0.003
21	THR	0.751 ± 0.0654	2.54 ± 0.0119	5.40 ± 0.0232	0.827 ± 0.004
22	SER	0.665 ± 0.0476	2.22 ± 0.0399	4.77 ± 0.0444	0.723 ± 0.013
23	VAL	0.738 ± 0.0832	2.57 ± 0.0197	5.40 ± 0.0361	0.838 ± 0.006
24	SER	0.692 ± 0.0623	2.53 ± 0.0145	5.29 ± 0.0259	0.823 ± 0.005
25	ASP	0.721 ± 0.0629	2.65 ± 0.0600	5.79 ± 0.0461	0.865 ± 0.020
26	LEU	0.673 ± 0.0497	2.66 ± 0.0162	5.48 ± 0.0343	0.872 ± 0.004
27	ASN	0.682 ± 0.0426	2.78 ± 0.00894	5.87 ± 0.0284	0.906 ± 0.003
28	VAL	0.728 ± 0.0441	2.71 ± 0.0177	5.82 ± 0.0313	0.883 ± 0.006
29	HIS	0.652 ± 0.0594	2.66 ± 0.0113	5.91 ± 0.0197	0.865 ± 0.004
30	GLY	0.713 ± 0.0497	2.73 ± 0.0181	5.60 ± 0.0159	0.896 ± 0.002
31	PHE	0.753 ± 0.0723	2.68 ± 0.00630	5.67 ± 0.0187	0.873 ± 0.002
32	ASN	0.727 ± 0.0493	2.76 ± 0.0155	5.74 ± 0.0255	0.898 ± 0.005
33	ASP	0.700 ± 0.0545	2.63 ± 0.0454	5.68 ± 0.0494	0.858 ± 0.015
34	ILE	0.737 ± 0.0845	2.66 ± 0.0537	5.79 ± 0.0491	0.867 ± 0.017
35	VAL	0.679 ± 0.0524	2.66 ± 0.0454	5.79 ± 0.0412	0.865 ± 0.014
36	SER	0.692 ± 0.0765	2.65 ± 0.0175	5.49 ± 0.0325	0.872 ± 0.004
37	SER	0.752 ± 0.0367	2.70 ± 0.0133	5.45 ± 0.0281	0.877 ± 0.003
38	ILE	0.739 ± 0.0620	2.69 ± 0.0141	5.55 ± 0.0288	0.883 ± 0.003
39	ILE	0.729 ± 0.0612	2.65 ± 0.0111	5.35 ± 0.0184	0.859 ± 0.002
40	VAL	0.741 ± 0.0653	2.64 ± 0.00863	5.37 ± 0.0294	0.859 ± 0.002
41	GLU	0.672 ± 0.0974	2.71 ± 0.0108	5.57 ± 0.0370	0.886 ± 0.003
42	SER	0.724 ± 0.0605	2.66 ± 0.0155	5.47 ± 0.0156	0.874 ± 0.002

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Table C.2 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
43	GLY	0.744 ± 0.111	2.62 ± 0.0567	5.43 ± 0.0476	0.866 ± 0.007
44	THR	0.695 ± 0.0797	2.37 ± 0.0179	4.96 ± 0.0329	0.773 ± 0.006
45	TRP	0.703 ± 0.0697	2.59 ± 0.0152	5.31 ± 0.0196	0.848 ± 0.003
46	PHE	0.741 ± 0.0722	2.57 ± 0.00892	5.32 ± 0.0229	0.838 ± 0.003
47	VAL	0.668 ± 0.0600	2.67 ± 0.0109	5.51 ± 0.0220	0.870 ± 0.003
48	PHE	0.683 ± 0.0595	2.68 ± 0.0184	5.60 ± 0.0252	0.873 ± 0.006
49	ASP	0.692 ± 0.0501	2.61 ± 0.0192	5.52 ± 0.0338	0.852 ± 0.006
50	ASP	0.763 ± 0.0689	2.81 ± 0.0226	6.00 ± 0.0209	0.914 ± 0.008
51	GLU	0.695 ± 0.0458	2.54 ± 0.0120	5.39 ± 0.0310	0.827 ± 0.004
52	GLY	0.673 ± 0.0575	2.72 ± 0.0154	5.50 ± 0.0210	0.881 ± 0.003
53	PHE	0.812 ± 0.0665	2.79 ± 0.0173	5.85 ± 0.0333	0.924 ± 0.004
54	SER	0.671 ± 0.0546	2.59 ± 0.0157	5.31 ± 0.0268	0.847 ± 0.003
55	GLY	0.719 ± 0.0519	2.48 ± 0.0137	5.23 ± 0.0308	0.807 ± 0.004
56	PRO	n/a	n/a	n/a	n/a
57	SER	0.638 ± 0.0527	2.59 ± 0.0171	5.19 ± 0.0196	0.834 ± 0.003
58	TYR	0.690 ± 0.0624	2.60 ± 0.0149	5.47 ± 0.0244	0.848 ± 0.005
59	LYS	0.583 ± 0.0505	2.48 ± 0.0105	4.99 ± 0.0179	0.788 ± 0.005
60	LEU	0.704 ± 0.0664	2.60 ± 0.0109	5.27 ± 0.0302	0.846 ± 0.003
61	THR	0.720 ± 0.0634	2.40 ± 0.0225	4.90 ± 0.0304	0.780 ± 0.004
62	PRO	n/a	n/a	n/a	n/a
63	GLY	0.679 ± 0.0506	2.68 ± 0.0134	5.43 ± 0.0308	0.872 ± 0.003
64	LYS	0.665 ± 0.0895	2.34 ± 0.0243	4.98 ± 0.0355	0.763 ± 0.008
65	TYR	0.767 ± 0.0927	2.81 ± 0.00981	5.69 ± 0.0227	0.914 ± 0.002
66	PRO	n/a	n/a	n/a	n/a

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Table C.2 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
67	ASN	0.731 ± 0.0367	2.70 ± 0.0376	5.67 ± 0.0331	0.903 ± 0.005
68	PRO	n/a	n/a	n/a	n/a
69	GLY	0.698 ± 0.0477	2.60 ± 0.0422	5.52 ± 0.0353	0.846 ± 0.014
70	SER	0.765 ± 0.0426	2.64 ± 0.0355	5.78 ± 0.0367	0.861 ± 0.012
71	TRP	0.747 ± 0.0657	2.64 ± 0.0217	5.84 ± 0.0322	0.860 ± 0.007
72	GLY	0.748 ± 0.0554	2.61 ± 0.0177	5.45 ± 0.0364	0.849 ± 0.006
73	GLY	0.734 ± 0.0544	2.56 ± 0.0187	5.39 ± 0.0309	0.832 ± 0.006
74	ASN	0.644 ± 0.0702	2.38 ± 0.0534	5.19 ± 0.0405	0.776 ± 0.017
75	ASP	0.824 ± 0.170	2.31 ± 0.0871	5.39 ± 0.0518	0.753 ± 0.030
76	ASP	0.717 ± 0.0491	2.58 ± 0.0495	5.61 ± 0.0402	0.839 ± 0.017
77	GLU	0.749 ± 0.0893	2.63 ± 0.0551	5.68 ± 0.0464	0.858 ± 0.018
78	LEU	0.702 ± 0.0554	2.61 ± 0.0469	5.78 ± 0.0376	0.849 ± 0.016
79	SER	0.622 ± 0.0733	2.60 ± 0.0192	5.55 ± 0.0239	0.846 ± 0.006
80	SER	0.719 ± 0.0436	2.74 ± 0.0164	5.62 ± 0.0300	0.897 ± 0.004
81	VAL	0.693 ± 0.0834	2.65 ± 0.0105	5.53 ± 0.0296	0.864 ± 0.004
82	LYS	0.719 ± 0.0674	2.66 ± 0.0119	5.36 ± 0.0330	0.865 ± 0.003
83	GLN	0.606 ± 0.0823	2.60 ± 0.0295	5.26 ± 0.0703	0.846 ± 0.008
84	GLN	0.625 ± 0.0661	2.50 ± 0.0108	5.15 ± 0.0222	0.820 ± 0.003

C.2 800 Mhz relaxation and ModelFree data for *Ci*- $\beta\gamma$ in apo and holo form

Table C.3: Apo 800 MHz relaxation and ModelFree data of $Ci\text{-}\beta\gamma$

Sequence	Residue	NOE	R ₁	R ₂	S ²
2	GLY	0.773 ± 0.022	1.790 ± 0.024	6.20 ± 0.13	0.877 ± 0.011
3	LYS	0.842 ± 0.062	1.680 ± 0.037	5.44 ± 0.23	0.807 ± 0.016
4	ILE	0.796 ± 0.025	1.710 ± 0.015	5.60 ± 0.06	0.821 ± 0.006
5	ILE	0.782 ± 0.029	1.880 ± 0.004	6.18 ± 0.03	0.907 ± 0.002
6	LEU	0.800 ± 0.028	1.860 ± 0.008	5.91 ± 0.03	0.824 ± 0.017
7	PHE	0.817 ± 0.027	1.780 ± 0.006	6.00 ± 0.03	0.865 ± 0.003
8	GLU	0.759 ± 0.021	1.820 ± 0.007	6.18 ± 0.03	0.887 ± 0.002
9	ASP	0.779 ± 0.020	1.900 ± 0.005	6.40 ± 0.03	0.922 ± 0.002
10	VAL	0.745 ± 0.021	1.700 ± 0.020	5.92 ± 0.04	0.806 ± 0.011
11	GLU	0.855 ± 0.022	1.910 ± 0.008	6.35 ± 0.03	0.923 ± 0.003
12	PHE	0.864 ± 0.024	1.990 ± 0.012	6.61 ± 0.03	0.961 ± 0.004
13	GLY	0.799 ± 0.021	1.890 ± 0.004	6.32 ± 0.02	0.915 ± 0.002
14	GLY	0.728 ± 0.020	1.740 ± 0.006	5.94 ± 0.02	0.824 ± 0.005
15	LYS	0.915 ± 0.069	1.830 ± 0.010	6.46 ± 0.04	0.885 ± 0.005
16	LYS	0.767 ± 0.068	1.870 ± 0.009	6.29 ± 0.05	0.908 ± 0.004
17	LEU	0.781 ± 0.025	1.850 ± 0.004	5.94 ± 0.03	n/a
18	GLU	0.737 ± 0.023	1.750 ± 0.008	5.66 ± 0.02	n/a
19	LEU	0.805 ± 0.033	1.740 ± 0.008	5.96 ± 0.04	0.844 ± 0.004
20	GLU	0.835 ± 0.034	1.670 ± 0.010	5.54 ± 0.05	0.807 ± 0.004
21	THR	0.833 ± 0.027	1.720 ± 0.017	6.07 ± 0.07	0.835 ± 0.008
22	SER	0.711 ± 0.020	1.550 ± 0.046	5.13 ± 0.22	0.731 ± 0.019
23	VAL	0.831 ± 0.037	1.830 ± 0.021	6.15 ± 0.10	0.888 ± 0.008
24	SER	0.793 ± 0.032	1.690 ± 0.080	5.86 ± 0.36	0.831 ± 0.030

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Table C.3 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
25	ASP	0.840 ± 0.023	1.830 ± 0.045	6.34 ± 0.26	0.893 ± 0.019
26	LEU	0.825 ± 0.025	1.820 ± 0.016	6.00 ± 0.11	0.879 ± 0.007
27	ASN	0.860 ± 0.020	1.950 ± 0.019	6.97 ± 0.09	n/a
28	VAL	0.840 ± 0.023	1.930 ± 0.018	6.66 ± 0.07	0.946 ± 0.006
29	HIS	0.733 ± 0.033	1.800 ± 0.015	8.05 ± 0.09	0.852 ± 0.011
30	GLY	0.775 ± 0.020	1.830 ± 0.016	6.66 ± 0.08	0.878 ± 0.009
31	PHE	0.772 ± 0.023	1.810 ± 0.015	6.39 ± 0.06	0.865 ± 0.009
32	ASN	0.781 ± 0.019	1.950 ± 0.019	6.85 ± 0.10	0.961 ± 0.008
33	ASP	0.786 ± 0.023	1.830 ± 0.041	7.11 ± 0.25	0.888 ± 0.020
34	ILE	0.732 ± 0.040	1.840 ± 0.034	7.44 ± 0.25	0.873 ± 0.018
35	VAL	0.842 ± 0.029	1.760 ± 0.034	6.02 ± 0.17	0.858 ± 0.015
36	SER	0.826 ± 0.030	1.720 ± 0.062	5.81 ± 0.30	0.835 ± 0.025
37	SER	0.848 ± 0.014	1.880 ± 0.011	6.33 ± 0.07	0.914 ± 0.005
38	ILE	0.829 ± 0.029	1.800 ± 0.014	6.08 ± 0.05	0.876 ± 0.005
39	ILE	0.849 ± 0.029	1.820 ± 0.010	5.97 ± 0.04	0.876 ± 0.004
40	VAL	0.840 ± 0.033	1.810 ± 0.011	6.15 ± 0.05	0.881 ± 0.005
41	GLU	0.758 ± 0.034	1.860 ± 0.024	5.93 ± 0.14	0.889 ± 0.010
42	SER	0.718 ± 0.055	1.740 ± 0.022	6.30 ± 0.09	0.819 ± 0.016
43	GLY	0.857 ± 0.050	1.760 ± 0.048	5.52 ± 0.27	0.839 ± 0.021
44	THR	0.780 ± 0.032	1.640 ± 0.023	5.29 ± 0.12	0.787 ± 0.009
45	TRP	0.763 ± 0.030	1.740 ± 0.014	5.80 ± 0.07	0.840 ± 0.006
46	PHE	0.764 ± 0.028	1.750 ± 0.011	5.84 ± 0.06	0.847 ± 0.005
47	VAL	0.811 ± 0.031	1.850 ± 0.009	6.03 ± 0.06	0.890 ± 0.004
48	PHE	0.797 ± 0.027	1.850 ± 0.013	6.21 ± 0.06	0.897 ± 0.005

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Table C.3 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
49	ASP	0.768 ± 0.021	1.750 ± 0.016	5.87 ± 0.08	0.849 ± 0.006
50	ASP	0.872 ± 0.028	1.790 ± 0.029	6.86 ± 0.20	0.890 ± 0.013
51	GLU	0.762 ± 0.022	1.740 ± 0.017	6.47 ± 0.09	0.832 ± 0.009
52	GLY	0.828 ± 0.027	1.900 ± 0.024	6.40 ± 0.07	0.923 ± 0.008
53	PHE	0.775 ± 0.021	1.950 ± 0.020	6.71 ± 0.10	0.954 ± 0.008
54	SER	0.769 ± 0.028	1.780 ± 0.021	6.12 ± 0.10	0.869 ± 0.008
55	GLY	0.786 ± 0.024	1.690 ± 0.016	7.38 ± 0.11	0.818 ± 0.007
56	PRO	n/a	n/a	n/a	n/a
57	SER	0.814 ± 0.021	1.780 ± 0.019	5.86 ± 0.08	0.856 ± 0.007
58	TYR	0.767 ± 0.023	1.770 ± 0.013	6.14 ± 0.08	0.864 ± 0.005
59	LYS	0.861 ± 0.061	1.780 ± 0.013	5.57 ± 0.08	0.848 ± 0.006
60	LEU	0.828 ± 0.033	1.790 ± 0.014	6.01 ± 0.06	0.869 ± 0.005
61	THR	0.819 ± 0.021	1.640 ± 0.025	5.21 ± 0.13	0.784 ± 0.010
62	PRO	n/a	n/a	n/a	n/a
63	GLY	0.805 ± 0.023	1.830 ± 0.014	6.03 ± 0.06	0.883 ± 0.005
64	LYS	0.651 ± 0.087	1.590 ± 0.030	4.79 ± 0.13	0.741 ± 0.011
65	TYR	0.839 ± 0.043	1.970 ± 0.012	7.11 ± 0.05	0.956 ± 0.006
66	PRO	n/a	n/a	n/a	n/a
67	ASN	0.866 ± 0.018	1.840 ± 0.035	6.18 ± 0.17	0.892 ± 0.014
68	PRO	n/a	n/a	n/a	n/a
69	GLY	0.854 ± 0.022	1.840 ± 0.039	5.94 ± 0.20	0.881 ± 0.016
70	SER	0.836 ± 0.020	1.890 ± 0.040	6.35 ± 0.19	0.917 ± 0.016
71	TRP	0.908 ± 0.025	1.870 ± 0.027	6.55 ± 0.13	0.920 ± 0.011
72	GLY	0.837 ± 0.020	1.750 ± 0.022	6.10 ± 0.08	0.865 ± 0.008

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Table C.3 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
73	GLY	0.752 ± 0.018	1.750 ± 0.023	6.05 ± 0.09	0.859 ± 0.008
74	ASN	0.771 ± 0.026	1.630 ± 0.036	5.38 ± 0.23	0.789 ± 0.015
75	ASP	0.848 ± 0.035	1.810 ± 0.021	6.10 ± 0.09	0.880 ± 0.008
76	ASP	0.837 ± 0.022	1.730 ± 0.035	6.27 ± 0.21	0.856 ± 0.015
77	GLU	0.820 ± 0.032	1.790 ± 0.041	7.03 ± 0.26	0.865 ± 0.019
78	LEU	0.678 ± 0.030	1.330 ± 0.081	3.24 ± 0.55	0.610 ± 0.035
79	SER	0.870 ± 0.039	1.710 ± 0.022	6.02 ± 0.13	0.837 ± 0.009
80	SER	0.809 ± 0.019	1.900 ± 0.026	7.77 ± 0.12	0.918 ± 0.013
81	VAL	0.811 ± 0.046	1.820 ± 0.010	6.01 ± 0.09	0.882 ± 0.004
82	LYS	0.815 ± 0.087	1.880 ± 0.014	6.39 ± 0.09	0.914 ± 0.006
83	GLN	0.882 ± 0.037	1.830 ± 0.009	6.36 ± 0.06	0.888 ± 0.004
84	GLN	0.679 ± 0.030	1.760 ± 0.010	5.78 ± 0.06	0.824 ± 0.007

Table C.4: Holo 800 MHz relaxation and ModelFree data of $Ci\text{-}\beta\gamma$

Sequence	Residue	NOE	R ₁	R ₂	S ²
2	GLY	0.833 ± 0.045	1.800 ± 0.027	6.08 ± 0.13	0.874 ± 0.011
3	LYS	0.829 ± 0.046	1.700 ± 0.026	5.56 ± 0.10	0.817 ± 0.010
4	ILE	0.829 ± 0.048	1.700 ± 0.010	5.73 ± 0.04	0.824 ± 0.004
5	ILE	0.800 ± 0.048	1.940 ± 0.009	6.16 ± 0.02	n/a
6	LEU	0.831 ± 0.047	1.870 ± 0.006	6.01 ± 0.02	n/a
7	PHE	0.809 ± 0.047	1.710 ± 0.024	6.03 ± 0.04	0.822 ± 0.012
8	GLU	0.784 ± 0.036	1.740 ± 0.025	6.29 ± 0.03	0.839 ± 0.012
9	ASP	0.852 ± 0.037	1.970 ± 0.018	6.71 ± 0.04	0.950 ± 0.008
10	VAL	0.802 ± 0.035	1.770 ± 0.010	6.18 ± 0.03	0.853 ± 0.005

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Table C.4 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
11	GLU	0.876 ± 0.045	1.950 ± 0.009	6.45 ± 0.03	0.941 ± 0.003
12	PHE	0.903 ± 0.047	1.970 ± 0.020	6.77 ± 0.03	0.982 ± 0.004
13	GLY	0.895 ± 0.040	1.840 ± 0.015	6.39 ± 0.008	n/a
14	GLY	0.855 ± 0.041	1.760 ± 0.005	6.21 ± 0.02	0.850 ± 0.003
15	LYS	0.780 ± 0.046	1.790 ± 0.012	6.58 ± 0.022	0.860 ± 0.006
16	LYS	0.745 ± 0.047	1.820 ± 0.006	6.28 ± 0.015	0.876 ± 0.003
17	LEU	0.811 ± 0.044	1.880 ± 0.015	6.03 ± 0.024	0.884 ± 0.003
18	GLU	0.785 ± 0.060	1.780 ± 0.005	5.74 ± 0.020	0.831 ± 0.004
19	LEU	0.799 ± 0.061	1.760 ± 0.011	5.73 ± 0.047	0.845 ± 0.004
20	GLU	0.834 ± 0.074	1.650 ± 0.015	5.57 ± 0.031	0.807 ± 0.004
21	THR	0.785 ± 0.052	1.790 ± 0.010	6.17 ± 0.051	0.860 ± 0.005
22	SER	0.711 ± 0.043	1.460 ± 0.054	5.13 ± 0.15	0.730 ± 0.017
23	VAL	0.898 ± 0.066	1.800 ± 0.016	5.82 ± 0.081	0.863 ± 0.006
24	SER	0.808 ± 0.063	1.750 ± 0.011	5.86 ± 0.035	0.849 ± 0.004
25	ASP	0.870 ± 0.051	1.850 ± 0.052	6.19 ± 0.22	0.895 ± 0.018
26	LEU	0.766 ± 0.042	1.870 ± 0.017	5.88 ± 0.088	0.889 ± 0.006
27	ASN	0.830 ± 0.040	1.960 ± 0.040	6.74 ± 0.074	0.974 ± 0.010
28	VAL	0.788 ± 0.038	1.910 ± 0.022	6.86 ± 0.049	0.920 ± 0.010
29	HIS	0.819 ± 0.045	1.850 ± 0.009	7.15 ± 0.054	0.891 ± 0.005
30	GLY	0.795 ± 0.043	1.780 ± 0.031	6.57 ± 0.058	0.855 ± 0.015
31	PHE	0.806 ± 0.042	1.770 ± 0.024	6.33 ± 0.067	0.851 ± 0.012
32	ASN	0.783 ± 0.039	1.930 ± 0.021	6.50 ± 0.091	0.937 ± 0.008
33	ASP	0.829 ± 0.044	1.780 ± 0.045	6.03 ± 0.19	0.867 ± 0.018
34	ILE	0.845 ± 0.062	1.800 ± 0.046	6.52 ± 0.19	0.866 ± 0.022

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Table C.4 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
35	VAL	0.786 ± 0.049	1.790 ± 0.049	6.28 ± 0.18	0.886 ± 0.018
36	SER	0.825 ± 0.054	1.790 ± 0.019	5.87 ± 0.083	0.862 ± 0.007
37	SER	0.811 ± 0.033	1.790 ± 0.036	6.53 ± 0.049	0.862 ± 0.018
38	ILE	0.782 ± 0.042	1.720 ± 0.023	6.23 ± 0.047	0.830 ± 0.012
39	ILE	0.780 ± 0.047	1.800 ± 0.011	6.00 ± 0.030	0.872 ± 0.003
40	VAL	0.864 ± 0.057	1.850 ± 0.009	6.13 ± 0.032	0.892 ± 0.003
41	GLU	0.804 ± 0.074	1.800 ± 0.026	6.41 ± 0.051	0.869 ± 0.012
42	SER	0.852 ± 0.060	1.790 ± 0.023	6.33 ± 0.084	0.864 ± 0.011
43	GLY	0.798 ± 0.090	1.790 ± 0.045	5.65 ± 0.18	0.847 ± 0.016
44	THR	0.739 ± 0.059	1.620 ± 0.019	5.31 ± 0.086	0.780 ± 0.007
45	TRP	0.789 ± 0.051	1.790 ± 0.008	5.76 ± 0.061	0.858 ± 0.003
46	PHE	0.854 ± 0.063	1.760 ± 0.007	5.78 ± 0.053	0.846 ± 0.003
47	VAL	0.818 ± 0.048	1.770 ± 0.018	6.06 ± 0.041	0.853 ± 0.009
48	PHE	0.837 ± 0.051	1.770 ± 0.023	6.21 ± 0.053	0.850 ± 0.011
49	ASP	0.878 ± 0.049	1.810 ± 0.022	5.97 ± 0.099	0.872 ± 0.009
50	ASP	0.899 ± 0.047	1.820 ± 0.051	6.83 ± 0.093	0.878 ± 0.026
51	GLU	0.841 ± 0.042	1.810 ± 0.012	6.14 ± 0.068	0.876 ± 0.005
52	GLY	0.841 ± 0.055	1.860 ± 0.020	6.42 ± 0.060	0.896 ± 0.009
53	PHE	0.822 ± 0.045	1.840 ± 0.038	6.56 ± 0.078	0.887 ± 0.018
54	SER	0.813 ± 0.037	1.790 ± 0.021	5.80 ± 0.075	0.855 ± 0.008
55	GLY	0.793 ± 0.043	1.670 ± 0.016	6.07 ± 0.073	0.805 ± 0.008
56	PRO	n/a	n/a	n/a	n/a
57	SER	0.778 ± 0.040	1.720 ± 0.029	5.83 ± 0.074	0.841 ± 0.009
58	TYR	0.820 ± 0.042	1.790 ± 0.015	5.96 ± 0.062	0.864 ± 0.006

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Table C.4 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
59	LYS	0.785 ± 0.047	1.770 ± 0.011	5.70 ± 0.046	0.844 ± 0.004
60	LEU	0.841 ± 0.057	1.760 ± 0.023	6.07 ± 0.045	0.847 ± 0.011
61	THR	0.783 ± 0.040	1.630 ± 0.030	5.25 ± 0.11	0.776 ± 0.011
62	PRO	n/a	n/a	n/a	n/a
63	GLY	0.795 ± 0.044	1.800 ± 0.016	6.07 ± 0.048	0.878 ± 0.005
64	LYS	0.755 ± 0.060	1.610 ± 0.035	5.34 ± 0.10	0.778 ± 0.011
65	TYR	0.834 ± 0.075	2.060 ± 0.011	6.70 ± 0.063	0.989 ± 0.005
66	PRO	n/a	n/a	n/a	n/a
67	ASN	0.812 ± 0.028	1.860 ± 0.035	6.23 ± 0.13	0.902 ± 0.012
68	PRO	n/a	n/a	n/a	n/a
69	GLY	0.803 ± 0.042	1.750 ± 0.048	6.09 ± 0.14	0.868 ± 0.015
70	SER	0.829 ± 0.037	1.750 ± 0.066	6.44 ± 0.17	0.841 ± 0.032
71	TRP	0.856 ± 0.041	1.850 ± 0.025	6.58 ± 0.12	0.893 ± 0.012
72	GLY	0.824 ± 0.040	1.800 ± 0.027	6.16 ± 0.085	0.882 ± 0.009
73	GLY	0.827 ± 0.040	1.840 ± 0.019	6.20 ± 0.084	0.893 ± 0.007
74	ASN	0.775 ± 0.053	1.620 ± 0.042	5.41 ± 0.20	0.782 ± 0.016
75	ASP	0.825 ± 0.127	1.650 ± 0.072	5.52 ± 0.27	0.801 ± 0.027
76	ASP	0.826 ± 0.037	1.780 ± 0.043	5.88 ± 0.18	0.858 ± 0.016
77	GLU	0.789 ± 0.057	1.820 ± 0.046	5.90 ± 0.21	0.872 ± 0.018
78	LEU	0.800 ± 0.042	1.710 ± 0.044	6.08 ± 0.19	0.848 ± 0.017
79	SER	0.821 ± 0.056	1.720 ± 0.019	5.70 ± 0.090	0.831 ± 0.008
80	SER	0.781 ± 0.033	1.930 ± 0.028	6.29 ± 0.084	0.922 ± 0.009
81	VAL	0.825 ± 0.070	1.890 ± 0.013	6.07 ± 0.072	0.903 ± 0.005
82	LYS	0.788 ± 0.057	1.950 ± 0.005	6.34 ± 0.053	0.940 ± 0.002

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Table C.4 – continued from previous page

Sequence	Residue	NOE	R ₁	R ₂	S ²
83	GLN	0.787 ± 0.063	1.780 ± 0.036	5.84 ± 0.11	0.856 ± 0.012
84	GLN	0.783 ± 0.064	1.810 ± 0.006	5.80 ± 0.029	0.839 ± 0.006