NMR SPECTROSCOPY OF SMALL SIZED PROTEINS

THESIS SUBMITTED TO THE CENTRAL UNIVERSITY OF PUNJAB

FOR THE AWARD OF

MASTERS DEGREE

IN

CHEMICAL SCIENCES

BY

VISHAL SHARMA

SUPERVISOR

Dr. RAJESH KUMAR

Associate Professor

DEPARTMENT OF CHEMICAL SCIENCES

SCHOOL OF BASIC AND APPLIED SCIENCES

CENTRAL UNIVERSITY OF PUNJAB, BATHINDA

DECLARATION

I declare that the dissertation/thesis entitled "NMR SPECTROSCOPY OF SMALL SIZED PROTEINS" has been prepared by me under the guidance of Dr. Rajesh Kumar, Associate Professor, Chemical Sciences, Basic and Applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Vishal Sharma
Department of Chemical Sciences,
Basic and Applied Sciences
Central University of Punjab, Bathinda
Date:

CERTIFICATE

I certify that VISHAL SHARMA has prepared his thesis entitled "NMR SPECTROSCOPY OF SMALL SIZED PROTEINS", for the award of, M.Sc. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Chemical Sciences, Basic and Applied Sciences, Central University of Punjab.

Dr. Rajesh Kumar
Associate Professor

Department of Chemical Sciences
Basic and Applied Sciences
Central University of Punjab, Bathinda
Date:

ABSTARCT

NMR SPECTROSCOPY OF SMALL SIZED PROTEINS

Name of Student	Vishal Sharma
Registration Number	16mscchm05
Degree for Which Submitted	M.Sc. Chemistry
Name of Supervisor	Dr. Rajesh Kumar
Name of Department	Chemical Sciences
Name of School	Basic and Applied Sciences

In this project, the NMR spectra of proteins is discussed and the one dimensional ^{1}H NMR protein spectra of ferricytochrome and lysozome is collected to study the conformational changes in them by varying the pH values from neutral to alkaline. In this thesis it is focused on that why we aren't able to study larger and complex protein molecules with NMR spectra, what are the advantages of NMR spectra in the study of proteins and why we need multidimensions in protein NMR.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my advisor Prof. **Dr. Rajesh Kumar** for the continuous support of my M.Sc study and related research, for his motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor for my M.Sc project.

Last but not the least, I would like to thank my family, other professors of the department and friends for supporting me spiritually throughout writing this thesis and my life in general.

TABLE OF CONTENTS

TABLE OF ABBERIVATIONS

Sr. No.	FULL FORM	ABBERIVATION
1	NUCLEAR MAGNETIC RESONANCE	NMR
2	NUCLEAR OVERHAUSER EFFECT	NOE
3	HETERONUCLEAR SINGLE QUANTUM CO-RELATION SPECTROSCOPY	HSQC
4	HETERONUCLEAR MULTIDIMENSIONAL QUANTUM CO-RELATION SPECTROSCOPY	HMQC
5	TRANSVERSE RELAXATION OPTIMIZED SPECTROSCOPY	TROSY
6	CO-RELATIONOVERHAUSER SPECTROSCOPY	COSY
7	NUCLEAR OVERHAUSER SPECTROSCOPY	NOESY
8	TOTALCO-RELATION SPECTROSCOPY	TOCSY
9	INSESNSITIVE NUCLEI ENHANCED BY POLARIZATION	INEPT
10	PHOTOCHEMICALLY INDUCED DYNAMIC NUCLEAR POLARIZATION	PHOTO-CIDNP

LIST OF FIGURES

CHAPTER-I

INTRODUCTION

INTRODUCTION

The NMR spectroscopy is a very important technique to investigate the structure and dynamics of biomolecules (Sattler *et al.* 1999). Biological NMR can also assist in determining the subtle information about the chemical activity of proteins. NMR technology is invented by physicists. It is an important technique for organic chemists to elucidate the structure of organic molecules. Biochemist and biophysics scientist applied NMR spectroscopy in structural biology area to find out the NMR structures of biomolecules (Jacobsen 2007). In the 1980s, scientist developed solution NMR techniques to elucidate the three dimensional structure of biomolecules. Prof. Kurt Wuthrich won the Nobel Prize in chemistry (2002) for developing NMR methods for studying biological macromolecules (Rule *et al.* 2006). Within a molecule, NMR relies on ^{1}H, ^{13}C, and ^{15}N nuclei. By estimating the perturbations of chemical shifts, NMR can be implied to map the binding of small sized molecules onto the surface of a protein. Recent development in the NMR technology has focused on methods for studding the protein up to 60 kD by increasing the field strength from 600 MHz to 900 MHz and using new technologies such as gradients and shaped pulses in NMR (Kumar *et al.* 2006) (Rao *et al.* 2006). Protein NMR experiments can be carried out by both solution state and sold state NMR. In general, the directionally dependent interactions are present sold state NMR but these interactions are subdued by Brownian motion in solution NMR. The solution state NMR generally used to study the comprehensive molecular motion of biomolecules. The diffusion along any three axes as well as the direction and speed of flow can be measured by Pulse-field gradients. Rotation of the whole biomolecule could be monitored by its effect on the relaxation parameters of any of the nuclei within the molecule.

Multidimensional biological NMR techniques can be used to study structure and dynamics of proteins (Pervushin *et al.* 1997). The major problem in multidimensional biological NMR is that it limits the size of protein under investigation due to complexity and linewidth problems. The complexity problem

can be subdued using the NMR active nuclei and 3D and and 4D experiments while the linewidth problem can be resolved through replacing all the N-H protons with deuterium. In general, the determination of 3D structure of proteins depends on two factors, including (i) the measurement of nuclear overhauser effect (NOE) distances, and (ii) dihedral angles.

CHAPTERT-II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The 3D structures of proteins at atomic resolution can be resolved or determined by NMR and X-ray crystallographic techniques. If one can able to prepare the crystal of a protein than X-ray crystallographic techniques can be implied to determine the 3D protein structure. However, if a protein is unable give crystal than the NMR spectroscopy is the only technique that can solved the 3D structure of protein in solution.

2.1 LABELING IN NMR

In 1D experiments, for studying the protein molecules, the stable isotope labeling technique is regarded a powerful method. In general, for isotopic labeling, the carbon and nitrogen nucleus are replaced by ^{13}C and ^{15}N. The labeling of a protein can bring another chemical shift scale and that can decrease the size and overlap of protein. Isotopic labeling can make simpler overlapped spectra through either diluting the NMR active nuclei or allowing the resonances to be separated in multiple dimensions

2.2 SENSITIVITY IN NMR

To perform the protein NMR, the sensitivity is a major issue due to concentrated protein sample (e.g 10^2 uM to 1 mM). New technique, such as Laser-assisted NMR or photochemically induced dynamic nuclear polarization (Photo-CIDNP) in combinations with cryogenic probe can be used to overcome these sensitivity issues. Photo-CIDNP heteronuclear correlation spectroscopy in combination with cryogenic probe gives better signal-to-noise ratio relative to the constant-time SOFAST HMQC and SE-HSQC pulse sequences.

2.3 Protein NMR initially (1D NMR)

The solving of protein structure using solution state NMR requires one has to measure many of short proton-proton distances (measured by NOE) and angles, and restraining the protein structure with these computationally.

2.4 RECENT ADVANCES IN PROTEIN NMR

The protein NMR continues to be a powerful technique to study the structure, physicochemical and biological properties of protein molecules (Macomber 1998). Till now several advances were took place in protein NMR, including the its advancement from single to multidimensional NMR and combination of NMR with new techniques such as stopped flow (real time NMR). Recent, protein NMR techniques are based on multidimensional experiments involving the ^{1}H, ^{13}C, and ^{15}N nuclei in isotopically labeled proteins. NOE is the very fundamental method to estimate the proton-proton distance. Because all the protons do not have unique chemical shift, therefore the peaks of these protons spectra overlap (Frydman *et al.* 2002). To overcome this overlap, the higher dimensions (3D NOESY and 4D NOESY) can be include in the NOESY spectroscopy. In 4D NOESY spectroscopy, the proton–proton cross peaks can be separated by the chemical shifts of hetero-atoms attached to each proton. The 4D NMR experiment used to seprate NOE interactions between ^{13}C attached protons.

2.5 HOW MANY DIMENSIONS WE NEED IN A PROTEIN NMR

While the dimensions in protein NMR are increased from 1D to 4D but the basic question in protein NMR spectroscopy is that what is the correct dimensions are required is not clear. According to Sorensen, 3D or 4D NMR spectra are regarded as mathematical products of their corresponding 2D building blocks. As a result, the 3D or 4D experiments do not provide the new information but merely resolve overlap problems present in the 2D spectra (Sanders *et al.* 1988). The main outcome due to increase in dimensions in NMR spectrum is to decrease the

spectral overlap. The resolution in the 2D spectrum may be improved by taking longer acquisition times in the orthogonal time dimensions. In addition, the inherent sensitivity of the sample can be reduced sharply by extending the acquisition time far beyond the transverse relaxation time T_2.

CHAPTER-III

EXPERIMENTAL SECTION

3.1 Materials and methods

Horse heart cytochrome *c* (Cyt *c*; type VI), salts of buffer (sodium phosphate, and 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS)) were purchased from Sigma. Hen egg white lysozyme (Lyz) was purchased from Calbiochem. All other used chemicals were of analytical grade. The desired pH of the samples solutions was adjusted by using the concentrated DCl and NaOD solutions.

3.2 Measurement of ^{1}H NMR spectra of native and base-denatured Ferricytochrome- c and Lysozyme

For preparation of native states, D_2O solution containing 1.0 mM protein (Ferricyt c or Lyz) was adjusted to pH 7.0 (0.05 M phosphate) using concentrated DCl or NaOD. For preparation of base-denatured states, D_2O solution containing 1.0 mM protein (Ferricyt *c* or Lyz) and 1.0 mM CAPS, was adjusted to pH 12.9 ($\pm$0.1) using concentrated NaOD.

CHAPTER-IV

Results and Discussion Section

Result and Discussion

The 1H NMR spectra of lysozyme and ferri-cytochrome are collected to investigate the effect of alkaline pH on secondary and tertiary structures of these proteins. Increase in the pH from neutral to extreme alkaline can alter the stability of these proteins due to alkaline pH-denaturation.

The NMR spectra of ferrricytochrome at pH 7.0 and at pH 13.7 are shown in Fig. 1A and Fig. 1B, respectively.

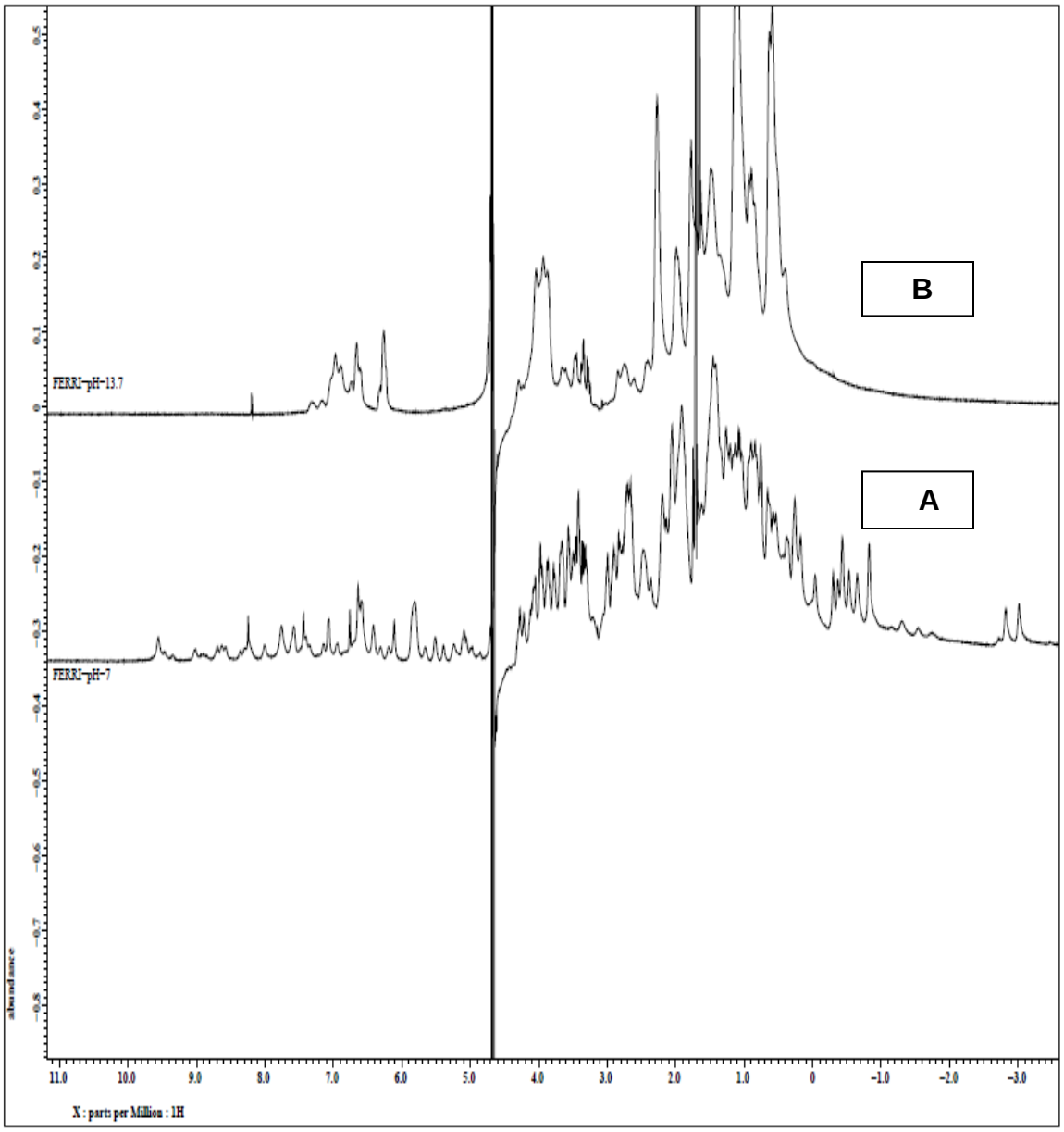

Fig 1. The comparison of NMR spectra of ferricytochrome in the native sate (pH 7) and base-denatured state (pH 13.7), 25 °C. In this figure the spectra mentioned as A represents the folded sate or native state of ferricytochrome at pH-7 and the spectra mentioned as B represents the denatured structure of the ferri-cytochrome at the pH-13.7. The vertical axis represents the abundance of protein backbone residues and the horizontal axis represents the chemical shift in ppm of proton attached to the protein backbone residues.

The NMR spectra of lysozyme at pH 7.0 and at pH 13.7 are shown in Fig. 2A and Fig. 2B, respectively. By comparing the panel (A) and (B) in Fig 1 and Fig. 2, it has been concluded that as pH is increased from pH 7.0 to pH 13.7, (i) the proton resonances observed at pH 7.0 is disappeared at pH 13.7 in the aromatic region and as well as in the aliphatic region, and (ii) the proton signal intensity increases at pH 13.7. This result reveals that at neutral pH 7.0 the protein exists in native folded state but as the pH increases to alkaline and reaches a value of 13.7 the protein gets denatured due unfavorable charge repulsion.

.

The intensity of protons in denatured states of ferricytochrome c and lysozyme at pH 13.7 increases because as protein denatures due to unfavorable charge repulsion it approaches towards the primary structure where proton peaks overlap due to the same chemical environment.

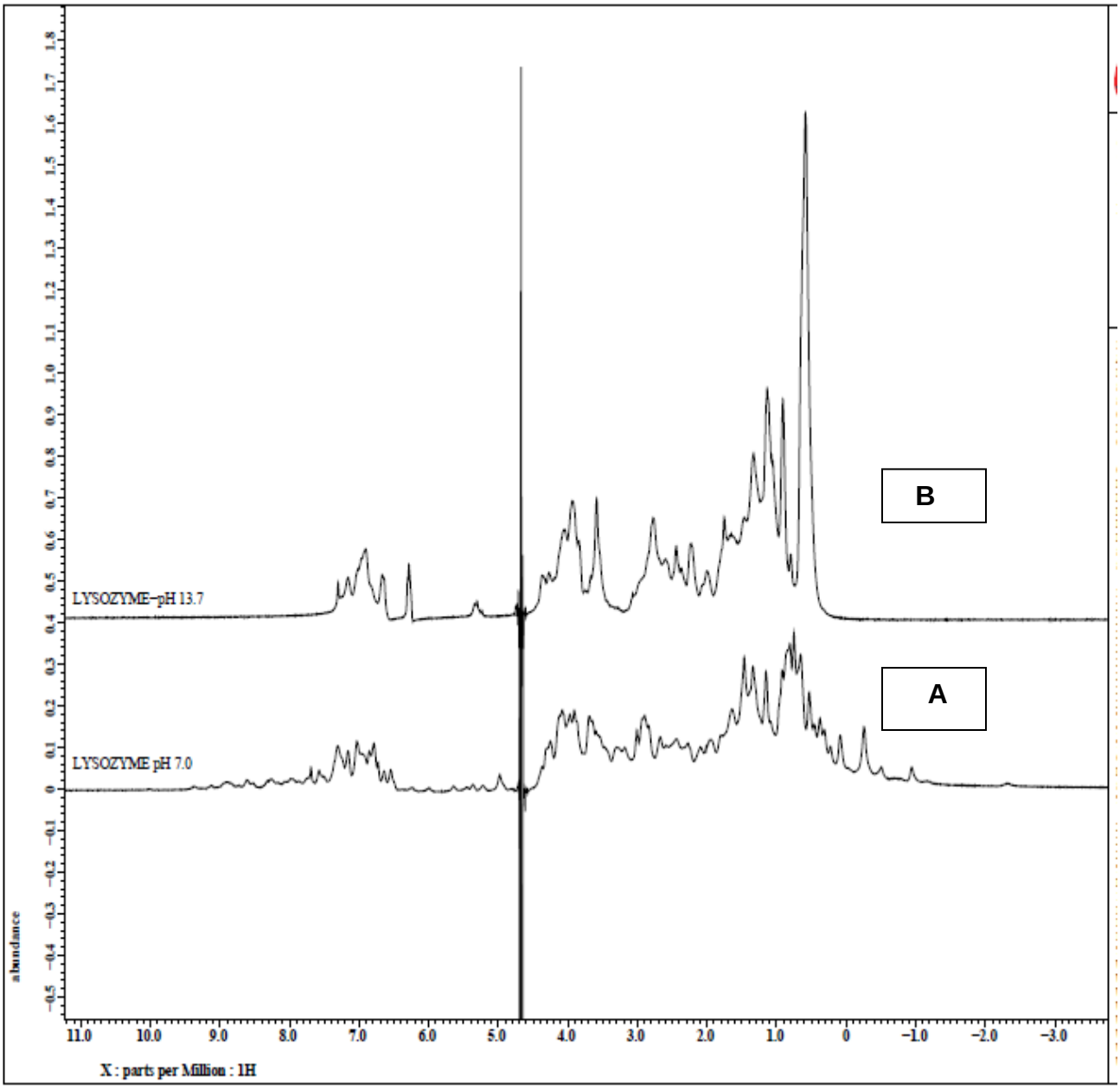

Fig.2 The comparison of NMR spectra of lysozyme at pH 7.0 and at pH 13.7. The spectra mentioned as A represents the native state of lysozyme at pH-7 and the spectra mentioned as B represents the denatured state of lysozyme at pH-13.7. The vertical axis represents the abundance of protein backbone residues and the horizontal axis

represents the chemical shift in ppm of proton attached to the protein backbone residues.

CHAPTER-V

CONCLUSION

CONCLUSION

From all the discussion and the experimental section it has been concluded that there is dispersion and sharpness of resonance in the native state of ferricytochrome *c* and lysozyme at pH-7, but as the pH of these samples increases from pH 7.0 to pH 13.7, the sharpness of resonances and dispersion is lost, which indicates that at the pH 13.7, ferricytochrome c and lysozyme were denatured at alkaline pH conditions due to the interactions among amino acids has been broken in the native sate to achieve the denatured state. This study in future can be extended for large size proteins by increasing the dimensions or the acquisition time in the protein NMR spectroscopy and by increasing the sensitivity of the sample and labeling the nucleus in proteins.

REFERENCES

Frydman L, Scherf T,Lupulescu A. (2002). The Acquisition of Multidimensional Nmr Spectra within a Single Scan. Proceedings of the National Academy of Sciences, **99**, 15858-15862.

Jacobsen N E. (2007). Nmr Spectroscopy Explained: Simplified Theory, Applications and Examples for Organic Chemistry and Structural Biology: John Wiley & Sons.

Kumar R, Prabhu N P, Rao D K,Bhuyan A K. (2006). The Alkali Molten Globule State of Horse Ferricytochrome C: Observation of Cold Denaturation. Journal of molecular biology, **364**, 483-495.

Macomber R S. (1998). A Complete Introduction to Modern Nmr Spectroscopy: Wiley New York.

Pervushin K, Riek R, Wider G,Wüthrich K. (1997). Attenuated T2 Relaxation by Mutual Cancellation of Dipole–Dipole Coupling and Chemical Shift Anisotropy Indicates an Avenue to Nmr Structures of Very Large Biological Macromolecules in Solution. Proceedings of the National Academy of Sciences, **94**, 12366-12371.

Rao D K, Kumar R, Yadaiah M,Bhuyan A K. (2006). The Alkali Molten Globule State of Ferrocytochrome C: Extraordinary Stability, Persistent Structure, and Constrained Overall Dynamics. Biochemistry, **45**, 3412-3420.

Rule G S,Hitchens T K. (2006). Fundamentals of Protein Nmr Spectroscopy (Vol. 5): Springer Science & Business Media.

Sanders J K,Hunter B K. (1988). Modern Nmr Spectroscopy: A Guide for Chemists.

Sattler M, Schleucher J,Griesinger C. (1999). Heteronuclear Multidimensional Nmr Experiments for the Structure Determination of Proteins in Solution. Progress in nuclear magnetic resonance spectroscopy, **34**, 93-158.