

**SPECTROSCOPIC AND THERMODYNAMIC STUDIES OF THE NON-  
NATIVE CONFORMATIONS OF HORSE CYTOCHROME C AND THEIR  
INTERACTION WITH NUCLEOTIDES**

BY

**UNNATI AHLUWALIA**

**Department of Chemistry**

SUBMITTED

IN FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF

**DOCTOR OF PHILOSOPHY**

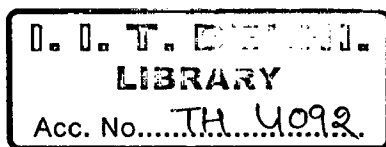
TO THE



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

APRIL 2011

Nucleotides; spectroscopic analysis.



TH

577.113.3

AHL-5

## CERTIFICATE

This is to certify that the thesis entitled, “**Spectroscopic and thermodynamic studies of the non-native conformations of horse cytochrome c and their interaction with nucleotides**”, being submitted by **Ms. Unnati Ahluwalia** to the Indian Institute of Technology Delhi for the award of the degree of Doctor of Philosophy in Chemistry is a record of bonafide research work carried out by her. **Ms. Unnati Ahluwalia** has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

  
**Dr. Shashank Deep**

Department of Chemistry

Indian Institute of Technology Delhi

New Delhi - 110016

## ACKNOWLEDGEMENT

*If I have seen a little further, it is by standing on the shoulders of Giants* -- Isaac Newton

*I have not walked alone in this task, and there have been so many who, have inspired and motivated me. I stand humbled before all those who make me worthwhile. Firstly, my supervisor Dr. Shashank Deep, the backbone of this work, has made me familiar with the world of physical chemistry of proteins. He as a versatile scientist has helped me ambitiously plan my work. His pragmatic approach, eagerness to explore new areas, kind heartedness and patience has made my thesis pursuit a spiritual experience. As my supervisor he has allowed me to take up new challenges and stood by in all the difficulties we faced.*

*I gratefully acknowledge Prof Rajiv Bhat, School of Biotechnology, JNU, as my external supervisor. His contribution in my research spans extensive discussions around my work and interesting observations which have been very helpful for this study. His encouragement and guidance have provided a good basis for the present thesis. I am grateful to the JNU–Advanced instrumentation and research facility for the instrumentation support.*

*I would like to thank the members of my student research committee, as well as the past and present Heads of the Department and DRC Chairpersons for their kind support. I gratefully acknowledge the IIT Delhi central library, for the journals and the loan facility. I am also grateful for the infrastructure facilities and lucid atmosphere in the department. I thank the staff of Department of Chemistry, Instrumentation and NMR facility, and Stores for the efficiency and timely help. I am grateful to CSIR for the financial assistance.*

*I would like to express gratitude to Prof. Nand Kishore at the Department of Chemistry IIT B Mumbai, for his help in carrying out the ITC experiments, helpful trouble shooting and the beneficial stay at his facility. The experiments, discussions and students at the laboratory have*

*enriched me with knowledge. I also thank Dr. Faizan Ahmad at Jamia Milia Islamia for the use of the Dynamic light scattering facility. These people have been benevolent, and have made my research path a possibility.*

*I would like to thank Prof Sunil Kumar Khare Prof. Siddharth Pandey, Dr. N. G. Ramesh, Dr. Pramit Chaudhry, and Dr. Sameer Sapra for the extending me access to their laboratory as well as their encouragement. I would extent my heartfelt gratitude to Prof. Charusita Charavarty and Prof. Ashok K Ganguli, for being a source of positive energy and inspiration. I look up to them as my ideals. I thank Dr. Tapan Chaudhuri for initiating me to the field of structural biology.*

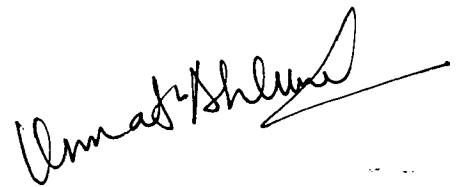
*I am happy to have Md. Shahid Nayeem, Ashhar and Komal, as my colleagues who have shared their endeavours to build the laboratory as a wonderful progressive work place and for the wonderful scientific discussions. The students Rakhi, Vinay, Preeti and Shivnetra, who have made it a vibrant experience. My interaction with Akash, Chetan, Aritrika, have made research and life in laboratory animated. The holistic support of the students Shruti Trivedi, Dr. Ram Karan, Rajeshwari, Shadrack, Nagraj, Vikas and Ashima is fondly appreciated. I would thank Dr. Neeraj Mishra and Dr. Saurabh Singh at JNU-SBT for sharing their expertise in thermodynamics, as well as Mr. Manish and Dr. Swapnil Sharma for generously accommodating me in their laboratory. I would thank Dr. Niki Shweta Jha at IIT B for her expertise in ITC, and the time we could explore the campus and the city The coolest bunch of people with whom I shared happy moments Archana, Geeta, Usha, Rachna, Jency and Priyanka for being the coolest bunch.*

*My friends Chetna Joshi and Manish Agarwal have given me spiritual and intelligent support and many helpful conversations over coffee, their friendship is cherished. Chetna has been my anchor and reason to show up in laboratory every day. They saw me through the crests and troughs. I would like to mention my friend Gaurav for enthusing me to explore this career, as well as my*

*friends, Svati and Ajay for being the source of stability and sanity in my life and to listen to my dreams, plans and problems.*

*The blessings of my Grandparents have kept me nurtured in all times. I cannot thank enough to my parents for their faith and support. I owe them my abilities, my principles, my hard work and good fortune to be educated and always providing the best environment during my growth. A “thank you” would be less for my brother Rajat for the trips he made to provide me home food when I was working in JNU and for my car maintenance (The beloved blue Sienna). My Uncles and Aunts and beloved cousins who remembered me in their prayers. I am also reminded of Ruby for she has taken away all the stress from me.*

*I thank God almighty for health of all and this life, ,the good opportunities and good people that he has sent along the path,.*



**Unnati Ahluwalia**

2005 CYZ8249

## ABSTRACT

Structural information on the intermediates/non-native conformation in protein folding/unfolding pathway is critical for the understanding of the molecular basis of protein folding. Cytochrome c (cyt c) has long been serving as a model protein for developing new concepts and approaches in protein folding. Cyt c unfolds and refolds by a pathway consisting of same number of intermediates via step-by-step folding of cooperative units. Structural and thermodynamic characterization of these intermediates helps in understanding of the folding mechanism of the proteins. Non-native conformations of proteins are also considered to play an important role in many physiological events. Intermediates/altered forms of cytochrome c (cyt c) are particularly important considering their participation in electron transfer and programmed cell death (apoptosis). Understanding such a complex system requires a thorough investigation of the entire biophysical aspect of these non-native conformations such as their structure, stability and thermodynamics of interaction. The thesis entitled **'SPECTROSCOPIC AND THERMODYNAMIC STUDIES OF THE NON NATIVE CONFORMATIONS OF HORSE CYTOCHROME C AND THEIR INTERACTION WITH NUCLEOTIDES'** is concerned with the characterization of the non-native conformations of cyt c in different environments. Another important aspect of this thesis is the study of interaction of such non-native conformations with nucleotides since these interactions are the basis of many cellular events.

The thesis has been divided into seven chapters. Chapter 1 provides a brief introduction to models of folding and a brief review of different types of non-native conformations described in literature and their significance in metabolic pathways. It also

describes the various tools available in literature to differentiate between different types of non-native conformation of proteins. The chapter also provides an introduction to cytochrome c and a detailed review of progress made in understanding the various non-native conformations of cyt c and their biological significance in processes like electron transfer and apoptosis. Chapter 2 titled “Materials and Methodologies” is about protein and chemical procurement, purification as well as techniques and methodologies used in the investigation. The chapter also describes the procedures for analyzing the data. Chapter 3 (**Sugar and phosphate induced non-native conformations of acid denatured cytochrome c**) discusses the effect of sucrose, a sugar, and phosphate anion on the induction of intermediate conformation in acid denatured cyt c. Based on the data obtained, similarity and subtle differences among the sucrose and phosphate induced conformations were discussed. Chapter 4 (**Interaction of nucleotides with acid denatured cytochrome c and structural and thermal characterization of resulting non-native conformations**) presents the investigation of the effect of the adenosine nucleotides (ATP/ADP/AMP) on the acid denatured cyt c. The chapter also discuss the effect of other nucleotides on the acid-denatured cyt c. Effect of Nucleotides on cyt c was discussed in terms of coupled binding-folding equilibria. Chapter 5 (**The non-native conformations of cytochrome c in sodium dodecyl sulfate and their modulation by ATP**) presents the investigation of the effect of SDS, an anionic detergent, on the conformation of the cyt c. The effect of nucleotides on the conformation of SDS bound cyt c was also investigated. Docking of cyt c with SDS and ATP/GTP was carried out to characterize the putative binding sites of these molecules on cyt c. Information on the docking sites, thus obtained, was used to explain the experimental data obtained from the

binding of cyt c with SDS and the effect of ATP/GTP on the SDS bound cyt c. **Chapter 6 (The non-native conformations of cytochrome c in Cardiolipin/Oleic acid and their modulation by ATP)** deals with the characterization of non-native conformation of cyt c obtained by interaction of native cyt c with lipid cardiolipin/oleic acid and its comparison with other non-native conformations of cyt c. Based on the structural data, the unfolding of the cyt c was explained in terms of various domains. The effect of nucleotides on the cardiolipin/oleic acid induced conformation was also discussed. Information on the docking sites obtained from docking of cyt c and cardiolipin, thus obtained, was used to explain the experimental data obtained from the binding of cyt c with cardiolipin/oleic acid and the effect of ATP on the cardiolipin/oleic acid bound cyt c. **Chapter 7 (Summary and Future Perspectives)** contains salient features of this work. Taken together, the findings/observation of this thesis will help develop a much needed fundamental and molecular-level view of the non-native conformations of the cyt c. In brief, it is concluded that the different non-native conformations of protein may be obtained depending on the protein environment. The effect of nucleotides is different on the different non-native conformation of the protein.

# CONTENTS

	Page No.
<b>Certificate</b>	I
<b>Acknowledgements</b>	II
<b>Abstract</b>	V
<b>Contents</b>	VIII
<b>List of Figures</b>	XVII
<b>List of Tables</b>	XXV
<b>Chapter 1: Introduction</b>	1
<b>1.1. Introduction</b>	2
<b>1.2. The protein folding</b>	3
1.2.1    Levinthal paradox-presence of intermediate	3
1.2.2    Models of folding	5
1.2.2.1 <i>Classical view: Folding Pathways</i>	5
1.2.2.1.1 <i>Nucleation and Growth model</i>	5
1.2.2.1.2. <i>Sequential protein folding Model</i>	5
1.2.2.1.3 <i>Diffusion-Collision Mechanism</i>	6
1.2.2.1.4 <i>Hydrophobic Collapse model</i>	7
1.2.2.2 <i>New View: Energy landscape and folding funnel</i>	7
1.2.2.3 <i>Old versus New view of Protein Folding</i>	10
<b>1.3. Proteins conformational states: stability and dynamics</b>	11
1.3.1    The Globular proteins	11
1.3.1.1 <i>The native state</i>	13

1.3.1.2	<i>The molten globule state</i>	15
1.3.1.3	<i>The unfolded states</i>	18
1.3.2.	Intrinsically disordered protein	19
<b>1.4.</b>	<b>Biological function of different conformations of protein</b>	<b>21</b>
<b>1.5.</b>	<b>Methods to probe the structure of folding-unfolding intermediates/ intrinsically disordered proteins.</b>	<b>29</b>
<b>1.6.</b>	<b>Cytochrome c: A model protein for folding studies and well known for its physiologically relevant non-native conformations</b>	<b>35</b>
1.6.1	Structural features of cyt c	35
1.6.2	Importance of Cyt c to study the protein folding mechanism	36
1.6.3	Biological functions of Cytochrome c	39
1.6.3.1	<i>Electron transfer</i>	30
1.6.3.2	<i>Apoptotic function</i>	40
<b>1.7.</b>	<b>Origin of problem in the context of this thesis</b>	<b>41</b>
<b>1.8.</b>	<b>Aim and Outline of the present research problem</b>	<b>43</b>
<b>1.9.</b>	<b>References</b>	<b>45</b>
<b>Chapter 2.</b>	<b>Materials and Methodologies</b>	<b>72</b>
<b>2.1.</b>	<b>Introduction</b>	<b>73</b>
<b>2.1.</b>	<b>Materials</b>	<b>73</b>
<b>2.3.</b>	<b>Techniques</b>	<b>77</b>
2.3.1.	Techniques used for structural characterization	77
2.3.1.1	<i>Circular dichroism</i>	77

2.3.1.2	<i>UV-Vis absorbance</i>	80
2.3.1.3	<i>Intrinsic Fluorescence</i>	82
2.3.1.4	<i>Extrinsic fluorescence</i>	83
2.3.2	Techniques employed for exploring the stability of protein	84
2.3.2.1	<i>Spectroscopic methods</i>	84
2.3.2.1.1	<i>Thermal UV-Vis profiles</i>	85
2.3.2.1.2	<i>Thermal intrinsic fluorescence profiles</i>	85
2.3.2.1.3	<i>Thermal CD profiles</i>	86
2.3.2.1.4.	<i>Analysis of thermal profiles obtained by spectroscopic techniques</i>	86
2.3.2.2	<i>Differential Scanning Calorimetry</i>	87
2.3.3	ITC The techniques employed to determine the ligand binding	92
<b>2.4.</b>	<b>References</b>	99
<b>Chapter 3</b>	<b>Characterization of cytochrome c folding intermediates in the presence of sucrose and phosphate</b>	101
<b>3.1.</b>	<b>Introduction</b>	102
<b>3.2.</b>	<b>Experimental Section</b>	103
3.2.1	Materials	103
3.2.2	Methods	104
3.2.2.1	<i>Absorbance measurements</i>	104
3.2.2.2	<i>Fluorescence measurements</i>	104
3.2.2.3	<i>CD measurements</i>	104

3.2.2.4	<i>DSC measurements</i>	104
<b>3.3.</b>	<b>Results</b>	105
3.3.1	Acid denaturation of cyt c	105
3.3.2	Structural characterization of Sucrose and phosphate stabilized intermediates	110
3.3.3	Comparison of phosphate and sucrose stabilized cyt c intermediate with similar UV-Vis spectra	114
3.3.4	Thermodynamic characterization of sucrose and phosphate stabilized intermediates	117
<b>3.4.</b>	<b>Discussion</b>	121
<b>3.5.</b>	<b>Conclusions</b>	127
<b>3.6.</b>	<b>References</b>	127
<b>Chapter 4.</b>	<b>Characterization of ATP induced molten globules in denatured cytochrome c</b>	131
<b>4.1.</b>	<b>Introduction</b>	132
<b>4.3.</b>	<b>Experimental Section</b>	133
4.3.1	Materials	133
4.3.2	Methods	134
4.3.2.1	<i>Absorbance measurements</i>	134
4.3.2.2	<i>CD measurements</i>	134
4.3.2.3	<i>Differential Scanning Calorimetry</i>	134
4.3.2.4	<i>Isothermal titration calorimetry</i>	135

4.3.2.5	<i>Peroxidase Activity Measurement</i>	135
<b>4.4.</b>	<b>Results</b>	136
4.4.1	Titration of cyt c with ATP/ADP/AMP	136
4.4.1.1	<i>Effect of nucleotides on Soret Band</i>	136
4.4.1.2	<i>Effect of nucleotides on Q band of cyt c:</i>	138
4.4.1.3	<i>Effect of nucleotides on Far-UV CD spectrum of Cytochrome c</i>	139
4.4.1.4	<i>Effect of nucleotides on Cotton CD spectrum of cytochrome c</i>	141
4.4.1.5	<i>Effect of nucleotides on the DSC thermograms of cyt c</i>	142
4.4.1.6	<i>Effect of the interaction of cyt c with nucleotides probed by Isothermal Calorimetry</i>	145
4.4.2	Interaction of cyt c with different poly-phosphate nucleotides (ATP/CTP/GTP)	147
4.4.2.1	<i>Effect of nucleotides ATP/GTP/CTP on the Soret spectra of cyt c</i>	147
4.4.2.2	<i>Effect of nucleotides ATP/GTP/CTP on the Q band of cyt c</i>	148
4.4.2.3	<i>Effect of nucleotides ATP/GTP/CTP on the Far-UV CD spectra of cyt c</i>	149
4.4.2.4	<i>Effect of nucleotides ATP/GTP/CTP on the Soret CD spectra of cyt c</i>	149
4.4.2.5	<i>Effect of nucleotides ATP/GTP/CTP on the DSC thermogram of cyt c</i>	150
4.4.2.6	<i>Interaction of nucleotides ATP/GTP/CTP with cyt c as probed by ITC</i>	151

<b>4.5.</b>	<b>Discussion</b>	152
<b>4.6.</b>	<b>Conclusion</b>	156
<b>4.7.</b>	<b>References</b>	157
<b>Chapter 5</b>	<b>The non-native conformations of cytochrome c in sodium dodecyl sulfate and their modulation by ATP</b>	159
<b>5.1.</b>	<b>Introduction</b>	160
<b>5.2.</b>	<b>Experimental Section</b>	161
5.2.1	Materials	161
5.2.2	Methods	162
5.2.2.1	<i>Absorbance measurements</i>	162
5.2.2.2	<i>CD measurements</i>	162
5.2.2.3	<i>Fluorescence Spectroscopy</i>	163
5.2.2.4	<i>Peroxidase Activity Measurement</i>	163
5.2.2.5	<i>Docking simulations</i>	163
<b>5.3.</b>	<b>Results</b>	164
5.3.1	<i>Change in fluorescence parameters of cyt c on the addition of SDS</i>	164
5.3.2	<i>Change in Soret absorbance of cyt c with the addition of SDS</i>	166
5.3.3	<i>Change in the Far-UV CD spectrum of cyt c on the addition of SDS</i>	169
5.3.4	<i>Change in near-UV CD and Soret CD of cyt c with the addition of SDS</i>	170

5.3.5	<i>Change in the peroxidase activity of cyt c in presence of SDS</i>	172
5.3.6	<i>Effect of salt on the cyt c-SDS interaction</i>	172
5.3.7	<i>Change in Soret absorbance of C1/C2 on the addition of ATP</i>	173
5.3.8	<i>Change in CD spectra of cyt c-SDS system on the addition of ATP</i>	175
5.3.9	<i>Peroxidase activity of C1/C2 in presence of ATP</i>	177
5.3.10	<i>Docking of cyt c with SDS: SDS was docked on cyt c</i>	177
5.3.11	<i>Docking of cyt c with ATP and GTP</i>	178
<b>5.4.</b>	<b>Discussion</b>	181
<b>5.5.</b>	<b>Conclusions</b>	186
<b>5.6.</b>	<b>References</b>	186
<b>Chapter 6</b>	<b>The non-native conformations of cytochrome c in cardiolipin/oleic acid and their modulation by ATP</b>	193
<b>6.1.</b>	<b>Introduction</b>	194
<b>6.2.</b>	<b>Experimental Section</b>	197
6.2.1	Materials	197
6.2.2	Methods	198
6.2.2.1	<i>Absorbance spectroscopy</i>	198
6.2.2.2	<i>Circular dichroism (CD)</i>	198
6.2.2.3	<i>Fluorescence Spectroscopy</i>	198
6.2.2.4	<i>Peroxidase Activity Measurement</i>	199
6.2.2.5	<i>Nile red-cardiolipin/oleic acid interaction and its modulation with cyt c</i>	199

6.2.2.6	<i>Docking simulations</i>	201
<b>6.3.</b>	<b>Results</b>	201
6.3.1	Change in fluorescence parameters of cyt c on the addition of cardiolipin/oleic acid	201
6.3.2	Change in the absorbance of cyt c with the addition of cardiolipin/oleic acid	204
6.3.3	Change in the Far-UV CD spectrum of cyt c on the addition of cardiolipin	206
6.3.4	Change in near-UV CD and Soret CD of cyt c with the addition of cardiolipin/oleic acid	207
6.3.5	Change in the peroxidase activity of cyt c in presence of cardiolipin/oleic acid	208
6.3.6	Change in CD spectra of cardiolipin induced intermediates on the addition of ATP	209
6.3.7	Change in peroxidase activity of cardiolipin induced intermediates on the addition of ATP	210
6.3.8	Nile red-cardiolipin interaction and its modulation by cyt c	211
6.3.9	Nile red-oleic acid interaction and its modulation by cyt c	214
6.3.10	Docking of cyt c with Cardiolipin	216
6.3.11	Docking of cyt c with oleic acid	218
<b>6.4.</b>	<b>Discussion</b>	219
<b>6.5.</b>	<b>Conclusions</b>	221
<b>6.6.</b>	<b>References</b>	222

**BIODATA OF THE AUTHOR**