BIOCHEMICAL AND BIOPHYSICAL INVESTIGATION OF THE MOLECULAR MECHANISM OF ATP SYNTHESIS

by

BHAWANA AGARWAL

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

Submitted

in fulfillment of the requirements of the degree of

Doctor of Philosophy

to the

Indian Institute of Technology, Delhi

DECEMBER, 2008

Dedicated

to

My beloved Parents

CERTIFICATE

This is to certify that the thesis entitled "Biochemical and Biophysical Investigation of the Molecular Mechanism of ATP Synthesis", being submitted by **Ms. Bhawana Agarwal** to the Indian Institute of Technology, Delhi, for the award of degree of **Doctor of Philosophy**, is a record of bonafide research work carried out by her under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology, Delhi. The research reports and results presented in the thesis have not been submitted to any other University or Institute for the award of any other degree or diploma.

Sunil nath

(Dr. Sunil Nath)
Professor and Head of the Department,
Department of Biochemical Engineering and Biotechnology,
Indian institute of Technology, Delhi
Hauz Khas, New Delhi-110016.

Acknowledgements

"Sow the seeds of your ambition, Water them with hardwork, Manure them with patience and success will be surely yours"

With the culmination of five most remarkable years of my academic career, I have finally tasted the sweet fruits of my endeavors in these bygone years whereby I have been accompanied and supported by many in this venture. I have now the opportunity to pleasantly remember their contributions and express my sincere gratitude to all of them.

"Great heights achieved by men, Were not attained by sudden flight, But they while the world slept, Were tailing upward in the night"

This is indeed true for my research supervisor Dr. Sunil Nath to whom I first and foremost express my deepest gratitude and reverence for his expert guidance and support. It is very satisfying to state that his encouraging attitude, patience, sensitivity, perpetual inspiration and good spirits has helped me in making innumerable value laden decisions and choices and sail through all dilemma of life, which I encountered, with a positive attitude. I owe him lots of gratitude for having shown me this way of research. His deep knowledge of his project and his logical way of thinking has been of great value for me. I am glad sir that you were always there when I needed a family the most, consoling me, motivating me, providing me a good company and also appreciating me. I shall remain indebted to him all my life.

"All men think they can do it alone but a real man knows there is no substitute for support and encouragement as a crew"

I also owe my profound thanks to my research committee members: Prof. Saroj Mishra, Dr. nalin Pant and Dr. Prashant Mishra for their valuable suggestions and critical comments during the course of my PhD tenure. The committee has indeed been instrumental in helping me take a direct approach towards my topic and enhance my research skills to a great extent. They took efforts in reading my work and providing me with valuable comments and required facilities to carry out my work.

I would like to specially acknowledge Prof. Saroj Mishra, the former head of the department, for providing the basic facilities that helped me a lot in settling the lab. I am also thankful to the

Swarnajayanti Research Grant that was awarded to my supervisor that helped in procuring the sophisticated equipments needed to carry out my research work.

I thank my Department of Biochemical Engineering and Biotechnology for providing me the technical expertise and other sophisticated analytical instruments. I am also thankful to the office staff of this department, Mrs. Meena Mathur, Mrs. Sunita Dang and Mr. Rajeev who were helpful and friendly. I sincerely thank Ranaji, Renu m'am, Neera m'am, Bhagwansingh ji, Yadav ji, Khan sahib and Swapan sir for their timely help.

I am extremely thankful for having met my senior, Dr. Vibha Bansal, as my friend here, to whom I shall remain highly indebted for inspiring me to take up this work of research scholar. Her optimistic attitude and, constant and cheerful support encouraged me to see light in the face of darkness.

My sincere thanks to another senior friend of mine Dr. Shilpa Khaparde for her unconditional support and affection because of which I could remain composed during the crucial stages of my life.

I am especially grateful to my friends Roohi Gupta and Richa Singh who made my stay at IIT, Delhi a pleasant experience. I owe my profound thanks to them for being a constant support and wonderful friends who were always ready to listen to me whenever and whatever I wanted to share with them at any time of the day.

I shall cherish in a special way the care and guidance bequeathed upon me by my seniors Dr. Sunita Jarkya, Dr. Rumpa Biswas and Dr. Ruchi Shukla.

I am extremely blessed to have a strong support of my in-laws who have always been on my side and never pressurizing me and always making a warm and cordial home environment.

How can I forget the constant support extended by my family especially my mother who has been a pillar on whom I could rest. I am thankful to my father who stands on a point 'studies never go waste' that always gave me the perseverance to do PhD and work hard to accomplish it. I also thank my brother and sister and their families for encouraging and supporting me in moments of profound grief. My special and loving thank to my sister who knows my psyche so well that it could never take

her any time to understand me during the discussion on any of the issues related to my life and thus could render me her best advice.

"A journey is easier when you travel together. Interdependence is certainly more valuable than independence".

Last but not the least I would like to acknowledge the unrelenting support and love of my husband, Dinesh, without whose help the completion of my PhD thesis would not have been possible. He has been there hearing all my problems, coping with all ups and downs and giving meaningful remedies. I am genuinely overjoyed on having him as a life partner and for bringing joyful dimensions in my life.

I acknowledge the awarded Institute Doctoral Fellowship of IIT, Delhi under the PhD program. For having awarded me, Grant of Partial Financial Assistance from IIT, Delhi, Partial Travel Support, as a full air fair refund, from Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, and ICMAT 2007 Financial Assistance in the form of Hostel Accommodation at National University of Singapore (NUS) during the scheduled time of my stay for helping me attend the international conference on Materials for Advanced Technologies (ICMAT), at Suntec, Singapore.

I would certainly not forget to thank God Almighty for showering me with all his blessings and bestowing me with the ability to complete this work.

In the end as I depart from IIT, Delhi, I wish to bid adieu to all my soulmates, my companions, my mentors and all my well wishers.

"The saddest has a morrow; The darkest night has a dawn, So turn from yesterdays sorrow and press courageously on"!

Bhawana

Bhawana Agarwal

ABSTRACT

The synthesis of ATP by the central enzyme F_1F_0-ATP synthase in the energy-transducing membranes of animal mitochondria, plant chloroplasts and bacteria is a most fundamental process in the biological world. A lot of information has been accumulated on this fundamental process during more than 50 years of research; yet true mechanistic insights are still lacking and the details of the molecular mechanism of ATP synthesis are still unanswered. In this work, comprehensive biochemical and biophysical studies have been carried out to elucidate the molecular mechanism of ATP synthesis in the F_0 portion of the ATP synthase in the model system of chloroplast thylakoids from spinach. A novel role for anions in making ATP has been discovered. Our experimental evidence in favor of this novel scenario is discussed. In particular, new biochemical data has been reported on the inhibition of ATP synthesis at nanomolar concentrations by the potent, specific anion channel blockers 4,4'-diisothiocyanostilbene-2, 2'-disulphonic acid (DIDS) and tributyltin chloride (TBTCl). Based on these inhibition studies, attention has been drawn to anion translocation (in addition to proton translocation) as a requirement for ATP synthesis. The type of inhibition has been quantified and an overall kinetic scheme for mixed inhibition that explains the data has been evolved. The inhibitors are also tested for specificity of the access channels in F_0 for different anions. The experimental data and the type of inhibition found have been interpreted in the light of the torsional mechanism of energy transduction and ATP synthesis (Nath, 2002; Nath, 2003; Nath, 2004), which postulates a role for both protons and membrane-permeable anions. This detailed and unified mechanism resolves long-standing problems and inconsistencies in the prevalent chemiosmotic theory, makes several novel predictions that are experimentally verifiable,

and provides us with a new and fruitful paradigm in bioenergetics. Methods to estimate both ΔpH and $\Delta\Psi$ components by the amino probe 9-aminoacridine and the quinolinium probe Oxonol-VI have been presented. Further, to attempt validation of these results, biophysical studies with the help of fluorophores, in particular, time-resolved fluorescence changes during the entire acid-base transition process of making ATP have been measured and led to important findings. The interpretation of these results inferred a biphasic distribution of absorbance change of 9-AA in thylakoids. These findings were interpreted based on the assumption that the neutral and cationic forms of the probe partitioned between the bulk solution and the membrane domain. The mechanistic details of the oxonol-VI probe partitioning in the membrane and further its response indicated a coupled transport of anion and proton through the F_0 portion of ATP synthase. In this work, a ΔpH of 1.4 units across the membrane and a $\Delta\psi$ of 85 mV per ion-binding site were measured during physiological ATP synthesis. Finally, ion transport phenomena based on real time bio-imaging of our thylakoid model system by fluorescence microscopy has been recorded. It has been shown that mechanistic interpretation of the experimental data requires substantial addition to available conceptual foundations such that present concepts, theories, and mechanisms must be revised. Indeed, it is even possible to make predictions of mechanistic details of ATP synthesis in the F_0 portion of ATP synthase, and to offer detailed microscopic explanations of such data in the light of the torsional mechanism, which is certainly a very satisfying outcome in bioenergetics, membrane biology, biochemistry and biophysics. Thus, the torsional mechanism appears to be a useful theory/framework for guiding future experimentation and logical thought in the field.

CONTENTS