

**CHAPERONE ASSISTED FOLDING
OF
YEAST MITOCHONDRIAL ACONITASE
IN
*ESCHERICHIA COLI***

By

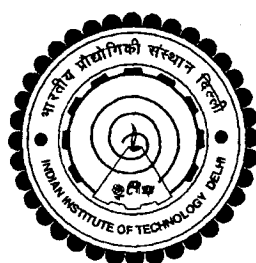
PARUL GUPTA

Department of Biochemical Engineering and Biotechnology

*Submitted
in fulfillment of the requirement of the degree of*

DOCTOR OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

APRIL 2008

I. I. T. DELHI.
LIBRARY
Acc. No. TH-3606

TH

602.641:579.8

Group - C



DEDICATION

*to my dear parents, Brother, Bhabhi and Tishya
for their everlasting love and patience....*

CERTIFICATE

This is to certify that the thesis entitled “Chaperone assisted protein folding of yeast mitochondrial aconitase in *Escherichia coli*” being submitted by Ms. Parul Gupta to the Indian Institute of Technology Delhi, for the award of the degree of ‘Doctor of Philosophy’, is a record of the bonafide research work carried out by her, which has been prepared under our supervision in conformity with the rules and regulations of the “Indian Institute of Technology Delhi”. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

Tapan K. Chaudhuri
Dr. Tapan Kumar Chaudhuri

Saroj Mishra
Prof. Saroj Mishra

ACKNOWLEDGEMENT

Even though this PhD dissertation can yield at most one doctor's title to at most one person, it has involved the help, interest, and support of many. Since I value these people very highly, it is appropriately iconic to thank them at the very beginning of this thesis.

My supervisor, Dr. Tapan Kumar Chaudhuri, has contributed enormously to the genesis of this doctorate both in terms of intellectual input and immense support and encouragement. Even though his teaching load is considerable and his research and administrative responsibilities manifold, he has always given generously of his time and insights, and not a word of mine has appeared in print or in this dissertation which he has not read and commented on. I feel deeply grateful and privileged to have been his student. I warmly thank my co-supervisor, Prof. Saroj Mishra, not only because of her sharp scientific judgment and often crucial hints, but also because of the never failing kindness and moral and intellectual honesty with which she handles challenges and problems of any kind. I will forever remain grateful for her interest and encouragements expressed at various occasions.

For having accepted to act as members of my doctoral examination board, as well as for putting up with my dabbings in research, I most sincerely thank Prof. V. S. Bisaria, Prof. Sunil Nath and Dr. S. K. Khare. I owe a special debt of gratitude to Prof. Faizan Ahmad (Director, Centre of Interdisciplinary Research in Basic Sciences, Jamia Milia Islamia), who not only consented to be the expert member of my research evaluation committee, but also provided me with the most useful and extensive insights into the fundamentals of unraveling the mysteries of protein folding. I especially thank Dr.

Gomes for training a novice like me for the use of the fermentor in his lab for biomass generation.

I thank the Council of Scientific and Industrial Research for providing me the scholarship in order to be able to work for five years. As well, I thank the Department of Biochemical Engineering and Biotechnology, all faculty members and Indian Institute of Technology Delhi for the endless help in terms of excellent infrastructure and resources required for carrying out the experimental work.

The time and effort invested in the editorial work is not always valued sufficiently in academia. What I have found stimulating in my contacts with various editors and referees is the ways in which they help to complement one's analyses from, sometimes, very different theoretical perspectives. While I cannot thank by name the anonymous referees for the time they have spent on my behalf, I can at least thank them in principle. As well, I thank the editors of journals in which I have published for their role in the process of publishing, but especially for the concrete suggestions for improvement they have made in aspects of form, as well as content.

Even if the big bad world out there may ultimately function competitively, I have been lucky in meeting a host of people more than willing to take an active interest in my work or to provide practical help in such various ways, as asking questions or adding comments at conference talks, reading and commenting on draft versions of papers, passing on references which could be of interest to me, or simply wishing me good luck. For all these kinds of things I thank numerous un-named people for being there.

Without people seeing to it that practical problems are solved, work would become difficult in any workplace. I thank all the administrative and technical staff of the

department. To Mr. V. K. Ghosh, Ranaji, Sharmaji, Mukesh Anandji and Renu madam for making things work around the Labs. To Neera madam, for being a minefield of documentation information. To Sunita, Pushplata, Meena madam, Tarzan, Rajeev and Swapan for making administrative working smooth. Some people who tend to go unacknowledged are once who work in numerous ways to make our environment so much more comfortable and efficient. In this regard, I acknowledge the assistance provided by Kishanji, Ram Gopalji, Yadavji, Hardeepji, Harishji, Sitaramji, Ratanji, and Bhagwansinghji. Special thanks to Sohrabji, who was an excellent 'Mr. Fix-it' that any lab could have wanted. His Biryani, jalebi and samosa parties will be forever etched in my memories.

I thank all my colleagues in the Department of Biochemical Engineering and Biotechnology for the (real or virtual) chats about all things science and especially non-science. For their regular expressions of best wishes I warmly thank Raju, Ashish, Richa, Roohi, Asif, Rajib, Alok, and Saurabh. I will forever be indebted to my seniors, Salony, Pranita, Rumpa, Shilpi, for teaching me the basic ropes of experimental techniques. No acknowledgement of mine can be complete without the mention of my labmates, who not only shared various experiences but also made the learning of both good and bad memorable. Special thanks to Pravin who patiently taught me enough basic to survive the often unfathomable depths of *in vivo* of protein folding, to Subhankar for exposing me to various invaluable computer skills, to Aditi and Vikas, for making the long hours spent in lab full of laughter and fun. The role that the various M. Tech./SURA students of Structural Biology Lab, Nishtha, Pragya, Vatsala, Jaskaran and Vivek played in enriching my research experience goes a long way to prove that learning and teaching has very

little to do with age and more to do with willingness to share and learn. Finally, I thank all my seniors, Amalendu, Gunjan, Ruchi, and Snehashish for lending help whenever required. I also thank Gupteshwarji, Lalit, Rahul, and Smita for their help and presence whenever needed.

Much as I enjoyed the rigors of working on the project, I am very thankful to people who have truly insulated me against the frustrations of often failed results and other unavoidable occurrences. To all those who cheered me up, listened to me whine, or made me laugh while I was doing my PhD. To Anand, for inquiries into the progress I was or was not making, or for simply listening to my rants about the latter. If not for your ideas and efforts for protein purifications, I would not have reached to thesis submission so soon. With you I have shared a lab, but also many laughs, occasional writer's block frustrations, and a lot of dear friendship. To Anjali, for being there right through the adolescent years of graduation to the, often stuck up, doctoral years. The trips back to yester years with you were instrumental in keeping me sane. To Bhawna, for bringing back colors of often forgotten joyful 'masti' of youth and for disagreeing with me on basic fundamental issues. You surely kept the surprises and unpredictable moments of what is 'life' alive. PhD became an unforgettable experience thanks to their friendship. Thank you all and most significantly to Shijo, for having regularly, over the past year or so, taken me out of my scientists ivory tower through visits to golf course, shooting range (the temptation to shoot you was often irresistible), having dinner or going to plays or films together. I cant imagine how dull life was without your presence.

On a different note, I would like to thank: the coffee producers of Nescafe for keeping me thinking, and the Holistic Food Centre for making me feel happier about all those coffee cups by drinking healthy glasses of juice.

No thanks can describe the level of gratitude I feel for my mother and my father, for having made 'home' far more than just a place. Their unending love, strength and patience for my lack of attention, and tolerance, for often late nights have been invaluable. Their profound love for education inspired me to take up the daunting task of a doctoral thesis. My deepest love for my brother, Mayank, and his better half, Ruchika, for putting up with my idiosyncrasies. Finally, to darling Tishya, my 2 year old niece whose toothy grin and fantastic antics never failed to wipe out the frustrating memories at the end of a bad day.

Finally, to God almighty and being there for me at all turns and rough terrains of life.

Of course, despite all the assistance provided by all, I alone remain responsible for the errors or omissions which may unwittingly remain.


Parul Gupta

Abstract

Over last two decades many researchers have demonstrated the mechanisms of how the *Escherichia coli* chaperonin GroEL and GroES work in the binding and folding of different aggregation prone substrate proteins both *in vivo* and *in vitro*. However, preliminary aspects, such as influence of co-expressing GroEL and GroES on the over expression of other recombinant proteins in *E. coli* cells and subsequent growth aspects, as well as the conditions for optimum production of recombinant proteins in presence of recombinant chaperones, which significantly affect the yields of recombinant proteins, have not been properly investigated. In the present study we have demonstrated the temperature dependent growth characteristics of *E. coli* cells, which are over expressing recombinant aconitase and *E. coli* chaperonin GroEL and GroES. For the aconitase over-expressing cells the changes in the growth rate indicated the participation of endogenous chaperonin in the folding of a fraction of over expressed aconitase. However, in presence of co-expressed GroEL and GroES the growth rate of aconitase producing cells confirmed the assistance of exogenous chaperone system for the folding of recombinant aconitase. *In vivo* folding of aconitase required co-production of complete *E. coli* chaperonin machinery GroEL and GroES, together.

Apo-aconitase, the Fe₄S₄ cluster free form of Tri carboxylic acid cycle enzyme aconitase, binds with GroEL and dissociates itself, upon maturation, through the insertion of the cluster. It is not quite clear why the apo-protein binds with GroEL. In order to explore the possibility, that the stability is a factor responsible for the aggregation of apo-form and hence, the non-native protein associates with GroEL to avoid the unfavorable event, we carried out the equilibrium and kinetic unfolding studies with holo- and apo-

aconitase. By probing the unfolding process through the changes in secondary structural element, exposed surface hydrophobicity, and the microenvironment around tryptophan residues, we were able to calculate various relevant parameters associated with the event. Results indicate that the lower ground state stability and higher solvent exposed hydrophobic surface make the apo-form aggregation prone. Based on the present observation and earlier findings, we propose that the binding of apo-aconitase to GroEL, not only rescues it from the aggregation, but also assists in the final stage of maturation by orienting the cluster insertion site on GroEL bound apo-protein. This information sheds new light on the potential role of GroEL in the biosynthetic pathway of the metallo proteins.

Osmolytes are known to stabilize proteins against aggregation. The present study also investigates, the *in vitro* chemical assisted activity restoration of aconitase. Our study suggests a strategy to enhance the percentage of correct aconitase refolding through the addition of some chemical folding aids into the refolding buffer. As is commonly recognized, the primary refolding problem is aggregation, either *in vitro* or *in vivo*. The simple treatments with added osmolytes applied in this study provide strong evidence that some osmolytes acting as folding aid reagents can effectively aid aconitase on the path to correct folding.

CONTENTS

CERTIFICATE	i
ACKNOWLEDGEMENT	ii
ABSTRACT	vii
CONTENTS	vxi
LIST OF FIGURES	xv
LIST OF TABLES	xviii
LIST OF ABBREVIATIONS AND SYMBOLS USED	xxi

1. Introduction and Objectives	1-6
---------------------------------------	------------

2. Review of Literature	7-36
2.1. Protein folding	7
2.2. The Chaperonin: GroE	8
2.2.1. Structure of GroEL	9
2.2.2. Structure of subunits	9
2.2.3. The central cavity	12
2.2.4. Structure of GroES	13
2.2.5. Structural basis for GroEL function	16
2.2.6. Domain shifts in the <i>cis</i> ring	17
2.3. Mechanism of GroEL/GroES assisted folding	20
2.3.1. <i>Cis</i> mechanism of folding	22
2.3.2. <i>Trans</i> mechanism of folding	24

2.4. Applications of chaperone-mediated protein folding	29
2.4.1. Molecular chaperone assisted folding of recombinant proteins	29
2.4.2. Importance of molecular chaperones in treatment of protein misfolding diseases	30
2.5. Aconitase: an overview	33
<hr/>	
3. <i>In vivo</i> folding of aconitase by co-expressed GroEL/GroES in <i>E. coli</i>	37-64
3.1. Materials	37
3.1.1. Strains, Plasmids and Culture conditions	37
3.2. Methods	38
3.2.1. Expression of aconitase in M15 <i>E. coli</i> strain on transformation with pQE60Aco	38
3.2.2. Co-expression of aconitase, GroEL and GroES	39
3.2.3. Effect of inducer concentration on the efficiency of induction	39
3.2.4. Time course of aconitase induction	40
3.2.5. Optimum starting time of induction	40
3.2.6. Growth of recombinant <i>E. coli</i> cells	40
3.2.7. <i>In vivo</i> folding of aconitase	41
3.3. Analytical methods	42
3.3.1. Determination of specific growth rate for bacterial growth	42

3.3.2. Estimation of relative intensities of the bands in SDS-PAGE	42
3.3.3. Aconitase assay	43
3.3.4. Protein estimation	43
3.4. Experimental organization	43
3.5. Results	45
3.5.1. Optimization of parameters for maximum expression of recombinant aconitase in <i>E. coli</i>	45
3.5.1.1. Inducer concentration required for maximum expression of recombinant aconitase	45
3.5.1.2. Change in the expression of aconitase in M15 cells with time of induction in the absence and presence of over expressing chaperonin GroEL/ES	45
3.5.1.3. Optimum starting time of induction	49
3.5.1.4. Over expression of aconitase in <i>E. coli</i> M15 cells in presence and absence pACYCEL/pACYCELS	51
3.5.2. Changes in growth characteristics of <i>E. coli</i> on transformation with various recombinant plasmids	53
3.5.2.1. Effect of plasmid characteristics on growth rate of transformed cells	53
3.5.2.2. Effect of IPTG induction on growth at different temperatures	54
3.5.2.3. Effect of temperature on specific growth rate	

of transformed strains	55
3.5.3. <i>In vivo</i> folding of aconitase in absence and presence of co-expressed GroEL and GroES in <i>E. coli</i> cells	55
3.6. Discussion	58
3.7. Conclusion	63
<hr/>	
4. <i>In vitro</i> unfolding of aconitase and role of GroEL in aconitase maturation	65-106
4.1. Materials	65
4.1.1. Strains, plasmids and culture conditions	65
4.2. Methods	66
4.2.1. Purification of various recombinant proteins	66
4.2.1.1. Aconitase purification	66
4.2.1.2. GroEL purification	67
4.2.1.3. GroES purification	70
4.2.1.4. Preparation of apo-aconitase	71
4.2.1.5. Aconitase activity	72
4.2.2. Equilibrium unfolding of holo-aconitase and apo-aconitase	72
4.2.2.1. Guanidine hydrochloride induced denaturation of aconitase	72
4.2.2.2. Change in aconitase activity on denaturation	73
4.2.2.3. Change in secondary structure of aconitase, monitored by far UV circular dichroism	73

4.2.2.4. Change in tertiary structure of aconitase, monitored by intrinsic tryptophan fluorescence spectroscopy	75
4.2.2.5. Change in surface hydrophobicity of aconitase, monitored by extrinsic fluorescence measurement using ANS probe	76
4.2.3. Kinetic measurements of aconitase unfolding	77
4.2.4. Encapsulation of GroEL bound apo-aconitase by GroES	78
4.2.4.1. Formation of binary complexes between GroEL and denatured aconitase or apo-aconitase	78
4.2.4.2. Proteolytic digestion of aconitase/GroEL complexes with Proteinase K	79
4.3. Results	79
4.3.1. Conformational properties and secondary structure of holo- and apo-aconitase	79
4.3.2. Equilibrium unfolding of aconitase	84
4.3.3. Kinetics of aconitase unfolding	91
4.3.4. GroEL forms stable binary complexes with apo-aconitase	94
4.3.5. 82 kDa apo-aconitase in GroEL cavity cannot be capped by GroES	97
4.4. Discussion	99
4.5. Conclusion	106

5. Refolding of aconitase	107-116
5.1. Materials	107
5.2. Methods	107
5.2.1. Refolding of aconitase	107
5.2.1.1. Spontaneous refolding studies of aconitase	107
5.2.1.2. Chemical chaperone assisted refolding studies of aconitase	107
5.2.1.3. GroEL-GroES assisted refolding studies of aconitase	108
5.2.1.4. Reconstitution of apo-acnitase to holo-form	108
5.3. Results	109
5.3.1. Spontaneous refolding of denatured aconitase	109
5.3.2. Chemical chaperone assisted refolding of denatured aconitase	110
5.3.3. Molecular chaperonin GroEL/GroES assisted refolding of denatured aconitase	112
5.4. Discussion	112
5.5. Conclusions	116
<hr/> Summary	117-118
<hr/> References	119-148
<hr/> Annexures	149-156
<hr/> Biodata of author	157-160
<hr/>	