SIZE AND SHAPE OF CROWDING CONTROL THE SECONDARY STRUCTURE FOLDING OF ACID-DENATURED CYTOCHROME *C*

A Project Work submitted to the Central University of Punjab

For the award of

Master of Science

In

Chemical Sciences

By

Manoj Kumar Sharma

Supervisor

Dr. Rajesh Kumar

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

CERTIFICATE

I declare that the project entitled "SIZE AND SHAPE OF CROWDING CONTROL THE SECONDARY STRUCTURE FOLDING OF ACID-DENATURED CYTOCHROME *C*" has been prepared by me under the guidance of Dr. Rajesh Kumar, Associate Professor, Department of Chemical Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

Manoj Kumar Sharma
Department of Chemical Sciences
School of Basic and Applied Sciences
Central University of Punjab, Bathinda - 151001.
Date:

CERTIFICATE

I certify that MANOJ KUMAR SHARMA has prepared his project entitled “SIZE AND SHAPE OF CROWDING CONTROL THE SECONDARY STRUCTURE FOLDING OF ACID-DENATURED CYTOCHROME *C*”**,** for the award of M.Sc. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Department of Chemical Sciences, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Rajesh Kumar,
Associate Professor,
Department of Chemical Sciences,
School of Basic and Applied Sciences,
Central University of Punjab
Date:

Abstract

Size and Shape of Crowding Control the Secondary Structure Folding of Acid-Denatured Cytochrome *C*

Name of Student	:	Manoj Kumar Sharma
Registration Number	:	16MSCCHM15
Degree for which submitted	:	M.Sc. Chemical Sciences
Supervisor	:	Dr. Rajesh Kumar
Centre	:	Chemical Sciences
School of studies	:	Basic and Applied Sciences
Key words	:	Macromolecular crowding, Cytochrome *c*, Circular Dichroism, Molten Globule, Dextran 40, Dextran 70, Ficoll 70

Stability and refolding of Acid-denatured cytochrome *c* at pH 2 were examined under the influence of size, shape and varying concentration of synthetic macromolecular crowding agents (Dextran 40, Dextran 70, ficoll 70). Analysis of far UV-CD data revealed that (1) higher concentration of crowding agents induces the secondary structure in acid-denatured cyt *c* (2) shape and size of similar shaped crowding control the crowding-induced secondary structure of acid denatured cyt *c.* The far UV-CD data further revealed that the salt presence also induces the secondary structure in acid-denatured cyt *c* due to charge screening mechanism.

(Manoj Kumar Sharma) (Dr. Rajesh Kumar)

ACKNOWLEDGEMENTS

The successful completion of this project was made possible through the invaluable contribution of a number of people. I specially want to thank my supervisor **Dr. Rajesh Kumar**, Associate professor, Head of department, Department of Chemical sciences, for generously providing guidance on the technical aspect of this project, for continuously encouraging me and pushing me to my limits. It is indeed my pleasure to work under him.

I am also thankful to other faculty members **Dr. J.N. Babu, Dr. K.K. Haldar, Dr. R.S. Dhayal, Dr. Rakesh Kumar, Dr. Biplab Banerjee** and all non-teaching staff for their invaluable cooperation and help during the entire tenure of my studies in the department.

I am deeply thankful to the research fellow **Mansi Garg** for her help and encouragement. Without her help completion of this project is quite difficult. I wish to express my deep sense of gratitude to my friends Rohtash, Ram Singh, Purushottam for their cooperation and support at each and every step of my studies in Central University of Punjab.

In the end I wish to express my gratitude to my sister Monika and my family. It is the power of their blessing, which has given me the courage, confidence and zeal to do hard work in every odd situation.

(Manoj Kumar Sharma)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

S.NO.	Full form	Abbreviation
1	Ribo Nucleic Acid	RNA
2	Molten Globule	MG
3	Cytochrome *c*	Cyt *c*
4	Circular Dichroism	CD
5	Ultra violet	UV
6	Nuclear Magnetic Resonance	NMR
7	Polyethylene glycol	PEG
8	Polyvinyl pyrrolidine	PVP

CHAPTER 1

INTRODUCTION

1. Introduction

Protein plays vital roles in biological processes. It is necessary to know about how protein functioning and behaving. Proteins are encoded by genes, transcribed into ribonucleic acid (RNA) and translated into polypeptide with the help of ribosomes. Protein folding is a physical process in which unorganized polypeptide chain is transformed into a compact folded state. In general, the smaller sized single domain protein folding occurs in two state processes while the folding of longer sized proteins involves more intermediate states. Traditionally, the folded state has a define state with higher flexibility. Several molecular forces such hydrogen-bonding, Vander wall forces, ionic interactions and hydrophobic interactions are responsible for correct folding and stability of proteins (Benjwal *et al.* 2005; Dominy *et al.* 2002; Karshikoff *et al.* 2007; Pace 1995).

The folded protein has several structural elements such as primary, secondary, tertiary and quaternary. Primary structure defines sequence of amino acids. Secondary structure further consists of α-helices and β-strands. Tertiary structure is the 3-dimensional structure. When a folded protein combined with the other folded proteins, it forms a multimeric assembly which is termed as quaternary structure. Metal ions presence in metalloproteins also plays important role in correct folding, stability and biological function (Whitford 2013).

In general, there are three thermodynamic states of proteins, including folding, unfolding and molten globule(MG) states. Unfolded state is difficult to identify as compared to folded state. There is a small energy barrier between folded and folded state.(Whitford 2013) Tanford explained that the unfolded state acquires a disordered coil conformation. Generally, no residual secondary structure elements or tertiary structure elements are apparent in unfolded state.(Greene *et al.* 1974) MG state is a molecular compact state that has native like secondary structure and hydrodynamic radii but lacks or disordered tertiary structure.(Ptitsyn 1995)

Cytochrome *c* (Cyt *c*) is an electron transfer mitochondrial globular protein and which is associated to the outside of the inner membrane. Cyt *c* also plays an important role in the oxidation inside the cells.(Dix *et al.* 2008). It is typically known as catalyst of respiration. It forms an electron bridge between

oxygen and respirable substrates. Cyt *c* typically transfers electron from Cyt *c* reductase to Cyt *c* oxidase. (Liu *et al.* 1996; Yang *et al.* 1997). Cyt *c* has about 104 amino acids in length with α-helical structure. It also contains a prosthetic type C heme group and that is attached to the polypeptide through cysteine residues. The coordinated iron in heme plays a vital role for its function as an electron relay between complexes in the electron transport chain. It has been reported that Cyt *c* unfolds reversibly under dilute buffer conditions. Furthermore, the reduced form of Cyt *c* (Ferrocyt *c*) is thermodynamically more stable than the oxidized form (Ferricyt *c*). It has been reported that under various solvent condtions (presence of salts, osmolytes, crowding agents) Cyt *c* form molten globules (MGs) at both acidic and alkaline conditions (Goto *et al.* 1991; Kumar *et al.* 2006). In general, the MG-states can be characterized by various spectroscopy techniques like circular dichroism(CD), NMR, Fluorescence.(Kelly *et al.* 2000; Kuroda *et al.* 2001) etc.

Native state of proteins is generally compact and can be easily disrupted. Unfolded protein does not exhibit biological functions. To function properly, the unfolded protein must fold to three-dimensional shape. Proteins can be denatured at extreme conditions, such as at extreme acidic and basic pH conditions, high temperature of pressure, low temperature (cold denaturation) and high concentration of denaturants (alcohols, urea, SDS, guanidine hydrochloride). The commonly use denaturants are urea and guanidine hydrochloride.(Tanford 1970)

Protein folding is a spontaneous physical process. Upon folding, the free energy of the system lowers down. Overall free energy change of the system is reliant on two thermodynamic factors including, enthalpy and entropy. Entropy of unfolded state is greater than that of folded state. Typically, the folded state is stabilized enthalpically by H-bonding in secondary structure and hydrophobic interactions in protein core. Earlier in-vitro studies were carried out under ideal dilute conditions. However, *in-vivo* the protein folds in highly crowded cellular environment (Ellis 2001). *In-vivo*, the concentration range of macromolecules varies from ~80-340 mg/ml. Typically, the crowding environment reduces the excluded volume occupy by unfolded protein. The extent of excluded volume depends on the shape and size of crowding agents.

Generally, non-spherical molecules show larger magnitudes. To determine the exclude volume effects, there is no requirement of considering any electrostatic interactions between crowding agents and proteins. Macromolecular crowding typically exhibits excluded volume effect, viscosity changes and change in non-specific interactions. Several research groups also estimated the difference between thermodynamic parameters *in vivo* and *in vitro*. It is expected that the chemical potential for unfolded proteins increases and which resulting to destabilize the unfolded state. Researchers already investigated the concentration effect of sugar on protein stability and got affirmative results (Sasahara *et al.* 2003).

This research project work investigated the role of concentration, shape and size of synthetic crowding agents (dextran 40, dextran 70, ficoll 70) on secondary structure of acid-denatured Cyt *c* at pH 2.0, 25 °C. For comparison, this research project work also investigated the effect of salt on secondary structure of acid-denatured Cyt *c* at pH 2.0, 25 °C.

CHAPTER 2

LITERATURE REVIEW

2. Literature review

Excluded volume theory plays a great role on stability and folding of native and denatured proteins (Sasahara *et al.* 2003; Tokuriki *et al.* 2004). Most of the protein folding studies select synthetic crowding agents. Crowding agents are highly soluble in solvent and have definite shape and size and they do not exhibit attractive interactions with protein. In general, the protein crowders are not soluble in high concentrations and they produce several charge-charge interactions. It is necessary to suppress these charges by inclusion of high concentrations of salts or by using low concentrations of proteins. Circular dichroism (CD) spectroscopy depicts the ability of a molecule for differential absorption of left- and right-circularly polarized light. Typically, the type of secondary structure and their relative abundance in the molecule depend on the absorption maxima and line shape in the far-UV CD spectrum of protein. Experimentally, secondary structure composition of a protein can be estimated by comparing a given CD spectrum against spectra of proteins with known secondary structures.(Davidson *et al.* 1988; Granath 1958) In native state of Cyt *c*, the two negative bands at 210 nm and 222 nm confirm the presence of secondary structure. At pH 2 in absence of any salt and crowding protein, Cyt *c* significantly lost these peptide bands and thus substantially lost native structure (Sasahara *et al.* 2003). It was found that that high concentration of dextran 35 or salt (KCl) induces the secondary structure in acid-denatured Cyt *c* at pH 2.0 (Sasahara *et al.* 2003). The mechanism of dextran and KCl is quite different. KCl stabilized secondary structure of acid denatured Cyt *c* due to chloride ion interactions with positive charge on Cyt *c.* On the other hand, dextran 35 stabilized the secondary structure by decreasing the volume available to macro solute. There are few reports available which showed that both enthalpic and entropic effects contribute to crowding mediated stabilization of proteins. Further few reports showed that even enthalpic effect is dominant in crowding-mediated stabilization of proteins (Benton *et al.* 2012; Kumar, Sharma *et al.* 2015).

The free energy barrier to folding decreases in presence of macromolecular crowding.(Ellis 2001). It is known that the MG and non-native states of protein exist in the cell and these influence the protein insertion and migration through membranes. MG state of protein generally forms at extreme acidic or alkaline pH in presence salts, crowding, and sugars salts (Sasahara *et al.* 2003)

CHAPTER 3

MATERIAL AND METHODS

3. Material and methods:

3.1 Material

Protein (Horse heart cytochrome *c*), buffer salt (glycine), common salt (NaCl) were purchased from Sigma Aldrich. Synthetic crowding agents such as dextran 40 and dextran 70 were purchased from HI Media while ficoll 70 was purchased from GE Healthcare. All these chemicals/ proteins were used without further purification.

3.2 Methods

3.2.1. Measurement of Far-UV CD spectra of Cyt *c* in the absence and presence of crowding (dextran 40, dextran 70, ficoll 70) and NaCl.

Far-UV CD spectra for acid-denatured Cyt *c* (pH 2.0) were collected by MOS 500 CD spectropolarimeter (Biologic France) in 10 mM glycine buffer in the absence and presence of 300 mg/ml crowding agent (dextran 40, dextran 70, ficoll 70) or 2.0 M NaCl, 25 °C. For comparison, the far-UV CD spectra for native Cyt *c* (pH 7.0) was collected in 50 mM phosphate buffer at 25 °C. The incubation time prior to sample collection is about 3 hrs. The final concentration of protein in these sample was 10 μM.

3.2.2. Measurement of Far-UV CD spectra of acid-denatured Cyt *c* (pH 2.0) in presence of different concentrations crowding agents and salt.

Far-UV CD spectra for acid-denatured Cyt *c* (pH 2.0) were collected in the range of 0.0 to 300 mg/ml crowding agents **(**dextran 40, dextran 70, ficoll 70) or 0.0 to 2.0 M NaCl, 25 °C. The incubation time prior to sample collection is about 3 hrs. The final concentration of protein was 10 μM.

CHAPTER 4

RESULT

4. Result:

4.1. Crowding and salt presence induces the secondary structure in acid-denatured Cyt *c*

In the far-UV CD spectra, the negative peaks at 210 and 222 nm reflects the secondary structures of native proteins (Holzwarth *et al.* 1965).Upon denaturation, the loss of these peaks revealed the disruption of secondary structure of proteins. To test the effect crowding and salt on secondary structure of acid-denatured protein, the far-UV CD spectra of Cyt *c* were collected in the absence and presence of 300 mg/ml crowding agents (dextran 40, dextran 70, ficoll 70) or 2.0 M NaCl at pH 2.0, 25 °C

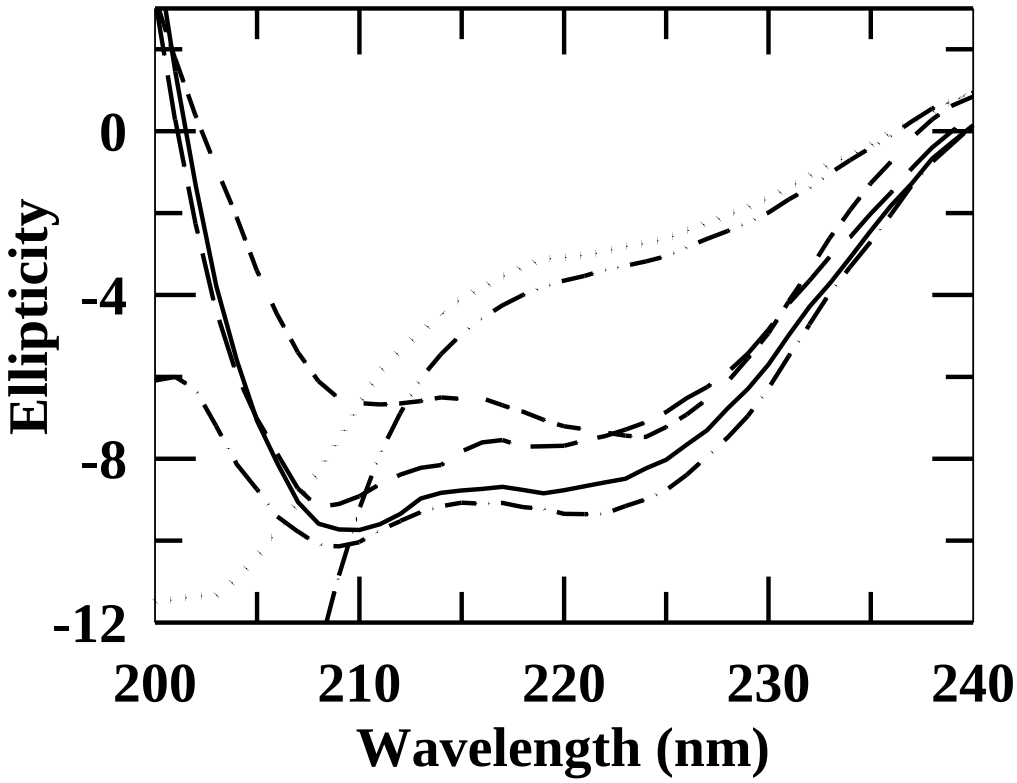

Fig. 1: Far-UV CD data of of Cyt *c*: pH 7, native state (short-short-short line); pH 2, denatured state (dotted line); 300 mg/ml dextran 40 induce secondary structure gain (solid line); 300 mg/ml dextran 70 induce secondary structure gain (long dash line); Ficoll 70 induce secondary structure gain (dash-dot-dot line); 2M NaCl induce secondary structure gain (dash-dot line).

For comparison, the far-UV CD spectrum of native Cyt *c* was also collected at pH 7.0, 25 °C. Clearly, the native Cyt *c* at pH 7.0 show two negative peaks at 210 and

222 nm (Fig. 1a), indicative of substantial secondary structure in the native protein. When pH is decreased from 7.0 to 2.0, the CD signals of these two negative peaks are lost (Fig. 1 dotted line), indicative of secondary structure disruption of protein. However, upon addition of 300 mg/ml of crowding agent () or 2.0 M NaCl, the acid-denatured protein gains substantial CD signals at 210 and 222 nm (Fig. 1 (dextran 40 (sold line), dextran 70 (long dash line), ficoll 70 (dash-dot-dot line), 2.0 M NaCl (dash dot line)) indicative of crowding or salt-induced formation of secondary structures.

4.2. Macromolecular crowding effect controls the refolding of acid-denatured Cyt *c*.

To test the effect concentration, size and shape of crowding agents on secondary structure of acid-denatured protein, the far-UV CD spectra of Cyt *c* were collected in the absence and presence of different concentrations of crowding agents (dextran 40, dextran 70, ficoll 70) or 2.0 M NaCl at pH 2.0, 25 °C. Under solution conditions, the ficoll is considered as a spherical shaped crowder while dextran is regarded rod-shaped [(Christiansen *et al.* 2010)]. The three synthetic crowding agents used in this study are related by Stokes radii in the given order as: dextran 70 (58 Å) >ficoll 70 (49.5 Å) >dextran 40 (45 Å) (http://tdbcons.com/images/pdf/FITCFicoll.pdf.). Fig. 2 presents far-UV CD monitored (222 nm) crowding-induced gain of secondary structure of acid denatured Cyt *c* at pH 2.0 (dextran 40 (filled triangle), dextran 70 (filled circle), ficoll 70 (filled squire). The extent of crowding-mediated gains in far-UV CD signals at 222 nm of acid-denatured Cyt *c* (Fig. 2a) typically follows the trend as dextran 40 > dextran 70 > ficoll 70 (Fig. 2), which reveals that the nature and shape of crowding agents or size of crowding agents of similar shape control the refolding of acid-denatured Cyt *c* at pH 2. The present project work also investigated the salt-mediated gains in far-UV CD signals at 222 nm of acid-denatured Cyt *c*. The data in Fig, 2b clearly indicates ionic screening of electrostatic interactions also prompt formation of secondary structure in acid-denatured Cyt *c*.

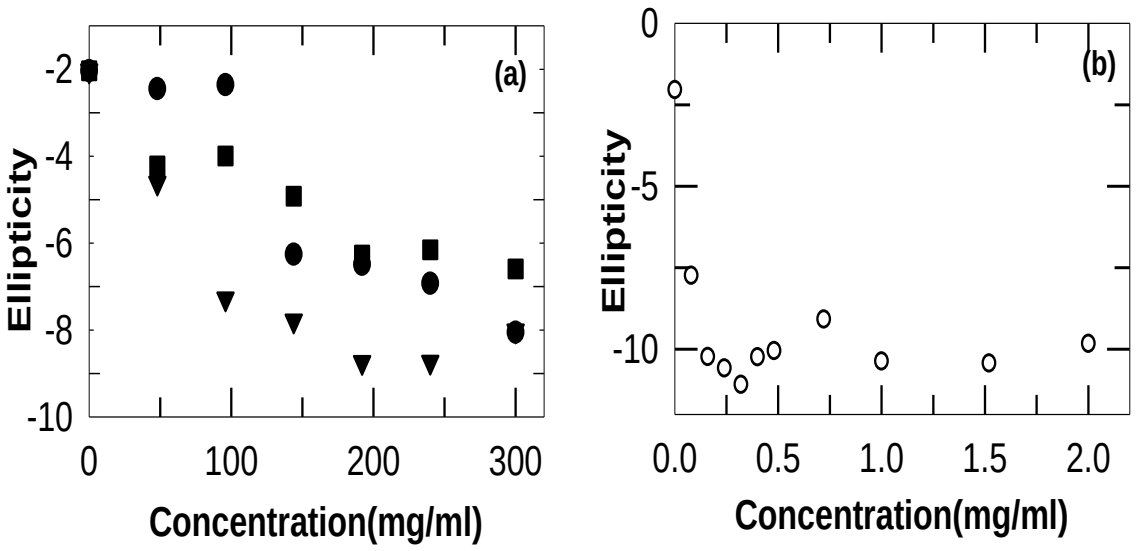

Fig. 2. (a) The change in ellipticity (222 nm) with [crowding agents] (mg/ml). Filled down triangle as a function of dextran 40, filled square as a function of ficoll 70, filled circle as a function of dextran 70. (b) Change in ellipticity (222 nm) with [NaCl] (M) at pH 2.0.

CHAPTER 5

DISCUSSION

5. Discussion:

The present project work investigates the role of shape, size and concentration of crowding on the secondary structure of acid-denatured Cyt *c* at pH 2. It is observed that as compared to native protein secondary structure at pH 7, the secondary structure of Cyt *c* is significantly disrupted at pH 2 due to positive charge repulsion in protein. However, inclusion of high concentration of crowding agents or salt in acid-denatured Cyt *c* at pH 2.0 induces significant content of secondary structures. The formation of secondary structure of acid-denatured Cyt *c* by salt is likely to ionic screening between negative charge of anions and positive charge of protein amino acid residues (Sasahara *et al.* 2003). The formation of secondary structure of acid-denatured Cyt *c* by crowding agent is likely due either entropic effect (exclude volume effects) or enthalpic effect (interaction between crowder and protein) or by both entropic and enthalpic effects. Dextran and ficoll are neutral molecules so ionic interactions are not expected; therefore, excluded volume effect should play major role in the crowding-mediated induced secondary of acid-denatured Cyt *c*. However, few recent reports showed that enthalpic effect also involves in crowding-mediated stabilization of native and denatured proteins (Benton *et al.* 2012; Kumar *et al.* 2015; Senske *et al.* 2014). To evaluate the exact thermodynamic parameter by which the crowding presence stabilizes the protein structure one should investigate the effect of macromolecular crowding on thermodynamic properties of acid-denatured Cyt *c*. The current results showed that the rod shaped crowder (dextran 70) produced greater content of secondary structure in acid-denatured Cyt *c* than the spherical shaped crowder (ficoll 70). The current results further showed that for similar shaped crowder, the smaller sized crowder (dextran 40) produced greater content of secondary structure in acid-denatured Cyt *c* than larger sized crowder (dextran 70). These results thus revealed that the crowding-mediated gain in secondary structure of acid-denatured Cyt *c* is modulated by concentration, size and shape of crowding agents.

The investigation of effect of macromolecular crowding on the denatured protein is very crucial for the study of the globular proteins not follows two state models but exhibit a compact intermediate state MG. The results in the figure **1** and **2** implicate that crowding agents continue to exert molecular force on Cyt *c* to

gain secondary structure that would not be found in the ideal diluted environment. These results have a vast biological significance in *in-vivo* highly crowded environment.

CHAPTER 6

CONCLUSIONS

6. Conclusions:

This study sought to elucidate that the high concentration of synthetic crowding agent induces secondary structure in acid-denatured Cyt *c*. The extent of crowding-induced secondary structure is dependent on the concentration, shape and size of crowder. With similar shaped, the smaller sized crowder (dextran 40) favors the more gain of secondary structure in acid-denatured Cyt *c* than the larger sized crowder (dextran 70). Furthermore, the rod shaped crowder (dextran 70) favors the more gain of secondary structure in acid-denatured Cyt *c* than the spherical shaped crowder (ficoll 70).

CHAPTER 7

REFERENCES

7. References:

Benjwal S, Jayaraman S,Gursky O. (2005). Electrostatic effects on the stability of discoidal high-density lipoproteins. Biochemistry, **44**, 10218-10226.

Benton L A, Smith A E, Young G B,Pielak G J. (2012). Unexpected effects of macromolecular crowding on protein stability. Biochemistry, **51**, 9773-9775.

Christiansen A, Wang Q, Samiotakis A, Cheung M S,Wittung-Stafshede P. (2010). Factors defining effects of macromolecular crowding on protein stability: an *in vitro/in silico* case study using cytochrome c. Biochemistry, **49**, 6519-6530.

Davidson M G,Deen W M. (1988). Hindered diffusion of water-soluble macromolecules in membranes. Macromolecules, **21**, 3474-3481.

Dix J A,Verkman A. (2008). Crowding effects on diffusion in solutions and cells. Annual Review of Biophysics, **37**, 247-263.

Dominy B N, Perl D, Schmid F X,Brooks III C L. (2002). The effects of ionic strength on protein stability: the cold shock protein family. Journal of Molecular Biology, **319**, 541-554.

Ellis R J. (2001). Macromolecular crowding: an important but neglected aspect of the intracellular environment. Current Opinion in Structural Biology, **11**, 114-119.

Ellis R J. (2001). Macromolecular crowding: obvious but underappreciated. Trends in Biochemical Sciences, **26**, 597-604.

Goto Y,Nishikiori S. (1991). Role of electrostatic repulsion in the acidic molten globule of cytochrome c. Journal of Molecular Biology, **222**, 679-686.

Granath K A. (1958). Solution properties of branched dextrans. Journal of Colloid Science, **13**, 308-328.

Greene R F,Pace C N. (1974). Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α-chymotrypsin, and β-lactoglobulin. Journal of Biological Chemistry, **249**, 5388-5393.

Holzwarth G,Doty P. (1965). The ultraviolet circular dichroism of polypeptides1. Journal of the American Chemical Society, **87**, 218-228.

Karshikoff A,Ladenstein R. (2007). The role of electrostatic interactions in the stabilization of proteins from thermophiles pp. 71: Nova Biomedical Books, New York.

Kelly S M,Price N C. (2000). The use of circular dichroism in the investigation of protein structure and function. Current Protein and Peptide Science, **1**, 349-384.

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.

Kumar R, Sharma D, Jain R,Kumar S. (2015). Role of macromolecular crowding and salt ions on the structural-fluctuation of a highly compact configuration of carbonmonoxycytochrome *c*. Biophysical chemistry, **207**, 61-73.

Kuroda R, Harada T,Shindo Y. (2001). A solid-state dedicated circular dichroism spectrophotometer: Development and application. Review of Scientific Instruments, **72**, 3802-3810.

Liu X, Kim C N, Yang J, Jemmerson R,Wang X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell, **86**, 147-157.

Pace C. (1995). [24] Evaluating contribution of hydrogen bonding and hydrophobic bonding to protein folding *Methods in Enzymology* **259**, 538-554: Elsevier.

Ptitsyn O. (1995). Molten globule and protein folding Advances in Protein Chemistry 47, 83-229: Elsevier.

Sasahara K, McPhie P,Minton A P. (2003). Effect of dextran on protein stability and conformation attributed to macromolecular crowding. Journal of Molecular Biology, **326**, 1227-1237.

Senske M, Törk L, Born B, Havenith M, Herrmann C,Ebbinghaus S. (2014). Protein stabilization by macromolecular crowding through enthalpy rather than entropy. Journal of the American Chemical Society, **136**, 9036-9041.

Tanford C. (1970). Protein Denaturation: Part C. Theoretical Models for the Mechanism of Denaturation Advances in Protein Chemistry **24**, 1-95: Elsevier.

Tokuriki N, Kinjo M, Negi S, Hoshino M,. (2004). Protein folding by the effects of macromolecular crowding. Protein Science, **13**, 125-133.

Whitford D. (2013). Proteins: structure and function, John Wiley & Sons.

Yang J, Liu X, Bhalla K, Kim C N,. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science, **275**, 1129-1132.

Urkund Analysis Result

Analysed Document: manoj16mscchm15thesis.pdf (D39600181)
Submitted: 5/31/2018 12:02:00 PM
Submitted By: rajendra.dhayal@gmail.com
Significance: 3 %

Sources included in the report:

http://dspace.thapar.edu:8080/jspui/handle/10266/4446
https://kundoc.com/pdf-role-of-macromolecular-crowding-and-salt-ions-on-the-structural-fluctuation-of-a.html

Instances where selected sources appear:

3

1 CHAPTER 1 INTRODUCTION

2

3 1. Introduction Protein plays vital roles in biological processes. It is necessary to know about how protein functioning and behaving. Proteins are encoded by genes, transcribed into ribonucleic acid (RNA) and translated into polypeptide with the help of ribosomes. Protein folding is a physical process in which unorganized polypeptide chain is transformed into a compact folded state. In general, the smaller sized single domain protein folding occurs in two state processes while the folding of longer sized proteins involves more intermediate states. Traditionally, the folded state has a define state with higher flexibility. Several molecular forces such hydrogen-bonding, Vander wall forces, ionic interactions and hydrophobic interactions are responsible for correct folding and stability of proteins (Benjwal et al. 2005; Dominy et al. 2002; Karshikoff et al. 2007; Pace 1995). The folded protein has several structural elements such as primary, secondary, tertiary and quaternary. Primary structure defines sequence of amino acids. Secondary structure further consists of α-helices and β-strands. Tertiary structure is the 3-dimensional structure. When a folded protein combined with the other folded proteins, it forms a multimeric assembly which is termed as quaternary structure. Metal ions presence in metalloproteins also plays important role in correct folding, stability and biological function (Whitford 2013). In general, there are three thermodynamic states of proteins, including folding, unfolding and molten globule(MG) states. Unfolded state is difficult to identify as compared to folded state. There is a small energy barrier between folded and folded state.(Whitford 2013) Tanford explained that the unfolded state acquires a disordered coil conformation. Generally, no residual secondary structure elements or tertiary structure elements are apparent in unfolded state.(Greene et al. 1974) MG state is a molecular compact state that has native like secondary structure and hydrodynamic radii but lacks or disordered tertiary structure.(Ptitsyn 1995) Cytochrome c (Cyt c) is an electron transfer mitochondrial globular protein and which is associated to the outside of the inner membrane. Cyt c also plays an important role in the oxidation inside the cells.(Dix et al. 2008). It is typically known as catalyst of respiration. It forms an electron bridge between oxygen and respirable substrates. Cyt c typically transfers electron from Cyt c reductase to Cyt c oxidase.

4 (Liu et al. 1996; Yang et al. 1997). Cyt c has about 104 amino acids in length with α-helical structure. It also contains a prosthetic type C heme group and that is attached to the polypeptide through cysteine residues. The coordinated iron in heme plays a vital role for its function as an electron relay between complexes in the electron transport chain. It has been reported that Cyt c unfolds reversibly under dilute buffer conditions. Furthermore, the reduced form of Cyt c (Ferrocyt c) is thermodynamically more stable than the oxidized form (Ferricyt c). It has been reported that under various solvent condtions (presence of salts, osmolytes, crowding agents) Cyt c form molten globules (MGs) at both acidic and alkaline conditions (Goto et al. 1991; Kumar et al. 2006). In general, the MG-states can be characterized by various spectroscopy techniques like circular dichroism(CD), NMR, Fluorescence.(Kelly et al. 2000; Kuroda et al. 2001) etc. Native state of proteins is generally compact and can be easily disrupted. Unfolded protein does not exhibit biological functions. To function properly, the unfolded protein must fold to three-dimensional shape. Proteins can be denatured at extreme

conditions, such as at extreme acidic and basic pH conditions, high temperature of pressure, low temperature (cold denaturation) and high concentration of denaturants (alcohols, urea, SDS, guanidine hydrochloride). The commonly use denaturants are urea and guanidine hydrochloride.(Tanford 1970) Protein folding is a spontaneous physical process. Upon folding, the free energy of the system lowers down. Overall free energy change of the system is reliant on two thermodynamic factors including, enthalpy and entropy. Entropy of unfolded state is greater than that of folded state. Typically, the folded state is stabilized enthalpically by H-bonding in secondary structure and hydrophobic interactions in protein core. Earlier in-vitro studies were carried out under ideal dilute conditions. However, in-vivo the protein folds in highly crowded cellular environment (Ellis 2001). In-vivo, the concentration range of macromolecules varies from ~80- 340 mg/ml. Typically, the crowding environment reduces the excluded volume occupy by unfolded protein. The extent of excluded volume depends on the shape and size of crowding agents. Generally, non-spherical molecules show larger magnitudes. To determine the exclude volume effects, there is no requirement of considering any electrostatic

5 interactions between crowding agents and proteins. Macromolecular crowding typically exhibits excluded volume effect, viscosity changes and change in non- specific interactions. Several research groups also estimated the difference between thermodynamic parameters in vivo and in vitro. It is expected that the chemical potential for unfolded proteins increases and which resulting to destabilize the unfolded state. Researchers already investigated the concentration effect of sugar on protein stability and got affirmative results (Sasahara et al. 2003). This research project work investigated the role of concentration, shape and size of synthetic crowding agents (dextran 40, dextran 70, ficoll 70) on secondary structure of acid-denatured Cyt c at pH 2.0, 25 ?C. For comparison, this research project work also investigated the effect of salt on secondary structure of acid- denatured Cyt c at pH 2.0, 25 ?C.

6

7 CHAPTER 2 LITERATURE REVIEW

8 2. Literature review Excluded volume theory plays a great role on stability and folding of native and denatured proteins (Sasahara et al. 2003; Tokuriki et al. 2004). Most of the protein folding studies select synthetic crowding agents. Crowding agents are highly

9 soluble in solvent and have definite shape and size and they do not exhibit attractive interactions with protein. In general, the protein crowders are not soluble in high concentrations and they produce several charge-charge interactions. It is necessary to suppress these charges by inclusion of high concentrations of salts or by using low concentrations of proteins. Circular dichroism (CD) spectroscopy depicts the ability of a molecule for differential absorption of left- and right-circularly polarized light. Typically, the type of secondary structure and their relative abundance in the molecule depend on the absorption maxima and line shape in the far-UV CD spectrum of protein. Experimentally, secondary structure composition of a protein can be estimated by comparing a given CD spectrum against spectra of proteins with known secondary structures.(Davidson et al. 1988; Granath 1958) In native state of Cyt c, the two negative bands at 210 nm and 222 nm confirm

the presence of secondary structure. At pH 2 in absence of any salt and crowding protein, Cyt c significantly lost these peptide bands and thus substantially lost native structure (Sasahara et al. 2003). It was found that that high concentration of dextran 35 or salt (KCl) induces the secondary structure in acid-denatured Cyt c at pH 2.0 (Sasahara et al. 2003). The mechanism of dextran and KCl is quite different. KCl stabilized secondary structure of acid denatured Cyt c due to chloride ion interactions with positive charge on Cyt c. On the other hand, dextran 35 stabilized the secondary structure by decreasing the volume available to macro solute. There are few reports available which showed that both enthalpic and entropic effects contribute to crowding mediated stabilization of proteins. Further few reports showed that even enthalpic effect is dominant in crowding-mediated stabilization of proteins (Benton et al. 2012; Kumar, Sharma et al. 2015). The free energy barrier to folding decreases in presence of macromolecular crowding.(Ellis 2001). It is known that the MG and non-native states of protein exist in the cell and these influence the protein insertion and migration through membranes. MG state of protein generally forms at extreme acidic or alkaline pH in presence salts, crowding, and sugars salts (Sasahara et al. 2003)

10

11 CHAPTER 3 MATERIAL AND METHODS

12 3. Material and methods: 3.1 Material Protein (Horse heart cytochrome c), buffer salt (glycine), common salt (NaCl) were purchased from Sigma Aldrich. Synthetic crowding agents such as dextran 40 and

13 dextran 70 were purchased from HI Media while ficoll 70 was purchased from GE Healthcare. All these chemicals/ proteins were used without further purification. 3.2 Methods 3.2.1. Measurement of Far-UV CD spectra of Cyt c in the absence and presence of crowding (dextran 40, dextran 70, ficoll 70) and NaCl. Far-UV CD spectra for acid-denatured Cyt c (pH 2.0) were collected by MOS 500 CD spectropolarimeter (Biologic France) in 10 mM glycine buffer

0: https://kundoc.com/pdf-role-of-macromolecular-crowding-and-salt-ions-on-the-structural-fluctuation-of-a.html 92%

in the absence and presence of 300 mg/ml crowding agent (dextran 40, dextran 70, ficoll 70)

or 2.0 M NaCl, 25 ?C. For comparison, the far-UV CD spectra for native Cyt c (pH 7.0) was collected in 50 mM phosphate buffer at 25 ?C. The incubation time prior to sample collection is about 3 hrs. The final concentration of protein in these sample was 10 ?M. 3.2.2. Measurement of Far-UV CD spectra of acid-denatured Cyt c (pH 2.0) in presence of different concentrations crowding agents and salt. Far-UV CD spectra for acid-denatured Cyt c (pH 2.0) were collected in the range of 0.0 to 300 mg/ml crowding agents (dextran 40, dextran 70, ficoll 70) or 0.0 to 2.0 M NaCl, 25 ?C. The incubation time prior to sample collection is about 3 hrs. The final concentration of protein was 10 µM.

14

15 CHAPTER 4 RESULT

16 4. Result: 4.1. Crowding and salt presence induces the secondary structure in acid-denatured Cyt c

17 In the far-UV CD spectra, the negative peaks at 210 and 222 nm reflects the secondary structures of native proteins (Holzwarth et al. 1965).Upon denaturation, the loss of these peaks revealed the disruption of secondary structure of proteins. To test the effect crowding and salt on secondary structure of acid-denatured protein,

0: http://dspace.thapar.edu:8080/jspui/handle/10266/4446 54%

the far-UV CD spectra of Cyt c

were collected

in the absence and presence of 300 mg/ml crowding agents (dextran 40, dextran 70, ficoll 70)

or 2.0 M NaCl at pH 2.0, 25 ?C Fig. 1:

Far-UV CD data of of Cyt c: pH 7, native state (short-short-short line); pH 2, denatured state (dotted line); 300 mg/ml dextran 40 induce secondary structure gain (solid line); 300 mg/ml dextran 70 induce secondary structure gain (long dash line); Ficoll 70 induce secondary structure gain (dash-dot-dot line); 2M NaCl induce secondary structure gain (dash-dot line). For comparison, the far-UV CD spectrum of native Cyt c was also collected at pH 7.0, 25 ?C. Clearly, the native Cyt c at pH 7.0 show two negative peaks at 210 and 222 nm (Fig. 1a), indicative of substantial secondary structure in the native protein. When pH is decreased from 7.0 to 2.0, the CD signals of these two negative peaks are lost (Fig. 1 dotted line), indicative of secondary structure disruption of protein. Wavelength (nm) 200 210 220 230 240 Ellipticity -12 -8 -4 0

18 However, upon addition of 300 mg/ml of crowding agent () or 2.0 M NaCl, the acid-denatured protein gains substantial CD signals at 210 and 222 nm (Fig. 1 (dextran 40 (sold line), dextran 70 (long dash line), ficoll 70 (dash-dot-dot line), 2.0 M NaCl (dash dot line)) indicative of crowding or salt-induced formation of secondary structures. 4.2. Macromolecular crowding effect controls the refolding of acid-denatured Cyt c. To test the effect concentration, size and shape of crowding agents on secondary structure of acid-denatured protein, the far-UV CD spectra of Cyt c were collected

0: http://dspace.thapar.edu:8080/jspui/handle/10266/4446 89%

in the absence and presence of different concentrations of crowding agents (dextran 40, dextran 70, ficoll 70)

or 2.0 M NaCl at pH 2.0, 25 ?C. Under solution conditions, the ficoll is considered as a spherical shaped crowder while dextran is regarded rod- shaped [(Christiansen et al. 2010)]. The three

synthetic crowding agents used in this study are related by Stokes radii in the given order as: dextran 70 (58 Å) <ficoll 70 (49.5 Å) <dextran 40 (45 Å) (http://tdbcons.com/images/pdf/FITCFicoll.pdf.). Fig. 2 presents far-UV CD monitored (222 nm) crowding-induced gain of secondary structure of acid denatured Cyt c at pH 2.0 (dextran 40 (filled triangle), dextran 70 (filled circle), ficoll 70 (filled squire). The extent of crowding-mediated gains in far- UV CD signals at 222 nm of acid-denatured Cyt c (Fig. 2a) typically follows the trend as dextran 40 < dextran 70 < ficoll 70 (Fig. 2), which reveals that the nature and shape of crowding agents or size of crowding agents of similar shape control the refolding of acid-denatured Cyt c at pH 2. The present project work also investigated the salt-mediated gains in far-UV CD signals at 222 nm of acid-denatured Cyt c. The data in Fig, 2b clearly indicates ionic screening of electrostatic interactions also prompt formation of secondary structure in acid-denatured Cyt c.

19 Fig. 2. (a) The change in ellipticity (222 nm) with [crowding agents] (mg/ml). Filled down triangle as a function of dextran 40, filled square as a function of ficoll 70, filled circle as a function of dextran 70. (b) Change in ellipticity (222 nm) with [NaCl] (M) at pH 2.0. Concentration(mg/ml) 0 100 200 300 Ellipticity -10 -8 -6 -4 -2 Concentration(mg/ml) 0.0 0.5 1.0 1.5 2.0 Ellipticity -10 -5 0 (a) (b)

20

21 CHAPTER 5 DISCUSSION

22

23 5. Discussion: The present project work investigates the role of shape, size and concentration of crowding on the secondary structure of acid-denatured Cyt c at pH 2. It is observed that as compared to native protein secondary structure at pH 7, the secondary structure of Cyt c is significantly disrupted at pH 2 due to positive charge repulsion in protein. However, inclusion of high concentration of crowding agents or salt in acid-denatured Cyt c at pH 2.0 induces significant content of secondary structures. The formation of secondary structure of acid-denatured Cyt c by salt is likely to ionic screening between negative charge of anions and positive charge of protein amino acid residues (Sasahara et al. 2003). The formation of secondary structure of acid- denatured Cyt c by crowding agent is likely due either entropic effect (exclude volume effects) or enthalpic effect (interaction between crowder and protein) or by both entropic and enthalpic effects. Dextran and ficoll are neutral molecules so ionic interactions are not expected; therefore, excluded volume effect should play major role in the crowding-mediated induced secondary of acid-denatured Cyt c. However, few recent reports showed that enthalpic effect also involves in crowding-mediated stabilization of native and denatured proteins (Benton et al. 2012; Kumar et al. 2015; Senske et al. 2014). To evaluate the exact thermodynamic parameter by which the crowding presence stabilizes the protein structure one should investigate the effect of macromolecular crowding on thermodynamic properties of acid-denatured Cyt c. The current results showed that the rod shaped crowder (dextran 70) produced greater content of secondary structure in acid-denatured Cyt c than the spherical shaped crowder (ficoll 70). The current results further showed that for similar shaped crowder, the smaller sized crowder (dextran 40) produced greater content of secondary structure in acid-denatured Cyt c than larger sized crowder

(dextran 70). These results thus revealed that the crowding-mediated gain in secondary structure of acid-denatured Cyt c is modulated by concentration, size and shape of crowding agents. The investigation of effect of macromolecular crowding on the denatured protein is very crucial for the study of the globular proteins not follows two state models but exhibit a compact intermediate state MG. The results in the figure 1 and 2 implicate that crowding agents continue to exert molecular force on Cyt c to gain

24 secondary structure that would not be found in the ideal diluted environment. These results have a vast biological significance in in-vivo highly crowded environment.

25 CHAPTER 6 CONCLUSIONS

26 6. Conclusions:

27 This study sought to elucidate that the high concentration of synthetic crowding agent induces secondary structure in acid-denatured Cyt c. The extent of crowding- induced secondary structure is dependent on the concentration, shape and size of crowder. With similar shaped, the smaller sized crowder (dextran 40) favors the more gain of secondary structure in acid-denatured Cyt c than the larger sized crowder (dextran 70). Furthermore, the rod shaped crowder (dextran 70) favors the more gain of secondary structure in acid-denatured Cyt c than the spherical shaped crowder (ficoll 70).

28

29 CHAPTER 7 REFERENCES

30

Hit and source - focused comparison, Side by Side:

Left side: As student entered the text in the submitted document.
Right side: As the text appears in the source.

Instances from: http://dspace.thapar.edu:8080/jspui/handle/10266/4446

1: http://dspace.thapar.edu:8080/jspui/handle/10266/4446 54%	1: http://dspace.thapar.edu:8080/jspui/handle/10266/4446 54%
the far-UV CD spectra of Cyt c were collected in the absence and presence of 300 mg/ml crowding agents (dextran 40, dextran 70, ficoll 70)	the far UV-CD (222 nm) monitored thermal denaturation curves of base-denatured Cyt c, apoMb and Lyz measured in the absence and presence of 300 mg ml-1 of crowding agents (dextran 70 and ficoll 70)
2: http://dspace.thapar.edu:8080/jspui/handle/10266/4446 89%	**2: http://dspace.thapar.edu:8080/jspui/handle/10266/4446 89%**
in the absence and presence of different concentrations of crowding agents (dextran 40, dextran 70, ficoll 70)	in the absence and presence of different concentrations of crowding agents (dextran 70 and ficoll 70)

Instances from: https://kundoc.com/pdf-role-of-macromolecular-crowding-and-salt-ions-on-the-structural-fluctuation-of-a.html

0: https://kundoc.com/pdf-role-of-macromolecular-crowding-and-salt-ions-on-the-structural-fluctuation-of-a.html 92%

in the absence and presence of 300 mg/ml crowding agent (dextran 40, dextran 70, ficoll 70)

0: https://kundoc.com/pdf-role-of-macromolecular-crowding-and-salt-ions-on-the-structural-fluctuation-of-a.html 92%

in the absence and presence of 200 mg ml-1 crowding agent

NU

(dextran 40, dextran 70 and ficoll 70).