

STUDY OF HEME PROTEINS IN PRESENCE OF MACROMOLECULAR CROWDING AGENTS

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STUDY OF HEME PROTEINS IN PRESENCE OF MACROMOLECULAR CROWDING AGENTS

by

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Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to the



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Dedicated to my

Pujyapada Mej Da

(Rev. Dr. Alok Kumar Chakraborty)

CERTIFICATE

This is to certify that the thesis titled “**Study of Heme Proteins in Presence of Macromolecular Crowding Agents**” being submitted by **Mr. Jayanta Kundu** 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 him.

Mr. Jayanta Kundu 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 thesis are original and have not been submitted, in part or full, to any other University or Institute for the award of any other degree or diploma.

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Abstract

The thesis entitled "**Study of Heme Proteins in Presence of Macromolecular Crowding Agents**" focuses on the understanding of the effects of macromolecular crowding agents on the stability, structure and functions of heme proteins. This thesis features a detailed investigation of the stability and the changes in the local environment of the functional part, that is, the heme prosthetic group and environment around the heme of three heme proteins Myoglobin (Mb), *Synechocystis* Hemoglobin (SynHb) and Cytochrome *c*, in presence of macromolecular crowding agents utilizing various spectroscopic techniques. In addition the micro environments of various mixed macromolecular crowding agents have been investigated using fluorescence correlation spectroscopy (FCS).

Chapter 1 entitled '**Introduction**' provides a brief introduction to the structure - function relationship of heme proteins and the phenomenon of macromolecular crowding agents and its influence on the protein structure and function. It also describes the objectives of the studies carried out in this thesis and the strategy devised to achieve the same.

Chapter 2 entitled '**Materials and Methods**' describes chemical procurement, purification and storage along with the techniques used during the investigation. Specifically, UV-VIS spectroscopy, circular dichroism (CD), steady-state and time resolved fluorescence, flash photolysis and fluorescence correlation spectroscopy (FCS) were used to carry out the requisite characterization.

Chapter 3 entitled '**Unusual Effects of Crowders on Heme Retention in Myoglobin**' shows a detailed investigation of the heme stability of chemically denatured myoglobin in presence of various macromolecular crowding agents like Ficoll 70, Dextrans (Dextran 70, Dextran 40 and Dextran 6), Polyethylene glycols (PEG 8,00 and 35,000) and their respective monomers (Sucrose, Glucose and Ethylene glycol) and protein based crowders, BSA and lysozyme. Myoglobin (Mb) undergoes pronounced heme loss under denaturing conditions wherein the proximal histidine gets protonated. Our data show that macromolecular crowding agents (both synthetic and protein based) can appreciably influence the extent of heme retention in Mb. Interestingly, glucose and sucrose, the monomeric constituents of dextran and ficoll-based crowders were much more effective in preventing heme dissociation of Mb, albeit, at much higher concentrations. The protein crowders BSA and lysozyme show very interesting results with BSA bringing about the maximum heme retention amongst all the

crowding agents used while lysozyme induced heme dissociation even in the native state of Mb. The stark difference that these protein crowders exhibit when interacting with the heme protein is a testament to the varied interaction potentials that a test protein might be exposed to in the physiological (crowded) milieu.

Chapter 4 entitled '**Effects of Macromolecular Crowding on a Hexacoordinate Heme Protein: Structural and Functional Aspects**' describes the detailed investigation of the effects of synthetic macromolecular crowding agents on the conformational changes and functional properties of a hexacoordinated heme protein *Synechocystis* hemoglobin (SynHb) *in vitro*. We observed that both the folded and unfolded states of SynHb became more compact in presence of macromolecular crowding agents. Moreover, the CO ligand binding activity of SynHb is remarkably increased in presence of macromolecular crowding agents both in absence and presence of denaturants (urea and GdmCl). In addition, macromolecular crowding agents brought about appreciable local structural modulation, which we probed by using the Stern-Volmer quenching analyses of the emission of the tyrosine residues of the heme protein .

Chapter 5 entitled '**Effect of both individual and mixed crowding agents on (A) unfolding of myoglobin, (B) electrochemical behaviour of myoglobin and (C) soret band spectra of Cytochrome c**' describes the effects of mixed crowding agents on thermal unfolding of myoglobin. Our results are in contrast to our popular belief where synthetic crowding agents are always stabilizing in nature, that is, the effect of mixed crowding agents on myoglobin unfolding were destabilizing. Interestingly, the extent of destabilization decreases when myoglobin unfolding was carried out as a function of urea. Our electrochemical measurements show that macromolecular crowding agents are able to protect the heme environment of Mb from external perturbation, that is, chemical denaturation. Our results also reveals that macromolecular crowding agents can effectively modulate the heme moiety and its surrounding environment of another heme protein cytochrome c, giving rise to breakdown of the axial symmetry of the heme environment

Chapter 6 entitled '**Fluorescence correlation spectroscopy (FCS) studies of translational diffusion of FITC (Fluorescein isothiocyanate) in presence of mixed macromolecular crowding agents**' provides a detailed investigation of the diffusion characteristics of the fluorescent probe FITC (fluorescein iso thiocyanate) in crowded media (both individual and

mixed) by using the single-molecule based technique of FCS (fluorescence correlation spectroscopy). Our FCS measurements show that the microviscosity of the mixtures is always higher than the sum of the microviscosities of the individual crowding agents at their respective mixture concentrations. This observation implies that the effect of mixed macromolecular crowding is non-additive, that is, deviates from that of the sum of the individual components of the respective mixtures. This deviation depends on the sizes and nature of the crowding agents.

Chapter 7 entitled '**Conclusion and future prospects**' presents the conclusions drawn from the overall investigation including how the macromolecular crowding agents protect heme prosthetic group, the key factor of heme proteins, of myoglobin against dissociation, affect ligand binding properties and conformational changes of *synechocystis* hemoglobin and modulate the heme moiety of cytochrome *c*. This thesis opens the door to a host of future directions that will contribute significantly to the fundamental understanding of structure-function relationship of heme proteins in crowded environment.

"मैक्रोमोलेक्यूलर क्राउडिंग एजेंटों की उपस्थिति में हेम प्रोटीन का अध्ययन" शीर्षक वाले सिद्धांत में हेम प्रोटीन की स्थिरता, संरचना और कार्य पर macromolecular भीड़ के एजेंटों के प्रभाव की समझ पर केंद्रित है। इस थीसिस में स्थिरता की विस्तृत जांच और कार्यात्मक भाग के स्थानीय परिवेश में परिवर्तन, अर्थात्, हेम कृत्रिम समूह और तीन हेम प्रोटीन माइोग्लोबिन (एमबी), सिनीकोसिस्टिस हेमोग्लोबिन (सिंहिहुब) और सिटोक्रोम सी, विभिन्न स्पेक्ट्रोस्कोपिक तकनीकों का उपयोग करने वाले मैक्रोमोलेकुलर भीड़ एजेंटों की उपस्थिति में इसके अतिरिक्त फ्लोरोसेंस सहसंबंध स्पेक्ट्रोस्कोपी (एफसीएस) का उपयोग करके विभिन्न मिश्रित macromolecular भीड़ के एजेंटों के सूक्ष्म वातावरण की जांच की गई है।

अध्याय 1 का हकदार 'परिचय' संरचना के लिए एक संक्षिप्त परिचय प्रदान करता है- हेम प्रोटीन के कार्य संबंध और मैक्रोमोलेकुलर भीड़ वाले एजेंटों की घटना और प्रोटीन संरचना और फ़ंक्शन पर इसका प्रभाव। यह इस शोध में किए गए अध्ययनों के उद्देश्यों और उसी को प्राप्त करने के लिए तैयार की गई रणनीति का भी वर्णन करता है।

अध्याय 2 शीर्षक 'सामग्री और तरीके' में जांच के दौरान उपयोग की जाने वाली तकनीकों के साथ रासायनिक खरीद, शुद्धि और भंडारण का वर्णन किया गया है। विशेष रूप से, यूवी-वीआईएस स्पेक्ट्रोस्कोपी, सर्कुलर डिचोरिज्म (सीडी), स्थिर-राज्य और समय का समाधान किया गया फ्लोरोसेंट, फ्लैश फोटोलिसिस और फ्लोरोसेंस सहसंबंध स्पेक्ट्रोस्कोपी (एफसीएस) का उपयोग आवश्यक लक्षण वर्णन को पूरा करने के लिए किया गया था।

अध्याय 3 हकदार 'माइोग्लोबिन में हेम रिटेनेंट पर क्राउडर के असामान्य प्रभाव' में फैकोल 70, डेक्सट्रांस (डेक्सट्रान 70, डेक्सट्रान 40 और डेक्सट्रान 6), पॉलिथिलीन जैसे विभिन्न मैक्रोमोलेकुलर भीड़ वाले एजेंटों की उपस्थिति में रासायनिक रूप से विकृत माइओग्लोबिन की स्थिरता की विस्तृत जांच का पता चलता है। ग्लाइकोल (पीईजी 8,00 और 35,000) और उनके संबंधित मोनोमर्स (सुक्रोज, ग्लूकोज और इथाइलीन ग्लाइकॉल) और प्रोटीन आधारित भीड़, बीएसए और लाइसोसिम माइोग्लोबिन (एमबी) ने परिभाषित शर्तों के तहत हेमी हानि का सामना किया है जिसमें समीपस्थ हिस्टीडाइन प्रोटोनेट हो जाता है। हमारे आंकड़े बताते हैं कि मैक्रोमोलेकुलर भीड़ वाले एजेंट (दोनों सिंथेटिक और प्रोटीन आधारित) एमबी में हीम अवधारण की सीमा को सहाय्य रूप से प्रभावित कर सकते हैं। दिलचस्प बात यह है कि, ग्लूकोज और सुक्रोज, डेक्सट्रान के मोनोमेरिक घटकों और

एफसीओएल-आधारित भीड़ वाले एमबी के असंतुलन को रोकने में ज्यादा प्रभावी थे, यद्यपि अधिक मात्रा में सांद्रता में। प्रोटीन भीड़ बीएसए और लाइसोसिम बीएसए के साथ बहुत ही दिलचस्प परिणाम बताते हैं कि सभी भीड़-भाड़ एजेंटों में अधिकतम हेम अवधारण के रूप में इस्तेमाल किया जाता है जबकि लासोमाइम ने एमबी के मूल अवस्था में हीम विस्थापन भी प्रेरित किया था। हेम प्रोटीन के साथ बातचीत करते समय ये प्रोटीन भीड़ दर्शाने वाले अंतर में विभिन्न अंतर-क्षमता क्षमता के लिए एक वसीयतनामा है कि एक परीक्षण प्रोटीन को शारीरिक (भीड़ भरे) परिवेश में दिखाया जा सकता है।

अध्याय 4 हकदार 'एक Hexacoordinate हीम प्रोटीन पर Macromolecular भीड़ के प्रभाव: संरचनात्मक और कार्यात्मक पहलुओं' गठनात्मक परिवर्तन और एक hexacoordinated हीम प्रोटीन Synechocystis हीमोग्लोबिन के कार्यात्मक संपत्तियों पर सिंथेटिक macromolecular भीड़ एजेंटों के प्रभाव (SynHb) इन विट्रो की विस्तृत जांच का वर्णन करता है। हमने देखा कि मैक्रोमोलेकुलर भीड़ के एजेंटों की उपस्थिति में दोनों सीएनएचबी के मुड़ा हुआ और सामने हुए राज्य अधिक कॉम्पैक्ट बन गए। इसके अलावा, कंपनी ligand SynHb के बंधन गतिविधि उल्लेखनीय दोनों अभाव और denaturants की उपस्थिति (यूरिया और GdmCl) में macromolecular भीड़ एजेंटों की उपस्थिति में वृद्धि हुई है। इसके अलावा, macromolecular भीड़ एजेंटों सराहनीय स्थानीय संरचनात्मक मॉड्यूलन है, जो हम हीम प्रोटीन की tyrosine अवशेषों के उत्सर्जन के स्टर्न-Volmer बुझाने विश्लेषण का उपयोग करके जांच के बारे में लाया।

अध्याय 5 हकदार '(ए) (बी) मायोग्लोबिन की विद्युत व्यवहार और (सी) solet बैंड साइटोक्रोम ग के स्पेक्ट्रा, मायोग्लोबिन के खुलासा पर दोनों अलग-अलग और मिश्रित भीड़ एजेंटों के प्रभाव' मायोग्लोबिन के थर्मल खुलासा पर मिश्रित भीड़ एजेंटों के प्रभावों का वर्णन। हमारे परिणाम हमारे लोकप्रिय विश्वास के विपरीत हैं, जहां सिंथेटिक भीड़ एजेंट हमेशा प्रकृति में स्थिर होते हैं, अर्थात्, मैओग्लोबिन पर प्रकट मिश्रित एजेंटों का असर अस्थिर कर रहा था। दिलचस्प बात यह है कि जब मैओलोग्लोबिन फैलाना यूरिया के एक समारोह के रूप में किया जाता था तो अस्थिरता की सीमा घट जाती है। हमारे इलेक्ट्रोकेमिकल मापन से पता चलता है कि मैक्रोमोलेकुलर भीड़ वाले एजेंट बाहरी परेशानी से एमईएम के हेम पर्यावरण की रक्षा करने में सक्षम हैं, जो कि रासायनिक विकृति है। हमारे परिणाम भी पता चलता है कि macromolecular भीड़ एजेंटों को प्रभावी ढंग से हीम आधा भाग और एक अन्य हीम प्रोटीन साइटोक्रोम ग की उसके आस-पास के माहौल मिलाना कर सकते हैं, हीम वातावरण का अक्षीय समरूपता के टूटने को जन्म दे रही

अध्याय 6 हकदार 'Fluorescence सहसंबंध स्पेक्ट्रोस्कोपी (FCS) मिश्रित macromolecular भीड़ एजेंटों की उपस्थिति में FITC (fluorescein आइसोथियोसाइनेट) की अनुवादकीय प्रसार की पढ़ाई' एक विस्तृत भीड़ मीडिया में फ्लोरोसेंट जांच FITC के प्रसार विशेषताओं की जांच (fluorescein isothiocyanate) (प्रदान करता है एफसीएस (प्रतिदीप्ति सहसंबंध स्पेक्ट्रोस्कोपी) की एकल-अणु आधारित तकनीक का उपयोग करके व्यक्तिगत और मिश्रित दोनों)। हमारे एफसीएस मापन दर्शाते हैं कि मिश्रित पदार्थों की सूक्ष्मदर्शिता व्यक्तिगत एकत्रित एजेंटों की सूक्ष्मदर्शिताओं की तुलना में उनके संबंधित मिश्रण सांद्रता से हमेशा अधिक होती है। यह अवलोकन दर्शाता है कि मिश्रित मैक्रोमोलेकुलर भीड़ का प्रभाव गैर-योजक है, जो कि संबंधित मिश्रणों के व्यक्तिगत घटकों के योग से भटक जाता है। यह विचलन भीड़ वाले एजेंटों के आकार और प्रकृति पर निर्भर करता है।

'निष्कर्ष और भविष्य की संभावनाओं' के अध्याय 7 में समग्र जांच से प्राप्त निष्कर्ष प्रस्तुत किए गए हैं जिनमें शामिल हैं कि मैक्रोमोलेकुलर भीड़ वाले एजेंटों से हेम कृत्रिम समूह की रक्षा कैसे की जाती है, हेम प्रोटीन का मुख्य घटक, पृथक्करण के विरुद्ध माओलोग्लोबिन, लिगैंड बाध्यकारी गुणों को प्रभावित करता है और सिनेकोसिस्टिस हीमोग्लोबिन के गठनात्मक परिवर्तन को प्रभावित करता है। और साइटोक्रोम सी के हीम अंश को नियंत्रित करते हैं। यह शोध भविष्य के दिशा-निर्देशों के लिए दरवाजे खोलता है जो भीड़ वाले परिवेश में हेम प्रोटीन के संरचना-समारोह संबंधों की मौलिक समझ में महत्वपूर्ण योगदान देगा।

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