

**RESIDUE LEVEL INVESTIGATION OF PROTEIN
CONFORMATIONS IN THE CROWDED MEDIUM: A
FLUORESCENCE QUENCHING APPROACH**

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INDIAN INSTITUTE OF TECHNOLOGY DELHI
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CONFORMATIONS IN THE CROWDED MEDIUM: A
FLUORESCENCE QUENCHING APPROACH**

by

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Department of Chemistry

Submitted

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



INDIAN INSTITUTE OF TECHNOLOGY DELHI

OCTOBER 2019

Dedicated to my mother

Smt. Mamta Singh

Certificate

This is to certify that the thesis titled “**Residue Level Investigation of Protein Conformations in the Crowded Medium: A Fluorescence Quenching Approach**” being submitted by **Mrs. Priyanka Singh** 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.

Mrs. Priyanka Singh 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 “**Residue Level Investigation of Protein Conformations in the Crowded Medium: A Fluorescence Quenching Approach**” describes the novel approach of using the sensitivity of the tryptophan (Trp) fluorescence of different proteins to its (Trp) surroundings to monitor the local perturbations in the immediate proximity of the intrinsic fluorophore. The thesis features detailed investigation of the Fluorescence quenching using the intrinsic tryptophan residues as the local reporters of modulations of protein conformations in the crowded milieu, in presence of a variety of external conditions such as changes in pH, thermal and/ or chemical denaturation. Moreover we have also included traditional quenchers like acrylamide and KI to interrogate the local conformational changes taking place in different proteins under these aforementioned conditions. Our work highlights the importance of using local probes in understanding alterations in protein structure in greater detail in presence of crowding agents.

Chapter 1 entitled (**Introduction**) anticipate how macromolecular crowding has been known to influence the conformational landscape of proteins both globally and locally. The chapter gives an account of the research work that has been carried out by various research groups to understand and overcome the limitations and challenges for the local perturbation and dynamics of proteins using various site specific IR probes and nonnatural amino acid. Quenching studies using Trp (Tryptophan) as local reporters have been focused. The chapter further discusses macromolecular crowding, that has also been shown to modulate the protein conformation.

Chapter 2 entitled (**Materials and Methods**) describes chemical procurement, purification and storage along with techniques used for carrying out experiments have been summarized. Specifically, steady-state and time-resolved fluorescence, UV-VIS spectroscopy, Circular Dichroism (CD) and Dynamic light scattering (DLS) measurements were used to carry out the requisite characterization. The techniques of fluorescence (steady-state and time-resolved) and CD spectroscopy have been employed to study the conformational changes of the proteins.

Chapter 3 entitled "**Crowding-Induced Quenching of Intrinsic Tryptophans of Serum Albumins: A Residue-Level Investigation of Different Conformations**" In this chapter, we have used the intrinsic fluorescence of the tryptophan (Trp) residues of two homologous proteins, BSA (bovine serum albumin) and HSA (human serum albumin), to provide residue-level interrogation of the protein conformations in the presence of different crowding agents (e.g., Dextran 6, Dextran 40, Ficoll 70, and PEG 8000). Our results hint at significant modulation in local structure as addressed by the Trp residues, with almost all of the crowding agents bringing about quenching of the Trp fluorescence. The nature of perturbation was observed to be largely dependent on the specific crowding agent used, with Dextran 6 showing the maximum effect while PEG 8000 showed the least. Additionally the extent of local structure modulation was found to be the largest either in the native state of the proteins or under near-native conditions, signifying the important role that the

surrounding amino acids play in determining the fluorescence of the Trp residues. Although BSA and HSA show ~76% sequence homology and have almost identical structural disposition in the native state, their individual responses to the crowder-induced perturbation were found to be quite different. The observed quenching occurs via a static mechanism that we hypothesize to be resulting from ground-state complex formation between Trp and the neighboring amino acid residues in response to the enhanced excluded volume effect as the crowder concentration is increased.

Chapter 4 entitled "**Unravelling the Intricacy of the Crowded Environment Through Tryptophan Quenching in Lysozyme**" In this work, using the intrinsic tryptophan (Trp) residues of lysozyme, we have studied the effect of the crowding agents on the local conformation of this biomolecule. In presence of the macromolecular crowder Dextran 6, considerable quenching of tryptophan fluorescence was observed, which we have attributed to the enhanced proximity of the surrounding charged residues arising from local perturbation of protein structure. Accessibility of the Trp residues in presence of crowders as a function of thermal and chemical denaturation has also been monitored using the traditional quenchers, acrylamide and iodide. Quenching in presence of the crowding agents had to be modelled predominantly using the sphere-of-action model, with the sphere-of-volume ' V ' being postulated to be a signature of the cage-like environments that can exist in the solutions of such polymeric macromolecular crowding agents. Moreover, percolation of the quencher molecules through the entangled crowder systems was observed to be dependent on the micropolarity of the specific crowding agent being studied, with the neutral quencher acrylamide exhibiting maximum quenching in presence of Dextran 6, while iodide being charged, exhibits higher quenching efficiency when Dextran 70 was the crowder. Additionally, control studies with the free amino acid tryptophan suggest that the variation in quenching so observed is not only due to the changes in the conformation of lysozyme and hence accessibility of the Trp residues but is also dictated by the underlying details and complexity of the crowder solutions to an appreciable extent.

Chapter 5 entitled "**Effect of Macromolecular Crowding on the Homodimeric protein, Beta Lactoglobulin: Local Interactions and Hydrophobic Changes**" In this chapter we have compared the modulations in the local conformations of Beta lactoglobulin (β -LG) in its two different forms by probing how the intrinsic Trp fluorescence is affected in presence of different crowding agents namely, Dextran 6, Dextran 40, Dextran 70, Ficoll 70 and monomers like glucose and sucrose, ethylene glycol (EG) and PEG 8000. Using crowder induced quenching Dextran 6 was the most dominating and potent amongst all the crowding agents. Thermal studies in presence of traditional quencher acrylamide have been carried out under crowded media leading to dramatic modulations of protein conformation. To reveal the changes in hydrophobic patches as a function of the crowders, we have also performed ANS binding assays for both the monomeric and dimeric forms of the protein. It was evident that for the high molecular weight crowders, hydrophobic patches become increasingly more accessible for the dimeric form while the reverse happens for the monomer.

Chapter 6 entitled "**Probing the Accessibility of Intrinsic Tryptophans of Bovine Serum Albumin in Crowded Environments using Fluorescence Quenching**" In this chapter we have focused on intrinsic tryptophan fluorescence quenching to monitor the changes in a multidomain protein Bovine serum albumin (BSA) in the presence of various crowding agents like Dextran 6, 40, 70, Ficoll 70 and PEG 8000. Temperature dependent quenching experiments were carried out in buffer and in presence of different concentrations of the commonly used chemical denaturants, Urea and Guanidium Hydrochloride (GdmHCl). Interestingly enough, BSA only (in absence of the crowders) exhibited the lowest exposure of its Trp residues. This implies that the crowders induced greater accessibility to the intrinsic fluorophore, an aspect that reveals significant changes in the immediate vicinity of the tryptophans. GdmHCl proved to be more potent in bringing into deciphering changes in Trp fluorescence as compared to urea under crowded media. The data analysis based on quenching mechanism for the crowding agents can differ considerably (linear, sphere of action, or Lehrer's plot) based on the solvent conditions thereby further alludes to the complexity of the energy landscape of proteins.

Chapter 7 entitled "**Conclusion and future prospects**" represents the conclusions drawn from all the studies mentioned above and the future prospects of the work carried out so far in this thesis. Local probes are thus incredibly useful tools poised to play a tremendous role in deciphering the future challenges that the crowded medium has to offer.

सारांश

"मध्यम स्तर पर प्रोटीन के जमाव के अवशेष स्तर की जांच: एक प्रतिदीप्ति शमन दृष्टिकोण" शीर्षक वाली थीसिस स्थानीय तृप्ति की निगरानी करने के लिए विभिन्न प्रोटीनों की ट्रिप्टोफैन (टीआरपी) की संवेदनशीलता का उपयोग करने के महान दृष्टिकोण का वर्णन करती है। आंतरिक फ्लोरोफोरे की तत्काल निकटता। थीसिस पीएच, थर्मल और/या रासायनिक विकृतीकरण में परिवर्तन जैसे बाहरी परिस्थितियों की उपस्थिति में, भीड़ वाले मील में प्रोटीन के परिवर्तन के स्थानीय संवाददाताओं के रूप में आंतरिक ट्रिप्टोफैन अवशेषों का उपयोग करके प्रतिदीप्ति शमन की विस्तृत जांच की सुविधा है। इसके अलावा हमने इन पूर्वोक्त स्थितियों के तहत विभिन्न प्रोटीनों में होने वाले स्थानीय रूपात्मक परिवर्तनों से पूछताछ करने के लिए एक्रिलामाइड और केआई जैसे पारंपरिक क्वैशचरों को भी शामिल किया है। हमारा काम भीड़ एजेंटों की उपस्थिति में प्रोटीन संरचना में परिवर्तन को समझने में स्थानीय जांच का उपयोग करने के महत्व पर प्रकाश डालता है।

अध्याय 1 शीर्षक (परिचय) अनुमान लगाता है कि वैश्विक और स्थानीय स्तर पर मैक्रोलेक्यूलर मांसपेशियों को प्रोटीन के विरूपण परिदृश्य को प्रभावित करने के लिए कैसे जाना जाता है। अध्याय विभिन्न अनुसंधान समूहों द्वारा विभिन्न साइट विशिष्ट आईआर जांच और गैर-अलौकिक अमीनो एसिड का उपयोग करके प्रोटीन के स्थानीय गड़बड़ी और गतिशीलता के लिए सीमाओं और चुनौतियों को समझने और दूर करने के लिए किए गए शोध कार्यों का एक खाता देता है। स्थानीय संवाददाताओं के रूप में ट्रप (ट्रिप्टोफैन) का उपयोग करके शमन करने वाले अध्ययनों पर ध्यान केंद्रित किया गया है। अध्याय में मैक्रोमोलेक्यूलर भीड़ पर चर्चा की गई है, जिसे प्रोटीन विरूपण को संशोधित करने के लिए भी दिखाया गया है।

अध्याय 2 शीर्षक (सामग्री और विधियाँ) रासायनिक खरीद, शुद्धि और भंडारण का वर्णन करता है और साथ ही प्रयोगों को करने के लिए उपयोग की जाने वाली तकनीकों को संक्षेप में प्रस्तुत किया गया है। विशेष रूप से, स्थिर-राज्य और समय-संकल्प प्रतिदीप्ति, यूवी-विज़ स्पेक्ट्रोस्कोपी, परिपत्र द्विवर्णता (CD) और गतिशील प्रकाश बिखरने (DLS) माप का उपयोग अपेक्षित लक्षण वर्णन करने के लिए किया गया था। प्रतिदीप्ति (स्थिर-राज्य और समय-संकल्प) और सीडी स्पेक्ट्रोस्कोपी की तकनीकों को प्रोटीन के परिवर्तनकारी परिवर्तनों का अध्ययन करने के लिए नियोजित किया गया है।

अध्याय 3 शीर्षक "सीरम एल्बमों के आंतरिक ट्रिप्टोफैन की भीड़-प्रेरित शमन का शीर्षक: विभिन्न विकृतियों का एक अवशेष-स्तरीय जांच" इस अध्याय में, हमने दो समरूप प्रोटीन, बीएसए (गोजातीय सीरम) के ट्रिप्टोफैन (टीआरपी) अवशेषों के आंतरिक प्रतिदीप्ति का उपयोग किया है। एल्ब्यूमिन) और एचएसए (मानव सीरम एल्ब्यूमिन), विभिन्न भीड़भाड़ वाले एजेंटों (जैसे, डेक्सट्रान 6, डेक्सट्रान 40, फिकोल 70 और पेग 8000) की उपस्थिति में प्रोटीन के अवशेषों के स्तर की पूछताछ प्रदान करने के लिए। हमारे परिणाम स्थानीय संरचना में महत्वपूर्ण मॉड्यूलेशन पर संकेत देते हैं, जैसा कि ट्रप अवशेषों द्वारा संबोधित किया गया है, लगभग सभी क्राउडिंग एजेंटों ने टीआरपी प्रतिदीप्ति को बुझाने के लिए लाया है। गड़बड़ी की प्रकृति का उपयोग बड़े पैमाने पर विशिष्ट भीड़ एजेंट पर निर्भर होने के लिए किया गया था, जिसमें डेक्सट्रान 6 अधिकतम प्रभाव दिखाते हैं जबकि पीईजी 8000 सबसे कम दिखा। इसके अतिरिक्त स्थानीय संरचना मॉड्यूलेशन की सीमा या तो प्रोटीन की मूल स्थिति में या निकट-देशी परिस्थितियों में सबसे बड़ी पाई गई, जो महत्वपूर्ण भूमिका को दर्शाती है कि आसपास के अमीनो एसिड ट्रापी अवशेषों के प्रतिदीप्ति का निर्धारण करने में खेलते हैं। यद्यपि बीएसए और एचएसए 76% अनुक्रम होमोलॉजी दिखाते हैं और मूल राज्य में लगभग समान संरचनात्मक स्वभाव रखते हैं, क्राउडर-प्रेरित गड़बड़ी के लिए उनकी व्यक्तिगत प्रतिक्रियाएं काफी भिन्न पाई गईं। मनाया शमन एक स्थैतिक तंत्र के माध्यम से होता है जिसे हम ट्रप और पड़ोसी अमीनो एसिड अवशेषों के बीच ग्राउंड-स्टेट कॉम्प्लेक्स गठन के परिणामस्वरूप परिकल्पित करते हैं ताकि बढी हुई अपवर्जित मात्रा प्रभाव के जवाब में पड़ोसी अमीनो एसिड अवशेषों के रूप में भीड़ एकाग्रता बढ़े।

अध्याय 4 शीर्षक "लाइसोजाइम में ट्रिप्टोफैन शमन के माध्यम से भीड़ भरे वातावरण की गहनता को उजागर करना" शीर्षक है, इस काम में, लाइसोजाइम के आंतरिक ट्रिप्टोफैन (टीआरपी) अवशेषों का उपयोग करते हुए, हमने इस बायोमोलेक्यूल के स्थानीय संवहन पर भीड़ एजेंटों के प्रभाव का अध्ययन किया है। मैक्रोमोलेक्यूलर क्राउडर डेक्सट्रान 6 की उपस्थिति में, ट्रिप्टोफैन प्रतिदीप्ति की काफी शमन क्रिया देखी गई, जिसे हमने प्रोटीन संरचना के स्थानीय गड़बड़ी से उत्पन्न होने वाले आसपास के आवेशित अवशेषों की बढी हुई निकटता के लिए जिम्मेदार ठहराया है। थर्मल और रासायनिक विकृतीकरण के कार्य के रूप में भीड़ की उपस्थिति में ट्रप अवशेषों की पहुंच पर भी पारंपरिक क्वेंचर्स, एक्रिलामाइड और आयोडाइड का उपयोग करके निगरानी की गई है। भीड़ एजेंटों की उपस्थिति में शमन मुख्य रूप से क्षेत्र-ए-एक्शन मॉडल का उपयोग करके किया जाना था, जिसमें गोले की मात्रा 'V' को पिंजरे जैसे वातावरण के हस्ताक्षर के रूप में जाना जाता है जो समाधानों में मौजूद हो सकते हैं इस तरह के बहुलक मैक्रोमोलेक्यूलर भीड़ एजेंट। इसके अलावा, उलझा हुआ क्राउडर सिस्टम के माध्यम से क्वेन्चर अणुओं का परावर्तन अध्ययन किया जा रहा विशिष्ट क्राउडिंग एजेंट की सूक्ष्मता पर निर्भर था, तटस्थ क्वेंकर एक्रिलामाइड के साथ डेक्सट्रान 6 की उपस्थिति में अधिकतम शमन का प्रदर्शन किया गया, जबकि आयोडाइड का आरोप लगाया जा रहा है, उच्च शमन का प्रदर्शन करता है। दक्षता जब डेक्सट्रान 70 भीड़ थी। इसके अतिरिक्त,

मुक्त अमीनो एसिड ट्रिप्टोफैन के साथ नियंत्रण अध्ययन से पता चलता है कि बुझाने में भिन्नता न केवल लाइसोजाइम के संचलन में परिवर्तन के कारण होती है और इसलिए टीआरपी अवशेषों की पहुंच में कमी होती है, लेकिन यह भीड़ के अंतर्निहित विवरण और जटिलता से निर्धारित होती है। एक प्रशंसनीय सीमा तक समाधान।

अध्याय 5 शीर्षक "होमोडिमरिक प्रोटीन, बीटा लैक्टोग्लोबुलिन पर मैक्रोमोलेक्यूलर क्राउडिंग का प्रभाव: स्थानीय सहभागिता और हाइड्रोफोबिक परिवर्तन" इस अध्याय में हमने बीटा लैक्टोग्लोबुलिन (β -LG) के स्थानीय अनुरूपणों में संशोधन की तुलना अपने दो अलग-अलग रूपों में जांच करके की है कि कैसे। आंतरिक ट्रेप प्रतिदीप्ति अलग-अलग भीड़ एजेंटों की उपस्थिति में प्रभावित होती है, जैसे कि डेक्सट्रान 6, डेक्सट्रान 40, डेक्सट्रान 70, फिकॉल 70 और ग्लूकोज और सुक्रोज, एथिलीन ग्लाइकॉल (ईजी) और खूंटी 8000 जैसे मोनोमर। क्राउडर प्रेरित शमन Dextran 6 का उपयोग करना सबसे अधिक था। सभी भीड़ एजेंटों के बीच हावी और शक्तिशाली। पारंपरिक क्वेनर एक्रिलामाइड की उपस्थिति में थर्मल अध्ययन भीड़ मीडिया के तहत किया गया है, जिससे प्रोटीन विरूपण के नाटकीय संशोधन होते हैं। भीड़ के एक समारोह के रूप में हाइड्रोफोबिक पैच में परिवर्तन को प्रकट करने के लिए, हमने प्रोटीन के मोनोमरिक और डिमरिक दोनों रूपों के लिए एएनएस बाध्यकारी assays का प्रदर्शन किया है। यह स्पष्ट था कि उच्च आणविक भार भीड़ के लिए, मंदक के लिए हाइड्रोफोबिक पैच तेजी से अधिक सुलभ हो जाते हैं, जबकि रिवर्स मोनोमर के लिए होता है।

अध्याय 6 शीर्षक "प्रतिदीप्ति शमन का उपयोग करते हुए भीड़ भरे वातावरण में गोजातीय सीरम एल्बुमिन के आंतरिक ट्रिप्टोफैन की पहुंच की क्षमता" शीर्षक इस अध्याय में हमने एक मल्टीडोमैन प्रोटीन गोजातीय सीरम एल्बुमिन (बीएसए) की उपस्थिति में परिवर्तन की निगरानी के लिए आंतरिक ट्रिप्टोफैन प्रतिदीप्ति शमन पर ध्यान केंद्रित किया है। Dextran 6, 40, 70, Ficoll 70 और PEG 8000 जैसे विभिन्न क्राउडिंग एजेंट। बफर में तापमान पर निर्भर शमन प्रयोग किए गए और आमतौर पर इस्तेमाल किए जाने वाले रासायनिक डिनोट्रानेंट्स, यूरिया और गनीडेन हाइड्रोक्लोराइड (GdmHCl) के विभिन्न सांद्रता की उपस्थिति में। दिलचस्प रूप से पर्याप्त है, बीएसए ने केवल (भीड़ की अनुपस्थिति में) अपने ट्रेप अवशेषों के सबसे कम प्रदर्शन का प्रदर्शन किया। इसका मतलब यह है कि भीड़ ने आंतरिक फ्लोरोफोर के लिए अधिक पहुंच को प्रेरित किया, एक ऐसा पहलू जो ट्रिप्टोफैन के तत्काल आसपास के क्षेत्र में महत्वपूर्ण परिवर्तनों को प्रकट करता है। GdmHCl भीड़ मीडिया के तहत यूरिया की तुलना में Trp प्रतिदीप्ति में निर्णायक परिवर्तन लाने में अधिक शक्तिशाली साबित हुआ। भीड़ एजेंटों के लिए शमन तंत्र पर आधारित डेटा विश्लेषण विलायक के आधार पर काफी भिन्न हो सकता है (रैखिक, कार्वाई के क्षेत्र, या लेहरर की साजिश)। इस तरह की स्थितियाँ प्रोटीन के ऊर्जा परिदृश्य की जटिलता को और बढ़ाती हैं।

अध्याय 7 शीर्षक "निष्कर्ष और भविष्य की संभावनाएं" शीर्षक से ऊपर उल्लिखित सभी अध्ययनों और इस थीसिस में अब तक किए गए कार्य की भविष्य की संभावनाओं से प्राप्त निष्कर्षों का प्रतिनिधित्व करता है। स्थानीय जांच इस प्रकार अविश्वसनीय रूप से उपयोगी उपकरण हैं जो भविष्य की चुनौतियों का सामना करने में एक जबरदस्त भूमिका निभाने के लिए तैयार की जाती हैं जो कि भीड़-भाड़ वाले माध्यम को पेश करना पड़ता है।

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