

**PROTEIN DYNAMICS, ENERGY FLOW, AND
AGGREGATION MODULATION IN THE
ULTRAFAST TIMESCALE**

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by

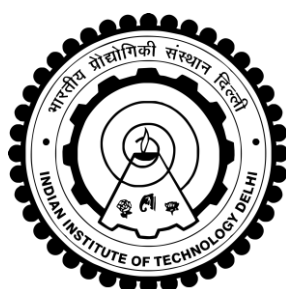
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Department Of Chemistry

Submitted

in fulfilment of the requirements of the degree of

Doctor of Philosophy



INDIAN INSTITUTE OF TECHNOLOGY DELHI

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Dedicated to

My Parents,

Mrs Shelley Majumdar and Mr Bijan Majumdar

and

In loving memory of my grandparents

Late Mrs Rama Chakraborty, Late Mr Anil Baran Chakraborty,

Late Mrs Aparna Majumdar and Late Mr Binay Krishna Majumdar

CERTIFICATE

This is to certify that the thesis titled “**Protein Dynamics, Energy Flow, and Aggregation Modulation in the Ultrafast Timescale**” being submitted by **Miss Shubhangi Majumdar** to the **Indian Institute of Technology Delhi**, for the award of the degree of **Doctor of Philosophy in Chemistry**, is a record of bonafide research work carried out by her.

Miss Shubhangi Majumdar 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 full or in parts, to any other University or Institute for the award of any other degree or diploma.

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Shubhangi Majumdar

Abstract

Proteins are increasingly recognised as highly dynamic molecules that continuously communicate with their surrounding environment to execute their biological functions with precision and efficiency. What sets this thesis, **Protein Dynamics, Energy Flow, and Aggregation Modulation in the Ultrafast Timescale**, apart is its unified investigation of protein dynamics, by sequentially tracking how proteins fluctuate, reorganise, and respond to perturbations arising from a crowded milieu. A key focus lies in uncovering the driving force behind these dynamics and conformational fluctuations—specifically, how energy travels through protein frameworks and how this flow maintains a delicate balance between the internal cavity and the surrounding environment. Probing this balance clarifies why proteins remain stable and functional under physiological conditions and what triggers their transition toward aggregation when this equilibrium breaks down. The thesis further elucidates the mechanistic pathways through which small molecules modulate aggregation and mitigate the associated toxicity. Throughout, ultrafast spectroscopy serves as the central tool, capturing the earliest molecular events that ultimately shape long-term protein behaviour.

Chapter 1, entitled **Introduction**, establishes the conceptual foundation of the thesis by discussing how protein dynamics underpin biological function and how these motions are influenced by macromolecular crowding. It highlights the roles of excluded-volume effects and soft interactions in reshaping conformational landscapes and altering protein behaviour in physiologically relevant environments. The chapter then introduces the concept of energy flow within proteins, explains its significance, and reviews key studies spanning heme systems, small peptides, and non-heme proteins to situate the present work within the broader field. A detailed description of protein aggregation, including its molecular steps, its contribution to various diseases, and current inhibition strategies, is provided, with special emphasis on the role of natural polyphenols and their ESIP-T-based photophysical mechanisms. The chapter concludes with an overview of the six proteins investigated in this thesis, namely HSA (PDB: 1AO6), BSA (PDB: 3VO3), β -Lactoglobulin (PDB: 2AKQ), Cytochrome c (PDB: 2B4Z), SOD1 (PDB: 1HL5), and the intrinsically disordered Huntingtin exon-1 mutant HD39Q.

Chapter 2, entitled **Materials and Methods**, lists all chemicals, proteins, ligands, and reagents used, and describes the recombinant expression and purification of the Huntingtin HD39Q mutant and SOD1 proteins. It outlines the use of UV–Vis spectroscopy for concentration measurements and binding studies, steady-state fluorescence instruments (FLS920 and

Fluoromax) for emission to explore global conformation and binding interactions, circular dichroism (CD) for secondary and tertiary structural intrusion, Raman spectroscopy for probing vibrational signatures, and the Ultrafast transient absorption setup for tracking excited state dynamics. Molecular docking procedures used to analyse ligand–protein interactions are also summarised. Together, these techniques provide a comprehensive framework for probing structure, dynamics, and interactions across all investigated systems.

Chapter 3, entitled **Bridging Soft Interaction and Excluded Volume in Crowded Milieu through Subtle Protein Dynamics**, demonstrates how macromolecular crowding affects protein dynamics, revealing a transition from soft–interaction–dominated behaviour at low crowder concentrations to excluded–volume–dominated effects at higher ones. Using temperature-dependent measurements on human serum albumin (HSA), it is shown that both local and global protein motions are modulated by the nature and concentration of the crowders, with asymmetric domain responses that reveal a “bridging” regime largely independent of crowder identity. Interestingly, this study distinguishes between solvent-coupled and decoupled motions, indicating a heterogeneous dynamic landscape in crowded environments. Finally, it proposes a link between increased global stability and relaxed, finely tuned local dynamics, suggesting that crowding-induced stabilisation can paradoxically facilitate subtle internal fluctuations.

Chapter 4, entitled **Capturing Ultrafast Energy Flow of a Heme Protein in Crowded Milieu**, reveals how macromolecular crowding alters the ultrafast relaxation pathways of the heme protein, Cytochrome c, by modulating the efficiency and directionality of energy dissipation. Using femtosecond transient absorption spectroscopy, the study shows that both through-space (ferric) energy transfer and methionine-rebinding (ferrous) kinetics channels are strongly solvent-coupled and exhibit pronounced slowing as crowding increases. The crowders induce microstructural organisation—mainly transient, cluster formation—around the protein, creating a heterogeneous environment that reshapes vibrational energy transport. This study demonstrates that synthetic crowders cause only modest delays, whereas protein-based crowders produce a significantly larger slowdown due to their more intimate and complex interactions with the protein surface, which disrupt the native hydrogen-bonding network of biological water. Together, these results demonstrate that the qualitative nature of the crowded environment, not just the degree of crowding, dictates how effectively energy migrates through the protein framework.

Chapter 5, entitled **Dynamic Balance Between Solvent Shell and Protein Core: Ultrafast Hemin Dynamics Mapping Energy Flow in Non-Heme Proteins**, highlights how the interplay between the protein core and its surrounding solvent shell governs the energy flow in non-heme proteins through the relaxation of the excited hemin. Using femtosecond transient absorption spectroscopy, the study reveals that proteins with buried binding pockets, such as HSA, facilitate rapid sub-ps relaxation, whereas proteins with solvent-exposed pockets, like BSA and β -LG, display delayed dynamics extending into the nanosecond regime. These striking differences highlight how microenvironmental heterogeneity—particularly solvent accessibility, hydrophobicity, and local structural organisation—dictates the energy-dissipation pathways. The results demonstrate that the surrounding solvent often plays a louder, more decisive role than the protein core itself in controlling the fate of energy flow.

Chapter 6, entitled **Polyphenols at the Helm: Excited State Photophysics and Mechanistic Insights into Protein Aggregation Modulation through Ultrafast Studies**, explores how natural polyphenols delay protein aggregation by mapping their excited-state behaviour. Ultrafast spectroscopy reveals ESPT-driven and related photophysical pathways that dictate how polyphenols interact with early oligomeric species. A clear correlation emerges that polyphenols that bind strongly to the native protein exhibit weaker inhibition, whereas those that fail to bind tightly to the native state show markedly stronger aggregation suppression by targeting oligomers instead. These findings demonstrate how excited-state reactivity guides selective binding to aggregation-prone intermediates. Together, the chapter provides a mechanistic framework that links transient photophysics to the long-timescale modulation of protein aggregation, whether globular or intrinsically disordered.

Chapter 7, entitled **Conclusion and Future Scope**, synthesises how ultrafast spectroscopy disentangles the combined influence of macromolecular crowding, solvent dynamics, and small-molecule modulators in regulating protein behaviour under physiologically relevant conditions. It highlights how crowding-driven soft interactions, heterogeneous caging, and solvent coupling collectively shape protein dynamics and energy flow, and ESPT-mediated polyphenol activity modulate aggregation across diverse protein systems. The chapter proposes extending these insights to cofactor-containing proteins other than heme, probing LLPS-induced heterogeneity using hemin as a microenvironmental reporter, and integrating ultrafast spectroscopy with in-cell measurements. Together, these future directions aim to achieve real-time mapping of energy dissipation and aggregation pathways directly within living, crowded environments.

सारांश

प्रोटीन को अत्यधिक गतिशील अणुओं के रूप में तेजी से पहचाना जा रहा है जो अपने जैविक कार्यों को सटीकता और दक्षता के साथ निष्पादित करने के लिए अपने आसपास के वातावरण के साथ निरंतर संचार करते हैं। इस शोध-प्रबंध, 'अल्ट्राफास्ट टाइमस्केल में प्रोटीन गतिकी, ऊर्जा प्रवाह और एकत्रीकरण मॉडुलन' को प्रोटीन गतिकी की एकीकृत जाँच द्वारा विशिष्ट बनाया गया है, जो क्रमिक रूप से यह पता लगाता है कि प्रोटीन कैसे उतार-चढ़ाव करते हैं, पुनर्गठित होते हैं और एक भीड़भाड़ वाले वातावरण से उत्पन्न होने वाले विक्षोभों पर प्रतिक्रिया करते हैं। मुख्य ध्यान इन गतिकी और संरूपणीय उतार-चढ़ावों के पीछे की प्रेरक शक्ति को उजागर करने पर है—विशेष रूप से, कैसे ऊर्जा प्रोटीन ढाँचों से होकर गुजरती है और कैसे यह प्रवाह आंतरिक गुहा और आसपास के वातावरण के बीच एक नाजुक संतुलन बनाए रखता है। इस संतुलन की जाँच यह स्पष्ट करती है कि शारीरिक परिस्थितियों में प्रोटीन स्थिर और क्रियाशील क्यों रहते हैं और जब यह संतुलन टूट जाता है तो एकत्रीकरण की ओर उनके संक्रमण को क्या प्रेरित करता है। यह शोध-प्रबंध उन यांत्रिक मार्गों को और स्पष्ट करता है जिनके माध्यम से छोटे अणु एकत्रीकरण को नियंत्रित करते हैं और संबंधित विषाक्तता को कम करते हैं। संपूर्ण प्रक्रिया में, अल्ट्राफास्ट स्पेक्ट्रोस्कोपी केन्द्रीय उपकरण के रूप में कार्य करती है, जो प्रारंभिक आणविक घटनाओं को पकड़ती है, जो अंततः दीर्घकालिक प्रोटीन व्यवहार को आकार देती हैं।

अध्याय 1, जिसका शीर्षक **परिचय** है, इस शोध-प्रबंध का वैचारिक आधार स्थापित करता है कि प्रोटीन गतिकी जैविक कार्य को कैसे आधार प्रदान करती है और ये गतियाँ वृहत् आणविक संकुलन से कैसे प्रभावित होती हैं। यह संरूपण परिदृश्यों को पुनः आकार देने और शारीरिक रूप से प्रासंगिक वातावरणों में प्रोटीन व्यवहार को बदलने में बहिष्कृत-आयतन प्रभावों और मृदु अंतःक्रियाओं की भूमिकाओं पर प्रकाश डालता है। इसके बाद, यह अध्याय प्रोटीन के भीतर ऊर्जा प्रवाह की अवधारणा का परिचय देता है, इसके महत्व की व्याख्या करता है, और हीम प्रणालियों, लघु पेप्टाइड्स और गैर-हीम प्रोटीनों पर किए गए प्रमुख अध्ययनों की समीक्षा करता है ताकि वर्तमान कार्य को व्यापक क्षेत्र में रखा जा सके। प्रोटीन एकत्रीकरण का विस्तृत विवरण, जिसमें इसके आणविक चरण, विभिन्न रोगों में इसका योगदान और वर्तमान अवरोध रणनीतियाँ शामिल हैं, प्रदान किया गया है, जिसमें प्राकृतिक पॉलीफेनोल्स की भूमिका और उनके ESIP-आधारित प्रकाशभौतिक तंत्रों पर विशेष जोर दिया गया है। अध्याय का समापन इस थीसिस में जांचे गए छह प्रोटीनों के अवलोकन के साथ होता है, अर्थात् HSA (PDB: 1AO6), BSA (PDB:

3VO3), β -Lactoglobulin (PDB: 2AKQ), Cytochrome c (PDB: 2B4Z), SOD1 (PDB: 1HL5), और आंतरिक रूप से अव्यवस्थित Huntingtin exon-1 उत्परिवर्ती HD39Q.

अध्याय 2, जिसका शीर्षक **सामग्री और विधियाँ** है, सभी प्रयुक्त रसायनों, प्रोटीनों, लिगैंडों और अभिकर्मकों की सूची प्रदान करता है, और हंटिंग्टिन HD39Q उत्परिवर्ती और SOD1 प्रोटीनों की पुनः संयोजक अभिव्यक्ति और शुद्धिकरण का वर्णन करता इसमें सांद्रता मापन और बंधन अध्ययन के लिए UV-Vis spectroscopy, वैश्विक अनुरूपता और बंधन अंतःक्रियाओं का पता लगाने के लिए उत्सर्जन हेतु स्थिर-अवस्था प्रतिदीप्ति उपकरणों (FLS920 and Fluoromax), द्वितीयक और तृतीयक संरचनात्मक घुसपैठ के लिए सर्कुलर डाइक्रोइज्म (CD), कंपन संकेतों की जांच के लिए Raman spectroscopy, और उत्तेजित अवस्था गतिशीलता पर नज़र रखने के लिए Ultrafast transient absorption सेटअप के उपयोग की रूपरेखा दी गई है। लिगैंड-प्रोटीन अंतःक्रियाओं का विश्लेषण करने के लिए प्रयुक्त आणविक डॉकिंग (Molecular docking) प्रक्रियाओं का भी सारांश दिया गया है। साथ में, ये तकनीकें सभी जाँची गई प्रणालियों में संरचना, गतिकी और अंतःक्रियाओं की जाँच के लिए एक व्यापक ढाँचा प्रदान करती हैं।

अध्याय 3, जिसका शीर्षक है **सूक्ष्म प्रोटीन गतिकी के माध्यम से भीड़ भरे वातावरण में मृदु अंतःक्रिया और अपवर्जित आयतन को जोड़ना**, यह दर्शाता है कि वृहद आणविक भीड़ प्रोटीन गतिकी को कैसे प्रभावित करती है, तथा कम भीड़ सांद्रता पर मृदु-अंतःक्रिया-प्रधान व्यवहार से लेकर उच्च सांद्रता पर अपवर्जित-आयतन-प्रधान प्रभावों में परिवर्तन को प्रकट करती है। मानव सीरम एल्ब्यूमिन (HSA) पर तापमान-निर्भर मापों का उपयोग करते हुए, यह दिखाया गया है कि स्थानीय और वैश्विक दोनों प्रोटीन गतियाँ भीड़ की प्रकृति और सांद्रता द्वारा नियंत्रित होती हैं, जिसमें असममित डोमेन प्रतिक्रियाएँ होती हैं जो एक "ब्रिजिंग" व्यवस्था को प्रकट करती हैं जो काफी हद तक भीड़ की पहचान से स्वतंत्र होती है। दिलचस्प बात यह है कि यह अध्ययन विलायक-युग्मित और अपयुग्मित गतियों के बीच अंतर करता है, जो भीड़ भरे वातावरण में एक विषम गतिशील परिदृश्य का संकेत देता है।

अध्याय 4, जिसका शीर्षक है **भीड़ भरे वातावरण में एक हीम प्रोटीन के अल्ट्राफास्ट ऊर्जा प्रवाह को कैप्चर करना**, यह बताता है कि मैक्रोमोलिक्युलर क्राउडिंग, ऊर्जा अपव्यय की दक्षता और दिशा को संशोधित करके हीम प्रोटीन, Cytochrome c के अल्ट्राफास्ट विश्राम मार्गों को कैसे बदल देती है। फेमटोसेकंड क्षणिक अवशोषण स्पेक्ट्रोस्कोपी का उपयोग करते हुए, अध्ययन से पता चलता है कि

अंतरिक्ष के माध्यम से (फेरिक) ऊर्जा हस्तांतरण और मेथिओनिन-रीबाइंडिंग (फेरस) कैनेटीक्स चैनल दोनों दृढ़ता से विलायक-युग्मित होते हैं और क्राउडिंग बढ़ने पर स्पष्ट धीमापन प्रदर्शित करते हैं। क्राउडर प्रोटीन के चारों ओर सूक्ष्म संरचनात्मक संगठन—मुख्य रूप से क्षणिक, क्लस्टर गठन—को प्रेरित करते हैं, जिससे एक विषम वातावरण बनता है जो कंपन ऊर्जा परिवहन को नया रूप देता है। यह अध्ययन दर्शाता है कि सिंथेटिक क्राउडर केवल मामूली देरी का कारण बनते हैं, जबकि प्रोटीन आधारित क्राउडर प्रोटीन सतह के साथ अपनी अधिक घनिष्ठ और जटिल अंतःक्रियाओं के कारण काफी बड़ी मंदी पैदा करते हैं, जो जैविक जल के मूल हाइड्रोजन-बंधन नेटवर्क को बाधित करते हैं। कुल मिलाकर, ये परिणाम दर्शाते हैं कि भीड़ भरे वातावरण की गुणात्मक प्रकृति, न कि केवल भीड़ की मात्रा, यह निर्धारित करती है कि प्रोटीन ढांचे के माध्यम से ऊर्जा कितनी प्रभावी रूप से प्रवास करती है।

अध्याय 5, जिसका शीर्षक है **विलायक आवरण और प्रोटीन कोर के बीच गतिशील संतुलन: गैर-हीम प्रोटीन में ऊर्जा प्रवाह का अति तीव्र हेमिन गतिकी मानचित्रण**, इस बात पर प्रकाश डालता है कि प्रोटीन कोर और उसके आसपास के विलायक आवरण के बीच परस्पर क्रिया उत्तेजित हेमिन के शिथिलन के माध्यम से गैर-हीम प्रोटीनों में ऊर्जा प्रवाह को कैसे नियंत्रित करती है। फेमटोसेकंड क्षणिक अवशोषण स्पेक्ट्रोस्कोपी का उपयोग करते हुए, अध्ययन से पता चलता है कि दबे हुए बंधन पॉकेट्स वाले प्रोटीन, जैसे HSA, तीव्र उप-अवशोषण शिथिलन को सुगम बनाते हैं, जबकि विलायक-उजागर पॉकेट्स वाले प्रोटीन, जैसे BSA और β -LG, नैनोसेकंड शासन तक विस्तारित विलंबित गतिशीलता प्रदर्शित करते हैं। ये उल्लेखनीय अंतर इस बात पर प्रकाश डालते हैं कि कैसे सूक्ष्म-पर्यावरणीय विविधता—विशेषकर विलायक की सुगमता, जलभीति, और स्थानीय संरचनात्मक संगठन—ऊर्जा-क्षय मार्गों को निर्धारित करती है। परिणाम दर्शाते हैं कि ऊर्जा प्रवाह के भाग्य को नियंत्रित करने में आसपास का विलायक अक्सर प्रोटीन कोर की तुलना में अधिक प्रभावशाली, अधिक निर्णायक भूमिका निभाता है।

अध्याय 6, जिसका शीर्षक है **हेल्म पर पॉलीफेनोल्स : अल्ट्राफास्ट अध्ययनों के माध्यम से प्रोटीन एकत्रीकरण मॉड्यूलेशन में उत्साहित राज्य फोटोफिजिक्स और यांत्रिक अंतर्दृष्टि**, यह पता लगाता है कि प्राकृतिक पॉलीफेनोल्स अपने उत्तेजित-अवस्था व्यवहार का मानचित्रण करके प्रोटीन एकत्रीकरण में देरी कैसे करते हैं। अल्ट्राफास्ट स्पेक्ट्रोस्कोपी ईएसआईपीटी-चालित और संबंधित फोटोफिजिकल मार्गों को प्रकट करती है जो यह निर्धारित करते हैं कि पॉलीफेनोल्स प्रारंभिक ओलिगोमेरिक प्रजातियों के साथ कैसे अंतःक्रिया करते हैं। एक स्पष्ट सहसंबंध उभरता है कि जो पॉलीफेनोल्स मूल प्रोटीन से

मजबूती से बंधते हैं वे कमजोर अवरोध प्रदर्शित करते हैं, जबकि जो मूल अवस्था से कसकर बंध नहीं पाते हैं वे इसके बजाय ओलिगोमर्स को लक्षित करके एकत्रीकरण दमन को स्पष्ट रूप से मजबूत दिखाते हैं। ये निष्कर्ष दर्शाते हैं कि कैसे उत्तेजित-अवस्था प्रतिक्रियाशीलता एकत्रीकरण-प्रवण मध्यवर्ती के लिए चयनात्मक बंधन को निर्देशित करती है। कुल मिलाकर, यह अध्याय एक यांत्रिक रूपरेखा प्रदान करता है जो क्षणिक प्रकाशभौतिकी को प्रोटीन एकत्रीकरण के दीर्घ-कालिक मॉड्यूलेशन से जोड़ता है, चाहे वह गोलाकार हो या आंतरिक रूप से अव्यवस्थित।

अध्याय 7, जिसका शीर्षक है **निष्कर्ष और भविष्य का दायरा**, यह संश्लेषित करता है कि कैसे अल्ट्राफास्ट स्पेक्ट्रोस्कोपी शारीरिक रूप से प्रासंगिक परिस्थितियों में प्रोटीन व्यवहार को विनियमित करने में मैक्रोमॉलिक्युलर क्राउडिंग, विलायक गतिकी और लघु-अणु मॉड्यूलैटर के संयुक्त प्रभाव को अलग करती है। यह इस बात पर प्रकाश डालता है कि कैसे क्राउडिंग-चालित मृदु अंतःक्रियाएँ, विषमांगी केजिंग और विलायक युग्मन सामूहिक रूप से प्रोटीन गतिकी और ऊर्जा प्रवाह को आकार देते हैं, और ESIPT-मध्यस्थ पॉलीफेनोल गतिविधि विविध प्रोटीन प्रणालियों में एकत्रीकरण को नियंत्रित करती है। अध्याय इन अंतर्दृष्टियों को हीम के अलावा अन्य सहकारक-युक्त प्रोटीनों तक विस्तारित करने, हेमिन को सूक्ष्म-पर्यावरणीय रिपोर्टर के रूप में उपयोग करके LLPS-प्रेरित विषमता की जाँच करने, और अल्ट्राफास्ट स्पेक्ट्रोस्कोपी को कोशिका-अंतर्गत मापों के साथ एकीकृत करने का प्रस्ताव करता है। साथ में, इन भविष्य की दिशाओं का उद्देश्य जीवित, भीड़-भाड़ वाले वातावरणों में सीधे ऊर्जा अपव्यय और एकत्रीकरण मार्गों का वास्तविक समय मानचित्रण प्राप्त करना है।

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