

**BIOPHYSICAL INVESTIGATIONS OF
ADENYLATE KINASE (AK3L1) IN THE CROWDED MILIEU-
CORRELATING ACTIVITY, CONFORMATION, STRUCTURE
AND DYNAMICS**

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**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY DELHI
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CORRELATING ACTIVITY, CONFORMATION, STRUCTURE
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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 Beloved Husband

CERTIFICATE

This is to certify that the thesis entitled, “**Biophysical Investigations of Adenylate Kinase (AK3L1) in the Crowded Milieu – Correlating Activity, Conformation, Structure and Dynamics**”, being submitted by **Ms. Harshita Rastogi** 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.

Ms. Harshita Rastogi 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 dissertation are original and have not been submitted, in part or full, to any other University or Institute for the award of any degree or diploma.

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Abstract

The emerging view considers enzymes to be highly flexible and dynamic biomolecules, in contrast to the conventional view of enzymes as rigid structural scaffolds. The interplay of structure and dynamics with the enzyme function has recently garnered much attention. The thesis titled **‘Biophysical Investigations of Adenylate Kinase (AK3L1) in the Crowded Milieu – Correlating Activity, Conformation, Structure and Dynamics’** is focused on understanding the correlation between activity, conformational flexibility, structure and dynamics of *Escherichia coli* adenylate kinase (AK3L1, UniProt Id: Q9UIJ7) under cell-mimicking crowded environments. The thesis entails a comprehensive investigation of the aspects that link enzyme catalysis to the dynamics at sub-nanosecond timescale and hence modulation of the overall energy landscape of the enzyme under different conditions, through fluorescence based spectroscopic studies.

Chapter 1 entitled **‘Introduction’** includes a brief introduction to the biophysical view of the enzymes, emphasizing mainly on the conformational motions at different timescales affecting the enzyme catalytic turnover. The energetic coupling of the enzyme dynamics with solvent has been discussed from different perspectives. The implications of the complex cellular interior mimicking macromolecular crowders on enzyme kinetics, conformational changes and dynamics have been talked about. Later in this chapter, these aspects have been discussed with regards to the multidomain enzyme, adenylate kinase (AK3L1).

Chapter 2 entitled **‘Materials and Methodologies’** describes chemical procurement, sample preparation along with different spectroscopic techniques used during the investigation. Specifically, UV-Vis spectroscopy, steady-state and time-resolved fluorescence, circular dichroism were used to carry out the characterization.

Chapter 3 entitled **‘Expression, Purification, Mutations and Enzymatic Activity Assay of AK3L1’** describes recombinant expression and purification of recombinant *E. Coli* adenylate kinase (AK3L1). The expressed protein was subsequently characterized using spectroscopic techniques, like UV-Visible spectroscopy, fluorescence measurements and CD scans for secondary structure determination. The construction of (alanine to cysteine) three single point mutants using site directed mutagenesis has been discussed. Activity assay and analysis of kinetic parameters of AK3L1 as well as its mutants have also been described.

Chapter 4 entitled **‘Understanding enzyme behavior in a crowded scenario through modulation in activity, conformation and dynamics’** addresses the different aspects by which

the crowded environment can affect the overall landscape of the multidomain enzyme AK3L1, through modulation in activity, structure and dynamics. Macromolecular crowders of different sizes and morphology have been used to mimic cellular conditions, namely, Ficoll 70, Dextran 40, Dextran 70 and PEG 8. The chapter focuses on the effect of crowders on the equilibrium properties of the enzyme, activity enhancement, domain movement monitored through FRET and local dynamics at sub-nanosecond timescale through solvation studies. Through this multipronged approach, a distinct correlation between activity, structure and dynamics has been observed, where crowders not only increased activity manifolds, but higher crowder concentration, mainly Ficoll 70, above 100 g/L, led to reduction in activity due to excess structural compaction and rigidity.

Chapter 5 entitled '**Correlating Local and Global Dynamics of an Enzyme in the Crowded Milieu**' describes an extensive study comparing the sub-nanosecond time-scale local dynamics with the global structural fluctuations of AK3L1 using solvation dynamics and quenching studies respectively, in the absence and presence of different concentrations of macromolecular crowders. Activation energy profiles of local dynamics were determined through temperature-dependent solvation studies at three different sites along the polypeptide backbone spanning different domains, while the activation energy for global dynamics was monitored using tryptophan quenching studies at different temperatures. In spite of the fact that solvation times increased as a function of crowding, the activation energies associated with the local dynamics undergoes a significant decrease as we increase the crowder concentrations. The crowded environment also aids in enhancing coupling between the local and global dynamics of multidomain enzyme. The study not only provides new insights into how crowding affects internal protein dynamics, but also mirrors the role of local motions as mechanical precursors in controlling the global motions when subjected to crowded conditions.

Chapter 6 entitled '**Towards the Energy Landscape of Adenylate Kinase in Crowded Milieu: Activity, Conformation, Structure and Dynamics in Sequence**' describes the activity, structure and dynamics of AK3L1 under crowded conditions as a function of urea induced chemical denaturation. The enzyme exhibited an initial increase in activity as a function of urea, in contrast to the expected decline in activity as a result of structural unfolding. The study presents interesting aspects, wherein a sequential trend of events was observed, with activity getting affected the first followed by structure, conformation and local dynamics. Higher urea concentration leads to appreciable alteration in structure (through CD) and conformation (through FRET). Surprisingly,

the increase in enzyme dynamics up to around 3-4 M urea shows that the enzyme gains structure around the local mutated site (A132C) even when it undergoes a significant decrease in global conformation. The results further prove that the observed protein dynamics are highly local in nature and are not necessarily coupled to the global structure of enzyme.

Chapter 7 entitled ‘**Conclusions and Future Perspectives**’ encompasses the conclusions drawn from the overall investigations carried out and the future scope of the research work. In brief, the thesis presents an insightful experimental investigation of the modulation in activity, structure, conformation and dynamics of the enzyme under the effect of macromolecular crowders. The enhancement in enzyme activity, reduced activation energy barrier of local dynamics and overall enzyme landscape alterations are the observed effects of cell-mimicking crowders. The interplay of intrinsic factors of proteins along with the extrinsic solvent factors have also been highlighted. The overall findings of this work provide a solid groundwork for a number of future directions, such as similar investigations in mixed macromolecular crowders, *in vivo* (cell based) studies probing conformational changes using FLIM and FRET approaches, single molecule studies using FCS and smFRET and much more.

सार

उभरता हुआ दृष्टिकोण एंजाइमों को कठोर संरचनात्मक मचानों के रूप में एंजाइमों के पारंपरिक दृष्टिकोण के विपरीत अत्यधिक लचीला और गतिशील जैव-अणुओं के रूप में मानता है। एंजाइम फंक्शन के साथ संरचना और गतिकी के परस्पर क्रिया ने हाल ही में बहुत ध्यान आकर्षित किया है। क्राउडेड मिलियू में 'बायोफिजिकल इन्वेस्टिगेशन ऑफ एडिनाइलेट किनेज (AK3L1) - सहसंबंधी गतिविधि, संरचना, संरचना और गतिशीलता' शीर्षक वाली थीसिस एस्चेरिचिया कोलाई एडिनाइलेट किनेज (AK3L1, UniProt Id: Q9UII7) की गतिविधि, संरचना के लचीलेपन, संरचना और गतिशीलता के बीच संबंध को समझने पर केंद्रित है। सेल-नकल भीड़-भाड़ वाले वातावरण में। थीसिस में उन पहलुओं की एक व्यापक जांच शामिल है जो एंजाइम उत्प्रेरण को उप-नैनोसेकंड टाइमस्केल पर गतिशीलता से जोड़ते हैं और इसलिए फ्लोरोसेंस आधारित स्पेक्ट्रोस्कोपिक अध्ययनों के माध्यम से विभिन्न परिस्थितियों में एंजाइम के समग्र ऊर्जा परिदृश्य का मॉड्यूलेशन करते हैं।

'परिचय' शीर्षक वाले अध्याय 1 में एंजाइमों के जैवभौतिक दृष्टिकोण का एक संक्षिप्त परिचय शामिल है, जो मुख्य रूप से एंजाइम उत्प्रेरक कारोबार को प्रभावित करने वाले विभिन्न समय-सीमाओं पर गठनात्मक गतियों पर जोर देता है। विलायक के साथ एंजाइम गतिकी के ऊर्जावान युग्मन पर विभिन्न दृष्टिकोणों से चर्चा की गई है। एंजाइम कैनेटीक्स, गठनात्मक परिवर्तन और गतिकी पर मैक्रोमोलेक्यूलर क्राउडर्स की नकल करने वाले जटिल सेलुलर इंटीरियर के निहितार्थ के बारे में बात की गई है। बाद में इस अध्याय में, इन पहलुओं पर मल्टीडोमेन एंजाइम, एडिनाइलेट किनेज (AK3L1) के संबंध में चर्चा की गई है।

'सामग्री और कार्यप्रणाली' शीर्षक वाले अध्याय 2 में जांच के दौरान उपयोग की जाने वाली विभिन्न स्पेक्ट्रोस्कोपिक तकनीकों के साथ-साथ रासायनिक खरीद, नमूना तैयार करने का वर्णन किया गया है। विशेष रूप से, यूवी-विज़ स्पेक्ट्रोस्कोपी, स्थिर-अवस्था और समय-समाधान प्रतिदीप्ति, वृत्ताकार द्वैतवाद का उपयोग लक्षण वर्णन करने के लिए किया गया था।

अध्याय 3 शीर्षक 'एके3एल1 की अभिव्यक्ति, शुद्धिकरण, उत्परिवर्तन और एंजाइमेटिक गतिविधि परख' पुनः संयोजक ई. कोलाई एडिनाइलेट किनेज (AK3L1) की पुनः संयोजक अभिव्यक्ति और शुद्धि का वर्णन करता है। व्यक्त प्रोटीन को बाद में स्पेक्ट्रोस्कोपिक तकनीकों का उपयोग करने की विशेषता थी, जैसे कि यूवी-विज़िबल स्पेक्ट्रोस्कोपी, प्रतिदीप्ति माप और माध्यमिक संरचना निर्धारण के लिए सीडी स्कैन। साइट निर्देशित उत्परिवर्तन का उपयोग करते हुए (एलानिन से सिस्टीन) तीन एकल बिंदु म्यूटेंट के निर्माण पर

चर्चा की गई है। गतिविधि परख और AK3L1 के गतिज मापदंडों के विश्लेषण के साथ-साथ इसके म्यूटेंट का भी वर्णन किया गया है।

‘गतिविधि, संरचना और गतिशीलता में मॉड्यूलेशन के माध्यम से भीड़ भरे परिदृश्य में एंजाइम व्यवहार को समझना’ शीर्षक वाला अध्याय 4 उन विभिन्न पहलुओं को संबोधित करता है जिनके द्वारा भीड़-भाड़ वाला वातावरण गतिविधि, संरचना और गतिशीलता में मॉड्यूलेशन के माध्यम से मल्टीडोमेन एंजाइम AK3L1 के समग्र परिदृश्य को प्रभावित कर सकता है। विभिन्न आकारों और आकारिकी के मैक्रोमोलेक्यूलर क्राउडर्स का उपयोग सेलुलर स्थितियों की नकल करने के लिए किया गया है, अर्थात्, फिकोल 70, डेक्सट्रान 40, डेक्सट्रान 70 और पीईजी 8। यह अध्याय एंजाइम, गतिविधि वृद्धि, डोमेन आंदोलन के संतुलन गुणों पर भीड़ के प्रभाव पर केंद्रित है। सॉल्वेशन अध्ययन के माध्यम से सब-नैनोसेकंड टाइमस्केल पर FRET और स्थानीय गतिकी के माध्यम से निगरानी की जाती है। इस बहुआयामी दृष्टिकोण के माध्यम से, गतिविधि, संरचना और गतिशीलता के बीच एक अलग सहसंबंध देखा गया है, जहां भीड़ ने न केवल गतिविधि को कई गुना बढ़ा दिया है, बल्कि उच्च भीड़ एकाग्रता, मुख्य रूप से 100 ग्राम / एल से ऊपर फिकोल 70, अतिरिक्त संरचनात्मक के कारण गतिविधि में कमी आई है। संघनन और कठोरता।

अध्याय 5 शीर्षक **‘भीड़ वाले वातावरण में एक एंजाइम के स्थानीय और वैश्विक गतिशीलता को सहसंबंधित करना’** एक व्यापक अध्ययन का वर्णन करता है जिसमें उप-नैनोसेकंड समय-पैमाने पर स्थानीय गतिशीलता की तुलना AK3L1 के वैश्विक संरचनात्मक उतार-चढ़ाव के साथ क्रमशः सॉल्वेशन डायनेमिक्स और शमन अध्ययनों का उपयोग करके की जाती है। मैक्रोमोलेक्यूलर क्राउडर्स के विभिन्न सांद्रता की उपस्थिति। स्थानीय गतिशीलता के सक्रियण ऊर्जा प्रोफाइल को अलग-अलग डोमेन में फैले पॉलीपेप्टाइड रीढ़ के साथ तीन अलग-अलग साइटों पर तापमान-निर्भर सॉल्वेशन अध्ययन के माध्यम से निर्धारित किया गया था, जबकि वैश्विक गतिशीलता के लिए सक्रियण ऊर्जा की निगरानी विभिन्न तापमानों पर ट्रिप्टोफैन शमन अध्ययन का उपयोग करके की गई थी। इस तथ्य के बावजूद कि भीड़ के एक समारोह के रूप में सॉल्वेशन समय में वृद्धि हुई है, स्थानीय गतिकी से जुड़ी सक्रियण ऊर्जा एक महत्वपूर्ण कमी से गुजरती है क्योंकि हम भीड़ की सांद्रता बढ़ाते हैं। भीड़-भाड़ वाला वातावरण मल्टीडोमेन एंजाइम की स्थानीय और वैश्विक गतिशीलता के बीच युग्मन को बढ़ाने में भी सहायता करता है। अध्ययन न केवल नई अंतर्दृष्टि प्रदान करता है कि कैसे भीड़ आंतरिक प्रोटीन गतिशीलता को प्रभावित करती है, बल्कि भीड़ की स्थिति के अधीन होने पर वैश्विक

गति को नियंत्रित करने में यांत्रिक अग्रदूतों के रूप में स्थानीय गतियों की भूमिका को भी प्रतिबिंबित करती है।

अध्याय 6 शीर्षक 'टुवार्ड्स द एनर्जी लैंडस्केप ऑफ एडिनाइलेट किनेज इन क्राउडेड मिलियु: एक्टिविटी, कंफॉर्मेशन, स्ट्रक्चर एंड डायनेमिक्स इन सीकेंस' यूरिया प्रेरित रासायनिक विकृतीकरण के कार्य के रूप में भीड़-भाड़ वाली परिस्थितियों में AK3L1 की गतिविधि, संरचना और गतिशीलता का वर्णन करता है। संरचनात्मक खुलासा के परिणामस्वरूप गतिविधि में अपेक्षित गिरावट के विपरीत, एंजाइम ने यूरिया के एक समारोह के रूप में गतिविधि में प्रारंभिक वृद्धि का प्रदर्शन किया। अध्ययन दिलचस्प पहलुओं को प्रस्तुत करता है, जिसमें घटनाओं की एक अनुक्रमिक प्रवृत्ति देखी गई थी, जिसमें गतिविधि पहले प्रभावित हुई, उसके बाद संरचना, संरचना और स्थानीय गतिशीलता। उच्च यूरिया सांद्रता संरचना (सीडी के माध्यम से) और संरचना (एफआरईटी के माध्यम से) में उल्लेखनीय परिवर्तन की ओर ले जाती है। आश्चर्यजनक रूप से, लगभग 3-4 एम यूरिया तक एंजाइम की गतिशीलता में वृद्धि से पता चलता है कि एंजाइम स्थानीय उत्परिवर्तित साइट (A132C) के आसपास संरचना प्राप्त करता है, तब भी जब यह वैश्विक संरचना में उल्लेखनीय कमी से गुजरता है। परिणाम आगे साबित करते हैं कि देखे गए प्रोटीन गतिकी प्रकृति में अत्यधिक स्थानीय हैं और जरूरी नहीं कि एंजाइम की वैश्विक संरचना से जुड़े हों।

अध्याय 7 शीर्षक 'निष्कर्ष और भविष्य के परिप्रेक्ष्य' में किए गए समग्र जांच और शोध कार्य के भविष्य के दायरे से निकाले गए निष्कर्ष शामिल हैं। संक्षेप में, थीसिस मैक्रोमोलेक्यूलर क्राउडर्स के प्रभाव में एंजाइम की गतिविधि, संरचना, संरचना और गतिशीलता में मॉड्यूलेशन की एक व्यावहारिक प्रयोगात्मक जांच प्रस्तुत करती है। एंजाइम गतिविधि में वृद्धि, स्थानीय गतिशीलता के सक्रियण ऊर्जा अवरोध को कम करना और समग्र एंजाइम परिदृश्य परिवर्तन सेल-नकल भीड़ के देखे गए प्रभाव हैं। बाह्य विलायक कारकों के साथ प्रोटीन के आंतरिक कारकों की परस्पर क्रिया पर भी प्रकाश डाला गया है। इस काम के समग्र निष्कर्ष भविष्य की कई दिशाओं के लिए एक ठोस आधार प्रदान करते हैं, जैसे कि मिश्रित मैक्रोमोलेक्यूलर क्राउडर्स में समान जांच, विवो (सेल आधारित) अध्ययनों में एफएलआईएम और एफआरईटी दृष्टिकोणों का उपयोग करते हुए गठनात्मक परिवर्तनों की जांच, एफसीएस और एसएमएफआरईटी का उपयोग करके एकल अणु अध्ययन और बहुत अधिक।

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