Structural Energy Bioscience Research Section

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Introduction

We investigate how biomolecules such as proteins (including enzymes) and functional nucleic acids (DNA and RNA) operate at atomic resolution using structural biology approaches with NMR. By determining both static and dynamic structures, supported by the development of novel methodologies, we elucidate the mechanisms underlying their functions. We also apply structural biology to analyze enzymes involved in degradation of woody biomass at atomic resolution. This analysis contributes to the development of methods for extracting energy and valuable compounds from woody biomass, which can serve as raw materials for a wide range of products. Through these efforts, we aim to support the paradigm shift from oil-based refineries to biorefineries. The following are the main research achievements of 2024.

1. Engineering a hypoxia-tolerant *Saccharomyces cerevisiae* for efficient ethanol production through co-utilization of glucose and acetic acid

Improving the robustness of microbial cell factories is essential for advancing bioethanol production. *Saccharomyces cerevisiae* often encounters fermentation stress from acetic acid, a by-product of both yeast metabolism and lignocellulosic biomass pretreatment, which inhibits growth and reduces fermentation efficiency. To overcome these challenges, we engineered a hypoxia-tolerant *S. cerevisiae* strain capable of rapid ethanol production while co-utilizing glucose and acetic acid under oxygen-limited conditions. Using CRISPR-Cas9, we sequentially deleted four key genes (*GPD1*, *ALD6*, *NDE1*, and *NDE2*) involved in glycerol synthesis and NADH oxidation, while introducing the acetylating acetaldehyde dehydrogenase gene from *Salmonella enterica* (*SeEutE*). The resulting strain, E5, achieved more than a 343% faster fermentation rate than the parent strain (C1) when grown under hypoxic conditions with 10% glucose and 0.4% acetic acid. E5 consumed approximately 25% of the acetic acid, reached 98% of the theoretical ethanol yield, and exhibited a 9% higher fermentation rate under hypoxic conditions than under hyperoxic conditions. Notably, flocculation in E5 was induced by ethanol accumulation, beginning as ethanol levels increased and reaching approximately 3%. By the end of fermentation, 75% of the cells were flocculated, likely enhancing both stress tolerance and fermentation performance. This flocculation occurred without intentional overexpression of flocculin genes, suggesting indirect induction mechanisms linked to metabolic engineering. These results present a novel strategy for optimizing *S. cerevisiae* as a cell factory for bioethanol production, providing a promising approach to efficiently co-ferment glucose and acetic acid while maintaining high performance under hypoxic conditions, with potential applications in both first- and second-generation bioethanol processes.

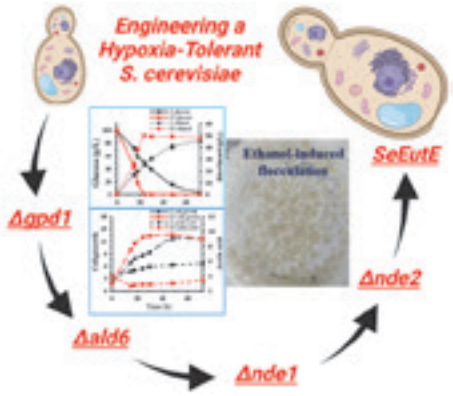

Figure 1. Engineering a hypoxia-tolerant *Saccharomyces cerevisiae* for rapid ethanol production via co-utilization of glucose and acetic acid and redox-enhanced flocculation.

2. Molecular crowding distinctly modulates base-pair dynamics in DNA triplex structures

This study sheds light on how molecular crowding (MC), a key feature of the intracellular environment, modulates the base-pair opening and closing (BPOC) dynamics of DNA triplex structures. DNA triplexes, composed of Hoogsteen base pairs (HBPs) and Watson–Crick base pairs (WCBPs), play crucial roles in gene regulation and genomic stability. However, how crowded environments affect their dynamics at the base-pair level has remained unclear. Using advanced NMR techniques, we determined, for the first time, the opening (k_{open}) and closing (k_{close}) rate constants of individual base pairs in a DNA triplex under MC conditions mimicked by Ficoll PM 70 and

PEG 200, representing excluded-volume effects and reduced water activity, respectively. Our findings reveal contrasting effects of these crowders. Ficoll PM 70 stabilized the triplex by prolonging the lifetime of closed base pairs, increasing Gibbs energies ($\Delta G^{\circ}_{\mathrm{open}}$) and thermal stability. In contrast, PEG 200 destabilized the triplex by shortening closed-state lifetimes and extending open state lifetimes near the strand ends, reducing $\Delta G^{\circ}_{\mathrm{open}}$ and thermal stability. Notably, PEG 200 induced residue-specific effects, suggesting localized base-pair openings that may create transient binding sites for regulatory proteins. These results highlight how DNA triplex dynamics respond to changing intracellular environments, potentially contributing to the regulation of gene expression and genomic stability during processes such as the cell cycle and cellular stress.

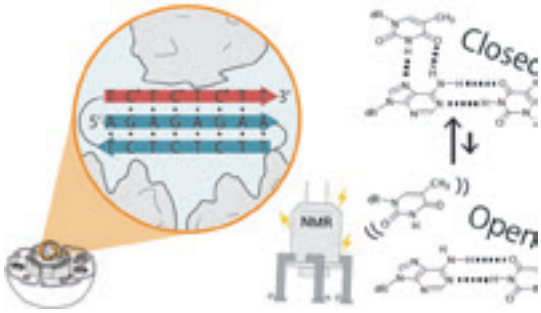

Figure 2. Base-pair opening and closing dynamics of DNA triplex structures under molecular crowding conditions.

3. Substrate preference of the anti-HIV factor APOBEC3C and the inhibitory effects of the HIV-1 Vif–human E3 ubiquitin ligase complex on its activity

APOBEC3 (A3) family proteins are cytidine deaminases that convert deoxycytidine to deoxyuridine in single-stranded (ss) DNA. APOBEC3C (A3C) restricts HIV-1 replication by introducing mutations into the viral reverse-transcribed DNA. In infected cells, the viral infectivity factor (Vif) forms the VβBCC complex, consisting of CBFβ and the E3 ubiquitin ligase components Elongin B, Elongin C, and Cullin 5, which promotes the ubiquitination and degradation of A3 proteins. Our previous studies demonstrated that VβBCC can also inhibit APOBEC3G (A3G) activity independently of degradation, but its effects on A3C remained unclear. In this study, we evaluated A3C deaminase activity using a uracil-DNA glycosylase assay and examined its inhibition by VβBCC. A3C showed the highest activity at pH 5.5 and displayed a preference for target sequences (TC) located near the 5′ or 3′ ends of ssDNA rather than the center. Furthermore, increasing concentrations of VβBCC progressively inhibited A3C activity. Ongoing analyses are investigating the interactions between A3C, ssDNA, and VβBCC to clarify the mechanism of inhibition. These findings reveal key features of A3C enzymatic activity and its regulation by HIV-1 Vif, enhancing our understanding of viral strategies to evade host defenses.

4. Enhancement of APOBEC3A deaminase activity by the HIV-1 Vif–human E3 ubiquitin ligase complex

APOBEC3A (A3A) is a cytidine deaminase that introduces mutations into single-stranded (ss) DNA and contributes to innate antiviral defense. We have previously demonstrated that the HIV-1 Vif–human E3 ubiquitin ligase complex (VβBCC) inhibits the deaminase activities of APOBEC3B, 3F, 3G, and 3C. Unexpectedly, in the case of A3A, we found that VβBCC enhances its deaminase activity. Using a uracil-DNA glycosylase assay, we confirmed that A3A deaminase activity increases in the presence of VβBCC. Furthermore, fluorescence polarization experiments revealed that A3A, ssDNA, and VβBCC form a complex, suggesting a direct interaction among these components. We are currently investigating the molecular mechanism underlying this unique enhancement of A3A activity by VβBCC. These findings may provide new insights into the diverse regulatory roles of the Vif complex on different APOBEC3 family members and offer clues to understanding the complex interplay between HIV-1 and host defense mechanisms.

5. Applying an RNase inhibitor cocktail to extend the available time for in-cell NMR experiments on RNA

The intracellular environment is highly crowded with macromolecules, influencing RNA structure and interactions. Although in-cell NMR spectroscopy enables atomic-resolution analysis of RNA in living human cells, its application is challenging due to rapid RNA degradation. To address this issue, we introduced an RNase inhibitor cocktail into cells along with an RNA aptamer and successfully obtained in-cell NMR spectra of the RNA aptamer, which binds strongly to the HIV-1 Tat protein, both in the presence and absence of a Tat-derived peptide. The inhibitor effectively suppressed RNA degradation, extending the RNA's lifetime and allowing the acquisition of intact spectra at physiological temperatures. The resulting spectra provided structural insights into RNA before and after ligand binding without requiring chemical modifications. Notably, the inhibitor was essential for detecting the peptide-free aptamer, which appears to be highly RNase-sensitive. This cost-effective approach enhances in-cell NMR applications for RNA, offering new opportunities to investigate cellular processes and develop RNA-based therapeutics.

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