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ADVANCING PROTEIN ENGINEERING VIA ORGANIC CHEMISTRY

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Abstract. Proteins are central to nearly all biological processes; their functions are tightly regulated by dynamic mechanisms such as covalent alterations; e.g., post-translational modifications (PTMs). These modifications can influence the protein's structure, localization, and activity. Inspired by this diversity and regulation, advances in synthetic organic chemistry have enabled the production of a plethora of novel proteins for both basic research and biomedical applications. Recent progress in structural elucidation technologies and modern organic chemistry has enabled atom-level modifications, significantly enhancing our ability to tailor protein function. These approaches greatly expand the toolkit currently available for generating complex proteins with unique structural and functional properties. In this review, we summarize recent progress in chemical protein engineering and highlight its emerging applications in catalysis, functional studies, and drug development.

Keywords: protein engineering, chemical protein modification, post-translational modifications (PTMs), synthetic organic chemistry, protein structure–function relationship, covalent crosslinking, unnatural amino acids, protein folding, bioconjugation, structural biology, catalytic applications, drug development.

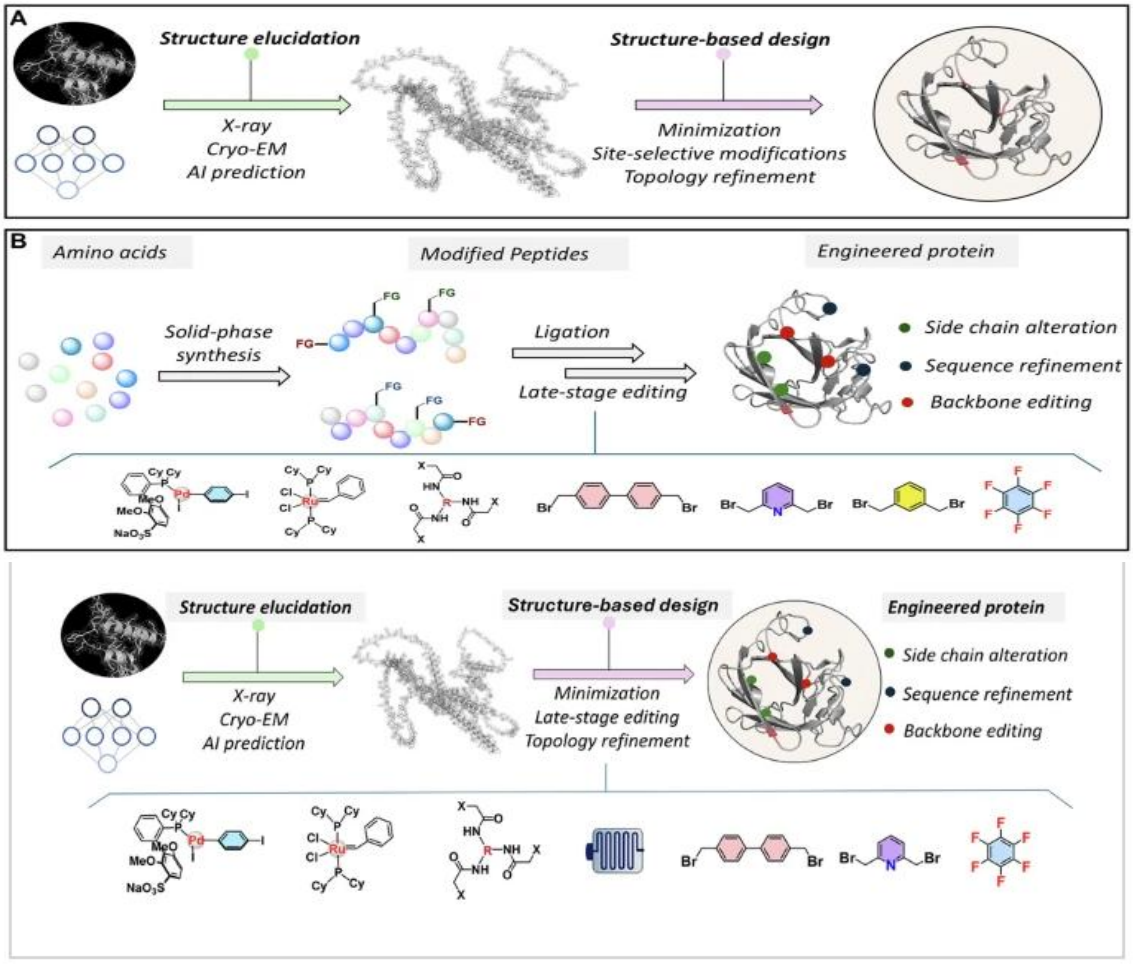
Introduction. Nature has developed sophisticated protein machinery to control nearly every cellular process in living organisms. Proteins function as receptors, signaling molecules, catalysts, transporters of molecules and ions, as well as structural scaffolds that maintain cell integrity. Post-translational modifications (PTMs) expand the structural and functional diversity of proteins, allowing a single protein to carry out multiple functions and increasing the complexity and dynamics of biological systems. Inspired by this natural versatility, chemists have sought to engineer advanced proteins with tailored properties and tuned activity. By introducing modifications such as side-chain alterations, sequence refinements, or backbone editing, key protein properties, e.g., shape, stability, and function, can be optimized. Combining structure-based design with targeted protein modification enables the production of novel biomolecules with enhanced properties and customized activities for a wide range of biomedical and therapeutic applications.

Protein folding is largely dictated by intramolecular interactions and environmental conditions; covalent cross-links between secondary structural elements play a central role in maintaining structural and functional integrity (Fig. 1). Disulfide bonds, for example, formed between Cys residues, are critical for stabilizing native protein structures by influencing folding thermodynamics. Inspired by these natural features, researchers have introduced non-native disulfide bridges to reinforce protein architecture. Moreover, structure-guided covalent crosslinking strategies—such as macrocyclization, bicyclization, stapling, and controlled oligomerization—have emerged as effective tools to enhance protein stability and folding. On the other hand, the incorporation of unnatural amino acids and side-chain modifications was also introduced and investigated to refine protein function predictions. Together with advances in structural biology, modern organic chemistry enables atom-level control over protein structure and activity, expanding the scope of protein engineering. Although novel synthetic reactivities are well established in peptides, selectively functionalizing proteins remains challenging, requiring advanced methods that operate without disrupting their complex structures or native folding and function. high precision. Such advances have contributed markedly to elucidating protein structure, function, and the development of new therapeutics.

Fig. 1: Overview of protein design and chemical engineering

A Schematic representation of rational protein design. **B** Schematic representation of chemical protein engineering. (PDB; 1ija).

Key methodologies—including solid-phase peptide synthesis (SPPS), chemoselective ligation techniques, and late-stage bioconjugation technologies—



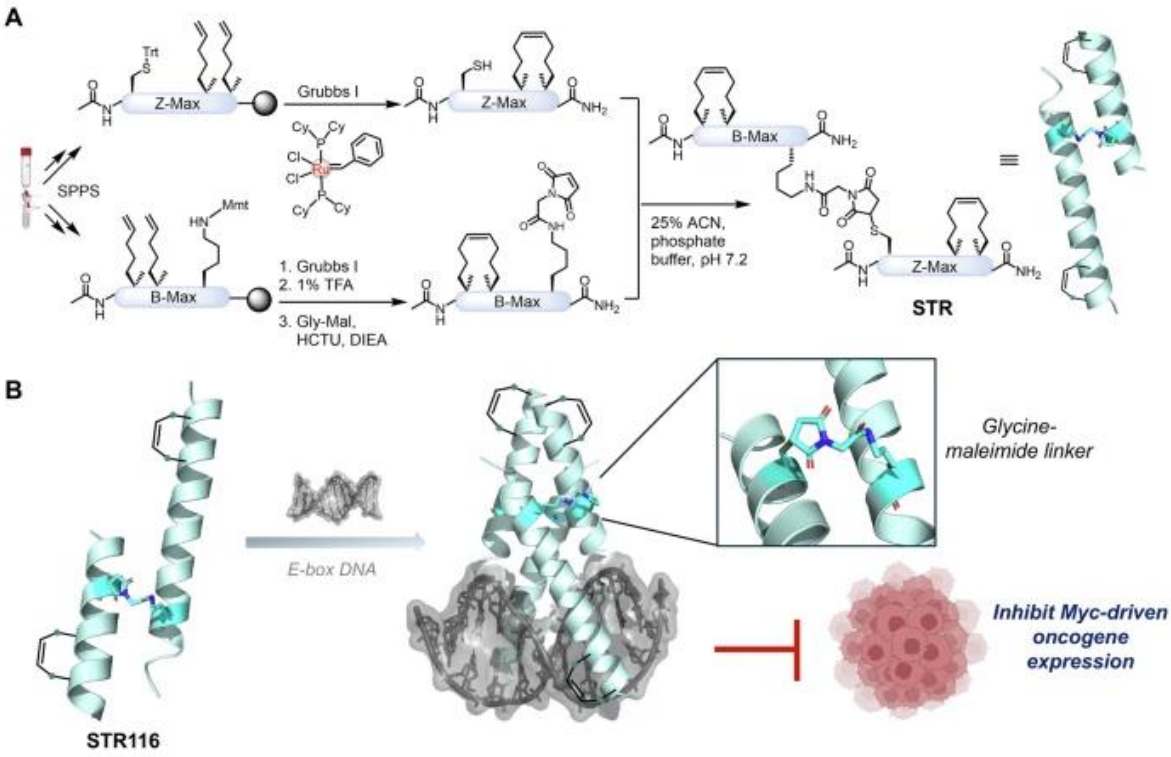
have enabled the generation of novel protein analogs with enhanced stability and new functionalities. Further innovations in orthogonal protecting group strategies and late-stage modification chemistries have greatly expanded the repertoire of accessible protein topologies. These advances have opened unprecedented opportunities to explore new chemical reactivities not found in nature, significantly expanding the functional space of proteins. All together, these innovations have enabled the production of tailor-made protein analogs with novel physicochemical properties and programmable activities for fundamental research and therapeutic applications.

In this review, we highlight recent advances in chemical protein engineering through the lens of organic chemistry and discuss their applications in catalysis, functional studies, and drug development. It should be noted that engineered proteins generated through recombinant expression are not covered in this review. Readers are encouraged to refer to other relevant reviews that focus on this important area.

Protein engineering through strategic side chain alteration

The intrinsic reactivity of various amino acid side chains has been widely explored to fine-tune the physicochemical properties of proteins. This includes deliberate modification of functional groups to modulate key non-covalent interactions, such as electrostatic interactions and hydrogen bonding, which are essential for proper protein folding and stability. In addition, covalent cross-linking at strategic positions within or between proteins has been successfully employed to enhance the structural stability. These covalent modifications are often achieved using orthogonal reactivities, such as the thiol group in Cys, or by incorporating non-canonical amino acids (ncAAs). This strategy is commonly used in site-specific cross-linking with bis or tri-electrophilic linkers and has proven to be a powerful method for reinforcing protein secondary and tertiary structures. Importantly, over the past decade, the synthesis of several stabilized proteins with tailored activity has been achieved through strategic side chain alteration. Of particular interest are transcription factor (TF) proteins, which play a key role in human health and disease. TFs bind to specific DNA sequences and regulate cellular processes by controlling the rate of gene expression. The intrinsically disordered nature of many TFs makes them particularly challenging drug targets. Engineered synthetic DNA-binding proteins have emerged as powerful modulators to target oncogenic TFs. In 2022, the Moellering group reported an intriguing strategy to target Myc-driven gene expression using chemically stabilized TF mimetics called synthetic transcriptional repressors (STRs) derived from the basic helix-loop-helix (bHLH) domain of Max. STRs were designed to compete with the endogenous Myc/Max system for the E-box sites to block Myc-driven oncogene expression

Fig. 2: Chemical synthesis of synthetic transcriptional repressors (STRs) to inhibit Myc-driven oncogene expression.



Engineering advanced protein analogs often entails introducing multiple synthetic modifications to tune their activity. However, achieving site selectivity and executing multiple transformations remains a significant challenge. Recently, Jbara and coworkers introduced orthogonal palladium(II) chemistry to engineer artificial TFs with enhanced stability and potent DNA binding activity[42,43]. Their group leveraged palladium(II) chemistry for high-resolution protein functionalization on the thiolate residue of Cys to allow sequential and site-specific insertion of novel transformations.

nhancing the thermal and chemical tolerance of proteins, particularly in enzymes, has emerged as a powerful strategy to produce new functional analogs in complex settings. Recently, Grossmann and coworkers reported a structure-based stabilization strategy involving the in-situ cyclization of proteins (INCYPRO) composed entirely of proteinogenic amino acids[53]. The group initially chose *Staphylococcus aureus* sortase A (SrtA, 60-206) transpeptidase as a target protein. SrtA is often used as a biomolecular tool for the specific labeling of proteins for various applications. Given the drop in its activity at elevated temperatures or under denaturation conditions, the group employed a crosslinking strategy that uses biselectrophiles that target pairs of Cys to enhance its stability. Notably, since SrtA already contains a Cys (C184) that is crucial for catalytic activity and may undergo undesired reactions with electrophiles, they tested several electrophiles and identified 2-chloroacetamide, which was unreactive with the native Cys184. Using 2-chloroacetamide, the group designed a set of

biselectrophilic linkers with 8-17 bridging atoms spanning a broad range of distances (up to 21 Å). Multiple analogs of SrtA (**SrtA S1-S6**) were then engineered by introducing different Cys pairs at surface-exposed positions, which are not relevant for catalytic activity (Fig. 3A). These variants were expressed and subjected to crosslinking with different biselectrophiles. Cyclization was confirmed in most of the analogs, and melting point analysis showed that cross-linking increased thermal stability.

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