



Protein Regulation Hot Paper

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A General Supramolecular Approach to Regulate Protein Functions by Cucurbit[7]uril and Unnatural Amino Acid Recognition

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Abstract: Regulation of specific protein function is of great importance for both research and therapeutic development. Many small or large molecules have been developed to control specific protein function, but there is a lack of a universal approach to regulate the function of any given protein. We report a general host–guest molecular recognition approach involving modification of the protein functional surfaces with genetically encoded unnatural amino acids bearing guest side chains that can be specifically recognized by cucurbit[7]uril. Using two enzymes and a cytokine as models, we showed that the activity of proteins bearing unnatural amino acid could be turned off by host molecule binding, which blocked its functional binding surface. Protein activity can be switched back by treatment with a competitive guest molecule. Our approach provides a general tool for reversibly regulating protein function through molecular recognition and can be expected to be valuable for studying protein functions.

Proteins are key regulators of biological processes, and molecules that precisely control protein functions are of great importance for protein functional studies. Molecular recognition based on host–guest chemistry resembles protein-based recognition, such as antibody–antigen and biotin–streptavidin binding, and has already been used for protein modification, regulation, and assembly.^[1] For example, Finbloom et al. used cucurbit[6]uril-catalyzed click chemistry to site-specifically modify proteins and synthesize protein conjugates.^[2] In addition, cucurbit[7]uril (CB[7]) and PEG-modified CB[7] can recognize N-terminal phenylalanine residues of target proteins, thus inhibiting their function^[3] and enhancing their pharmacokinetic properties.^[4] Cucurbit[8]uril can recognize of proteins with N-terminal L-phenylalanyl-glycylglycine (FGG) motifs and methionine-terminated peptides,^[5] and this behavior has been used to regulate protein dimerization,^[6] oligomerization,^[7] and ordered assembly.^[8] Modified calixarenes^[9] and molecular tweezers^[10] can recognize the

surface of proteins and inhibit their functions. However, the aforementioned methods are limited to specific proteins or to proteins with a specific sequence motif. A universal host–guest molecular recognition approach for targeting the binding surface of any protein and thus reversibly regulating its function, would be highly desirable.

Nearly all naturally occurring proteins are composed of combinations of the 20 canonical amino acids, and achieving high-affinity, high-specificity host–guest molecular recognition on protein surfaces with this limited set of building blocks is challenging. We hypothesized that this challenge could be overcome by using genetic code expansion, whereby synthetic unnatural amino acids can be genetically encoded into a protein in place of any naturally occurring residue via site-directed mutagenesis with nonsense codon suppression.^[11] Genetic code expansion has previously been used to precisely control protein function at the single-residue level by means of photoregulation^[12] or chemical decaging.^[13] Despite their power, however, these methods cannot regulate protein function in a reversible manner. Although unnatural amino acid containing azobenzene group provide a reversible layer for protein function regulation, the design is difficult and may not be applied to any given protein.^[14] We reasoned that site-specific replacement of residues in proximity to a protein's functional surface—such as the substrate entry site for an enzyme or the receptor binding site for a cytokine—with an unnatural amino acid bearing a guest side chain would allow residue-specific recognition by a host molecule and thus permit reversible, on-demand control of the protein's function (Scheme 1).

An ideal unnatural amino acid for this purpose would closely resemble canonical amino acids, to minimize any deleterious effects on protein activity after mutation. Aromatic residues are natural guests for many host molecules, and the interaction of these pairs have been well-documented.^[15] Logsdon et al. demonstrated that among a series of phenylalanine analogs, 4-*tert*-butyl-L-phenylalanine (tBuF) and 4-(aminomethyl)-L-phenylalanine (pAMF) (Supporting Information, Figure S1) are the best guest molecules recognized by CB[7] with high affinity.^[15a] CB[7] is one of the most commonly used host molecules in biological systems with low cytotoxicity.^[4a,b,16] The reported K_d values for binding of CB[7] to tBuF and pAMF are 0.25 μ M and 0.46 μ M, indicating that CB[7] binds these residues 35 and 19 times as selectively as phenylalanine^[15a] and a few hundred to a few thousand times as selectively as tyrosine and tryptophan.^[17]

To genetically incorporate tBuF and pAMF into proteins, we utilized two previously reported *Methanococcus jannaschii* TyrRS tRNA^{CUA} pairs.^[18] Nonsense codon suppression

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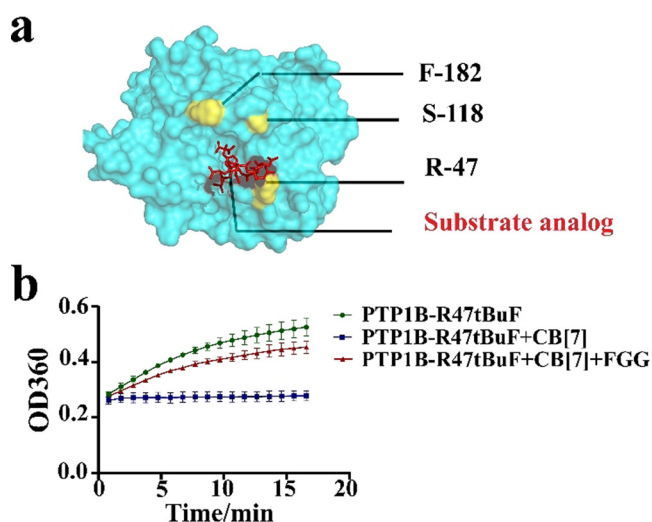


Figure 2. a) Crystal structure of PTP1B (yellow) bound to an analogue of the peptide substrate (red) (PDB ID: 1bzh). Sites selected for incorporation of tBuF are indicated in green. b) Protein tyrosine phosphate assay of the PTP1B-R47tBuF mutant. The activity of the enzyme (500 nM) was assayed in the absence of CB[7], in the presence of 1 mM CB[7], or in the presence of 1 mM CB and 1.2 mM FGG in Tris buffer (pH 7.4). Assays were performed in triplicate. Data are presented as the mean and error bars represent the standard deviation.

idea that enzyme function can be efficiently and reversibly regulated by supramolecular host–guest recognition and binding with a single-residue resolution.

To confirm that the observed inhibitory effects were due to specific molecular recognition, we used isothermal titration calorimetry to measure the binding affinities between CB[7] and two of the GST mutants. The K_d values for binding of CB[7] to GST-R108tBuF and GST-Q207tBuF were determined to be 24.2 and 4.45 μM , respectively (Figure 1c; Figure S10a), whereas no binding between wild-type GST and CB[7] could be detected (Figure S10b). These findings suggest that the observed inhibition was indeed due to specific molecular-recognition-induced binding between the host molecule CB[7] and the site-specifically encoded guest tBuF.

Protein tyrosine phosphorylation is an important post-translational modification that regulates various cellular processes, and its malfunction results in many human diseases. Our laboratory has a long-term interest in studying protein tyrosine phosphatases, a family of enzymes that regulate the dephosphorylation of phosphotyrosine residues.^[18b] To determine whether our tBuF-CB[7] recognition system could be generalized to other enzymes, we selected protein tyrosine phosphatase 1B (PTP1B) as our next target protein. PTP1B, which has been extensively studied, is an important drug target because it regulates numerous signaling cascades. Upon thorough inspection of the crystal structure of PTP1B bound to an analogue of the peptide substrate, we identified three potential mutation sites near the binding pockets: R47, S118, and F182 (Figure 2a). The corresponding mutant proteins were constructed and expressed in *E. coli* BL21, and expression yields were determined to be 8, 15 and 6 mg L^{-1} for PTP1B-R47tBuF, PTP1B-S118tBuF, and

PTP1B-F182tBuF, respectively (Figure S11). It is possible that mutation into the bulky tBuF could have an impact on protein stability and yield,^[20] and therefore more positions could be tested. After purification of the enzymes, their activities were assessed by means of a colorimetry assay kit that detects the release of phosphate from a peptide substrate (Figure S12). In the absence of CB[7], all three mutants showed activities similar to the wild-type activity (Figure S13). However, in the presence of 1 mM CB[7], the activity of PTP1B-R47tBuF was completely inhibited (Figure 2b); whereas the activities of the other enzymes were only slightly affected by the addition of the host molecule (Figures S14 and S15). Again, these results are consistent with the structural analysis showing that of the mutated residues, R47 is closest to the substrate binding pocket. Addition of FGG (1.2 mM) restored more than 70% of the enzyme activity, while the FGG itself at different concentrations does not influence the enzyme activity (Figure S16). To convey a more in depth understanding of the system, different ratios of protein and CB[7], effects on K_{cat} and K_{M} of CB[7], and a titration of FGG inhibitor were studied. A decrease of enzymatic activity was observed with increasing concentrations of CB[7] (Figure S17). A decrease of K_{M} and apparent K_{cat} was also observed with the addition of inhibitors (Figure S18). The enzymatic activities were restored with increasing concentrations of competitors (Figure S19). Collectively, these data indicating that the function of PTP1B-R47tBuF could indeed be reversibly controlled by means of our host–guest molecule recognition approach.

To further demonstrate the utility of this approach, we extended it to the regulation of cytokines. Cytokines are important regulatory proteins that play crucial roles in cell signaling and cytokine engineering is a hot topic in the therapeutic protein field. We wondered whether our host–guest system could be used to regulate cytokine function, which could in turn allow for regulation of cellular activities. To evaluate this possibility, we selected the pleiotropic cytokine tumor necrosis factor alpha (TNF α)-which induces many cellular processes, including apoptosis and inflammation-as a model cytokine.

TNF α binds to TNFR1 (tumor necrosis factor receptor 1) and induces cell death through the caspase pathway (Figure S20). To design a controllable TNF α , we examined the crystal structure of a TNF α trimer and data obtained by mutation analysis of TNF α bound to TNFR1.^[21] On this basis, we chose four residues near the binding interface for mutation to tBuF (Figure 3a): Q21, Q25, Q31, and A145. The mutant and wild-type proteins were obtained by recombinant expression in *E. coli* BL21, and their cytotoxicities to L929 cells were evaluated. The measured bioactivities of TNF α -Q21tBuF, TNF α -Q25tBuF, TNF α -Q31tBuF, and TNF α -A145tBuF were, respectively, 92 %, 97 %, 87 %, and 7 % of the wild-type bioactivity (Figure 3b). The biocompatibilities of the host (CB[7]) and the competitive guest (FGG) were evaluated on cells at concentrations ranging from 0.25 to 2.5 mM and from 0.5 to 2.5 mM, respectively. No cytotoxicity was observed for either compound in the tested concentration ranges (Figure S21), confirming that they are safe to use on living cells, as has previously been reported in the litera-

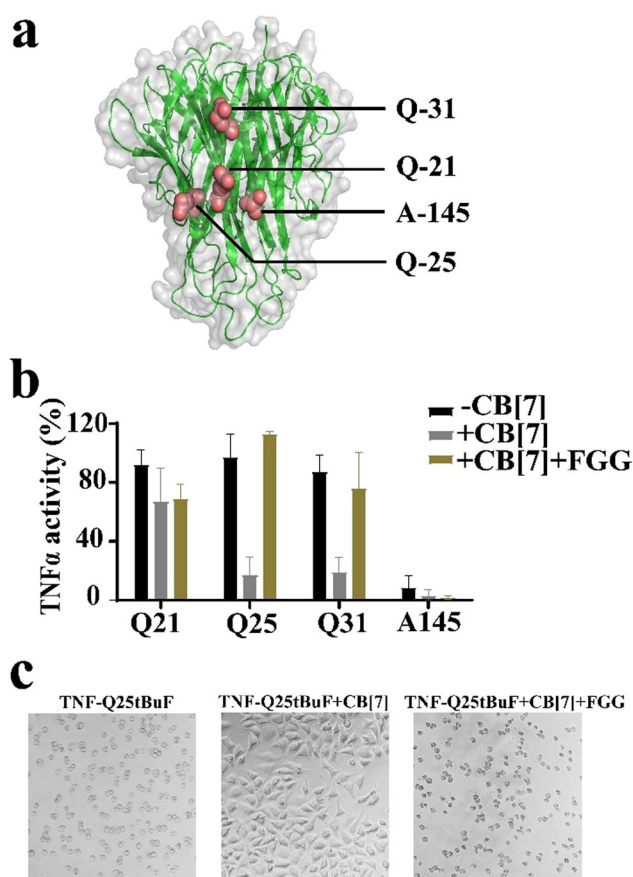


Figure 3. a) Crystal structure of TNF α trimer (PDB ID: 2tnf). Sites selected for incorporation of tBuF are indicated in red. b) Cytotoxicity assay of TNF α mutants. L929 cells were treated with cytokines (2 ng mL⁻¹) in the absence of CB[7], in the presence of 1 mM CB[7], or in the presence of 1 mM CB[7] and 1.2 mM FGG, along with actinomycin D (1 μ g mL⁻¹). Cytotoxicity was measured with a CCK8 kit. Assays were performed in triplicate. Data are presented as the mean and error bars represent the standard deviation. c) Images of L929 cells after treatment with TNF α -Q25tBuF (2 ng mL⁻¹), TNF α -Q25tBuF (2 ng mL⁻¹) with 1 mM CB[7], and TNF α -Q25tBuF (2 ng mL⁻¹) with 1 mM CB[7] and 1.2 mM FGG, respectively.

ture.^[16] The cytotoxicities of wild-type TNF α in the absence and presence of CB[7] (1 mM), as well as in the presence of both CB[7] (1 mM) and FGG (1.2 mM), were also assayed; no obvious cytotoxicity was observed (Figure S22). Next, we evaluated the bioactivities of the four TNF α mutants in the presence of 1 mM CB[7]. As shown in Figure 3b, in the presence of 1 mM CB[7], the bioactivities of TNF α -Q21tBuF, TNF α -Q25tBuF, and TNF α -Q31tBuF were inhibited 27%, 83%, and 78%, respectively. Subsequent treatment with 1.2 mM FGG restored the activities of the Q25 and Q31 mutants to 100% and 87%, respectively (Figure 3b,c). These findings indicate that by introducing tBuF in proximity to the receptor binding interface, we could precisely and reversibly regulate the bioactivity of TNF α by host–guest molecular interactions, and could in principle be extended to other cytokines.

Conclusion

In summary, we have developed a simple, universal supramolecular host–guest interaction approach to reversibly regulate protein function. Specifically, we introduced a recognition element to the surfaces of several proteins by genetically encoding the unnatural amino acid tBuF containing a guest side chain. Guided by structural information, we installed tBuF at selected positions in proximity to each protein's functional interface, and we found that the resulting mutant proteins could be specifically recognized by the biocompatible macrocyclic host molecule CB[7] with high affinities. Using two enzymes and a cytokine as model proteins, we showed that active proteins containing the built-in guest residue at carefully selected sites could be reversibly regulated by the host molecule and by a competitive guest molecule. The components of the system showed great biocompatibility and could be used on living cells. Therefore, the system can serve as a general tool for reversible regulation of protein function through molecular recognition and can be expected to be valuable for the study of protein functions. This proof-of-principle study opens up design and application possibilities where host macrocycles and guest amino acid pairs feature. Higher binding affinity and selectivity on proteins could be accessible in the future. The development of novel pairs would also overcome some current limitations and allow targeting of intracellular proteins as well as achieving reversibility with multiple cycles.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cytokines · enzymes · protein function · supramolecular chemistry · unnatural amino acids

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