Affinity Agents

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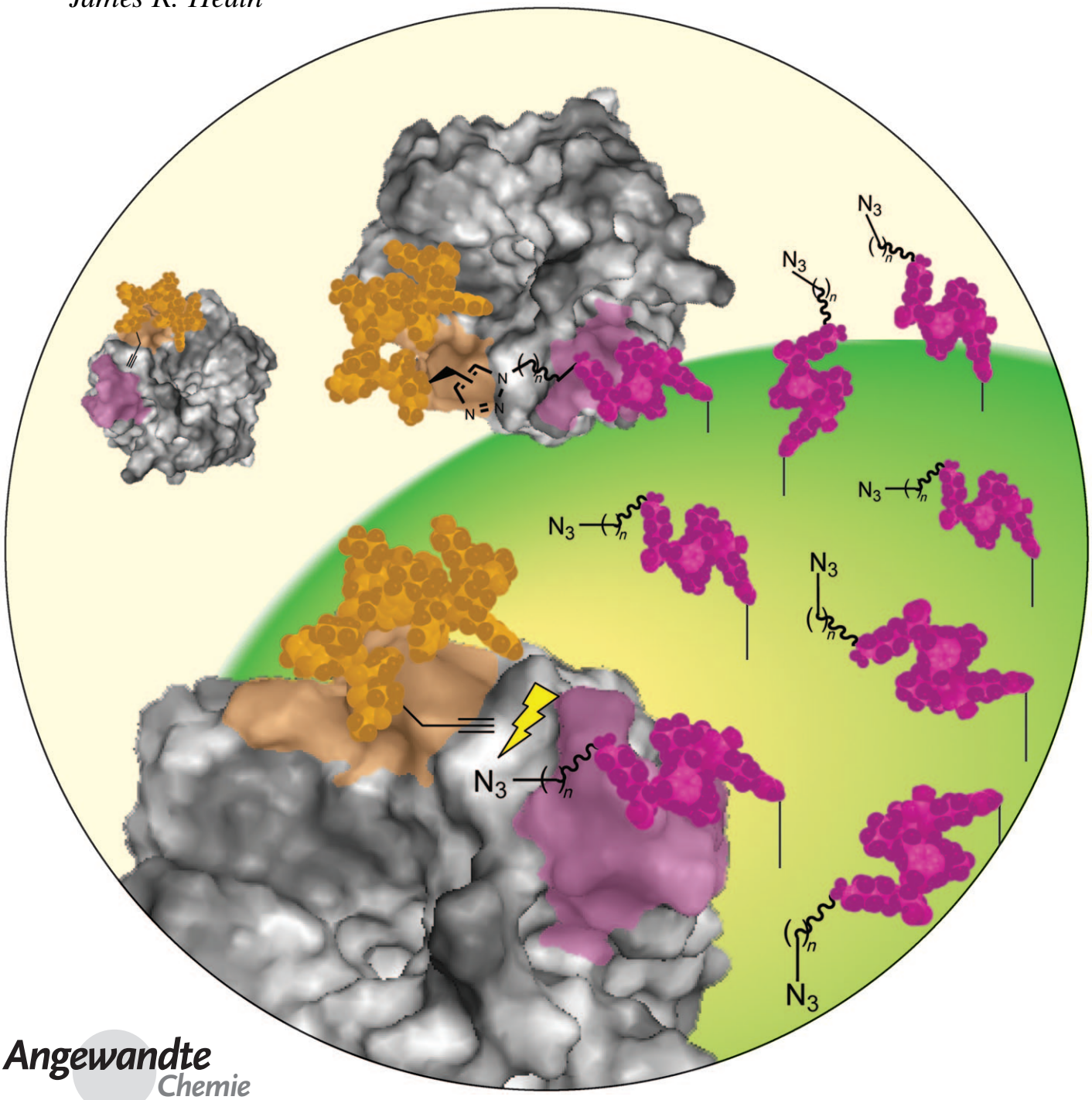
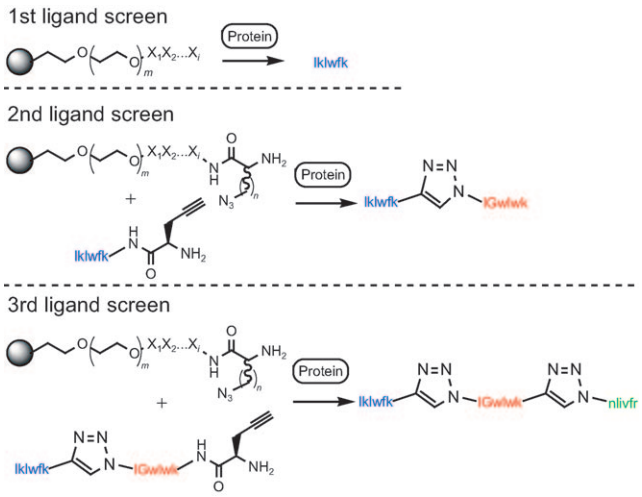
# Iterative In Situ Click Chemistry Creates Antibody-like Protein-Capture Agents**

*Heather D. Agnew, Rosemary D. Rohde, Steven W. Millward, Arundhati Nag, Woon-Seok Yeo, Jason E. Hein, Suresh M. Pitram, Abdul Ahad Tariq, Vanessa M. Burns, Russell J. Krom, Valery V. Fokin, K. Barry Sharpless, and James R. Heath**



Most protein-detection methods rely upon antibody-based capture agents.[1] A high-quality antibody exhibits high affinity and selectivity for its cognate protein. However, antibodies are expensive and can be unstable towards dehydration, pH variation, thermal shock, and many other chemical and biochemical processes.[2,3] Several alternative protein-capture agents, including oligonucleotide aptamers and phage-display peptides, have been reported, each of which has advantages as well as significant limitations.[4–10] A further alternative is the use of one-bead–one-compound (OBOC) peptide or peptide-mimetic libraries.[11–15] An advantage of OBOC libraries is that chemical stability, water solubility, and other desired properties may be designed into the compounds. However, OBOC libraries contain typically only $10^4$–$10^6$ elements, and so significant trade-offs are made between peptide length and library chemical diversity. Herein we report the use of in situ click chemistry as a screening approach towards the construction of multiligand protein-capture agents (Scheme 1). We harnessed the method to produce a triligand capture agent against human and bovine carbonic anhydrase II (h(b)CAII) as a model system.

In situ click chemistry has been utilized previously for the rapid identification of small-molecule enzymatic inhibitors.[16–20] These studies implemented libraries of small-molecule building blocks functionalized with either azide or acetylene groups. During the screening of the target protein with the molecular libraries, the protein plays an active role in the selection and covalent assembly of a new inhibitor. In these systems, the protein accelerates the Huisgen 1,3-dipolar cycloaddition by holding the two fragments—azide and acetylene—in proximity. The protein exhibits exquisite selectivity; it only promotes the formation of a 1,2,3-triazole (Tz) between those library elements that can be brought into a precise relative molecular orientation on the protein surface. The result is a biligand inhibitor with an affinity that approaches the full product of the affinities of the individual molecular components. Furthermore, the triazole itself can contribute to the binding affinity observed for this inhibitor.




***Scheme 1.*** Representation of an in situ screen based on click chemistry for the preparation of a multiligand protein-capture agent. 1st: A comprehensive OBOC peptide library on TentaGel (TG) beads ($x_i$: variable region) is incubated together with a fluorescently labeled protein target. Hit beads are identified on the basis of their fluorescence intensity. 2nd: A hit peptide from the 1st screen is employed as the anchor ligand and incubated in the presence of the OBOC peptide library, in which the peptides are now appended with an azide linker ($n = 4$, 8). 3rd: The process is repeated, but with the biligand from the 2nd screen as the new anchor unit to enable the rapid identification of higher-order multiligand capture agents.

The advances we report herein are manifold. First, the production of the capture agent does not require prior knowledge of affinity agents against the target protein. Our anchor ligand was a relatively weakly binding short heptapeptide comprised of non-natural D-amino acids and a terminal, acetylene-containing amino acid (D-propargylglycine, D-Pra). It was identified by using a standard, two-generation OBOC screen against bCAII; the peptide sequence on the hit beads was identified by Edman degradation (see the Supporting Information). This first anchor ligand, lklwfk-(D-Pra), exhibited an approximately 500 μM affinity for bCAII (see the Supporting Information). The second advance is that the in situ click screen (Scheme 1) samples a very large chemical space. Our OBOC library consisted of short-chain peptides and was comprehensive. We utilized five copies of a $2 \times 10^7$-element library of D stereoisomers: $Az_n$-$x_2$-$x_3$-$x_4$-$x_5$-$x_6$-$Az_n$ ($Az_n$ = azide-containing amino acids ($n = 4$, 8); $x_i$ = any D-amino acid except Cys). $Az_n$ building blocks were prepared by published methods (see the Supporting Information).[21–23]

The third advance is that the process can be repeated. Once a biligand has been identified, that biligand can serve as the anchor ligand. The same OBOC library is employed to identify a triligand, and so forth (Scheme 1). Upon the addition of each ligand to the capture agent, the affinity and the selectivity of the capture agent for its cognate protein

[*] H. D. Agnew, R. D. Rohde, Dr. S. W. Millward, A. Nag, Dr. W.-S. Yeo,[+] A. A. Tariq, V. M. Burns, R. J. Krom, Prof. J. R. Heath
Division of Chemistry and Chemical Engineering
California Institute of Technology
1200 East California Boulevard, Pasadena, CA 91125 (USA)
Fax: (+1) 626-395-2355
E-mail: heath@caltech.edu
Homepage: http://www.its.caltech.edu/~heathgrp
Dr. J. E. Hein, Dr. S. M. Pitram, Prof. V. V. Fokin, Prof. K. B. Sharpless
Department of Chemistry and
The Skaggs Institute for Chemical Biology
The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
[+] Current address: Department of Bioscience and Biotechnology
Konkuk University, Seoul, 143-701 (Korea)

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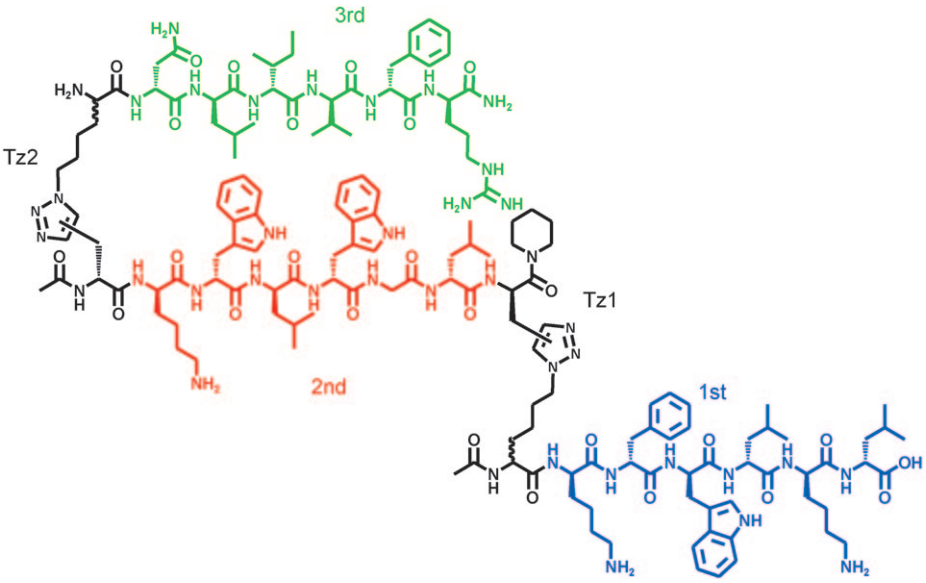
increase rapidly. With lklwfk-(D-Pra) as the anchor ligand, we used the screen in Scheme 1, followed by a more focused screen against a much smaller OBOC library, to identify the biligand (D-Pra)-kwlwGl-Tz1-kfwlkl against bCAII. This biligand exhibited a 3 μM binding affinity for bCAII, as measured by surface plasmon resonance (SPR). With this biligand as the new anchor unit, we repeated the screen in Scheme 1, followed again by a focused screen in situ, to identify a triligand, rfviln-Tz2-kwlwGl-Tz1-kfwlkl (Scheme 2), which exhibited 64 and 45 nM binding affinities against bCAII and hCAII, respectively, as determined by SPR.[24] The triligand can be prepared in bulk quantities by standard solid-phase synthesis of the individual heptapeptides followed by ligation through the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC).[25] Details of all screening conditions and OBOC libraries are in the Supporting Information.




***Scheme 2.*** Triligand capture agent for the protein b(h)CAII. The triazoles (Tz1, Tz2) can be either 1,4 (*anti*) or 1,5 (*syn*) isomers since the protein-templated reaction can produce both products.

In the case of previously reported screens based on in situ click chemistry, the triazole product, or biligand inhibitor, was identified by chromatographic separation followed by mass spectrometry.[16–20] In the screen in Scheme 1, the triazole product represents a very small fraction of the peptide on the bead, and so only the variable region of the peptide is identified during the peptide-sequencing step. Thus, we sought to confirm the validity of the in situ screen in multiple ways.

For triligand screens, we generated a histogram to chart the position-dependent frequency of amino acids observed on the hit beads (Figure 1). On the basis of this histogram, we constructed two focused OBOC libraries. The first library contained only the 3rd-ligand variable region and was used in an in situ screen. The second library contained the same 3rd-ligand variable region and was coupled by CuAAC (Tz2;

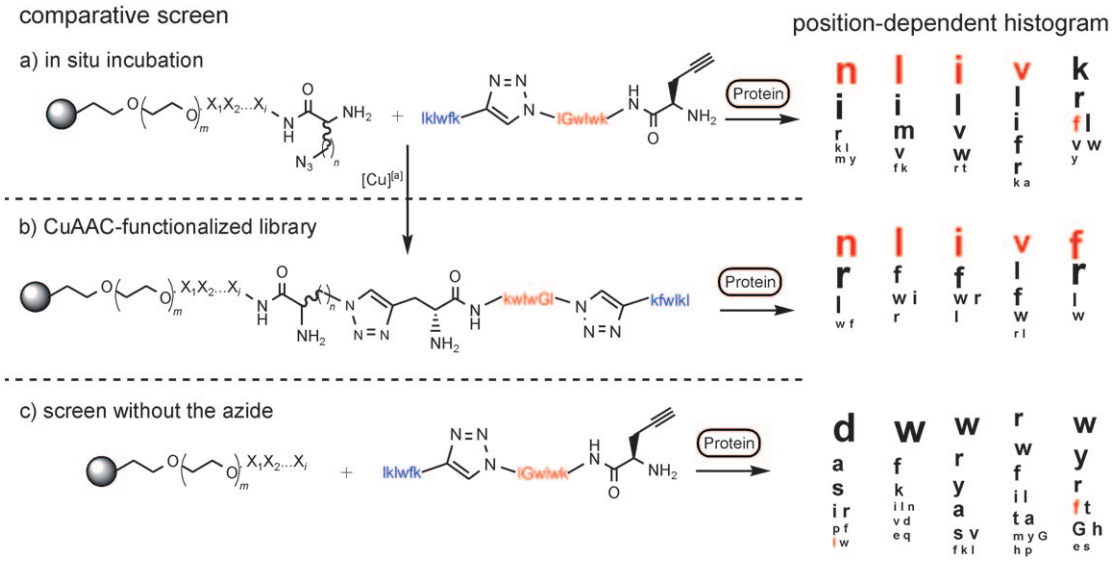



***Figure 1.*** Position-dependent histograms for the first-generation in situ click screens (for peptides with (a) and without (c) an azide-containing amino acid) to generate a triligand. a) For the in situ screen, a third of the beads had no azide group at the $x_1$ and $x_7$ positions, but all hit beads contained an azide group. b) First- and second-generation CuAAC-library screens yielded independent validation of the result obtained in the in situ screens. The final, consensus triligand sequence is indicated by red font. c) In the absence of the azide functionality, completely different hit sequences were obtained. Sample size: in situ, 25 hits; in situ, no azide, 24 hits; CuAAC library, 21 hits. [a] See the Supporting Information for CuAAC conditions.

Scheme 2), to the biligand. This on-bead triligand screen and the in situ screen both yielded the same consensus sequence. This result confirmed the equivalence of the two screen types. We also carried out a third in situ screen in which the $Az_n$ (azide-containing) amino acid was not included in the OBOC library. The formation of a triazole linkage was thus prohibited. This screen generated a very different, and much less homologous, set of hit sequences (Figure 1). This result confirmed the importance of the triazole linkage in the formation of a multiligand species.

Finally, we developed an enzyme-linked colorimetric assay for detecting the on-bead, protein-templated multiligand inhibitor (Figure 2a). For this assay, we prepared a biotin conjugate of the biligand anchor (biotin-$(EG)_5$-(D-Pra)-kwlwGl-Tz1-kfwlkl; EG = ethylene glycol), which was then employed in an in situ OBOC screen (Scheme 1) with beads appended with the single consensus 3rd ligand Az4-nlivfr. After the screen, alkaline phosphatase–streptavidin (AP–SA) was introduced to bind to any potential bead-bound biotinylated triligand. Excess AP–SA was removed, and the beads were incubated with 5-bromo-4-chloro-3-indoyl phosphate (BCIP), a chromogenic substrate for AP (Figure 2b; see the Supporting Information for details).[26] The purple color is a positive indicator for an on-bead triligand. The triligand was only formed in the presence of the protein b(h)CAII, and not when the protein substrate was transferrin (Tf), bovine serum albumin (BSA), or absent. Similarly, the on-bead triligand was not obtained when the incorrect biligand anchor sequence was used.

For the first-generation biligand and triligand screens, a striking result was the extremely high sequence homology that was observed for the hit beads. For example, for the first 17 hit beads sequenced from the initial biligand in situ screen (with five copies of a $2\times10^7$-element OBOC library), two peptides were identical, and a third peptide differed by only a single amino acid (see the Supporting Information). For the initial triligand screen (against the same library), the most commonly observed amino acids by position (Figure 1) reflect the consensus sequence identified in the second-generation (focused) screen almost exactly. Such sequence homology is unique to the in situ screens and suggests that these screens generate highly selective hits. Thus, multiligand capture agents identified in this way should exhibit high selectivity. In a dot-blot experiment, b(h)CAII was detected selectively by the triligand in 10% porcine serum with a detection limit of 20 ng of the protein (Figure 3; see the Supporting Information for details). The sequence identity of the proteins bCAII and hCAII is greater than 80% (PDB ID: 1CA2, 1V9E).

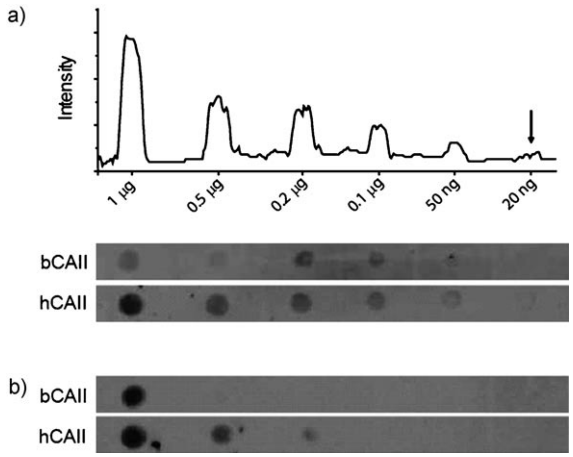



***Figure 3.*** a) Dot blot illustrating the limit of detection by the triligand for b(h)CAII in 10% porcine serum. b) When the biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl was used as the capture agent in 0.1% serum, the sensitivity was reduced more than 10-fold.

The protein bCAII is also known to have intrinsic esterase activity. It catalyzes the hydrolysis of 4-nitrophenyl acetate (4-NPA) to the chromophore 4-nitrophenol (4-NP).[27] Thus, we utilized the 4-NPA assay to determine whether the triligand binds to the active site (see the Supporting Information). The

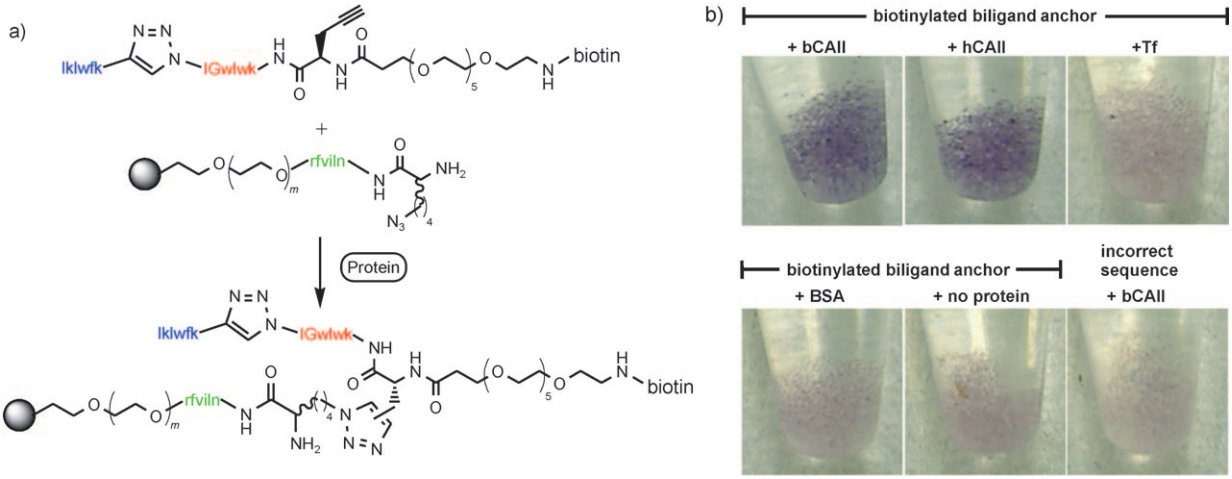



***Figure 2.*** a) Scheme for the in situ click assay for on-bead triazole formation with a biotinylated biligand anchor (biotin-$(EG)_5$-(D-Pra)-kwlwGl-Tz1-kfwlkl). b) After treatment with AP–SA then BCIP, purple beads are a positive indicator of triazole formation. The triligand was only formed by the in situ process in the presence of b(h)CAII, and not when the protein was Tf, BSA, or absent. The on-bead triligand was not observed when the biligand anchor sequence was incorrect.

triligand did not interfere with the enzyme activity of bCAII; it apparently binds away from the active site, or at least does not interfere with the normal catalysis of the active site. Such off-site, yet highly selective, binding is common for natural antibodies raised against proteins and bodes well for the scope of the technique we have described.

We are currently exploring the limits of the binding affinity that can be attained with these multiligand inhibitors and developing multiligand capture agents against other proteins so as to demonstrate the generality and/or limitations of this approach.

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