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A Genetically Encoded Direct Sensor of Antibody-Antigen Interactions

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Abstract

To overcome the limitations imposed by the genetic code on the engineering of proteins with new or enhanced physical, chemical, or biological properties, we developed a general method for the site specific incorporation of unnatural amino acids into proteins directly in living cells.[1] An orthogonal tRNA/aminoacyl-tRNA synthetase pair (one that does not crossreact with host tRNAs and aminoacyl-tRNA synthetases) is evolved to deliver a specific non-natural amino acid into a protein in response to a nonsense or frameshift codon. To date, we have selectively incorporated over 40 unnatural amino acids into a diverse set of proteins in both prokaryotic and eukaryotic organisms using this methodology.[2]

Keywords

unnatural amino acid incorporation; protein-protein interactions; fluorescence; antibodies

We have previously used the genetically encoded fluorescent amino acid, L-(7-hydroxycoumarin-4-yl) ethylglycine[3] **1** and 2-amino-3-(5-(dimethylamino)naphthalene-1 sulfonamide) propanoic acid (dansylalanine)[4], as well as the bio-orthogonal chemical handles *p*-acetylphenylalanine[5], and alkyne and azide containing amino acids[6] to introduce fluorescent reporters at specifically defined locations in proteins.[3,7] This allowed us to exploit the high sensitivities of fluorescent techniques, and the ability to selectively introduce a relatively small fluorescent probe at virtually any site in a protein. We hoped to extend this method by using the 7-hydroxycoumarin amino acid as a direct reporter of protein-protein interactions without the need for protein immobilization or a displacement assay. The fluorescent properties of hydroxycoumarins are well characterized, and have been shown to exhibit solvent dependent changes in fluorescence.[8,9] Because the pK_{As} of hydroxycoumarins are around 7.8, in many biologically relevant solvents they will exist in both neutral and ionized species[9] and can therefore also be used to probe pH in the environment of the protein.

We and others have previously shown that antibodies that are chemically modified with small molecule fluorophores near the combining site fluorescently signal the interaction of the antibody and its ligand.[10,11] We sought to expand this method to the interaction of an antibody with a protein antigen using a one step genetic method rather than chemical modification to incorporate the fluorophore.

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We chose to study the interaction of the protein CD40 ligand (CD40L), with a neutralizing α -CD40L antibody, 5c8. CD40L is a tumor necrosis factor (TNF) homologue that is expressed on the surface of T-cells. It has been found to be involved in B-cell proliferation, and isotype switching,[12] as well as hyper-IgM syndrome.[13] Crystallographic characterization of the interaction of 5c8 and CD40L has been reported[14] (PDB id 1I9R). In order to monitor a protein-protein interaction by fluorescence, the fluorophore should be close enough to the interface that its environment is substantially modified on binding, but not so close that it significantly affects the binding interaction.[11] Analysis of the 5c8-CD40L co-crystal structure revealed a number of candidate sites for incorporation of **1** at the antibody-antigen interface which appear to fit the criteria above (Figure 1) including Ile98(L) which lies ~5.0 Å from the nearest CD40L residue.

To incorporate the coumarin containing amino acid, the codon for Ile98(L) was mutated to TAG. *E. coli* cells were co-transformed with plasmids encoding the mutant antibody as a humanized Fab in which the bicistronic heavy and light chains were under the control of a single araBAD promoter, and the previously engineered aminoacyl-tRNA synthetase (pEB-CouRS), and *Mj*tRNA^{Tyr}_{CUA}.

The purified Fab was dialyzed into 150 mM sodium phosphate buffer at pH 7.4, and the fluorescent properties of the coumarin containing mutant were analyzed in the presence and absence of CD40L which was obtained by expression in *Pichia pastoris*. Replacement of the side chain of Ile 98 in the light chain of 5c8 with a 7-hydroxycoumarin moiety yielded a fluorescent antibody with an emission maximum at 450 nm as expected.[8] This residue is in proximity to, but does not directly contact the antigen in the co-crystal structure[14] suggesting that the fluorescent antibody would still bind CD40L. Interestingly, the emission signal of this mutant exhibited a 2–3 fold increase in intensity (depending on the excitation wavelength) in the presence of saturating concentrations of the antigen, but λ_{max} did not change (Figure 3). To examine whether or not binding was affected, a titration of CD40L over a range of concentrations (50 nM – 7 μ M) that spanned the dissociation constant (K_d) of 5c8 for CD40L was carried out. The fluorescent signal increased sigmoidally; a nonlinear fit of the binding curve (Figure S1) yielded a K_d of 120 nM. The K_d s of wt 5c8 and I98(L) $\rightarrow$ **1** for CD40L were analyzed by Biacore, and found to be 7.0 nM and 28 nM, respectively. Although the K_d of the I98(L) $\rightarrow$ **1** mutant determined from Biacore analysis and fluorescence quenching differ (likely due to surface interactions which increase affinity in the former case) these data show that introduction of the hydroxycoumarin group leads to an ~4 fold decrease in CD40L binding affinity. In general such an effect is not expected to adversely affect the use of **1** as a direct sensor of antibody-antigen interactions, but will likely vary depending on the specific complex under investigation, and the site of modification (which can be varied by simple mutagenesis). Finally, the effect was shown to be antigen specific as the CD40L homologue TNF- α , (which binds 5c8 with 100 fold lower affinity than CD40L in an enzyme-linked immunosorbent assay) did not result in changes in fluorescence (Figure 4).

The fact that 7-hydroxycoumarins exist in both acid and base forms with different absorption maxima allows analysis of the local environment surrounding the fluorophore. Addition of saturating concentrations of antigen resulted in an increase in fluorescence of similar magnitude when the fluorophore was excited at 316 or 370 (2.1 and 2.3 fold respectively) suggesting no significant perturbation of the pKa of the phenolic proton of the 7-hydroxycoumarin occurs on addition of CD40L.

Antibodies have found widespread application as bioanalytical reagents and as therapeutics. [15] Current methods for fluorescent labeling of proteins often rely on nucleophilic lysines or cysteines as handles for fluorophore attachment. Unfortunately, lysine conjugation is generally non-specific, resulting in high background fluorescence. Further, the presence of a number of

disulfide bonds in the antibody scaffold (which are essential for correct folding) renders the application of cysteine conjugation chemistries difficult if not impossible in this system. Thus, by genetically encoding the fluorophore, we remove the necessity for chemical modification of the protein, and have shown that the fluorescent properties of 7-hydroxycoumarins can be exploited to monitor protein-protein interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Crystal structure of the 5c8 antibody in complex with CD40L antigen (PDB id 1I9R). The heavy and light chains are shown in grey and light blue respectively, and the protein antigen is shown in purple. The side chain of I98(L) is shown in sticks, and the structure of **1** is shown in the inset.

Figure 2.

Non-reducing and reducing SDS PAGE analysis of 5c8 I98(L) → **1** stained with coomassie **A**) and visualized with UV light **B**). Addition of dithiothreitol results in the separation of the Fab into heavy and light chains which are seen as distinct bands in **A**) (right lane in each gel). Only the light chain is visible under ultraviolet light **B**) as it contains the fluorophore. Convolved and deconvoluted (inset) ESI-MS spectra of the coumarin containing antibody are seen in **C**). The deconvoluted mass of 48012 corresponds to the expected mass of 5c8 in which residue I98(L) has been mutated to **1** (expected mass 48014). No other protein species were detected by MS analysis.

Figure 3.

Addition of CD40L to 5c8 I98(L) $\rightarrow$ **1**. Spectra shown are at CD40L concentrations of 0, 250 nM, 550 nM, 850 nM, and 1 μ M. Excitation was at 316 nm. Fluorescence signal intensity at 450 nm increases with increasing concentrations of CD40L.

Figure 4. 5c8 I98(L)→1 alone (dashed line), in the presence of 1 μM TNF-α (dotted line), and in the presence of 1 μM TNF-α and 1 μM CD40L (solid line). Excitation was at 316 nm. Only addition of CD40L results in an increase in fluorescence.