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Application of non-canonical crosslinking amino acids to study protein–protein interactions in live cells

Irene Coin

The genetic incorporation of non-canonical amino acids (ncAAs) equipped with photo-crosslinking and chemical crosslinking moieties has found broad application in the study of protein–protein interactions from a unique perspective in live cells. We highlight here applications of photo-activatable ncAAs to map protein interaction surfaces and to capture protein–protein interactions, and we describe recent efforts to efficiently couple photo-crosslinking with mass spectrometric analysis. In addition, we describe recent advances in the development and application of ncAAs for chemical crosslinking, including protein stapling, photo-control of protein conformation, two-dimensional crosslinking, and stabilization of transient and low-affinity protein–protein interactions. We expect that the field will keep growing in the near future and enable the tackling of ambitious biological questions.

Address
University of Leipzig, Faculty of Life Sciences, Institute of Biochemistry, Brüderstr. 34, 04301 Leipzig, Germany

Corresponding author: Coin, Irene (irene.coin@uni-leipzig.de)

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Introduction

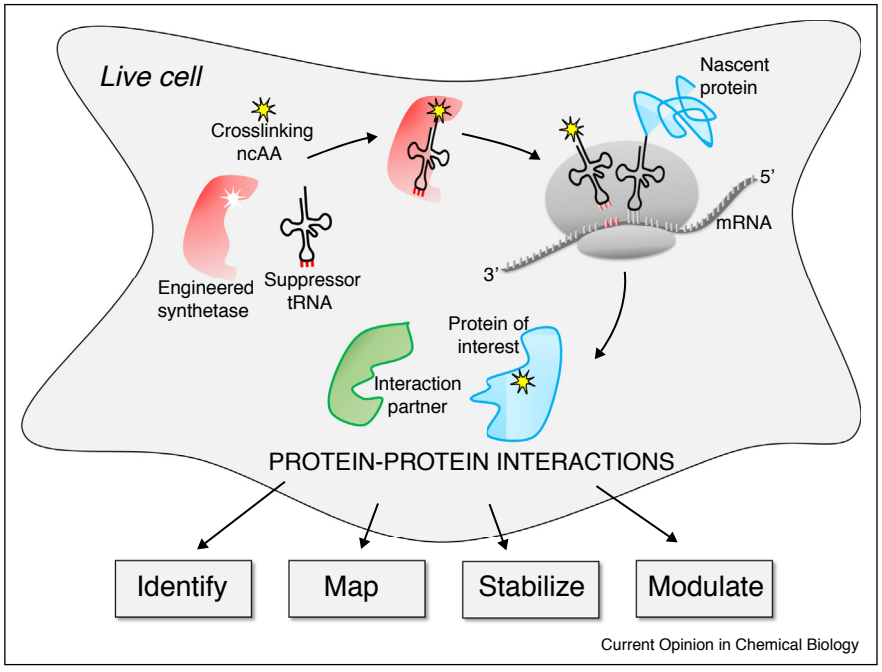
The genetic incorporation of chemical probes into proteins provides the biologist unique tools to study protein–protein interactions both *in vitro* and in live cells, including bacteria, yeast and mammalian cells. Artificial amino acids are site-specifically incorporated into proteins using expanded genetic code technologies (Figure 1) [1–4]. These methods import into the host organism an exogenous orthogonal amino-acyl-tRNA synthetase/tRNA (AARS/tRNA) pair, which does not cross-talk with the endogenous pairs and is engineered to recognize only a desired ncAA. The ncAARS charges the ncAA on the tRNA, usually an amber suppressor tRNA ($tRNA_{CUA}$), which incorporates the ncAA in response to an in-frame amber stop codon (UAG). Among the hundreds of ncAAs that have been developed to date, we will summarize here recent achievements obtained using photo-crosslinking and chemical crosslinking ncAAs to capture, identify, characterize and modulate protein–protein interactions.

Photo-crosslinking ncAAs

Photo-crosslinkers are photo-activatable moieties that are chemically inert in the physiological milieu, but convert into highly reactive groups when irradiated with UV light (Figure 2). The activated crosslinker covalently captures, via quick non-specific radical reactions, organic molecules within a radius of a few Å [5]. Three types of photo-crosslinkers have been installed on genetically encoded ncAAs: benzophenone (Bpa, **1**), azide (Azi, **2**) and diazirine crosslinkers (TmdPhe, **3**; AbK, **4**; DizPK, **5**), all activatable by biocompatible 365-nm light (Figure 2a). They are used either to search for putative and unknown partners of a protein or to map interaction surfaces between proteins. In the first case, the idea is to capture with a covalent bond low-affinity or transient protein–protein interactions that would be otherwise lost in standard immunoprecipitation procedures. In the second case, the photo-crosslinker is used as a 'proximity-sensor' to reveal which positions of the investigated protein are close to the interaction partner in the associated complex [6,7,8•]. Both applications are ideally combined with mass spectrometric (MS) analysis of the crosslinked product to identify either the identity of the captured protein (the prey), or the site of crosslinking. In fact, while the first examples of ncAA photo-crosslinking in living cells were reported over 10 years ago [9,10], coupling genetically encoded photo-crosslinking with mass spectrometry (MS) remains challenging. One major obstacle is that the yield of photo-crosslinking is usually low. In turn, MS analysis of crosslinked samples is biased by high background, which gives rise to false positives in proteomic analysis and significantly hinders the identification of the site of crosslinking via MS/MS. The problem is especially acute for experiments in mammalian cells, where small amounts of proteins are usually involved.

Lately, a big effort has been invested into the development of new photo-crosslinking ncAAs that facilitate MS analysis. Particularly interesting is a series of photo-crosslinking ncAAs bearing cleavable and modifiable linkers (Figure 2b) that enable selective enrichment and labeling of prey molecules after crosslinking (Figure 3a). The three ncAAs DiZSeK (**6**) [11], DiZHSeC (**7**) [12], and DizASeC (**8**) [13•], which are based on the scaffold of DizPK (**5**) [14], contain a selenium atom either at the γ or δ position, where the ncAA

Figure 1



Genetically encoded ncAA for crosslinking studies of protein–protein interactions. Scheme for ribosomal incorporation of ncAAs in live cells. The orthogonal AARS charges the ncAA onto the suppressor tRNA, which delivers it to the ribosome, where it is incorporated into the nascent protein in response to an in-frame amber codon. In this way, the protein of interest is equipped with a crosslinking ncAA to identify, map, stabilize or modulate its interaction with endogenous or overexpressed protein partners.

is cleaved upon treatment with mild oxidizing agents. After enriching the crosslinked product using a tag on the protein bearing the crosslinker (the bait), prey and bait are separated. A bioorthogonal chemical anchor is left on the prey (Figure 2b, red boxes) that can be chemoselectively modified with a unique second tag. In this way, the prey can be isolated prior to MS analysis. To guarantee the cleanest bioorthogonality, DizAseC (**8**) bears an independent alkyne moiety that can be modified with robust copper catalyzed azido-alkyne cycloaddition (CuAAC) chemistry [13•]. Alternatively, the pentyl-acylamide moiety from cleavage of DiZHSeC (**7**) adds onto the prey a unique weight than can directly serve as a label for MS analysis. Combined with 2D difference gel electrophoresis [15], this approach led to identifying distinct and specific pools of client proteins for the acid-activated *Escherichia coli* chaperones HdeA and HdeB [16,17] and contributed to the elucidation of the bacterial mechanism of acid resistance [18•]. However, as successful as the application of these amino acids has been in *E. coli* cells, their usage in mammalian cells is still quite preliminary [12].

By contrast, the application of photo-crosslinking ncAAs to map peptide–protein and protein–protein interactions is well established in *E. coli*, yeast and mammalian cell hosts (Figure 3b) [6]. In this case, the ncAA (usually Bpa, **1** or Azi, **2**) is systematically incorporated throughout a putative domain of interaction between the examined protein of interest (POI) and a known interaction partner (the prey). Crosslinking is triggered on the associated complex and the occurrence of crosslinking is analyzed via immunoblotting using an antibody targeted to the prey. Crosslinking hits identify the residues of the POI that come into proximity of the bound prey and reveal the shape of the interaction surface. Contrary to classic biophysical methods (crystallography, NMR, EM), photo-crosslinking mapping requires only very little amounts of protein (< μg per position) and, most importantly, allows investigating the interaction on the intact proteins in live cells.

The method was first applied to map ligand binding sites on G-protein coupled receptors (GPCR), such as the orthosteric binding pocket for the neuropeptide Urocortin I on the corticotropin releasing factor receptor CRF1R [7,19], the binding site of the 16-mer peptide T140 on the CXC chemokine receptor 4 [20], the binding site of substance P on the neurokinin-1 receptor [21], and very recently the binding site of exendin-4 on the glucagon-like peptide-1 receptor [22]. The technique also allows distinguishing binding modes of pharmacologically distinct ligands on the same receptor [8•]. Photo-crosslinking mapping of membrane receptors also includes the study of the insulin–insulin receptor complex [23] and recently the identification of high and low affinity binding sites of antidepressant drugs on the human serotonin transporter [24,25].