In Vivo Photocrosslinking with Unnatural Amino Acid Mutagenesis

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mutagenesis · photoaffinity labeling · protein engineering · proteins · proteomics

An emerging challenge for proteomics is the enumeration of the interactions between any given protein and all other molecules in a cell. The yeast two-hybrid system[1, 2] and protein arrays[3] have facilitated the discovery of protein–protein interactions. However, these methods, whether in vivo or in vitro, largely consider protein–protein interactions in a pairwise, albeit parallel fashion. It is clear that protein interactions within a cell are context dependent and the precise interactions depend upon numerous interacting biological networks.[4] Deciphering these interactions in their native context, in which a given protein has the opportunity to "see" all other cellular components simultaneously, is key to defining the cellular roles of many protein interactions.

The characterization of protein interactions in vivo is facilitated by the purification of intact complexes. While a battery of techniques have emerged to analyze isolated complexes,[5–7] less progress has been made on isolation of intact protein complexes from intact cells. Current methods rely on affinity purification of noncovalently associated proteins, which may dissociate during cell lysis or during affinity purification.[4, 8] As a result, the protein interaction maps produced from these experiments may be incomplete and may provide a distorted picture of the protein interaction network in cells, with interactions that have particular thermodynamic or kinetic profiles systematically absent.

We have previously demonstrated that addition of new components to the translational machinery of *Escherichia coli* allows several additional amino acids to be site-specifically incorporated into proteins in vivo with high translational fidelity.[9–11] We recently evolved a mutant *Methanococcus Jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) that works with a mutant MjtRNA and the *E. coli* translational machinery to incorporate the photocrosslinking amino acid *p*-benzoyl-L-phenylalanine[12] (*p*Bpa) into proteins with high translational fidelity and efficiency in response to the amber codon, TAG.[13]

*p*Bpa has been widely used to map protein–peptide interactions in vitro, is chemically stable, and can be routinely manipulated under ambient lighting.[14] The benzophenone group of *p*Bpa preferentially reacts with C–H bonds upon excitation in the near-UV at 350–365 nm, wavelengths that avoid protein and nucleic acid damage. We have shown that this amino acid can be used to efficiently crosslink interacting proteins in vitro.[13] It should also be possible to use the genetic incorporation of *p*Bpa to define the interaction partners for a given surface of a protein within the cell. Our strategy (Figure 1) involves: 1) replacement of a single amino acid in a protein with *p*Bpa in vivo; 2) irradiation of the cell with near-UV light to crosslink proteins proximal to the surface of the *p*Bpa-containing protein; 3) cell lysis, purification, and identification of the complex or complexes formed.

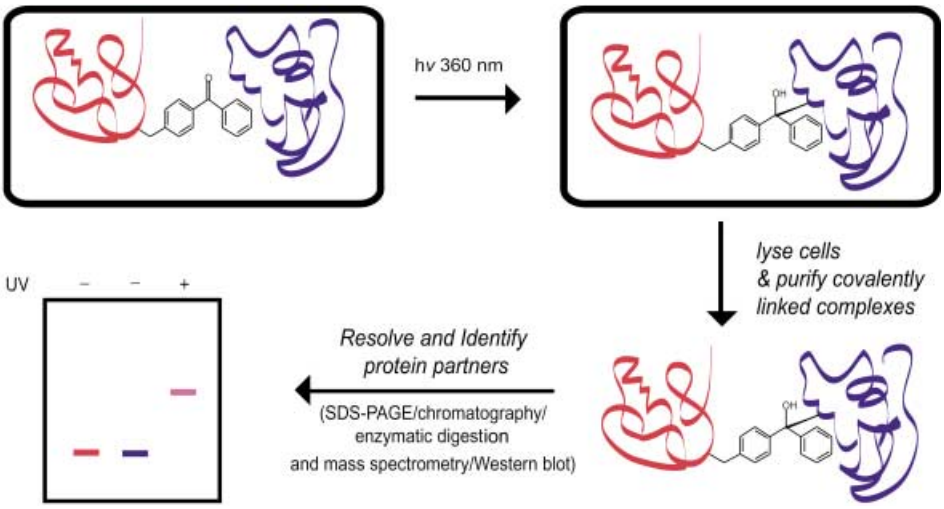

Figure 1. *A method for the creation of covalent bonds between protein surfaces in vivo.*

To begin to test the feasibility of this approach, we studied the association of glutathione *S*-transferase within the context of the *E. coli* proteome. This protein crystallizes as a dimer of two identical subunits that have previously been crosslinked both specifically[13, 15] and nonspecifically[16] in vitro. We previously generated a Phe52*p*Bpa mutant of SjGST[13] in which *p*Bpa lies at the protein–protein interface of the dimer by using the crystal structure of *Schistosoma Japonica* glutathione S-transferase (SjGST)[17] and showed that the mutant could be efficiently crosslinked in vitro.[13]

SjGST (Phe52*p*Bpa) was expressed by cotransformation of an amber mutant of the SjGST gene with an orthogonal aminoacyl tRNA synthetase/tRNA pair for *p*Bpa incorporation. SjGST (Phe52*p*Bpa) was expressed as less than 1% of the soluble protein in the cell. This level of expression is comparable to that of many endogenous proteins and favors, by mass action, nonspecific interactions with the vast number of competing

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protein surfaces on the approximately 2500 cytoplasmic proteins present in the cell.[18] To assess the covalent complexes formed with SjGST in vivo, we lysed the cells, purified SjGST and the complexes it forms after exposure to increasing doses of UV light, and performed Western blots with an anti-GST antibody (Figure 2B). This assay is capable of detecting soluble complexes formed with SjGST in vivo but comparison with in vitro crosslinking of purified SjGST (Phe52*p*Bpa)[13] reveals that only the SjGST dimer is formed. The dimerization of SjGST in vivo is therefore highly specific, as indicated by the absence of any other complexes detectable by Western blot.

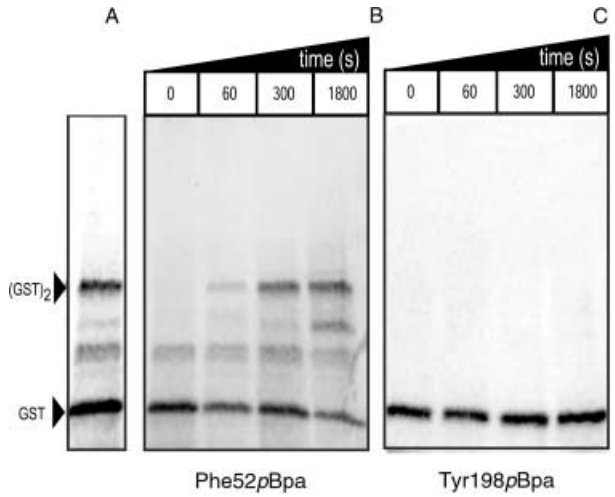

Figure 2. *In vivo surface-specific protein crosslinking.* ***A****. Purified SjGST (Phe52pBpa) was crosslinked in vitro and detected by Western blot with an anti-GST antibody.* ***B****. The covalent dimerization of SjGST (Phe52pBpa) in vivo as a function of cellular irradiation at 365 nm, detected as in A.* ***C****. As in B, but with cells expressing SjGST (Tyr198pBpa).*

To further investigate the specificity of this approach for isolation of specific protein–protein interactions, we repeated the in vivo crosslinking experiment with SjGST (Tyr198*p*Bpa). This mutant does not crosslink in vitro[13] and displays a benzophenone group on the protein surface outside the protein–protein interface. We find that SjGST (Tyr198*p*Bpa) does not form detectable, soluble crosslinked complexes with proteins in *E. coli* even after irradiation for 30 minutes at 365 nm (Figure 2C). Wild-type SjGST is also refractory to photocrosslinking (data not shown). These results underscore the specificity of this approach for isolation of only specific protein complexes.

These experiments provide the basis for the isolation of covalent complexes formed between a chosen surface on any protein in the cell and its cellular partners. In general, more sophisticated methods for the separation and analysis of protein complexes, such as shotgun mass spectrometry[6] or 2D SDS-PAGE coupled to mass spectrometry, will of course be required to decipher the interaction partners of a protein that has several interactions within the proteome.

In conclusion, we have used unnatural amino acid mutagenesis to examine properties inside a cell. We have demonstrated the in vivo, photoactivated, surface-specific crosslinking of interacting proteins in a model system. These experiments highlight the power of in vivo, site-specific, unnatural amino acid mutagenesis for both better understanding the cell and altering its properties at will.[19] We are currently applying this methodology to the study of the in vivo properties of macromolecular cell machinery,[20] as well as developing similar approaches to the study and manipulation of cells from higher organisms.

Experimental Section

Plasmids pYC/SjGST52TAG or pYC/SjGST52TAG, which contain a tetracycline resistance marker and an SjGST gene (with amber mutation at codon 52 or 198) on an arabinose promoter with a rrnB terminator, and mutRNA$^{Tyr}_{CUA}$ on an lpp promoter with an rrnC terminator were cotransformed into DH10B *E. coli* with a pBK vector that expresses BpaRS-1.[13] Cells were amplified in 2xYT (10 mL) that contained kanamycin (30 mg L^{-1}) and tetracycline (25 mg L^{-1}), washed in 1xM9 media, and used to inoculate GMML (glycerol minimal media with leucine) with the appropriate antibiotics and *p*Bpa (1 mM). Protein expression was induced at an optical density of 0.55 at 600 nm by the addition of arabinose to 0.02%. After induction, cells were harvested and washed twice in phosphate buffered saline (PBS; 500 mL) before resuspension in PBS (3 mL). Cells were transferred to a single well of a six-well tissue culture plate (Costar, Corning Inc. N.Y.) at 4 °C. To crosslink proteins in vivo the culture was irradiated at 365 nm with a hand-held UV lamp (11 V, 60 Hz, 0.2 A, Spectronics EC-240) placed 2.5 cm from the surface of the cell suspension. Cells were removed from the wells after irradiation for 0 min, 1 min, 5 min, 15 min, or 30 min and harvested by centrifugation before lysis by sonication. SjGST (Phe52*p*Bpa) or SjGST (Tyr198*p*Bpa) and their soluble complexes were purified by virtue of the presence of a C-terminal hexahistidine residue by using nickel–nitrilo triacetate (Ni-NTA) chromatography (Qiagen) according to the manufacturers instructions. Elution fractions from the Ni-NTA column were diluted with SDS loading buffer and the products of in vivo crosslinking were resolved by SDS-PAGE on a 10–20% gradient gel. Proteins were transferred to a Trans-Blot nitrocellulose membrane (Biorad). The membrane was blocked for 12 hrs in 5% nonfat milk in PBS with 0.1% Tween-20 (PBS-T) and then incubated with goat anti-GST (1:1000, Pharmacia) in the same buffer for 1 hour. The membrane was washed for 3×10 min with PBS-T before addition of a secondary donkey-antigoat–alkaline-phosphotase conjugate (Santa Cruz Biotech) at 1:1000 dilution in PBS-T with 5% nonfat milk. The membrane was then washed (as above) and rinsed in PBS and the signal was developed by addition of Vistra ECF (1 mL, Pharmacia) and incubation for 1 min. The signal was visualized on a Storm phosphoimager (Molecular Dynamics) by scanning with a blue laser at a photomultiplier voltage of 500 V. In vitro crosslinking reactions were performed as previously described[13] but analyzed as described above.

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[19] The near-UV irradiation per unit area (fluence) at 365 nm used in these studies for a five-minute exposure was approximately $10-30\ kJ\ m^{-2}$. Classic studies on the effects of bacterial exposure at this wavelength have shown that lethal effects of such radiation on bacterial cells require fluences one to two orders of magnitude greater than this value. This observation raises the possibility that the phenotypes of live cells may be altered by photocrosslinking between protein surfaces in vivo.
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De Novo Design, Synthesis, and In Vitro Evaluation of a New Class of Nonpeptidic Inhibitors of the Malarial Enzyme Plasmepsin II

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Malaria, a life-threatening disease caused by parasites of the genus *Plasmodium*, affects 500 million people annually, of which more than one million die.[1] The emergence of multi-drug-resistant strains of *Plasmodium falciparum*, the parasite that causes the deadliest form of malaria, exacerbates the situation and necessitates new medicines with novel modes of action.[2] Plasmepsin II (PII; EC 3.4.23.39),[3] a parasitic aspartic protease involved in the hemoglobin degradation process that takes place in an acidic vacuole, has been identified as a potential target for antimalarial therapy. Several groups reported PII inhibitors that mimic the natural substrate and display up to single-digit nanomolar activity.[4] Inhibition of PII is expected to block the life cycle of the parasite.[5] Here we report the synthesis and in vitro evaluation of a new class of nonpeptidic PII inhibitors developed with the help of structure-based de novo design that show up to single-digit micromolar inhibitory activities.[6–8]

A major conformational change around the active site of the human aspartic protease renin (EC 3.4.23.15) upon complexation of 3,4-disubstituted piperidines has been observed, which unveils unexpected flexibility of the enzyme.[9] The flap that lies over the catalytic dyad, and a tryptophan side chain of the core domain, move and thereby unlock a new hydrophobic pocket (flap pocket). The high sequence homology between renin and PII prompted us to hypothesize that an induced-fit adaptation such as that of the active site of renin might also be operative

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