Unnatural Amino Acids (UAAs) and the mirror world: potentials and implications of rewriting biology

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Introduction

A universal algorithm to relate nucleotide triplets to protein sequences exists as nature's ever evolving elegant machinery of time and selectivity occurring before the split of the tree of life into three separate kingdoms: Eukarya, Bacteria and Archaea. Recently these translational machineries are being modified to incorporate unnatural amino acids (UAAs) and quadruplet codons. Not all of these strategies are novel; in fact, evolution has formed multiple translational pathways that are being modified to unnatural functions beyond natural translation. Recently, unnatural peptides are being constructed by utilizing so called UAA that may offer novel physicochemical and biological structure functionality in proteins. However, changes may result unpredictable outcomes such as inactivation of cellular proteome pathways that involve responding to a cells needs or interrupting the intrinsic translation system of cells overall.

Biochemistry of translation

Translation is information transfer: linear sequence of ribonucleotides are translated into amino acids through 3-mers that universally specify a residue or a termination signal (4). Transfer RNAs are adaptor molecules of ~80 nucleotides with double helical regions that bridge connection between nucleic acid and polypeptide sequence by translating mRNA to protein (4). In the 3D structure of tRNAs or cloverleaf depiction, there are generally three loops that do not hydrogen bond with the polynucleotide chain and anticodon loops consist of 3-bases that are complementary to mRNA sequences called codons (4,13). Since several codons can correspond to an amino acid, the genetic code is redundant and redundancy exists as amino acids may require complementary 5'-codon base pairing with either just the first 3'-anticodon on the tRNA or through the second and tolerate a mismatch in the other position(s) (4,13). The universal genetic code may have been constructed with genes encoding tRNA which were duplicated and underwent anticodon mutations and so resulted in different recognition sequences (18).

Efficiency of protein translation depends on precise biochemical and genetic factors. First, a tRNA-synthetase recognizes its cognate amino acid through high affinity for the enzyme's active site and also the amino acid's cognate tRNA by recognizing the matching anticodon (4,13). This initiates binding of a specific AA~tRNA through a two-step sequential process starting with an aminoacylation reaction that converts an amino acid to an adenylated amino acid through ATP hydrolysis: ATP donates an AMP which is linked to the carboxyl end of an amino acid (4,13). Next, aminoacyl-tRNA synthetase catalyzed reaction product aminoacyl-tRNA is formed when the carboxyl end of the amino acid (linked to AMP) is transferred to a hydroxyl-group of the cognate tRNA's 3'-ribose end thereby activating the molecule through an ester-linkage whose hydrolysis associated with a favorable change in free energy (4,11,13). Base pairing between the mRNA codon and ester linked AA~tRNA complex anticodon is corrected by tRNA hydrolytic editing in case of incorrect attached amino acids (4,11,13). Upon passing, ester-linked activated complex is delivered to the ribosomal A-site – important for maintaining fidelity of triplet decoding (4,11).

Mutations change the sequence of DNA. Nonsense mutations are intragenic termination codons that halt the translation of polypeptide molecules prematurely and are key to UUA incorporation (4,13). Deleterious nonsense mutations can be rescued and nullified with nonsense suppressors: either the second mutation converts a termination codon to an amino acid or suppresses the effects of the termination codon (4,13). Mutant tRNA suppressor genes alter anticodon loops such that tRNAs can recognize termination codons but are charged with an amino acid so poly-

peptide elongation continues past the triplet codon (4,11,13). For example, a mutant tyrosine tRNA or tRNAtyr contains the anticodon 3'-AUC-5' that recognizes and aligns to the termination codon 5'-UAG-3' instead of a tyrosine codon 5'-UAC-3' thereby bypassing intragenic termination codon UAG and appending tyrosine to the nascent polypeptide chain (4,13).

Strategies for decoupling translation already found in nature

Current progress exists in decoupling translation from host machinery to orthogonal translation machinery that opens opportunities for a parallel genetic code which can run alongside wild-type translation (11,13,14). Construction of orthogonal translational machinery is reliant on non-cross-talk between wild-type/endogenous and neo-functional molecules; subsequently, careful tailoring of the ribosome and it's mRNA binding sites so much as the relation becomes predetermined, modular, predictable and thereby reproducible (6,14).

Lessons from selenocysteine: UAA that nature kept through genetic code evolution by means of specific translational machinery

First discovered in 1986, selenocysteine (Sec) is a selenium containing amino acid that is co-translated through an in frame UGA termination codon that is recognized exclusively by a special serine-tRNA or $tRNA^{(Ser)Sec}$(15). Sec biosynthesis involves dedicated orthogonal machinery which incorporates selenocysteine without the cognate aminoacyl-tRNA synthetase for selenocysteine that is otherwise found in ~20% of Eubacteria and ~10% of Archaea genomes sequenced (15). Novel evolutionary technology utilizes the codon UGA that has dual roles as a termination codon and for amino acid incorporation and the former occurs if the downstream region contains a stem-loop secondary structure which folds back to attenuate translation; incidentally, the theme of having dual uses for termination codons is exploited to construct synthetic orthogonal machinery (15,18). Selenocysteine biosynthesis begins with $tRNA^{(Ser)Sec}$, which is then charged with the respective canonical amino acid serine facilitated by serine aminoacyl-tRNA synthetase (SerS), ATP and Mg^{+2} (15,16,18). In *E. coli*, this serine is enzymatically converted to selenocysteine before it is incorporated into the ribosome by *Sec synthetase* (selenocysteine synthesis enzyme), that is a pyridoxal phosphate dependent protein which removes a hydroxyl to generate a seryl-$tRNA^{(Ser)Sec}$ intermediate which then accepts a monoselenophosphate to form selenocysteyl-$tRNA^{(Ser)Sec}$: minor type of seryl-tRNA (15).

Although very rare, pyrrolysine existence among methanogens also makes double use of its termination codon

Methanogens are primitive bacteria that frequently reduce methylated amines, thiols, methanol, acetate or carbon dioxide compounds to methane through process of methanogenesis that requires substrate specific methylamino-, dimethylamino-, and trimethylamino-transferases — all which are known to incorporate the 22nd amino acid pyrrolysine (pyl) (1,2,18). The gene encoding monomethylamine-methyltransferase contains an in-frame termination codon that is used by anaerobic archaea *Methanosarcina barkeri*. To fully transcribe this protein transcript, a special amber suppressor tRNA , Pyl ($tRNA^{Pyl}$) is required that has an anticodon complementary to UAG that can then read and align to UAG (16). The gene *PylS* encodes $tRNA^{Pyl}$ that is aminoacylated with *Pyl*, an "aminoacyl-tRNA synthetase-like" enzyme called pyrrolysyl-tRNA synthetase or PyIRS, pyrrolysine insertion elements (Figure 1.b) (1,16). *PyRIS* is specific for pyrrolysine and its cognate tRNA and not lysine along with its cognate tRNA (1). Polycarpo et al.

(2004) has shown incorporation of pyrrolysine in *E. coli* involves only PyIRS and a mRNA encoding a methyltransferase (1).

Artificially expanding the genetic code of prokaryotic through tyrosyl-tRNA incorporation

Orthogonal translation is nonessential for host viability and can be utilized for novel functionality which is beyond the reach for wild type translation by constructing a system that can run parallel to host translation (14). As previous discussed, necessary ingredients for successful incorporation of an UUA that corresponds to a new codon assignment includes the UAA itself along with its aminoacyl tRNA synthetase and its cognate tRNA which should be both highly specific to each other and orthogonal to aminoacyl tRNA synthetase and its cognate tRNA inside the host organism (3,11,14,16,18). Furthermore, the UUA must not be a substrate for endogenous aminoacyl tRNA synthetases of the host and the UAA must confer intracellular stability once inside the host system (3,11). To construct such a system, first a tRNA codon and its cognate amino acyl tRNA synthetase pair have to be recognized (21).

MjtRNA system uses an orthogonal amber suppressor

Also a methanogenic archaea, *Methanococcus jannaschii* uses an odd tyrosyl-tRNA synthetase (MjTyrRS) and cognate $tRNA^{tyrosyl}$ pair where the synthetase does not bind to the anticodon loop of its cognate $tRNA^{tyrosyl}$ (11,21).This orthologous translational pair was further mutated to recognize codon CUA to decode amber-suppressor TAG and placed in orthologous *E. coli* system to make use of differences in translational machinery between prokaryotic and archeal synthetase and tRNA pair (11,21). Next, novel *Mj*$tRNA^{tyr}$ were generated by randomly mutating anticodon-loop tRNA libraries precisely to interfere with non-orthogonal synthetases but not between tRNA and MjTyrRS or the ribosome (22). Variants were selected if their aminoacylation to *Mj*$tRNA^{tyr}$ was with the supplied and modified *MjTyrRS* that would recognize the new amino acid in response to the amber codon UAG. Selection was against non-orthogonal or endogenous synthetases that would otherwise aminoacylate *Mj*$tRNA^{tyr}$ with endogenous amino acids (4,21,22).

A negative selection procedure introduces amber codon(s) to barnase gene at nonessential positions and checks if endogenous *E. coli* amino-acyl-tRNA synthetases aminoacylate *Mj*$tRNA^{tyr}$: the amber codon is suppressed and toxic barnase is expressed which leads to cell death (11,21,22). Consequently, positive selection collects cells which contain orthogonal tRNAs: *Mj*$tRNA^{tyr}$ variants that would be putatively aminoacylated by *Mj*TyrRS only or non-functional tRNAs and would survive due to β-*lac* or *ampR* drug resistance transcript containing amber codons at nonessential position is co-transformed into cells along with *Mj*TyrRS gene (11,21,22). In short, survivors contain (MjTyrRS) and cognate $tRNA^{tyrosyl}$ pair that directs the incorporation of *O*-methyl-l-tyrosine in response to the amber codon, UAG (11,21,22).

Unnatural amino acids can also be encoded in mammalian cells through genetic engineering. Archeal *Mj*TyRS/*Mj*$tRNA^{tyr}$ pair would not be orthogonal to mammalian cells due to a difference of essential identity elements; however, yeast translational machinery is homologous to higher-organisms and offers a means to transfer machinery to mammalian cells with larger success (23). Liu et. al (2007) have developed a technique that site-specifically incorporates UAAs using a mutant *E. coli* aminoacyl-tRNA synthetase (*aaRS*) evolved from yeast to selectively aminoacylate it's cognate amber suppressor tRNA from prokaryotic thermophile *Bacillus stearothermophilus* with UAA of interest (23). However, unlike prokaryotes, mammalian genomes

contain a two-fold higher occurrence of amber stop codons and this would likely interfere with positive/negative selection procedures (23).

Further alterations in the translational machinery allow for quadruplet codons

Limits of orthogonal translation based on dual roles of amber suppressors is such that only one UAA can be appended to a polypeptide at a time (11,14). Development of ribosome that can process quadruplet codons instead of triplets increases information-theoric content of proteins: the available codons are increased from 64 (4^3) to 256 (4^4) thereby allowing more complex protein sequences and also constructing "blank codons" that do not relate to an amino acid (5,11,12). The naturally expanded genetic code includes the previously mentioned amino acids selenocysteine and pyrrolysine, involve a similar process of using an amber stop codon with dual use (12).

Directed evolution of mRNA to incorporate quadruplet codons and engineered ribosomes

Muranaka et al. (2006) developed a four-base codon strategy by first constructing a non-natural amino acid peptide library through mRNA display, a protocol used to evolve transcript that binds to a target of choice (24). Three previously successful quadruplet codons that would be decoded by *E. coli* host tRNAs which contained cognate anticodons were incorporated into an mRNA transcript that also sometimes contained an amber codon in the form $(NNK)_n$ (24). In this case, streptavidin (that is targeted by biocytin), became a component of peptides evolved in the library through incorporation by one of three quadruplet amino acids or amber codon (24). Conformation of biocytin incorporation is determined by mass-spectrometry of unnatural quadruplet amino acids: bphAla, bzoPhe, azoAla and napAla (24). Results indicate incorporation of multiple non-natural amino acids encoded by quadruplet codons were achieved through the protocol thus opening doors to protein structural diversity (24). More recently, Neumann et al. (2010) constructed orthogonal translational machinery by synthetic evolution of a ribosome that can decode quadruplet codons through extended anticodon tRNAs and an amber suppressor in which translational kinetic efficiency is comparable to that of wild type translation unlike previous similar methods that lack substantial yields of the desired product (11).

Function of unnatural amino acids within proteins

Many approaches are taken to incorporate unnatural amino acids into proteins. In one example, as mentioned above selenocysteine can be used to de novo synthesize cysteine, an essential amino acid as the only difference in structure occurs at the selenium which can be replaced with sulfur to form cysteine (15). UAAs consist of unique functional or chemical groups that have novel properties once incorporated within proteins including usefulness for biochemical and cellular studies and include, fluorescent, glycosylated, sulfated, heavy atom metal ion-binding, long-chain amino acids along with novel amino acids with specific chemical reactivity that utilize ketone, azide, or photocrosslinker: *p*-azidophenylalanine, *p*-benzoylphenylalanine, *p*-(3-trifluoromethyl-3H-diazirin-3-yl)-phenylalanine functional groups (3,11,23). Such additions open opportunities in tailoring substrate and enzyme specificity for general synthetic biology and metabolic engineering (14).

Photo cross-linkers

Photo-crosslinkers such as *p*-benzoylphenylalanine (Bpa) can be inserted between carbon-hydrogen bonds when activated around 300nm through the benzophenone moiety (3,8). In one

example, light is use to cross-link benzo-Phe or azido-Phe to ligands of transmembrane protein complexes (Figure 3). GPCRs and ion channels undergo a biophysical conformational change between active (ligand-bound) and resting (unbound) states that cumulate triggering of intracellular signaling cascades (3,7). Understanding binding partners elucidates biochemical structure and function of ligand receptors (3). In another example, Hino et al. (2005) utilized site-specific incorporation of photoreactive *p*-benzoyl-L-phenylalanine (*p*Bpa) in the adaptor protein Grb2 Src homology 2 (SH2) domain of mammalian cells which upon irradiation cross-linked to epidermal growth factor (EGF) receptor thereby allowing precision in protein-protein interaction experiments (3,10).

Subtle cation-π interactions

Cation-π interactions are as significant as salt bridges and hydrogen bonds, but subtle as they are formed under stringent geometrical side chain interaction. Methods to study these interactions utilize an all or nothing strategy by replacing high π-electron density aromatic residues with non-aromatic residues (3,17). This changes physicochemical properties and does not accurately report strength of cation-π interaction (3,17). Common sites for such interactions are found in cys-loop receptors which are arranged around an ion-conducting pore with five subunits in the periphery (3,17). Stepwise fluorination of phenylalanine and tryptophan residues produces monofluoro-, difluoro-, trifluoro-, etc. derivatives that decrease electronegativity and therefore cation-π binding (3,17). Binding pocket for agonist follows cation-interaction which can be studied through UUA site-specific mutagenesis of fluorinated phenylalanine (3,17). Lummis et al. (2013) has shown through site-specific UUA Phe-206 and Tyr-254 form meaningful cation-π interaction as stepwise addition of fluorine atoms to UUA derivatives leading to stepwise increase of GABA EC_{50} in *Xenopus* oocytes (3,17). This methodology thereby elucidates a correlation between receptor function and specific cation-π bonding and offers a means of experimental control that has highlighted prevalence of interactions between cationic receptors and aromatic side chains which has been previously been underestimated (7).

Photocaging and photoactivating UUAs

Cysteine, serine and tyrosine residues undergo unique reactivity when blocked by substituted nitrobenzyl groups on their thiol or hydroxyl functional groups (3,8). These so-called "photocaged" amino acids have their substituted nitrobenzyl groups cleaved with 365-nm light thereby allowing functionality of specific amino acids through irradiation (3,8). Photo-regulation of specific amino acids have allowed for highly useful synthetic proteins with UUA(s) (3,8). In one example, photocaged tyrosine is site-specifically inserted to replace a tyrosine substrate residue for a protein kinase (Figure 3). Irradiation would remove the blocking group and allow phosphorylation to occur (3,8). Arbely et al. (2013) has shown photocontrol of STAT1 Tyr-701 phosphorylation in mammalian cells opens possibilities to photo-activate specific tyrosine residues in multicellular organisms through evolution of the orthogonal pyrrolysyl-tRNA synthetase (PylRS/$tRNA_{CUA}$) system (3,8). Similarly, Chou et al. (2009), has shown photoactivation of *Taq* DNA polymerase catalytic activity through site-specific incorporation of UUA caged tyrosine derivative at Tyr601 (Figure 5) (3,8,9). Upon 365nm irradiation, 71% of wild-type *Taq* activity of DNA polymerization is restored (9). A photocaged UUA encoded *Taq* is utilized to develop light-triggered hot-start PCR which allows for spatial control of PCR (9).

Mirror Image Cells

Chemical scope of protein translation is limited to α-L amino acids and close analogs as biological systems have evolved to depend exclusively on the L-form enantiomers of amino acids; or in other words there is only one biologically active stereoisomer (11). With the exception of glycine, the four groups attached to the central carbon are different signifying a "handedness" of amino acids due to the chiral stereo-center of the central carbon and their molecular configurations can only be changed through breaking and reforming covalent bond. Mirror image cells theoretically can be constructed with opposite to chiral molecules found in wild type cells and would therefore confer complete incompatibility wild type cells. One approach as proposed by the George Church Lab at Harvard University seeks to find a mutant ribosome that permits translation of mirror image amino acids consequently allowing production of mirror image proteins (25). Furthermore, these mirror image components can then survive in a synthetic cellular membrane or a proto cellular membrane that would be able to "boot-up" in a mirror world (25). Aside from biological challenges, ethical challenges exist such that mirror image cells have essentially no predators: their growth and prosperity would compete with wild type cells. Overall, mirror image cells would win and cause a potential evolutionary reboot on earth (25).

Figures

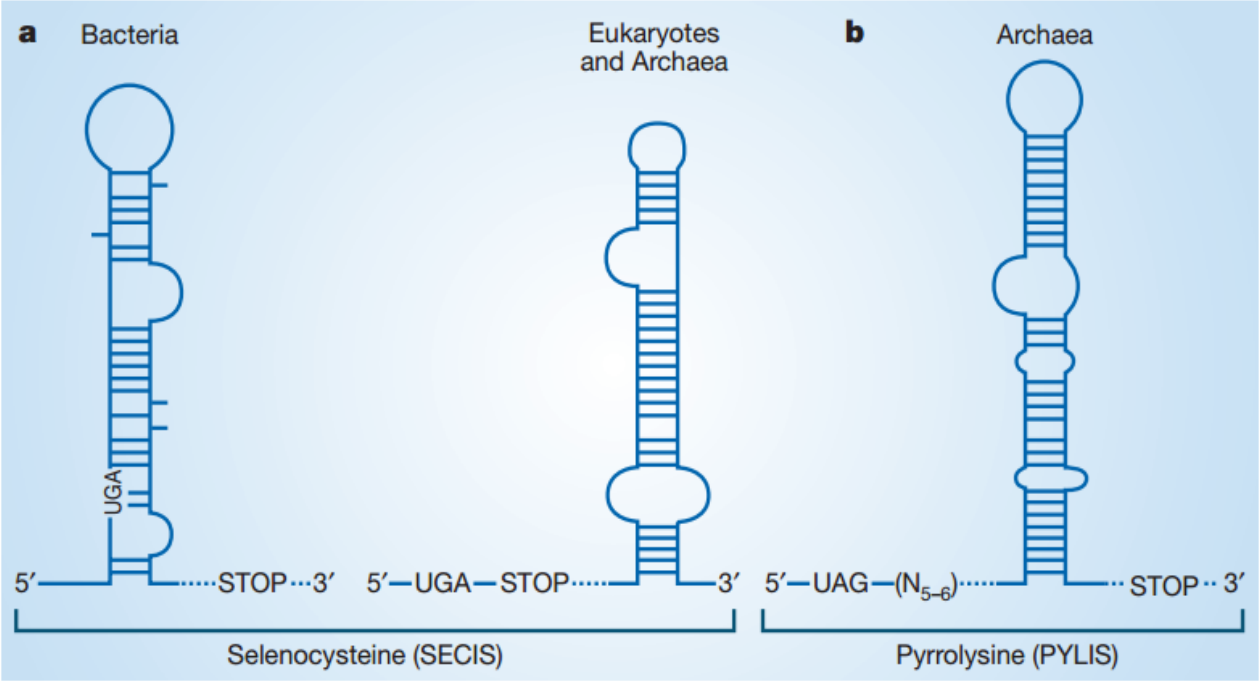

Figure 1. UAA RNA stem-loop structures. a. The Sec insertion sequence (SECIS) element and SECIS-binding protein-2 (SBP2) are constituents of successful Sec incorporation whereby a complex is constructed between the bound SBP2 to SECIS element and elongation factors; moreover, the SECIS element is found in the 3'-UTR of selenoprotein mRNA (15,16). **b.** Predicted structure of PYLIS (pyrrolysine insertion element) from Archaea, perhaps such as *Methanosarcina barkeri,* which uses amber suppressor UAG codon to incorporate pyrrolysine: similar to Sec insertion (16). Figure adapted from Schimmel and Beebe, 2004 (16).

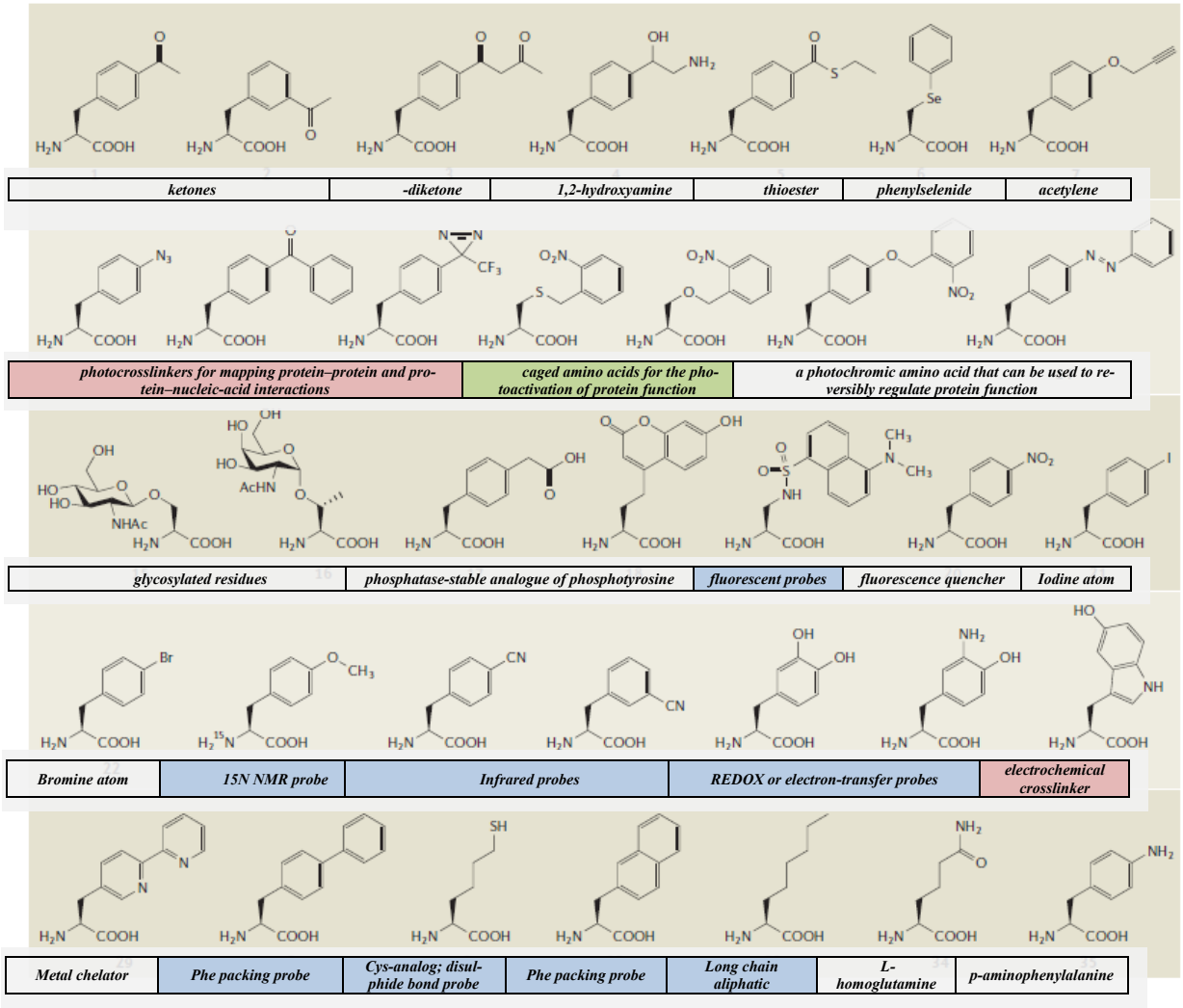

Figure 2. Types of unnatural amino acids that can be incorporated into proteins. In general, blue boxes represent probes, red are cross-linkers and green are used for photo-caging. Figure is adapted from Xie & Schultz (2006) (3).

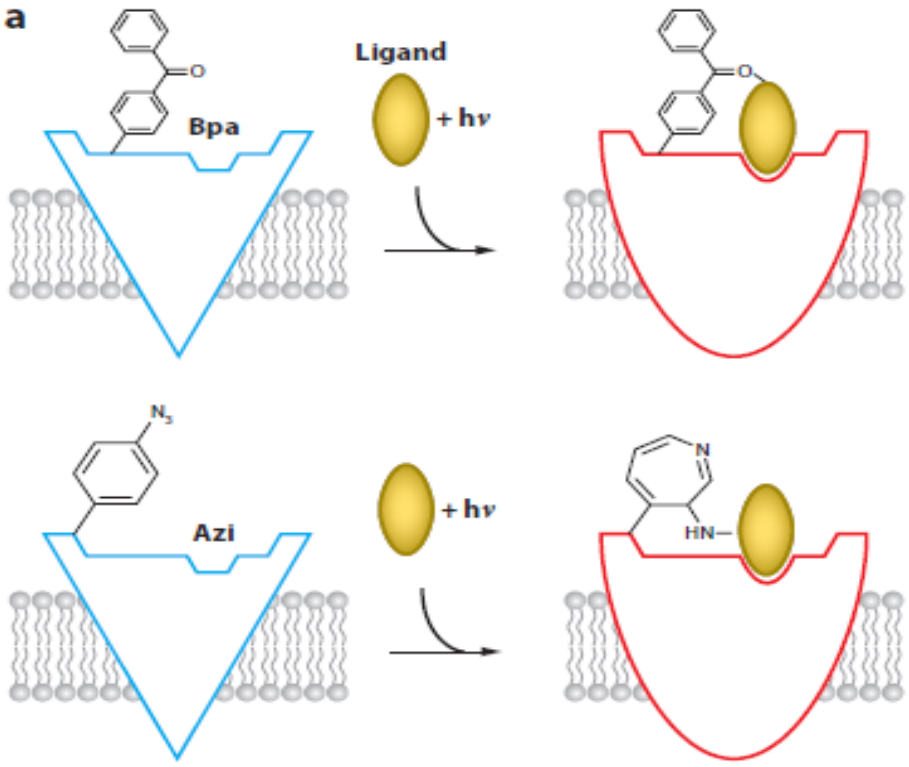

Figure 3. Strategies for light activated cross-linking UUAs benzo-Phe (Bpa) and azido-Phe (Azi). Figure adapted from Pless and Ahern (2013) (7).

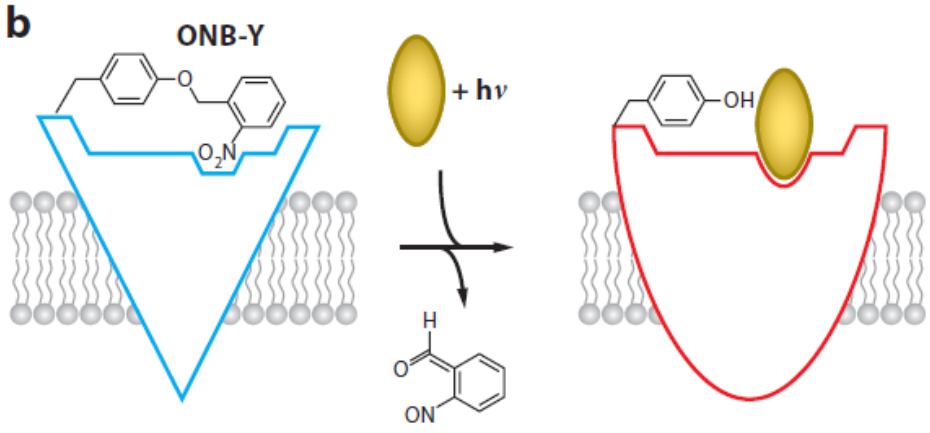

Figure 4. UUA tyrosine derivative ortho-nitrobenzyl Tyr (ONB-Tyr) allows identification of aromatic side chains involved in binding. Figure adapted from Pless and Ahern (2013) (7).

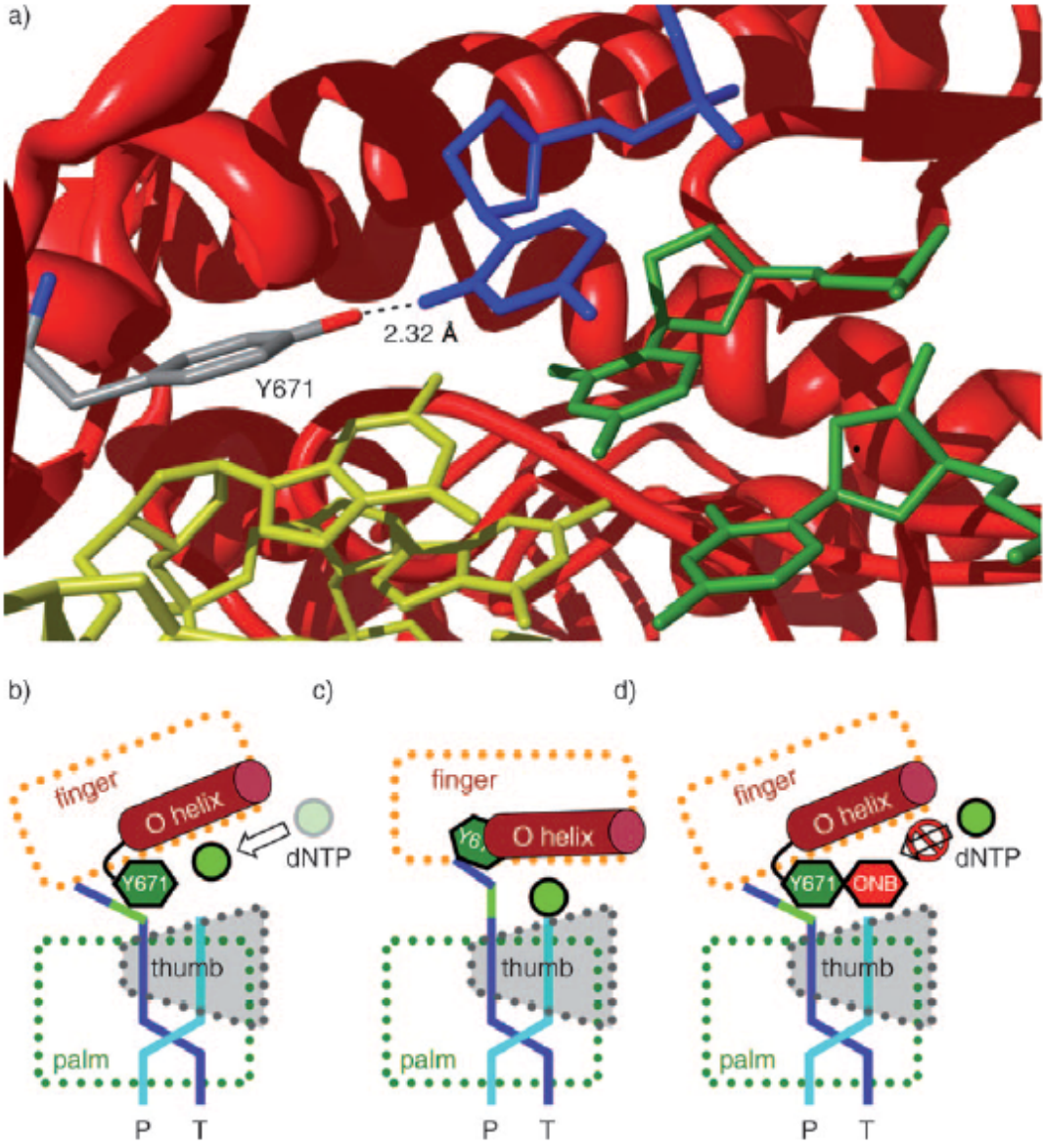

Figure 5 Light activated DNA polymerase. a. Photo-caged tyrosine derivative is site-specifically inserted to *Taq* polymerase Tyr671 residue. **b.** Wild type *Taq* polymerase. **c.** Prior to irradiation, dNTP cannot access active site thereby disruption DNA polymerization **d.** proceeding irradiation the blocking *o*-nitrobenzyl (ONB) is cleaved, restoring wild type function of DNA polymerization. Figure adapted from Chou et al. (2009) (9).

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