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The Incorporation of a Photoisomerizable Amino Acid into Proteins in *E. coli*

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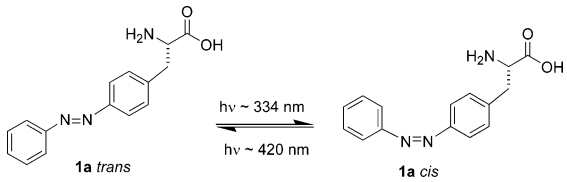
Photochromic and photocleavable groups can be used to spatially and temporally control a variety of biological processes, either by directly regulating the activity of enzymes,[1] receptors,[2] or ion channels[3] or by modulating the intracellular concentrations of various signaling molecules.[4] In general, this requires the chemical modification of either a protein or small molecule with a photoactive ligand, such as azobenzene or a nitrobenzyl group. The ability to genetically incorporate photoresponsive amino acids into proteins at defined sites directly in living organisms would significantly extend the scope of this technique.[5] Here we report the generation of an orthogonal tRNA−aminoacyl tRNA synthetase pair that allows the selective incorporation of the photoisomerizable amino acid phenylalanine-4′-azobenzene (AzoPhe) into proteins in *E. coli* in response to the amber codon, TAG. Furthermore, we show that AzoPhe can be used to photomodulate the binding affinity of an *E. coli* transcription factor to its promoter.

Azobenzene undergoes a reversible *cis*−*trans* photochemical isomerization. Irradiation at 320−340 nm converts the thermodynamically more stable *trans* isomer to the *cis* isomer; the *cis* form reverts thermally or upon irradiation at ≥420 nm.[6] Because the two isomers differ in geometry and dipole, placement of an azobenzene moiety in close proximity to a substrate or ligand binding site in an enzyme, receptor, or ion channel allows one to reversibly modulate the binding affinity and, consequently, the activity of a protein. To biosynthetically incorporate the azobenzene moiety into proteins, the unnatural amino acid AzoPhe **1a** was used. AzoPhe was synthesized by coupling of nitrosobenzene to N-Boc-*p*-aminophenylalanine followed by Boc deprotection. The short distance between the azobenzene moiety and C_α carbon of **1a** minimizes the number of conformational isomers of the side chain, which should result in a greater differential effect of *cis*−*trans* isomerization on the active site structure.

To selectively incorporate AzoPhe at defined sites in proteins in *E. coli*, an orthogonal tRNA−aminoacyl tRNA synthetase pair was evolved that uniquely specifies **1a** in response to the amber TAG codon. Previously, it has been reported that the *M. jannaschii* tyrosyl−tRNA synthetase (MjTyrRS) and a mutant tyrosyl amber suppressor tRNA ($MjtRNA^{Tyr}_{CUA}$) function efficiently in protein translation in *E. coli*, but do not cross react with endogenous tRNAs or synthetases.[7] To alter the specificity of the MjTyrRS synthetase to selectively recognize AzoPhe (and not an endogenous amino acid), a library of 10^9 TyrRS mutants was generated by randomizing six residues (Tyr-32, Leu-65, Phe-108, Gln-109, Asp-158, and Leu-162) in the tyrosine binding site of TyrRS. The X-ray crystal structures[8] of the *M. jannaschii* TyrRS show that these residues are all close to the aryl ring of bound tyrosine (and include Tyr-32 and Asp-158, which form hydrogen bonds with the hydroxyl group of tyrosine). Both positive and negative selections were then applied to the MjTyrRS library (pBK-lib2).[9] In the positive selection, cell survival is dependent on the suppression of an amber codon introduced at a permissive site in the chloramphenicol acyltransferase gene when cells cotransformed with pBK-lib2 and $tRNA^{Tyr}$ are grown in the presence of 1 mM AzoPhe and chloramphenicol. Synthetase clones from surviving cells are then transformed into cells containing the orthogonal tRNA and a gene encoding the toxic barnase protein with amber mutations introduced at three permissive sites. These cells are grown in the absence of AzoPhe to remove any clones that utilize endogenous amino acids.



After five rounds of selection (three positive and two negative), 10 synthetase clones were identified that allow cells to survive up to 120 μg/mL chloramphenicol in the presence of AzoPhe and up to 20 μg/mL chloramphenicol in the absence of the unnatural amino acid. All 10 active synthetase clones converged to one sequence with the same mutations: Tyr32Gly, Leu65Glu, Phe108Ala, Gln109Glu, Asp158Gly, and Leu162His. On the basis of the wild-type synthetase structure, these mutations likely create an enlarged binding pocket to accommodate the azobenzene moiety. For example, the bulky amino acid side chains of Tyr32 and Phe108 of the wild-type synthetase have been replaced by glycine or alanine residues in AzoPheRS. In addition, the side chains of both Tyr32 and Asp158, which are involved in hydrogen bonding to the phenolic hydroxyl of the bound tyrosine, are substituted by glycine.

To determine the fidelity and the efficiency of incorporation of AzoPhe into proteins, an amber codon was substituted for residue 75 in sperm whale myoglobin containing a C-terminal His_6 tag. Protein expression was carried out in the presence of the selected synthetase (AzoPheRS) and $MjtRNA^{Tyr}_{CUA}$ in *E. coli* grown in minimal media supplemented with 1 mM of AzoPhe. As a negative control, protein expression was simultaneously carried out in absence of AzoPhe. Analysis of the purified protein by SDS−PAGE and Western blot showed that protein was expressed only in the presence of **1a** (Figure S2a). ESI (electrospray ionization) mass spectrometric analyses of the mutant myoglobin gave an observed average mass of 18 535 Da, with expected mass of 18 535.3 Da (Figure S3). No other peaks resulting from incorporation of any common amino acid into myoglobin-75TAG were observed in the mass spectrum. In addition, mutant myoglobin having AzoPhe substituted for amino acid 4 was purified and subjected to N-terminal protein sequencing. Edman degradation showed that the fourth position was occupied by a nonstandard amino acid (and none of the common 20 amino acids), whose elution time matched that of the independently synthesized phenylthiohydantoin derivative of AzoPhe.

To further characterize the photochemical properties of proteins containing AzoPhe, this amino acid was introduced into the *E. coli* catabolite activator protein, CAP. CAP is a homodimeric bacterial

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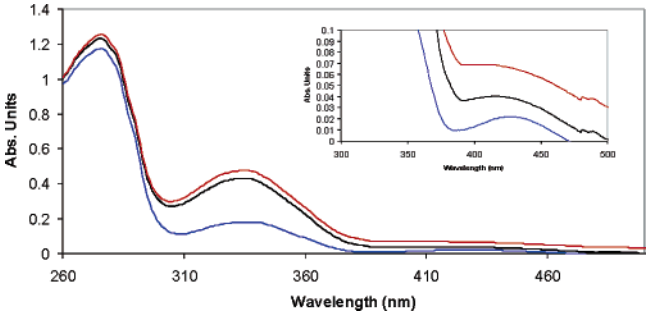

Figure 1. Absorption spectra of the mutant CAP (CAP125TAG; 50 μM) in 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0 buffer: red, after Ni−NTA purification and prior to irradiation; blue, irradiation at 334 nm, 40 min; black, subsequent irradiation with 420 nm light, 40 min.

transcriptional activator that regulates a number of catabolite-sensitive operons in *E. coli*.[10] Binding of cAMP to CAP results in conformational changes in the protein that increase its binding affinity to its promoter, resulting in enhanced transcription from CAP-dependent promoters. An amber codon was introduced for Leu125, a residue at the dimerization interface. A C-terminal His_6 tag was added, and this mutant was expressed in rich media in the presence of 1 mM AzoPhe, AzoPheRS, and $MjtRNA^{Tyr}_{CUA}$. Upon Ni−NTA purification, about 1.5 mg of mutant CAP was obtained per liter of cultured cells, compared to about 3 mg/L for wild-type CAP. A UV−visible spectrum (Figure 1) of this mutant protein shows a distinct absorbance peak at 334 nm corresponding to the *trans*-azobenzene chromophore **1a**. Irradiation of the mutant CAP at 25 °C with 334 nm light led to a decrease in the 334 peak and an increased absorbance at 420 nm, consistent with a photostationary state of approximately 45% *trans*-, 55% *cis*-azobenzene (Figure 1). The isomerized mutant was then irradiated with 420 nm light, resulting in complete conversion back to the 334 nm band. These results show that AzoPhe can be selectively incorporated into proteins with high fidelity and undergo reversible *cis*−*trans* photoisomerization.

To determine whether AzoPhe could be used to photoregulate the DNA binding affinity of CAP, AzoPhe was substituted for Ile71. The crystal structure of the CAP−cAMP−DNA complex[11] shows that Ile71 lies in close proximity to residues Glu72, Arg82, Thr27, and Ser128, which form an intricate network of interactions with cAMP.[12] Thus the *trans* and *cis* isomers might differentially affect the binding affinity of cAMP to CAP and, as a consequence, the affinity of CAP for its promoter. The mutant protein was expressed and purified on Ni−NTA followed by FPLC purification with a mono-S column with a gradient of 25 mM NaCl to 1 M NaCl over 20 min. The binding constant (K_b) of the mutant CAP protein to a purified lac promoter containing the primary CAP binding site was then determined,[12] both before and after irradiation at 334 nm (Figure 2). Using the serial-dilution technique and gel shift assays, a 4-fold lower K_b was observed for the *trans* CAP71AzoPhe mutant ($K_b \sim 4.0 \times 10^6\ M^{-1}$) compared to wt CAP ($K_b \sim 1.6 \times 10^7\ M^{-1}$) in the presence of cAMP (20 μM). Following photoirradiation at 334 nm, the K_b of the mutant CAP decreased 4-fold to $1.0 \times 10^6\ M^{-1}$. This is consistent with a photostationary state of 50% *cis*, in which 75% of the homodimers have at least one subunit in the *cis* form which has a significantly reduced affinity for cAMP (both subunits must have a cAMP bound to form the CAP−promoter complex).[13] This was verified with a cAMP binding assay (Figure S4), which demonstrated at the protein and cAMP concentration used in this study that wt CAP binds roughly twice as much cAMP as the *trans* CAP71AzoPhe, which binds around 40% less cAMP upon *trans*-to-*cis* isomerization. The isomerized CAP71AzoPhe

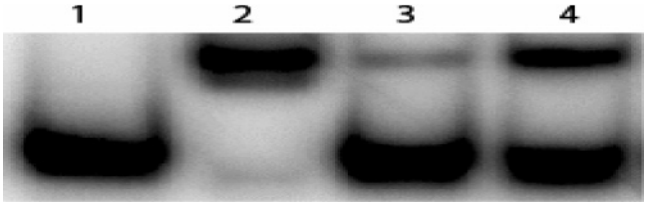

Figure 2. Gel mobility shift assay to determine CAP binding affinity to 33 nM lactose promoter fragment (wild-type or mutant CAP71TAG, 160 nM; buffer, 10 mM Tris, 50 mM NaCl, 500 μM EDTA, 500 μM DTT, 1 mM $MgCl_2$, 4% glycerol, and 20 μM cAMP, pH 7.5). Lane 1, DNA only. Lane 2, DNA + CAP wild-type. Lane 3, DNA + CAP71TAG (irradiated at 334 nm). Lane 4, DNA + CAP71TAG (prior to irradiation at 3334 nm).

sample was then switched back to a predominantly *trans* state by irradiation at ≥420 nm and allowed to incubate at 4 °C. Analysis by gel shift assays of this sample resulted in complete recovery of the *trans* form of CAP with a K_b of $4.0 \times 10^6\ M^{-1}$. These results show that AzoPhe can be used to photoregulate the binding affinity of a transcription factor to its promoter sequence. Although complete conversion of *trans* AzoPhe to the *cis* form cannot be obtained due to their overlapping absorption spectra,[14] these results suggest that the genetic incorporation of AzoPhe into proteins should be useful for temporally regulating a variety of biological processes in vitro and in living cells.

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Supporting Information Available: Materials and methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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