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The Genetic Incorporation of a Distance Probe into Proteins in *Escherichia coli*

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The unnatural amino acid *p*-nitrophenylalanine (pNO_2-Phe) is a useful biochemical probe of protein structure and activity.[1−4] When introduced into proteins either chemically or by in vitro biosynthesis,[3−6] the pNO_2-Phe group can quench the intrinsic fluorescence of nearby tryptophan (Trp) residues. The ability to genetically encode this amino acid in vivo would greatly facilitate the generation of pNO_2-Phe mutants for both biochemical and cellular studies of protein structure and function. Previously, it was shown that unnatural amino acids can be site-specifically introduced into proteins in bacteria and yeast in response to unique triplet (nonsense) and quadruplet (frameshift) codons.[7−12] Here we use this methodology to genetically encode pNO_2-Phe in bacteria with high fidelity and efficiency. In addition, we illustrate the use of the pNO_2-Phe/Trp pair as a distance probe in a coiled-coil protein.

pNO_2-Phe was incorporated into proteins in *Escherichia coli* by means of a unique amber suppressor tRNA ($mutRNA^{Tyr}_{CUA}$)/aminoacyl tRNA synthetase (MjTyrRS) pair derived from a *Methanococcus jannaschii* $tRNA^{Tyr}$/TyrRS pair. The specificity of MjTyrRS was altered so that the synthetase specifically charges $mutRNA^{Tyr}_{CUA}$ with pNO_2-Phe and no endogenous amino acid. A MjTyrRS library was constructed based on the crystal structure of a mutant MjTyrRS that selectively charges $mutRNA^{Tyr}_{CUA}$ with *p*-bromophenylalanine.[13] The Ser^{107}, Pro^{158}, Leu^{159}, and Glu^{162} active site mutations were preserved in this library, and random mutations were introduced at Leu^{32}, Leu^{65}, His^{70}, Gln^{109}, His^{160}, and Tyr^{161}. To identify a synthetase specific for pNO_2-Phe, alternating rounds of positive selection (based on suppression of an amber stop codon in the chloramphenicol acetyltransferase (CAT) gene in the presence of 1 mM unnatural amino acid) and negative selection (based on suppression of amber nonsense mutations in the toxic barnase gene)[14] were carried out. After several rounds of positive and negative selection, a clone was evolved whose survival at high concentration of chloroamphenicol (120 μg/mL) was dependent on the presence of pNO_2-Phe. These results suggest that the evolved synthetase has higher specificity for pNO_2-Phe than for endogenous amino acids. Sequencing revealed the following mutations in this evolved synthetase compared to the wild-type MjTyrRS: Tyr^{32}→Leu, Glu^{107}→Ser, Asp^{158}→Pro, Ile^{159}→Leu, His^{160}→Asn, and Leu^{162}→Glu.

To test the ability of the evolved synthetase ($mutNO_2$-PheRS) and $mutRNA^{Tyr}_{CUA}$ to selectively incorporate pNO_2-Phe into proteins, an amber stop codon was substituted at a permissive site (Lys^7) in the gene for the Z domain protein with a C-terminal hexameric His tag.[15] Cells transformed with $mutNO_2$-PheRS, $mutRNA^{Tyr}_{CUA}$ and the mutant Z domain gene were grown in the presence of 1 mM pNO_2-Phe in minimal medium containing 1% glycerol and 0.3 mM leucine (GMML medium). The mutant protein was purified by Ni^{2+} affinity column and subsequently analyzed by SDS-PAGE and MALDI-TOF (Figure 1). The yield of mutant Z-domain protein is 2 mg/L in the presence of pNO_2-Phe, but is insignificant in the absence of pNO_2-Phe (Figure 1a), indicating a very high fidelity for the incorporation of the unnatural amino acid. Moreover, the MALDI-TOF spectrum shows two peaks at m/z = 7958 and 7828 (Figure 1b), which match the expected molecular weight for the pNO_2-Phe Z-domain mutant (m/z = 7958) and the molecular weight for this mutant protein with the loss of its first methionine (m/z = 7826).[16]

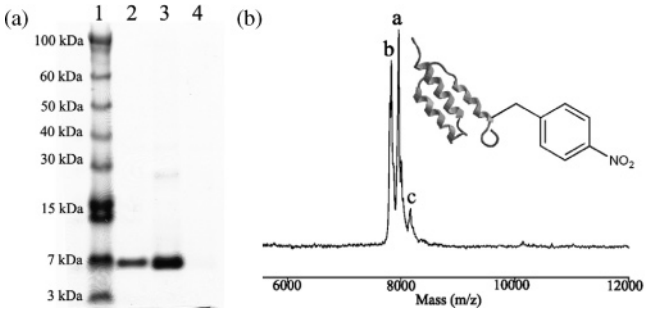
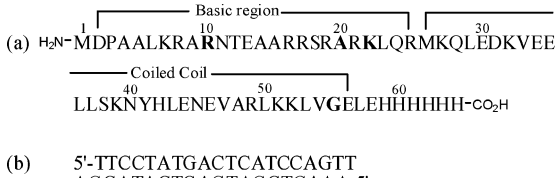


Figure 1. (a) SDS-PAGE analysis of Lys^7→TAG Z-domain protein expressed under different conditions. Lane 1: molecular mass marker; lane 2: expression with WT MjTyrRS; lane 3: expression with $mutNO_2$-PheRS in the presence of pNO_2-Phe; lane 4: expression with $mutNO_2$-PheRS in the absence of pNO_2-Phe. The SDS-PAGE gel was stained with GelCode Blue stain reagent. (b) MALDI-TOF analysis of pNO_2-Phe incorporated Z-domain protein: peak a can be assigned to the full length mutant Z domain, peak b is assigned to the Z domain protein without the first Met, peak c is the matrix adduct.

Next, we examined the utility of pNO_2-Phe as a distance probe through its ability to quench the intrinsic fluorescence of tryptophan residues in proteins. This Trp/pNO_2-Phe fluorophore−quencher pair was incorporated into a model GCN4 leucine zipper protein, which forms a parallel coiled-coil homodimer.[17,18] The DNA binding region of the GCN4 gene (676−840 bp, bZIP), which does not encode any tryptophans, was cloned from the yeast genome into the protein expression vector pET-26b with an additional N-terminal Met and a C-terminus $Leu^{57}Glu^{58}$ (encoding a Xho I restriction site) followed by a 6XHis tag (Scheme 1a). Site-directed mutagenesis was then used to substitute amino acids at selected sites with either Trp or pNO_2-Phe (TAG codon). The bZIP expression vector as well as a plasmid containing both $mutNO_2$-PheRS and $mutRNA^{Tyr}_{CUA}$ were cotransformed into *E. coli* BL21(DE3) cells,

Scheme 1



(a) Sequence of the model GCN4 bZIP protein; mutation sites are indicated in bold. (b) 20-mer bZIP duplex DNA binding site.

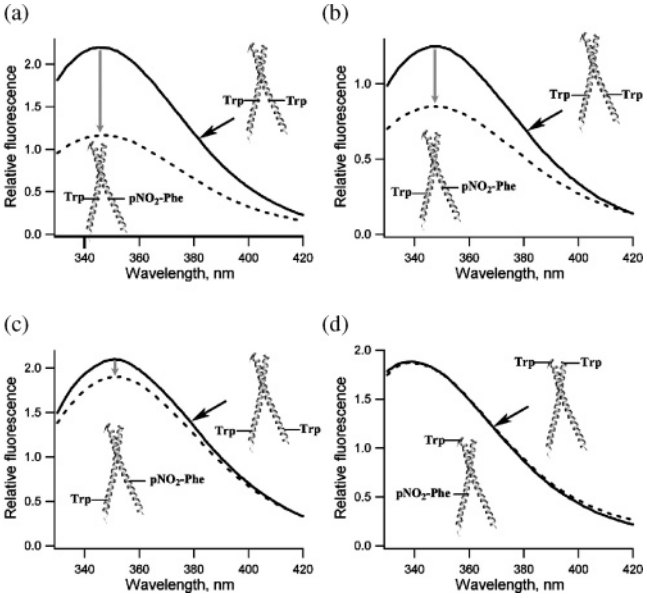


Figure 2. Steady-state fluorescence spectra of tryptophan containing bZIP proteins with the Trp mutation introduced at residue (a) Trp^{22}, (b) Trp^{20}, (c) Trp^{10} and (d) Trp^{55}. The spectra were recorded with 20 μM of bZIP duplex DNA binding site in the absence (solid lines) and the presence (broken lines) of 10 μM pNO_2-Phe^{22} bZIP mutant.

which were then grown in the presence of 1 mM pNO_2-Phe in GMML minimal media. The 64 residue mutant bZIP proteins were purified by Ni^{2+} affinity column and characterized by SDS-PAGE and MALDI-TOF analyses (see Supporting Information).

The coiled-coil region of bZIP consists of residues 26−56 with the leucine heptad repeat at positions 29, 36, 43, and 50. A Trp was substituted for Lys^{22} at a surface accessible site in the noninteracting region of the coiled-coil; a Lys^{22} to pNO_2-Phe mutant was also generated at this position. The fluorescence spectrum of the Trp^{22} bZIP mutant (10 μM) was measured at 22 °C with 295 nm excitation in 50 mM phosphate-buffered 300 mM saline solution (pH 8.0) with 20 μM of the bZIP DNA recognition site (Scheme 1b)[19] in both the absence and presence of stoichiometric amounts of the pNO_2-Phe^{22} mutant (Figure 2a). The observed fluorescence intensity of the Trp^{22} mutant was reduced by 47% in the presence of stoichiometric pNO_2-Phe^{22} mutant protein. Because the substitutions are made in a noninteracting region of the protein, there should be a statistical mixture of a 2:1:1 ratio of Trp^{22}/pNO_2-Phe^{22}, pNO_2-Phe^{22}/pNO_2-Phe^{22}, and Trp^{22}/Trp^{22} bZIP dimers. On the basis of the reported K_d of 0.5 μM for the coiled-coil homodimer,[20] the maximum fluorescence quenching efficiency can be estimated to be ~45%, which is very close to that observed for the Trp^{22}/pNO_2-Phe^{22} pair. This result indicates that the pNO_2-Phe group is indeed an efficient quencher of tryptophan fluorescence. Similar results were obtained when quenching experiments were carried out under the same conditions in the absence of the duplex DNA.

To further investigate the distance-dependence of the fluorescence-quenching interaction, Trp^{10}, Trp^{20}, and Trp^{55} mutant proteins were also generated, and their fluorescence spectra were again measured in the absence and presence of stoichiometric amounts of the pNO_2-Phe^{22} mutant (Figure 2, curves b−d). The distance separations between Trp and pNO_2-Phe in the Trp/pNO_2Phe dimeric proteins were estimated to be 6, 15, 26, and 49 Å for the Trp^{22}, Trp^{20}, Trp^{10}, and Trp^{55} mutants, respectively, based on the crystal structure of the wild-type bZIP dimer.[19] Due to the larger spatial separation between Trp and pNO_2-Phe, the presence of pNO_2-Phe^{22} bZIP results in a smaller quenching efficiency (32%) for the Trp^{20} mutant compared to that for the Trp^{22} mutant. The pNO_2-Phe^{22} bZIP mutant has minimal effect on the fluorescence quantum yield of either the Trp^{10} mutant or the Trp^{55} mutant. This result clearly shows that the strength of the fluorophor−quencher interaction between Trp and pNO_2-Phe is related to the distance between these two moieties.

In conclusion, we have evolved a mutant MjTyrRS synthetase to genetically encode *p*-nitrophenylalanine (pNO_2-Phe) in *E. coli* with high fidelity and efficiency. The applicability of pNO_2-Phe as a biophysical probe was demonstrated using a model GCN4 bZIP leucine zipper protein, in which pNO_2-Phe quenched the fluorescence of Trp in a distance dependent fashion. Thus, the site-specific incorporation of pNO_2-Phe into proteins should be a useful tool to study protein folding and conformational changes as well as protein−protein interactions. In addition, we are currently exploring the utility of the pNO_2-Phe moiety for the production of immunogenic proteins for vaccine production.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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