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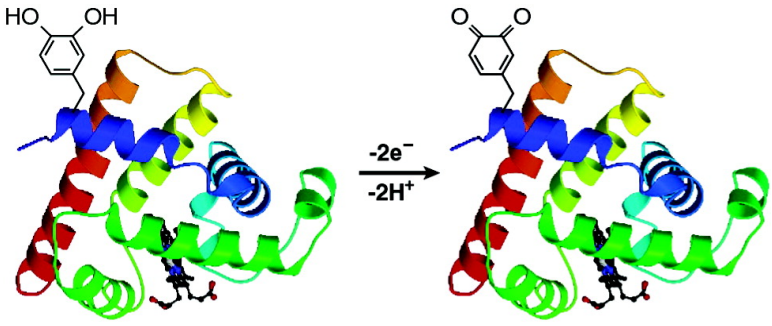

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Site-Specific Incorporation of a Redox-Active Amino Acid into Proteins

Lital Alfonta,† Zhiwen Zhang,† Sean Uryu,† Joseph A. Loo,‡ and Peter G. Schultz*,†

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, and Department of Biochemistry, University of California Los Angeles, 405 Hilgard Avenue, Los Angeles, California 90095

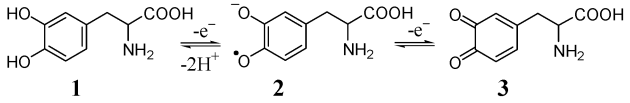
Received August 31, 2003; E-mail: schultz@scripps.edu

Among the 20 common genetically encoded amino acids, only cysteine undergoes facile redox chemistry.[1] Consequently, most biological redox processes require cofactors such as flavins, nicotinamides, and metal ions. In rare cases, quinones, derived from the post-translational modification of tyrosine and tryptophan side chains, are used as the redox cofactor.[2] For example, bovine plasma copper amine oxidase uses 3,4,6-trihydroxy-L-phenylalanine (TOPA) in the conversion of primary amines and molecular oxygen to aldehydes and hydrogen peroxide, respectively.[3] Clearly, the ability to genetically encode additional redox-active amino acids, rather than generate them by complex post-translational mechanisms,[4] would significantly enhance our ability to both study and engineer electron-transfer processes in proteins. To this end, we report that the redox-active amino acid 3,4-dihydroxy-L-phenylalanine (DHP), which can undergo one- or two-electron oxidation to the semiquinone radical or quinone state, (Scheme 1), respectively, can be genetically incorporated selectively and efficiently into proteins in *Escherichia coli* in response to a TAG codon.

Recently it has been reported that a number of unnatural amino acids can be incorporated selectively into proteins in *E. coli* and yeast[5,6] using orthogonal tRNA-aminoacyl tRNA synthetase pairs. These orthogonal pairs do not cross-react with endogenous components of the translational machinery of the host cell, but recognize the desired unnatural amino acid and incorporate it into proteins in response to the amber nonsense codon, TAG.[7] To genetically encode DHP in *E. coli*, the specificity of an orthogonal *Methanococcus jannaschii* tRNA-synthetase (MjTyrRS) was altered so that the synthetase aminoacylates the mutant tyrosine amber suppressor tRNA (mut $tRNA^{Tyr}_{CUA}$) with **1** and not with any of the common 20 amino acids. These mutant synthetases were selected from two mutant MjTyrRS libraries.[5a,8] In the first library, which is based on an analysis of the crystal structure of the homologous TyrRS from *Bacillus stearothermophilus*,[9] five residues (Tyr 32, Glu 107, Asp 158, Ile 159, Leu 162) in the active site of MjTyrRS that are within 6.5 Å of the para position of the aryl ring of tyrosine were randomly mutated (encoded on plasmid pBK-lib). In the second library, six residues (Tyr32, Ala 67, His 70, Gln 155, Asp 158, Ala 167) within 6.9 Å of the meta position of the tyrosine aryl ring were randomly mutated (encoded on plasmid pBK-lib-m).

To alter the specificity of the TyrRS so it specifically incorporates **1** and none of the other natural amino acids, several rounds of positive and negative selection were applied. In the positive selection, both libraries of mutant TyrRS were subjected to a selection scheme based on the suppression of an amber codon introduced at a permissive position (Asp112) in the chloroamphenicol acetyl transferase (CAT) gene (pRep(2)/YC). Cells transformed with the mutant TyrRS libraries, the $mutRNA^{Tyr}_{CUA}$ gene, and the amber mutant CAT gene were grown in minimal media containing 1 mM **1** and 70 μg/mL of chloramphenicol under reducing conditions to avoid the oxidation of compound **1**. Surviving cells contain mutant TyrRS's that aminoacylate the mut $tRNA^{Tyr}_{CUA}$ with either **1** or endogenous amino acids. Next, a negative selection was applied to remove the mutant TyrRS's that charge natural amino acids. The negative selection is based on suppression of three amber codons introduced at permissive positions (Gln2, Asp44, Gly55) in the toxic barnase gene (pLWJ17B3). Cells harboring the mutant TyrRS's from the previous positive selection, the $mutRNA_{CUA}$, and the amber mutant barnase gene were grown in Luria−Bertani media in the absence of **1**. Under these conditions, cells encoding mutant TyrRS's with specificity for endogenous amino acids will produce full-length barnase and die. Only those cells containing mutant TyrRS's with specificity for **1** can survive. After three rounds of positive selection alternating with two rounds of negative selection, a clone was evolved whose survival at high concentrations of chloroamphenicol (90 μg/mL) was dependent on the presence of **1**, the selected mutant TyrRS gene (DHPRS), mut $tRNA^{Tyr}_{CUA}$, and the Asp112TAG CAT gene. However, in the absence of **1**, the same cells survived only in 20 μg/mL chloroamphenicol. This result suggests that the mutant DHPRS (mutDHPRS) enzyme has higher specificity for **1** than for natural amino acids. Sequencing revealed the following mutations in the mutDHPRS: Tyr32 → Leu, Ala67 → Ser, His70 → Asn, and Ala167 → Gln.

Scheme 1. Oxidation Products of DHP **1** to DHP-Semiquinone Radical **2**, Which Is Readily Oxidized to DHP-Quinone **3**



To measure the fidelity and efficiency of DHP incorporation, we incorporated DHP in response to an amber codon at the surface-exposed fourth residue in C-terminally hexahistidine tagged mutant sperm whale myoglobin (Mb).[10] Full-length myoglobin containing DHP (DHPMb) was expressed using glycerol minimal media with leucine as the growth medium and under reducing conditions (100 μM dithiothreitol, DTT) to prevent oxidation of DHP (in the absence of DTT most cells died due to the toxicity of the oxidized quinone **3**). In the presence of mutDHPRS, mut $tRNA^{Tyr}_{CUA}$, and DHP, the yield of mutant protein was approximately 1 mg/L (the yield of wild-type Mb, wtMb, under the same conditions is 5.4 mg/L). No full-length Mb was expressed in the absence of either DHP or mut $tRNA^{Tyr}_{CUA}$ as determined by silver staining and Western analysis with an anti-His_6-tag antibody. A full-length DHPMb was purified using cobalt-based immobilized metal affinity chromatography resin. Electrospray ionization (ESI) with a quadrupole−quadrupole time-of-flight (QqTOF) mass spectrometer afforded a mass of 18 448.5 Da for DHPMb (Figure 1). This is within 70 ppm of the calculated mass of 18 447.2 Da for the DHP containing Mb.[11] Taken

† The Scripps Research Institute.
‡ University of California Los Angeles.

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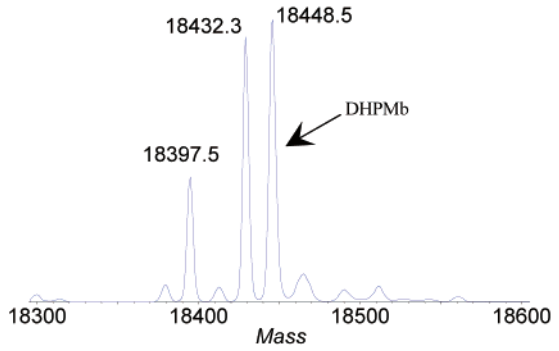

Figure 1. ESI-QqTOF mass spectrum of DHPMb.

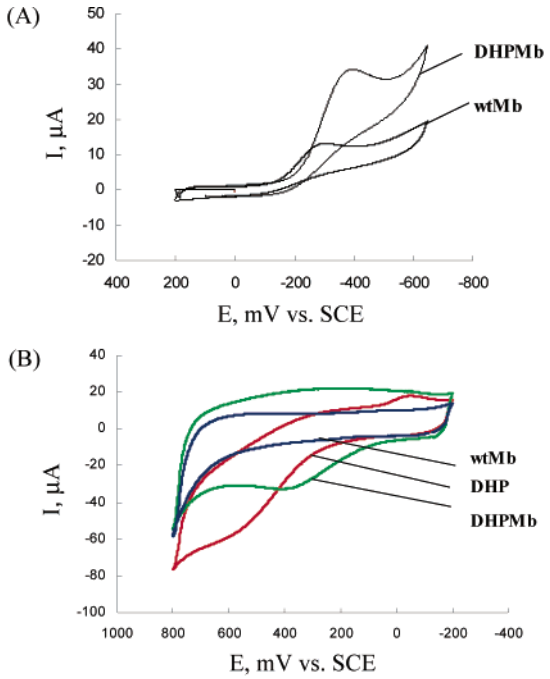

Figure 2. (A) Cyclic voltammograms of the heme group in wtMb and DHPMb. (B) Cyclic voltammograms of DHP for different solutions containing 10 nM (i) DHP, (ii) wtMb, or (iii) DHPMb. All voltammograms were recorded in 0.1 M phosphate buffer, pH 7.4, under argon; scan rate: 1 V s^{-1} vs SCE.

together, these results show that DHP is efficiently and selectively incorporated into myoglobin in response to the TAG codon.

Cyclic voltammetry was used to determine whether the redox wave of the oxidized hydroquinone could be observed when a bare gold electrode was immersed in a solution containing the DHPMb. Figure 2A shows an irreversible voltammetric response of a solution containing the wtMB and that of the DHPMb under anaerobic conditions.[12] The reductive peak potential originating from the wtMbFe(III) is observed at $E = -320$ mV, whereas the reductive peak potential of the mutant protein is shifted to a more negative potential, $E = -400$ mV. This shift is attributed to the presence of DHP, which results in a lower reduction potential of Fe(III) than in the absence of DHP. The irreversible voltammograms reflect a slow electron transfer rate which likely results from the limited accessibility of the electron to the electrode. Figure 2B shows the voltammetric responses of different solutions containing 100 μM of (i) DHP, (ii) wtMb, or (iii) DHPMb. The current originating from DHP oxidation appears only in the presence of the mutated Mb or in a solution of free DHP with $E = 580$ mV and $E = 385$ mV, respectively. These results show clearly that there is a significant influence of the presence of DHP in Mb on the redox potential of the Fe(III) heme group and vice versa. The nature of this effect will be investigated in our future work.

In conclusion, we have shown that the redox-active amino acid DHP can be efficiently and selectively incorporated into proteins in *E. coli*. We have demonstrated that we are able to electrochemically oxidize this amino acid within the protein. The ability to incorporate redox-active amino acid site specifically into proteins should facilitate the study of electron transfer in proteins, as well as enable the engineering of redox proteins with novel properties. Currently, we are attempting to utilize the site-specific incorporation of **1** into various sites in Mb to study electron-transfer pathways in this protein.[13]

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