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Selection of an Eevolved Orthogonal Ne-Acetyllysine tRNAsynthetase that can Incorporate the Unnantural Amino Acid L-homocitrulline Site-specifically into Proteins

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Selection of an evolved orthogonal N^{e}-Acetyllysine tRNAsynthetase that can incorporate the unnantural amino acid L-homocitrulline site-specifically into proteins

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Abstract

Oxidatively damaged apolipoprotein A1 (apoA1, the main protein constituent of high density lipoprotein (HDL) - the "good cholesterol") has been isolated from circulating plasma and atherosclerosis plaque with modification of lysine amino acid residues to homocittruline (carbamyllysine) in apoA1 identified as a modification found increased in smokers that correlates with elevated cardiovascular risk for myocardial infarction (MI), stoke or death (1). This dysfunctional HDL molecule has been shown to be proinflammatory in nature and can activate the expression of cellular adhesion molecules in endothelial cells via NF-κB transcription factor activation (2). Here we designed ApoA1 to include *specifically* carbamylated lysine at predefined sites to further investigate the role of oxidative damage in atherosclerosis, leading to dysfunctional HDL. Current chemical and enzymatic oxidation of proteins (e.g. apoA1) results in *incomplete* and *rarely* site-specific oxidation of amino acids. Thus, *genetic encoding* of oxidized amino acids through orthogonal tRNA/aminoacyl-tRNA synthetase (RS) pairs offers the most reliable method for producing *site specific* oxidized proteins (3). We used an orthogonal tRNA/RS pair from *Methanosarcina barkeri that has been evolved previously to incorporate the unnatural amino acid N^{ε}-acetyllysine instead of pyrolysine* (4*)* as a starting point to create a library of synthetase mutants that could utilize homocittruline (carbamyllysine) to specifically incorporate this uAA into proteins at defined sites. Using X-ray crystallographic information provided for the pyrolyisne tRNA synthetase (4) we replaced the pyrolysine with acetyllysine and observed which mutations were successful. Using this information and then replacing acetyllysine with carbamyllysine into the

catalytic pocket we identified likely key residues that were within 6.5 angstroms of carbamyl lysine as it would sit in the catalytic pocket that might enhance hydrogen bonding or salt bridge electrostatic interactions between carbamyllysine and the catalytic pocket backbone amino acids. From the *N^{ε}-acetyllysine* we identified A267(All), Leu270(All), Tyr 271(All), L274(All), F312(All) and C313(All). We used inverse PCR mutagenesis (4) to mutate each of these residues using two inverse PCR reactions and two mutagenic primers. A total of 6.4 $x10^7$ possible mutants could be made. After relegation of dilute isolated Bsa I restricted PCR plasmid DNA a library with a total of 1.5 x 10^8 was obtained. The library is in the process of being positively selected for incorporation of carbamyllysine into the kanamycin kinase gene at position 15 to suppress the encoded amber suppressor codon. Positive selected clones that grow on kanamycin sulfate (150μg/ml) in the presence of 1mM carbamyllysine will be negatively selected against incorporation of any non-carbamyllysine amino acid in the absence of carbamyllysine using suppression of uracil phophoribosyl transferase at two amber codon sites (5 and 125) to incorporate 5-fluorouracil into cellular DNA, resulting in cell death. Clones which grow after being negatively selected for will then be positively selected a final time on kanaymcin sulfate plates in the presence of 1mM carbamyllysine for a second round of positive selection. Individual clones from this round of selection will be isolated and tested the clone encoding the synthase which best incorporates carbamyllysine at the lowest uAA concentration. The selected carbamyllysine tRNA synthetase will then be used to express a series of human apoA1 alleles containing amber codons in place of various lysine residues and assayed for its ability to form nascent HDL particles and to activate NF-κB and vascular adhesion molecule expression on endothelial cells.