Asp Mutants (write-up for Liviu and Hagan)

Construction of pT_7αHL-RL3

pT_7-αHL-RL_3 encodes the wild type αHL polypeptide but contains six silent restriction sites that encompass DNA encoding the transmembrane β-barrel. These sites are 5' to 3': SacII, HpaI, BsiWI, StuI, AflII and XhoI. pT_7-αHL-RL_3 was derived from pT_7-αHL-RL_2[1] in two separate ligation steps. For the first step, pT7-αHL-RL_2 was digested with BsiWI and AflII and the purified vector was then ligated with two double-stranded oligonucleotide cassettes. The first cassette was formed from SC197: 5' GTACGGATTCAATGGTAATGTTACTGGTGATGATACAGGAAAA and phosphorylated SC198: 5' AATTTTTCCTGTATCATCACCAGTAACATTACCATTGAATCC, while the second cassette was formed from phosphorylated SC199: 5' ATTGGAGGCCTTATTGGTGCAAATGTTTCGATTCGTCATACAC and SC200: 5' TTAAGTGTATGACGAATCGAAACATTTGCACCAATAAGGCCTCC. The resulting three-way ligation yielded pT_7-αHL-RL_3-K8A. In the second ligation step, the engineered Ala codon present in the αHL-RL2 gene at position 8 was replaced with the wild type Lys codon. To restore the wild type residue, pT_7-αHL-RL_3-K8A was digested with AgeI and MfeI and the resulting fragment was ligated with the wild type gene in the T7 vector (pT7-αHL-WT) that had been cut with the same enzymes to yield pT_7-αHL-RL_3. All genes were verified by DNA sequencing.

Construction of β-Barrel Mutants: αHL-K131D, αHL-K147D and αHL-K131D/K147D

Mutant genes, αHL-K131D and αHL-K147D, were constructed by PCR-based recombination as previously described [2]. To construct αHL-K131D, pT7-αHL-RL_3 (see above) was used as the template for PCR with mutagenic primers: SC807: 5' GGTGATGATACAGGAgacATTGGAGGCCTTATTGG (forward) and 5' SC808: 5' CCAATAAGGCCTCCAATgtcTCCTGTATCATCACC (reverse). The mutant codon and anticodon are in small type. For αHL-K147D, the mutagenic primers were SC809: 5' GGTCATACACTTgacTATGTTCAACCTG (forward) and SC810: 5' CAGGTTGAACATAgtcAAGTGTATGACC (reverse). The non-mutagenic primers SC46: 5' ATAAAGTTGCAGGACCACTTCTG (forward) and SC47: 5' CAGAAGTGGTCCTGCAACTTTAT (reverse) were used for both sets of PCR reactions. To construct the double aspartate mutant, αHL-K131D/K147D, pT7-αHL-K147D was digested with StuI and HindIII and the resulting small fragment was ligated to the large fragment purified from pT7-αHL-K131D after digestion with the same enzymes. Successful replacement was screened by cutting plasmid isolates with AflII, since this site is removed by the K147D mutation. All mutant genes were verified by DNA sequencing.

Mutant αHL polypeptides

[^{35}S]methionine-labeled, mutant polypeptides were synthesized and assembled *in vitro* by coupled transcription and translation (IVTT) in the presence of rabbit erythrocyte membranes as previously described.[1] The labeled, membrane-bound homoheptamers were washed in MBSA buffer (10 mM MOPS; titrated with NaOH, 150 mM NaCl, containing 0.1% (wt/v) BSA, pH 6.8), solubilized in sample buffer[2] and then purified on an 8% SDS-polyacrylamide gel. The gel was then dried between two sheets of plastic film (Cat # V713B; Promega Corporation) at 50ºC for 2 h using a Bio-Rad drying system ("GelAir" Cat. No. 165-1771, Bio-Rad Laboratories). After autoradiography of the dried gel, bands corresponding to each mutant oligomer were excised with scissors and rehydrated in 0.5 ml of ultapure, distilled and deionized water. The adherent, plastic gel-backings were released from the slices after rehydration and removed with flamed metal forceps prior to maceration with a disposable plastic pestle (Cat. No. 749521-1590; Nalge Nunc International). Gel fragments were removed with a spin filter ("QIAshredder", Cat. No. 79654; Qiagen) and the resulting filtrate was stored frozen in 50 µl aliquots at –80ºC.

References

1 Cheley, S., Braha, O., Lu, X., Conlan, S. and Bayley, H. Protein Science 8, 1257-1267 (1999).

2 Howorka and Bayley Biotechniques 25, 766-772 (1998).

3 Laemmli, U.K. Nature 227, 680-685 (1970).