Cell-free expression with the toxic amino acid canavanine

Emanuel G. Worst[a#], Matthias P. Exner[b#] ,Alessandro De Simone[b], Marc Schenkelberger[a], Vincent Noireaux[c], Nediljko Budisa[b] and Albrecht Ott[a]

[a]*Universität des Saarlandes, Department of Experimental Physics, Campus Saarbruecken, 66123 Saarbruecken, Germany*
[b]*TechnischeUniversität Berlin, Institute of Chemistry, Mueller-Breslau-Strasse 10, 10623 Berlin, Germany*
[c]*University of Minnesota, School of Physics and Astronomy, 115 union street se, Minneapolis Mn 55455, USA*
[#]*Authors contributed equally*

Experimental details

In vivo incorporation of canavanine

In vivo incorporation was performed following the well-established SPI method[1]. Briefly, the arginine auxotrophic strain AH5 JE7094 was transformed with plasmid pQE80L-EGFP and cultivated in 4 L of NMM (7.5 mM NH_4SO_4, 8.5 mM NaCl, 22 mM KH_2PO_4, 50 mM K_2HPO_4, 1 mM $MgSO_4$, 20 mM D-Glucose, 50 mg mL^{-1} of every amino acid but arginine, 1 µg mL^{-1} $CaCl_2$, 1 µg mL^{-1} $FeCl_2$, 0.01 µg mL^{-1} of each $CuCl_2$, $ZnCl_2$, $MnCl_2$, $MoOHCl_2$, 10 µg mL^{-1} biotin, 10 µg mL^{-1} thiamine) with limiting concentration of arginine (0.12 mM). After 12-13 hours, the culture reached a stable OD of app. 0.7, indicating depletion of arginine. Canavanine was added at a final concentration of 0.5 mM and the expression of EGFP was induced by adding 1 mM IPTG for 4 hours. Expressed EGFP(Can) was purified via immobilized nickel affinity chromatography and subsequent size-exclusion chromatography on an ÄKTA purifier (GE Healthcare, Munich, D) to >95% purity and concentrated.

In vitro incorporation of canavanine

For cell-free expression, the plasmid pBest-p15a-OR2-OR1-Pr-UTR1-eGFP-Del6-229-T500 (based on pBest-Luc, Promega, USA) for expression of truncated EGFP (amino acids 6 – 229) was constructed and amplified in *E. coli* strain KL740 (Yale CGSC#:4382). Plasmid features are the p15A origin of replication, the strong lambda phage promoter Pr flanked by the operons OR1 and OR2[2], the untranslated region UTR1 containing the T7 g10 leader sequence for highly efficient translation initiation[3] [GenBank: M35614.1] and the T500 transcriptional terminator[4]. A polyhistidine-tag was inserted in front of the stop codon by Gibson assembly (New England Biolabs, Ipswich, USA).

The cell-free system used in this work was adapted from Shin *et al.*[2], a modification of the protocol by Kigawa *et al.*[5]. A typical cell-free reaction consists of 33% (v/v) crude extract (9-9.5 mg/ml of proteins) and 66% (v/v) reaction buffer including the PGA energy regenerating system, plasmid DNA and amino acids without added arginine. The concentration for magnesium glutamate (6 mM), potassium glutamate (10 mM) and plasmid DNA (10 nM) was optimized for high-level protein synthesis. For a cell free expression reaction, 270 µl of reaction mix were prepared. 1 mM of canavanine was added. The reaction mix was aliquotet to 6 µl in 1.5-mL reaction tubes to allow sufficient oxygen diffusion and incubated at 29 °C for at least 12 hours. Control reactions with added arginine or without either canavanine or arginine were prepared the same way.

After incubation, split reaction solutions were pooled and proteins were purified using a His-Spin Protein Miniprep Kit (Zymo Research, Germany) to >95% purity in a single step and concentrated.

Protein analysis

All proteins were dissolved and stored in storage buffer (50 mM Tris-Cl pH8, 100 mM NaCl, 10% glycerol) at -80 to -20 °C.

Purified proteins were analyzed with SDS-PAGE and HPLC-ESI mass spectrometry using a C5 column (Supelco analytical, Sigma-Aldrich, St. Lois, USA) on an Agilent 1260 for separation and a coupled Agilent QTOF 6530 (Agilent, Santa Clara, Ca, USA) or Exactive Orbitrap (Thermo Scientific, Waltham, MA, USA for mass analysis. Measured mass spectra were deconvoluted using Agilent software supplied with the instrument or the program MagTran, using the maximum entropy algorithm.

Fluorescence spectra of samples were measured on a LS 55 (PerkinElmer Life Sciences, Boston, USA) with an excitation/emission slit of 5 nm at 20 °C. Absoption spectra of samples were measured on a Lambda 35 (PerkinElmer Life Sciences, Boston, USA) UV/VIS spectrophotometer at 20 °C. Five spectra were accumulated for each sample.

Supplementary figures

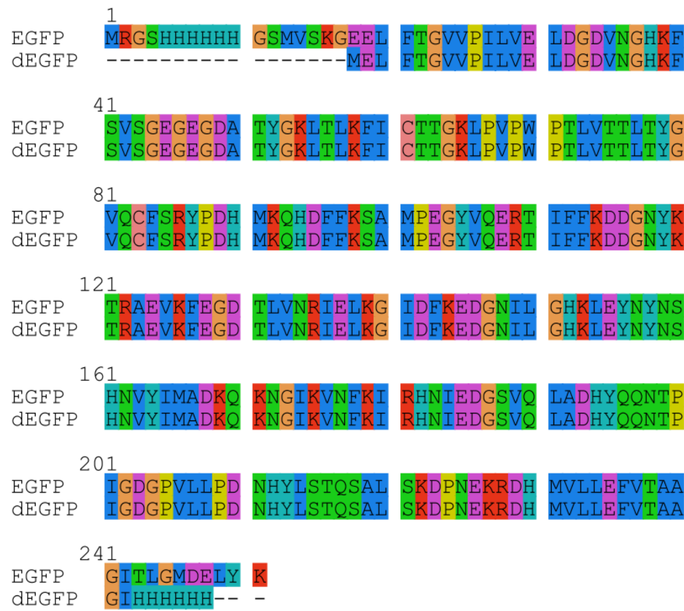

Figure S1: Sequence alignment of EGFP and dEGFP. Compared to dEGFP, EGFP has an additional arginine position upstream of the hexahistidine tag (Arg2).

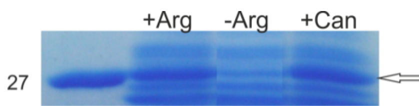

Figure S2: ***In vitro*** **expression of dEGFP with added arginine, without arginine and with canavanine instead of arginine. The gel was loaded with unpurified cell-free expression mixtures.** The position of dEGFP is indicated by the arrow. No protein is produced in absence of both arginine and canavanine. The residual band at the can-position is due to other proteins of similar molecular weight from the extract. The additional bands in each lane also correspond to proteins from the extract.

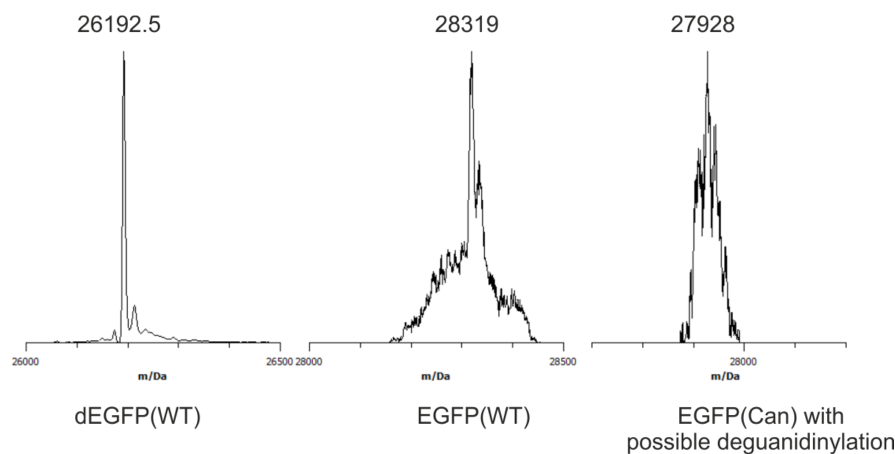

Figure S3: ESI-MS analysis of dEGFP(WT) (expected mass 26193 Da), EGFP(WT) (expected mass 28319 Da) and EGFP(Can) with putative deguanidinylated canavanines (i. e. homoserine) incorporated at arginine positions. The expected mass for EGFP with all seven arginines replaced by homoserine (27933 Da) was not found, however, the measured mass differs only by 5 Da which can be attributed to the high noise due to low protein amounts and impurities**.**

Supplementary references

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