SUPPLEMENTARY MATERIALS AND METHODS

Characterization of the commercial isoleucine samples and utilization in various assays

Kinetic analyses in combination with NMR spectroscopy (Figures S1 and S2) revealed that leucine content in the different lots of Sigma Bioultra isoleucine may vary from 0.0019% to 0.38%. Several other commercial isoleucine samples (Serva, Roth) were also tested, and the observed levels of leucine contamination were between 0.01-0.02% (Fig S2). As isoleucine lots significantly vary in the leucine content, a purification procedure was established to produce an isoleucine with the tolerable level of leucine (see below). The purified samples were tested with the cognate IleRS and $tRNA^{Ile}$, and no inhibition of isoleucylation was observed (Fig S6). The isoleucine sample with 0.0019% of leucine was suitable for most assays where tRNA was used in the concentration highly above the leucine contamination. A requirement for highly purified Ile (0.00035% of Leu) was demonstrated in assays performed in the absence of tRNA, such as amino acid activation (ATP-PP_i exchange) since use of less pure isoleucine led to severe kinetic artefacts (Table S1).

Interestingly, we did not observe significant cognate amino acid contamination in the commercial samples of several other amino acids: norvaline, valine and threonine were subjected to kinetic analysis with LeuRS, IleRS and ValRS and no significant contaminations with leucine, isoleucine and valine were detected, respectively.

Purification of isoleucine

Purification of isoleucine from traces of leucine was achieved by transforming the amino acids into their sesquisulphates analogously to the description given in the patent application by Bertholet and Hirsbrunner. The sesquisulphate of leucine is more soluble then the sesquisulphate of isoleucine in a solution of sulphuric acid and a low-polarity solvent like ethyl-methyl ketone. This provides the basis for purification of Ile. The sesquisulphate contains three isoleucinium cations per one sulphate anion and one hydrogensulphate anion, its formula being $(HIle)_3(SO_4)(HSO_4)$ where HIle designates the protonated Ile. The commercial isoleucine was purified in several steps after which the pure amino acid was recovered from the sesquisulphate by precipitation of the sulphate anions with aqueous solution of barium hydroxide. The number of repeated purification steps may vary, depending on the purity of the starting material.

Step 1: 3.957 g of Ile (Sigma lot 2) was suspended in 80 mL of ethyl-methyl ketone (EMK) and 2.4 mL of concentrated sulphuric acid was added drop wise over the period of 10 minutes while the suspension was stirred using a magnetic stirrer. Formation of the isoleucinium sesquisulphate could be visually observed. The mixture was sonicated for 30 minutes. The resulting suspension was left stirring overnight. The following day the mixture was sonicated for 1 h leading to complete dissolution of the precipitate which reformed upon further stirring. The precipitate was filtered under reduced pressure and ~1.8 g of the sesquisulphate was collected.

Step 2: In the next step, 1.711 g of the sesquisulphate was suspended in 25 mL of EMK and 0.21 mL of concentrated H_2SO_4 was added drop wise. It was stirred for an hour, then sonicated for 1 hour and left stirring overnight. The next day the suspension was sonicated for 1 hour, left stirring for 2 hours and filtered under reduced pressure.

Step 3: In the next step, 1.157 g of the sesquisulphate was suspended in 20 mL of EMK and 0.20 mL of concentrated H_2SO_4 was added drop-wise. The formed suspension was treated by the procedure described above.

Step 4: In the next step, 0.630 g of the sesquisulphate was suspended in 12 mL of EMK and 0.14 mL of concentrated H_2SO_4 was added drop wise. The formed suspension was treated by the procedure described above.

Final recovery: 244 mg of sesquisulphate was finally collected from which Ile was recovered by precipitation of the sulphate anions with barium hydroxide. Aqueous solution of $Ba(OH)_2$ was added drop-wise and the finely suspended precipitate of $BaSO_4$ was each time removed from solution by centrifugation (5000 g, 5 minutes). $Ba(OH)_2$ has been added until no further precipitate formed and the solution pH was 6. Volume of the water solution of Ile was then reduced to ~1/4 of its initial volume by allowing the water to evaporate in a furnace kept at 80 °C. Ile was precipitated from the water solution by drop-wise addition of 100 mL of acetone while stirring. The precipitate was filtered using a sinter funnel (B3 porosity), washed with acetone, dried on vacuum and weighted directly into sterile tubes. Total of 122 mg of Ile was collected.

The purity of the starting commercial Ile (Sigma lot 2), and the final purified Ile could not be estimated by NMR analysis, as the level of contamination was below the sensitivity threshold of NMR (Fig S2C). However, the kinetic analysis showed that a reduction by a factor of 5.4 in Leu content was achieved in the Ile sample - the starting commercial Ile (Sigma lot 2) contained 0.0019 %, while the final purified Ile contained 0.00035 % of leucine.

Construction of the editing-deficient D345A-LeuRS *E. coli* strain

The pKOV vector contains a temperature-sensitive origin of replication and markers for positive (Cam^R) and negative (sacB) selection for chromosome integration and excision (Link et al 1997). The gene for D345A LeuRS was cloned as a NotI-XbaI cassette into pKOV. *E. coli* MG1655 strain was transformed with the pKOV-D345A-LeuRS plasmid, a single transformed colony was resuspended in 100 µl of 10 % $MgSO_4$ and 100 µl decimal dilutions (3^{rd}–5^{th}) were plated onto LB^{Cam} plates followed by incubation overnight at 42 °C. Six colonies from 42 °C plates were resuspended in 500 µl LB broth without NaCl, and 100 µl decimal dilutions (3^{rd}–5^{th}) were plated onto LB plates without NaCl and with 5 % sucrose (w/v). Individual colonies were tested on LB^{Cam} plates to confirm the loss of the pKOV-D345A-LeuRS plasmid. The putative positives (D345A LeuRS strain) were selected by their sensitivity to norvaline. Individual colonies were grown to saturation at 37 °C in LB media. Each culture was diluted 1:50 in M9 medium supplemented with 50 µg/ml of each proteinogenic amino acids and 40 µg/ml of both uracil and adenine, with or without the addition of 10 mM norvaline. The cultures were grown at 37 °C, 200 rpm, and OD_{600} was measured after 6-8 h. The presence of the gene for D345A LeuRS in the colonies sensitive to norvaline, was confirmed by DNA sequencing. Two independent clones were generated and used as biological replicates. The clones insensitive to norvaline were also isolated, and the presence of the chromosomal WT *leuS* gene was confirmed by DNA sequencing. Such strains were used as the WT strains.

Structure determination and refinement

The diffraction data were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and were integrated with XDS and scaled with XSCALE (Kabsch, 1993). Further data analysis was performed with the CCP4 suite (Bailey, 1994). The structure was solved by molecular replacement with PHASER (McCoy et al, 2005) using the core protein from the *E. coli* LeuRS:$tRNA^{Leu}$:LeuAMS aminoacylation complex (Pdb: 4aq7). Subsequent cycles of search with PHASER combined with manual adjustments in COOT (Emsley & Cowtan, 2004) were used to place the $tRNA^{Leu}$. The final model was refined using REFMAC5 with TLS. Interfaces were analysed with the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Structure quality was analysed with MOLPROBITY (http://molprobity.biochem.duke.edu/), showing all residues in allowed regions (> 97%), and the figures were drawn with PYMOL (http://www.pymol.org/).

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