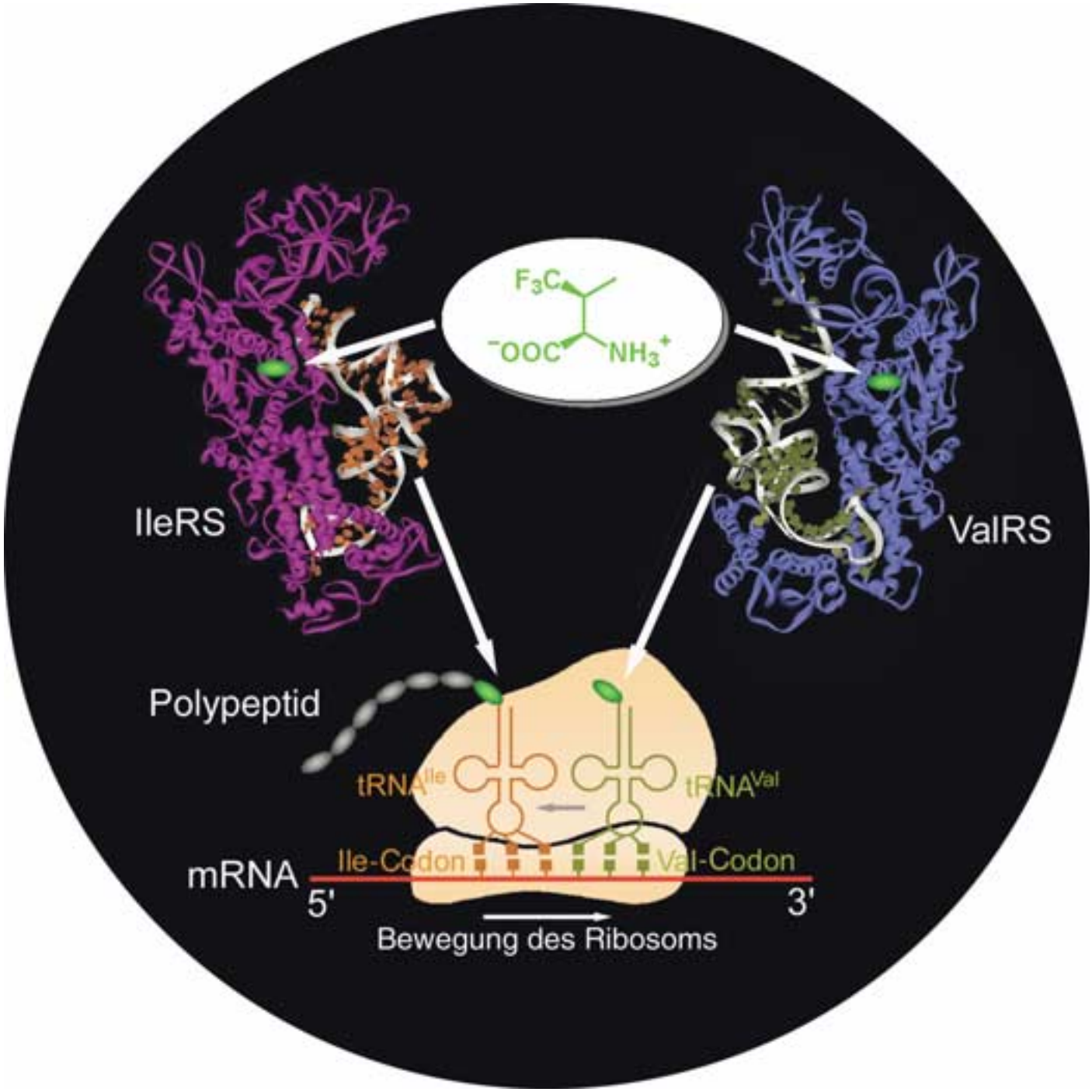
Zuschriften



Eine Proteinsequenz ist unter normalen Umständen durch die Sequenz ihrer mRNA eindeutig festgelegt. In der folgenden Zuschrift belegen Tirrell et al., dass das nicht sein muss: 4,4,4-Trifluorvalin kann in Abhängigkeit von der Aktivität der entsprechenden Aminoacyl-tRNA-Synthetasen im Expressionswirt sowohl Valin- als auch Isoleucincondons zugeordnet werden.

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RNA Translation

Alternative Translations of a Single RNA Message: An Identity Switch of (2*S*,3*R*)-4,4,4-Trifluorovaline between Valine and Isoleucine Codons**

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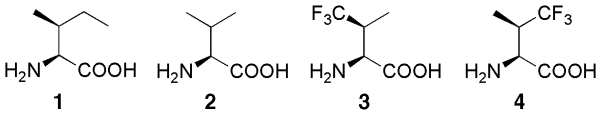
The genetic code links the sequence of a messenger RNA to the sequence of the corresponding protein. Codon assignments are established in the aminoacylation step, wherein the aminoacyl-tRNA synthetases (aaRSs) catalyze the attachment of the amino acids to their cognate tRNAs.[1,2] Here we show that engineering of bacterial expression hosts can allow a single RNA message to be translated in different ways depending on the relative rates of competing aminoacylation reactions. Specifically, we show that the identity of the noncanonical amino acid (2*S*,3*R*)-4,4,4-trifluorovaline can be changed by its assignment either to isoleucine or to valine codons according to the intracellular levels of the canonical amino acids and their cognate synthetases. It has been known for many years that relative aminoacylation rates play a critical role in determining translational fidelity.[3]

This work was motivated by previous demonstrations that fluorinated amino acids can be used to engineer the stability and dimerization specificity of coiled-coil peptides and proteins.[4–10] Trifluoroleucine and hexafluoroleucine can be introduced into recombinant proteins by using a leucine auxotroph as the bacterial expression host.[7,8] High-level incorporation of either of the fluorinated amino acids requires depletion of leucine in the culture medium prior to induction of gene expression. For hexafluoroleucine, depletion of the natural amino acid is not enough; efficient incorporation demands the use of an engineered bacterial host with enhanced leucyl-tRNA synthetase activity.[7] One of our continuing objectives is to expand the availability of novel amino acids for use in protein engineering.

There are two stereoisomeric forms of (2*S*)-4,4,4-trifluorovaline (2*S*-Tfv; Scheme 1): the 2*S*,3*S* form (**3**) and the 2*S*,3*R* form (**4**). Previously we found that both the 2*S*,4*S* and the 2*S*,4*R* forms of 5,5,5-trifluoroleucine are readily incorporated into recombinant proteins; the leucyl-tRNA synthetase of *Escherichia coli* apparently activates both isomers.[8] In evaluating 2*S*-Tfv as a substrate for protein synthesis, we considered two related questions. First, would 2*S*-Tfv function more efficiently as a surrogate for valine (**2**) or for isoleucine (**1**)? Second, would either the valyl-tRNA synthetase or the isoleucyl-tRNA synthetase discriminate between the two diastereomeric forms of 2*S*-Tfv?



Scheme 1. Amino acids used in this work: (2*S*)-isoleucine (**1**), (2*S*)-valine (**2**), (2*S*,3*S*)-4,4,4-trifluorovaline (2*S*,3*S*-Tfv, **3**), and (2*S*,3*R*)-4,4,4-trifluorovaline (2*S*,3*R*-Tfv, **4**).

Initial efforts to incorporate **3** and **4** into recombinant proteins failed. Neither **3** nor **4** supported protein synthesis in *E. coli* cultures depleted of either isoleucine or valine. We then examined the kinetics of activation of **3** and **4** by the valyl- and isoleucyl-tRNA synthetases (ValRS and IleRS, respectively). The requisite genes were obtained by PCR amplification of chromosomal DNA of *E. coli*. A hexahistidine tag was placed at the amino terminus of each enzyme, and the His-tagged variants of the wild-type IleRS and ValRS were expressed in *E. coli* and purified by immobilized-metal affinity chromatography. Amino acid activation kinetics were determined in vitro by using the adenosine triphosphate–inorganic pyrophosphate (ATP–PP_i) exchange assay.[11,12] The results were striking; **4** is activated by both enzymes, **3** by neither (Table 1). When we compared the rates of activation by IleRS, we found the specificity constant, k_{cat}/K_m, for **4** to be reduced approximately 600-fold with respect to the native substrate **1**; for ValRS, the k_{cat}/K_m value was reduced approximately 2500-fold with respect to **2**.

Table 1: Kinetic parameters for activation of amino acids **1**–**4** by *E. coli* IleRS and ValRS.[a]

Substrate	Enzyme	k_{cat} [S^{-1}]	K_m [M]	k_{cat}/K_m (rel.)
1	IleRS	3.00	6.8	1
3	IleRS	n.a.[b]	n.a.[b]	n.a.[b]
4	IleRS	0.21	282	1/590[c]
2	ValRS	5.96	36.6	1
3	ValRS	n.a.[b]	n.a.[b]	n.a.[b]
4	ValRS	0.36	5542	1/2490[d]

[a] Substrates **1** and **2** were used as the L isomers, **3** was the 2*S*,3*S* form, and **4** was the 2*S*,3*R* form. [b] n.a. = not activated. [c] Relative to k_{cat}/K_m for activation of **1** by IleRS. [d] Relative to k_{cat}/K_m for activation of **2** by ValRS.

In our previous work on the incorporation of methionine and leucine analogues into recombinant proteins, we found that elevation of the cellular activity of aminoacyl-tRNA synthetase could facilitate introduction of amino acids that are poor substrates for the wild-type synthetases.[7,13] The

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results shown in Table 1 encouraged us to test this approach with **4**. Expression plasmids harboring either of the synthetase genes *ileS* and *valS*, each outfitted with its endogenous promoter, were constructed from pQE15 and designated pQE15-ileS and pQE15-valS, respectively. Assays of whole-cell lysates from the corresponding transformants (designated AIV-IQ[pQE15-ileS] and AIV-IQ[pQE15-valS], respectively[14]) showed enhanced activity in the ATP-PP_i exchange assay. (For AIV-IQ[pQE15-ileS], total synthetase activity was elevated approximately eightfold; for AIV-IQ[pQE15-valS], total synthetase activity was elevated approximately sixfold.)

Murine dihydrofolate reductase (mDHFR), a marker protein encoded in pQE15, served as the test protein for experiments on in vivo incorporation of **3** and **4**. Assays were performed in *E. coli* cultures depleted in the canonical amino acid and supplemented with the analogue of interest. As shown in Figure 1, strains bearing either pQE15-ileS or pQE15-valS supported protein biosynthesis in media supplemented with **4**; cultures enriched in **3** showed only background levels of mDHFR.[15] Amino acid analysis indicated that approximately 92% of the encoded isoleucine residues were replaced by **4** in mDHFR produced by the strain outfitted with additional copies of IleRS; approximately 86% of encoded valine residues were replaced by **4** in the strain outfitted with additional copies of ValRS. "Reassignment" of the isoleucine and valine codons was confirmed by MALDI MS analysis of tryptic fragments of mDHFR (Figure 2). The fragment (**5**) corresponding to residues 124–147 contains a single residue encoded as isoleucine and four encoded as valine. When this fragment is derived from mDHFR produced in pQE15-ileS cultures supplemented with **4**, reassignment of the isoleucine codons to **4** causes the expected mass shift of +40 Da (Figure 2a). In contrast, reassignment of the valine codons causes a mass increase of 54 Da for fragment **6** (corresponding to residues 62–69) derived from mDHFR expressed in the strain bearing pQE15-valS. MALDI mass spectra of the intact proteins were consistent with the results of the amino acid analyses and analyses of tryptic peptide fragments. mDHFR contains 14 sites each for valine and for isoleucine. Quantitative replacement of valine by **4** would shift the mass of the protein by +756 Da; quantitative replacement of isoleucine would give a shift of +560 Da. The observed shifts were +690 and +569 Da, respectively, a result indicating efficient—although perhaps not quantitative—codon reassignment.

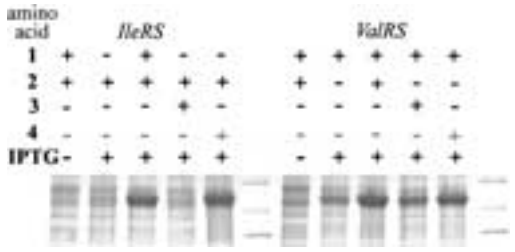

Figure 1. Sodium dodecylsulfate PAGE assay for translational incorporation of noncanonical amino acids into recombinant mDHFR. Analyses were conducted on whole-cell lysates; expression conditions are noted above each lane. IPTG = isopropyl-β,D-thiogalactoside.

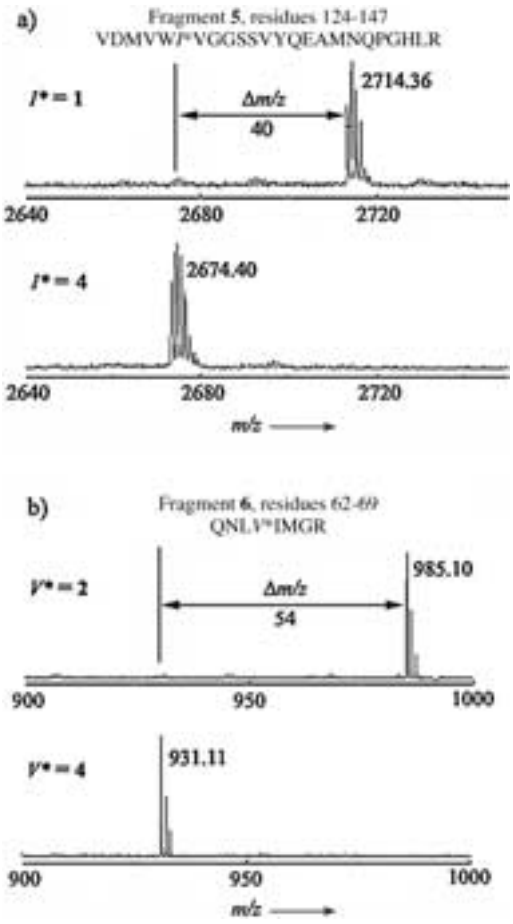

Figure 2. Reassignment of isoleucine and valine codons can be detected by MALDI MS analysis of tryptic fragments of mDHFR. a) Spectrum for fragment **5** (Val124–Arg147) from mDHFR produced by strain pQE15-ileS. The mass of this fragment is 2674.40 when the single isoleucine codon is assigned to **1** (bottom panel; mDHFR from culture supplemented with **1**). The mass shifts to 2714.36 Da when this codon is assigned to **4** (top panel; mDHFR from culture supplemented with **4**). b) Spectrum for fragment **6** (Gln62–Arg69) from mDHFR produced by strain pQE15-valS. The mass of this fragment is 931.11 Da when the single valine codon is assigned to **2** (bottom panel; mDHFR from culture supplemented with **2**). The mass shifts to 985.10 Da when this codon is assigned to **4** (top panel; mDHFR from culture supplemented with **4**).

In conclusion, these results demonstrate efficient, stereospecific introduction of 2*S*-Tfv into recombinant proteins in *E. coli*. We find that **4** can be assigned either to isoleucine codons or to valine codons depending on whether the host is engineered with enhanced IleRS or ValRS activity. Alternative translations of a single RNA message can thus be accomplished by control of the aminoacyl-tRNA synthetase activity and the amino acid pools of the expression host.

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[1] P. Schimmel, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4521.
[2] L. R. de Pouplana, P. Schimmel, *Trends Biochem. Sci.* **2001**, *26*, 591.
[3] D. Soll, U. L. Rajbhandary, *tRNA: Structure, Biosynthesis, and Function*, ASM Press, Washington, DC, **1995**.
[4] B. Bilgicer, X. Xing, K. Kumar, *J. Am. Chem. Soc.* **2001**, *123*, 11815.
[5] B. Bilgicer, A. Fichera, K. Kumar, *J. Am. Chem. Soc.* **2001**, *123*, 4393.
[6] N. C. Yoder, K. Kumar, *Chem. Soc. Rev.* **2002**, *31*, 335.
[7] Y. Tang, D. A. Tirrell, *J. Am. Chem. Soc.* **2001**, *123*, 11089.
[8] Y. Tang, G. Ghirlanda, W. A. Petka, T. Nakajima, W. F. DeGrado, D. A. Tirrell, *Angew. Chem.* **2001**, *113*, 1542; *Angew. Chem. Int. Ed.* **2001**, *40*, 1494.
[9] Y. Tang, G. Ghirlanda, N. Vaidehi, J. Kua, D. T. Mainz, W. A. Goddard, W. F. DeGrado, D. A. Tirrell, *Biochemistry* **2001**, *40*, 2790.
[10] A. J. Link, M. L. Mock, D. A. Tirrell, *Curr. Opin. Biotechnol.* **2003**, *14*, 603.
[11] T. L. Hendrickson, T. K. Nomanbhoy, P. Schimmel, *Biochemistry* **2000**, *39*, 8180.
[12] Y. Tang, D. A. Tirrell, *Biochemistry* **2002**, *41*, 10635.
[13] K. L. Kiick, J. C. M. van Hest, D. A. Tirrell, *Angew. Chem.* **2000**, *112*, 2232; *Angew. Chem. Int. Ed.* **2000**, *39*, 2148.
[14] Expression strains were derived from the isoleucine and valine double-auxotrophic strain AIV-IQ (*E. coli* $BF{-}ompThsdS(r_B^- m_B^-)\,gal\,dcm\,L(DE3)\,ilvD691$), which has been described previously (P. Wang, Y. Tang, D. A. Tirrell, *J. Am. Chem. Soc.* **2003**, *125*, 6900).
[15] Our medium-shift protocol does not completely exhaust the intracellular pool of **2**, so significant background expression was observed in the strain bearing pQE15-valS in cultures supplemented with **3**. MALDI MS analysis of tryptic peptides from these background proteins showed the valine codons in mDHFR were assigned exclusively to **2**.

