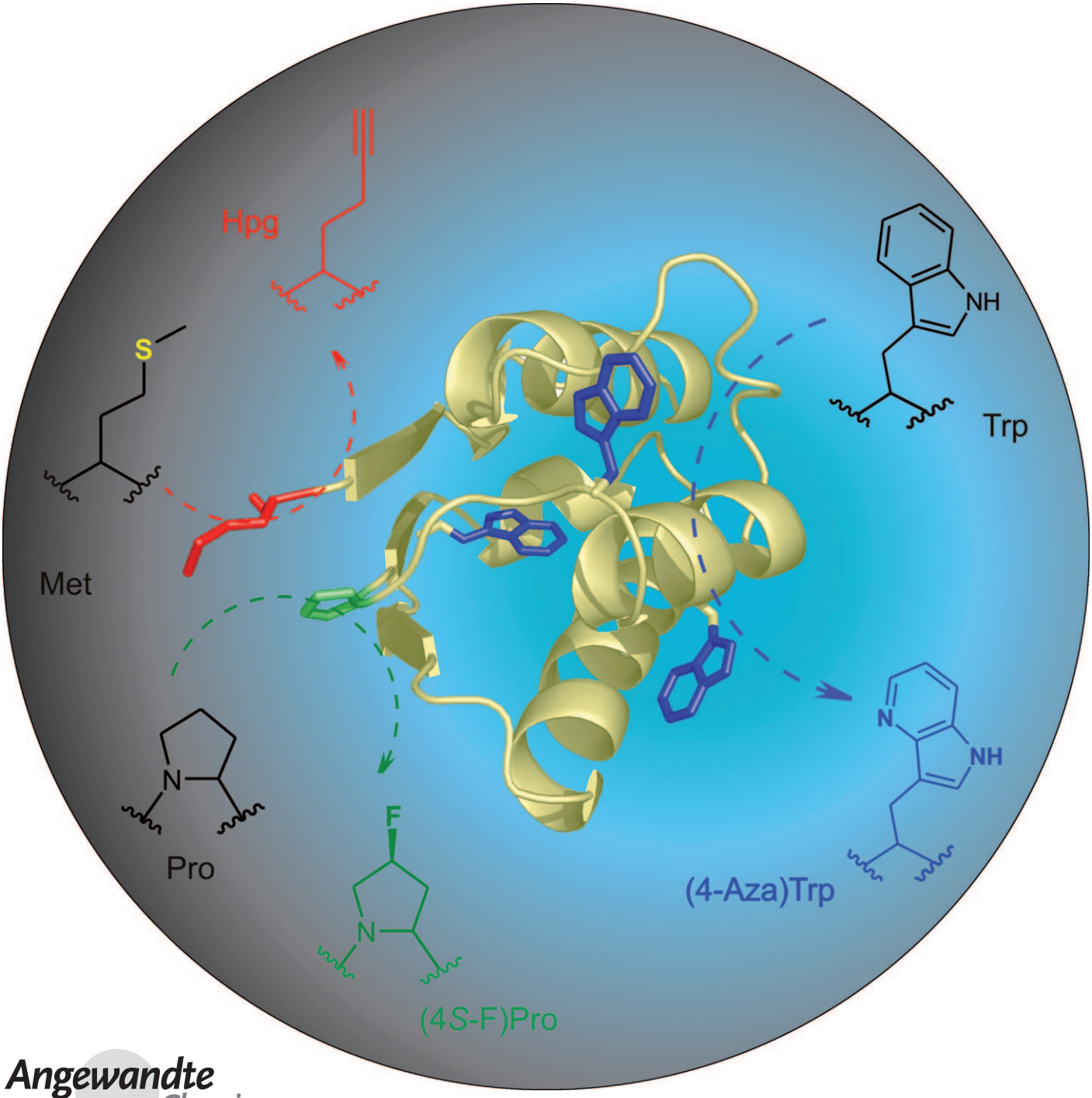
Engineering the Genetic Code

DOI: 10.1002/anie.201000439

In Vivo Double and Triple Labeling of Proteins Using Synthetic Amino Acids**

*Sandra Lepthien, Lars Merkel, and Nediljko Budisa**



Synthetic amino acids exhibit features distinct from those of the canonical amino acids, and their incorporation into proteins or peptides usually endows these with novel structural and functional features.[1,2] In general, there are few approaches for in vivo incorporation of synthetic amino acids into target proteins in the frame of recombinant DNA technology. One approach, "genetic code engineering", is based on the reassignment of the sense codon[3,4] through the supplementation incorporation method (SPI).[5] By exploiting the substrate tolerance of cellular uptake as well as the endogenous translation system, synthetic amino acids can be successfully translated into expressed proteins. This methodology allows the residue-specific replacement of a particular amino acid at any position in the protein sequence without the need for DNA mutagenesis. Such replacements are highly relevant since many structural/biological features, such as conformational stability and folding properties,[6] are based on synergistic effects of different amino acids at multiple positions in the protein sequence, as has been demonstrated recently.[7,8]

In contrast, "expanded genetic code" methodologies use DNA mutagenesis to introduce in-frame termination triplets (e.g. the amber stop codon) or quadruplets that are considered as blank codons for the expansion of the cellular code.[9] Synthetic amino acids are incorporated into single recombinant proteins by means of nonsense or frameshift suppression using genetically engineered components of translational machinery (e.g., aminoacyl-tRNA synthetases, tRNAs).[10] Although these approaches are quite popular in academic communities, their practical usefulness is still limited for several reasons. For example, these approaches typically a) result in low protein yields due to cellular toxicity,[11] b) suffer from context effects, and c) compete with the highly specialized termination machinery developed by evolution.[12] In addition, in some instances experimental reproducibility is difficult.[13] Very recently, improved systems were reported for double amino acid incorporation using normal and mutated ribosomes in the frame of an expanded genetic code.[9,14] Although further progress in this area can be expected, it is reasonable to suppose that increasing the number of stop or non-triplet codons in the coding sequence will significantly decrease the efficiency of translation. Thus, nonsense or frameshift suppression is still not optimal for efficient in vivo multiple incorporation of amino acids in a protein.[15] A promising alternative approach is certainly "expressed protein ligation", which successfully combines natural intein-mediated protein self-splicing with peptide ligation, allowing for the generation of semisynthetic proteins with a practically unlimited number of noncanonical amino acids.[16] The major drawback of this methodology is that the synthetic amino acid can be delivered only into the peptide part of the tailored protein molecule.

In contrast, SPI for the modification of expressed proteins is a straightforward method that requires neither prior genetic engineering nor extensive system optimization. This approach has been used to produce proteins with novel spectroscopic properties,[18] changed pH sensitivity,[19] enhanced stability,[8] or enzymatic activity[20] in yields comparable to those of the parent protein. Until now, SPI has been limited to one-dimensional improvements resulting from the incorporation of only one type of synthetic amino acid per target protein. In this way the biophysical properties of a protein were changed, for example, its fluorescence[21] and folding behaviour;[7] in another example a bioorthogonal reactive handle[22] was introduced for subsequent protein modifications. However, it would be highly desirable to combine all these properties in one single protein variant.

To explore this possibility, we engineered a model protein by multiple incorporation of two or three chemically distinct synthetic amino acids in a single expression experiment, as outlined in Figure 1. Cysteine-free "pseudo-wild-type barstar" (ψ-b*; mutations P28A/C41A/C83A)[17] from *Bacillus amyloliquefaciens* was selected as the target protein. ψ-b* is a 10 kDa protein that contains one methionine (Met), three tryptophan (Trp), and one proline (Pro) residue. In our previous studies, we used ψ-b* for individualized modifications through copper(I)-catalyzed azide–alkyne Huisgen cycloaddition (CuAAC, "click chemistry")[23,24] with azide/alkyne-containing ligands. In particular, iodination[25] and glycosylation[26] of ψ-b* containing homopropargylglycine (Hpg) or azidohomoalanine (Aha) were achieved. However, the general utility of such protein conjugates in cell systems would be substantially improved if they could be made intrinsically fluorescent in a noninvasive manner. Recently, we have shown that the isosteric Trp analogue 4-azatrypto-

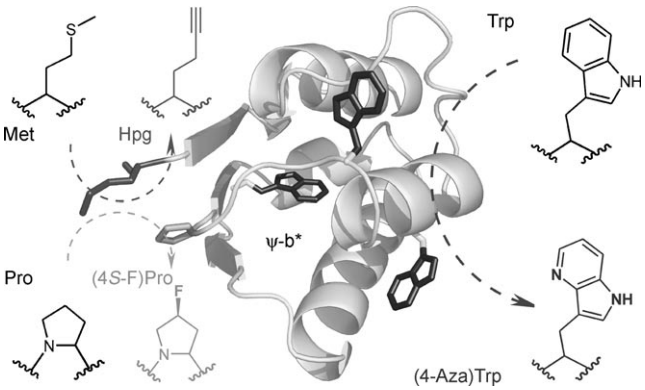


Figure 1. General concept of simultaneous in vivo multiple labeling of a protein in a single expression experiment. In the structure of ψ-b* Met1, Trp39, Trp45, Trp54, and Pro48 and the related analogues Hpg, (4-Aza)Trp, and(4*S*-F)Pro are depicted.

[*] Dr. S. Lepthien, Dr. L. Merkel, Prof. Dr. N. Budisa
Max Planck Institute of Biochemistry, Molecular Biotechnology
Am Klopferspitz 18, 82152 Martinsried (Germany)
Fax: (+49) 89-8578-3557
E-mail: budisa@biochem.mpg.de
Homepage: http://www.biochem.mpg.de/budisa

[**] We thank Prof. Luis Moroder for useful discussions and advice, Elisabeth Weyher-Stingl and the Microchemistry Core Facility for excellent assistance in analytics, and Waltraud Wenger for excellent technical assistance. The National BioResource Project (Japan) is acknowledged for providing bacterial strains. This work was supported by the BioFuture Program of the Federal Ministry of Education and Research of Germany.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201000439.

phan ((4-Aza)Trp) displays a strongly red-shifted fluorescence upon excitation with UV light ($\lambda_{max,em} > 415$ nm)[27] and thus has great potential for biological imaging studies, for example, by single-molecule spectroscopy.

To combine bioorthogonal chemistry and intrinsic fluorescence it is necessary to simultaneously incorporate (4-Aza)Trp and Hpg into ψ-b*. However, since said Trp analogue is more hydrophilic than Trp itself, its incorporation into a protein is expected to reduce protein stability, as has already been observed in the case of aminotryptophans.[28] Thus, incorporation of a third synthetic amino acid into ψ-b* should compensate for the possible loss of protein stability. The proline analogue *cis*-4-fluoroproline (4*S*-F)Pro was previously shown to exert a thermodynamically stabilizing effect on ψ-b*.[29] This effect has been attributed to the increase of conformational stability caused by 4*S*-fluorination which stabilizes the C^{γ}-*endo* pucker of (4*S*-F)Pro in the ψ-b* structure.[30] Through the rational application of this stereoelectronic effect, we have recently dramatically improved the folding properties of green fluorescent protein by incorporation of (4*S*-F)Pro.[7]

The *Escherichia coli* (*E. coli*) polyauxotrophic strain JE 7345 (*ileS, ara, proC, galK, trp, his, argG, xyl, mtl, metA* or *B*; National BioResource Project, Japan) was used for double-incorporation experiments and strain JE 5630 (*dacA1191, dacB12, metA* or *B, thi, ile, mtl, xyl, str, his, trp, gal, tsx, proC, lacY*; National BioResource Project, Japan) for triple-incorporation experiments. For the expression of the recombinant protein, cells were transformed with the ampicillin-resistance plasmid pQE-80 L (Qiagen, Hilden, Germany), which harbors the gene of ψ-b* under the control of a T5 promotor. Transformed cells were grown in New Minimal Medium (NMM)[31] with 100 μg mL^{-1} ampicillin and 4 μM Trp and 30 μM Met as canonical amino acid substrates for double incorporation and with 6 μM Trp, 35 μM Met, and 58 μM Pro for triple incorporation at 37 °C and 220 rpm until depletion of Met, Trp, and Pro. Defined concentrations of these canonical amino acids allow the production of cell mass up to an OD_{600} value of 0.6–0.8. For double incorporation, cells were provided with 100 mg D,L-Hpg and 100 mg pre-incubated 4-azaindole in 1 L NMM followed by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) after 30 min to induce target protein expression. For triple incorporation the culture was additionally supplemented with 100 mg (4*S*-F)Pro per 1 L NMM. Protein expression was performed for 6 h at 27 °C and 220 rpm and checked by loading 0.3 OD_{600} of total cell lysates on a 20% SDS polyacrylamide gel. Fluorescent bands at roughly 10 kDa were clearly visible in unstained gels exposed to UV light (Figure 2A) providing a first qualitative indication of the incorporation of (4-Aza)Trp in ψ-b*. As expected, these UV bands correspond exactly to the Coomassie stained bands of ψ-b* containing (4-Aza)Trp.

Tag-free ψ-b* and its variants were expressed in inclusion bodies and were routinely refolded prior to purification as described elsewhere.[28] Interestingly, initial experiments showed incomplete deformylation of N-terminal formyl-Hpg[32] in both double- and triple-labeled samples. To achieve more homogeneous protein samples the fermentation procedure was modified by a media-shift. First cells were grown in

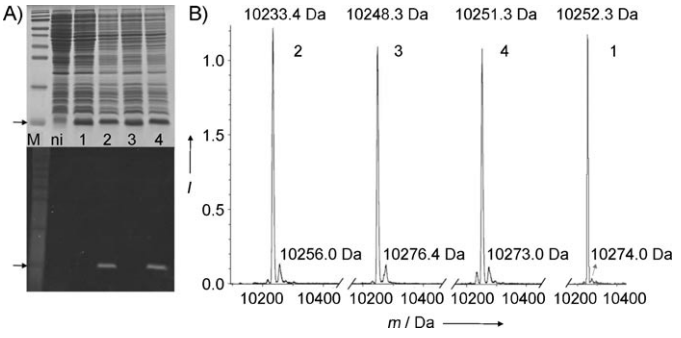


Figure 2. Expression and mass spectrometric profiles of wild-type ψ-b* and related variants. A) Cell lysates after Coomassie staining (top) and under UV light (bottom); arrows indicate a molecular mass of roughly 10 kDa; M: molecular weight standard, ni: noninduced cell lysate, 1: ψ-b* (wild-type protein), 2: ψ-b*[Hpg]/[(4-Aza)Trp], 3: ψ-b*[Hpg]/[(4*S*-F)Pro], 4: ψ-b*[Hpg]/[(4-Aza)Trp]/[(4*S*-F)Pro] (triple-labeled ψ-b*). Fluorescence bands of (4-Aza)Trp-containing ψ-b* are easily detectable (bands in lanes 2 and 4 in the bottom picture). B) In the mass spectra the perfect match between the calculated and found masses indicates quantitative incorporation; calculated masses for wild-type protein ψ-b* (1): 10252.6 Da; double-labeled fluorescent ψ-b*[Hpg]/[(4-Aza)Trp] (2): 10233.6 Da; double-labeled nonfluorescent ψ-b*[Hpg]/[(4*S*-F)Pro] (3): 10248.6 Da; triple-labeled ψ-b*[Hpg]/[(4-Aza)Trp]/[(4*S*-F)Pro] (4): 10251.5 Da. Minor side peaks (ca. +22 Da) arise from sodium adducts.

Luria–Bertani (LB) medium until $OD_{600} \approx 0.5$; second they were washed and resuspended in 0.5 L NMM. After about 30 minutes of incubation without canonical amino acids, analogues and IPTG were added as described above. In our earlier experiments we demonstrated that ψ-b*[Hpg] was expressed in yields of about 8 mg L^{-1}.[22] The co-incorporation of (4-Aza)Trp reduced the yield of the resulting ψ-b*-[Hpg]/[(4-Aza)Trp] by about 50% (ca. 3.8 mg L^{-1}). Expectedly, the co-incorporation of (4*S*-F)Pro substantially increased the expression yield: 10.8 mg L^{-1} of ψ-b*-[Hpg]/[(4*S*-F)Pro] formed. A markedly increased yield for the expression of the recombinant protein was also achieved for ψ-b*[Hpg]/[(4-Aza)Trp]/[(4*S*-F)Pro] (ca. 5 mg L^{-1}).

Purified proteins were kept at +4 °C in 50 mM sodium phosphate buffer (pH 7.4) and analyzed for purity and homogeneity by SDS polyacrylamide gel electrophoresis (see the Supporting Information) and ESI mass spectrometry (Figure 2B). In addition to high homogeneity, the ESI-MS profiles also confirmed that in all examined samples (and all combinations of synthetic amino acids) the canonical amino acids have been quantitatively replaced by the synthetic amino acids. A high level of Met→Hpg substitution (at least 95%) in sequence position 1 was independently confirmed by N-terminal sequencing (see the Supporting Information). Finally, enzymatic digestion (Glu-C) of all recombinant proteins and subsequent Orbitrap MS analyses unambiguously confirmed the high level of substitutions at all sequence positions (see the Supporting Information).

After MS characterization the protein samples were examined by spectroscopy (Figure 3). Substitutions Met1→Hpg1 and Pro48→(4*S*-F)Pro48 did neither affect the UV absorbance ($\lambda_{max,UV} = 280$ nm; see the Supporting Information) nor the fluorescence profiles ($\lambda_{max,em} = 340$ nm) of the protein variants. In contrast, the replacement of all three Trp

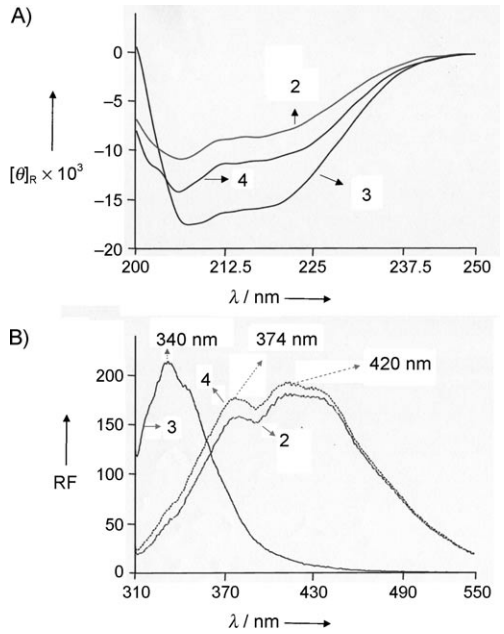


Figure 3. A) CD and B) fluorescence spectra of double- and triple-substituted ψ-b* variants; the protein variants are numbered as in Figure 2. Note the strong red-shift (80 nm) in the fluorescence emission spectrum of the (4-Aza)Trp-containing protein species ($\lambda_{max,em}$(2,4) = 420 nm) relative to the spectrum of the wild-type protein (not shown) and ψ-b*[Hpg]/[(4*S*-F)Pro] ($\lambda_{max,em}$(3) = 340 nm). The shoulder at about 374 nm corresponds to (4-Aza)Trp53 buried in the hydrophobic interior of the protein. Far-UV CD spectra (200–260 nm) were recorded at 20 °C; mean residual ellipticity ($[\theta]_R$) is expressed in deg cm^2 dmol^{-1}; RF: relative fluorescence intensity. Fluorescence spectra of ψ-b* and related variants were measured upon excitation at 295 nm.

residues in ψ-b* with (4-Aza)Trp led to a distinct change in the UV and fluorescence profiles, as depicted in Figure 3B. Both the UV absorbance ($\lambda_{max,UV}$ = 288 nm) and the fluorescence maxima ($\lambda_{max,em}$ = 420 nm) are strongly red-shifted. The presence of (4-Aza)Trp in ψ-b* endows the protein with a unique blue fluorescence suitable for spectroscopic studies in biological systems.

The analyses of secondary structure of the (4-Aza)Trp-containing ψ-b* variants revealed reduced structural stability (Figure 3A): The far-UV CD spectrum of ψ-b*[Hpg]/[(4*S*-F)Pro] (3) at 20 °C in PBS is identical to that of the parent ψ-b*. The CD profile exhibits two minima at 222 nm and 208 nm, typical for largely α-helical proteins (Figure 3A, curve 3). Conversely, the dichroic properties of ψ-b*-[Hpg]/[(4-Aza)Trp] are significantly different (curve 2). The shift of the minimum towards 205 nm and the significant decrease of the intensities at both wavelengths (222 and 208 nm) indicate a loss of ordered protein structure and a reduced contribution of the α-helical structure to the overall secondary structure of the protein. Compared to ψ-b*-[Hpg]/[(4-Aza)Trp], the spectrum of ψ-b*[Hpg]/[(4-Aza)Trp]/[(4*S*-F)Pro] (curve 4) shows increased intensities at the two minima by about 20%, indicating stabilization of the native state resulting from the presence of (4*S*-F)Pro in the protein structure.

Recently, we could demonstrate that weak signals in the region between 210 and 225 nm in far-UV CD profiles of aminotryptophan-containing ψ-b* are due to the protein's secondary structure rather than to the intrinsic contribution of aminotryptophan residues.[28] Most probably the same is true for ψ-b* with (4-Aza)Trp since the physicochemical properties of 4-azatryptophan are similar to those of 4-aminotryptophan (hydrophilicity, pH sensitivity, intramolecular charge transfer).[18] Our model protein ψ-b* contains three Trp residues: buried Trp53, fully or partially solvent-exposed Trp38, and Trp44 which is directly involved in tight interactions with barnase.[33] Not surprisingly, both (4-Aza)Trp-containing variants were unable to inhibit barnase as efficiently as the parent ψ-b* and the ψ-b*[Hpg]/[(4*S*-F)Pro] variant (see the Supporting Information).

In general, by co-translation of a third noncanonical amino acid here (4*S*-F)Pro, into ψ-b* the loss of conformational stability was at least partially compensated. This was also confirmed by the temperature-induced unfolding experiments monitored by recording the CD intensities at 222 nm (see the Supporting Information). In particular, we could demonstrate that the presence of (4*S*-F)Pro in ψ-b*-[Hpg]/[(4*S*-F)Pro] increased the melting temperature (T_m = 64.6 °C) to a value comparable to that of the parent ψ-b* protein (T_m = 66.3 °C). Unfortunately, thermal unfolding of (4-Aza)Trp-containing ψ-b* variants led to sample aggregation over 85 °C; thus thermodynamic parameters could not be derived and only denaturation midpoints (T_m) were compared. In ψ-b*, Trp53 is completely buried inside the hydrophobic core and essential for folding and stability of the whole protein. Therefore it is reasonable to expect that the replacement Trp53→(4-Aza)Trp53 increases the hydrophilicity of the protein core and destabilizes the whole structure. Indeed, the T_m value (42 °C) of ψ-b*[Hpg]/[(4-Aza)Trp] is lowered by about 20 °C (see the Supporting Information). Expectedly, the co-translation of (4*S*-F)Pro induced a substantial increase in the T_m value by more than 8 °C in ψ-b*[Hpg]/[(4-Aza)Trp]/[(4*S*-F)Pro] (T_m = 50.6 °C). This protein variant proved to be more suitable for storage and exhibits much better tolerance than ψ-b*[Hpg]/[(4-Aza)Trp] to the reaction conditions for click chemistry.

To illustrate the suitability of our triple-labeled protein for click chemistry, the CuAAC reactions were performed with two ligands, that is, an azido-sugar (1-azido-1-deoxy-β-D-glucopyranoside) and azido-PEG570 (azido-polyethyleneglycol, 570 Da). The conjugation reactions were very efficient with the azido-sugar and yielded the expected fluorinated blue-fluorescent glycoprotein (Figure 4A). Click chemistry with azido-PEG570, although less efficient, yielded a PEGylated, fluorinated, blue-fluorescent protein (Figure 4B). Both reactions confirm that triple-labeled ψ-b* is well suited for bioorthogonal transformations.

To our knowledge, this is the first demonstration of a triple incorporation of synthetic amino acids in vivo into a recombinant protein. Through the simultaneous double and triple replacement with chemically distinct synthetic amino acids, the number of steps required for multiple labeling of a protein is reduced to one. Thus we have access to single proteins with specific properties for a variety of applications

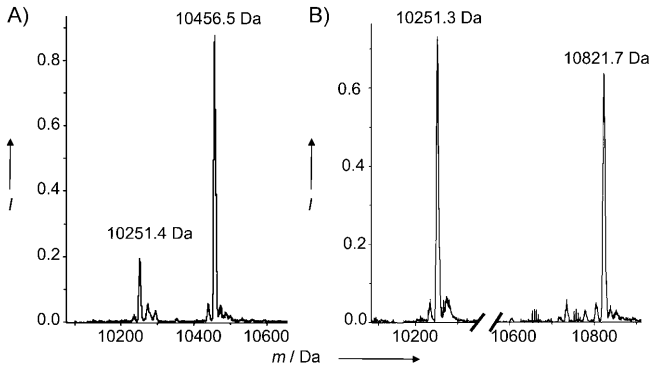


Figure 4. Mass spectrometric analysis of the click reaction products of the triple-substituted protein (10251 Da). A) Glycoconjugation reaction with 1-azido-1-deoxy-β-D-glucopyranoside very efficiently (ca. 80%) yielded blue-fluorescent ψ-b*[Hpg]/[(4-Aza)Trp]/[(4S-F)Pro]-{triazole}sugar (m_{calcd} = 10456.6 Da). B) The PEGylation reaction was less efficient (ca. 45–50%), resulting in blue-fluorescent ψ-b*-[Hpg]/[(4-Aza)Trp]/[(4S-F)Pro]{triazole}PEG570 (m_{calcd} = 10821.6 Da).

(imaging, bio-orthogonal transformations, fluorinations, etc.). This technology of multiple labeling in vivo will expand the use of synthetic amino acids for individual protein modifications in protein engineering and biotechnology. In the future, flexible and application-oriented multiple labeling in vivo may serve as a convenient and efficient tool to meet the specific demands of both academic and industrial applications.

Experimental Section

Expression and fermentation: The *Escherichia coli* strain JE 7345 with stable auxotrophy for Met and Trp was identified as the optimal expression host for double-incorporation experiments. For this purpose, the cells were grown overnight at 37°C in NMM[5] with defined concentrations of Trp and Met; 4 μM L-Trp and 30 μM L-Met were identified as ideal limiting concentrations to enable cell growth up to an OD_{600} value between 0.6 and 0.8. The *Escherichia coli* strain JE 5630 was identified as the optimal expression strain for triple-incorporation experiments. The calibration and optimization of fermentation and expression conditions for triple incorporation were similar to those used for double incorporation (see above). Concentrations of 6 μM L-Trp, 35 μM L-Met, and 58 μM L-Pro allowed cell growth and depletion of the canonical amino acids at an OD_{600} value between 0.6–0.8.

CuAAC reactions: Hpg-containing ψ-b* variants (1 mg mL^{-1} in 50 mM sodium phosphate buffer, pH 7.4) were incubated for 18 h at room temperature with 3.75 mM $CuSO_4$, 3.75 mM ascorbic acid, 200 mM sodium phosphate buffer, pH 7.4, and required amounts of the respective azide ligand (1-azido-1-deoxy-β-D-glucopyranoside: 2.5 mg mL^{-1}; azido-PEG570: 5 mg mL^{-1}; reaction batch volume 200 μL).

Other methods and experiments: UV, fluorescence, CD, and mass spectrometry measurements as well as other experiments are described in the Supporting Information.

Received: January 25, 2010
Revised: May 7, 2010
Published online: June 23, 2010

Keywords: amino acid analogues · genetic code · methionine · proline · tryptophan

[1] N. Budisa, *Angew. Chem.* **2004**, *116*, 6586; *Angew. Chem. Int. Ed.* **2004**, *43*, 6426.
[2] A. J. Link, M. L. Mock, D. A. Tirrell, *Curr. Opin. Biotechnol.* **2003**, *14*, 603.
[3] N. Budisa, C. Minks, S. Alefelder, W. Wenger, F. M. Dong, L. Moroder, R. Huber, *FASEB J.* **1999**, *13*, 41.
[4] A. J. Link, D. A. Tirrell, *Methods* **2005**, *36*, 291.
[5] C. Minks, S. Alefelder, L. Moroder, R. Huber, N. Budisa, *Tetrahedron* **2000**, *56*, 9431.
[6] L. Moroder, N. Budisa, *ChemPhysChem* **2010**, *11*, 1181.
[7] T. Steiner, P. Hess, J. H. Bae, B. Wiltschi, L. Moroder, N. Budisa, *PLoS ONE* **2008**, *3*, e1680.
[8] C. Wolschner, A. Giese, H. A. Kretzschmar, R. Huber, L. Moroder, N. Budisa, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 7756.
[9] H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin, *Nature* **2010**, DOI: 10.1038/nature08817.
[10] H. Neumann, A. L. Slusarczyk, J. W. Chin, *J. Am. Chem. Soc.* **2010**, *132*, 2142.
[11] R. E. Doerig, B. Suter, M. Gray, E. Kubli, *EMBO J.* **1988**, *7*, 2579–2584.
[12] T. S. Young, I. Ahmad, J. A. Yin, P. G. Schultz, *J. Mol. Biol.* **2010**, *395*, 361.
[13] A. K. Antonczak, Z. Simova, E. M. Tippmann, *J. Biol. Chem.* **2009**, *284*, 28795.
[14] W. Wan, Y. Huang, Z. Wang, W. K. Russell, P.-J. Pai, D. R. Russell, W. Liu, *Angew. Chem.* **2010**, *122*, 3279; *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 3211.
[15] E. A. Rodriguez, H. A. Lester, D. A. Dougherty, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 8650.
[16] T. W. Muir, J. N. Abelson (Eds.), *Methods Enzymol.* **2009**, *462*.
[17] B. Nolting, R. Golbik, A. R. Fersht, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10668.
[18] L. Merkel, M. G. Hoesl, M. Albrecht, A. Schmidt, N. Budisa, *ChemBioChem* **2010**, *11*, 305.
[19] N. Budisa, M. Rubini, J. H. Bae, E. Weyher, W. Wenger, R. Golbik, R. Huber, L. Moroder, *Angew. Chem.* **2002**, *114*, 4238; *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 4066.
[20] P. C. Cirino, Y. Tang, K. Takahashi, D. A. Tirrell, F. H. Arnold, *Biotechnol. Bioeng.* **2003**, *83*, 729.
[21] J. H. Bae, M. Rubini, G. Jung, G. Wiegand, M. H. J. Seifert, M. K. Azim, J.-S. Kim, A. Zumbusch, T. A. Holak, L. Moroder, R. Huber, N. Budisa, *J. Mol. Biol.* **2003**, *328*, 1071.
[22] S. L. Dong, L. Merkel, L. Moroder, N. Budisa, *J. Pept. Sci.* **2008**, *14*, 1148.
[23] V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596.
[24] C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057.
[25] S. L. Dong, L. Moroder, N. Budisa, *ChemBioChem* **2009**, *10*, 1149.
[26] L. Merkel, H. S. G. Beckmann, V. Wittmann, N. Budisa, *ChemBioChem* **2008**, *9*, 1220.
[27] S. Lepthien, M. G. Hoesl, L. Merkel, N. Budisa, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 16095.
[28] M. Rubini, S. Lepthien, R. Golbik, N. Budisa, *Biochim. Biophys. Acta Proteins Proteomics* **2006**, *1764*, 1147.
[29] C. Renner, S. Alefelder, J. H. Bae, N. Budisa, R. Huber, L. Moroder, *Angew. Chem.* **2001**, *113*, 949; *Angew. Chem. Int. Ed.* **2001**, *40*, 923.
[30] M. D. Shoulders, K. A. Satyshur, K. T. Forest, R. T. Raines, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 559.
[31] N. Budisa, P. P. Pal, *Biol. Chem.* **2004**, *385*, 893.
[32] J. Z. Tang, G. Hernandez, D. M. LeMaster, *Protein Expression Purif.* **2004**, *36*, 100.
[33] R. W. Hartley, *Methods Enzymol.* **2001**, *341*, 599.

