Artificial Proteins

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# Multiple-Site Labeling of Proteins with Unnatural Amino Acids**

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The advent of efficient systems using genetic encoding for the site-specific incorporation of unnatural amino acids (UAA) into proteins has opened countless new possibilities for studying the structure, dynamics, and interactions of proteins.[1] In particular, orthogonal pairs of amber suppressor tRNA (*Mj*tRNA) and tyrosyl-tRNA synthetase (*Mj*TyrRS) of *Methanocaldococcus jannaschii* have been evolved that specifically recognize amber stop codons for the incorporation of over 40 different UAAs.[1] Although these systems can produce mutant protein with yields as high as for the wild-type,[2,3] the protein yields are strongly context dependent. Thus, the presence of a single amber stop codon in the gene of a target protein can lead to unacceptably poor expression yields.[3–6]

Many efforts have been directed at biasing the competition between the *Escherichia coli* release factor RF1 that recognizes the amber stop codon and the suppressor tRNA in favor of the production of full-length protein. Thus, the incorporation of UAAs can be enhanced by omission of RF1 from a cell-free (CF) synthesis system reconstituted from the individually purified enzyme components.[7] CF systems were also used to eliminate RF1 with anti-RF1 antibodies,[8,9] or by deploying an RNA aptamer against RF1.[10] Unfortunately, all these approaches are affordable only for small-scale sample preparations, and protein yields can be compromised by antibodies,[9] while aptamers still yield truncated protein as the predominant product.[10] Heat-shock inactivation of a thermosensitive mutant of RF1 in a low-yield *E. coli* CF system increased the incorporation efficiencies of UAAs to at most 75% ($<50$% in most cases),[11] while the fidelity of translation was compromised by prolonged heat treatment. Depletion of tagged RF1 from a cell-free S30 extract by affinity chromatography was reported, but the identity of the tag was not revealed and anti-RF1 antibodies were required for complete elimination.[9] In a different approach, truncation of the ribosomal protein L11 was shown to weaken the binding of RF1, but the resulting protein yields with UAAs were only moderately enhanced.[12] Initial efforts to produce RF1-deficient *E. coli* strains led to compromised strains that depended on unnatural amino acids for growth (making protein expression expensive)[13] or strains that were not entirely independent of RF1.[14] Recently, an enhanced version of release factor RF2 enabled the production of an RF1-free *E. coli* DH10β strain and the incorporation of UAAs at multiple sites in vivo, but the protein yields obtained with UAAs were significantly reduced compared with those obtained with the natural amino acid (tyrosine).[6]

In view of the cost of many of the most attractive UAAs and the difficulty to tailor the concentrations of the aminoacyl-tRNA synthetase (RS) and suppressor tRNA in in vivo expression systems to the requirements of different UAAs and incorporation sites, we developed a continuous exchange cell-free (CECF) system that allows facile, inexpensive, and complete removal of the release factor RF1 from an S30 extract derived from the widely used high-yielding and protease-deficient *E. coli* strain BL21 Star (DE3) (Invitrogen). The approach relies on replacing wild-type RF1 by a mutant with a C-terminal affinity tag consisting of three consecutive chitin-binding domains (RF1-$CBD_3$) for selective removal by filtration through a chitin column after production of an S30 extract in the usual way. The chitin-binding-domain tag allows the removal of RF1 under conditions that maintain the full activity of the S30 extract and at the same time delivers dramatically improved incorporation yields of difficult UAAs at difficult positions, suppresses the production of truncated protein, and allows the incorporation of UAAs at multiple sites in the same protein. Conveniently, the modified strain is fully compatible with protein expression from pET vectors,[15] which are the most frequently used vectors for protein production in structural biology.

The yields of wild-type protein obtained with chitin-treated ($S30^{RF1-}$) and untreated ($S30^{RF1\text{-}CBD}$) extracts were indistinguishable from the yields obtained with S30 extracts prepared from the original BL21 Star (DE3) strain (Figure S3 in the Supporting Information). To assess and optimize the expression yields with UAAs without having to purify the proteins, we used the *Mj*tRNA/*Mj*TyrRS pair evolved for incorporation of the fluorescent UAA L-(7-hydroxy-coumarin-4-yl)ethylglycine (Hco).[16] Optimization employed the West Nile virus NS2B-NS3 protease (WNVpro), a 27 kDa protein that is also an established drug target.[17] Optimization of the concentration of aminoacyl-tRNA syn-

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thetase (CouRS-D8;[16] Figure S2 in the Supporting Information) was sufficient to obtain expression yields of Hco mutants of WNVpro comparable to those of wild-type WNVpro (Figure S3 in the Supporting Information). Interestingly, the expression yields decreased for overly high CouRS-D8 concentrations.

Figure 1 compares the expression yields of WNVpro with amber stop codons at positions 53 and 132 in the amino acid sequence, using conventional S30 and RF1-depleted S30 extracts. While the mutant WNVpro(Trp53Hco) was

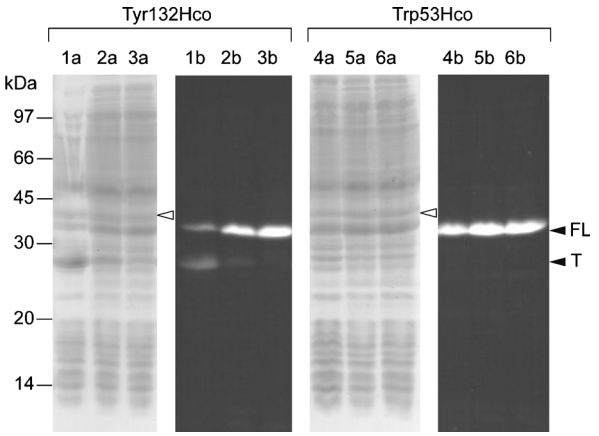


***Figure 1.*** Cell-free expression of Hco mutants of WNVpro. a) and b) show the same SDS-PAGE (15%) gel after staining with Coomassie blue and as a fluorescence image (UV irradiation at 312 nm), respectively. Lanes 1–3 (Tyr132Hco) and 4–6 (Trp53Hco): using conventional S30 extract (1 and 4), S30 extract without chitin treatment ($S30^{RF1\text{-}CBD}$; 2 and 5), and S30 extract after removal of RF1-$CBD_3$ ($S30^{RF1-}$; 3 and 6). The positions of the bands of WNVpro(1–131) (T), full-length WNVpro (FL) and CouRS-D8 (open triangle) are indicated.

expressed in high yield by any of the S30 extracts, the mutant WNVpro(Tyr132Hco) was produced poorly by the conventional S30 extract, which yielded mostly truncated WNVpro(1–131). In contrast, the $S30^{RF1-}$ extract produced about 1 mg of full-length protein per mL of reaction mixture (Figure 1, lanes 3a and 3b). For comparison, an attempt to express WNVpro(Tyr132Hco) by using the published pEVOL system[3] in vivo produced only about 30 μg of protein per mL cell culture (Figure S4 in the Supporting Information). Similar improvements in protein yield were obtained with other proteins and mutants (Figure S5 in the Supporting Information).

Interestingly, the use of the $S30^{RF1\text{-}CBD}$ extract already improved the production of full-length protein without treatment with chitin (Figure 1). This effect appears to be due to precipitation of RF1-$CBD_3$ during S30-extract preparation, because western blots of conventional S30 extract and $S30^{RF1\text{-}CBD}$ revealed significantly higher concentrations of release factor in the conventional S30 extract (Figure S6 in the Supporting Information).

The improved yields of UAA incorporation obtained by the $S30^{RF1-}$ extract facilitate the production of proteins containing UAAs at multiple positions in a single protein. To demonstrate this feature we incorporated 4-trifluoromethyl phenylalanine (tfmF) simultaneously in positions 86, 112, 120, and 160 of WNVpro. The side chains of all four residues are highly solvent-exposed in the crystal structures of the protease.[18–20] The unnatural amino acid tfmF was incorporated by using the enzyme *p*CNF-RS.[21,22] One-dimensional $^{19}F$ NMR spectroscopy revealed three signals for the 86/112/120/160 quadruple mutant, with the central signal being two times more intense than the outer signals. To assign the resonances to individual tfmF residues, we produced the two additional double mutants 86/112 and 86/120. These three samples allowed the assignment of the four tfmF signals to the corresponding residues (Figures 2 and 3).

The samples used for the spectra of Figure 2 were produced by cell-free protein synthesis from linear PCR-amplified DNA.[25] The ease with which multiple amber stop codons can be placed into a gene at different positions by

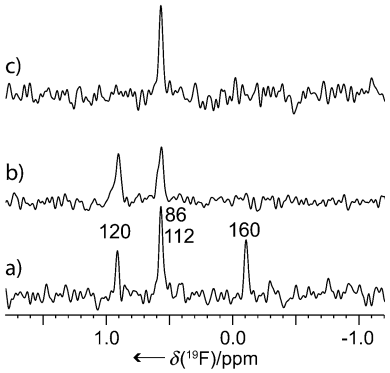


***Figure 2.*** 1D $^{19}F$ NMR spectra of WNVpro with combinatorial tfmF labeling. The spectra were recorded at 25 °C in a buffer of 90% $H_2O$/10% $D_2O$ containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.9, and 1 mM tris-(2-carboxyethyl)phosphine (TCEP) on an Agilent 400 MHz NMR spectrometer with a room-temperature probehead that uses an outer coil for $^{19}F$. The enzyme was inhibited by a fivefold excess of *p*-nitrophenyl-*p*-guanidino benzoate.[23] The spectra were calibrated relative to an external reference of free tfmF. a) Quadruple mutant with tfmF at positions 86/112/120/160. The assignments are indicated. The protein concentration was 8 μM, total recording time four hours. b) Double mutant 86/120. 30 μM, 0.5 h. c) Double mutant 86/112. 20 μM, 0.5 h.

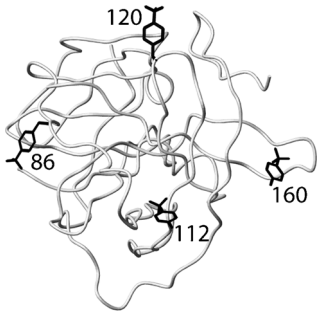


***Figure 3.*** Sites of tfmF incorporation into WNVpro. The tfmF residues were modeled onto the crystal structure of WNVpro (PDB ID: 2FP7).[18] All tfmF residues are located at least 15 Å from the active site. The figure was created with Molmol.[24]

  

PCR enables the use of combinatorial labeling, where $N$ samples allow the assignment of up to $2^N$-1 NMR signals,[26] provided that the protein structure tolerates the simultaneous presence of $2^{(N-1)}$ UAAs. In the case of WNVpro, the sample with four tfmF residues started to precipitate during sample preparation and NMR spectroscopy measurements, whereas the double mutants were perfectly stable.

The unnatural amino acid tfmF is particularly promising in protein NMR spectroscopy, owing to the high sensitivity of $^{19}$F NMR spectroscopy, the absence of fluorine from biomacromolecules, and the specific properties of the trifluoromethyl group, which allows the observation of $^{19}$F NMR spectra for large proteins in vitro and proteins in living cells.[27,28,33] In the case of WNVpro, the $^{19}$F signal of the tfmF residue at position 120 proved to be a probe for ligand binding. Figure 4 shows that in the absence of an inhibitor, only the signal of

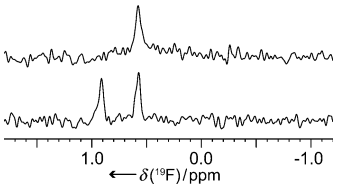


***Figure 4.*** 1D $^{19}$F NMR spectra of the WNVpro(86/120) double mutant with (bottom) and without inhibitor (top). The spectra were acquired at 25 °C of 30 μM protein solutions under the same conditions as described in Figure 2. Each spectrum took 0.5 h to record on a 400 MHz NMR spectrometer.

residue tfmF86 can be observed, whereas the signal of residue tfmF120 is broadened beyond the detection limit. WNVpro is known to display severe line broadening in large parts of the protein owing to conformational exchange, which can be suppressed by inhibitors bound to the substrate binding site.[17] Residue 86 is located in the most stable part of the protein, thus explaining why the resonance of residue tfmF86 is insensitive to the conformational exchange, whereas the appearance of the resonance of tfmF120 indicates inhibitor binding. The signal of tfm86 can thus serve as a control for the integrity of the protein and the experiment.

In conclusion, the removal of RF1 from an *E. coli* cell extract offers a generic approach to poor expression yields with UAAs. This approach enables 1) the incorporation of UAAs at locations that otherwise do not accept UAAs by conventional cell-free or in vivo methods, 2) the incorporation of UAAs at multiple positions of the target protein without any need for orthogonal ribosomes[29] or extensively reengineered *E. coli* strains,[6,13,14] 3) the use of constructs without C-terminal purification tags, since truncation products are suppressed, and 4) the production of proteins with UAAs that are difficult to incorporate. For example, Hco is among the unnatural amino acids that incorporates least well.[3] This property may in part explain the scarcity of experimental articles ($<10$) reporting the successful incorporation of Hco into proteins, despite the enormous potential of this UAA and its publication more than five years ago.[16]

For many practical reasons, our approach to the incorporation of UAAs employs cell-free protein synthesis. The advantages include the facile incorporation of amber stop codons, because proteins can be made from linear DNA produced by PCR; the sparing use of the UAAs;[30,31] and the easy optimization of the concentrations of different reagents for best expression yields. CECF systems are particularly well-suited for producing the small quantities of dual-tagged proteins required for distance measurements by electron paramagnetic resonance (EPR) spectroscopy.[32] The simple preparation of amber mutants by PCR also opens the door to combinatorial labeling of proteins with multiple tfmF residues for $^{19}$F NMR resonance assignments with a minimal number of samples, as demonstrated above. Such samples are highly attractive for screening compound libraries for specifically binding ligands. High throughput could be achieved with a dedicated $^{19}$F probehead.[33]

## *Experimental Section*

Materials: *E. coli* strain BL21 Star (DE3) (Invitrogen) was genomically modified to BL21 Star (DE3)::RF1-CBD$_3$ to prepare the S30 extracts S30$^{RF1\text{-}CBD}$ and S30$^{RF1-}$ (see the Supporting Information). Conventional S30 extract was prepared by using BL21 Star (DE3).[34] CouRS-D8 and *p*CNF-RS were cloned into the pETMCSIII vector with N-terminal His$_6$ tag[35] and produced as described in the Supporting Information. Total tRNA containing optimized suppressor tRNA was prepared following a described procedure.[36] Hco was synthesized as described.[37] The UAA tfmF and 4-nitrophenyl-4-guanidinobenzoate were purchased from PepTech and Sigma Aldrich, respectively.

Cell-free protein synthesis: CECF reactions were carried out at 30 °C for 14 h as described.[25,34,38] The final concentrations were 0.263 mg mL$^{-1}$ total tRNA containing optimized suppressor tRNA[3] in the inner buffer, 1 mM UAA (inner and outer buffer), and 0.14 mg mL$^{-1}$ CouRS-D8 and 0.84 mg mL$^{-1}$ *p*CNF-RS (inner buffer) for the experiments with Hco and tfmF, respectively. Reaction volumes were 0.2 mL (inner buffer) and 2 mL (outer buffer) for incorporating Hco into different amber mutants. Each sample for NMR spectroscopy of multiply tfmF-labeled WNVpro was prepared using 1.2 mL inner buffer and 12 mL outer buffer. Wild-type WNVpro[39] and amber mutants of WNVpro and sortase A were produced from linear PCR-amplified DNA templates;[25] WNVpro-(Trp53Hco),[36] WNVpro(Tyr132Hco),[36] WNVpro(His87Hco), and ERp29(Gly147Hco) were expressed from plasmid DNA. All genes were under control of the T7 promoter. The identity of WNVpro-(Tyr132Hco), the tfmF double mutants of WNVpro, and WNVpro(1–131) was confirmed by mass spectrometry (Figures S7 and S8 in the Supporting Information). The yields of unpurified proteins containing Hco were determined by quantifying the band intensities in the Coomassie blue stained SDS-PAGE gels and in the fluorescence images of the SDS-PAGE gels using the program ImageJ[40] and by a subsequent comparison with the known concentration of the carbonic anhydrase (30 kDa) in the lane with the low-molecular-weight markers (Table S3 in the Supporting Information).

Preparation of samples for NMR spectroscopy: The three WNVpro mutants with tfmF in multiple positions were prepared with C-terminal His$_6$ tags for purification over Ni-NTA spin columns (Nalge Nunc International USA). The eluted proteins were washed with buffer used for NMR spectroscopy (20 mM MES, pH 6.9, 1 mM TCEP in 90 % $H_2O$/10 % $D_2O$) and concentrated to a final volume of 0.5 mL by ultrafiltration using Amicon centrifugal filter devices (10000 MWCO). The protein concentrations of the samples for NMR

  

spectroscopy were determined by UV absorption, using the absorption coefficient $\varepsilon_{280} = 55\,920\,\text{M}^{-1}\,\text{cm}^{-1}$.

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# Communications

## Artificial Proteins

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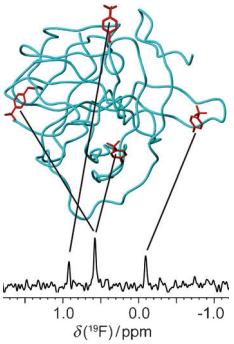
Multiple-Site Labeling of Proteins with Unnatural Amino Acids




**A cell-free protein synthesis system** from which the release factor RF1 has been selectively removed enables the facile incorporation of unnatural amino acids into proteins at difficult and multiple sites by optimized use of orthogonal tRNA/aminoacyl-tRNA synthetase systems. $^{19}$F NMR spectroscopy of a protein labeled combinatorially with trifluoromethyl phenylalanine (red in picture) at multiple sites establishes resonance assignments with a minimal number of samples.