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A Kinetic Safety Gate Controlling the Delivery of Unnatural Amino Acids to the Ribosome

Joerg Mittelstaet,[†] Andrey L. Konevega,[†,‡] and Marina V. Rodnina*[,†]

[†]Department of Physical Biochemistry, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

[‡]Molecular and Radiation Biophysics Department, B. P. Konstantinov Petersburg Nuclear Physics Institute, 188300 Gatchina, Russia

Supporting Information

ABSTRACT: Improving the yield of unnatural amino acid incorporation is an important challenge in producing novel designer proteins with unique chemical properties. Here we examine the mechanisms that restrict the incorporation of the fluorescent unnatural amino acid εNH_2-Bodipy576/589-lysine (BOP-Lys) into a model protein. While the delivery of BOP-Lys-tRNA^{Lys} to the ribosome is limited by its poor binding to elongation factor Tu (EF-Tu), the yield of incorporation into peptide is additionally controlled at the step of BOP-Lys-tRNA release from EF-Tu into the ribosome. The unnatural amino acid appears to disrupt the interactions that balance the strength of tRNA binding to EF-Tu-GTP with the velocity of tRNA dissociation from EF-Tu-GDP on the ribosome, which ensure uniform incorporation of standard amino acids. Circumventing this potential quality control checkpoint that specifically prevents incorporation of unnatural amino acids into proteins may provide a new strategy to increase yields of unnatural polymers.

INTRODUCTION

The discovery of how to reprogram the translation machinery from using the standard proteinogenic amino acids to unnatural amino acids is a major breakthrough development of synthetic biology in recent years.[1−4] Utilization of unnatural amino acids provided tools to produce designer proteins with novel functions for numerous diverse applications in protein science and biotechnology. However, despite the great potential for the development of new materials and therapeutics, progress in the synthesis of designer proteins is often limited by the low yield of unnatural amino acid incorporation during mRNA translation on the ribosome. In addition to restricting the yield of synthesis, impaired delivery of unnatural amino acids to the ribosome may cause undesired translation pausing that may lead to ribosome drop-off or altered cotranslational protein folding.[5,6] To solve these problems, it is of eminent importance to understand the reasons for impaired incorporation of unnatural amino acids and to find ways to improve yields to the levels attained by the standard amino acids.

Protein biosynthesis is evolutionarily optimized for the incorporation of the standard amino acids[7,8] and for the rejection of incorrect substrates.[9] Aminoacyl-tRNA (aa-tRNA) synthetases, which charge tRNAs with their cognate amino acids, and the ribosome, which selects cognate aa-tRNAs according to the codon in the decoding site, employ sophisticated mechanisms to recognize the cognate substrates and discriminate against the bulk of noncognate ones. The same mechanisms that contribute to the fidelity of tRNA aminoacylation and protein synthesis may limit the incorporation of unnatural amino acids. To overcome the substrate-specificity of aminoacylation, a number of orthogonal aa-tRNA synthetase/tRNA pairs have been designed to change the substrate specificity of a tRNA from its cognate amino acid to an unnatural amino acid. Examples are the leucyl-tRNA synthetase/tRNA^{Leu} pair[10] or the pyrrolysyl-tRNA synthetase/tRNA^{Pyl} pair,[11] which were engineered to accept a broad spectrum of unnatural amino acids. Furthermore, unnatural aa-tRNA (uaa-tRNA) can be produced using the flexizyme technology which allows to charge tRNAs with the help of an RNA enzyme.[1] In general, the efficiency of orthogonal aminoacylation is very good; however, the following steps on the way of unnatural amino acids incorporation into peptides are often inhibited. Inefficient incorporation may be due to impaired binding of uaa-tRNA by elongation factor Tu (EF-Tu).[12] To overcome the low affinity of EF-Tu for uaa-tRNAs, the amino acid binding pocket of EF-Tu can be altered to remove steric clashes with bulky unnatural amino acids.[13−15] On the ribosome, uaa-tRNA may interfere with the conformational changes of EF-Tu on the ribosome that are induced by correct codon-anticodon recognition and lead to the GTPase activation of EF-Tu. Following GTP hydrolysis, the release of uaa-tRNA from EF-Tu and the accommodation of uaa-tRNA in the A site might be impaired. Finally, unnatural amino acids may be slow in peptide bond formation. Previous work suggested that aa-tRNA selection is not the primary step governing the amino acid specificity of the ribosome.[16] In fact, synthetically evolved orthogonal ribosomes have been generated which show improved decoding of UAG stop codons[4] or quadruplet codons[17] by unnatural amino acids. Thus, the reasons for inefficient incorporation of unnatural amino acids remain elusive.

Here we have analyzed the hurdles for the incorporation of a fluorescent analog of lysine, εNH_2-Bodipy576/589-lysine

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(BOP-Lys; Figure 1a). We have chosen BOP-Lys because it can be incorporated into proteins, albeit with low yield, and proved

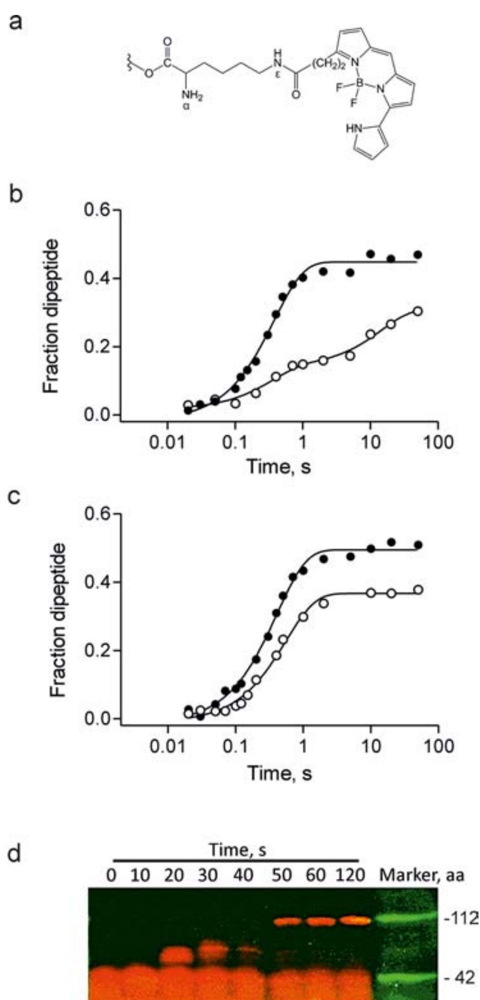

Figure 1. Incorporation of BOP-Lys into peptides. (a) Chemical formula of BOP-Lys. (b,c) Formation of fMet-BOP-Lys (○) and fMet-Lys (●) dipeptides in the presence of EF-Tu at low (0.2 μM; (b)) or high (4 μM; (c)) concentration. Thin lines represent the following fitting results: a double-exponential fit with 2.9 ± 0.9 s^{-1} and 0.07 ± 0.02 s^{-1} for BOP-Lys (b) or single-exponential fits with rate constants of 2.7 ± 0.2 s^{-1} (fMet-Lys; (b)), 1.8 ± 0.1 s^{-1} (BOP-Lys; (c)) and 2.4 ± 0.1 s^{-1} (fMet-Lys; (c)). (d) Translation of the N-terminal fragment (112 amino acids) of PrmC followed by BOP fluorescence (red). BOP-Lys is incorporated at a Lys codon (AAA) at position 34. The wide band at the bottom of the gel (at and below the 42 amino acids marker band) is due to the bromphenol blue dye. The bands at short incubation times (20−40 s) are translational intermediates (with the length between 42 and 112 aa) which transiently accumulate before the final product is synthesized after 50 s . Markers are PrmC peptides of specified length (in amino acids) obtained by in vitro translation and visualized by the fluorescent reporter BODIPY-FL attached to the N-terminus of the peptides.[22]

to be a useful tool in studies of cotranslational protein folding and membrane targeting.[18] BOP-Lys can be considered an example of a bulky, hydrophobic unnatural amino acids that may impair (i) binding to EF-Tu, (ii) any of the reactions during the delivery of uaa-tRNA to the ribosome, or (iii) peptide bond formation (Supplementary Figure 1, Supporting Information). We have investigated each of these steps in a fully reconstituted translation system in real time by rapid kinetics monitoring the fluorescence of the BOP group as well as GTP hydrolysis by EF-Tu and peptide bond formation.

■ RESULTS

Inefficient BOP-tRNA^{Lys} Binding by EF-Tu. To test whether BOP-Lys-tRNA^{Lys} is incorporated into proteins with the same velocity as unmodified Lys-tRNA^{Lys}, we measured the rates of peptide bond formation on ribosomes with fMet-tRNA^{fMet} in the P site and a cognate lysine codon (AAA) in the A site. EF-Tu-GTP was formed in excess of GTP, incubated with either BOP-Lys-tRNA^{Lys} or Lys-tRNA^{Lys} to form the ternary complex, and then rapidly mixed with the ribosomes in a quench-flow apparatus. The concentration of EF-Tu used was saturating for the incorporation of the natural amino acid; the reaction with Lys-tRNA^{Lys} was monophasic because preformed ternary complexes were rapidly delivered to the ribosome and Lys incorporated into dipeptides; the reaction was complete within 1 s (k_{pep} = 2.7 s^{-1}). In contrast, the reaction with the BOP-Lys-tRNA^{Lys} ternary complex was much slower (Figure 1b). The time courses with BOP-Lys-tRNA^{Lys} were biphasic with an initial rapid step of 2.9 s^{-1}, similar to the rate of peptide bond formation with the canonical substrate, and a slow step of 0.07 s^{-1}, much slower than the average incorporation rate of most natural amino acids.[19−21]

The fast phase was likely due to a burst of preformed ternary complex reacting rapidly with the ribosome, while the slow phase may represent the slow formation of active ternary complex caused by the shift in equilibrium after the consumption of the preformed ternary complex.[12] To test whether this was the case for BOP-Lys-tRNA, we measured the rate of peptide bond formation at very high concentration of EF-Tu, much higher than the saturating concentration required for Lys-tRNA^{Lys} delivery. This increased the incorporation rate of BOP-Lys-tRNA^{Lys} to 1.8 s^{-1} (Figure 1c), which is close to the rate of 2.4 s^{-1} measured for the natural amino acid under the same conditions and comparable to the average rate of protein biosynthesis in our in vitro translation system (~3−5 s^{-1}). This indicates that the concentration of EF-Tu is an important parameter for the incorporation of unnatural amino acids, in line with a recent report.[12] This was further verified in an in vitro translation assay. 70S ribosomes were programmed with an mRNA coding for the 112 amino acid-long N-terminal fragment of PrmC with a Lys codon at position 34. The initiation complexes were mixed with EF-Tu, purified amino-acyl-tRNAs including BOP-Lys-tRNA^{Lys}, EF-G, and GTP and the appearance of the peptide products monitored over time (Figure 1d). Translation products released from tRNA were separated by Tris-Tricine SDS-PAGE[22] and visualized using BOP fluorescence. Using an excess of EF-Tu over total aa-tRNA allowed for the efficient incorporation of BOP-Lys, indicating that increasing EF-Tu concentration in vitro or in vivo may improve the efficiency of unnatural amino acids incorporation into proteins.

Restoring the Stability of the Ternary Complex. One approach to overcome the low affinity of the interaction of EF-Tu with uaa-tRNAs containing bulky aromatic side chains is to replace residues E215 and D216 in the amino acid binding pocket of EF-Tu with alanines (Figure 2a).[15] We prepared those EF-Tu mutants with single (E215A or D216A) and double (E215A/D216A) replacements and measured the affinity of BOP-Lys-tRNA binding (K_d) and the kinetic parameters for the formation (k_{on}) and dissociation (k_{off}) of the ternary complex. Upon rapid mixing of EF-Tu-GTP with

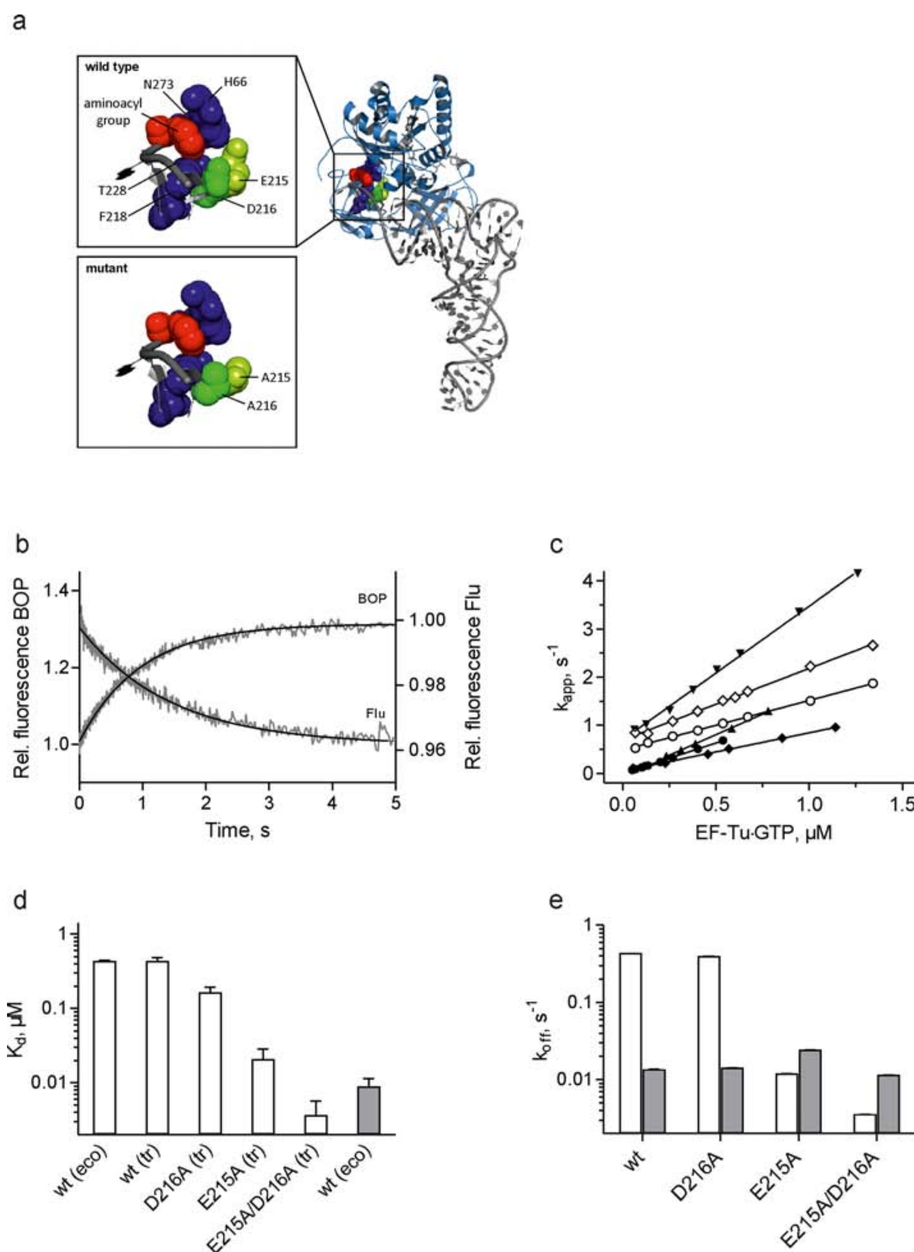

Figure 2. Effect of replacements in the amino acid binding pocket of EF-Tu on the interaction with BOP-Lys-tRNA^{Lys}. (a) Residues at the amino acid binding pocket of EF-Tu (from *E. coli*, PDB 1OB2). The amino acid of Phe-tRNA^{Phe} is shown in red. Mutations E215A and D216A may enlarge the binding pocket.[15] (b) Time-resolved fluorescence change reflecting the binding of BOP-Lys-tRNA^{Lys} (0.05 μM) (BOP, left axis) or Lys-tRNA^{Lys}(Flu) (0.05 μM) (Flu, right axis) to EF-Tu(wt) (0.8 μM). Thin lines represent single-exponential fits. (c) Concentration dependence of k_{app} values estimated for BOP-Lys-tRNA^{Lys} (native tRNA^{Lys}) binding to EF-Tu(wt) (○); BOP-Lys-tRNA^{Lys} (unmodified transcript) to EF-Tu(wt) (◊), EF-Tu(E215A) (♦), EF-Tu(D216A) (▼), and EF-Tu(E215A/D216A) (▲); and Lys-tRNA^{Lys}(Flu) binding to EF-Tu (wt) (●). (d) Equilibrium dissociation constants (K_d) of ternary complexes formed with BOP-Lys-tRNA^{Lys} (white bars) and Lys-tRNA^{Lys}(Flu) (gray bar) using native tRNA^{Lys} (eco) or tRNA transcript (tr). (e) Dissociation rate constants (k_{off}) of EF-Tu complexes formed with BOP-Lys-tRNA^{Lys} (white bars) and Lys-tRNA^{Lys}(Flu) (gray bars).

BOP-Lys-tRNA^{Lys} in a stopped-flow apparatus, the fluorescence of BOP increased in a single-exponential manner (Figure 2b). The apparent rate constants of the reaction (k_{app}) increased linearly with the concentration of EF-Tu-GTP (Figure 2c), suggesting a simple one-step reversible binding mechanism. The k_{on} and k_{off} values were determined from the slope and *Y*-axis intercept, respectively, of the linear concentration dependence. The K_d values were estimated from the concentration dependence of fluorescence amplitudes (Figure 2d, Supplementary Figure 2a−d, Supporting Information) or calculated from the k_{off}/k_{on} values (Supplementary Table 1, Supporting Information). In addition, the k_{off} values were measured in independent dissociation experiments (Figure 2e, Supplementary Figure 2e,f, Supporting Information) in which the release of uaa-tRNA from the ternary complex was initiated by rapid mixing with a large excess of unlabeled aa-tRNA. We note that the binding parameters of native, fully modified tRNA^{Lys} and of unmodified tRNA^{Lys} prepared by in vitro transcription were very similar, which allowed us to use the tRNA^{Lys} transcript in those cases where using the native tRNA would be prohibitively

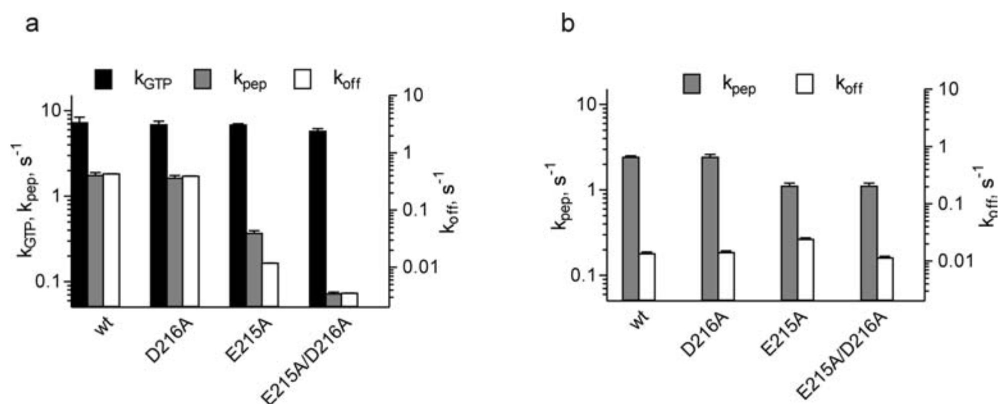

Figure 3. Rates of reactions on the ribosome. The rates of GTP hydrolysis (k_{GTP}, black bars) and peptide bond formation (k_{pep}, gray bars) upon interaction of BOP-Lys-tRNA^{Lys}-EF-Tu-GTP (a) or Lys-tRNA^{Lys}-EF-Tu−GTP (b) with ribosomes carrying fMet-tRNA in the P site and displaying a Lys codon (AAA) in the A site. The rate of dissociation from unbound EF-Tu (k_{off}, white bars) is shown for comparison.

expensive, e.g., in titration experiments. However, crucial parameters, such as the k_{off} values, were always additionally verified with native tRNA^{Lys}. EF-Tu carrying the E215A or E215A/D216A mutations exhibited increased affinity for BOP-Lys-tRNA^{Lys}, whereas the D216A mutation alone had little effect. The change in the K_d values was mainly due to the k_{off} effect, while the k_{on} values were similar (Supplementary Table 1, Supporting Information).

For comparison, the binding affinities and kinetics of interaction with EF-Tu were measured with native Lys-tRNA^{Lys} labeled with fluorescein (Flu) at acp^{3}U47 (3-(3-amino-3-carboxypropyl)uridine). acp^{3}U47 is located in the variable region of tRNA which does not interact with EF-Tu in the ternary complex;[23] hence, modifications at position 47 do not interfere with complex formation.[24] The fluorescence of Flu decreased upon ternary complex formation in a single-exponential fashion (Figure 2b) with the k_{app} values increasing linearly with the concentration of EF-Tu (Figure 2c). The affinity of wild-type (wt) EF-Tu for Lys-tRNA^{Lys} was 30-fold higher than that for BOP-Lys-tRNA^{Lys}, which was mainly due to a slower dissociation of the complex with natural Lys-tRNA^{Lys} (Figure 2d, Supplementary Tables 1 and 2, Supporting Information). The affinity of EF-Tu(E215A/D216A) binding to BOP-Lys-tRNA^{Lys} was similar to that of EF-Tu(wt) to Lys-tRNA^{Lys} (see below), i.e., in the <10 nM range, due to similarly low dissociation rates. The E215A and D216A mutations in EF-Tu did not affect the dissociation rate of Lys-tRNA^{Lys} (Figure 2e; Supplementary Table 2, Supporting Information). Thus, mutations of critical residues in the amino acid-binding pocket of EF-Tu can greatly stabilize the binding of uaa-tRNA to EF-Tu both kinetically and thermodynamically.

Identification of Steps Restricting BOP-Lys Incorporation into Proteins. Because the binding of BOP-Lys-tRNA^{Lys} to EF-Tu(E215A/D216A) was almost as efficient as that of EF-Tu(wt) to native Lys-tRNA^{Lys}, we tested whether the two complexes performed equally well on the ribosome. During decoding, aa-tRNAs can be rejected prior to GTP hydrolysis by EF-Tu ("initial selection") or after GTP hydrolysis prior to peptide bond formation ("proofreading") (Supplementary Figure 1, Supporting Information). To test the performance of BOP-tRNA^{Lys} on the ribosome, we measured the rates of GTP hydrolysis (k_{GTP}) and peptide bond formation (k_{pep}) at saturating concentrations of either BOP-Lys-tRNA^{Lys} or EF-Tu. The concentration of the limiting component, which defined the actual concentration of ternary complex, was 0.1 μM. For maximum sensitivity, the concentration of the ribosomes used in these experiments, 0.3 μM, was chosen to be in the linear range of the concentration dependence (k_{cat}/K_M conditions). In the complex of BOP-Lys-tRNA^{Lys} with EF-Tu(wt) or any of the three EF-Tu mutants, the rates of GTP hydrolysis were essentially identical (Figure 3a, Supplementary Figure 3a, Supplementary Table 1, Supporting Information). Thus, GTP hydrolysis by EF-Tu on the ribosome as well as the step(s) preceding GTP hydrolysis (initial binding, codon reading, and codon recognition) were not appreciably affected by the presence of unnatural amino acids or the mutations in the amino acid-binding pocket of EF-Tu.

By contrast, the rates of peptide bond formation with BOP-Lys-tRNA^{Lys} were strongly affected by the mutations in EF-Tu, even when the concentration of EF-Tu was saturating, and reduced to about 0.4 s^{-1} with EF-Tu(E215A), or to 0.07 s^{-1} with EF-Tu(E215A/D216A) (Figure 3a, Supplementary Figure 3b, Supplementary Table 1, Supporting Information). The effect on k_{pep} correlated with the reduced dissociation rate (k_{off}) of BOP-Lys-tRNA^{Lys} from EF-Tu-GTP in solution. Notably, the effect is specific for BOP-Lys-tRNA^{Lys}, because both k_{pep} and k_{off} of Lys-tRNA^{Lys} are largely insensitive to the replacements in the amino acid binding pocket of EF-Tu (Figure 3b). Thus, mutations at the amino acid binding pocket that rescued the binding of uaa-tRNA to EF-Tu allowed for rapid recruitment of the complex to the ribosome and efficient GTPase activation, but led to inhibition of unnatural amino acids incorporation into the protein at a later step, presumably due to slow release of BOP-Lys-tRNA^{Lys} from EF-Tu after GTP hydrolysis. This is tested in the following.

Release of aa-tRNA from EF-Tu on the Ribosome. To dissect which steps impair BOP-Lys incorporation in the presence of tightly binding EF-Tu mutants, we first used a FRET assay in which the EF-Tu-GTP-BOP-Lys-tRNA^{Lys} complex was rapidly mixed with ribosomes containing fluorescent αBodipyFL-Met-tRNA^{fMet} (BOF-Met-tRNA^{fMet}) in the P site and a Lys codon in the A site; the replacement of the formyl group of initiator tRNA by BodipyFL does not affect the functional activity of the tRNA,[22] including the rate of peptide bond formation (Wohlgemuth, I.; Holtkamp, W.; Rodnina, M. V., unpublished results). The Förster radius (R_0) for the donor−acceptor pair used in these experiments was 54 Å, assuming freely rotating fluorophores. Before accommodation, the BOP group (FRET acceptor) of BOP-tRNA^{Lys} is bound to EF-Tu in the A/T state and is located approximately 90 Å away

from the peptidyltransferase center where the BOF label (FRET donor) is located (Figure 4a); at such a large distance, a small FRET efficiency <0.05 is expected. The accommodation of the acceptor-labeled tRNA in the A site would bring the two fluorophores into close proximity, resulting in a FRET efficiency of close to 1 as indicated by a large decrease of the donor fluorescence. Thus, a major decrease of donor fluorescence likely reflects the movement of BOP-Lys-tRNA^{Lys} into the A site following its release from EF-Tu. In fact, the fluorescence of BOF decreased upon rapid mixing of ribosome complex with ternary complex containing BOP-Lys-tRNA^{Lys} (Figure 4b) in a EF-Tu-dependent manner (Supplementary Figure S4a, Supporting Information). To compare the kinetics of peptide bond formation with the observed changes of FRET, the incorporation of BOP-Lys into the dipeptide was plotted against the fluorescence change of the donor (Figure 4b). With EF-Tu(wt) and EF-Tu(D216A), both accommodation, as measured by FRET, and peptide bond formation were rapid. With EF-Tu(E215A) or EF-Tu(E215A/D216A) the accommodation was slower by about 5- or 25-fold, respectively, but in both cases the kinetics of accommodation coincided with the kinetics of peptide bond formation. This indicates that either accommodation itself or a step between GTP hydrolysis (which was not affected by the mutations in EF-Tu) and accommodation limits the rate of BOP-Lys incorporation, whereas the chemistry of peptide-bond formation is not appreciably affected by the unnatural amino acid.

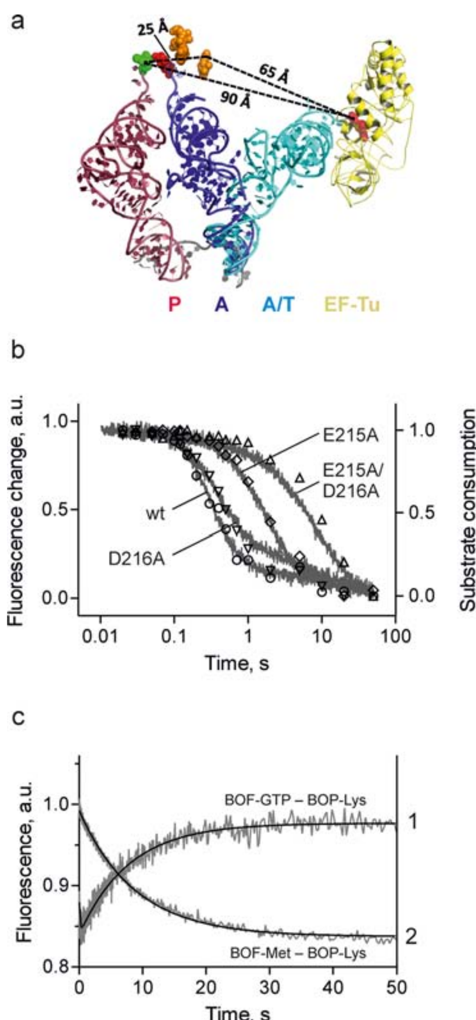

Figure 4. Safety gate at the step of BOP-Lys-tRNA^{Lys} release from EF-Tu to the ribosome. (a) Schematic of movement of aa-tRNA from the EF-Tu-bound A/T state to the A/A site of the ribosome. EF-Tu (yellow), aa-tRNA in the A/T state (cyan) and in the A/A state (dark blue), peptidyl-tRNA (magenta); mRNA (gray). The 3′ends of aa-tRNA and peptidyl-tRNAs are highlighted to show the approximate positions of the acceptor (red) and the donor (green) FRET pairs. Bases C2556, U2492, and C2573 of 23S rRNA forming the accommodation gate[25] are highlighted in orange. The picture was generated from[26] (PDB: 2WRN, 2WRO), except for aa-tRNA in the A/A state which was taken from[27] (PDB: 1JGQ) and aligned using 16S rRNA. (b) Quenching of the donor (BOF) fluorescence upon binding of the ternary complex of EF-Tu-GTP (4 μM) with BOP-Lys-tRNA^{Lys} (0.1 μM) to initiation complexes containing BOF-Met-tRNA^{fMet} in the P site (0.3 μM) (left *Y*-axis, stopped-flow traces). Consumption of substrate (BOP-Lys-tRNA) upon peptide bond formation at the same concentrations (right axis, symbols). Left to right: EF-Tu(wt) (○), EF-Tu(D216A) (▽), EF-Tu(E215A) (◊), EF-Tu(E215A/D216A) (Δ). (c) Release of BOP-Lys-tRNA^{Lys} (0.1 μM) from EF-Tu(E215A/D216A)-BOF-GTP (4 μM) upon binding to initiation complexes (0.3 μM) monitored by FRET (trace 1); the rate is 0.13 ± 0.01 s^{-1}. The accommodation measured by BOF-to-BOP FRET (0.11 ± 0.01 s^{-1}) is shown for comparison (trace 2, taken from b).

To identify which step is impaired, we analyzed the release of BOP-Lys-tRNA^{Lys} from EF-Tu(E215A/D216A), this time measuring FRET between BOF-GTP bound to EF-Tu and BOP-Lys-tRNA^{Lys}. In the ternary complex, BOF-GTP (FRET donor) and BOP-Lys (FRET acceptor) on the tRNA are located in close proximity (approximately 25 Å apart) which is expected to result in an efficient FRET signal ($E \approx 0.99$). Upon release of the tRNA from EF-Tu on the ribosome, aa-tRNA moves into the A site while EF-Tu-GDP dissociates from the ribosome.[28] As a result, the donor fluorescence should increase because the two FRET labels move apart, which is exactly what is observed when both donor and acceptor are present (Figure 4c).[28] The rate of tRNA release from EF-Tu(E215A/D216A) was 0.13 s^{-1}, which was very similar to the rate of accommodation, 0.11 s^{-1} (Figure 4c), measured at the same concentrations of components, and to the rate of peptide bond formation, 0.07 s^{-1} (Supplementary Table 1, Supporting Information). The observed effect was due to a separation between the two reporters because binding of the ternary complex of EF-Tu–BOF-GTP with unlabeled Lys-tRNA (donor alone control) to the ribosome results in a very slow fluorescence decrease (0.02 s^{-1}) (Supplementary Figure S4b, Supporting Information), which is due to dissociation of BOF-GDP from EF-Tu in solution, whereas acceptor alone showed essentially no signal in the absence of BOF. The similarity of the rates of tRNA release from EF-Tu, accommodation, and peptide bond formation indicates that the incorporation of BOP-Lys from the ternary complex with EF-Tu(E215A/D216A) is limited by the release of BOP-Lys-tRNA from EF-Tu, whereas the rates of the following steps of accommodation and peptide bond formation are comparable to the rates observed with natural amino acids.

■ DISCUSSION

Our results suggest the existence of two independent control mechanisms related to EF-Tu function that may impair the incorporation of unnatural amino acids into peptides. An uaa-tRNA may be excluded from translation because it is impaired either in binding to EF-Tu or in the release from EF-Tu on the ribosome. EF-Tu has evolved to bind all natural aa-tRNAs

uniformly by idiosyncratic tuning of the individual contributions of the tRNA and amino acid moieties to the overall binding affinity.[7] With the unnatural amino acid used here, the affinity of EF-Tu binding is decreased 50-fold, which disfavors the delivery of unnatural amino acids to the ribosome. Similarly, bulky, hydrophobic unnatural amino acids were suggested to cause steric clashes within the amino acid binding pocket of EF-Tu, thereby lowering the affinity of uaa-tRNA binding, compared to natural aa-tRNAs.[12,15,29]

Identification of EF-Tu as a potential cause for inefficient incorporation of unnatural amino acids into proteins suggests several strategies for improving the yields of modified peptides. In the simplest case, the unnatural amino acid incorporation may be enhanced by increasing the effective concentration of EF-Tu by, e.g., overexpression (in vivo) or adding EF-Tu in large excess over aa-tRNA (in vitro). Notably, if the concentration of EF-Tu is limiting, e.g., as in *E. coli* where the concentrations of EF-Tu and of natural aa-tRNAs are similar and hence little EF-Tu is free under conditions of normal growth,[30] the addition of excess uaa-tRNA is unlikely to improve the utilization of unnatural amino acids because natural aa-tRNA will efficiently compete with the unnatural ones for the EF-Tu binding. Alternatively, the increase in affinity of uaa-tRNA binding may be achieved by lowering the incubation temperature,[12] as the K_d values of aa-tRNA binding to EF-Tu decrease with temperature.[31] Finally, the affinity of uaa-tRNA to EF-Tu may be improved by introducing mutations.[14,15] Our results show that the latter strategy may backfire: If the stabilization of uaa-tRNA binding to EF-Tu is achieved by reducing the k_{off} value of the complex (as we find for the E215A and E215A/D216A mutants), the tight binding may result in an inefficient release of uaa-tRNA from EF-Tu-GDP on the ribosome, thereby impairing the incorporation of unnatural amino acids into the protein by yet another mechanism. The E215A or D216A replacements in EF-Tu, which resulted in a moderate affinity increase, improved the incorporation of L-2-anthraquinonylalanine, L-2-pyrenylalanine and L-1-pyrenylalanine into protein, whereas the double mutant showed no activity,[15] in line with the present findings for BOP-Lys.

One attractive alternative strategy is to search for EF-Tu mutants with an improved uaa-tRNA binding due to faster recruitment to EF-Tu, rather than slower dissociation of the complex, or to engineer tRNAs which would compensate for the altered affinity of unnatural amino acids to EF-Tu. The latter approach has been successfully applied to improve the delivery of unnatural amino acids by an amber suppressor, $tRNA^{Tyr}{}_{CUA}$ (ref 32). Alternatively, some amino acid replacements in EF-Tu may tune the affinity for an unnatural amino acids in a very selective way,[13] which might be useful to harmonize the contributions of the tRNA and the unnatural amino acids to the affinity of binding to EF-Tu and thus increase the yield of unnatural amino acids incorporation.

The fidelity of aa-tRNA selection on the ribosome is monitored at three stages: initial selection, proofreading, and post-transfer editing.[9,33] In principle, the incorporation of unnatural amino acids can be impaired at each of these steps. For example, unnatural amino acids could disturb the conformational transitions of EF-Tu that account for efficient, selective GTPase activation by correct codon−anticodon recognition,[34−36] thereby leading to aggressive discrimination of uaa-tRNA during the initial selection step. Our data indicate that the BOP modification or the mutations at the amino acid binding pocket of EF-Tu do not affect the GTPase activation step, although such an inhibitory effect cannot be excluded for other unnatural amino acids or EF-Tu mutations. Another potential checkpoint is the tRNA accommodation step at the proofreading stage, when the 3′end of the tRNA with amino acid moves through a narrow corridor of the large ribosomal subunit into the peptidyl transferase center.[25] Unnatural amino acids may slow down accommodation and cause enhanced drop-off of uaa-tRNAs. Although this mechanism cannot be excluded for other unnatural amino acids, our experiments with BOP-Lys and previous results with other unnatural amino acids[12] suggest that neither the accommodation step nor the following peptide bond formation are affected, in line with the notion that the ribosome is a versatile chemical catalyst that can accept a variety of substrates.[37,38] Rather, stable binding of uaa-tRNA to EF-Tu, e.g., induced by mutations in the amino acid binding pocket of EF-Tu, causes the delayed release of uaa-tRNA to the ribosome, leading to slow peptide bond formation. Thus, the release of aa-tRNA from EF-Tu appears to constitute yet another quality control checkpoint which may hinder the incorporation of unnatural amino acids into proteins.

Interactions between aa-tRNAs and EF-Tu are constrained by the need to bind tightly enough to form a stable ternary complex but weakly enough to be released from EF-Tu following GTP hydrolysis.[8] Uhlenbeck et al. carried out a systematic comparison of k_{off} and k_{pep} values for a number of aa-tRNAs, including misacylated tRNAs and tRNAs with T-stem mutations which affect the stability of binding to EF-Tu. They found a striking proportionality between k_{off} and k_{pep} over a wide range of values, which suggested that the interface between EF-Tu and aa-tRNA is thermodynamically similar both on and off the ribosome.[8] We note that the values obtained here for the native Lys-$tRNA^{Lys}$ and EF-Tu(wt) also lie on the Uhlenbeck's proportionality line. The BOP-Lys-$tRNA^{Lys}$ complex with EF-Tu(wt) or EF-Tu(D216A) fall into the part of the plot that is characteristic for weak binders, for which k_{pep} does not change with k_{off}, probably because a step other than the dissociation from EF-Tu becomes rate-limiting.[8] However, the BOP-Lys-tRNA−EF-Tu(E215A) and in particular BOP-Lys-tRNA−EF-Tu(E215A/D216A) complexes deviate dramatically from the proportionality line. While the K_d and k_{off} values of the BOP-Lys-tRNA−EF-Tu(E215A/D216A) complex are only 3-fold lower than of the native Lys-$tRNA^{Lys}$−EF-Tu(wt), the k_{pep} values of the two complexes differ by a factor of 30. At the same time, the EF-Tu mutations alone have only minor effects on the k_{off} or k_{pep} values for the complex with Lys-$tRNA^{Lys}$, suggesting a specific effect on decoding with the unnatural amino acids. Thermodynamic tuning within the ternary complex, which ensures the proportionality of k_{off} and k_{pep} for natural aa-tRNAs, is insufficient to ensure the efficient delivery of uaa-tRNA to the ribosome, implying the existence of an additional control mechanism that inhibits uaa-tRNA release from EF-Tu. Thus, the release of aa-tRNA from EF-Tu after GTP hydrolysis may constitute an additional safety gate that contributes to controlling the fidelity of tRNA selection during protein synthesis.

■ MATERIALS AND METHODS

Buffers and Reagents. All chemicals were purchased from Merck, Sigma Aldrich, and Life Technologies. Radioactive amino acids were from Hartmann Analytic and Perkin-Elmer. BOF-GTP was from Life Technologies. Messenger RNA (5′-GGCAAGGAGGUAAAUAAU-GAAA UUCGUUAC-3′, sequence coding for fMet-Lys is underlined)

was purchased from IBA. Buffer A: 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$. Buffer B: 50 mM Hepes−KOH, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 20 mM $MgCl_2$. Buffer C: 50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermidine, 8 mM putrescine, 2 mM DTT.

Preparation of fMet-tRNA^{fMet}, BOF-Met-tRNA^{fMet}, BOP-Lys-tRNA^{Lys}, and Lys-tRNA^{Lys}(Flu). fMet-tRNA^{fMet} from *E. coli* was prepared as described.[39] Met-tRNA^{fMet} was prepared in essentially the same way except that N^{10}-formyltetrahydrofolate was omitted in the aminoacylation reaction. Met-tRNA^{fMet} was modified using the sulfosuccinimidyl ester derivative of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BodipyFL or BOF, Life Technologies, D6140) following the described protocol.[40] After labeling, BOF-Met-tRNA^{fMet} was ethanol precipitated at least three times to remove the excess of unreacted free dye. Labeling was quantitative as tested by a Biosuite 250 HR (Waters) gel filtration chromatography using an isocratic elution in buffer A.

tRNA^{Lys} was prepared from *E. coli* total tRNA (Roche) by purification on Sepharose 4B and Phenyl-Sepharose.[41] Aminoacylation of tRNA^{Lys} (30 μM) was carried out in buffer B containing DTT (2 mM), ATP (3 mM), [^{14}C]Lys (40 μM), and *E. coli* Lys-tRNA synthetase (1% v/v) (the plasmid coding for LysRS was kindly provided by R. Green, Johns Hopkins University) for 1 h at 37 °C. The charged tRNA was purified by phenol extraction and ethanol precipitation. Labeling of Lys-tRNA^{Lys} with the succinimidyl ester of 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (Bodipy 576/589 or BOP, Life Technologies D2225) was performed as described.[18] εNH_2-modified Lys-tRNA^{Lys} was separated from the αNH_2- and $\alpha/\varepsilon NH_2$-modified and unlabeled species using an ethanol gradient (5−40%) on a reversed-phase C-18 resin (LiChrospher WP300, Merck).

To label Lys-tRNA^{Lys} with Flu at acp^{3}U47, tRNA^{Lys} (80 A_{260} units/mL) was incubated with 5-carboxyfluorescein succinimidyl ester (Life Technologies C2210) (4.5 mM) in Hepes-KOH pH 8.2 (0.1 M) and 70% (v/v) DMSO for 16 h at 37 °C. Free dye was removed by ethanol precipitation followed by phenol extraction. tRNA^{Lys}(Flu) was aminoacylated and purified on reversed-phase chromatography (RP-18 LiChrospher WP300, Merck) using an ethanol gradient (5−30%). After ethanol precipitation, all tRNA preparations were dissolved in H_2O and stored in aliquots at −80 °C.

Ribosomes and Translation Factors. Ribosomes from *E. coli* MRE 600, initiation factors, and EF-G were prepared as described.[39,42,43] Site-directed mutagenesis of EF-Tu to introduce the mutations E215A, D216A, and E215A/D216A was carried out following standard QuikChange site-directed mutagenesis protocols. EF-Tu (wt and mutants) was expressed and purified as described.[44]

Initiation Complex Formation. Initiation complexes were prepared in buffer A supplemented with DTT (2 mM) and GTP (1 mM) by incubating 70S ribosomes (1 μM) in the presence of IF 1, 2, and 3 (1.5 μM each), mRNA (3 μM), and f[^{3}H]Met-tRNA^{fMet} or BOF-[^{3}H]Met-tRNA^{fMet} (1.5 μM) for 30 min at 37 °C and purified by centrifugation through a sucrose cushion (1.1 M) in buffer A. Centrifugation was performed in a TLS 55 swing-out rotor in a Beckman Optima XP ultracentrifuge at 4 °C and 259000*g* for 2 h. After centrifugation, the supernatant was discarded, and the ribosome pellets were dissolved in buffer A on ice, frozen in small aliquots in liquid nitrogen, and stored at −80 °C. Initiation complex formation was monitored by nitrocellulose filtration.

EF-Tu−GTP. Complexes of EF-Tu (wt and mutants) and GTP were formed by incubating EF-Tu−GDP (5−25 μM) with GTP (1 mM), DTT (2 mM), phosophoenol pyruvate (3 mM), and pyruvate kinase (0.05 mg/mL) in buffer A at 37 °C for 15 min. The EF-Tu complex with BOF-GTP (G12411, Life Technologies) was formed in essentially the same way, except that 0.3 mM BOF-GTP and EF-Ts (0.02 μM) were present. To remove excess BOF-GTP, the complex was purified on a NAP-5 column (Sephadex G-25, GE Healthcare). EF-Tu−[γ-^{32}P]GTP containing EF-Tu(wt or mutants) (20 μM) was prepared in the same way, except that [γ-^{32}P]GTP was added (4 μL of the undiluted commercial stock solution) and the complex was purified by gel filtration on a NAP-5 column (Sephadex G-25, GE Healthcare).

Stopped-Flow Measurements. Ternary complex formation was measured in a SX-20MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK), monitoring the fluorescence changes upon rapid mixing of BOP-Lys-tRNA^{Lys} (0.05 μM) with variable amounts of EF-Tu−GTP in buffer A at 20 °C. Excitation was at 560 nm, and emission was monitored after passing a KV590 cutoff filter (Schott). Fluorescence changes were analyzed by fitting the data to a single-exponential function $F = F_\infty + A \times \exp(-k_{app}t)$. The K_d values were obtained by plotting the fluorescence amplitudes against the concentration of EF-Tu−GTP and fitting the data to a quadratic equation $C = M \times [(A + X + K_d)/2 - ((A + X + K_d)^2/4 - AX)^{1/2}]$. Association ($k_{on}$) and dissociation rates (k_{off}) (Supplementary Tables 1 and 2, Supporting Information) were determined from the linear concentration dependence of k_{app} values (Figure 2c). Alternatively, dissociation rates were obtained from chase experiments in which the dissociation of the ternary complex of EF-Tu·GTP (2 μM) with BOP-Lys-tRNA^{Lys} (0.05 μM) was induced by adding an excess (40 μM) of unlabeled total aa-tRNA; k_{off} was evaluated from the time course of fluorescence decrease by single-exponential fitting. Kinetics of the interaction of EF-Tu−GTP−BOP-Lys-tRNA^{Lys} and ribosomes with an AAA codon in the A site and BOF-[^{3}H]Met-tRNA^{fMet} in the P site, as well as the reaction of EF-Tu−BOF-GTP−BOP-Lys-tRNA^{Lys} in the presence of ribosomes with f[^{3}H]Met-tRNA^{fMet} in the P site were monitored by FRET in the stopped-flow apparatus. The donor (BOF) was excited at 470 nm and the fluorescence of the FRET donor was monitored after passing a 500-nm cutoff filter (with the 590-nm cutoff filter, e.g., at the emission wavelength of the FRET acceptor BOP, the sensitivity of the stopped-flow device is reduced). Error bars are standard deviations of measured values.

Quench-Flow Measurements. To measure GTP hydrolysis by EF-Tu on the ribosome, EF-Tu−[γ-^{32}P]GTP (0.1 μM) was incubated with BOP-Lys-tRNA^{Lys} (1 μM) for 1 min at 20 °C and rapidly mixed with initiated ribosomes (0.3 μM) in a quench-flow apparatus (KinTek) (14 μL each). Samples were quenched with 25% (v/v) formic acid and analyzed by thin-layer chromatography on polyethyleneimine−cellulose plates (CEL 300 PEI, Macherey Nagel) run in 0.5 M potassium phosphate pH 3.5.

To measure dipeptide formation, of EF-Tu−GTP−BOP-[^{14}C]-Lys-tRNA^{Lys} and initiation complexes (0.3 μM) were rapidly mixed in the quench-flow apparatus. The reaction was quenched by potassium hydroxide (0.5 M). Peptides were released by alkaline hydrolysis at 37 °C for 30 min. Subsequently, the pH was adjusted by the addition of 1/10 volume of acetic acid and all samples were brought to the same volume by the addition of trifluoroacetic acid (0.1%, v/v). Dipeptides were analyzed by reversed-phase HPLC (LiChrospher RP-8, Merck) using a two-step linear gradient from 0 to 65% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Fractions were analyzed by liquid scintillation counting (Tri-Carb 3110 TR, Perkin-Elmer). The portion of dipeptide formed was calculated from the ratio of Met-Lys/(Met+Met-Lys). The rate of peptide bond formation was determined by exponential fitting of time courses using one or two exponential terms.

In Vitro Translation. Translation of the 112 amino acid long N-terminal part of PrmC was carried out in buffer C essentially as described[22] except that no EF-P was added to the reaction mix. After temperature equilibration for 10 s at 37 °C, translation was started by the addition of ternary complexes (40 μM) and EF-G (2 μM) to the purified initiation complexes (20 nM). Reactions were stopped by flash-freezing in liquid nitrogen. The frozen samples were then thawed on ice in the presence of RNase A (0.4 mg/mL, Fermentas) and digested for 30 min at 37 °C. Samples were then incubated in loading buffer (50 mM Tris-HCl, pH 6.8, 12% (w/v) glycerol, 2% β-mercaptoethanol, 4% SDS, 0.01% bromophenol blue) at 40 °C for 30 min. Tris-Tricine SDS−PAGE was performed according to the Schägger and von Jagow protocol using a 4% stacking, 10% spacer, and 16.5% separation gel (49.5% T, 3% C).

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures 1−4 and Supplementary Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org."

■ AUTHOR INFORMATION

Corresponding Author

rodnina@mpibpc.mpg.de

Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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