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A Genetically Encoded Spin Label for Electron Paramagnetic Resonance Distance Measurements

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Supporting Information

ABSTRACT: We report the genetic encoding of a noncanonical, spin-labeled amino acid in *Escherichia coli*. This enables the intracellular biosynthesis of spin-labeled proteins and obviates the need for any chemical labeling step usually required for protein electron paramagnetic resonance (EPR) studies. The amino acid can be introduced at multiple, user-defined sites of a protein and is stable in *E. coli* even for prolonged expression times. It can report intramolecular distance distributions in proteins by double-electron electron resonance measurements. Moreover, the signal of spin-labeled protein can be selectively detected in cells. This provides elegant new perspectives for in-cell EPR studies of endogenous proteins.

Electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL) is a powerful tool to study structure, dynamics, and interactions of proteins.[1,2] Of particular interest are SDSL-EPR distance measurements that can be performed using two identical, small spin labels. Such measurements rely on the dipole−dipole coupling between the spin labels (that is inversely proportional to the cube of their distances) and are precise over a broad range of distances.[3−5] These dipole−dipole interactions can be separated from other contributions of the spin Hamiltonian using pulsed methods[6] such as the widely used double-electron electron resonance [DEER, an acronym synonymously used with PELDOR (pulsed electron double resonance)].

SDSL traditionally relies on the chemical conjugation of unique cysteine residues in proteins with sulfhydryl-reactive reagents such as the methanethiosulfonate spin label (MTSSL).[7] Alternatively, nitroxide amino acids can be introduced by protein total synthesis or semisynthesis,[8] nonsense codon suppression with chemically aminoacylated tRNAs,[9,10] or oxime formation with the noncanonical amino acid (ncAA) *p*-acetyl-L-phenylalanine.[11] However, these approaches rely on obligate chemical labeling steps that require multistep procedures and result in limitations such as the need for cystein-free mutants and for surface-accessible sites of the protein.

To obviate the need for chemical labeling in SDSL-EPR studies of proteins, we aimed to directly biosynthesize spin-labeled proteins *in vivo*. Our approach is based on the genetic encoding of nitroxide amino acids in response to the amber stop codon (TAG)[12] by evolutionary design of orthogonal tRNA^{Pyl}/pyrrolysyl-tRNA-synthetase (PylRS) pairs in *E. coli*.[13−24] Compared to previously genetically encoded ncAA, a particular challenge of this approach is the reported reactivity of nitroxides *in vivo*, which results in lifetimes far below the requirements of typical protein expressions in *E. coli*.[25−28] We chose the five-membered 2,2,5,5-tetramethyl-pyrrolin-1-oxyl moiety as spin-label for amino acid design (**1a** and **2a**, Figure 1A).[26,27] To avoid reactions that might interfere with the design process, we employed the corresponding, expectedly stable hydroxylamines **1b** and **2b** as surrogates for evolution of PylRS mutants with potential cross-reactivity toward nitroxides **1a** and **2a**.

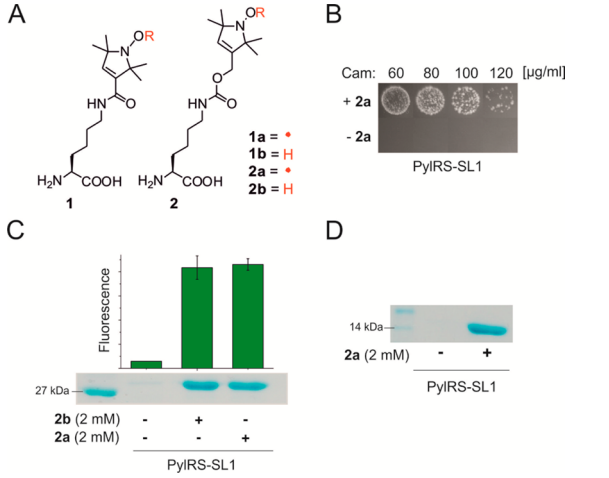

Figure 1. Genetic encoding of spin-labeled amino acids. (A) Structures of nitroxide and hydroxylamine amino acids. (B) Growth assay of *E. coli* expressing CAT_Q98TAG and tRNA^{Pyl}/PylRS-SL1 in the presence and absence of **2a** on Cam media. (C) Expression of GFP-Y39→**2a** and GFP-Y39→**2b** with C-terminal His6 tag under coexpression of tRNA^{Pyl}/PylRS-SL1. Top: Cellular GFP fluorescence. Bottom: SDS-PAGE analysis of proteins purified by Ni-NTA chromatography. (D) SDS-PAGE analysis of TRX-R74→**2a** expressions under coexpression of tRNA^{Pyl}/PylRS-SL1 purified as in panel C.

We subjected a library of ∼10^8 PylRS active site mutants to an *in vivo* selection process [(see the Supporting Information (SI)].[12,22] No PylRS mutant was identified for **1b**, whereas for **2b**, the sampled diversity converged to the three mutants PylRS-SL1−3 (SI Table 1). Clones coexpressing tRNA^{Pyl}

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/PylRS-SL1−3 and an amber mutant of chloramphenicol (Cam)-acetyltransferase (CAT_Q98TAG) exhibited growth on Cam media in the presence of both **2b** and **2a**, but did not grow in their absence (SI Figure 6). PylRS-SL1 exhibited the strongest growth (Figure 1B), suggesting that it indeed exhibits the envisaged cross-reactivity for **2a** and **2b** and promotes their incorporation into proteins with high efficiency and fidelity. For quantitative analysis, we coexpressed $tRNA^{Pyl}$/PylRS-SL1 with an amber mutant of green fluorescent protein with a C-terminal His6 tag (GFP-Y39TAG)[21] in the presence or absence of **2a** or **2b**. Cellular fluorescence as well as SDS-PAGE analysis of the protein (purified by Ni-NTA chromatography via the C-terminal His6 tag) revealed the expression of full-length protein only in the presence of **2a** or **2b** to similar yields (4.6 and 4.3 mg/L, Figure 1C). Moreover, electrospray ionization tandem mass spectrometry (ESI-MS/MS) of DTT-reduced, trypsin-digested GFP-Y39→**2a** revealed the expected presence of the reduced form **2b** at position 39 (SI Figure 7). In addition, an equivalent expression/purification employing **2a** and different amber mutants of *E. coli* thioredoxin revealed expression of full-length protein only in the presence of **2a** (Figure 1D and SI Figure 11). In summary, these data indicate high efficiency and fidelity of incorporation promoted by PylRS-SL1, i.e., protein labeling degrees of >99%.[12] PylRS-SL1 contains three mutations, Y306A, Y384F, and I413L. The Y306A mutation has previously been shown to facilitate the acceptance of large, Nε-carbamate-linked lysine derivatives,[18,21,22] presumably due to an opening of the binding pocket.[18,29] I413 is positioned behind Y306; hence, the mutation to leucine may further modulate the hydrophobic surface of the rear part of the opened pocket (SI Figure 5). The Y384F mutation has been shown to increase aminoacylation rates for distinct ncAA, possibly by facilitating access of $tRNA^{Pyl}$.[18]

Nitroxides have been reported to be highly unstable in all previously studied intracellular contexts,[26−28] but their reactivity in *E. coli* is unknown. Though high *labeling* degrees are offered by our approach, its applicability is determined by the *integrity* degree (i.e., the percentage of intact, paramagnetic **2a** present in a protein) as a function of time. We expressed GFP-Y39→**2a** in *E. coli* Top10 with time-resolved monitoring by SDS-PAGE analysis and EPR measurements (Figure 2A). In view of potential DEER measurements that require the incorporation of multiple ncAA, we also tested *E. coli* JX33 that provides increased amber suppression efficiencies.[30,31] Expression levels increased up to an induction time of 6 h, with higher levels for JX33. Likewise, the purified proteins featured a nitroxide-characteristic EPR signal that emerged at 3 h and reached its maximum at 6 h (Figure 2B). The spectral shape for **2a** incorporated into protein revealed a relatively low mobility of the nitroxide moiety (Figure 2C, rotational correlation time of τ = 250 ps). In contrast, the spectrum of free **2a** corresponded to a fast-tumbling small molecule (Figure 2D, τ = 40 ps; for full characterization of the EPR spectrum of **2a**, see SI). Quantification of the integrity degree by EPR measurements revealed the presence of 49% of intact spin label, even after 8 h induction time in the presence of 1 mM **2a** (Figure 2E).

When 3 mM **2a** was employed, integrity degrees of 68% after 8 h and 52% after 20 h were obtained (the partial loss of paramagnetism is likely due to a reduction of **2a** to the hydroxylamine **2b**; see SI Figure 8). This shows that our approach results in high yields of protein with a labeling degree of >99% and integrity degrees comparable to the ones observed

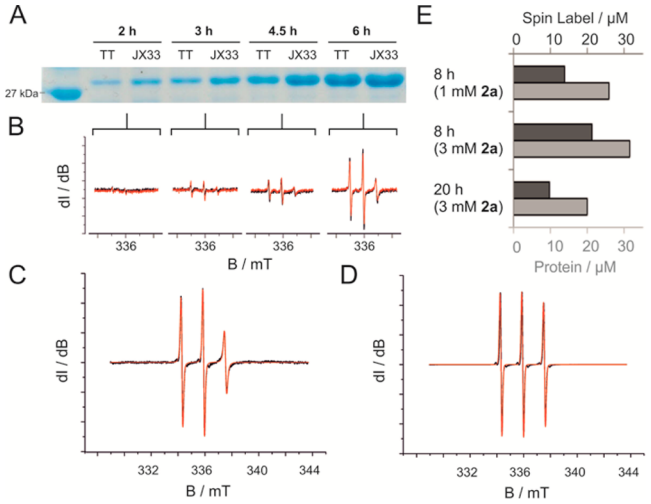

Figure 2. Direct biosynthesis of spin-labeled proteins in *E. coli*. (A) Expression kinetics of GFP-Y39→**2a** in *E. coli* strains Top10 (TT) and JX33 analyzed by SDS-PAGE. (B) EPR measurements of GFP-Y39→**2a** samples from panel A (from Top10 and JX33 shown in red and black, respectively). (C) EPR spectrum of purified TRX-D14→**2a** in buffer. (D) EPR spectrum of **2a** in buffer. Spectrum and spectral simulation for C and D are shown in red and black, respectively. (E) Quantification of the integrity degree of GFP-Y39→**2a**. Concentration of GFP protein was quantified with a BCA assay (light gray bars) and of **2a** by EPR measurements (dark gray bars). Integrity degree was obtained as the ratio of the concentration of **2a** and the concentration of GFP in percent.

in previous in-cell SDSL-EPR experiments.[26] The data further show that the stability of **2a** in *E. coli* is generally much higher than previously reported for nitroxides in other intracellular environments, possibly reflecting differences in the types and abundances of reducing agents between previously studied organisms and *E. coli*.[26−28] This suggests that the potential of nitroxides for *in vivo* applications has previously been underestimated and that our approach could be transferred to other organisms.

A particular advantage of DEER over fluorescence resonance energy transfer (FRET)-based methods for protein structural analysis is the ability to obtain precise, absolute distance distributions. Moreover, DEER should be more accessible in terms of label introduction by genetic encoding: In contrast to FRET studies that necessitate the selective incorporation of two different, large (and potentially perturbative) chromophores into a protein, DEER studies require only two small, identical spin labels. Since the scope of aminoacyl-tRNA-synthetases (aaRS) to accept large chromophores is still limited,[32−35] site-selective double labeling usually relies on the incorporation of small, reactive ncAA in response to individual codons followed by orthogonal conjugation *in vitro*.[14,36] In contrast, the direct *in vivo* double incorporation of **2a** requires only a single tRNA/aaRS pair and could be achieved with high efficiency under exclusive use of the amber codon in *E. coli* JX33. We expressed the doubly labeled protein mutant TRX-D14/R74→**2a** in JX33 (Figure 3A). The protein was purified as the single mutants and dialyzed into aqueous buffer. Following addition of 20% (v/v) glycerol, the protein solution was shock-frozen in order to trap the macromolecular conformation. The distance measurements were performed at a temperature of 50 K (for details see the SI). TRX-D14→**2a** was used as a single labeled control. The DEER data of TRX-D14→**2a** is in full agreement with a

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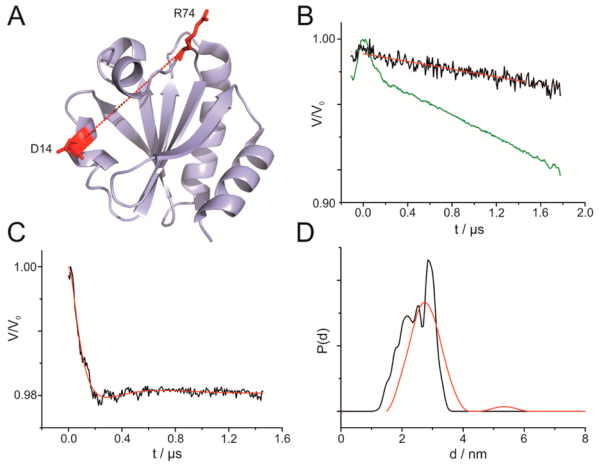

Figure 3. EPR distance measurements between two amino acids **2a** incorporated into *E. coli* thioredoxin (TRX). (A) Cartoon representation of the crystal structure of TRX (PDB entry 2TRX).[38] Amino acids at both incorporation sites of **2a** are shown as red sticks, and the corresponding C_α–C_α distance is indicated as a red line. (B) DEER raw data of doubly labeled TRX-D14/R74→**2a** (green) and TRX-D14→**2a** as control (black). (C) DEER data of TRX-D14/R74→**2a** after background correction (black) and fit based on a model-free analysis (red). (D) Distance distribution for TRX-D14/R74→**2a** corresponding to the fit shown in C (black) compared to the theoretically predicted distance distribution between conventional MTSSL labels (red).

homogeneous 3D distribution of spin labels as expected for a single labeled protein in solution (Figure 3B). For TRX-D14/R74→**2a**, the background-corrected DEER data (Figure 3C) resulted in the corresponding distance distribution by a model-free analysis using DEERAnalysis2013 (Figure 3D; see the SI).[37]

A systematic analysis of the influence of all steps of data postprocessing was performed (see the SI). In general, the width of the distance distribution reflects both the flexibility of TRX and the reorientational degree of freedom of **2a**, while the errors of EPR distance measurements are significantly smaller.[5] The influence of the linker flexibility of the spin labels can be predicted via a rotamer approach. The experimental distance distribution for TRX-D14/R74→**2a** is in full agreement with the theoretically predicted distance distribution between conventional MTSSL spin labels (Figure 3D; see the SI) and matches distances expected from published crystal structures (2.1 Å for the C_α–C_α distance, Figure 3A).[38] Hence, we can conclude that **2a** is a useful structural probe with characteristics similar to those of MTSSL.

The applicability of **2a** for *in vivo* protein biosynthesis raises the possibility, that the EPR signal of endogenous, spin-labeled proteins could even be selectively detected in cells? Since in-cell SDSL-EPR studies ususally rely on the microinjection of *in vitro* spin-labeled molecules into *Xenopus* oocytes,[26,39−42] this would provide a basis for future in-cell studies with increased biorelevance, i.e., for studying proteins directly in the natural host environment where they are biosynthesized and processed. However, this is not a trivial task: microinjection allows sensitive and background-free measurements because of high concentrations of the labeled and purified protein. In contrast, the encoded biosynthesis of spin-labeled proteins is limited in intracellular concentrations and results in particular selectivity challenges: signal of intracellular **2a** as free amino acid, as AMP-ester, esterified to $tRNA^{Pyl}$, and potentially incorporated into amber-terminated, genome-encoded host proteins could prevent a selective detection of the protein under study.

We performed expressions of *E. coli* TRX-R74→**2a** as above, subjected the cells to a short wash protocol and used the cell pellets for EPR measurements. To assess the individual signal intensities caused by the different forms of **2a**, we included controls for all three orthogonal translation components required for genetic encoding, i.e., for **2a**, the $tRNA^{Pyl}$/PylRS-SL1 pair and the amber codon at position 74 (Figure 4A). No

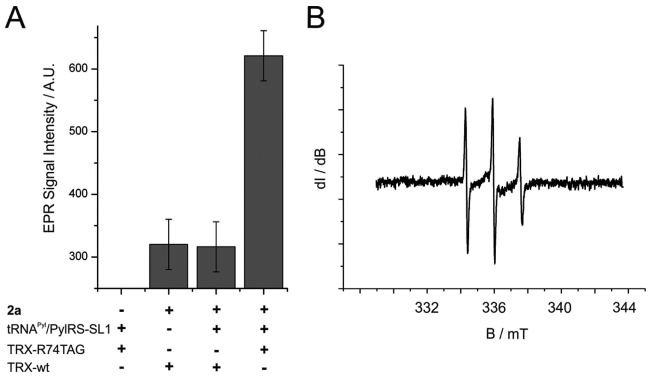

Figure 4. Selective detection of endogenous, spin-labeled proteins in *E. coli*. (A) EPR signal intensities for EPR measurements of washed *E. coli* cells with negative controls omitting **2a**, the amber codon at position 74, or the $tRNA^{Pyl}$/PylRS-SL1 pair as shown in the figure. A.U. = arbitrary units. (B) EPR spectrum of washed *E. coli* cells expressing *E. coli* TRX-R74→**2a** (column 4 of panel A) at 4 °C. The EPR signal corresponds to a spin-label concentration of 13.4 μM.

nitroxide signal was detected in the absence of **2a** (column 1). In the presence of **2a**, similar intensities were detected for cells that did not provide an amber codon in TRX (column 2 and 3). This was independent of the presence (column 3) or absence (column 2) of the $tRNA^{Pyl}$/PylRS-SL1 pair. In both cases, **2a** cannot be incorporated into TRX in response to the amber codon, and in the latter case, **2a** additionally cannot be activated or charged to $tRNA^{Pyl}$. These data reveal free **2a** as the major source of EPR signal in the absence of spin-labeled target protein. However, in its presence, i.e., in cells expressing TRX-R74→**2a** as a result of coexpression of the $tRNA^{Pyl}$/PylRS-SL1 pair and the presence of the amber codon and **2a**, the recorded EPR signal increased 2-fold (column 4).

This indicates that our approach indeed affords sufficient expression levels of endogenous, spin-labeled proteins for in-cell SDSL-EPR measurements and that free **2a** can be selectively washed out of cells under conditions that leave the spin-labeled protein intact and localized in the cells. Moreover, free **2a** and **2a** incorporated into a protein can be differentiated by distinct spectral shapes (Figure 2C,D). Figure 4B shows the first EPR spectrum of an endogenous, spin-labeled protein in its natural host (sample from Figure 4A, column 4).

In conclusion, we demonstrate the intracellular, genetically encoded biosynthesis of spin-labeled proteins. This eliminates the need for *in vitro* spin-labeling and thus overcomes current limitations of SDSL-EPR, including the restriction to cystein-free mutants and the need for multistep labeling protocols. **2a** can be incorporated at multiple, user-defined sites within a protein and exhibits characteristics similar to those of MTSSL, making it a valuable probe for the measurement of intramolecular distance distributions by DEER. Additionally, we

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make a first step toward future in-cell SDSL-EPR studies of endogenous proteins. We analyze fundamental aspects of EPR signal stability, sensitivity, and selectivity in *E. coli* and demonstrate that spin-labeled proteins can be selectively detected in living cells. Future efforts will focus on the transfer of the approach to other organisms, on *in vivo* DEER measurements, and on the development of nitroxide amino acids with further improved stabilities and conformational characteristics.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Syntheses and analytical data, construction of PylRS selection systems, libraries and selection, expression and purification of GFP and TRX proteins, cw EPR measurements, DEER measurements, and rotamer simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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