Performance Analysis of Orthogonal Pairs Designed for an Expanded Eukaryotic Genetic Code

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Abstract

Background: The suppression of amber stop codons with non-canonical amino acids (ncAAs) is used for the site-specific introduction of many unusual functions into proteins. Specific orthogonal aminoacyl-tRNA synthetase (o-aaRS)/amber suppressor tRNA_CUA pairs (o-pairs) for the incorporation of ncAAs in S. cerevisiae were previously selected from an E. coli tyrosyl-tRNA synthetase/tRNA_CUA mutant library. Incorporation fidelity relies on the specificity of the o-aaRSs for their ncAAs and the ability to effectively discriminate against their natural substrate Tyr or any other canonical amino acid.

Methodology/Principal Findings: We used o-pairs previously developed for ncAAs carrying reactive alkyne-, azido-, or photocrosslinker side chains to suppress an amber mutant of human superoxide dismutase 1 in S. cerevisiae. We found worse incorporation efficiencies of the alkyne- and the photocrosslinker ncAAs than reported earlier. In our hands, amber suppression with the ncAA containing the azido group did not occur at all. In addition to the incorporation experiments in S. cerevisiae, we analyzed the catalytic properties of the o-aaRSs in vitro. Surprisingly, all o-aaRSs showed much higher preference for their natural substrate Tyr than for any of the tested ncAAs. While it is unclear why efficiently recognized Tyr is not inserted at amber codons, we speculate that metabolically inert ncAAs accumulate in the cell, and for this reason they are incorporated despite being weak substrates for the o-aaRSs.

Conclusions/Significance: O-pairs have been developed for a whole plethora of ncAAs. However, a systematic and detailed analysis of their catalytic properties is still missing. Our study provides a comprehensive scrutiny of o-pairs developed for the site-specific incorporation of reactive ncAAs in S. cerevisiae. It suggests that future development of o-pairs as efficient biotechnological tools will greatly benefit from sound characterization in vivo and in vitro in parallel to monitoring intracellular ncAA levels.

Introduction

Protein engineering with non-canonical amino acids (ncAAs) that are not encoded by the standard genetic code has gained much attention in the recent years. The approach is particularly interesting because it provides for the introduction of unusual functions into target proteins directly by ribosomal translation. Especially the incorporation of amino acid analogs bearing azide or alkyne side chains for subsequent bioorthogonal copper(I)-catalyzed [3+2]-cycloaddition [1] with alkyne-, or azido-ligands, respectively, is a valuable technique for the artificial post-translational modification of proteins. We and others have successfully used the methionine analogs azidohomoalanine, azidonorleucine, and homopropargylglycine [2,3,4] for the global translational modification of Met residues in target proteins and subsequent orthogonal conjugation with fluorescent dyes, biotin, sugars, or PEG [5,6,7,8,9,10,11]. Quasi site-specific incorporation and conjugation, however, is only possible if the target protein contains a single Met.

Genuine site-specific incorporation of ncAAs is feasible at in-frame stop codons. Furter first demonstrated the introduction of p-fluoro-L-phenylalanine (pFF) at an amber stop codon in vivo by a “nonessential heterologous tRNA/synthetase pair” [12]. To specifically decode the amber stop codon with pFF, he chose the yeast phenylalanyl-tRNA synthetase (yPheRS) that is specific for Phe yet naturally tolerates pFF as a substrate. He introduced the yPheRS together with a compatible amber suppressor tRNA_CUA into a Phe-auxotrophic E. coli strain harboring an endogenous PheRS mutant with greatly reduced affinity for pFF [12]. Since the identity elements of PheRS/tRNA_Phe pairs differ in S. cerevisiae and E. coli [13], there is little cross-species aminoacylation, i.e., the pairs are orthogonal. However, the yPheRS did not have exclusive substrate specificity for pFF, therefore, the Phe-auxotrophy of the host was required to perform the incorporation in the presence of low amounts of Phe and excess pFF. This approach ensured that Phe was still incorporated at Phe codons while predominantly pFF appeared at the position of the amber stop codon.
Schultz and co-workers further developed the concept of orthogonal aminoacyl-tRNA synthetase/suppressor tRNA_CUA pairs (o-pairs) and devised an efficient screening system for the selection of orthogonal mutant aaRSs (o-aaRSs) with novel specificities for ncAAs [14,15]. The orthogonality is achieved by importing aaRSs together with appropriate suppressor tRNAs from distantly related organisms into the host, e.g., E. coli components in yeast. O-pairs are paramount with respect to site specificity as they allow the incorporation of an ncAA exactly at the position of an in-frame amber stop codon. During the last decade, the Schultz group and others established o-pairs for the incorporation of a vast number of mostly Tyr analogs in different expression hosts [16,17,18,19,20,21,22,23]. Tyr analogs with reactive side chains, such as p-azido-L-phenylalanine (AzF), or p-propargyloxy-L-phenylalanine (PxF; Figure 1) were successfully incorporated into target proteins at amber codons and used for bioorthogonal conjugation. Recently, a new generation of o-pairs has been developed that derive from naturally occurring pyrrolysl-tRNA synthetase (PylRS)/tRNA_CUA pairs from methanogenic archaea and allow the incorporation of lysine analogs at in-frame amber codons (see [15] and references therein).

As outlined above, Furter used a Phe-specific orthogonal yPheRS with a natural tolerance for pFF as the substrate, in combination with a host PheRS mutant that was inefficient for pFF recognition. The Schultz approach, however, employs ncAA-specific o-aaRSs derived from tyrosyl-tRNA synthetase (TyrRS), or PylRS by directed evolution of specific amino acid residues in the substrate binding pocket [17,18,24]. As such, the o-aaRS/tRNA_CUA pairs represent autonomous decoding units for amber stop codons with a particular ncAA, that neither cross-react with host tRNAs, nor with host aaRSs. The fidelity of the system relies on the inability of the o-aaRS to charge its cognate tRNA_CUA with the natural substrate, Tyr, or any other of the canonical amino acids.

Despite the vast number of o-pairs that have been described so far, a systematic analysis of their catalytic properties has not yet been performed. In order to curtail this evidence gap we decided to perform a comprehensive characterization of o-pairs for the incorporation of ncAAs with reactive side chains at amber codons in yeast. The yeast S. cerevisiae is superior to bacterial expression hosts such as E. coli for the expression of integral membrane proteins and protein complexes, or for protein secretion [25,26]. Using appropriate AzRS/tRNA_CUA or PxRS/tRNA_CUA pairs we intended to functionalize a target protein in the yeast with reactive handles by incorporation of AzF or PxF, respectively. We also included the o-pair for the photocrosslinker, p-benzoyl-L-phenylalanine (Bpa; Figure 1) in our study. The expansion of the genetic code with these Tyr analogs in yeast has attracted much attention [18], however, we obtained only minute amounts of target protein labeled with PxF and Bpa while AzF was not incorporated at all. In order to systematically analyze the reason for the poor incorporation, we expressed the corresponding o-aaRSs in E. coli, purified them to homogeneity and analyzed their catalytic activities. We found that under our assay conditions, none of the ncAAs was demonstrably fortified while Tyr was recognized by all of them. We speculate that ncAAs that are poor substrates for the o-aaRSs can still be incorporated at amber codons if they accumulate in the cell due to metabolic inertness.

Results
Incorporation of tyrosine analogs into an amber mutant of hSOD1 in S. cerevisiae
For our performance analysis of o-pairs in yeast, we first intended to reproduce the site-specific introduction of azido or alkyne groups into a target protein in S. cerevisiae as described earlier [18]. To achieve this, we reconstructed the expression vectors pAz1/tRNA_CUA and pAz6/tRNA_CUA (Figure S1) for incorporation of AzF and PxF (Figure 1), respectively, as described in the relevant literature [18,19,27,28]. Though carefully reconstructed, our expression constructs might nevertheless have deviated from the original vectors in some unrecognized aspect. Thus, we expanded our o-pair vector collection with the original plasmids pAz3/3SUP-tRNA_CUA [29] and pPR1/3SUP-tRNA_CUA [29], which were a kind gift by P.G. Schultz. The o-pair for the photocrosslinker Bpa (Figure 1) was previously described in yeast [18] (kindly provided by S. Hahn and P.G. Schultz) as a positive control together with pTyr/tRNA_CUA (reconstructed according to [27]). A schematic map of the expression plasmids for the o-pairs

Figure 1. Structures of tyrosine and its analogs used in this study. Structures, names and abbreviations are shown. doi:10.1371/journal.pone.0031992.g001
can be found in Figure S1 of the supporting information. Figure S2 shows a sequence comparison of the different o-aaRSs.

Upon incorporation of AzF into their target protein, Chin et al. observed the reduced form, p-amino-L-phenylalanine (AmF; Figure 1) by tandem mass spectrometry [18] rather than AzF. This observation was attributed to the chemical reactivity and photoinstability of the azido group during mass analysis [21]. However, S. cerevisiae can be used as a biocatalyst to reduce azaldehydes to amines [33,34]. Therefore, we included AmF in our systematic studies in order to analyze whether AzF would use AmF as a substrate.

In accordance with reports from the Schultz lab, we chose the human superoxide dismutase hSOD1 as the target protein for analog incorporation; hSOD1 is a small, stable protein [35] that is well expressed in yeast. We first tested the expression efficiency of wild type hSOD1 from a construct similar to that of the Schultz group [18]. However, the expression of hSOD1 carrying a C-terminal hexahistidine-tag from the high copy, galactos inducible yeast-E. coli shuttle vector pYES2 was low and we did not obtain pure protein preparations (data not shown). For that reason, we replaced the hexahistidine-tag, which has been described as not ideally suitable for yeast [36], with a Strep-tag II. In addition, we exchanged the inducible GALI promoter for the strong constitutive PGK1 promoter. The strength of this promoter is comparable to that of the TDH3 promoter [26], which Chen et al. used for hSOD1 expression together with an improved orthogonal system for tyrosine analogs in yeast [29]. Using this modified expression construct, we obtained pure hSOD1 in high yield (unpublished observation). For the incorporation of the amino acids shown in Figure 1, we introduced a Trp (TGG) to amber stop codon (TAG) mutation into hSOD1 at position 33. Previous reports demonstrated the permisiveness of this position for analog incorporation by amber suppression [18,37,38,39,40]. The map of the resulting expression construct, as well as the coding DNA sequence and the protein sequence hSOD1/W33TAG with a C-terminal Strep-tag II are shown in Figure S3 and Figure S4 of the supporting information.

Similar to the original study [18], we performed all Tyr analog incorporations in the Saccharomyces cerevisiae strain InvSC1 (see Materials and Methods for experimental details). Each of the o-pairs listed in Table 1 was introduced into InvSC1 together with the hSOD1/W33TAG expression construct. We examined the efficiency of the amber suppression in hSOD1/W33TAG using the wild type E. coli TyrRS/tRNACUA o-pair with Tyr. In order to scrutinize a potential residual affinity of AzRS3 for the natural substrate Tyr, we used Tyr together with the AzRS3/3SUP-tRNACUA pair. For comparison of the incorporation efficiencies, hSOD1(W33TAG) was expressed in the presence of AzF and three o-pairs with corresponding substrate specificity, AzRS1/tRNACUA, AzRS6/3SUP-tRNACUA, or AzRS3/3SUP-tRNACUA. The same o-pairs were also used with AmF. In addition, we wanted to introduce PxF into hSOD1(W33TAG) by the AzRS6/3SUP-tRNACUA [18], or PxRS/tRNACUA [29] o-pairs. Finally, we introduced Bpa into hSOD1(W33TAG) with the corresponding BpaRS/tRNACUA o-pair.

Full-length hSOD1(W33X) variants, where X denotes Tyr or an Ala, are expressed only if the in-frame amber stop codon at position 33 is efficiently suppressed by Tyr or one of its analogs shown in Figure 1. In contrast to E. coli, expression of heterologous proteins in S. cerevisiae is very often too low for detection on SDS gels in spite of the use of high copy expression vectors. Usually, the foreign protein can be immunodetected in whole cell lysates using suitable antibodies. However, we were unable to detect the full-length hSOD1(W33X) variants in whole cell lysates by immunoblotting with an anti-Strep-tag II antibody. We observed specific bands on the immunoblot (Figure 2) only after purification and concentration of the tagged hSOD1 variants hSOD1(W33TAG).

We detected an hSOD1 signal on the immunoblot upon expression in the presence of the orthogonal TyrRS/tRNACUA o-pair together with Tyr, indicating successful amber stop codon suppression (Figure 2A and 2B, lanes 1). AzRS3/3SUP-tRNACUA and AzRS6/tRNACUA in combination with Tyr caused barely detectable expression of full-length hSOD1 (Figure 2B, lane 3), which suggests good fidelity of this o-pair. However, all our attempts to incorporate AzF into hSOD1(W33TAG) with any of the o-pairs involving AzRS failed (AzRS1/tRNACUA, Figure 2A, lane 2; AzRS3/3SUP-tRNACUA, Figure 2B, lane 2; AzRS6/3SUP-tRNACUA, Figure 2A, lane 4). Surprisingly, we observed strong signals on the immunoblot in two of three hSOD1(W33TAG) expressions with the AzRS3/3SUP-tRNACUA and AmF (Figure 2B, lanes 4–5). The third replicate yielded only a faint band (Figure 2B, lane 6) while no bands were detected with AmF and the o-pairs involving AzRS1 and AzRS6 (Figure 2A, lanes 3 and 6). Contrary to the original study, in which a dual specificity of AzRS6 for AzF and PxF was reported [19], we obtained an hSOD1 signal on the immunoblot only when PxF was used together with PxRS1 (Figure 2A, lane 7), and not with AzRS6 (Figure 2A, lane 5). This observation may be attributed to the improved expression of the suppressor tRNA from the pPR1/3SUP-tRNACUA vector. In this construct, the suppressor tRNA is inserted between short upstream and downstream regions of the yeast SUP4 gene. Three copies of this cassette are expressed in tandem from the strong PGK1 promoter.

| Orthogonal pair | aaRS | Specificity | 6xHis-tag on aaRS | tRNACUA copy number | tRNACUA promoter | Source |
|-----------------|------|------------|-------------------|--------------------|-----------------|-------|
| TyrRS/tRNACUA  | TyrRS| Tyr        | none              | 1                  | internal B-box  | [18]  |
| AzRS1/tRNACUA  | AzRS1| AzF       | none              | 1                  | internal B-box  | [18]  |
| AzRS3/3SUP-tRNACUA | AzRS3| AzF   | C-term.            | 3                  | improved*       | [29]  |
| AzRS6/tRNACUA  | AzRS6| AzF [18], PxF [19] | none              | 1                  | internal B-box  | [18,19] |
| PxRS1/3SUP-tRNACUA | PxRS1| PxF     | C-term.            | 3                  | improved*       | [29]  |
| BpaRS/tRNACUA  | BpaRS| Bpa       | none              | 1                  | internal B-box  | [18]  |

The pairs consist of E. coli TyrRS, or a mutant descendant, and E. coli amber suppressor tRNACUA. The aaRSs are expressed under the strong, constitutive ADH1 promoter (refer to Figure S1 for plasmid map details).

The improved promoter consists of a yeast PGK1 promoter followed by three copies of the E. coli tRNACUA gene (with an internal B-box), each flanked by 55 bp upstream and 30 bp downstream sequences of the yeast SUP4 gene [29].

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In summary, we observed suppression of the amber stop codon and hence expression of full-length hSOD1(W33X) with Tyr, Bpa, PxF, and AmF but not with AzF. The incorporation of Tyr, Bpa, and PxF into the target protein was confirmed by ESI-MS analysis. The incorporation of AmF could not be unambiguously revealed. PxF was only incorporated into the target protein with the improved PxsR1/3SUP-tRNA_CUA o-pair (Table 1). In our hands, the o-pairs for yeast performed less efficiently than expected although we exactly followed the published methods and used original expression constructs or reconstructed them according to previous reports.

In vitro activation of tyrosine analogs by the different o-aaRSs

Besides the in vivo performance analysis, another important aim of our study was to scrutinize the amino acid activation profiles of the o-aaRSs in vitro. These data provide important information about the substrate binding by the o-aaRSs. We performed in vitro ATP-PPi exchange assays [43,44] (see Materials and Methods for technical details) with the same o-aaRSs we had used for the in vivo incorporation of Tyr analogs into hSOD1(W33TAG). Basically, aminoacylation is indispensable for protein translation and occurs in a two-step process. The amino acid is first activated and then charged onto its cognate tRNA by a specific aaRS. The activation reaction consumes ATP and pyrophosphate is released. As this reaction is reversible, the amount of radioactive ATP that is formed from [32P]-pyrophosphate in the reverse reaction is a measure for the activation of an amino acid by an aaRS.

In order to prepare pure enzymes for the activation assay, we constructed His-tagged fusions of TyrRS, AzRS1, AzRS6, AzRS3, and BpaRS for expression in E. coli and subsequent purification by Ni-chelate chromatography (refer to Materials and Methods for details). The purified aaRSs were characterized by SDS-PAGE (Figure S6) and ESI-MS analysis (Table S1; Figure S7). In a first step we determined the optimal amino acid concentration range for the assay. To achieve this, we quantified the radioactive ATP produced by 1 μM TyrRS within 15 minutes with a range of Tyr concentrations (Figure S8). A steady increase in ATP formation and hence amino acid activation was found for Tyr concentrations between 5 μM and 100 μM. Tyr concentrations above 100 μM saturated the enzyme and did not further increase ATP formation.
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A

B

C

D

E

F

G

H
Next, we assayed the activation of Tyr and the analogs AzF and AmF by TyrRS and the ncAA-specific AzRS1, AzRS3, AzRS6, and BpaRS (Figure 4 and Figure 5). With the latter enzyme, an activation assay with Bpa was also performed. We suspected the Tyr analogs to be worse substrates for their cognate aaRSs than Tyr is for the TyrRS. Therefore, the amino acids were added in excess to a final concentration of 5 mM each. 1 µM TyrRS, 5 µM of the AzRS proteins, and 3 µM BpaRS were used in the assay. Tyr and its analogs are barely water soluble, thus, the substances were dissolved in 0.10 M HCl. Accordingly, the negative control (Figure 4 and Figure 5; w/o aa) contained no amino acid but the appropriate amount of HCl.

As expected, Tyr was efficiently activated by TyrRS (Figure 4A). The activation data are in good agreement with the successful in vivo (Figure 2A and 2B, lanes 1). TyrRS did not activate AzF nor AmF (Figure 4A), confirming the lack of natural tolerance for these substrates. However, we found no activation either of AzF or AmF by AzRS1, AzRS3, or AzRS6 (Figure 4B–D). On the contrary, all AzRSs activated Tyr (Figure 4B–D). Similarly, BpaRS did not activate Bpa but Tyr (Figure 4B–D). On the contrary, all AzRSs activated Tyr (Figure 4B–D). In contrast, hSOD1 was expressed in the presence of AmF (Figure 2B, lanes 4–6) and Bpa (Figure 2C, lane 1), yet the analogs were not activated in vitro. In order to exclude that the high concentration (5 mM) of the Tyr analogs caused an inhibition of the o-aaRSs, we assayed the activation of Tyr, AzF, and AmF also in the lower concentration range (500, 50, 5, and 1 µM; Figure S9). Once again, Tyr was activated in a concentration dependent manner while we did not detect activation of AzF nor of AmF.

In summary, under our experimental conditions the o-aaRSs did not demonstrably activate the Tyr analogs but properly activated their original substrate, Tyr.

### Discussion

We evaluated the incorporation of reactive ncAAs with existing o-pairs at the in-frame amber codon of an hSOD1(W33TAG) mutant in yeast. AzF-, PxF-, and Bpa-specific o-aaRSs for the corresponding o-pairs in S. cerevisiae were first evolved by Chin et al. [18]. The same o-aaRSs were used later to construct an improved expression system in yeast [29], and to establish the amber suppression incorporation of these ncAAs in P. pastoris [20].

The Schultz group reported incorporation of AzF and PxF into hSOD1 in yeast using the evolved o-pairs [18] and subsequent bioorthogonal conjugation to alkyn- or azido-functionalized fluorophores [19] and PEG [42]. Becker et al. used the Bpa-specific o-pair to introduce Bpa site-specifically into the G-protein coupled receptor (GPCR) Ste2p in yeast. The Bpa-labeled Ste2p could then be photo-crosslinked to its peptide ligand [31].

The groups of Sakmar and Wang used the AzF- and Bpa-specific o-pairs developed in yeast to incorporate these ncAAs into proteins expressed in mammalian cells [45,46]. Different GPCRs were labeled with AzF and Bpa for subsequent dynamics measurements by Fourier-transform infrared (FTIR) difference spectroscopy [47,48] and to analyze ligand binding [49,50] (for a comprehensive review on the incorporation of molecular probes to study GPCRs see [51]).

Although we used original or thoroughly reconstructed tandem expression constructs for the o-pairs and followed published methods, we obtained unexpected results. In our hands, none of the three different AzRS/tRNA\textsubscript{CUA} pairs (Table 1) previously described to work efficiently [18,19,29,42] promoted amber suppression with AzF.

PxF and Bpa were incorporated by their cognate o-pairs in response to the amber codon at position 33 of hSOD1, though less efficiently (Table 2) than previously reported [29]. This finding indicates that the failure to incorporate AzF with the AzRS/tRN\textsubscript{CUA} o-pairs did not originate from our experimental setup.

### Table 2. ESI-MS analyses of selected hSOD1 variants.

| Analog replicate | Orthogonal pair | Calculated mass (Da) | Detected mass (Da) | Interpretation | Protein per liter culture |
|------------------|-----------------|----------------------|--------------------|----------------|--------------------------|
| Tyr replicate 1  | TyrRS/tRNA\textsubscript{CUA} | 1702.6943            | 17043.0495         | +Na\textsuperscript{+}, S-S | 60 µg |
| Tyr replicate 2  | TyrRS/tRNA\textsubscript{CUA} | 1702.6943            | 17041.6996         | +Na\textsuperscript{+}, S-S | 120 µg |
| Tyr replicate 3  | AzRS3/3SUP-tRNA\textsubscript{CUA} | 17042.7675         | 21.0373           | +Na\textsuperscript{+}, S-S | 40 µg |
| AmF replicate 1  | AzRS3/3SUP-tRNA\textsubscript{CUA} | 17043.7254     | 23.016            | +Na\textsuperscript{+} | 78 µg |
| AmF replicate 2  | AzRS3/3SUP-tRNA\textsubscript{CUA} | 17042.3620          | 21.652           | +Na\textsuperscript{+}, S-S | 112 µg |
| AmF replicate 3  | AzRS3/3SUP-tRNA\textsubscript{CUA} | 17043.0344         | 22.325           | +Na\textsuperscript{+} | 90 µg |
| PxF             | PxFRS1/3SUP-tRNA\textsubscript{CUA} | 17057.9797        | 17057.9797        | −1.945 S-S | 96 µg |
| Bpa             | BpaRS/tRNA\textsubscript{CUA} | 17109.8012           | 17108.9558         | −0.845 S-S | 80 µg |

Only variant proteins for which defined mass spectra were obtained are shown. The same hSOD1 variants were detected on the immunoblot in Figure 2. The corresponding ESI-MS spectra are shown in Figure 3. All hSOD1 variants were found with the N-terminal methionine cleaved off and acetylated alanine at position 2, as reported in the literature [41]. The occasionally attached sodium ions (+2.99 Da) most probably originated from the Strep-Tactin elution buffer which contained 150 mM NaCl. The buffer was not exchanged during sample concentration in order to avoid protein loss. In some of the protein preparations we found a known disulfide bond (S-S, 22.99 Da) most probably originated from the Xaa-Xaa dipeptide in the sample.

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1. All hSOD1 masses were calculated without N-terminal methionine, acetylated alanine at position 2 and with completely reduced cysteines.

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We observed incorporation of PxF into hSOD1(W33TAG) with the tandem vector for optimized tRNA CUA expression (Table 1; [29]) while the originally described vector containing only one copy of the gene and no optimized promoter (Table 1; [18]) was inefficient. This confirms the published observation [29] that three copies of tRNACUA and the optimized promoter result in improved amber stop suppression.

Surprisingly, we obtained a rather strong immunoblot signal upon expression of hSOD1(W33TAG) in the presence of AmF and the AzRS3/3SUP-tRNACUA o-pair optimized for AzF (Figure 2B, lanes 4–5). Our attempts, however, to confirm incorporation of AmF by mass analysis were inconclusive owing to a calculated mass difference of only 1 Da between AmF and Tyr. The ESI-MS produced strong signals but the resolution was too low to unambiguously distinguish between intact hSOD1(-W33AmF) and hSOD1(W33Y) (see Table 2 for calculated and found masses; Figure 3D–F). Direct LC-MS/MS analysis of the trypsin-digested protein preparation was disappointing (data not shown), as we could not identify the specific peptide fragment carrying the ncAA. Yet, there is indirect evidence that we isolated an hSOD1(W33AmF) variant. The concentrations of Tyr and the other canonical amino acids in the expression medium were too low to support amber suppression with the AzRS3/3SUP-

Figure 4. Activation of Tyr, AzF, and AmF by TyrRS and the AzRSs. Tyr, AzF and AmF were used at a concentration of 5 mM. No amino acid was added to the negative control (w/o aa). TyrRS (A) was added at 1 μM and AzRS1 (B), AzRS3 (C), and AzRS6 (D) at a concentration of 5 μM. The data for each o-aaRS were collected in one series of experiments (see Materials and Methods for details). The average of duplicate determinations is shown; the bars indicate the discrete values. doi:10.1371/journal.pone.0031992.g004

Figure 5. Activation of Tyr, Bpa, AzF, and AmF by BpaRS. Tyr, Bpa, AzF and AmF were used at a concentration of 5 mM. In the negative control, the amino acid was omitted (w/o aa). BpaRS was added at a concentration of 3 μM. The data were all recorded in one row of experiments and each value was determined in duplicate. The bars denote the discrete values. doi:10.1371/journal.pone.0031992.g005
tRNA*CUA pair as can be concluded from the unsuccessful incorporation experiments with AzF. These observations strongly argue against the incorporation of Tyr or any other canonical amino acid in the presence of AmF.

Our finding indicates a hitherto unrecognized specificity of AzRS3 for AmF. Indeed, o-aaRSs with extended substrate tolerance have recently been described [52,53]. These o-aaRSs accept various ncAAs (up to 10 different species [53]) as their substrates yet they retain their ability to discriminate against the 20 canonical amino acids. The tolerance of AzRS3 for AmF might occur coincidentally as described for other o-aaRSs. It may, however, have been nurtured by the chemical properties of AzF. AmF is a reduced derivative of AzF. It was observed after however, have been nurtured by the chemical properties of AzF. AzRS3 for AmF. Indeed, o-aaRSs with extended substrate tolerance have recently been described [52,53]. These o-aaRSs...
Tyr is significantly activated at concentrations as low as 50 μM by the different AzRSs while the different nCAAs showed activation close to background at all tested concentrations (Figure 4, Figure 5, and Figure S9). Thus, from our results we would expect preferential activation of Tyr already at the normal concentration in the SC –Ura –Trp medium even in the presence of excess nCAAs.

It might be possible that Tyr is not or only insufficiently charged onto the tRNA_{CUA} although it is efficiently activated by an o-aaRS. However, charging of tRNA_{CUA} with Tyr or the nCAAs by an o-aaRS, such as AzRS, PxRS, or BpaRS, has not been systematically analyzed so far. Most probably, this owes to the fact that only a couple of radiolabeled nCAAs, that are required for the classical aminoaacylation assay, are commercially available. An alternative aminoaacylation assay that involves radioactive tRNA rather than labeled amino acids has recently been described [44] and could be used for a future, more detailed analysis of the aminoaacylation reaction of tRNA_{CUA} with nCAAs by the o-aaRSs.

Most strikingly, Bpa was incorporated into hSOD1 (Figure 2C) although the analog was not activated by BpaRS in vitro (Figure 5) when used at 5 mM. Due to the low solubility of Bpa in aqueous solution it was not possible to use higher concentrations in the activation assay. It is generally accepted that an amino acid must be activated before it can be charged onto a tRNA [2,61]. In vivo activation and subsequent charging onto suppressor tRNA_{CUA} by an unspecified BpaRS can plausibly occur if Bpa is efficiently taken up into the cells and accumulates intracellularly to levels above 5 mM. This is possible if the substance is actively imported into the cells and piled up because it is not metabolized. Indeed, Wang and co-workers observed roughly 9 mM intracellular dansylalanine upon esterification of the amino acid [62]. Giese et al. recently demonstrated that fluorinated tryptophan is actively taken up into mammalian cells to an intracellular concentration exceeding the extracellular by 70-fold [63]. A comparable accumulation could elevate the intracellular Bpa concentration above 5 mM when the cells are supplemented with 1 mM in the medium, as in our experiments. The assessment of the intracellular Bpa concentration would help to clarify this issue. In any case, the intracellular availability of the non-canonical amino acid is an important factor that governs the specificity of the o-aaRS and, thus, the fidelity of the incorporation [29].

In numerous studies, the site-specific incorporation of nCAAs into target proteins in response to amber codons was shown to occur with high fidelity and without significant amounts of canonical amino acids at the designated positions. However, reports on the catalytic properties of the evolved o-aaRSs as well as the intracellular availability and fate of the amino acid analogs are still rare exceptions [16,17,62]. If the modification of proteins by nCAAs is to extend beyond the proof-of-principle level, thorough characterization of the incorporation systems is urgently required. Polysubstrate specificity, i.e. broad substrate tolerance of aaRSs appears to be a general phenomenon. Naturally occurring enzymes usually show it, and this trait can be exploited for the global incorporation of amino acid analogs into proteins [64]. O-aaRSs evolved to recognize a specific nCAA can also tolerate alternative substrates [32,33]. This property could ease a broader applicability of the already existing o-pairs. However, natural enzymes and their cognate tRNAs are superior to the currently available orthogonal aaRS/tRNA_{CUA} pairs in terms of efficiency. In our opinion, the future strategies for the improvement of the o-pairs will enormously profit from the thorough characterization of the overall analog incorporation process. This involves the uptake of the nCAA into the cells, its cellular availability, the activation, and charging onto tRNA_{CUA} and finally the sequence context for the incorporation at the position of the amber stop codon [29,63,66]. The results of this thorough analysis will aid the design of o-pairs that can stand the comparison with the naturally occurring systems.

Materials and Methods

Materials

Unless otherwise indicated all chemicals were from Fluka (Neu-Ulm, Germany) or Merck (Darmstadt, Germany). Restriction endonucleases and T4 ligase were from New England Biolabs (Beverly, MA). Ex Taq and rtq DNA polymerases for proof-reading and standard PCR reactions, respectively, were from TaKaRa Bio Inc. (Saint-Germain-en-Laye, France), PfuUltra II HS-DNA-Polymerase for site-directed mutagenesis PCR was from Stratagene (La Jolla, CA).

Construction of the tandem expression vectors for orthogonal pairs

The reconstruction of the expression vectors pTyr/tRNA_{CUA}, pAz1/tRNA_{CUA}, and pAz6/tRNA_{CUA} (Figure S1) were performed as described previously [18,19,27,28]. Primers tRNA_{CUA}/template_fwd and tRNA_{CUA}/template_rev, containing the coding sequence for the E. coli tRNA_{CUA} gene, were annealed and the resulting double strand DNA was used for PCR amplification with primers trna5′ (contains an AgeI cleavage site) and trna3′ (contains an NdeI site, see Table S2 for primer sequences). The PCR product and the pESCTrp (Stratagene) target vector were both digested with NdeI and AgeI and ligated, which yielded pRNA_{CUA}. The ADH1-promoter for the expression of the E. coli TyrRS in S. cerevisiae was generated by PCR using the primers pADH (AgeI site) and pADHfr (EcoRI site) together with genomic S. cerevisiae DNA as the template. The PCR product was digested with EcoRI and AgeI. The pTyr/tRNA_{CUA} vector was digested with EcoRI and NodI. The pTyr/tRNA_{CUA} tandem vector was obtained by ligating AgeI/NodI digested pRNA_{CUA} with the AgeI/EcoRI digested ADH1-promoter and E. coli TyrRS cut with EcoRI and NodI. The pAz1/tRNA_{CUA} descendant encoding the TyrRS mutant AzRS1 was generated by site directed mutagenesis PCR using the pTyr/tRNA_{CUA} vector as the template. The primer pair 5′-3′Muta_N126 was used to introduce the silent mutation N126M. The additional mutations were introduced using the primer pairs 5′-3′Muta3306-1/pESCTrp2, 5′-3′Muta3306-2/pESCTrp2, and 5′-3′Muta3306-3/pESCTrp2. Each resulting mutant plasmid was used as the template for the subsequent mutagenesis PCR. AzRS6 was generated by introducing additional mutations into AzRS1 using mutagenesis primer pairs Thr371/Thr373, Ala183I/Ala183R, and Leu186I/Leu186R. Figure S2 shows the mutations that were introduced into TyrRS to generate the o-aaRSs. Mutagenesis primers are listed in Table S2. All vectors were verified by sequencing.

Construction of the hSOD1(W33TAG) expression vector

The gene for the human superoxide dismutase (hSOD1) was PCR-amplified from the cDNA vector pOTB7 (ATCC number MGC-2325; LGC Promochem GmbH, Wesel, Germany) with the primers hSODfp (MGC-2325; LGC Promochem GmbH, Wesel, Germany) with the primers hSODfp (see Table S2 for primer sequences). The PCR product and the high copy yeast-E. coli tandem vector was obtained by ligating AgeI/NodI digested pRNA_{CUA} with the AgeI/EcoRI digested ADH1-promoter and E. coli TyrRS cut with EcoRI and NodI. The pAz1/tRNA_{CUA} descendant encoding the TyrRS mutant AzRS1 was generated by site directed mutagenesis PCR using the pTyr/tRNA_{CUA} vector as the template. The primer pair 5′-3′Muta_N126 was used to introduce the silent mutation N126M. The additional mutations were introduced using the primer pairs 5′-3′Muta3306-1/pESCTrp2, 5′-3′Muta3306-2/pESCTrp2, and 5′-3′Muta3306-3/pESCTrp2. Each resulting mutant plasmid was used as the template for the subsequent mutagenesis PCR. AzRS6 was generated by introducing additional mutations into AzRS1 using mutagenesis primer pairs Thr371/Thr373, Ala183I/Ala183R, and Leu186I/Leu186R. Figure S2 shows the mutations that were introduced into TyrRS to generate the o-aaRSs. Mutagenesis primers are listed in Table S2. All vectors were verified by sequencing.
SpeI and ligated, yielding pYES2-hSOD1-6His. The tryptophan codon at position 33 of hSOD1 was mutated to an amber stop codon ATG by site-directed mutagenesis PCR with the primers SODmutf and SODmutr (Table S2). Furthermore, the inducible GAL promoter on pYES2-hSOD1(W33TAG)-6His was exchanged for the stronger constitutive PGK1 promoter via homologous recombination in yeast. To achieve this, the GAL promoter was excised from pYES2-hSOD1(W33TAG)-6His with SpeI and XhoII. The DNA sequence of the PGK1 promoter was amplified by PCR with primers PGK1fp and PGK1rp (Table S2) using genomic S. cerevisiae DNA as the template. In order to facilitate efficient homologous recombination, the primers PGK1fp and PGK1rp introduced flanking sequences of 43 nt and 35 nt, respectively to the PGK1 promoter sequence. These sequences were homologous to the ends of the cut pYES2 vector. The PGK1 PCR product with the flanking homology regions and the SpeI/XhoII digested pYES2-hSOD1(W33TAG)-6His were co-transformed into S. cerevisiae strain InvSc1 (MATa/α his3A1/α leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52; Invitrogen) by the lithium acetate method [67] and clones carrying successfully recombined plasmids, which were designated pYES2-hSOD1(W33TAG)-6His, were selected on synthetic complete medium lacking uracil (SC –Ura; 1% glucose, 0.67% yeast nitrogen base (Difco), 1.92 g/L yeast synthetic drop-out medium supplement) and 100 μM 6His, were selected on synthetic complete medium lacking uracil (SC –Ura; 1% glucose, 0.67% yeast nitrogen base (Difco), 1.4 g/L yeast synthetic drop-out medium supplement) containing Tyr analogs (SERVA Electrophoresis GmbH, Heidelberg, Germany) and probed with monoclonal mouse anti-Strep-tag II antibody MAB-Class (IBA BioTAGnology) as the first and horseradish peroxidase conjugated goat anti-mouse IgG (IBA BioTAGnology) as the second antibody. Tagged proteins were visualized by chemiluminescence detection (Pierce, Rockford, IL).

In a similar way, we exchanged the hexahistidine-tag for the Strep-tag II. The pYES2-hSOD1(W33TAG)-6His vector was linearized with EcoRI and the Strep-tag II coding sequence with appropriate homology hooks was amplified by PCR with primers hSOD1_Strep_fp and hSOD1_Strep_rp (see Table S2). Homologous recombination and screening of positive clones containing pYES2-hSOD1(W33TAG)-Strep was as described above for the promoter exchange.

Expression and purification of hSOD1(W33TAG) variants containing Tyr analogs

The S. cerevisiae expression strain InvSc1 was co-transformed with one of the o-pair expression vectors and pYES2-hSOD1(W33TAG)-Strep by the lithium acetate method [67]. Positive transformants were selected on synthetic complete medium lacking uracil and tryptophan, but containing all other amino acids (SC –Ura –Trp; 1% glucose, 0.67% yeast nitrogen base (Difco), 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan and uracil (Sigma), 76 mg/L histidine (Sigma), 380 mg/L leucine (Sigma); the medium contains 76 mg/L or 0.3 mM tyrosine disodium salt). For incorporation experiments, 5 mL SC –Ura –Trp medium were inoculated with the transformed expression strain and this starter culture was grown with vigorous shaking at 30°C over night. Subsequently, 50 mL SC –Ura –Trp medium were inoculated to an OD600 of 0.2 with the starter culture and incubated with shaking at 30°C for 24 h. Finally, we inoculated 500 mL SC –Ura –Trp medium supplemented with 1 mM of Tyr or one of the analogs with the pre-culture to an OD600 of 0.2. The amino acid was directly dissolved in the expression medium, which was then filter sterilized before inoculation. The hSOD1 variants were expressed with vigorous shaking at room temperature for 48 h. Cells were harvested by low speed centrifugation (3000×g, room temperature, 5 min) and the cell pellets were stored at −80°C until protein preparation.

To purify the hSOD1 variants, we lysed the yeast cells with Y-PER (Pierce, Rockford, IL) following the manufacturer’s instruction. The lysate was cleared by centrifugation (14000×g, room temperature, 10 min) and applied onto a Strep-Tactin-column (1 mL column volume (CV); IBA BioTAGnology, Gottingen, Germany). After protein binding, the column was washed with 5 CVs of washing buffer (100 mM Tris/Cl pH 8.0, 150 mM NaCl, 1 mM EDTA). Bound protein was eluted 6 times with 0.5 CVs of elution buffer (100 mM Tris/Cl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM desthiobiotin) each. Eluates were pooled and concentrated to a total volume of 150 μl by ultrafiltration (Centriprep YM-10, MWCO 10,000; Amicon, Beverly, MA).

Immunoblotting

Of each concentrated hSOD1 variant, 20 μl were run on a 12% Laemmli gel [68]. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany) for immunodetection [69]. The membranes were blocked with TBB5 (50 mM Tris/Cl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween20) containing 1.5% (v/v) bovine serum albumin (SERVA Electrophoresis GmbH, Heidelberg, Germany) and probed with monoclonal mouse anti-Strep-tag II antibody MAB-Class (IBA BioTAGnology) as the first and horseradish peroxidase conjugated goat anti-mouse IgG (IBA BioTAGnology) as the second antibody. Tagged proteins were visualized by chemiluminescence detection (Pierce, Rockford, IL).

Electro spray ionization mass spectrometry (ESI-MS)

For ESI-MS, 20 μl aliquots of the purified hSOD1 variants or the o-aaRSs were pre-separated on a Waters RP C18 column (300 A pore size; 3.5 μm particle size; 100×2.1 mm; Waters GmbH, Eschborn, Germany) by eluting with a gradient from 20 to 90% B in A within 20 min, where eluent B was 0.05% (v/v) TFA in water and eluent B was 0.05% (v/v) TFA in acetonitrile. A flow rate of 250 μl/min was used. The masses of the eluted fractions were analyzed on a MicroTOF ESI-MS (Bruker Daltonics, Bremen, Germany).

Cloning and expression of wild type TyrRS and the o-aaRSs for the ATP-PPi exchange assay

Cloning. The sequences encoding wild type TyrRS and the o-aaRSs were PCR amplified with primers pESCTrp1 and pESCTrp2 (Table S2) from corresponding o-pair expression vectors as templates. The primers contain an EcoRI and NotI cleavage site, respectively, flanked by additional nucleotides at their 5′-ends for efficient restriction. The PCR fragments were digested with EcoRI and NotI (New England Biolabs) and inserted into pET28a (Merck KGaA, Darmstadt, Germany) cleaved with the same enzymes, so that the hexahistidine-tag of the pET28a vector was attached to the N-terminus of the aaRSs. The resulting expression plasmids pET28a-H6-oaaRS were sequence-verified.

Protein expression. The pET28a-H6-oaaRS expression vectors were introduced into the E. coli strain B834 (DE3) (F ompT hsdSB(rB mB) gal dcm met proABCDE (DE3); Novagen Merck Chemicals, Nottingham, UK) by electroporation following standard laboratory procedures. Plasmid-harboring clones were selected and propagated in media containing 50 mg/L kanamycin. The sequences of the expression plasmids were verified by sequencing. For aaRS expression, the cells were grown in 1 liter LB (Luria Broth) medium at 37°C until they reached mid-log phase (OD600 0.6-0.8). Then, gene expression was induced by the addition of 1 mM isopropyl-Β-D-1-thiogalactopyranoside (IPTG; Application, Darmstadt, Germany) and was performed for 4–5 h at 25°C with vigorous shaking.

Protein purification. The cells were harvested by low speed centrifugation (3,200×g, 4°C, 20 min) and the cell pellet was resuspended in sodium phosphate buffer (50 mM NaH2PO4,
300 mM NaCl, pH 8.0) with 10 mM imidazole. After addition of 1 mg/mL DNase (Roche, Mannheim, Germany), RNAse (Sigma), and lysozyme (Sigma) each, cells were ruptured by sonication and the homogenate cleared from cell debris by high speed centrifugation (30,000 x g, 4°C, 30 min). The clear lysate was loaded onto a 5 mL HiTrap Chelating HP column (GE Healthcare, Munich, Germany), which was then washed with 10 CVs of sodium phosphate buffer containing 20 mM imidazole. Bound proteins were eluted by applying an imidazole gradient (20-250 mM) in sodium phosphate buffer. The elution fractions were analyzed by SDS-PAGE and those enriched in the desired aaRS were pooled and first dialyzed against 20 mM Tris/HCl pH 8.0 and then against aaRS-buffer (20 mM Tris/Cl pH 8.0, 150 mM KCl, 15 mM MgCl₂ and 5 mM β-mercaptoethanol). The dialyzed fractions were concentrated by ultrafiltration (Vivaspin 20 MWCO 10,000; Sartorius AG, Gottingen, Germany). Finally, the concentration of the protein samples was determined by recording the absorption at 280 nm and purity was analyzed by SDS-Page (Figure S6) and ESI-MS mass analysis (Figure S7).

**ATP-PPi exchange assay.** The reaction mix contained 100 mM Tris/Cl pH 8.0, 80 mM MgCl₂, 5 mM KF, 700 mM β-mercaptoethanol, 5.5 mM ATP, 0.1 mg/mL BSA, 2.2 mM [³²P]-PPi (0.2 cpm/µmol; PerkinElmer, Rodgau, Germany), 5 µM of the o-aaRS (or 1 µM of wild type TyrRS), and 5 mM [or less, as indicated] of Tyr or Tyr analogs in a final volume of 200 µL. For the assay, 20 µM stock solutions of the purified aaRSs in aaRS-buffer were used. All amino acids were dissolved in 0.01 M HCl at concentrations by the different AzRSs.

**Supporting Information**

**Figure S1** Plasmid map of the tandem expression vector for the orthogonal aaRS/tRNA_CUA pairs. The aaRS is expressed under the strong, constitutive ADH1 promoter on a yeast/E. coli shuttle vector containing an ampicillin resistance gene (AmpR) and the ColE1 origin of replication for selection and propagation in E. coli, respectively. The TRP1 auxotrophy marker and the 2µ origin of replication ensure plasmid maintenance in yeast. The amber suppressor tRNA(tRNA_CUA) expression cassettes are detailed in Table 1. The following tandem expression vectors were used in this work [the original o-aaRS nomenclature is given in brackets]: pTyr/tRNA_CUA for TyrRS (TyrRS [18]), pAz1/tRNA_CUA for AzRS1 (p-azidoPheRS1 [18]), pAz2/3SUP-tRNA_CUA for AzRS3 (p-azidoPheRS1 [29]), pAz6/tRNA_CUA for AzRS6 (p-azidoPheRS6 [18]), pPR1/3SUP-tRNA_CUA for PrRS1 (p-PpaRS1 [29]) and pBpa/tRNA_CUA for BpaRS (p-benzoyl-PheRS2 [18]).

**Figure S2** DNA and protein sequence alignment of TyrRS and the o-aaRSs. Mutated bases and exchanged amino acid residues are highlighted in color.

**Figure S3** The hSOD1(W33TAG) expression vector. Constitutive expression of hSOD1 with an in-frame amber stop codon (TAG) in position 33 and a C-terminal Strep-tag II is driven by the constitutive PGK1 promoter. The yeast/E. coli shuttle vector contains an ampicillin resistance gene (AmpR) and the ColE1 origin of replication for selection and propagation in E. coli, respectively. The UR3 auxotrophy marker and the 2µ origin of replication ensure plasmid maintenance in yeast.

**Figure S4** DNA and protein sequence of the hSOD1(W33TAG) open reading frame with a C-terminal Strep-tag II. The position of the amber stop codon (boxed) is indicated as X (bold black) in the protein sequence, hSOD1 is highlighted in green and the Strep-tag II in orange.

**Figure S5** Intracellular expression of the o-aaRSs from different orthogonal pairs. Sample preparation and immunodetection are described in Methods S1. For immunodetection, a C-terminal hexahistidine-tag was added to AzRS1 on pAz1/tRNA_CUA by homologous recombination as described in Methods S1. AzRS3 and PrRS1 on pAz2/3SUP-tRNA_CUA and pPR1/3SUP-tRNA_CUA, respectively, originally contained a C-terminal hexa-histidine-tag [29]. While only a single clone was analyzed for AzRS1(His) expression (lane 1), three different clones each were analyzed for expression of AzRS3 (lanes 2–4), and PrRS1 (lanes 5–7). The molecular weight of the o-aaRSs is 48 kDa. M, molecular weight marker; +, wild type hSOD1 with a C-terminal hexa-histidine-tag (positive control); -, empty lane.

**Figure S6** Purified E. coli wild type TyrRS and o-aaRSs. aaRSs were expressed in E. coli and purified by Ni-NTA affinity chromatography. The proteins were concentrated by ultrafiltration and analyzed by SDS-PAGE. The calculated molecular weight of TyrRS and the o-aaRSs is 51 kDa. 1, TyrRS; 2, AzRS1; 3, AzRS6; 4, AzRS3; 5, BpaRS; M, molecular weight marker. The gel was cut between lanes 4 and 5 to remove irrelevant lanes.

**Figure S7** ESI-MS analysis of E. coli wild type TyrRS and the o-aaRSs shown in Figure S6 and Table S1. TyrRS (A); AzRS1 (B); AzRS3 (C); AzRS6 (D); BpaRS (E).

**Figure S8** ATP-PPi exchange assay of TyrRS with different concentrations of Tyr. Different concentrations of Tyr were used to determine the substrate range for non-saturated enzymatic activity (A) and for substrate saturation (B). In the negative control, no amino acid was added to the reaction mix (w/o aa). TyrRS was used at a concentration of 1 µM. The data in (A) and (B) were collected in one series of experiments each and the ATP formation with each tyrosin concentration was determined in duplicate. Mean values are shown; the bars denote the discrete variations.

**Figure S9** Activation of Tyr, AzF and AmF at low concentrations by the different AzRSs. In order to exclude effects of substrate inhibition in the ATP-PPi assay, 5 µM each of AzRS1 (A), AzRS3 (B), and AzRS6 (C) were incubated with Tyr, AzF, and AmF at concentrations that had caused linear activation.
of Tyr by TyrRS (refer to Figure S8). In the negative control, no amino acid was added to the reaction mix (w/o aa). The data for each aaRS were all collected in one series of experiments. Mean values of duplicates are shown; the bars denote the discrete values. (TIF)

Figure S10  ESI mass spectrum of the PxF preparation. Among other unidentified impurities, approximately 1% Tyr (corresponding mass indicated in red in the lower panel) was present, most probably originating from the chemical synthesis [19]. The lower panel shows the same mass spectrum as the upper panel albeit at a magnified intensity scale (×106 in the upper panel, ×104 in the lower panel). (TIF)

Table S1  Mass analysis of E. coli wild type TyrRS and the different o-aaRs.

| Reference | Methodology |
|-----------|-------------|
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Author Contributions
Conceived and designed the experiments: NB BW. Performed the experiments: SN. Analyzed the data: SN NB BW. Wrote the paper: SN BW.
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