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In vivo Incorporation of Unnatural Amino Acids to Probe Structure, Dynamics, and Ligand Binding in a Large Protein by Nuclear Magnetic Resonance Spectroscopy

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Abstract: *In vivo* incorporation of isotopically labeled unnatural amino acids into large proteins drastically reduces the complexity of nuclear magnetic resonance (NMR) spectra. Incorporation is accomplished by coexpressing an orthogonal tRNA/aminoacyl−tRNA synthetase pair specific for the unnatural amino acid added to the media and the protein of interest with a TAG amber codon at the desired incorporation site. To demonstrate the utility of this approach for NMR studies, 2-amino-3-(4-(trifluoromethoxy)phenyl)propanoic acid (OCF_3Phe), $^{13}C/^{15}N$-labeled *p*-methoxyphenylalanine (OMePhe), and ^{15}N-labeled *o*-nitrobenzyl-tyrosine (oNBTyr) were incorporated individually into 11 positions around the active site of the 33 kDa thioesterase domain of human fatty acid synthase (FAS-TE). In the process, a novel tRNA synthetase was evolved for OCF_3Phe. Incorporation efficiencies and FAS-TE yields were improved by including an inducible copy of the respective aminoacyl-tRNA synthetase gene on each incorporation plasmid. Using only between 8 and 25 mg of unnatural amino acid, typically 2 mg of FAS-TE, sufficient for one 0.1 mM NMR sample, were produced from 50 mL of *Escherichia coli* culture grown in rich media. Singly labeled protein samples were then used to study the binding of a tool compound. Chemical shift changes in $^1H-^{15}N$ HSQC, $^1H-^{13}C$ HSQC, and ^{19}F NMR spectra of the different single site mutants consistently identified the binding site and the effect of ligand binding on conformational exchange of some of the residues. OMePhe or OCF_3Phe mutants of an active site tyrosine inhibited binding; incorporating ^{15}N-Tyr at this site through UV-cleavage of the nitrobenzyl-photocage from oNBTyr re-established binding. These data suggest not only robust methods for using unnatural amino acids to study large proteins by NMR but also establish a new avenue for the site-specific labeling of proteins at individual residues without altering the protein sequence, a feat that can currently not be accomplished with any other method.

Introduction

NMR spectroscopy is an established and very powerful biophysical method to study the structure, dynamics, and function of proteins.[1–3] In principle, molecular processes such as binding or conformational changes can be deciphered by NMR with atomic resolution. However, the complexity of NMR spectra of large proteins in practice often represents a formidable challenge. Simplification of spectra by amino acid-type specific isotope labeling[4] including labeling with fluorinated analogs of natural amino acids,[5–8] by incorporation of stereoarray isotope labeling (SAIL) amino acids,[9] or selective isotope labeling of methyl groups in perdeuterated proteins[10,11] is very helpful for many applications and required for larger systems. Recently developed methods for the *in vivo* incorporation of unnatural amino acids into proteins[12–18] provide an unique opportunity

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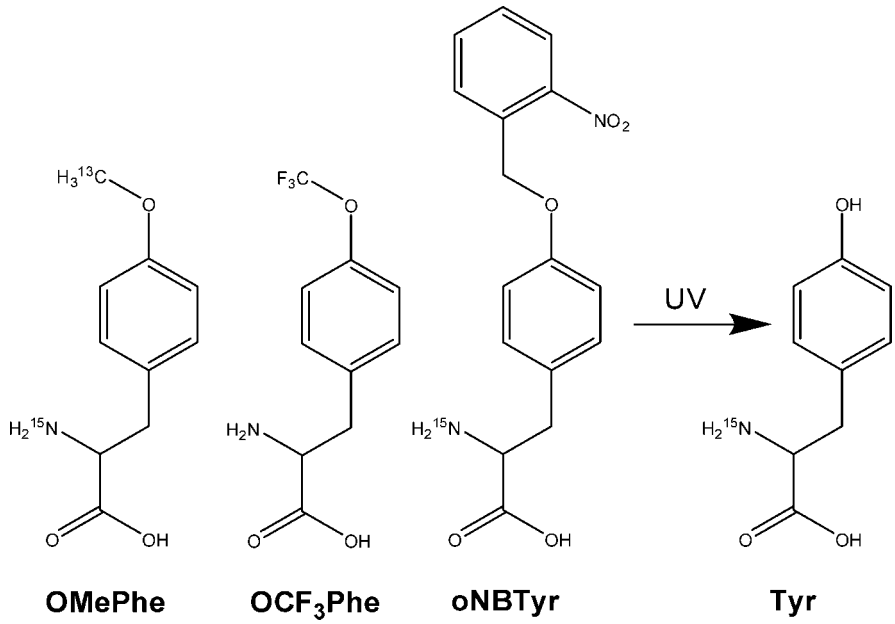

Figure 1. Isotopically labeled unnatural amino acids *O*-methyl-phenylalanine (OMePhe), its trifluorinated analog OCF_3Phe, and *o*-nitrobenzyl-tyrosine (oNBTyr), which is readily converted into tyrosine upon UV illumination.[23]

to introduce NMR-active labels at a single designated amino acid residue.[19]

Using an orthogonal amber tRNA/tRNA synthetase (tRNA/RS) pair that specifically amino-acylates an amber tRNA with the desired unnatural amino acid and incorporates it into a protein at the amber nonsense codon UAG, any desired protein residue can, at least in principle, be substituted *in vivo* by an unnatural amino acid. The incorporation of the unnatural amino acid at the desired location instantly provides an "assignment" for the NMR signal of the unnatural amino acid. Monitoring the chemical shift change of a single resonance opens an avenue for focused, site-directed screening for binders. This is particularly interesting for drug development because it can reduce the number of screening hits that bind to protein pockets of little interest. Furthermore, unnatural amino acids incorporated at different sites in multiple samples can be used to establish binding within an active site or could be used to triangulate binding of a small molecule or a biomacromolecule via NOE measurements. Similarly, saturation transfer techniques that utilize selective labeling strategies to study the structures of protein−ligand complexes[20] can utilize the specificity afforded by unnatural amino acid incorporation to determine both the protein residues and regions of the molecule that are in close proximity. Protein conformational changes and dynamics can also be monitored at specific sites of interest with minimal or no perturbation of sequence. Mixtures of proteins labeled with different unnatural amino acids can simplify the NMR characterization of symmetric oligomers. Selectively incorporating methyl labels can be very useful for examining the millisecond time scales associated with many biological processes, such as enzyme catalysis, product release, and protein folding.[21] The unnatural amino acid labeling technique presented here has the potential to extend NMR studies to many proteins that are currently not amendable because of their complexity, size, or physical characteristics.

Previously, we have shown that protein amounts sufficient for NMR studies can be produced in *Escherichia coli* using ^{15}N-labeled *O*-methyl-phenylalanine (OMePhe) (Figure 1).[19] Because the yields were modest, one of the primary goals of the current study was to improve the incorporation efficiency and consequently increase protein yields. This was accomplished by constructing a plasmid that overexpresses the RS specific for the unnatural amino acid at the time of induction. The second goal was to expand the number of NMR-active unnatural amino acids. Producing ^{15}N and ^{13}C-labeled OMePhe was an obvious first step (Figure 1). Developing an orthogonal tRNA/RS pair for the incorporation of a trifluorinated analog was our second choice. Fluorine represents an attractive NMR label[5–8] with high intrinsic sensitivity, 100% natural abundance, and the absence of any natural background. The chemical shift of ^{19}F is extremely sensitive to the local environment and can detect changes in van der Waals contacts, electrostatic fields and hydrogen bonding. In contrast to Mehl and co-workers who recently selected a specific tRNA/RS pair for trifluoromethyl-phenylalanine and demonstrated its utility for ^{19}F protein NMR studies,[22] we opted to develop such a system for OCF_3Phe (Figure 1) because of its structural similarity to OMePhe. As a third unnatural amino acid, we tested the incorporation of ^{15}N-labeled o-nitrobenzyl-tyrosine (oNBTyr) (Figure 1). It has been demonstrated that UV-photocleavage of this unnatural amino acid after incorporation converts oNBTyr back to the natural amino acid tyrosine.[23] In contrast to the other unnatural amino

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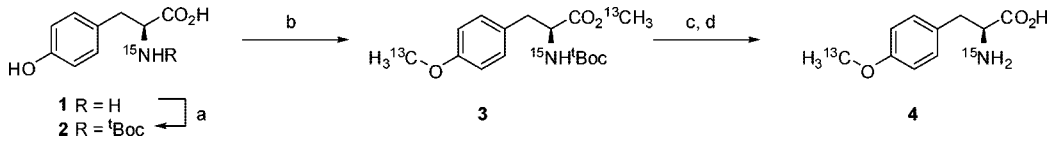
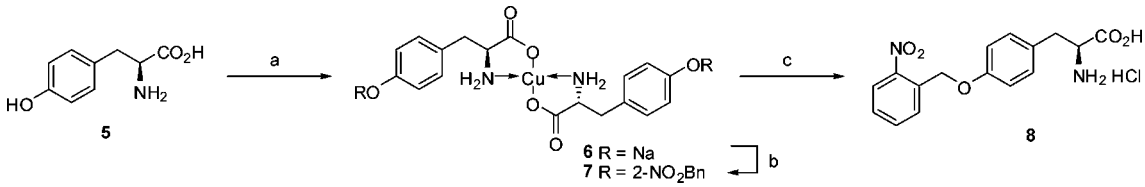
Scheme 1[a]



[a] Conditions: (a) $(^tBuOCO)_2O$, NEt_3, H_2O/1,4-dioxane (1:1 v/v), 23 °C, 16 h, 92%; (b) $^{13}CH_3I$, K_2CO_3, DMF, 0−23 °C, 97%; (c) TFA/DCM (1:4 v/v), 23 °C; (d) NaOH, H_2O/MeOH (1:1 v/v), 23 °C, 41% (2 steps).

Scheme 2[a]



[a] Conditions: (a) $CuSO_45H_2O$, NaOH, H_2O, 23 °C; (b) 2-nitrobenzyl chloride, NaOH, H_2O/MeOH (1:1 v/v), DMF, 23 °C, 46% (2 steps); (c) 1 M HCl, 23 °C, 83%.

acids, photocaged tyrosine facilitates the introduction of an NMR label at specific tyrosine residues without altering the protein sequence.

The three different NMR-active unnatural amino acids were used to study the binding of a small molecule ligand to the thioesterase domain of fatty acid synthase (FAS-TE), a 33 kDa protein of pharmaceutical interest. Fatty acid synthase (FAS) is a large, multidomain enzyme essential for the synthesis of long-chain fatty acids.[24] Because the sequence and architecture of FAS differ significantly between bacteria and mammals, bacterial FAS has long been considered a valuable target for the development of novel antibiotics (see ref 25 and references herein). In humans, FAS is overexpressed in many cancers[26] and is a drug target for obesity and related diseases.[27] Orlistat, an approved obesity drug, exhibits antitumor activity by inhibiting FAS-TE.[28,29] The molecular details of orlistat's interactions with FAS-TE have only been revealed in a recent X-ray crystallographic study.[30] Here we use a more soluble tool compound to evaluate the utility of unnatural amino acids for the characterization of protein−ligand interactions. Successful incorporation of three different NMR-active unnatural amino acids at 11 different positions around the proposed binding site in FAS-TE demonstrates the general utility of the approach to studies of protein structure, dynamics, function, and binding of small molecules in particular.

Materials and Methods

Unnatural Amino Acids, Tool Compound, and Other Chemicals. OCF_3-DL-Phe was purchased from JRD Fluorochemicals (Leatherhead, Surrey, UK) and used without further purification. OMe-L-Phe was purchased from Sigma-Aldrich and used without further purification. $^{13}C/^{15}N$-labeled OMePhe and ^{15}N-labeled oNBTyr were prepared from ^{15}N-labeled L-tyrosine (Cambridge Isotopes Laboratory) according to Schemes 1 and 2, respectively. Tool compound **17** was synthesized according to Scheme 3 (*vide infra*). Deuterated imidazole and D_2O were obtained from Cambridge Isotopes Laboratory and TCEP was obtained from Pierce. All other chemicals were obtained from Sigma.

Plasmids for the Expression of FAS-TE and Generation of Mutants. The coding sequence of our model protein, FAS-TE, was inserted into pMH4 (JCSG) using the PIPE cloning method.[31] TAG codons were mutated in at 11 specific sites using the same cloning technique. The sites of incorporation were chosen based on the solvent accessibility of the side chain in the FAS-TE apo-structure (1XKT.pdb).[32] To lower termination efficiency and subsequently reduce truncation at the TAG stop codon, the codon of the residue C-terminal of the incorporation site was optimized[33] for Tyr-2351 (Arg-2352: CGG to AGG, silent mutation) and for Leu-2222 (Leu-2223: CTG to ATT, resulting in a Leu-2223-Ile mutation). Cys-2359 was mutated to Ser to prevent disulphide mediated aggregation.

Plasmids for the Incorporation of OMePhe and oNBTyr and Improving the Expression Plasmid. Initial incorporation tests were performed with the previously described pSUP-OMePhe-3TRN plasmid.[13] This plasmid contains three copies of an amber tRNA and the mutant *Methanococcus jannaschii* tyrosyl-tRNA synthetase selected for OMePhe under the control of a glnS promotor. To coexpress the tRNA synthetase and therefore enhance amino acylation activity during protein production, we modified the plasmid as follows: For each synthetase of interest, the coding sequence was inserted into pMH4 by PIPE cloning.[31] The resulting plasmid was then used as template for a standard PCR amplification using the oligos KpnRS_F (5′-GGAGGTACCCAATTATGA-CAACTTGACGGCTACATC-3′) and SacRS_R (5′-GGAAC-GAGCTCACAAACAACAGATAAAACGAAAGGCCC-3′). The PCR product, containing the entire arabinose operon, was then prepared by restriction digest and inserted by ligation between the *Kpn*I and *Sac*I sites in the pSUP-RS-3TRN vector[13] with the same synthetase gene. The result is pSUPAR3-RS containing all of the elements of the pSUP-RS-3TRN vector plus an additional arabinose-inducible copy of the synthetase (Figure 2).

Plasmids and Libraries for the Selection of a OCF_3Phe-Specific tRNA Synthetase. All plasmids used in the selection of a OCF_3Phe-specific tRNA synthetase were described previ-

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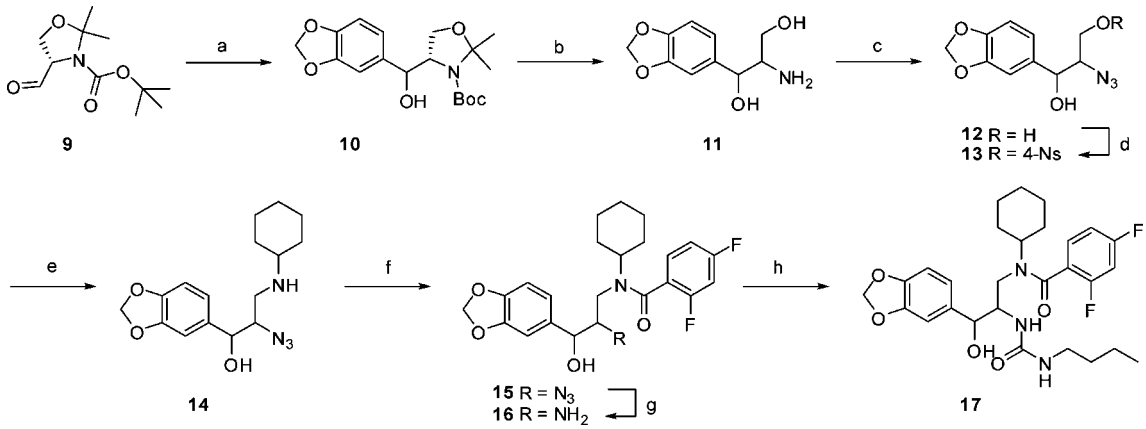
Scheme 3[a]



[a] Conditions: (a)3,4-(methylenedioxy)phenylmagnesium bromide, THF/toluene (1:1 v/v),−78 to 23 °C, 98%; (b) 1 M HCl, THF, 70 °C; (c) trifluoromethanesulfonyl azide, $CuSO_4$, K_2CO_3, H_2O/MeOH, 0−23 °C, 42% (2 steps); (d) 4-nitrobenzene-1-sulfonyl chloride, pyridine, cat. DMAP, DCM, 23 °C, 55%; (e) cyclohexanamine, NMP, 80 °C; (f) 2,4-difluorobenzoyl chloride, DIEA, DCM, 23 °C, 65%; (g) H_2, 5% Pd/C, MeOH, 23 °C; (h) *n*-butylisocyanate, DIEA, DCM, 23 °C, 28% (2 steps).

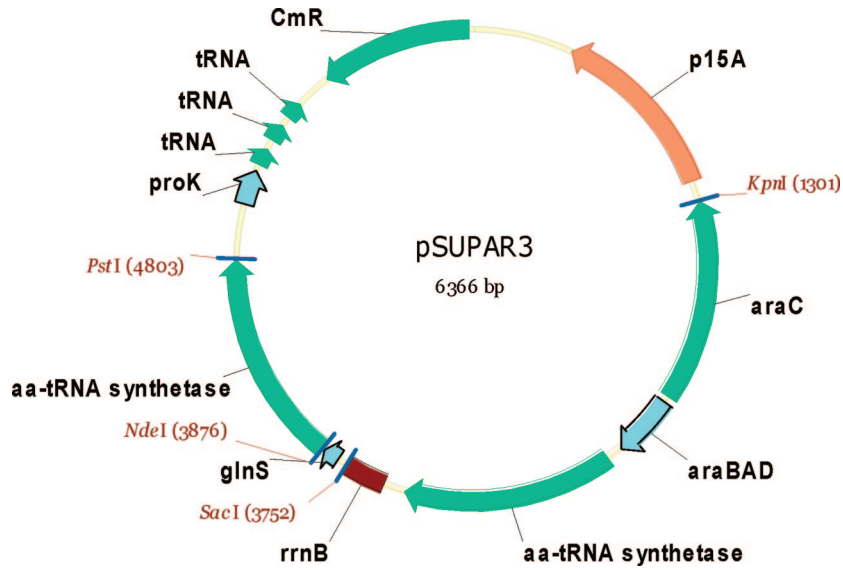


Figure 2. pSUPAR3 plasmid. Plasmid contains two copies of the amino-acyl tRNA synthetase specific for the unnatural amino acid (Uaa-RS), one under the control of a constitutively on glnS promoter and the second under the control of an arabinose-inducible ara promoter. The plasmid also encodes three copies of the unnatural amino acid specific amber tRNA with a proK promoter, a p15A origin and a chloramphenicol CmR resistance marker.

ously.[16,34] pREP2-YC-JYCUA encodes the mutated $tRNA^{Tyr}_{CUA}$ from *M. jannaschii*, a chloramphenicol resistance gene containing a TAG stop codon, and a tetracycline resistance gene. pNEG encodes the mutated $tRNA^{Tyr}_{CUA,}$ barnase with three TAG stop codons in the coding sequence, and β-lactamase. pBK-MJYRS-L1 (positions Tyr32, Leu65, His70, Gln155, Asp158, and Leu162 randomized), pBK-MJYRS-L2[34] (positions Tyr32, Leu65, Phe108, Gln109, Asp158, and Leu162 randomized), and pBK-MJYRS-L3D[35] (positions Tyr32, Leu65, His70, Phe108, Gln109, Gln155, Asp158, Ile159, and Leu162 randomized) encode the tyrosyl tRNA synthetase (aa RS) from *M. jannaschii* with different positions of the active site randomized to all 20 amino acids, as previously described.[34,35] pLeiZ encodes the Z-domain protein with a TAG codon at position 7 and C-terminal His_6-tag, the *Mj*$tRNA^{Tyr}_{CUA}$, and β-lactamase.[36]

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Selection of a Novel OCF_3Phe-Specific tRNA Synthetase. In the first round of selection, frozen aliquots, equivalent to $\sim 10^{10}$ cfu, of DH10B cells transformed with pREP2-YC-JYCUA and each of the pBK library plasmids were plated on GMML-Agar supplemented with 0.04% glucose; 50 μg/mL kanamycin; 0.25 mM OCF_3Phe; and 50 μg/mL chloramphenicol or on LB-Agar with 50 μg/mL kanamycin; 0.5 mM OCF_3Phe; and 50 μg/mL chloramphenicol. After 3 days, cells were scraped from the plates and the plasmids purified by Spin Miniprep Kit (Qiagen). The pREP2 and pBK plasmids were separated by gel electrophoresis on a 1% agarose gel, and the pBK plasmid pools were repurified by Minelute Gel Extraction Kit (Qiagen). For negative selections, the pBK pools were transformed by electroporation into HK100 cells (JCSG; derived from Genehogs by Invitrogen) harboring the pNEG plasmid and plated on LB-Agar supplemented with 50 μg/mL kanamycin, 100 μg/mL ampicillin, and 0.2% arabinose. After 12−14 h of growth, the pBK plasmid pools were isolated as before and transformed into HK100 cells with the pREP2 plasmid. After a total of four positive rounds (with 50, 50, 75, and 100 μg/mL chloramphenicol, with 0.25, 0.25, 0.5, and 0.5 mM OCF_3Phe, and with all defined media plates after the first round) and three negative

rounds, colonies were picked from the final positive round for further study. Following the positive selection recipe, a total of 296 colonies were replica plated onto agar plates either without OCF_3Phe but with 20, 35, or 50 μg/mL chloramphenicol; with 0.5 mM OCF_3Phe and 100, 125, or 150 μg/mL chloramphenicol; or with 1 mM OCF_3Phe and 100, 125, or 150 μg/mL chloramphenicol. The plasmids from 37 of these colonies that showed high growth with unnatural amino acid and poor growth without it were isolated and sequenced, revealing 14 novel sequences (Table S1 and S2). Five clones (A6, B7, B10, F6 and H4) were chosen for further evaluation based on the results of pooled replica plates and the number of occurrences of the clone.

Test Incorporation of OCF_3Phe into Z-Domain Protein and Evaluation of Misincorporation. HK100 cells cotransformed with pLeiZ and each of the five pBK-OCF_3Phe-RS were grown in 50 mL cultures of TB supplemented with 50 μg/mL kanamycin, 100 μg/mL ampicillin, and with or without 1 mM OCF_3Phe. Cells were harvested after 5 h induction with 1 mM IPTG at 30 °C. Lysates were prepared in 6 M guanidine by sonication and clarified by centrifugation at 20 000*g* for 20 min. The His-tagged proteins from each culture were purified by Ni-NTA (Qiagen) columns according to the manufacturer's protocol. Denaturant was removed by PD-10 columns (GE Healthcare). The protein samples were next evaluated by SDS-PAGE, Bradford assay (Pierce), and LC−ESI-MS (Supplemental Figures S2 and S3, Supporting Information). To test for miscorporation of natural amino acids at the TAG incorporation site, protein samples of Z-domain expressed in the absence or presence of OCF_3Phe with the five evolved OCF_3Phe-RS were digested with trypsin and subjected to LC−ESI-MS to evaluate the presence of the N-terminal peptide TSVDNXINK, where X represents the mutated position. The peptide masses for incorporation of OCF_3Phe, Tyr, Phe, and Trp were all monitored in MS experiments to evaluate misincorporation levels of the natural aromatic amino acids. The Z-domain protein produced with OCF_3Phe-RS clone A6 in the presence of 1 mM OCF_3Phe was used to verify the sequence of the peptide by MS−MS. In a separate experiment, the incorporation fidelity of the OCF_3Phe RS (clone F6) was further investigated at the Thr-26 site of FKBP12[37] by multiple-reaction monitoring as described previously.[38] The homogeneity of the protein was found to be better than 99.5%.

Expression of FAS-TE Mutants Containing OMePhe, OCF_3-Phe, and oNBTyr. OCF_3Phe-RS clone F6 was inserted into the pSUPAR3 plasmid. For each mutant, the expression plasmid and pSUPAR3-OMePhe, pSUPAR3-OCF_3Phe, or pSUPAR3-oNBTyr plasmid were cotransformed into HK100 cells. Multiple single colonies were picked for growth in 50 mL TB cultures (in 250 mL shaker flasks) with 100 μg/mL ampicillin and 35 μg/mL chloramphenicol. At $OD_{595} = 1.0$, cultures were moved to 30 °C and supplemented with 2.5 mM $^{13}C/^{15}N$-labeled OMePhe, 1 mM OCF_3Phe (both from 0.5 M stock solutions in 1 N HCl) or 0.5 mM ^{15}N-labeled oNBTyr (from a fresh 0.5 M stock in DMSO). Thirty minutes later, expression was induced by addition of 0.2% arabinose. After 20 h, cells were harvested. Lysates were prepared by sonication, clarified by centrifugation, and purified by Ni-NTA under native conditions according to the manufacturer's protocol. Protein samples were evaluated by SDS-PAGE, Bradford assay, and ESI-MS before being exchanged into NMR buffer (20 mM deuterated imidazole, 100 mM NaCl, 0.5 mM TCEP, pH 6.5) via PD-10 column and concentrated using Amicon-ultra units (Millipore). Expression yields after purification are given in Table 1. Photocleaveage of oNBTyr was accomplished by exposing the NMR samples for 10 min to UV light generated by an Oriel 500 W Hg light source (Newport Corporation; Stratford, CT).

Table 1. Incorporation of Three Unnatural Amino Acids into FAS-TE at 11 Positions Using pSUPAR3 and 2.5 mM $^{13}C/^{15}N$ -Labeled OMePhe, 1 mM OCF_3Phe, and 0.5 mM ^{15}N-Labeled oNBTyr[a]

FAS-TE	Yield per 50 mL of culture [mg/50 mL]			NMR sample concentration [mM]		
Mutant	OMePhe	OCF_3Phe	oNBTyr	OMePhe	OCF_3Phe	oNBTyr
Leu-2222	2.7	2.2	1.2	0.15	0.12	0.11
Thr-2255	2.2	3.1	2.1	0.12	0.17	0.080
Tyr-2307	1.6	2.0	0.94	0.088	0.11	0.10
Tyr-2343	0.47	2.2	1.9	0.051	0.12	0.13
Tyr-2347	1.3	2.3	2.5	0.072	0.13	0.27
Tyr-2351	1.7	1.4	1.9	0.095	0.077	0.16
Gln-2373	0.67	0.42	0.25	0.055	0.034	0.16
Phe-2375	2.4	1.8	4.5	0.096	0.096	0.11
His-2408	2.0	2.7	5.0	0.11	0.15	0.14
Thr-2450	0.84	2.5	1.9	0.099	0.14	0.052
Tyr-2454	2.1	1.2	7.2	0.11	0.066	0.12

[a] Yields after purification are given in milligram per 50 mL *E.coli* culture. The concentrations for the NMR samples shown in Figures 4, 5, and 6, respectively, are also listed.

Expression of FAS-TE Mutants Selectively Labeled with ^{15}N-Tyrosine. The tyrosine residues of wild-type and mutant FAS-TE were selectively labeled using the following protocol: 20 mL of an overnight TB culture was added per 1 L M9 media (Sigma) supplemented with 0.5% glycerol and 0.1 mM $FeCl_3$. Thirty minutes prior to induction a sterile 100 mL solution containing 100 mg ^{15}N-labeled tyrosine; 500 mg each of glutamic acid, aspartic acid, glutamine, and asparagine; 300 mg each of alanine, valine, isoleucine, leucine, tryptophan, phenylalanine, serine, and glycine; and 200 mg of each of the remaining amino acids was added. The culture was induced as described above and allowed to proceed for approximately one-fifth of the normal expression time to minimize scrambling.

NMR Sample Preparation and Data Collection. As a lock solvent, 50 μL of D_2O were added to 500 μL of protein solution resulting in a final buffer composition of 18 mM d-imidazole, 91 mM NaCl, 0.45 mM TCEP, pH 6.5. The final protein concentrations of all NMR samples are given in Table 1. Aliquots of the tool compound were added from a 50 mM stock in deuterated DMSO to a final concentration of 1.4 mol equiv unless mentioned otherwise. The final DMSO concentration was between 0.10% and 0.76% v.v. for all samples but typically less than 0.5% v.v. resulting in negligible chemical shift changes. All spectra were recorded at 300 K. ^{19}F spectra were recorded on an Avance 400 MHz instrument (Bruker Biospin, Billerica, MA) equipped with a $^1H/^{13}C/^{19}F/^{31}P$-QNP-cryoprobe. Spectra were typically recorded with 1024 scans, a recycle delay of 2 s, 8192 complex data points with a sweep width of 20 ppm. Proton decoupling was accomplished using Waltz-16. All other spectra were recorded on an Avance 600 MHz instrument equipped with a $^1H/^{13}C/^{15}N$-TXI-cryoprobe. $^1H-^{13}C$ HSQC[39] were typically recorded with 32 scans, 128 t_1 experiments at 300 K using a spectral width of 50 ppm in the carbon dimension and 13.97 ppm in the proton dimension. Similarly, $^1H-^{15}N$ HSQC spectra[40] were recorded using 48 scans, 256 t_1 experiments using a spectral width of 32 and 13.97 ppm in the nitrogen and proton dimension, respectively.

Results

Improving Incorporation Efficiency. A first incorporation test of OMePhe into the thioesterase domain of fatty acid synthase (FAS-TE) was performed in *E. coli* with the previously described mutant *M. jannaschii* tyrosyl tRNA/tRNA synthetase (tRNA/RS) pair encoded by the pSUP-OMePhe-6TRN plasmid.[13] Fifty milliliter bacterial cultures were grown in rich media

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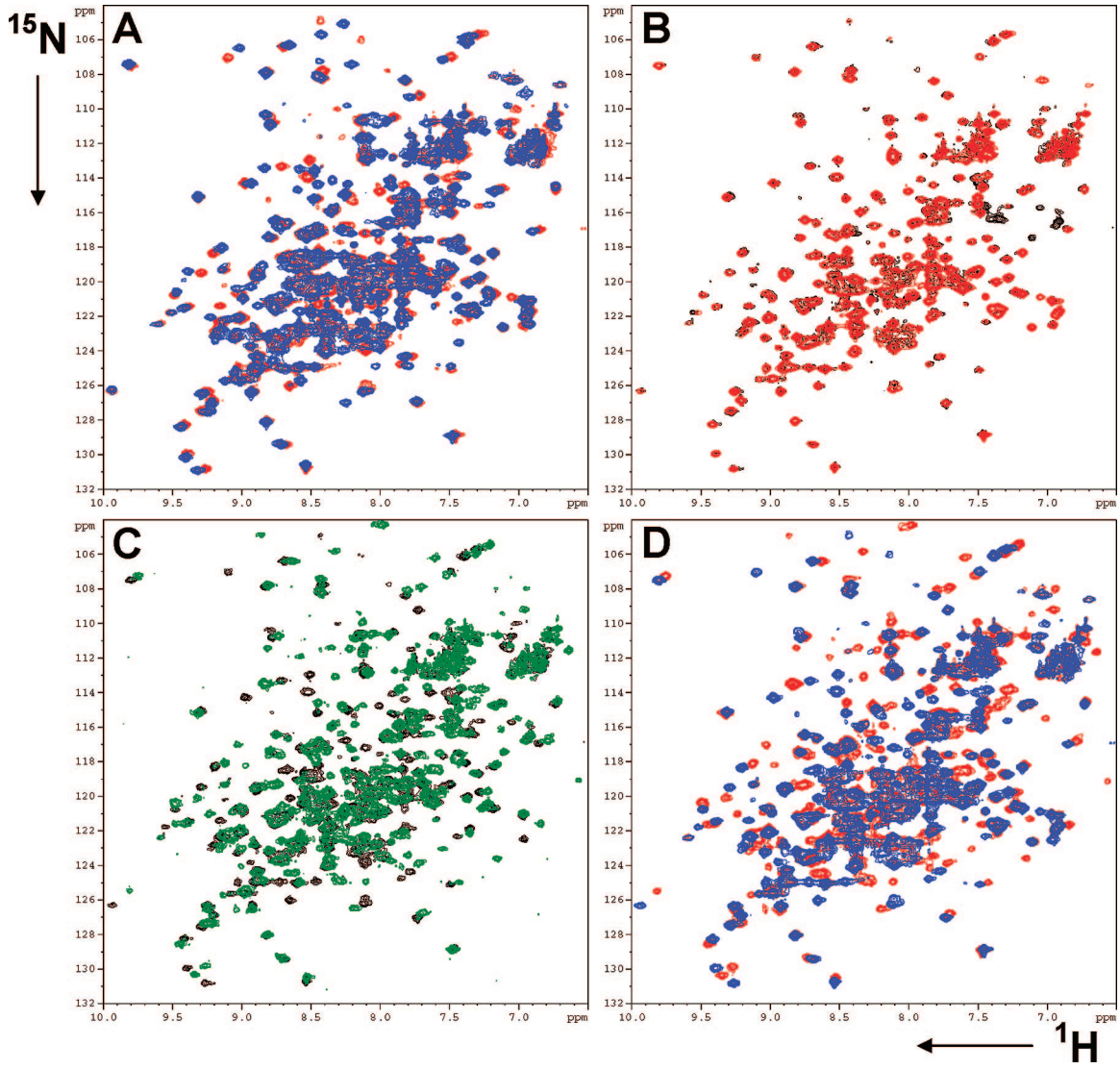

Figure 3. Incorporation of unnatural amino acids preserves structure of FAS-TE. $^{1}H-^{15}N$ HSQC of (A) "reverse-labeled" (unlabeled oNBTyr incorporated into uniformly ^{15}N-labeled protein) FAS-TE Tyr-2454-oNBTyr before (blue) and after UV cleavage (red); (B) the UV-cleaved sample (red) overlaid with uniformly ^{15}N-labeled wild-type FAS-TE (black); (C) Overlay of wild-type protein before (black) and after addition of tool compound (green); and (D) overlay of reverse labeled and UV-cleaved Tyr-2454-oNBTyr before (blue) and after addition of tool compound (red).

at OMePhe concentrations between 2.5 and 30 mM (between 25 and 300 mg per 50 mL) (Figure S1A and B, Supporting Information). Only between ~0.4 and 1.2 mg of full-length protein was produced, respectively, compared to ~3.8 mg for wild-type protein produced under the same conditions. The lower yield is caused by a high-rate of translation termination at the UAG amber codon as large amounts of truncated FAS-TE were detected in the insoluble fraction by SDS-gel and MS analysis. To increase the concentration and activity of the mutant tRNA synthetase, we constructed a plasmid pSUPAR3 that features an additional RS gene that is under the control of an arabinose inducible promoter (Figure 2). Simultaneous overexpression of RS and of the target protein dramatically reduced truncation and enhanced production of full-length FAS-TE (Figure S1C and D, Supporting Information). Optimization of the expression conditions showed that growth in TB media and induction at 30 °C for 20 h further increased yields of both wild-type and mutant proteins. Incorporation tests with the pSUPAR3 plasmid at different concentrations of OMePhe indicated saturation of protein production above 5 mM, suggesting that unnatural amino acid and synthetase are not limiting under these conditions. Comparisons of different versions of pSUPAR3 revealed that expression of full-length FAS-TE Tyr-2454-OMePhe was maximized by having 3 copies of the tRNA (vs 1 or 6) and both the inducible and the noninducible copies of the synthetase together (vs either copy alone). Changing the number of tRNA copies did not enhance yields suggesting that additional factors, that will be optimized in the future, are limiting to achieve wild-type yield. Using the pSUPAR3-OMePhe plasmid and only 2.5 mM OMePhe in 50 mL TB media, mutant FAS-TE with a single OMePhe incorporated can currently be produced with yields sufficient for a single 0.1 mM NMR sample. Subsequently, existing orthogonal tRNA synthetase for oNBTyr and a newly evolved tRNA synthetase for OCF_3Phe described below were also transferred to the pSUPAR3 plasmid and successfully used for protein expression.

Expanding the Number of NMR-Active Unnatural Amino Acids. Gram quantities of isotope labeled analogs of unnatural amino acids, namely a $^{13}C/^{15}N$-labeled analog of OMePhe and a ^{15}N-labeled analog of oNBTyr (Figure 1) were synthesized. $^{13}C/^{15}N$-labeled OMePhe **4** was prepared according to Scheme 1 whereas ^{15}N-labeled oNBTyr **8** was prepared according to

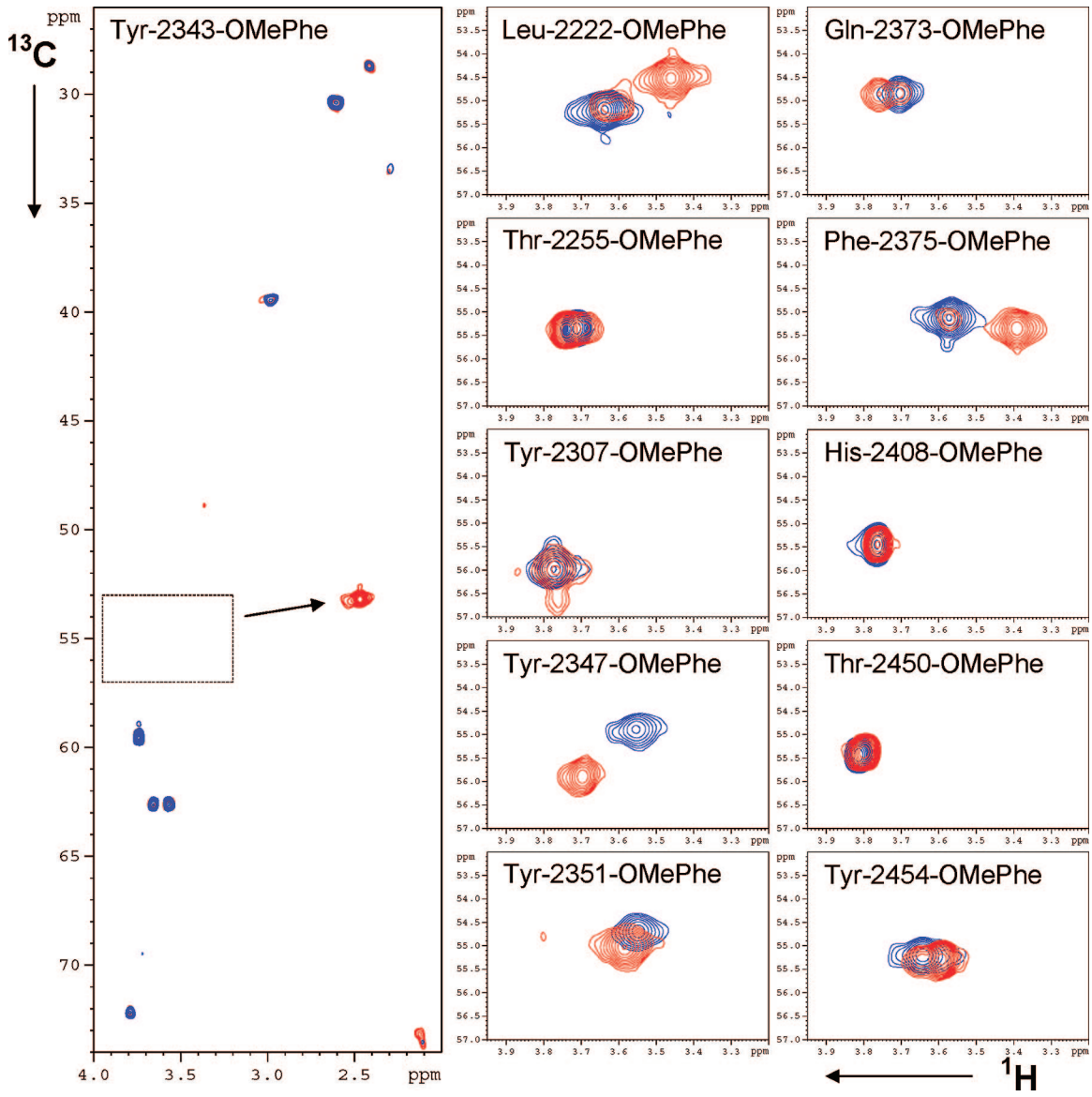

Figure 4. $^{1}H-^{13}C$ NMR spectra of FAS-TE mutants with $^{13}C/^{15}N$ -labeled OMePhe incorporated at 11 different positions: $^{1}H-^{13}C$ HSQC spectra before (blue) and after addition of tool compound (red). With the exception of the compound complex of Tyr-2343-OMePhe, all methoxy cross peaks of the unnatural amino acid have chemical shifts between 56.5 and 54.0 ppm for carbon and 3.85 and 3.35 ppm for protons. For Tyr-2343-OMePhe all observable peaks are shown. These include natural abundance peaks of TCEP, residual glycerol from concentration devices, residual TRIS from the purification buffer and other small molecule components of the NMR buffer. These peaks are also observed for all other mutants for which only the boxed region in the Tyr-2343-OMePhe mutant spectrum is shown.

Scheme 2 following procedures for the synthesis of *o*-benzyl-L-tyrosine ether.[41]

In addition, an incorporation system for a fluorinated unnatural amino acid was developed. Fluorinated analogs of natural amino acids, especially of Phe, Tyr, and Trp, can be incorporated into proteins at all respective aromatic residues and used for protein NMR studies.[5–8] A tRNA/RS pair for the site-specific *in vivo* incorporation of a fluorinated unnatural amino has so far been developed for only trifluoromethyl-phenylalanine.[22] Independently, we chose to develop such a system for OCF_3Phe (Figure 1) because (a) its structure is a conservative modification of the unnatural amino acid OMePhe that can be introduced without adverse effects on the structure of our test protein (see below), (b) three fluorine atoms virtually decoupled from a ring ($^{5}J_{HF}$ < 1 Hz, data not shown) should experience minimal fluorine-proton relaxation resulting in high intrinsic sensitivity for applications in large proteins, and (c) the tRNA/RS pair specific for OMePhe should be a good starting point for the evolution of a OCF_3Phe-specific RS. Initial tests to incorporate OCF_3Phe into proteins using the OMePhe-specific tRNA/RS pair failed and demonstrate the high selectivity of tRNA synthetases for their substrates which is consistent with previous suggestions that a CF_3 group should be bulkier and more hydrophobic than a CH_3 group.[42]

Randomizing amino acid residues in *M. jannaschii* TyrRS that are in contact with the substrate tyrosine side chain as illustrated in the X-ray structure[43] enabled the selection of a RS specific for OMePhe.[34] We successfully used similar libraries

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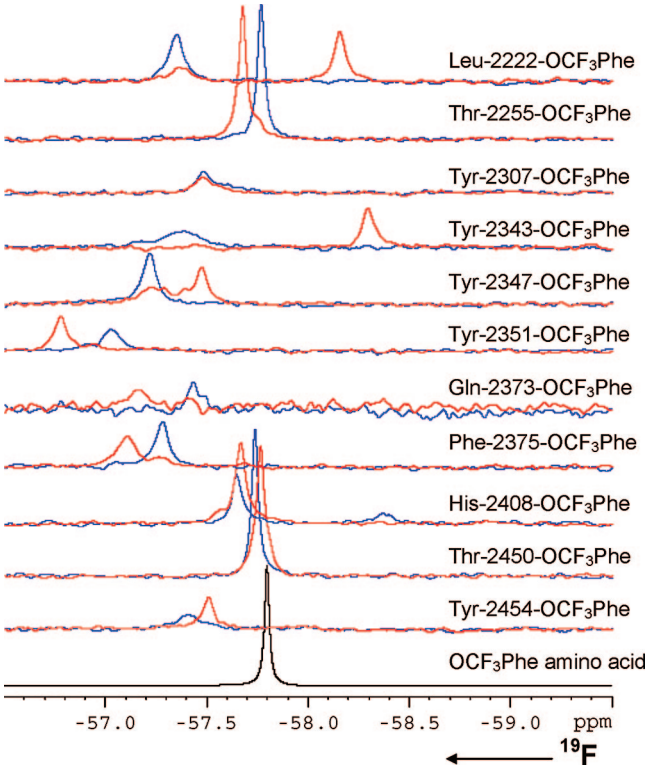

Figure 5. ^{19}F NMR spectra of FAS-TE mutants with OCF_3Phe incorporated at 11 different positions. Spectra in blue are of the uncomplexed protein while the spectra in red were recorded after the addition of tool compound. Significant line broadened, presumably because of conformational exchange, is observed for some but not all residues. For comparison, a spectrum of a 0.5 mM sample of OCF_3Phe is shown.

to select a mutant TyrRS specific for OCF_3Phe and illustrate that this RS selectively and specifically incorporates OCF_3Phe into Z-domain protein, FKBP12 and FAS-TE. Three libraries of mutants of the *M. jannaschii* tyrosyl-tRNA synthetase were subjected to alternating rounds of positive and negative selection in order to isolate clones that specifically recognized OCF_3Phe and not any natural amino acids. Five promising RS clones, A6 (Val32, Ala65, Gln108, Trp109, Ala158, Lys162), B7 (Val32, Ala108, Trp109, Gly158, Gln162), B10 (Ala32, Ala65, Trp108, Met109, Gly158, Asn159), F6 (Ala32, Ser65, Gln108, Ala109, Ala158, Tyr162) and H4 (Ile26, Val32, Gly65, His108, Tyr109, Ala158A, His162) (Tables S1 and S2, Supporting Information), were used to express Z-domain protein. In all five cases, the Z-domain was expressed only in the presence of OCF_3Phe, as measured by SDS-PAGE and LC-MS (Figure S2, Supporting Information). The expressed protein was confirmed to contain OCF_3Phe at the expected position by peptide sequencing. Based on LC−ESI-MS, the ratio of expressed protein containing a correct OCF_3Phe amino acid compared to a misincorporated Tyr, Phe, or Trp was greater than 300:1 for two of the amino acyl-RS clones, A6 and F6 (Figure S3, Supporting Information). Clone F6 was subsequently chosen for future use because it consistently produced higher expression yields.

FAS-TE Binding Tool Compound. Because orlistat is very insoluble in aqueous solutions, our binding experiments were performed with a FAS-TE binder **17** that was discovered in a high throughput screen (Caldwell et al., unpublished results). Compound **17**was synthesized according to Scheme 3. Briefly, Garner's aldehyde **9** was treated with a Grignard reagent, affording benzyl alcohol **10** quantitatively, followed by the global deprotecion by aqueous HCl. The amino group of **11** was masked as azide by trifluoromethanesulfonyl azide; the configuration of the stereo center of the amino group was retained under the condition.[44] The primary hydroxyl group of **12** was selectively converted to the nosylate, and then to the cyclohexylamino group by nucleophilic displacement. The resulting intermediate, **14**, was elaborated to the final compound, **17**, by following the sequence of benzoylation, azide reduction, and ureation. Although we were unable to determine the diastereo selectivity of the first reaction (**9** to **10**), the ^{1}H NMR and LC-MS analysis of **13** indicated that **13** comprises mainly one diastereomer. Because the synthesis started with homochiral aldehyde **9** and no steps were considered to involve racemization, we assume the final compound, **17**, also comprises mainly one species regarding the benzilic and homobenzylic positions.

Incorporation of OMePhe, OCF_3Phe, and oNBTyr into 11 FAS-TE Mutant Proteins. Using pSUPAR3 plasmids, 11 FAS-TE mutants were expressed with three unnatural amino acids substituted at single sites. Positions Leu-2222, Thr-2255, Tyr-2307, Tyr-2343, Gln-2373, Phe-2375, His-2408, and Thr-2450 were selected in order to place the unnatural amino acid in solvent exposed positions surrounding the active site. Tyr-2347, Tyr-2351 and Tyr-2454 were selected as additional incorporation sites to probe adjacent protein regions that are disordered in the crystal structures of apoprotein[32] and the orlistat complex.[30] Cells transformed with an expression plasmid for each mutant and pSUPAR3-OMePhe, pSUPAR3-OCF_3Phe or pSUPAR3-oNBTyr were fed the respective unnatural amino acid and induced to express the model proteins as described in Materials and Methods. Each mutant was purified using Ni-affinity and size-exclusion chromatography. The average yield of purified protein was ~2 mg per 50 mL culture (Table 1). With the exception of Gln-2373-OMePhe, Gln-2373-OCF_3Phe, Tyr-2307-oNBTyr, Tyr-2347-oNBTyr, Tyr-2351-oNBTyr, and Gln-2373-oNBTyr, purified protein from only one 50 mL culture was transferred to NMR buffer resulting in NMR samples of typically 0.1 mM in concentration (total volume 0.55 mL). The predicted molecular weight of each mutant protein was confirmed by ESI-MS. Generally, there is little difference in the incorporation yield for each of the three unnatural amino acids at the same incorporation site. However, protein yields of the different mutants varied significantly possibly because of differences in incorporation efficiencies and effects on protein stability during expression and purification.

Effects of Unnatural Amino Acid Incorporation on Protein Structure. NMR spectra of FAS-TE mutants individually labeled at 11 positions with three different unnatural amino acids were recorded in the absence and presence of a FAS-TE binding tool compound as described below. Individual incorporation of an unnatural amino acid does not grossly perturb the structure of any of these FAS-TE mutants as indicated by only small changes in the ^{1}H NMR spectra (Figures S4, S5 and S6, Supporting Information). All mutants retain well dispersed 1D ^{1}H NMR spectra indicative of well folded proteins. For some but not all proteins, structural integrity was further confirmed

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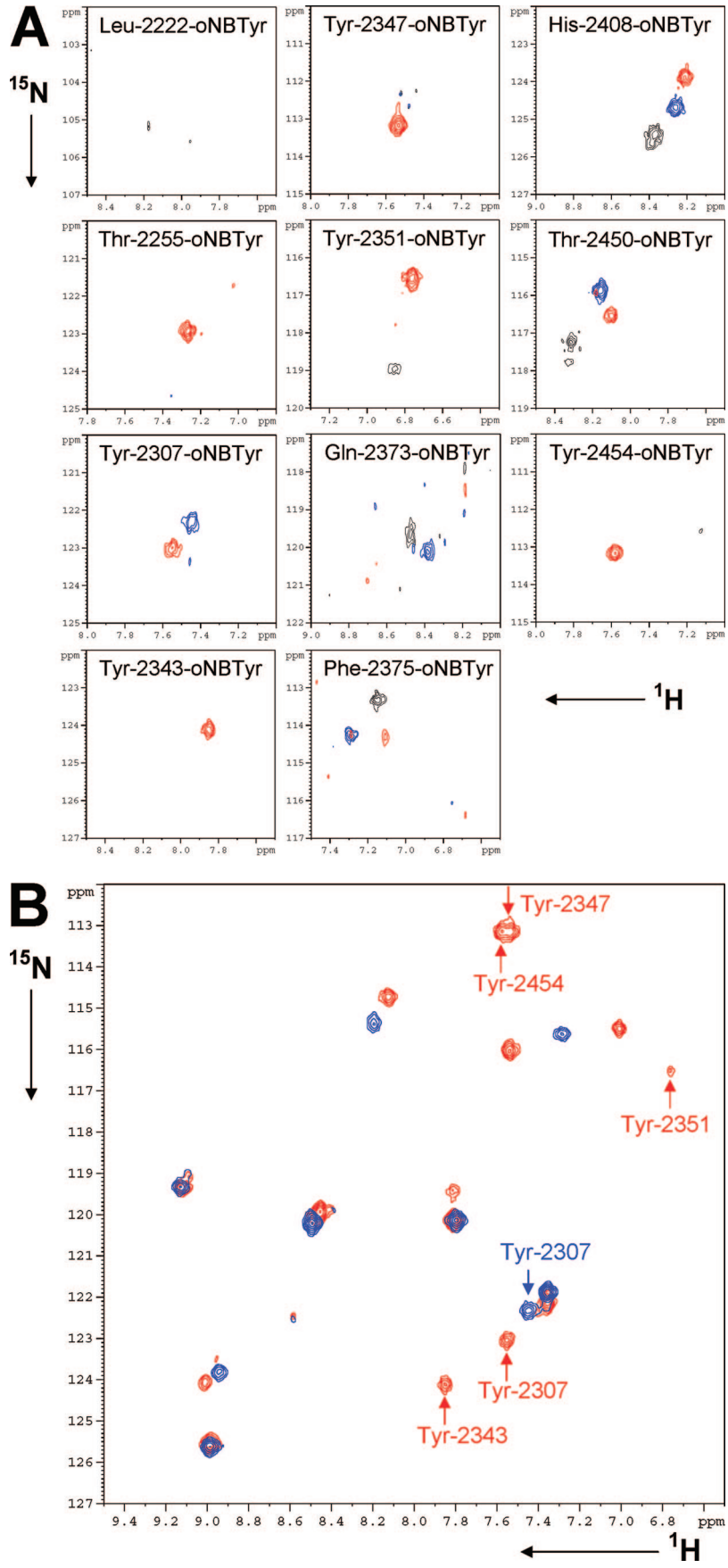

Figure 6. $^{1}H-^{15}N$ NMR spectra of FAS-TE mutants with ^{15}N-labeled oNBTyr incorporated at 11 different positions: (A) $^{1}H-^{15}N$ HSQC spectra before (black) and after UV-cleavage (blue), and after addition of tool compound (red). (B) $^{1}H-^{15}N$ HSQC spectra of wild-type FAS-TE selectively labeled with ^{15}N-tyrosine before (blue) and after addition of tool compound (red). Spectra of individually ^{15}N-oNBTyr labeled mutants facilitated signal assignments as indicated by residue numbers.

by $^{1}H-^{15}N$ HSQC spectra of uniformly ^{15}N-labeled mutant protein expressed in the presence of the unlabeled unnatural amino acid (Figure 3 and Figure S7, Supporting Information). For example, the "reverse-labeled" (^{15}N-labeled at all nitrogens except for oNBTyr) Tyr-2454-oNBTyr mutant differences in the amide cross peaks are restricted to a few resonances (Figure 3A) as would be expected for any well tolerated single site mutation. Upon UV cleavage, oNBTyr is converted to Tyr and as expected the $^{1}H-^{15}N$ HSQC spectrum becomes identical to that of wild-type FAS-TE (Figure 3B). The resonance of Tyr-2454 is, as discussed below, exchange broadened and consequently not visible as an additional peak in the wild-type spectrum. Both wild-type and reverse-labeled Tyr-2454-oNBTyr show identical chemical shift changes upon tool compound binding. Furthermore, a Tyr-2454-oNBTyr (unlabeled) mutant sample selectively labeled with ^{15}N-tyrosine gives rise to a $^{1}H-^{15}N$ spectrum identical to a ^{15}N-tyrosine wild-type protein sample (Figure S8, Supporting Information). Additional data for reverse labeled samples are shown in Figure S7 (Supporting Information) and all support the conclusion that incorporation of unnatural amino acids at the 11 sites chosen appears not to negatively affect the structural integrity of these FAS-TE mutants.

NMR Spectra of OMePhe Mutant Proteins. $^{1}H-^{13}C$ and $^{1}H-^{15}N$ HSQC spectra were recorded for each FAS-TE mutant labeled with $^{13}C/^{15}N$ OMePhe (Figure 4). With the exception of Tyr-2343-OMePhe, a single $^{1}H-^{13}C$ cross peak was observed for the methoxy group in a narrow spectral window from 3.55 to 3.82 ppm in the proton and from 54.8 to 56.0 ppm in the carbon dimension. There was no cross peak observed for Tyr-2343-OMePhe presumably because of conformational exchange. Upon addition of the tool compound, a sharp cross peak for the methoxy peak of Tyr-2343-OMePhe appears at approximately 2.45 ppm (^{1}H) and 53 ppm (^{13}C). Less dramatic chemical shift changes are apparent for Leu-2222-OMePhe, Tyr-2347-OMePhe, Tyr-2351-OMePhe, Gln-2373-OMePhe, Phe-2375-OMePhe, and Tyr-2454-OMePhe. Interestingly, Tyr-2307-OMePhe does not show any chemical shift changes despite its position at the center of the active site, adjacent to the catalytic residue Ser-2308 (see Figures 7 and 8). This suggests that the bulky methoxy group inhibits binding. Interestingly, for some of the proteins (Leu-2222-OMePhe, Gln-2373-OMePhe, Phe-2375-OMePhe and Tyr-2454-OMePhe) as much as 20% cannot be converted to a complex even in the presence of a 4-fold molar excess of the tool compound (data not shown). This is possibly the result of limited solubility of the tool compound.

The observations for Tyr-2343-OMePhe suggest that parts of FAS-TE undergo conformational exchange on the millisecond time scale. Tool compound binding apparently reduces conformational exchange but only partially. $^{1}H-^{15}N$ HSQC measurements of the same samples support this interpretation as an amide resonance peak was observed for only six of the 11 mutants even in the presence of 4-fold excess of the tool compound (Figure S9, Supporting Information). $^{1}H-^{15}N$ cross peaks were observed for Leu-2222-OMePhe, Thr-2255-OMePhe, Tyr-2347-OMePhe, His-2408-OMePhe, Thr-2450-OMePhe, and Tyr-2454-OMePhe; with the exception of Leu-2222 and Tyr-2347, these are mutants that show no or only small chemical shift changes upon compound binding in the $^{1}H-^{13}C$ HSQC data (Figure 4). Because of their superior relaxation behavior, methyl groups are generally more readily observable in large proteins than backbone amide resonances.[45,46] This seems to be the case for the OMePhe mutants of FAS-TE as well.

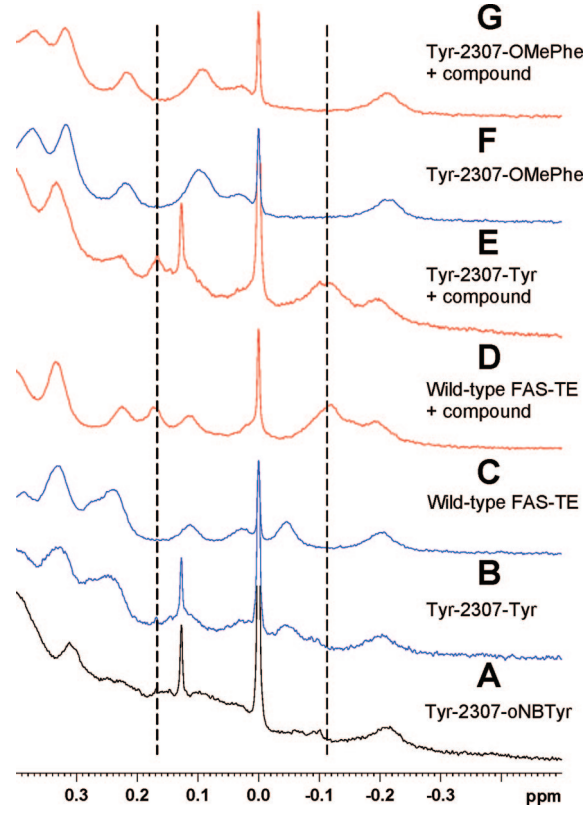

Figure 7. Site-directed labeling of active site residue Tyr-2307. After UV cleavage, ^{15}N-oNBTyr incorporated at Tyr-2307 does, in contrast to OMePhe and OCF_3Phe, not inhibit tool compound binding. Methyl region of ^{1}H NMR spectra of Tyr-2307-oNBTyr before (A) and after UV cleavage (B), of wild-type protein before (C) and after addition of tool compound (D), of Tyr-2307-oNBTyr after UV cleavage and addition of tool compound (E), and of Tyr-2307-OMePhe before (F) and after addition of tool compound (G). The position of methyl resonances characteristic of the complex are indicated by dashed lines.

NMR Spectra of OCF_3Phe Mutant Proteins. 1D ^{19}F-NMR spectra were recorded for each OCF_3Phe FAS-TE mutant (Figure 5). In each case, a single peak was observed within 0.9 ppm of a resonance line recorded for a 0.5 mM solution of the unnatural amino acid OCF_3Phe, dissolved in the same buffer and identical conditions. The width of the fluorine resonance line varied for each position again suggesting conformational exchange. The chemical shift of fluorine resonances is highly sensitive to changes in the environment,[5–7] and the line widths are a sensitive monitor of conformational exchange and reflect conformational fluctuations in the protein.[6,7,47]

Addition of tool compound results in significant chemical shift changes for residues near the binding site. In some cases, for example, for Tyr-2343-OCF_3Phe, Tyr-2351-OCF_3Phe, His-2408-OCF_3Phe, and Tyr-2454-OCF_3Phe compound addition results in sharpening of the fluorine line; for Gln-2373-OCF_3Phe and Phe-2375-OCF_3Phe the opposite is observed. As with Tyr-2307-

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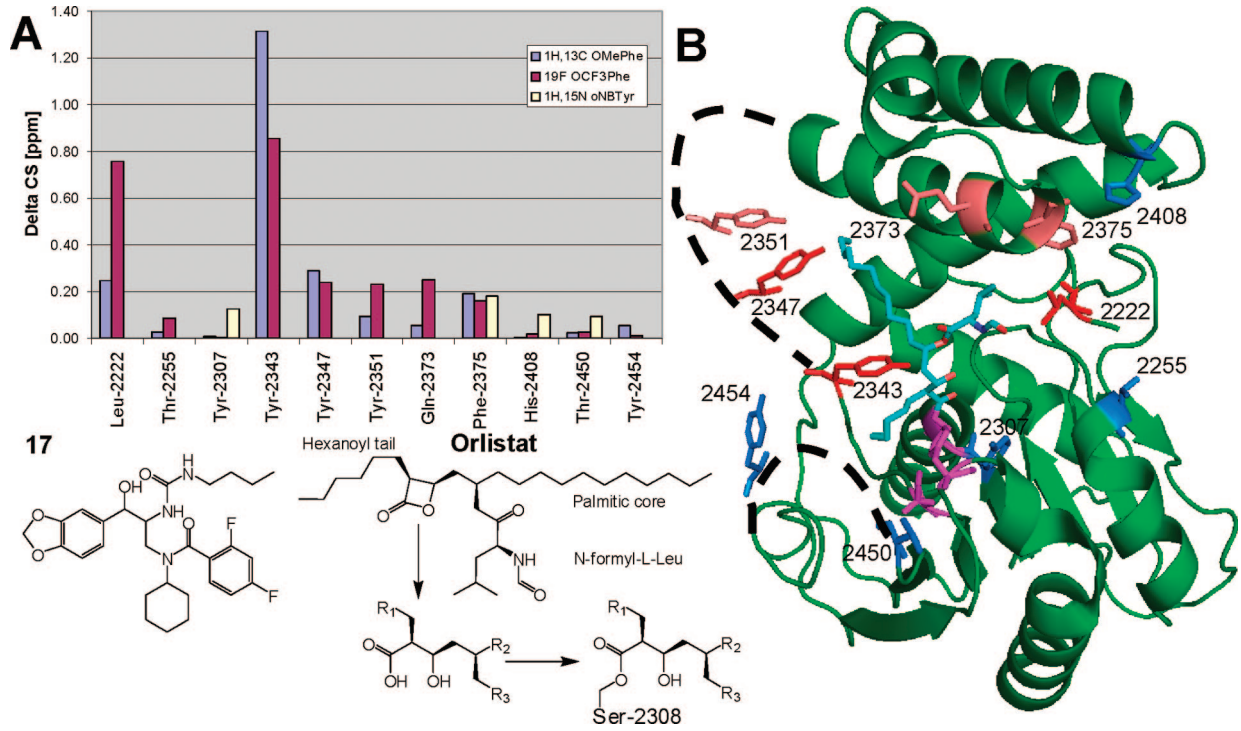

Figure 8. Chemical shift mapping of tool compound **17** binding to FAS-TE. (A) Combined chemical shift data. Chemicals shift changes ΔCS of ^{19}F resonances (OCF_3Phe; purple) were scaled relative to ^{1}H by the gyromagnetic ratio while ^{1}H−^{13}C (OMePhe; violet) and ^{1}H−^{15}N (oNBTyr; pale yellow) chemical shifts were combined using the expression $\Delta CS = [(\Delta^1H)^2 + (0.252*\Delta^{13}C)^2]^{0.5}$ and $\Delta CS = [(\Delta^1H)^2 + (0.102*\Delta^{15}N)^2]^{0.5}$, respectively. OMePhe and OCF_3Phe at Tyr-2307 block binding (see text). For oNBTyr mutants only a limited set of chemical shift changes could be compiled because of conformational exchange (see text). (B) Structure of the covalent orlistat complex (2PX6.pdb).[30] The average chemical shift changes induced by the binding of **17** are calculated for each unnatural amino acid and color-coded for each position: ΔCS < 0.1 ppm, blue; 0.1−0.2 ppm, salmon; > 0.2 ppm, red. Disordered loops are indicated by dashed lines. The active site residues Ser-2308, Asp-2338, and His-2481 are shown in magenta.

OMePhe, no changes are observed for Tyr-2307-OCF_3Phe suggesting again that the addition of the bulky OCF_3 (or OMe) group to this near active site residue blocks binding. The small chemical shift changes to Thr-2450-OCF_3Phe and Thr-2255-OCF_3Phe may be a solvent effect as the tool compound is added as DMSO stock (<0.5% final DMSO concentration).

NMR Spectra of oNBTyr Mutant Proteins. ^{1}H−^{15}N HSQC spectra were recorded for each FAS-TE mutant labeled with ^{15}N-oNBTyr (Figure 6) before and after photocleavage by UV light and after addition of tool compound. As expected from the ^{1}H−^{15}N HSQC data of OMePhe mutants (Supplemental Figure S9), cross peaks are observed for only a subset of mutants, namely Leu-2222-oNBTyr, Tyr-2351-oNBTyr, Gln-2373-oNBTyr, Phe-2375-oNBTyr, His-2408-oNBTyr, Thr-2450-oNBTyr, and Tyr-2454-oNBTyr. UV cleavage of the photocaging group results in modest chemical shift changes for four of these mutants while the signal for the three others disappears presumably because of conformational exchange (Figure 6). For the five mutants that are tyrosine in wild-type FAS-TE, only Tyr-2307 is observed in the UV-cleaved, uncomplexed form. Its chemical shift corresponds to an observable cross peak in the spectrum of wild-type apoprotein selectively labeled with ^{15}N-tyrosine (Figure 6B). In the latter spectrum, only nine of the 16 expected cross peaks are observed indicating that seven tyrosines undergo conformational exchange or rapid exchange with bulk solvent. After the addition of tool compound 15 of the 16 tyrosines (Figure 4D) are observed with various intensities and line widths suggesting that line broadening and absence of signals in the uncomplexed protein is indeed caused by conformational exchange (the last tyrosine is part of the presumably unstructured expression tag). The comparison with the individually ^{15}N-oNBTyr labeled mutant proteins allows the assignment of five of the tyrosine amide resonances.

As shown above, the data for OMePhe and OCF_3Phe indicate that unnatural amino acid incorporation at Tyr-2307, next to the active site residue Ser-2308, inhibits binding of the tool compound presumably because of steric interference by the added OCH_3 or OCF_3 group. UV photocleavage of Tyr-2307-oNBTyr re-establishes binding (Figure 6A) because the natural binding surface is regenerated as can be clearly seen from the comparison of 1D ^{1}H spectra of wild-type and mutant proteins (Figure 7).

Mapping the Binding Site of a Tool Compound. The tool compound binds tightly to all FAS-TE mutants (except for Tyr-2307-OMePhe and Tyr-2307-OCF_3Phe). Binding occurs in the slow exchange binding regime for all ^{1}H, ^{13}C, ^{15}N, and ^{19}F resonances. The chemical shift changes induced by compound addition map the binding site of the ligand to the active site of FAS-TE (Figure 8). Data obtained from ^{1}H−^{13}C, ^{1}H−^{15}N and ^{19}F NMR spectra are all consistent in that His-2408, Thr-2450 and Thr-2255 mutants are not affected by binding. The most pronounced chemical shift changes occur at residue Tyr-2343 that is apparently disordered in the uncomplexed proteins (by NMR but not in the crystal structures of apo FAS-TE[32] and of the orlistat complex[30]). Increasingly smaller chemical shift changes are observed for neighboring Tyr-2347 and Tyr-2351 mutants. Compound binding most likely stabilizes this flexible region of the protein as suggested by sharper fluorine resonance lines at residues Tyr-2343, Tyr-2351, and Tyr-2454 (Figure 5). Modest chemical shift changes are also observed for Gln-2373 and Phe-2375 mutants at the top surface of the binding pocket. At the far right site of the pocket, among the largest chemical

shift changes are observed for Leu-2222 mutants whereas Thr-2255 and His-2408 mutant resonances are not shifted by compound binding. On the other end, unnatural amino acids at residue Thr-2450 and Tyr-2454 are also not affected, observations that are consistent with the overall features of the orlistat complex.[30]

Discussion

Improving Incorporation Efficiency. NMR studies require milligram quantities of isotopically labeled protein. In our previous proof-of-concept experiment, we incorporated ^{15}N-labeled OMePhe using a mutant *M. jannaschii* tyrosyl tRNA/tRNA synthetase pair into myoglobin.[19] Approximately 0.5 mg of protein was produced from 0.5 L of *E. coli* culture in defined minimal media (GMML) using ~100 mg of the custom synthesized unnatural amino acid to yield a single 55 μM NMR sample.

Initial incorporation tests in rich rather than defined minimal media with a well-expressing FAS-TE construct indicated that most of the protein produced was truncated at the TAG incorporation codon (Figure S1, Supporting Information). This is in contrast to findings in GMML media where yields of full-length mutant protein can reach up to 70% of that of wild-type for some proteins.[34] At the higher expression levels achieved in rich media the amount of amber tRNA aminoacylated with the unnatural amino acid is apparently insufficient. To overcome this limitation, we modified the incorporation plasmid and added a copy of the orthogonal RS under the control of an inducible promoter (Figure 2). Simultaneous overexpression of the target protein and of the RS dramatically reduced truncation at the TAG incorporation codon (Figure S1, Supporting Information). This modification of the plasmid appears to be beneficial for the incorporation of at least three different unnatural amino acids: OMePhe, OCF_3Phe, and oNBTyr. Using standard shake flask cultures and rich media, single NMR samples from 50 mL of *E. coli* culture were obtained for most mutants using only 8 mg of oNBTyr, 12.5 mg of OCF_3Phe, or 25 mg of OMePhe (see details below). For well-expressing protein such as FAS-TE, the current system allows for the economical production of protein NMR samples in rich growth media and as such should be widely applicable.

Selecting a New tRNA Synthetase for a Fluorinated Unnatural Amino Acid. As discussed above, fluorinated unnatural amino acids and OCF_3Phe in particular represent attractive NMR labels. Consequently an expression system for incorporating OCF_3Phe into proteins was established. Initially, we attempted to express proteins with OCF_3Phe using the existing OMePhe RS and its amber tRNA,[13] but the two amino acids are sufficiently different that this RS does not facilitate incorporation of OCF_3Phe into proteins. In hindsight, this is not surprising as a CF_3 group should be ~70% bulkier than CH_3 (C−F bond length 1.39 Å vs C−H 1.09 Å; van der Waals radii of 1.35 Å for F vs 1.2 Å for H), and may be more hydrophobic as suggested by partitioning studies of hexafluoroleucine and leucine[48] (also see ref 42 and references therein).

Using three libraries that randomize positions Tyr32, Leu65, Asp158, Leu162, and either His70 and Gln155 (L1) or Phe108 and Gln109 (L2)[34] or His70, Phe108, Gln109, Gln155, and Ile159 (L3D)[35] several tRNA synthetases specific for OCF_3Phe were selected. His70 and Gln155 were conserved probably because they contact the ring and not the para substitution. As in other evolved tRNA synthetases, residues Tyr32 and Asp158 are mutated to remove H-bonds to the hydroxyl group of natural tyrosine. In the OMePhe RS, the mutations Tyr32Gln, Asp158Ala, and Leu162Pro increase the size of the binding pocket to accommodate the additional methyl group. Comparing the newly evolved OCF_3Phe RS to the OMePhe RS suggests that the OCF_3 group requires more space in the active site, as the OCF_3Phe RS repeats the Asp158Ala mutation but also has other mutations (Tyr32Ala, Leu65Ser) that further reduce the bulk of side chains near the OCF_3 group (based on the structure of the OMePhe RS).[49] Interestingly position 109 is mutated from a hydrophilic Gln to a hydrophobic residue of variable size, possibly because of the suggested higher hydrophobicity of the OCF_3 group (relative to OCH_3). Finally, residues 108 and 162, which point outward in the OMePhe structure, seem to be less important in the case of OCF_3Phe, as they are mutated to a wide variety of side chains in the selection winners.

The fidelity of amino acid incorporation of the newly evolved RS used in conjunction with the OMePhe tRNA[13] was evaluated by mass spectrometric methods[38] and found to be better than 99.5%. This suggests that it should be possible to evolve specific tRNA synthetases for other fluorinated, especially trifluoro-substituted amino acids. These unnatural amino acids do not have to be enantiomerically pure. Racemic mixtures of OCF_3Phe (this study) and of several other unnatural amino acids acids[50] have been used successfully to evolve selective RS mutants that retain their specificity for L-amino acids presumably because the residues randomized in the selection library are surrounding the end of the amino acid side chain rather than participating in backbone recognition. For FAS-TE, selective incorporation of L-OCF_3Phe is suggested by the unperturbed ^{1}H NMR spectra although DL-OCF_3Phe was used in the incorporation experiment (Figure S6, Supporting Information, see below).

Incorporation of Three Unnatural Amino Acids at 11 Different Sites. Our results indicate that all three unnatural amino acids (supplied as L-amino acids for OMePhe and oNBTyr and as DL-racemic mixture for OCF_3Phe) can be incorporated at all 11 sites in a 33 kDa protein with good yields (Table 1) and with little effect on the structure of the mutant proteins (Figure 3, Figures S4−S8, Supporting Information). As with single-site mutations using natural amino acids, unnatural amino acid incorporations can interfere with protein structure and stability. In the current study, care was taken to introduce the unnatural amino acids at solvent exposed sites. Subsequently, with the exception of incorporation at Tyr-2307 adjacent to the active site, no effect on the ability of FAS-TE mutants to bind a tool compound in the active site was observed (see below). However, the observed variations in expression yields may reflect destabilizing effects on some of the mutations or context dependent variations in incorporation efficiencies, and warrant future investigations.

Monitoring Conformational Exchange. At 33 kDa, FAS-TE is a sizable protein and normally deuterium labeling is employed in order to minimize spin diffusion and increase sensitivity and resolution.[51] With the exception of Tyr-2343-

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OMePhe, all of the methoxy groups of the 11 OMePhe mutants are observed (Figure 4). Solvent exposed methoxy groups may be less sensitive to exchanges processes because of smaller chemical shift differences between the exchanging forms, and may be more readily observed because of the generally favorable relaxation properties of methyl groups. The small $^{1}H-^{1}H$ dipole−dipole couplings of the methoxy protons to the ring protons may be beneficial as well. For Tyr-2343-OMePhe conformational exchange most likely causes line broadening as addition of the tool compound gives rise to a significantly shifted resonance line. This interpretation is supported by $^{1}H-^{15}N$ data obtained with $^{13}C/^{15}N$-OMePhe mutants (Figure S9, Supporting Information), ^{15}N-oNBTyr labeled mutant proteins and wild-type protein selectively labeled with ^{15}N-tyrosine (Figures 6 and S8, Supporting Information): Before addition of the tool compound amide signals of seven tyrosines including those of Tyr-2343, Tyr-2347, Tyr-2351 and Tyr-2454 are exchange broadened. Additional evidence for conformational exchange is also provided by ^{19}F NMR data with OCF_3Phe mutants. ^{19}F chemical shifts of fluorinated analogs of natural amino acids have long been known to be very sensitive to side chain dynamics.[6,7,47] Line broadening is evident for incorporation at the same FAS-TE location for different unnatural amino acids. Resonance lines that are most affected correspond to residues that have high B-factors or missing electron densities in the available crystal structures (residues 2325 to 2330, 2344 to 2356, 2452 to 2457, and 2487 are missing in the published X-ray structure of apo-FAS-TE, 1XKT.pdb,[32] and similarly in the orlistat complex, 2PX6.pdb[30]), suggesting conformational heterogeneity for these regions of the protein. This suggests that the NMR observations are indeed the result of conformational exchange processes in the natural protein and not the result of the unnatural amino acid incorporation into mutant FAS-TE. These observations in turn indicate that unnatural amino acids can be used to probe protein structure and dynamics at specific sites in large proteins.

Chemical Shift Mapping of a Small Molecule Binding Site in FAS-TE. Although different in absolute value, trends and magnitude of the chemical shift changes induced by tool compound binding in OMePhe, OCF_3Phe and oNBTyr mutants agree and identify the active site of FAS-TE as the binding pocket (Figure 8). Data obtained from $^{1}H-^{13}C$, $^{1}H-^{15}N$ and ^{19}F NMR spectra are all consistent in that residues His-2408, Thr-2450 and Thr-2255 mutants are not affected by binding. When compared to the crystal structure of the FAS-TE complex with orlistat,[30] Thr-2255 and His-2408 are at least 12 Å from the closest orlistat atom while the distance to Thr-2450 is 16 Å. Similarly, mutants of Thr-2450 exhibits minimal chemical shift changes as this residue is more than 13 Å from any orlistat atom. These observations indicate that the tool compound must bind in the active site not unlike orlistat. The similarities between the orlistat complexes and the complex of the tool compound are further supported by large chemical shift changes for Leu-2222 mutants. Leu-2222, therefore likely marks the extent of the compound binding pocket on the right side. The residue with the most pronounced chemical shift changes for its mutants, Tyr-2343, forms close contacts with the hexanoyl tail of orlistat in its hydrolyzed form and with both hexanoyl and the beginning of the palmitic core in the covalent orlistat complex (Figure 8). Tyr-2343, together with Tyr-2347 and Tyr-2351 are part of an α-helix that is observed in only one of two asymmetric units of an unpublished crystal structure (G. Spraggon, B. Bursulaya et al., unpublished data) and is absent in all published structures. Based on the sharper resonance lines, it is possible that tool compound binding stabilizes this helix but a more detail analysis must await a cocrystal structure or more detailed NMR studies. Because of the inherent flexibility of this part of the binding pocket we have not attempted to model binding of the tool compound but all preliminary evidence suggests a compound localization similar to that of orlistat.

The initial observations for Tyr-2307 mutants are misleading because introducing a bulky CH_3 or CF_3 group at the end of the unnatural amino acids instead of the natural tyrosine side chain apparently interferes with binding. UV cleavage of oNBTyr incorporated at Tyr-2307 restores binding (Figure 7) suggesting that the binding surface was perturbed in the Tyr-2307-OMePhe and Tyr-2307-OCF_3Phe mutant proteins. As with any type of mutation there is the need to carefully consider the sites of change because altering critical residues to unnatural amino acids may impact protein function. Clearly photocaged unnatural amino acids constitute an attractive and unique labeling strategy as, with for example oNBTyr, a single NMR label can be introduced at a selected tyrosine residue without perturbing the primary protein sequence.

Overall, the current study illustrates the robustness of our improved *in vivo* incorporation protocol. The three unnatural NMR-active amino acids can now potentially be employed to applications ranging from screening for site-selective binders to mapping small-molecule target interactions in the absence of a crystal structure. Most interesting will be applications with very large proteins with thousands of residues for which obtaining signal assignments by traditional approaches is a truly formidable challenge. A recent test incorporation of NMR-acitve unnatural amino acids into fully deuterated FAS-TE is a first step toward such applications.

Conclusions

Three different NMR-active unnatural amino acids were incorporated with high efficiency at different sites in a 33 kDa protein of pharmaceutical interest without adversely effecting protein structure and ligand binding. As expected, ^{13}C-labeled methyl groups introduced with the unnatural amino acid, because of their relaxation behavior, proved to be particularly advantageous for studies of a large protein. As illustrated here, unnatural amino acid labeling can be used to probe conformational exchange processes and small molecule ligand binding. More specifically, tool compound binding induces conformational rearrangements near the active site of FAS-TE and stabilizes part of the binding surface that is flexible in the X-ray structure of the orlistat complex. Although all mutations were well tolerated, introducing OMePhe or OCF_3Phe at one active site residue inhibited binding of a tool compound. The same site was probed with a photocaged oNBTyr that offers the advantage of introducing NMR labels at specific tyrosine residues without altering the protein sequence and therefore binding surface. Together with oNBCys (ref 52 and M. Jahnz, D. Summerer, B.H. Geierstanger, P.G. Schultz, unpublished results) and

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o-nitroveratryl-Ser,[53] the concept of photocaged unnatural amino acid incorporation opens new and exciting avenues for site-specific labeling and should therefore enable NMR studies of large proteins that can currently not be studies by traditional methods.

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Supporting Information Available: Sequence information of all newly selected tRNA synthetases, SDS gels of incorporation tests, gel and MS data of OCF_3Phe incorporation, and additional NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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