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Genetic incorporation of 4-fluorohistidine into peptides enables selective affinity purification†

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Due to the lowered pK_a of 4-fluorohistidine relative to histidine, peptides and proteins containing this amino acid are potentially endowed with novel properties. We report here the optimized synthesis of 4-fluorohistidine and show that it can efficiently replace histidine in *in vitro* translation reactions. Moreover, peptides containing 6×-fluorohistidine tags are able to be selectively captured and eluted from nickel resin in the presence of his-tagged protein mixtures.

The incorporation of non-canonical amino acids into proteins is a powerful tool for the manipulation of protein function and binding. One such non-canonical amino acid is 4-fluorohistidine. Because the van der Waals radius of fluorine is only slightly larger than that of hydrogen,[1] fluorohistidines can be considered isosteric with histidine. Indeed fluorohistidines can serve as substrates for histidyl-tRNA synthetase (HisRS), a feature that has been capitalized upon for the *in vivo* production of proteins containing these residues. Bann and co-workers have demonstrated that through the use of *E. coli* auxotrophs, both 2- and 4-fluorohistidine can be incorporated into proteins.[2,3] This can serve as a tool for the use of ^{19}F NMR to study protein conformational changes.[3,4] In addition, fluorohistidines can serve as mechanistic probes due to the fact that while the size of the molecule is not substantially altered, the electronics of the two systems (fluorohistidine *vs.* histidine) are changed. Addition of the electronegative fluorine lowers the pK_a from ~6 for histidine to ~2 for 4-fluorohistidine.[5] This reduction in pK_a can allow for elucidation of a histidine's role in a protein function or manipulation of protein function at variable pH,[6] and as we demonstrate here, selective affinity enrichment at lowered pH.

The hexa-histidine tag is one of the most ubiquitous methods of protein purification. With the small size of the hexa-histidine tag, high affinity to nickel resin, low cost, and use of easily-regenerated resin, capturing recombinant protein with a hexa-histidine tag is appealing. There are certain situations, however when the hexa-histidine tag is a disadvantage. In the Protein Synthesis using Recombinant Elements (PURE) system, *E. coli* translation proteins (elongation, initiation, and termination factors, and aminoacyl-tRNA synthetases) are expressed and purified *via* hexa-histidine tags.[7] These components are then combined along with necessary amino acids, ribosomes, and mRNA to biosynthesize peptides or proteins.[7] While this system is widely used for *in vitro* protein expression, it has also found a niche in experiments exploring the limits of the translation apparatus with noncanonical amino acids (ncAAs).[8–20] These experiments typically utilize an mRNA template that encodes for a short, FLAG-tagged peptide and use ^{35}S-methionine incorporation and MALDI-TOF respectively for analysis of yield and purity. The FLAG-tag presents three difficulties in this context: (1) the high cost of the anti-FLAG affinity resin, (2) the high negative charge of the FLAG peptide that reduces MALDI ionization, and (3) the requirement for using three amino acid/aminoacyl-tRNA synthetase enzymes for encoding of the tag (Asp/AspRS, Tyr/TyrRS, Lys/LysRS). A hexa-histidine tag would solve each of these three problems, but because the PURE system proteins are also hexa-histidine tagged, Ni-NTA purification of the translated peptide results in the co-purification of the PURE proteins alongside the desired peptide. These PURE proteins can compromise downstream applications, such as binding affinity determination. In this work we explore a potential solution to this problem through the use of a 4-FluoroHis tag.

In an effort to generate significant quantities of 4-fluorohistidine for protein biosynthesis projects we revisited a synthesis presented by Kirk and co-workers.[21] We experienced difficulty in reproducing the reported yields of intermediate and final compounds. In addition, full spectroscopic characterization of some intermediates was not reported in the original protocol, so we decided to optimize each step of the procedure. The resulting synthesis shown in Scheme 1. Importantly, we observed that conversion of **2** to **3** could be achieved in much

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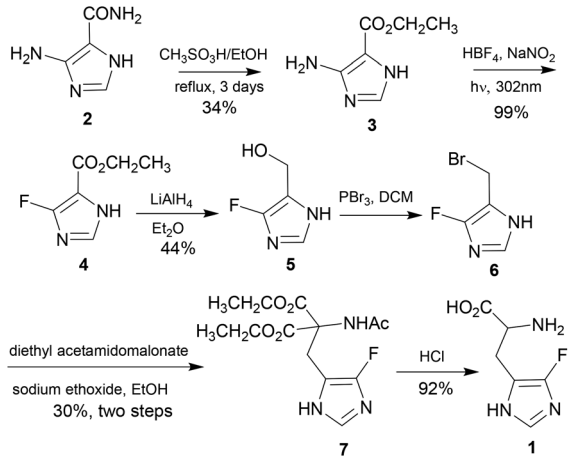

Scheme 1 Synthesis of 4-fluorohistidine.

less time that originally reported. We utilized the brominated intermediate **6** in place of a chlorinated analogue which resulted in higher yields of **7** over two steps. Finally we chose to purify compound **7** prior to final deprotections. These changes (detailed in the ESI†) provided an overall yield from starting material through six steps of 6%. While low, we were able to scale the synthesis to produce larger quantities (>100 mg) that could be used for protein expression experiments.

4-Fluorohistidine is a substrate for *in vitro* translation

With pure 4-FluoroHis (H_F) in hand we first verified that H_F is a substrate for wild type *E. coli* HisRS by using a charging assay based on MALDI.[22] HisRS readily accepted H_F as a substrate, resulting in charged $tRNA^{His}$ (Fig. 1A and B). Next we attempted to incorporate H_F into a peptide encoded by an mRNA template containing an encoded hexa-histidine tag (MHHHHHHMVEP), by excluding histidine in a cell-free translation system and replacing it with H_F. We were pleasantly surprised to find that the translation apparatus tolerates the addition of six sequential H_F residues (Fig. 1C and S1†). H_F translates with good fidelity, as measured by MALDI-TOF (Fig. 1C), and good yield, as measured by ^{35}S-methionine incorporation (Fig. 1D). From the MALDI-TOF data, we observed a major peak corresponding to a full hexa-fluorohisti-

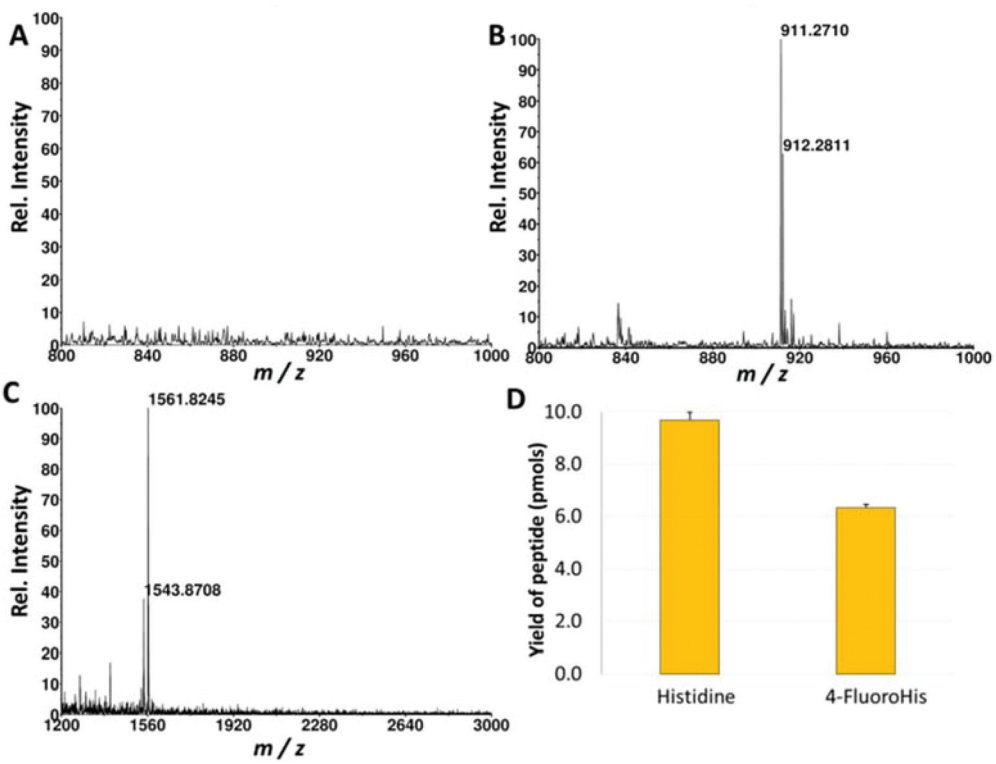

Fig. 1 4-Fluorohistidine is compatible with *in vitro* translation (A) MALDI-TOF aminoacyl-tRNA synthetase assay (AARS) with HisRS but with no added 4-FluoroHis. (B) MALDI-TOF AARS assay with HisRS and 4-FluoroHis. M^+ calcd 911.2738 obsd 911.2710. (C) *In vitro* translated reaction with template encoding the peptide $MH_{F6}MVEP$ in the presence of 4-FluoroHis. The major peak corresponds to six fluorohistidine incorporations: $(M - H)^-$ calcd 1562.5394, obsd 1561.8245, minor peak five fluorohistidines and one histidine $(M - H)^-$ calcd 1544.5488, obsd 1543.8708. (D) Translation yield in pmols for 50 μL translation reactions containing either 100 μM histidine or 600 μM 4-fluorohistidine. Experiments were performed in triplicate. Error bars represent the standard deviation from the mean.

dine tag being produced, along with a minor secondary peak corresponding to a peptide containing five H_F residues along with one histidine. This small contamination likely arose from a small amount of residual histidine present in the PURE reconstituted translation system.

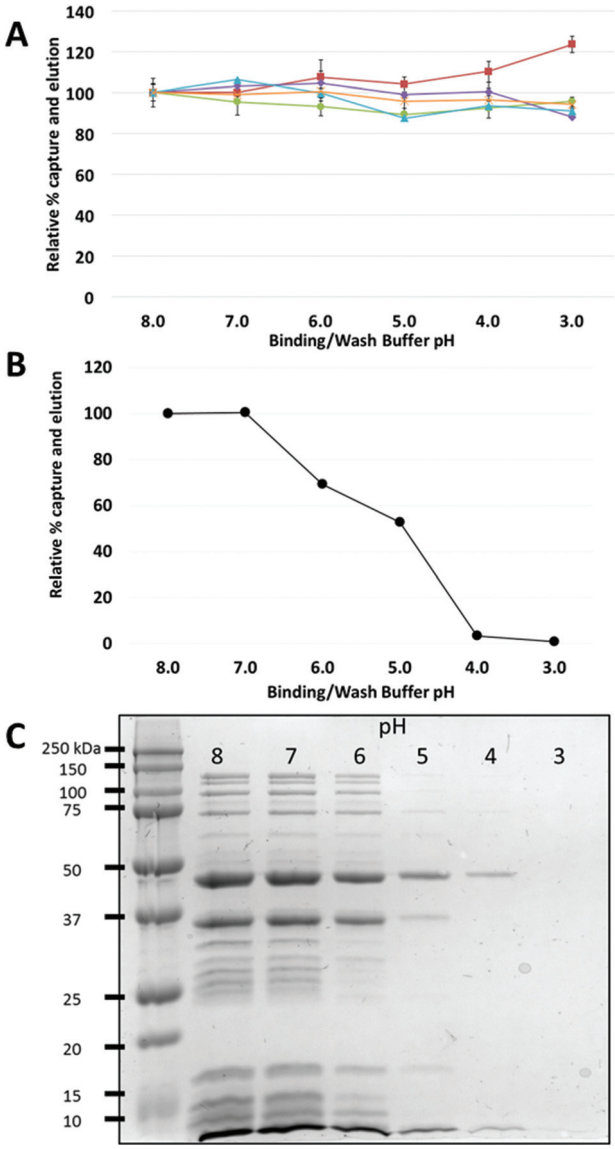

Fig. 2 Protein and peptide elution *vs.* binding/wash pH. (A) Fluorohis-tagged peptides MVM$(H_F)_6$ (X), M$(H_F)_6$MVEP (■), M$(H_F)_6$MVLT (●), M$(H_F)_6$MVAAAVEP (◆), MVTNSFVCTSVCGGG$(H_F)_6$ (▲), were bound and washed on Ni-NTA resin with various pH buffers. Peptides were eluted with 1% TFA and were quantified by scintillation counting of the ^{35}S-Met. Counts were relative to the experiment at pH = 8. Error bar represent standard deviations of experiments performed in triplicate. (B) Capture of PURE system His-tagged proteins as a function of pH buffers were the same as in (A). 1% TFA was used for elution, and the protein concentration at each pH was quantified by a Bradford Assay. Counts were relative to the experiment at pH = 8. (C) Coommassie-stained SDS-PAGE gel showing the capture of His-tagged PURE system proteins as a function of pH.

Selective capture of FluoroHis-tagged peptides in the presence of his-tagged proteins

Based on its lowered pK_a, we anticipated that H_{F6} tagged peptides would retain binding to Ni-NTA resin at low pH. To test this, we quenched *in vitro* translation reactions with 10 volumes of binding buffer adjusted to pH values between 8.0 and 3.0. Quenched reactions were bound to Ni-NTA resin, washed three times with buffer of the same pH, and finally were eluted with 1% TFA. Five hexafluorohistidine labeled peptides of different sizes (Fig. S1†) were retained on Ni-NTA resin at the lowest pH tested (pH = 3) (Fig. 2A).

Within the PURE translation system used in these experiments, there are 30 individual His-tagged proteins. We measured total His-tagged protein captured and eluted using a Bradford assay using the same binding/wash pH range used for the peptides (Fig. 2B). We observed that, indeed, most of the protein binding was lost at lower pH values. At pH of 3.0 the protein concentration was at background levels. SDS-PAGE analysis showed the identical trend (Fig. 2C). Interestingly, for one of the peptides (Fig. 2A) binding improved at lower pH—this could be due to the freeing up of resin binding sites previously occupied by proteins. Finally, the low pH capture/elution strategy did not lead to peptide degradation (Fig. S2†). Therefore, at low pH, hexa-fluorohis-tagged peptides can be selectively captured and eluted on Ni-NTA resin in the presence of his-tagged proteins.

Conclusions

In this work we have demonstrated that 4-fluorohistidine is not only a good substrate for protein biosynthesis, but that peptides containing the six sequential 4-fluorohistidines can be produced *in vitro*. Moreover, this tag can be used for selective enrichment of peptides in the presence of many other his-tagged proteins. In the work presented here we focused on short peptides in which we could capitalize on the reassignment of histidine codons using the relaxed substrate specificity of HisRS. If applied in the context of larger proteins, global incorporation of 4-fluorohistidine may be limited to those proteins which do not require a native histidine residue. Finally, the revised synthesis of 4-fluorohistidine presented here should enable the use of this unnatural amino acid in other biochemical experiments.

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