Controlling the incorporation of fluorinated amino acids in human cells and its structural impact

Azzurra Costantino, Lan B. T. Pham, Letizia Barbieri, Vito Calderone, Gili Ben-Nissan, Michal Sharon, Lucia Banci and Enrico Luchinat.

CERM–Magnetic Resonance Center, Università degli Studi di Firenze, Sesto Fiorentino, Italy.
Consorzio Interuniversitario Risonanze Magnetiche di Metallo Proteine CIRMMP, Sesto Fiorentino, Italy
Dipartimento di Chimica, Università degli Studi di Firenze, Sesto Fiorentino, Italy
Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel

INTRODUCTION

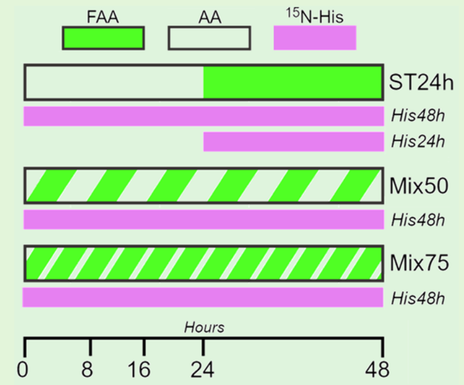
^{19}F NMR is a is a valuable tool for protein structural studies due to its high sensitivity and absence of natural background signals.[1] Incorporating fluorinated amino acids (FAAs) into proteins enables detailed analysis of specific sites, particularly in complex samples, such as intact cells, where traditional NMR approaches encounter difficulties with spectral overlap and complexity.[2,3] Here we investigate FAA incorporation into proteins expressed in human cells, to evaluate incorporation efficiency, structural integrity, and functional impact.[4] Optimizing FAA incorporation aims to improve ^{19}F NMR as a tool for studying protein dynamics, interactions, and conformational changes.

METHODS

Expression condition:

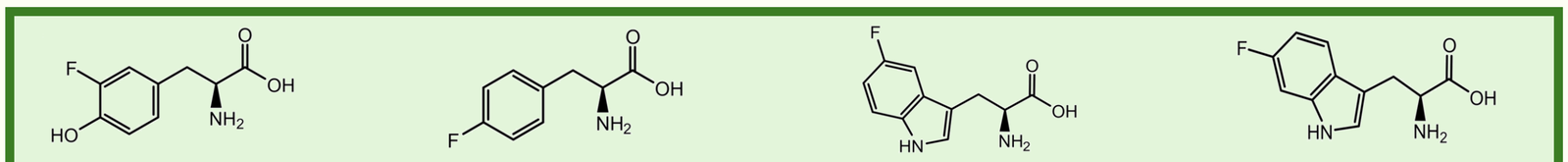
- **Media Switching**: cells were switched to FAA-containing media 24 hours after transfection (ST24h) to optimize FAA incorporation in protein expression and enhance ^{19}F NMR signal intensity.
- **Mixed Media**: proteins were expressed in a medium with 50% or 75% FAA content (Mix50 or Mix75) to achieve single FAA incorporation with minimal structural perturbation.

^{15}N-histidine was added immediately or 24 hours post-transfection (His48h or His24h), in conjunction with the FAA, to assess fluorine incorporation via ^{1}H-^{15}N NMR.



Schematic representation of the different expression conditions for FAA incorporation

SCAN for FULL WORK



RESULTS

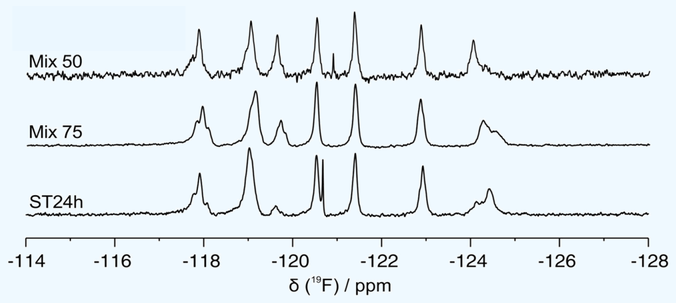
1D ^{19}F NMR

ST24h:

- Significant signal splitting and overlap.
- Higher but incomplete fluorine incorporation.

Mix50 and Mix75:

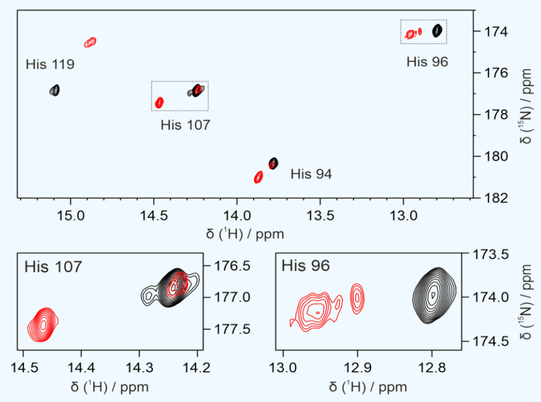
- Reduced signal intensity.
- Less signal overlap and spectral complexity.
- Lower fluorine incorporation.



1D ^{19}F NMR spectra of purified 6FW-CA2

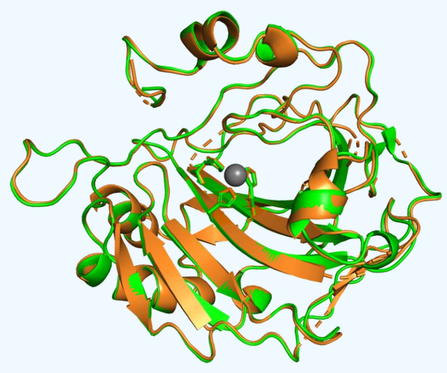
2D ^{1}H-^{15}N NMR

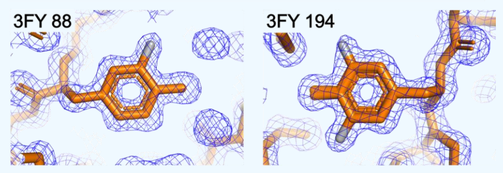
Each histidine side chain reveals the protein's fluorination state. Fluorine incorporation can be estimated by integrating signals from both fluorinated and non-fluorinated forms.



^{1}H-^{15}N NMR spectra of cell lysate: 6FW-CA2 (ST24h+His24h) (red) and CA2 expressed with ^{15}N-histidine (black)

X-Ray Crystallography





Structure of Carbonic Anhydrase II (CA2) with 3FY (orange) superimposed to that of native, nonfluorinated CA2 (green)

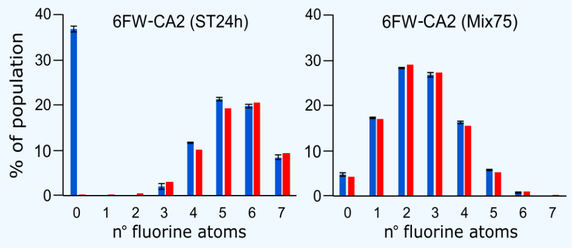
X-ray crystallography reveals different fluorine occupancies compared to MS data. Fluorination preserves CA2 folding, but crystallization may exclude molecules exhibiting minor conformational changes.

Mass Spectrometry

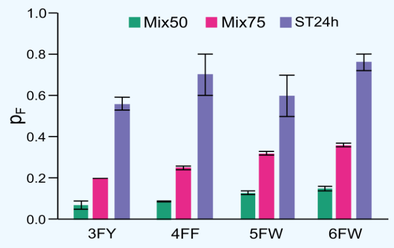
Probability of fluorine incorporation (p_F):

- Stochastic pattern with a binomial distribution.
- Lower in Mix samples compared to ST24h samples.

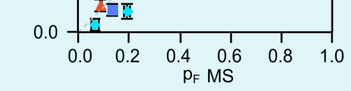
100% fluorine incorporation is not achieved.



Experimental fluorinated proteoforms (blue) and calculated binomial distribution (red)



p_F of MS data for FAA-CA2



Comparison of p_F values of FAA-CA2 obtained by direct MS and by NMR

CONCLUSION

FAA incorporation in human cells follows a predictable and stochastic pattern, regardless of amino acid position, and can be controlled to provide high-quality ^{19}F NMR data. The strong correlation between p_F values from NMR and MS confirms that FAA incorporation can be effectively estimated using ^{15}N-histidine or other amino acid-selective labeling. X-ray crystallography revealed that fluorine incorporation in certain positions leads to selective exclusion during crystallization, possibly due to subtle conformational changes. This work enhances the understanding of protein fluorination and its structural effects, advancing the applications of ^{19}F NMR in protein research.

1. Kitevski-LeBlanc, J.L.; Prosser, R.S. *Current Applications of ^{19}F NMR to Studies of Protein Structure and Dynamics.* Progress in Nuclear Magnetic Resonance Spectroscopy 2012, 62, 1–33.
2. Luchinat, E.; Cremonini, M.; Banci, L. *Radio Signals from Live Cells: The Coming of Age of In-Cell Solution NMR.* Chem. Rev. 2022, 122 (10), 9267–9306.
3. Pham, L. B. T.; Costantino, A.; Barbieri, L.; Calderone, V.; Luchinat, E.; Banci, L. *Direct Expression of Fluorinated Proteins in Human Cells for 19 F In-Cell NMR Spectroscopy.* J. Am. Chem. Soc. 2023, 145 (2), 1389–1399.
4. Costantino, A.; Pham, L.B.T.; Barbieri, L.; Calderone, V.; Ben-Nissan, G.; Sharon, M.; Banci, L.; Luchinat, E. *Controlling the Incorporation of Fluorinated Amino Acids in Human Cells and Its Structural Impact*. Protein Science 2024, 33 (3), e4910.