Bioorganic & Medicinal Chemistry Letters 19 (2009) 5449–5451

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Positional effects of monofluorinated phenylalanines on histone acetyltransferase stability and activity

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ARTICLE INFO

Article history:
Received 15 June 2009
Revised 17 July 2009
Accepted 20 July 2009
Available online 23 July 2009

Keywords:
Fluorinated amino acids
Histone acetyltransferase
Activity
Stability
Protein design

ABSTRACT

To explore the impact of global incorporation of fluorinated aromatic amino acids on protein function, we investigated the effects of three monofluorinated phenylalanine analogs *para*-fluorophenylalanine (pFF), *meta*-fluorophenylalanine (mFF), and *ortho*-fluorophenylalanine (oFF) on the stability and enzymatic activity of the histone acetyltransferase (HAT), tGCN5. We selected this set of fluorinated amino acids because they bear the same size and overall polarity but alter in side chain shape and dipole direction. Our experiments showed that among three fluorinated amino acids, the global incorporation of pFF affords the smallest perturbation to the structure and function of tGCN5.

The incorporation of amino acid analogs bearing new chemical functionalities expands the protein engineering capabilities with tremendous potential for biotechnology such as protein therapeutics.[1,2] Among the variety of unnatural amino acids incorporated into proteins, fluorinated residues demonstrate distinct properties dictated by the presence of a highly electronegative and hydrophobic fluorine atom.[3–5] Greater thermal stability and resistance to chemical denaturation has been demonstrated for proteins containing trifluorovaline,[6] trifluoroleucine,[6–8] trifluoroisoleucine,[9] fluoroproline[10] and hexafluoroleucine.[11,12] In some cases, however, the presence of fluorinated amino acids in certain large globular proteins, has led to reductions in stability or partial loss of function.[13–16] Thus, there is a context dependence of the influence of fluorinated analogs on proteins. Although the effects of global incorporation of saturated fluorocarbon analogs have been extensively studied, there are few examples in which the influence of fluorinated aromatic side chains on protein structure and function was systematically investigated.[17–20] To investigate the positional effects of fluorine substitution on aromatic side chains in biologically relevant globular proteins, we chose to explore the effects of monofluorinated phenylalanine analogs on histone acetyltransferases (HATs). Here we study the influence of residue-specific incorporation of *para*-fluorophenylalanine (pFF, 2), *meta*-fluorophenylalanine (mFF, 3), and *ortho*-fluorophenylalanine (oFF, 4),[19,20] on the stability, activity and selectivity of the HAT protein, tGCN5 (Fig. 1).[21–23]

First identified in *Tetrahymena thermophila*, tGCN5 is comprised of five α-helices and six β-strands, with N-terminal and C-terminal

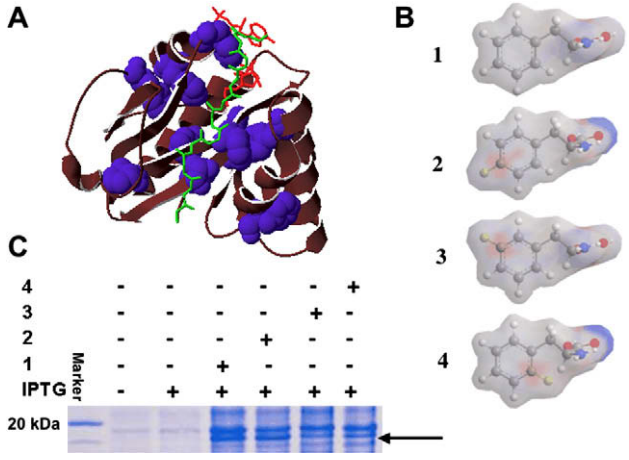

Figure 1. (A) Ribbon diagram of tGCN5 showing phenylalanines (purple) with CoA (red) and H3 peptide (green) at the active site (PDB: 1QSN). (B) Chemical structures of phenylalanine (**1**) and monofluorinated analogs: pFF (**2**), mFF (**3**), and oFF (**4**) with total charge densities calculated in Chem3DPro11.0. (C) SDS–PAGE analysis of tGCN5 overexpression. Expression conditions are shown above each lane. The first lane is molecular weight marker.

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doi:10.1016/j.bmcl.2009.07.093

regions separated by a deep hydrophobic cleft (Fig. 1A). It recognizes the histone H3 sequence QTARKSTGGK14APRKLASK and transfers the acetyl group from acetyl-coenzyme A (AcCoA) onto Lys 14.[21–23] The functional unit is comprised of 164 residues and is monomeric. There are 10 phenylalanine residues in tGCN5 that account for 6% of the total protein. All of the phenylalanines are buried with the exception of Phe 90 (Fig. 1A). Of the 10 residues, Phe 164 makes hydrophobic interactions with the C-terminal Pro 16, Arg 17, Lys 18 and Gln 19 of the H3 peptide.[21–23] Upon phosphorylation of H3, Phe 125 from tGCN5 makes additional van der Waals contacts to Gly 12 and 13 of the substrate. Although these two phenylalanines do interact with the substrate, they are not directly involved in the acetylation reaction; residues Glu 122, Val 123 and Tyr 160 are critical for catalysis.[21–23]

The monofluorinated phenylalanine analogs were incorporated into tGCN5 using a phenylalanine auxotrophic *Escherichia coli* strain AFIQ containing the plasmid IQ, which constitutively expresses the *lacI*^{q} repressor (see Supplementary data). The three analogs each bear the same overall polarity and size while altering the direction of the dipole and side chain shape (Fig. 1B). We sought to explore how the subtle changes in dipole and sterics can influence structure and function of tGCN5. Figure 1C shows SDS–PAGE analysis of cell lysates before induction (lane 2) or after induction (lanes 3–5) of tGCN5 expression. No tGCN5 expression was detected in the induced culture in the absence of **1**–**4** or in uninduced cultures supplemented with **1**, while tGCN5 expression was evident in the induced cells in the presence of **1** (0.3 mM) or **2**–**4** (3 mM) (Fig. 1C). The incorporation of the fluorinated amino acids were confirmed by tryptic peptide analysis of purified proteins. High yields of purified fluorine-containing tGCN5 proteins (88–181 mg/L) were obtained, notably higher than those reported in previous studies (Table 1).[24]

Based on MALDI MS of the peptide fragments, the extent of phenylalanine replacement was calculated to be 85.4% for pFF, 84.5% for mFF, and 83.8% for oFF (Fig. 2, Table 1). Percent incorporation determined by amino acid analysis was 88.9% for pFF and 81.3% for oFF, confirming MS data (tGCN5 expressed in mFF could not be determined due to signal overlap with phenylalanine).

Initially, the effect of fluorination (**2**–**4**) on the secondary structure and stability of tGCN5 was monitored by far-UV Circular Dichroism (CD) experiments (Fig. 3, S3). A loss in secondary structure was observed upon fluorination where the molar ellipticity values at 222 nm for tGCN5 bearing pFF, mFF and oFF were −2609 deg cm^2 M^{-1}, −2294 deg cm^2 M^{-1} and −2260 deg cm^2 M^{-1}, respectively, in comparison to wt (−4196 deg cm^2 M^{-1}) at 25 °C (Fig. 3A). In addition, the thermal melting temperatures for tGCN5 containing pFF, mFF and oFF were determined to be 43.3 ± 1.7 °C, 42.9 ± 1.1 °C, and 40.3 ± 0.5 °C, respectively (Fig. 3B, S3). Compared to wt (45.4 ± 1.3 °C), pFF exhibited the smallest decrease in T_m (ΔT_m = 2.1 °C), followed by mFF with 2.5 °C, while oFF demonstrated the largest destabilization by 5.1 °C. CD data suggest that as the monofluorinated substitution is situated closer to the Cα on the phenylalanine ring, steric repulsion is augmented when packed against other amino acids within the buried core.

Table 1
Levels of incorporation of fluorophenylalanines and protein yield

	% Incorporation		
tGCN5	MALDI-TOF MS	AAA	Yield (mg/L)
1			186
2	85.4	88.9	91
3	84.5	ND	181
4	83.8	81.3	88

AAA—amino acid analysis, ND—not determined.

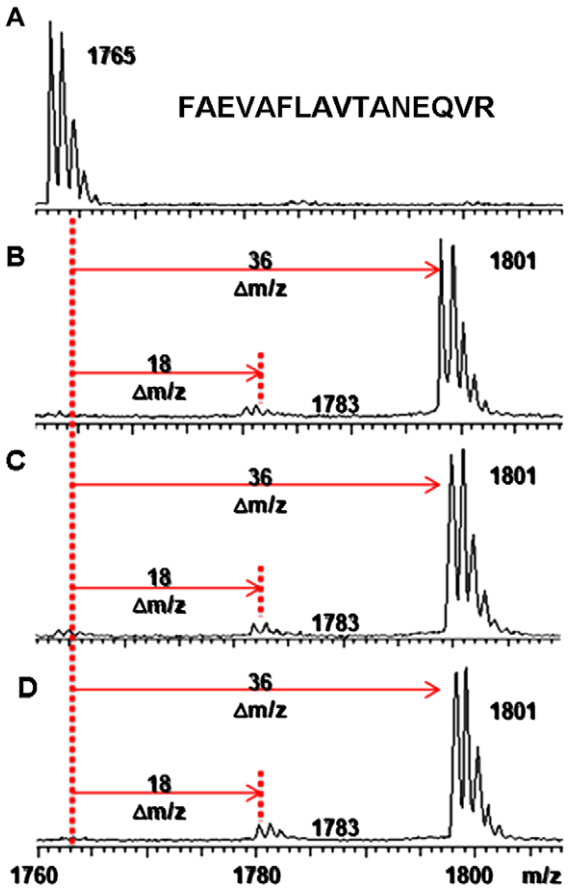

Figure 2. MALDI-TOF analysis of tGCN5 after trypsin digestion. (A) Sequence and mass peak of the trypsin peptide fragment FAEVAFLAVTANEQVR for wild type tGCN5; (B–D) mass peak of trypsin fragment for tGCN5 **2**, tGCN5 **3** and tGCN5 **4**, respectively.

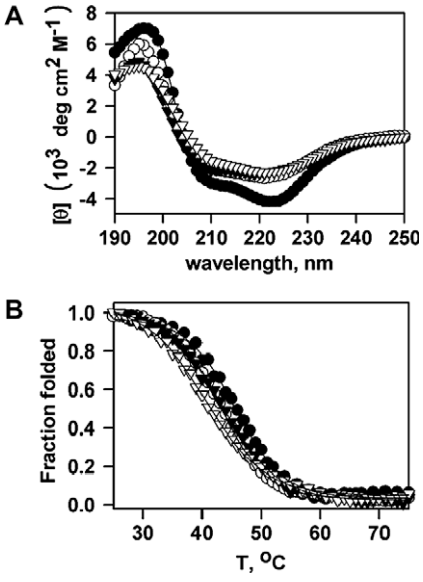

Figure 3. CD analysis of wt and fluorinated tGCN5 at 7 μM in 10 mM PBS, 0.1 mM DTT. (A) Wavelength spectra at 25 °C, (B) temperature scan at 222 nm. (●) wt tGCN5, (○) tGCN5 2, (▼) tGCN5 3, (▽) tGCN5 4.

Recent investigations have demonstrated that the incorporation of fluorinated residues can impart resistance to proteolysis.[25–27] Thus, we subjected tGCN5 bearing the monofluorophenylalanine

Table 2
Kinetic parameters and half-lives of fluorinated tGCN5

tGCN5	K_m ± s.d. (mM)	k_{cat}/K_m ± s.d. (mM^{-1} sec^{-1})	$t_{1/2}$ (min)
1	1.05 ± 0.24	0.87 ± 0.18	31.2 ± 8.8
2	0.69 ± 0.05	0.14 ± 0.02	27.5 ± 3.5
3	2.09 ± 0.74	0.25 ± 0.04	5.4 ± 0.6
4	0.80 ± 0.03	0.053 ± 0.001	6.8 ± 2.5

isomers (**2**–**4**) and wt to digestion with chymotrypsin (which cleaves after phenylalanine residues).[28] Proteins were incubated with chymotrypsin at various time intervals and monitored for degradation via SDS–PAGE. Presence of monofluorinated phenylalanines in tGCN5 resulted in reduced protease resistance in contrast to the previous studies. A minimal reduction in the half-life was observed for pFF, while mFF and oFF exhibited a six- and fivefold loss relative to wt (Table 2, Figs. S4, S5). Of the monofluorinated proteins, pFF maintained protease resistance and provided the smallest structural perturbation relative to mFF and oFF.

The activities of the wt and fluorinated proteins were determined for H3 peptide in vitro to assess the impact of fluorination on function (Table 2, Fig. S6). Relative to wt tGCN5, the presence of pFF exhibited enhanced H3 recognition with a K_m of 0.69 μM, which was followed by oFF (0.80 μM). By contrast, tGCN5 bearing mFF revealed a reduction in H3 recognition with a K_m of 2.09 μM (Table 2). An overall loss in catalytic rate was observed for all three fluorinated proteins; catalytic efficiency (k_{cat}/K_m) of tGCN5 containing pFF, mFF or oFF was reduced by 6, 3 and 16-fold, respectively, in comparison to wt (Table 2). Although the phenylalanines did not participate in tGCN5 mediated catalysis,[21–23] perturbations produced by incorporation of the fluorinated analogs could contribute to the observed reduction in k_{cat}/K_m. Based on these results, tGCN5 bearing pFF and mFF provides minimal perturbance to substrate recognition and activity.

Recently, Gellman and coworkers showed that pentafluorinated phenylalanine incorporation at defined positions in the subdomain of cVHD can lead to stabilization or destabilization.[17] They found that multiple substitutions with the pentafluorinated analog led to a loss in stability,[17] and similarly, we demonstrate that multiple replacement with monofluorophenylalanine isomers destabilize the protein. Our experiments reveal that even subtle positional changes of a single fluorine can influence the tGCN5 structure and function.[10] Proteolysis and kinetics results complement the research on fluorinated aliphatic amino acids[6,8,9,11,13,29] and demonstrate that pFF labeled tGCN5 is able to maintain stability and function, followed by mFF and oFF tGCN5. This provides a starting point for further engineering of artificial HAT-derived activators and investigating the structural dynamics.

Acknowledgements

This work was supported by start up funds, the Othmer Institute for Interdisciplinary Studies, ACS Division of Fluorine Chemistry, the Wechsler Award for Excellence (J.K.M.) and AFOSR.

Supplementary data

Experimental details for the expression of wild type and fluorinated tGCN5 proteins, MALDI-TOF characterization, CD, protease stability, and kinetics. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.093.

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