August 2010

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**Recommended Citation**

Kwon, Oh-Hoon; Yoo, Tae Hyeon; Othon, Christina M.; Van Deventer, James A.; Tirrell, David A.; and Zewail, Ahmed H., "Hydration dynamics at fluorinated protein surfaces" (2010). *Division III Faculty Publications*. 309.

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Hydration dynamics at fluorinated protein surfaces

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Contributed by David A. Tirrell, August 4, 2010 (sent for review May 10, 2010)

Water-protein interactions dictate many processes crucial to protein function including folding, dynamics, interactions with other biomolecules, and enzymatic catalysis. Here we examine the effect of surface fluorination on water-protein interactions. Modification of designed coiled-coil proteins by incorporation of 5,5,5-trifluoro- leucine or (4S)-2-amino-4-methylhexanoic acid enables systematic examination of the effects of side-chain volume and fluorination on solvation dynamics. Using ultrafast fluorescence spectroscopy, we find that fluorinated side chains exert electrostatic drag on neighboring water molecules, slowing water motion at the protein surface.

flourine | noncanonical amino acids | protein engineering | solvation dynamics | ultrafast hydration

The past decade has witnessed substantial expansion in the number and diversity of noncanonical amino acids that can be incorporated into recombinant proteins expressed in bacterial cells (1–3). Fluorinated amino acids have drawn special attention (4–16) because of the unusual solubility properties of fluorinated hydrocarbons. Several independent studies have shown that fluorination of coiled-coil and helix-bundle proteins leads to enhanced stability with respect to thermal or chemical denaturation (6–12), an effect attributed to the hyper-hydrophobic and fluorophilic character of fluorinated amino acid side chains. Although both classes of compounds are hydrophobic, hydrocarbons and fluorocarbons differ in important ways (17–22). The high electronegativity of fluorine renders the C-F bond both strongly polar and weakly polarizable (17, 21, 22). The dipole associated with the C-F bond exerts strong inductive effects on neighboring bonds (23) and can form reasonably strong electrostatic interactions with ionic or polar groups when the two moieties are appropriately positioned. The hydrophobic character of fluorinated compounds has been described as “polar hydrophobicity” (17), and is believed to play important roles in organic and medicinal chemistry. Furthermore, the C-F bond is significantly longer than the C-H bond, and the calculated volume of the trifluoromethyl group is about twice that of a methyl group (20). The studies described here constitute an attempt to understand more fully the interaction of water with fluorinated molecular surfaces, and to provide a sound basis for the use of fluorinated amino acids in the engineering of proteins with unique and useful physical properties.

The hydration layer adjacent to protein surfaces exhibits properties different from those of bulk water; the more rigid and denser structure of the hydration layer plays a crucial role in protein structure, folding, dynamics, and function (24–26). Elucidation of the dynamic features of this region, on the time scales of atomic and molecular motion, is essential in understanding protein hydration. In the past decade, the knowledge of hydration on protein surfaces has been extensively expanded by studying the dynamic properties of biological water for various proteins containing tryptophan (Trp) or synthetic fluorescent amino acids as local probes; the results have revealed multicomponent relaxation dynamics spanning a wide range of time scales (25, 27–29).

The nature of the protein hydration layer can be affected not only by the topographic and electrostatic properties of the protein surface (24), but also by the physical and chemical properties of individual surface-exposed residues (27, 30). In view of the unique properties of the C-F bond and of fluorocarbon—water interfaces (23, 31), we anticipated that fluorinated amino acid side chains might exhibit unusual hydration behavior. Here we report studies of local hydration dynamics at fluorinated protein surfaces, by monitoring the time-dependent fluorescence Stokes shifts of surface-exposed Trp residues in coiled-coil proteins with 5,5,5-trifluoroleucine (Tfl, I) residues adjacent to the probe. The results are compared to the hydration dynamics at hydrogenated protein surfaces with Leu (2) or (4S)-2-amino-4-methylhexanoic acid (homoisoleucine, Hil, 3) adjacent to the Trp probe. Hil has approximately the same volume as Tfl (20, 21), and although the shapes of the residues differ, the nearly identical side-chain volumes of Tfl and Hil allow us to differentiate changes due to fluorination from those that result from the increase in side-chain volume that accompanies replacement of Leu (Scheme 1).

Results

Coiled-Coil Protein System. The coiled-coil protein A1 (Fig. 1 A and B) was used as a model system to examine the effects of fluorinated amino acids on local hydration dynamics. The primary structure of A1 contains six copies of a heptad repeat (abcefg)6, where positions a and d are occupied by hydrophobic amino acids. Self-association of the peptide juxtaposes the a and d positions and results in the formation of a hydrophobic core. Fluorinated Leu analogues have previously been incorporated into the d positions of A1; the resulting proteins exhibited improved resistance to thermal and chemical denaturation with minimal differences in secondary structure (9, 11, 12). In this work, the surface-exposed Asp residue at the f position of the third heptad (position 34) was replaced by Trp, which serves as a fluorescence probe (Fig. 1C). The Trp variant of A1 was designated A1m. In order to examine the effects of fluorinated analogues on the local hydration dynamics, a Leu codon was introduced at one of two positions within A1m. Mutation of a serine residue at the c position of the third heptad (position 31) yielded a variant of A1 designated S31L (Fig. 1D), while replacement of an alanine residue at position b of the fourth heptad (position 37) gave the A1 variant A37L (Fig. 1E). Each protein was expressed in Tfl, Leu, and Hil form, yielding a total of nine different proteins that were examined in detail.*

Characterization of Global Structure. Analysis of each protein showed that the overall structural properties of the molecules...
were generally insensitive to genetic mutations and incorporation of noncanonical amino acids. Circular dichroism spectroscopy indicated that all nine proteins were helical, as determined from the molar ellipticity at 222 nanometers (Fig. 2) (32); an analysis with K2D2 software showed that the helicities of individual proteins range between approximately 40% and 48% (33). These results are consistent with the design of the A1 protein (34), in which approximately half of the amino acids are located within the heptad repeats expected to form α-helical secondary structure. The oligomerization states of the protein samples were determined by sedimentation velocity analysis (Fig. S1). Although A1 forms dimers and tetramers at neutral pH (11), the variants examined in this study form trimers or hexamers under mildly acidic conditions (pH 4). We suggest that protonation of Glu side chains at the e and g positions (Fig. 1A) of the proteins decreases the density of negative charges adjacent to the hydrophobic core and promotes formation of larger helical aggregates at pH 4. A1m, in which the single Trp residue occupies a surface-exposed position, is predominantly trimeric in Leu-, Tfl-, and Hil-forms, with a small fraction of hexamers (see Fig. S1). The majority of the S31L samples are present as hexamers, while the A37L samples appear to contain mixtures of trimers and hexamers.

Characterization of Local Structure. The steady-state fluorescence emission spectrum of Trp depends on the extent of exposure of the Trp side chain to water (35). All nine protein samples showed emission maxima between 349 nanometers and 352 nanometers, close to that of free Trp at 353 nanometers (Table 1 and Fig. S2). These observations indicate that the Trp residues are exposed to the aqueous environment (consistent with the original design), and not involved in oligomerization of the proteins. In addition, the steady-state UV-visible absorption and steady-state fluorescence emission spectra of each mutant containing Leu were nearly identical to the spectra of the corresponding mutant when it contained Tfl or Hil (Fig. S2), further confirming that perturbation of the protein structure upon replacement of Leu by Tfl or Hil was minimal.

The mobility of the probe residue was explored in each protein by measuring fs-resolved depolarization dynamics (Fig. S3). The anisotropic dynamics were found to consist of three components: ultrafast (≤500 fs), intermediate (20–80 ps), and slow (≥2 ns) decays. The ultrafast decays are attributed to fast internal conversion between the first two excited singlet states (τcays). The ultrafast decays to tumbling motions of the proteins (28). Similar values for the wobbling motions (φ) of all the other proteins. This result indicates that the organization of local residues or the flexibility of neighboring residues around the probe (28). All these features make it possible to compare the dynamic properties of the hydration layer. In many cases these properties are related to one another. The minimal change in the steady-state fluorescence spectrum caused by replacement of Leu by Tfl or Hil suggests similar features of the hydration region probed by Trp (e.g., the effective number of water molecules in the hydration shell). In addition, the similarity of the Trp wobbling angle of the Leu-, Tfl-, and Hil-forms of the proteins suggests similar organization and flexibility of neighboring residues around the probe (28).
observed by circular dichroism or sedimentation velocity mea-
surements. The abnormal behavior of the A1m-H variant is also
observed in the fluorescence lifetime measurements. Every pro-
tein except A1m-H displayed a short-lifetime component of a few
hundred ps, present at all wavelengths. These types of quenching
processes have been attributed to Trp interactions with nearby
charged residues (37–39), and the absence of such a feature in
A1m-H again indicates that this protein has a local structure dif-
ferent from those of the other eight proteins. The perturbation of
local structure and, thus, local solvent exposure can result in
different hydration dynamics, making it unreliable to compare
the dynamics of A1m-H to those of the other A1m proteins.
Accordingly, the dynamics obtained for A1m-H were not used in
the analysis that follows. Small shifts in the fluorescence emis-
sion maximum and Trp wobbling angle were observed for A37L-L
as compared to A37L-T and A37L-H (see Table 1 and Fig. S2).
These differences may be significant enough to alter the local en-
vironment surrounding the Trp probe, potentially complicating
assignment of changes observed in the hydration dynamics to
a particular effect (e.g., changes in an amino acid close to Trp).
Despite these concerns, the dynamics results for A37L-L remain
consistent with the conclusions of the paper (see below).

Our stringent standards for comparison of hydration dynamics
between modified proteins require that there be (i) no global
change in protein structure as measured by circular dichroism
spectroscopy and sedimentation velocity measurements; (ii) no
change in solvent exposure as measured by steady-state fluo-
estence maximum (±1 nm); and (iii) no change in local protein
structure or flexibility as measured by fluorescence anisotropy
(±1°). Seven of the nine proteins prepared in this study met
all of these criteria, and an additional protein, A37L-L, displayed
changes just outside the margin of error. Only one protein, A1m-
H, showed changes significant enough to require us to disregard
the hydration measurements observed. Given the subtle effects of
the chemical environment on hydration dynamics, we will com-
pare hydration results only within protein families. Thus, our
strongest conclusions will be drawn from observations made on
the S31L protein variants, and the data for A1m and A37L will be
used as corroborating evidence.

**Ultrafast Hydration Dynamics.** To investigate hydration dynamics at
the protein surfaces, we utilized a methodology developed by
Zhong and coworkers for the reconstruction of fs-resolved fluo-
estence spectra (28, 40). As an example, Fig. 3d shows several
representative fs-resolved fluorescence transients recorded for
A1m-T. The overall decay dynamics is retarded compared to that
of free Trp in buffer solution. Details of the results for all the
protein samples are presented in Table 1. The hydration dynamics
of the proteins were well represented by triple-exponential decays
with distinctive time scales of 0.2–0.8, 1.4–6.1, and 10–61 ps. Re-
laxation occurring on a time scale of a few hundred fs to several ps
is attributed to fast librational/rotational motions of bulk-type
and local water molecules around Trp. Observation of the fs com-
ponent suggests that the Trp probe in the test proteins is neither
crowded by neighboring residues nor protected from exposure to
water (27, 28, 41). The slowest phase of hydration dynamics (on the
time scale of tens of ps) is collective water network rearrange-
ment coupled to protein fluctuation dynamics (27, 41–43).

Several key features of the results (Fig. 4 and Table 1) are sum-
marized as follows. First, S31L-T and A37L-T, in which Tfl lies
close to Trp as well as in the hydrophobic core, showed slower
hydration dynamics than their Hil and Leu counterparts, indi-
cating that the fluorinated surface of the protein slows down the
hydration dynamics. For S31L-T, the time scales of local and col-
lective water motions (τ2 and τ3, respectively) are increased by
2–5 times (3.0 ps and 48 ps) from those of S31L-H (1.4 ps and
10 ps). The overall solvation of S31L-T is slower than that of
S31L-L, as well. However, this difference is manifested as an
increase in the contribution of the τ3 component to the overall
solvation of Trp. The 70% increase of the relaxation energy
(333 cm−1) of the slowest component (E3) compared to that of
the nonfluorinated S31L-L (194 cm−1) is an indication of the dra-
matic slowing of hydration dynamics near the fluorinated surface.
For A37L-T, τ2 and τ3 are also retarded to a greater extent (3–5
times slower) than those for A37L-H. These results suggest that
replacement of Leu by Tfl increases the residence time of water
molecules near the Trp probe and/or the number of water mole-
cules influenced by the amino acid side chain.

Second, S31L-H and A37L-H showed similar or even faster
hydration than their Leu counterparts. This result indicates that
in the comparison between hydrocarbon residues, increasing
the hydrophobic surface area results in faster motion of water
molecules around the residue. It should be noted that, for the S31L
series, hydration is greatly accelerated when Leu is replaced with
Hil. This pronounced hydrophobic effect (due to the increase in
the size of the residue) on the hydration is counteracted by fluo-
ration of leucine, resulting in slower dynamics for S31L-T. On
the other hand, for A37L, increasing the size of the hydrophobic
surface does not appear to affect the hydration dynamics as
greatly. Therefore, the retardation of the dynamics upon fluo-
nation is much more pronounced for A37L-T than for the corre-
sponding fluorination of S31L. Finally, A1m-L and A1m-H, which
differ from one another only in the nature of their hydrophobic
cores, exhibited almost identical hydration dynamics. Fluorina-
tion of the hydrophobic core of a helix-bundle protein can affect
protein dynamics (44). However, the nearly identical hydration
dynamics for A1m-L and A1m-T spanning a few hundred ps
dicates that modification of the hydrophobic core of A1m does
not affect protein motions that are coupled to local hydration

<table>
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<th>Sample</th>
<th>λmax (nanometers)</th>
<th>τ1, ps</th>
<th>τ2, ps</th>
<th>τ3, ps</th>
<th>ΔE1 (ps)</th>
<th>E1, cm−1</th>
<th>E2, cm−1</th>
<th>E3, cm−1</th>
<th>r(t)1</th>
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<td>2.1</td>
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<td>610</td>
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<td>171</td>
<td>0.049</td>
<td>0.056</td>
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<tr>
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<td>3.4</td>
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<td>2138</td>
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<td>3.6</td>
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<td>580</td>
<td>450</td>
<td>194</td>
<td>0.030</td>
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<tr>
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<td>3.0</td>
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<td>607</td>
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<td>333</td>
<td>0.044</td>
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<td>21</td>
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<tr>
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<td>1.4</td>
<td>10</td>
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<td>452</td>
<td>134</td>
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<td>522</td>
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<td>0.034</td>
<td>0.056</td>
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</table>

*All hydration-correlated energy relaxation dynamics were fitted to ΔEc(t) = E1 exp(−t/τ1) + E2 exp(−t/τ2) + E3 exp(−t/τ3).
*Refer to the SI Text for anisotropy analysis detail.

Table 1. Fluorescence emission maxima (λmax), hydration-correlated energy relaxation [ΔEc(t)], and depolarization dynamics [r(t)]
dynamics at the surface of the protein on the time scales examined here.†

Discussion

Protein surfaces are heterogeneous, consisting of polar, hydrophilic, and hydrophobic residues, and it is intriguing to consider how the heterogeneous surface chemistry affects the behavior of water molecules in the protein hydration layer. Head-Gordon and coworkers have reported heterogeneous water dynamics in the first hydration shell of model peptides (N-acetyl-leucine-methylamide and N-acetyl-glycine-methylamide), with faster water motions near the hydrophobic side chains than near the hydrophilic backbone (45, 46). Similar results have been reported for molecular dynamics simulation studies of a folded β-hairpin peptide (30). In addition, Qiu et al. showed that mutation of charged or polar residues of the enzyme staphylococcal nuclease into more hydrophobic residues (Ala), resulted in faster hydration dynamics; this result was attributed to the lack of strong interaction between the charges (or dipoles) of the mutated protein and the surrounding water (27). This observation can be understood in that the elimination of specific interactions between hydrophobic residues and water molecules causes a lower number of hydrogen bonds between water and a hydrophobic surface compared to those near a hydrophilic surface, thus allowing water molecules to reorient more freely. Computational studies have suggested that water layers adjacent to extended hydrophobic surfaces of low curvature are of lower density than those around hydrophilic and small hydrophobic molecules, and are dynamic rather than static (47–54). X-ray reflectivity experiments indicate submonolayer water depletion at hydrocarbon and fluorocarbon surfaces (55). Our observation of accelerated hydration dynamics around larger Thr residues as compared to the smaller Leu is consistent with these experimental and computational results, supporting the idea that water molecules neighboring hydrophobic side chains in the hydration layer of proteins are more dynamic than those around polar or hydrophilic residues.

Even though a simple comparison suggests that Thr should be more hydrophobic than Leu by virtue of its larger surface area, introduction of a Thr residue adjacent to the Trp probe caused retardation of the local hydration dynamics, in contrast to the results obtained when Leu was replaced with Thr (Fig. 4). These results suggest that hydration dynamics around fluorinated amino acid side chains cannot be explained exclusively by the increase in residue size. The C-F bond is assumed not to be involved in hydrogen bonding with liquid water, largely because of its low polarizability (17). However, replacement of Leu by Thr introduces a strong dipole at the fluorinated protein surface. Lee and coworkers have shown that introduction of CF₃ groups reduces the contact angle of water on self-assembled alkane-thiol monolayers (23), an effect that they attribute to dipolar interactions. Our results suggest that such dipolar interactions can also slow water motions at fluorinated molecular surfaces.

Fluorinated compounds are more hydrophobic than hydrogenated compounds of equal carbon number (4, 5, 17–21), and the increase in hydrophobic character of fluorocarbons has been ascribed to their increased molecular size (18, 56). This interpretation appears to be consistent with the observation that the melting temperature of A1-Thr is 13 °C higher than that of A1-Leu (11), while Tₘ for A1-Thr is increased by 17 °C in comparison to A1-Leu. However, the results reported here clearly indicate that the chemical nature of the protein surface dictates the dynamics of solvent-protein interactions, and that size effects alone

Fig. 3. Hydration dynamics. Experimental determination of local hydration dynamics at the surface of A1m-Th, excited at 295 nanometers. (A) Representative fs-resolved fluorescence upconversion transients. (B) Normalized time-resolved fluorescence spectra at different time delays. The steady-state emission spectrum is also depicted (dotted line). (C) Time-dependent spectral shift of the apparent emission maxima (νₛ) and the lifetime-associated (population) emission maxima (νₗ). (Inset) Entire evolution of νₛ and νₗ.

†We anticipated that A1m-H would also show hydration dynamics identical to those of A1m-L and A1m-Th. However as discussed before, the local protein packing and flexibility near the Trp probe appear to have been altered by substitution of Thr for Leu, reflected in the large Trp wobbling angle and total Stokes shift, ∆Es. Because the local structure has been changed, we would expect to observe altered hydration dynamics.
cannot explain the altered solvation dynamics observed at fluorinated protein surfaces.

Concluding Remarks

The results reported here show that fluorinated amino acids influence hydration dynamics at protein surfaces in a manner quite different from their hydrogenated counterparts. In general, water-protein interactions dictate many processes crucial to protein function including folding, dynamic motions, interactions with other biomolecules, and enzymatic catalysis (26). The slower hydration dynamics near fluorinated residues in proteins suggest that some of the water-mediated processes listed above may be changed upon fluorination. Tailoring the dynamics of protein-water interactions by the introduction of fluorinated residues may yield proteins with functional properties, such as binding, molecular recognition, or catalytic activities, that cannot be achieved with the canonical amino acids. Understanding hydration dynamics at fluorinated molecular surfaces is a critical step toward exploiting the properties of fluorine in biological systems.

Materials and Methods

Protein Expression and Characterization. A1 variants A1m, S31L, and A37L were expressed in 2xYT medium (which contains Leu) to yield proteins A1m-L, S31L-L, and A37L-L, respectively, in Leu-free M9 minimal medium (12.8 g/L NaHPO4 · 2H2O, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl) supplemented with 19 canonical amino acids plus TfI to give A1m-T, S31L-T, and A37L-T, and in Leu-free M9 medium supplemented with 19 canonical amino acids plus Hil to give A1m-H, S31L-H, and A37L-H. The proteins were purified under denaturing conditions and dialyzed against 10 mM acetate (pH4)/100 mM NaCl. The extent of replacement of Leu by TfI in A1m-T, S31L-T, and A37L-T, was determined by amino acid analysis to be 90%–91%. Leu replacement by Hil in A1m-H, S31L-H, and A37L-H was analyzed by liquid chromatography tandem mass spectrometry and determined to be at least 90% (Fig. S4). See SI Text for further details of expression, purification, and incorporation analysis.

Steady-State Measurements. Circular dichroism spectra were recorded on an Aviv 62DS spectropolarimeter (Lakewood, NJ). Absorption spectra were collected using a Cary 500 UV-Vis spectrophotometer and a 0.05-mm path length cuvette. Steady-state fluorescence emission spectra were measured using a FluoroMax-2 fluorimeter (ISA-Spek).

Time-Resolved Fluorescence Measurement. The experimental apparatus for time-resolved measurements are detailed in SI Text. All fluorescence spectra and transients were obtained by the excitation of samples (~350 μM) at 295 nanometers. The lifetime components were obtained by global analysis of fluorescence transients collected using a time-correlated single photon counting spectrometer. All transients show additional multiplexponential decay (at the blue side) and rise (at the red side) with time constants spanning from a few hundred fs to several tens of ps. In order to extract hydration dynamics precisely, we reconstructed apparent and lifetime-associated time-resolved fluorescence spectra with eight or nine transients at different wavelengths covering the blue and the red sides (Fig. 3B). By fitting these spectra to lognormal functions, we traced the time-dependent apparent emission maximum (νmax) and lifetime-associated emission maximum (νl) as plotted in Fig. 3C. Using Δν(t) = νl(t) − ν0(t), we correlated the extracted time-dependent spectral shift, Δν, to the hydration energy relaxation, ΔEmax, (Fig. 4).

ACKNOWLEDGMENTS. We thank Prof. Thomas Miller for helpful discussion, J.D. Fisk for synthesis of homoisoleucine, and the referees for their thoughtful comments on the original manuscript. This work is supported by National Institutes of Health (NIH) Grant GM62523, National Science Foundation (NSF) Grant DMR-0964886, Office of Naval Research (ONR) Grant N00014-03-10793, a Samsung Scholarship (to T.H.Y.), and a National Defense Science and Engineering Graduate Fellowship (to J.A.V.D.).


Supporting Information

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SI Text

SI Materials and Methods. Materials. All restriction enzymes were purchased from New England Biolabs (Beverly, MA). d.L-5,5.5-trifluoroleucine (Tfl) was purchased from Oakwood Products (West Columbia, SC). DNA oligomers were synthesized at Qiagen (Valencia, CA) or Integrated DNA Technologies (Corville, IA). (4S)-2-amino-4-methylhexanoic acid (homoisoleucine, Hil) was prepared according to the methods of O’Donnell and Eckrich (1) and Dorizon and coworkers (2).

Expression of fluorinated proteins. M9 medium supplemented with 0.4% glucose, 3.5 mg/L thiamine, 1 mM MgSO_4_, 0.1 mM CaCl_2_, 20 amino acids (40 mg/L), and 200 mg/L ampicillin was inoculated 1/50 with an overnight culture (M9) of Escherichia coli strain DH10B transformed with pQE-80L/A1m, pQE-80L/S31L, or pQE-80L/A37L, respectively. Plasmid pA1EL (4), which encodes both the protein A1 and a constitutively expressed leucyl-tRNA synthetase (LeuRS), was mutated using similar site-directed mutagenesis techniques. A Trp codon was introduced at first position 34 of A1, yielding pA1mEL. Introduction of leucine codons into either position 31 or position 37 resulted in the plasmids pS31LEL and pA37LEL, respectively.

Expression of proteins containing homoisoleucine. M9 medium supplemented with 0.4% glucose, 35 mg/L thiamine, 1 mM MgSO_4_, 0.1 mM CaCl_2_, 20 amino acids (40 mg/L), and 35 mg/L kanamycin was inoculated 1/50 with an overnight culture (M9) of E. coli strain LAM1000 transformed with pREP4 and pA1mEL, pS31LEL, or pA37LEL at 37 °C with shaking. After each culture reached OD600 = 0.9–1.0, the cells were harvested by centrifugation (6,000 × g, 4 °C, 10 min), and the cells were stored at −20 °C at least 12 h before purification. In the case of S31L-T, one sample was expressed using the cell strain LAM1000 containing pREP4 and pS31LEL and harvested using the procedure used for the production of proteins containing Hil.

Expression of hydrogenated proteins. 2xYT medium was used instead of supplemented M9 medium. When the culture reached OD600 = 0.9–1.0, IPTG was added to a final concentration of 1 mM. After 3 h, the cells were harvested by centrifugation (6,000 × g, 4 °C, 10 min), and the cells were stored at 20 °C at least 12 h before purification. In the case of S31L-L, one sample was expressed using the cell strain LAM1000 containing pREP4 and pS31LEL and harvested using the procedure used for the production of proteins containing Hil.

Protein purification. N-terminally histidine-tagged A1 variants were purified under denaturing conditions by affinity chromatography using Ni-NTA resin (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. For proteins containing Hil and for one batch each of S31L-L and S31L-T, the lysates in Qiagen Buffer B were thawed, sonicated, and then clarified using centrifugation (∼75,000 × g, 25 °C, 10 min). Imidazole was added to Qiagen Buffer C (50 mM) and Qiagen Buffer E (250 mM) in order to improve purification efficiency. The purified protein solutions were dialyzed against 10 mM sodium acetate (pH 4.0)/100 mM NaCl, and were concentrated by ultrafiltration (Amicon Ultra-15 devices, mwco: 10,000 or 3,000, Millipore, Billerica, MA). The protein concentration was determined as measured by the absorbance at 280 nanometers of solutions, assuming extinction coefficients of 5,500 M⁻¹ cm⁻¹ (5).

Amino acid analysis and sedimentation velocity analysis. Amino acid analysis of fluorinated proteins was performed at the W. M. Keck Facility at Yale University (New Haven, CT) on a Hitachi L-8900 amino acid analyzer (San Jose, CA) after hydrolysis at 115 °C in 70% formic acid. Sedimentation velocity analysis was performed at the National Analytical Ultracentrifugation Facility at the University of Connecticut (Storrs, CT) using a Beckman XL-I Analytical Ultracentrifuge at 20 °C. The rotor was accelerated to 55,000 rpm, and interference scans were acquired at 1 min intervals for 7 h. The data were analyzed by using the program Sedfit (6) to obtain normalized c(s) vs. sedimentation coefficient plots (Fig. S1).

Mass spectrometry. Liquid chromatography tandem mass spectrometry (LC/MS/MS) of proteins containing Hil or Tfl was performed at the Caltech Protein and Peptide Mass Analytical Laboratory. Trypsinized samples were subjected to liquid chromatography on an Eksigent (Dublin, CA) NanoLC-2D using a 6 cm long, 100 μm diameter C18 column, followed by MS/MS on an Applied Biosystems (Foster City, CA) QStar XL instrument. Data was analyzed using Analyst QS software provided by Applied Biosystems. Hil or Tfl incorporation levels were estimated using information contained within extracting ion currents (XIC) of trypsin-digested protein samples. For a given sample, a peak corresponding to a peptide globally substituted with the noncanonical amino acid and coding for multiple leucines was identified, and the related peak corresponding to replacement at a fraction of the leucine positions was also identified. Determination of the ratio of the partially substituted to globally substituted peak areas allowed for the estimation of amino acid incorporation levels assuming that leucines in the fragment were replaced statistically. An example calculation is shown in the section “LC/MS/MS Amino Acid Incorporation Estimates,” which can be found below.

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**Steady-state measurements.** Fluorescence absorption and emission spectra can be found in Fig. S2.

**Time-correlated single-photon counting (TCSPC).** The protein samples were prepared at 55 μM concentration in 10 mM acetate (pH 4)/100 mM NaCl solution. The TCSPC measurements were performed by using femtosecond pulses (<100 fs) from a Ti-sapphire oscillator (Spectra-Physics, Mai Tai HP). Laser output, of which the repetition rate was attenuated from 80 MHz to 8 MHz utilizing a pulse picker (Spectra-Physics, Model 3980-5), was tuned to 885 nanometers and frequency-tripled to 295 nanometers using a time-plate tripler (MiniOptic Technology, TP-2000B) for selective excitation of Trp. The UV beam, vertically polarized using a half waveplate, was introduced to a sample chamber and focused onto the sample cell. The residual frequency-doubled beam from the tripler was directed to a photodiode to trigger a TCSPC system (PicoQuant GmbH, FluTime 200). Typically, the energy of the excitation pulse (attenuated) at the sample was ∼10 picoJoules. In a right-angle geometry, the emitted fluorescence was collected at a magic angle (54.7°) with respect to the vertically polarized excitation beam and focused into a MCP-PMT (Hamamatsu, R3809U), which is attached to a double monochromator. The photomultiplier tube signal was separated from the gate beam and the fluorescence by using a constant fractional discriminator (PicoQuant GmbH, SPC 630). To avoid possible photobleaching and photodegradation, samples were kept stirring using a micro magnetic stirrer. In this configuration, the instrument response has a full width at half maximum of ∼30 ps. Multieponential decays convoluted with instrumental response functions were analyzed using the FluoFit software package (PicoQuant).

**Femtosecond fluorescence upconversion.** The protein samples were prepared at 550 μM concentration in 10 mM acetate (pH 4)/100 mM NaCl solution. We used an amplified Ti:sapphire laser system (Spectra-Physics, Hurricane X), which produces ∼110 fs pulses centered at 805 nanometers (fundamental), with a 1-kHz repetition rate and a 0.8-milliJoule energy. The output beam was split into equal parts to generate the pump and the gate pulse trains. For the pump, the fundamental light was used to pump an optical parametric amplifier (Spectra-Physics, OPA-800C), the infrared idler output of which was sum-frequency mixed with the residual fundamental in a 0.5-mm thick β-barium borate (BBO) crystal (type I), recompressed with a prism pair, and frequency-doubled to provide the 295-nanometers pulses in a 1.0-mm thick BBO crystal. The pump pulses were focused, with a 24 cm focal length lens, on the rotating circular cell (1-mm thickness) containing the sample. Typically, the energy of the pump pulse (attenuated) at the sample was ∼200 nJ. At these energies, the fluorescence signals from samples were linearly dependent on the pump energy. To check for sample degradation during experiments, fluorescence spectra were periodically measured right after the rotating cell by using a fiber-optic-coupled spectrometer (Acton Research, SpectraPro-300i) coupled to a charge-coupled device (Princeton Instruments, SpectraPro-MM-256iHB) before and after the collection of averaged transients for each sample. No difference between the spectra was observed. The forward-scattered fluorescence from excited samples was collected and focused by two off-axis parabolic mirrors into a 0.5-mm thick BBO crystal. Cutoff filters were placed between the mirrors to reject scattered laser light and pass the desired fluorescence wavelengths. The gate pulses, attenuated to 23 μJ/pulse, passed through a computer-controlled optical delay line and were noncollinearly overlapped with the fluorescence in the BBO crystal. After the crystal, the upconverted signal was separated from the gate beam and the fluorescence by using an iris, and was focused on the entrance slit of a 0.25-m double-grating monochromator (John Yvon, DH10) equipped with a photomultiplier tube at the exit slit. Upconversion efficiency was maximized by angle-tuning of the BBO crystal. The upconverted fluorescence transients were taken at the magic angle (54.7°) of the pump polarization relative to the gate polarization, parallel to the acceptance axis of the upconversion crystal, in order to eliminate the influence of induced sample anisotropy on the signal. The photomultiplier output was amplified (Stanford Research Systems (SRS), SR445) and processed by a gated integrator (SRS, SR250). The temporal response of the instrument was typically 350–450 fs. The observed fluorescence transients were fit to theoretical functions, using a Scientist nonlinear least-squares fitting program (Micromath), for the convolution of the Gaussian instrument response function with a sum of exponentials. All experiments were carried out at an ambient temperature of ∼24°C, and all fluorescence transients were obtained by the excitation of samples at 295 nanometers.

For fluorescence anisotropy measurements, the pump-beam polarization was rotated either parallel or perpendicular to the acceptance axis of the upconversion crystal to collect the parallel (Ip) and perpendicular (I⊥) signals, respectively. These transients were used to construct time-resolved anisotropy: r(τ) = (Ip − I⊥)/(Ip + 2I⊥). The results of the time-resolved anisotropy are shown in Fig. S3. The ultrafast depolarization time constant, τφ, attributed to fast internal conversion between the first two excited singlet states (1A∞ and 1A∞) of Trp, varies dramatically with the time resolution. This process has a time scale of ∼100 fs (7, 8). The limited resolution of our current apparatus (350–450 fs) does not allow us to fully resolve these dynamics and gives rise to a large uncertainty in the value of τφ. The variability of τφ will impact the fit of φ(τ). The uncertainty in the amplitude of the anisotropy, rA and rB, is not however affected by the limited time resolution of our data. Therefore we use the wobbling cone angle to reveal details about the local crowding near Trp. The wobbling cone angle is given by 1 − rB/Ip = (3 cos2θ − 1)/2Ip (9), and only depends upon the amplitude of the tryptophan wobbling motion and the anisotropy due to the rotation of the molecule.

**LC/MS/MS amino acid incorporation estimates.** LC/MS/MS was used to estimate the replacement levels of leucine in some protein samples. Fig. S4 depicts the total ion currents (TIC, Fig. S4A) and three extracted ion currents (XICs, Fig. S4B–D) from a digested A1m-H sample. The large peak in Fig. S4B corresponds to a peptide in which all of the Leu residues are replaced by Hil, the smaller peak in Fig. S4C corresponds to a mixture of two peptides containing one Leu and one Hil residue, and the very small peak in Fig. S4D corresponds to a peptide containing only Leu residues. The areas in the three XICs allow determination of the extent of incorporation of noncanonical amino acids in place of leucine. Assuming that there is a probability p of homoisoleucine substitution in place of leucine, the distribution of peak areas should correspond to the binominal distribution

\[ A[(1-p)^2 + 2(1-p)p + p^2], \]

where A is a multiplication factor equal to the total area of the three peaks and the three terms of the polynomial correspond to nonsubstituted, singly substituted, and doubly substituted peptides, respectively (The term for singly substituted peaks takes into account both positional isomers of singly substituted peptides). Because these three terms represent the only combinations of substitutions possible in the peptide, the relationship

\[ (1-p)^2 + 2(1-p)p + p^2 = 1 \]

also holds. The ratio between two peaks in a peptide series depends only on the probability of incorporation and not on the value of A. Therefore, the ratio of two peaks from experimental
data can be used in order to get an estimate of \( p \). The ratio of the peak areas of singly substituted to doubly substituted peptides is

\[
\frac{2(1-p)p}{p^2}.
\]

[S3]

Rearranging the above expression,

\[
\frac{2p - 2p^2}{p^2} = \frac{2(1-p)p}{p^2} = X.
\]

[S4]

where \( X \) is the experimentally observable ratio of singly substituted to doubly substituted peptides. Solving for \( p \) gives

\[
p = 0
\]

[S5]

or

\[
p = \frac{2}{X + 2}
\]

[S6]

with the root of interest being the nonzero root. Substituting for the ratio of peak areas gives an estimate of the incorporation level \( p \).

In some cases, peaks corresponding to peptides containing three leucine or leucine analogs were observed and used to quantify incorporation levels. In these cases, the peak area distribution is represented by

\[
A[(1-p)^3 + 3(1-p)^2p + 3(1-p)p^2 + p^3]
\]

[S7]

with

\[
(1-p)^3 + 3(1-p)^2p + 3(1-p)p^2 + p^3 = 1.
\]

[S8]

Defining the ratio of doubly substituted to triply substituted peak area as \( X \), substituting \( X \) into Eq. S8, and solving for \( p \) yields the nonzero root

\[
p = \frac{3}{X + 3}.
\]

[S9]

again enabling an estimation of the incorporation level of non-canonical amino acids in place of leucine.

In some cases, the tandem mass spectrometry did not enable positive identification of all possible positional isomers of a peptide. For example, in some cases, only two out of three of the possible doubly substituted positional isomers containing three possible substitution locations were identified in the tandem mass spectrometry data. In these cases, \( X \) was multiplied by an appropriate factor to account for peptides that were not observed (again invoking the assumption of completely random incorporation). Using the above example, when only two out of three doubly substituted peptides could be identified, \( X \) was multiplied by a factor of 1.5 in order to estimate what the peak area ratio would have been with all three peaks present in equal weights.

Using this methodology, the homoisoleucine-containing proteins used were found to have 90% or greater HIL in place of Leu, and the sample of S31L-T that was analyzed in this fashion contained approximately 99% Tfl in place of Leu. These results were obtained by examining three separate series of peptides from each protein sample. These peptides had the following sequences: AEIGDLNNTSIR, GSHHHHHHGSMASGDE-NEVAQLER, and SLEWEAELEQK (A1m), LLEWEAAELEQK (S31L), or SLEWEALELEQK (A37L).

**Fig. S1.** Normalized plots from the Sedfit c(s) analysis for A1m-L (A), S31L-L (B), A37L-L (C), A1m-T (D), S31L-T (E), A37L-T (F), A1m-H (G), S31L-H (H), and A37L-H (I). The protein samples were prepared at 550 μM concentration in 10 mM acetate (pH 4)/100 mM NaCl solution. The broad, single peak of the A37L-L trace may be the result of an equilibrium mixture between trimeric and higher-order species. It seems likely that the breadth of the traces derived from sedimentation velocity analysis of A1m-T, S31L-T, and A37L-T is a consequence of incomplete replacement of Leu with Tfl.

**Fig. S2.** Steady-state UV-visible absorption (red) and fluorescence emission spectra (black) of proteins excited at 295 nanometers. The protein samples were prepared at 550 μM concentration in 10 mM acetate (pH 4)/100 mM NaCl solution.
Fig. S3. Time-resolved anisotropy, $r(t)$, of the proteins. All anisotropy decays were fitted to $r(t) = r_I \exp(-t/\phi_I) + r_{Trp} \exp(-t/\phi_{Trp}) + r_\infty$, where $r_I$ is the initial ultrafast anisotropy, $r_{Trp}$ is the Trp motion-related anisotropy (value given in parentheses in figure), $r_\infty$ is the offset anisotropy, $\phi_I$ is the initial ultrafast internal-conversion time constant of Trp ($\leq 1$ ps), and $\phi_{Trp}$ is the Trp-rotational correlation time constant.

Fig. S4. LC/MS/MS of trypsinized A1m-H. (A) TIC of digested protein sample. (B)–(D) XICs of peptides containing two (B), one (C), or no (D) leucine to homoisoleucine substitutions in the peptide SLEWEAEELEQK. The ratios of the peak areas obtained in the XICs can be used to estimate the extent of leucine replacement in the protein sample. Peptide masses: SXEWEAEEXEQK: 2+ ion: 730.86 Da observed, 730.87 Da expected. SLEWEAEELEQK, SXEWEAEEXEQK: 2+ ion: 723.88 Da observed, 723.86 Da expected. SLEWEAEEALEQK 2+ ion: 716.87 Da observed, 716.85 expected.