CHAPTER 1

Introduction

Protein engineering via global incorporation of noncanonical amino acids

The genetic information in DNA is transcribed into mRNA using the specific base pairings, and then the ribosome synthesizes proteins based on the mRNA templates using amino acids linked to their cognate tRNAs. The charging reaction (aminoacylation) is catalyzed by enzymes called aminoacyl-tRNA synthetases (aaRSs). Considering the fact that the second decoding step, protein translation, is based on the interactions between codon of mRNA and anticodon of tRNA, it is clear that the reaction of charging amino acids to their corresponding tNRA is critical for the fidelity of protein translation. In general, there are twenty aaRSs specific to twenty canonical amino acids. However, these enzymes can sometimes demonstrate activity toward noncanonical amino acids. In particular, when an analogue is structurally similar to a natural one, the activity is high enough to support detectable protein synthesis in auxotrophic bacterial hosts starved for the natural amino acid and supplemented with the analog. Using the promiscuity of aaRSs, a number of noncanonical amino acids have been incorporated into proteins

instead of their canonical ones.[1,2] The method by which codons in mRNA are reassigned into noncanonical amino acids is called residue-specific incorporation of noncanonical amino acids. Recently, through introducing additional copies of wild-type or mutant aaRSs having modified amino acid-binding pockets, there has been an expansion in the repertoire of noncanonical amino acids for protein engineering.[1,2]

Contrary to site-specific incorporation using nonsense or frameshift suppression,[3] residue-specific incorporation of noncanonical amino acids enables global replacement of a particular amino acid with its unnatural analogue, which can induce substantial changes of physical properties of proteins and make available multiple sites for chemical modification.[1] Enhancing the stability of coiled-coil proteins by replacing residues of their hydrophobic core with fluorinated amino acids is a good example of this approach.[4-13] Budisa and coworkers reported that the replacement of two Trp residues of enhanced cyan fluorescent protein (ECFP) with (4-NH_2)-Trp resulted in a red shift in emission, which has not been obtained by site-directed mutagenesis or laboratory evolution using natural amino acids.[14] In addition, the thermostability was enhanced and the aggregation tendency decreased significantly. This example shows the power of this technique as a protein engineering tool when a target protein, the replaced residues in the protein, and analogues incorporated into the protein are carefully selected.

Installation of multiple copies of reactive moieties in proteins has also been demonstrated by residue-specific incorporation of noncanonical amino acids.[15-24] In particular, introducing chemical reactivity orthogonal to the 20 canonical amino acids into proteins is quite attractive. Noncanonical amino acids having an alkyne or azido group in their side chains were incorporated into proteins, and then the proteins were

selectively modified using the orthogonal reactivity between alkyne and azido groups through copper(I)-catalyzed reaction or Staudinger ligation.[16-18] Recently, this chemistry was employed to mimic the posttranslational modification of proteins in higher organisms.[19] The usefulness of the bioorthogonal amino acids was extended to study dynamic proteomics identifying and locating newly synthesized proteome in response to environmental stimuli.[20-22] After tagging newly synthesized proteins with noncanonical amino acids having alkyne or azido groups, the reactive moieties were labeled with affinity tags for specific separation and identification or with fluorescent tags for visualization. In addition, Tirrell and coworkers showed that intrinsically photoreactive proteins were synthesized using a photoreactive amino acid, para-azidophenylalanine, and films of these proteins were patterned using standard photolithographic techniques.[23-24]

A number of noncanonical amino acids have been incorporated into proteins using wild-type aaRSs so far, but the success is usually limited to analogues structurally similar to their natural ones. The specificity of aaRSs toward amino acids is provided by their amino acid-binding pockets. In order to alter the substrate specificities of aaRSs, rational,[25-27] computational,[15] and combinatorial[28-31] methods have been employed to engineer the amino acid binding pockets of several aaRSs. Based on structural studies of aaRSs complexed with amino acids, mutations in the amino acid binding pockets were introduced to enlarge the active sites. Alternatively, computational methods have been applied to synthetase design. Combinatorial strategies have been especially effective; Schultz and coworkers have developed powerful methods of selecting aaRS for site-specific incorporation,[28,29] and recently Tirrell and coworkers have reported an efficient

screening system for use in global replacement of amino acids.[30] Several residues in the amino acid-binding pockets were randomized, and then mutant synthetases that demonstrated activity toward noncanonical amino acids were selected or screened. In the case of the method of Tirrell and coworkers, outer membrane protein C (OmpC) was expressed in the presence of a noncanonical amino acid having bioorthogonal reactivity. Only cells harboring synthetases active toward the analogue can present OmpC with the reactive moiety on the cell surface, which was then labeled with a fluorescent probe. The method was successfully employed to engineer the *Escherichia coli* methionyl-tRNA synthetase (MetRS) to incorporate azidonorleucine. However, since this method relies on the bio-orthogonal derivatization of noncanonical amino acid side chains, a new approach is needed for the more general problem of activating noncanonical substrates that lack reactive side chain functionality. In chapter 4, we will describe a high-throughput method to screen *E. coil* MetRS variants that activate 6,6,6-trifluoronorleucine which does not have any reactivity in its side chain.[31]

Evolution of proteins with noncanonical amino acids

In many cases, the global incorporation of noncanonical amino acids will compromise protein folding and function,[32-36] since both protein structure and protein folding pathways have evolved in a context defined by the canonical amino acid side chains. Incorporation of norleucine in place of Met in cytochrome P450 BM-3 heme domain increased its peroxygenase activity, but the thermostability was reduced substantially.[32] In another report, Tirrell and coworkers incorporated fluorinated amino acids into chloramphenicol acetyl transferase (CAT).[34,35] In this work, the CAT enzyme,

whose Leu positions were occupied by 5,5,5-trifluoroleucine (Tfl), exhibited significantly reduced thermostability with little effect on activity.[34] On the other hand, replacement of Ile in CAT with 5,5,5-trifluoroisoleucine (Tfi) reduced the catalytic efficiency (kcal/Km) about 2.5-fold.[35]

These results have raised an intriguing question regarding the possibility of re-evolving proteins with novel composition. In order to address this question, our laboratory has employed laboratory evolution strategies combined with global incorporation of noncanonical amino acids. As a first attempt, Montclare and Tirrell recovered the reduced thermostability of fluorinated CAT by Tfl through a directed evolution method involving random mutagenesis using error-prone PCR and measuring remaining enzymatic activity after heat incubation.[34] Son and Tirrell also applied the similar directed evolution strategy to recover CAT's catalytic efficiency compromised through replacement of Ile with Tfi. In both cases, the recovered properties (thermostability and catalytic efficiency respectively) of fluorinated enzymes were similar to those of hydrogenated wild-type enzymes.[35]

These successes have demonstrated the possibility of producing functional proteins composed with abiological constituents. Even though CAT showed compromised properties due to fluorination, the enzyme was still able to fold with the non-standard amino acids. Motivated from this, we became interested in the case where proteins cannot fold into appropriate structure exhibiting their original functions with noncanonical amino acids. Replacement of Leu in green fluorescent protein (GFP) by Tfl caused a reduction of more than 500-fold in the median fluorescence of cells expressing the fluorinated protein, such that the observed fluorescence was comparable to that

characteristic of cells lacking the gene coding for GFP. The observed reduction in fluorescence is most probably a consequence of misfolding and aggregation of the fluorinated form of GFP based on lack of soluble GFP in cell lysate. In order to recover properly folded, fluorinated variants of GFP, we applied a directed evolution approach involving randomization of the sequence of GFP by error-prone PCR, expression of mutants in media depleted of Leu and supplemented with Tfl, and selection of highly fluorescent mutants by cell sorting (Chapter 2).[36]

Beyond proteins with novel composition, there have been efforts to evolve organisms to change their preferences for noncanonical amino acids by random mutagenesis and/or serial growth.[37-40] Wong reported that *Bacillus subtilis* strain QB928, a tryptophan auxotroph, was serially mutated in the presence of 4-fluorotryptophan, and the evolved strain preferred the analogue to tryptophan.[37] In similar experiments, Ellington and coworkders evolved *E. coli* variants and bacteriophage Qβ on fluorinated tryptophan analogues, 4-fluorotryptophan and 6-fluorotryptophan, respectively, through serial passages,[38,39] but the mutants still have preference of Trp to the analogues. Even though these examples have been restricted to the Trp codon, and only one hydrogen of Trp is replaced with fluorine, these results show the possibility of evolving organisms with non-standard chemistries to have novel properties.[40]

Fluorinated amino acids

A repertoire of noncanonical amino acids that can be incorporated into recombinant proteins has been expanded substantially, and now provides powerful tools to the protein engineering field.[1-3] Among the various analogues, fluorinated amino acids

have been given much attention due to the possibility of producing biological molecules with novel properties.[3-13, 41-51] Fluorine is small, and for this reason it is often thought to be isosteric with hydrogen. However, fluorinated compounds are more hydrophobic than their hydrogenated ones. For example, the trifluoromethyl group is almost twice as hydrophobic as a methyl group determined by partition constant, and the property has been used to increase the bioavailability of drugs by increasing their lipophilicity.[41] Despite the hydrophobicity, perfluorinated molecules are sometimes poorly soluble in hydrocarbon solvents.[41] Those properties can be described as "fluorophilic," rather than hydrophobic or lipophilic, which is called the fluorous effect. Several groups have utilized these novel properties of fluorinated amino acids by replacing residues in hydrophobic core with these noncanonical amino acids.[3-13, 46-48] Methyl groups in protein hydrophobic cores are replaced globally with trifluoromethyl groups, and fluorous cores would be formed. The replacement of Leu, Ile, or Val in the hydrophobic cores of coiled-coil proteins with trifluorinated or hexafluorinated analogs were able to increase the resistance to thermal and chemical denaturation.[3-13]

The fluorination of protein hydrophobic cores shows the possibilities of producing biological molecules with novel and useful properties. In addition to the hyper-hydrophobicity (and fluorophilicity), fluorinated compounds exhibit many other interesting properties which provide powerful tools to organic and medical chemistry. As mentioned above, fluorine is often thought of as isoteric with hydrogen. However, the van der Waals radius of fluorine is 1.47 Å, which is 0.27 Å larger than that of hydrogen, and the C-F bond (1.35 Å) is longer than a C-H bond (1.09 Å), which results in 0.53 Å difference in total size between the two bonds.[42] The trifluoromethyl group is often said

to be approximately as large as an isopropyl group.[44] However, the calculated molar volume of the trifluoromethyl group is 39.8 $Å^3$, which is much smaller than that of the isopropyl group (56.2 $Å^3$) and is about twice as large as that of a methyl group (21.6 $Å^3$).[43] Instead, the trifluoromethyl group is similar to an ethyl group (38.0 $Å^3$) in terms of occupied volume, even though they are not comparable in shape.[43]

Fluorine is the most electronegative element in the periodic table, and therefore the C-F bond has a high dipole moment compared to the C-H bond.[41-44] The dipole moment is strong enough to cause a polarization of C-H bonds of adjacent alky groups. In addition, the C-F bond is relatively nonpolarizable, and C-F bonds also reduce the overall molecular polarizability of organic molecules by increasing the hardness of the carbon framework.[42]

The strong dipole moment and the low polarizability endow the C-F bond with unique properties when interacting with other molecules. It is accepted that the dipolar C-F bonds can interact with ionic or dipolar groups by electrostatic (dipole-dipole or point-dipole) interactions in appropriately organized systems.[42,45] However, the C-F dipoles of fluorinated organic molecules are generally thought not to be involved with hydrogen bonding in polar solvent such as alcohols, amines, or water, though this concept is still controversial. DiMagno and coworkers explained the lack of hydrogen-bonding as follows:[42] time-dependent interactions involving induced dipole or dispersion are not so favorable for the relatively nonpolarizable C-F bond as they are for solvent heteroatoms, and the contribution of covalency to C-F hydrogen bonding is smaller than competing heteroatom hydrogen-bonding because of higher ionization potential of fluorine. That is, fluorinated compounds are hydrophobic (more hydrophobic than hydrogenated

compounds), but the dipolar C-F bond can be involved in the static dipole-dipole and charge-dipole interactions with appropriate orientation; these characteristic properties are explained as polar hydrophobic. Considering that many dipole moments are present in the backbone and side chains of proteins, we might expect other results by the fluorination of proteins in addition to hydrophobic or fluorophilic effect. Likewise, these properties can be incorporated into new strategies to engineer proteins with fluorinated amino acids.

Hydration of protein surfaces

Almost all proteins, with several exceptions including membrane proteins, fold into their final three-dimensional structure in aqueous environments. Water molecules play critical roles in the functions of the folded proteins, even in the case of membrane proteins. In addition, considering non-static properties of a random, fluctuating, three-dimensional network of hydrogen bonds, the dynamic features of proteins cannot be separated from those of protein-associated water molecules. Therefore, the characterization of interactions between water molecules and proteins is necessary for understanding protein structure, folding, dynamics, and function.

Properties of the hydration layer around proteins have been studied both experimentally and theoretically.[52-64] Using small-angle scattering of x-rays in H_2O and neutrons in H_2O and D_2O, Svergun and coworkers showed that the density of water molecules in the first hydration shell is higher than that of bulk water for several model proteins.[52] Merzel and Smith used molecular dynamics simulation to study these results, and showed that both the topological and electrostatic properties of the protein surface determine the density of the surface water layer.[54] Compared to bulk water, the dipoles

of water molecules in the hydration layer align in a more parallel fashion to each other, and the number of hydrogen bonds per water molecule is higher.

Structure of proteins fluctuates and dynamic features play an important role in protein function such as catalysis and interactions with other molecules. Considering the fact that the protein hydration layer interacts strongly with the protein surface, the dynamic properties of water molecules in the region are critical to understanding the nature of proteins. As mentioned previously, the structure of water in the hydration layer is rigid, and thus water molecules in the region exhibit slower dynamic features than bulk water. Zewail and coworkers pioneered femtosecond-resolved analyses of solvation on protein surfaces using intrinsic Trp as a local optical probe.[55-63] The relaxation process monitored through the fluorescence Stokes shift of the probe upon excitation is composed of a biphasic distribution. They proposed a model to explain the observed results: the faster dynamics observed in picoseconds are contributed by fast motions of local water molecules which are involved in the structure similar to that of bulk water, and the slower relaxation (which takes place in tens of picoseconds) is associated with rearrangement of the relatively rigid structure of water molecules. Later, the model was developed further to include protein-coupled dynamics of water molecules. Recently, Zewail and coworkers examined the effects of neighboring residues on relaxation of the dipole in the Trp indole ring using the enzyme staphylococcal nuclease.[63] Mutations of charged residues around the Trp into Ala resulted in faster hydration dynamics, which was explained by a less rigid structure around the hydrophobic Ala residue than that around the charged residues. Because of the strong interactions between local water and charges, the motion of water molecules is more restricted and involved in the dipole relaxation process in a slower

time compared the case of Ala. In the case of a mutation to Cys (hydrophilic residue), the time scale of the dynamics lies between those of the charged residue and Ala.

The nature of hydration of pure hydrophobic surfaces is dependent on the length scale of the molecules considered.[65,66] Water molecules can reorganize themselves to solvate small hydrophobic solutes without losing hydrogen bonds. However, in the case of large hydrophobic molecules with low curvature over areas larger than 1 nm^2, water molecules cannot maintain a hydrogen-bonding network with the surrounding liquid. In order to minimize the loss of hydrogen bonds, water molecules tend to move away from the surface and form an interface. This results in lower water density around the large hydrophobic surface. The properties of this region are similar to those of liquid-vapor interface,[67,68] and are affected by attraction forces between the large hydrophobic surface and the water molecules in the interface such as van der Waals attractions.[69-72] Actually, because of the weak attraction forces, the water density in the interface is fluctuating rather than static,[72-73] which might play an important role in the dynamics of large-surface hydrophobic molecules. Recently, in order to examine whether the same effects are observed at the protein surface, Smith and coworkers performed a simulation study on the folding of a β-hairpin peptide which has large solvent exposure of hydrophobic residues in the folded state.[64] As folding proceeds, the local water density around hydrophobic residues decreases while water density increases around hydrophilic residues; the folded peptide has higher hydration density around its hydrophilic residues than its hydrophobic ones. Similar effects were also reported in model peptides: water molecules around the Leu residue of a peptide showed faster dynamics than those around glycine.[74]

As described above, the structure of water molecules in the protein hydration layer can be affected not only by the topological and electrostatic properties of protein surface, but also by the physical and chemical properties of surface-exposed individual residues. In particular, the water molecules around surface-exposed hydrophobic residues can be involved in a less-rigid structure compared with those in the other region of the hydration layer, which therefore may influence the dynamic features of the hydration. Interestingly, the C-F bond of fluorinated compounds is not only hydrophobic but also has a strong dipole moment; these properties can influence the hydration dynamics in opposite ways. Motivated by this uniqueness, we have been interested in the nature of the local hydration layer around fluorinated amino acids in proteins. In order to address this question, we installed Tfl near a surface-exposed Trp residue of a coiled-coil protein and examined the effects of fluorination on the hydration dynamics (Chapter 3).

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