Molecular Evolution in Static and Dynamical Landscapes

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Abstract. In an attempt to understand protein evolution, we address the issues of how much variety in the sequences is needed to prompt the evolution of an enzyme from random polypeptides and how does cellular interaction affect the dynamics of molecular evolution to allow genetic diversity in population. The experimental evolution of phage-displayed random polypeptides of about 140 amino acid residues panned with transition state analogue for an esterase reaction showed that even with a population size as small as ten, not only could significant varieties be found but also the random polypeptides in each of the generation had great promise towards developing into functional proteins. Hence, it is evident that the enzyme evolution is prompted even within a small local area of the static landscape of the sequence space. Considering that interaction among living cells is an inevitable event in natural evolution, its role was investigated through three consecutive rounds of random mutagenesis on the glutamine synthetase gene and chemostat culture of the transformed *Escherichia coli* cells containing the mutated genes. The molecular phylogeny and population dynamics show the coexistence of some mutants having different level of glutamine synthetase at each generation. In addition, it was confirmed that cellular interaction via the medium influences the stability of the coexistence and bring forth fitness change to the coexisting members of the population, thereby, leading to a dynamical landscape. Based on experimental results reflecting the extent of interaction among members in population, here, I proposed that protein evolution could change its mode from the optimization on static landscape to diversification on dynamical landscape.

Key words: cellular interaction, diversification, fitness landscape, library size, optimization, protein evolution, sequence space

1. Introduction

Natural proteins have in no doubt utilizes different paths to acquire their fitness in accordance to functions. Theoreticians and experimentalists have been comparing notes in an attempt to understand the evolutionary paths. The fitness landscape analogy first described by Wright [1] was extended to the molecular world by describing the sequence space as a connected network of all protein sequences [2], after which several models of evolution were derived [3–6]. However, even with the wealth of theories as summarized by Voigt, et al. [7], frustration [7] and complexity catastrophe [5] attributable to mutation, selection, library size, entropy,

Though experimental motivation towards understanding evolutionary paths can be said to be still in its infancy, several studies providing different insights proved promising. Evolvability of primordial polypeptides to functional proteins with a frequency of roughly 1 in 10^{11} was demonstrated by Keefe and Szostak [8]. Since most of the polypeptides can lie in a sequence space completely devoid of function [7], to obtain new functional proteins from random polypeptides experimentally is to sample from a large library of about 10^{13} [8, 9]. Matsuura, et al. [10] showed that evolving sequences may take an alternative mode of evolution by means of elongation mutagenesis rather than being trapped in any local optimum after point mutagenesis. Bacterial cells evolving in identical environment diversified significantly in both morphology and mean fitness on the first 2000 generations but were nearly static for the last 5000 generations [11]. On the other hand, we have showed that cellular interaction via the environment could bring about plasticity of gene fitness and lead to diversification of species [12]. Based on experimental results, I, hereby, proposed the possible modes of protein evolution through addressing the issues of library size in search of evolvable primordial polypeptide and of cellular interaction in the role of diversification.

2.1. MATERIALS

Escherichia coli strains used were as follows: TG1 [K12, $\Delta(lac-pro)$ *supE thi hsdD5*, F⁺ *traD36 proAB lacI^q lacZ Δ M15*] [13], BL21(DE3) (Novagen), and *E. coli* YMC21 ($\Delta(glnA-glnG)$ 2000 $\Delta lacU169$ *endA1 hsdR17 thi-1 supE44*) lacking glutamine synthetase gene (Chen et al., 1982), which was kindly provided by Dr. Boris Magasanik (Massachusetts Institute of Technology). Phage library PL1, phagemid pCANSS, and the three phage clones displaying random polypeptides RP3-04, RP3-42, and RP3-45 were prepared previously [14]. Helper phage M13KO7 was purchased from Amersham Pharmacia Biotech. A vector for the expression of random polypeptides with a C-terminal His₆ tag, PET21aSH, was constructed by replacing the *HindIII/XhoI* region in the cloning site of pET21a(+) (Novagen) with *HindIII/XhoI* dsDNA fragment containing the *SfiI* site prepared by annealing 5'-AGCTTGGCCTCTGGGGCCGCACACCACCACCACCACCACTA AC-3' with 5'-TCGAGTTAGTGGTGGTGGTGGTGGTGGTGTGCGGCCCCAGAGGCCA-3'. Transition state analogue (TSA), *p*-nitrophenyl hydrogen 4-(hydroxycarbonyl)butylphosphonate [15], was kindly synthesized by Dojindo (Kumamoto, Japan). A hybrid plasmid pKGN containing *E. coli* glutamine synthetase gene was prepared previously (16). pKP1500 (17) was a generous gift from Dr. Takeyoshi Miki (Kyushu University). Glutaminase was a generous gift from Daiwa Kasei Co. Ltd. (Osaka). DNA manipulation and transformation of *E. coli* cells were carried out as described by Maniatis et al. (13).

2.2. *in vitro* SELECTION SYSTEM FOR PHAGE-DISPLAYED RANDOM POLYPEPTIDES

Random polypeptides displayed on the surface of M13 phage as fusion proteins with the pIII coat protein were selected based on their affinity towards TSA of the esterase reaction [15]. The affinity level of each clone was evaluated by the number of phage bound to the TSA plate. To prepare the TSA plate, an equal volume of 33 μ M TSA in 0.1 M MES (pH 4.7) containing 0.15 M NaCl and 26 mM water-soluble carbodiimide were added into the wells of the plate (CovaLink™ NH₂ Module, NalgeNunc International) and the plate was incubated overnight at 20 °C. After washing each well with 0.3 ml water three times, 0.3 ml of 1M glycine was added into each well, and the plate was again incubated at 20 °C. After incubating the plate for 1.5 h, the well was again washed with 0.3 ml of 50 mM Tris/HCl (pH 7.0) containing 150 mM NaCl three times.

Phage of about 10^{10} transducing unit (t.u.) suspended in 100 μ l of 50 mM Tris/HCl (pH 7.0) containing 150 mM NaCl and 0.5% skim milk were first allowed to bind onto the TSA plate upon incubation at 15 °C for 2 h. The well was washed 16 times by gentle addition of 200 μ l of 150 mM Tris/HCl (pH 7.0) containing 150 mM NaCl, and subsequent careful aspiration of 200 μ l of the solution in the well leaving 100 μ l of the solution to prevent the well from drying. After consecutive washing, the plate was again incubated at 15 °C for 2 h, before subjecting to the same washing procedures described above for 5 times more. After the final washing, the phage bound to the TSA plate was eluted by incubating the plate at 15 °C for 2 h after subsequent addition of 200 μ l of 250 μ M TSA dissolved in the same buffer and aspiration of 200 μ l of the solution. The number of phage in the eluate, expressed as t.u., was estimated by the number of ampicillin resistant colonies [18]. One selection round of the phage in the library, hence, consists of binding, washing, and the elution steps.

2.3. RANDOM MUTAGENESIS AND CONSTRUCTION OF PHAGE LIBRARY

The random polypeptide gene on the phagemid, prepared from *E. coli* TG1 cells infected with the selected phage, was amplified by mutagenic PCR as described previously [19] using the *Thermus thermophilus* DNA polymerase and the primers, 5'-CCGCCTTTGAGTGAGCTGAT-3' and 5'-TCGTCACCAGTACAAACCACA ACG-3'. The mutated products were isolated and further amplified by the standard PCR as described [14] using the primers, 5'-ATCCTCGCAACTGCGGCCACG-TGGCCATGGCTAGCATGACT GGTGGACAGCAAATGGGT-3' and 5'-AGTT-TAGGCCCCAGAGGCTAATCGCAGTCTGTCTGACTC-3'. The *Bgl*I fragment from the PCR products was ligated into pCANSS, and *E. coli* TG1 cells were transfected with the ligated DNA as described previously [14]. Phage library was prepared from the phagemid library as described previously [14].

2.4. EXPRESSION AND PURIFICATION OF RANDOM POLYPEPTIDES WITH A HIS-TAG

To express the random polypeptide of the selected phage, the random polypeptide gene on the phagemid was recloned into the *NheI/SfiI* site of the pET21aSH expression vector described above. *E. coli* BL21(DE3) cells harboring the hybrid plasmid were grown at 37 °C on LB medium [13] containing 50 µg/ml ampicillin and the culture at an OD₆₀₀ of 0.6–1.0 was induced with IPTG (final conc., 1 mM) for 5 h. The cells were harvested and disrupted in 50 mM potassium phosphate (pH 8.0) containing 8 M urea, 0.3 M NaCl, and 10 mM imidazole. The supernatant was applied to a Ni-NTA Superflow (Qiagen) column equilibrated with the same buffer. The column was washed with 50 mM potassium phosphate (pH 8.0) containing 8 M urea, 0.3 M NaCl, and 20 mM imidazole, and the random polypeptide was eluted with an imidazole gradient of 20–80 mM. All purified polypeptides were confirmed homogeneous by SDS-PAGE, and the amino acid compositions coincided well with those deduced from the nucleotide sequences. The molar absorption coefficients of the polypeptides at 280 nm, determined from the amino acid composition and A₂₈₀ value of the purified polypeptide solution as previously described [20], were as follows: RP3-42H, 37000 M⁻¹cm⁻¹; YSLP1-1, 63000 M⁻¹cm⁻¹; and YSLP3-1, 67000M⁻¹cm⁻¹.

2.5. MEASUREMENT OF ESTERASE ACTIVITY

The hydrolysis rate of *p*-nitrophenyl acetate was measured at 37 °C. Reaction mixture contains 20 µl of 2.0 mM *p*-nitrophenyl acetate dissolved in 1.25% methanol and 80 µl of a polypeptide solution in 62.5 mM sodium acetate (pH 5.0). After 1, 2, 3, or 4 h of incubation, the solution was chilled on ice before the pH was increased to 7.2 by the addition of 25 µl of 250 mM potassium phosphate (pH 8.0). The precipitate, formed while shifting the pH, was removed by centrifugation, and absorbance of the solution at 400 nm was measured. The sample at 0 h was prepared by the same procedures described above except that the substrate and polypeptide was mixed on ice without incubation at 37 °C. The concentration of the released *p*-nitrophenol was calculated using a molar absorption coefficient of 10400 M⁻¹cm⁻¹, which was determined experimentally at pH 7.2.

2.6. CHEMOSTAT CULTURE

Culture conditions of the mixture of transformants harboring varieties of glutamine synthetase genes were the same as those described previously [21, 22] except for the glutamate concentration in medium C and the value of dilution rate, which were fixed at 0.1 M and 0.075/h, respectively. Glutamate serves as the sole nitrogen source. By the established chemostat cultivation conditions, wall growth is ensured not to occur both in the chemostat and on the sampling tube.

2.7. CHARACTERIZATION OF MUTANT ENZYMES

Genes encoding mutant enzymes to be characterized were subcloned to modified pKP1500 having an additional *Xba*I site. *E. coli* YMC21 cells transformed with the hybrid plasmids were cultivated at 37 °C on medium C. The enzymes produced were purified as described previously [12]. Glutamine synthetase activity of the purified enzymes was measured as described previously [16].

2.8. ASSAY OF GLUTAMINE CONCENTRATION IN CHEMOSTAT CULTURE

Treatment of the 65 ml culture effluent sampled at different time intervals of the continuous chemostat culture before determining the glutamine concentration was as described previously [12]. Glutamine concentration was measured either by enzymatic method using glutaminase and glutamate dehydrogenase [23] or by PICO-TAG method [24] after chromatography of the treated sampled culture as previously described [12]. The glutamine concentration in the chemostat culture was estimated from the difference of the glutamate concentrations measured with and without glutaminase treatment.

3. Results and Discussion

Evolvability of polypeptides toward functional proteins has been commonly dealt by directed evolution that relieves researchers from exhaustive search for a new fitness in the huge sequence space. The library size and the variance among the members are tailored for an effective directed evolution. What then is the appropriate library size and how much variance among all possible sequences of a primordial polypeptides is required to effect evolution? Directed evolution was initiated with a library of random polypeptides of about 140 amino acid residues displayed on the surface of a filamentous bacteriophage as fusion proteins with the pIII coat protein [14]. The random DNA sequences of the polypeptides were designed to contain no stop codons in all the reading frames keeping the diversity as high as possible, such that any frame shift mutations which might occurred during genetic manipulation like PCR still allows the formation of long random polypeptides [25]. The selection system based on the affinity to a transition state analogue (TSA) for an esterase reaction was optimized to allow detection of the least minute affinity, if present. To address the issues on library size and operative variance, a library size as small as 10 from the generated phage library of mutagenized random polypeptides was employed.

Panning showed that even within a small library of the first generation consisting of members arbitrarily chosen from a big library or global sequence space, variance among the members could be well observed (Figure 1). Second and third generations obtained from mutagenesis of the clone with highest affinity in the first and second generations, respectively, derived some clones with higher affinity than the parent clone. These results indicate that enough diversity could also be

Figure 1. Binding affinity of each clone in the small phage libraries displaying random polypeptides towards the TSA for an esterase reaction. Binding affinity is expressed as the number of eluted phage bound to the TSA (see Materials and Methods). The values are arranged in the order of the lowest to the highest value for each generation. The black bars indicate the phage clones, YSLP1-1, YSLP2-1, and YSLP3-1, with the highest binding affinity value in the first, second, and third generations, respectively. The gray bar in the first generation shows the RP3-42-displaying clone, and those in the second and third generations show the parent clone of each generation.

found in a small population of 10 within the local sequence spaces. The increase in the affinity of the clones in each generation reflects an effective search of clones with higher property despite of the small fraction of sequences. Considering that about half of the clones in the mutant library had enhanced affinity than the parent clones, the parent random polypeptides in each generation are considered as primitive populace [26, 27]. Hence, the property has the allowance for improvement through the process of Darwinian evolution as theoretically proposed [27] and the evolutionary path of the random polypeptides followed an adaptive walk, thereby conferring to a static landscape.

Affinity for transition states is correlated with catalytic efficiency and libraries panned with TSA generate catalysts with improved or novel activities [28–31]. Likewise, when the genes of the three random polypeptides, as indicated in Figure 1, after detaching from the gIII gene of phage were expressed with a His₆ tag and the polypeptides purified, the esterase activities of the purified polypeptides, measured using *p*-nitrophenyl acetate as a substrate, showed an increase with an increase in the affinity of the phage clones to TSA (Figure 2). Negative control ruled out the possibility that the observed esterase activity is due to contamination of enzyme(s) present in the host cell. As the observed activity is much larger than the calculated esterase activity based on the sum of the individual activities of the monomer amino acid residues (Figure 2), the increase in the activity of the polypeptides on generations is not due to the change in the amino acid composition but due to the change in the sequence. These results indicate that enzyme evolution

Figure 2. Esterase activity of RP3-42, YSLP1-1 and YSLP3-1. The k_E value was calculated assuming that the activity is the sum of the individual activities of the amino acid residues contained in each peptide. Esterase activities of various amino acids and their derivatives were measured. Amino acid residues that gave insignificant k_E values were neglected, hence, only the following k_E values were used for calculations: His, $6.48 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$; Cys, $0.79 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$; Lys, $0.12 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$; and the N-terminal amino group, $0.28 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$. ■, Observed k_E values; □, Calculated k_E values.

is being prompted from random amino acid sequences even within a population size as small as ten.

Directed evolution commonly shows that a population, with a range of sequences evolves relentlessly uphill towards regions of a maximum, as in the adaptive walk models of evolution [5, 32, 33]. However, experimental studies had also shown that the fitness landscape changes over time, either due to alteration in the environment or due to coevolution of other interacting species [11, 12]. Kashiwagi et al. proposed that the plasticity of the fitness of a gene in consequence of cellular interaction via the environment had driven the population to diverge. Three generations derived from consecutive rounds of mutation and selection together with subsequent chemostat culture of the mixtures of transformants harboring mutant glutamine synthetase genes turned out to have different population structures with concurrent extinction of most of the mutants present in the initial stage of chemostat culture [12]. In addition, dominating member in one generation could become a minority in another generation, but could also regain its previous status, implying that the fitness landscape changes dynamically during the molecular evolution. Furthermore, the average enzyme activity over the population of the varieties of glutamine synthetase that coexisted in each of the generations fluctuated rather than converging to any fittest value, in contrast to the expectation that the average enzyme activity over the propagating population will converge towards the value of the fittest activity either on a monotonous increase or decrease, especially when a strain with mutant gene expressing a catalytic activity closer to the fittest value is dominating a heterogeneous population. The fluctuations relayed that instead of the adaptive walk towards optimization of catalytic functions, the population had un-

Figure 3. A. Schematic diagram of the source of glutamine in the chemostat mixed culture of W2 and A1 strains; B. Competition between the W2 and A1 genes. Each of the plasmid containing W2 gene or A1 gene of the same concentration was used to transform fresh host cells independently. The total resultant transformants from each transformation were mixed and an aliquot was used as inoculant for the chemostat run as described under Materials and Methods. Frequency of W2 was determined by double restriction endonuclease digestion analysis. W2 has only the *Pst*I site, while A1 has both the *Pst*I and *Bst*EII sites. (▲) and (●), parallel runs, A and B, respectively, of the competition experiments; (○), glutaminase (final 0.47 unit/ml) was added in the feeding medium. Glutamine concentration in the culture medium were measured at the indicated time (*) either by the PICO-TAG (for the A run) [24] or enzymatic method (for the B run) [23]. At 336h of the A run, 1.2 μ M glutamine was detected in the culture, while at 221h, 268h, 336h, and 410h of the B run, glutamine concentrations in the chemostat culture were 0.8 μ M, 1.9 μ M, 2.2 μ M, and 1.3 μ M, respectively.

dergone diversification. The changes in the fitness landscape temporarily suppress the optimization process, allow the coexisting state, and direct diversification.

Considering that glutamine synthetase gene is critical for the survival of the host strain, *E. coli* YMC21 deficient of the gene, under the culture conditions employed [21] and that the only difference among the transformants in the chemostat lies

Figure 4. A projection of the landscape without (A) and with (B) interactions among the members of the population. Circles in different presentation indicate different members of the population.

on their glutamine synthetase gene carried on the plasmid, the product of the enzyme catalyzed reaction glutamine could govern the coexistence of the diversified mutants. We have theoretically proposed that the essential mechanisms of coexistence involve the interplay between the internal metabolic network of competitors and the external variables of the environment, thereby allowing competitors to reach the same growth rate and coexist in the same environment [34]. Coexistence of the mutants, hence, might be brought about by the interaction among the members of the population via the glutamine, which leaked out from ruptured dead cells or from one strain with high glutamine synthetase activity. The leakage of glutamine into the medium influences the growth of the population, thereby allowing the mutants to change their fitness into a mean value (Figure 3A). In agreement, glutamine was detected in the culture of coexisting A1 and W2 mutants at different

Figure 5. The proposed modes of molecular evolution.

time intervals (Figure 3B). As glutamine was not added in the fresh medium fed to the chemostat, there could be a leakage of glutamine from the dead or live cells of both or either of the strains with the A1 or W2 gene. Addition of glutaminase, an enzyme that catalyzes the conversion of glutamine to glutamate, into the mixed culture resulted to the cessation of coexistence (Figure 3B), implying the influence of glutamine to the achievement of coexistence. In addition, when the population density was 1000 times diluted, the coexistence was lost resulting to the dominance of one member of the population, suggesting that cellular interaction could be too weak in a diluted population density to effect coexistence. These results indicate that the cellular interaction is an essential factor for the dynamical fitness change that allows the molecular diversification. The mechanisms underlying the coexistence of even closely related competitors in a minimal model system can be the provision for bio-diversification occurring in nature [12].

In an experimental evolution, if the interaction among the members in population is nil or scarce due to the experimental condition imposed, the fitness of each member is determined by its sequence and the condition employed, and the members of the population move along a static landscape in the sequence space. On a static landscape, though there will be some fluctuation brought by genetic drift [35], the cycle of mutation and selection still lead to an adaptive walk, thereby leading to an optimization of molecular sequences (Figure 4A). Directed evolution from the polypeptides with random sequences is a typical case where members of the population drew a static landscape. On the other hand, if there is prevailing interaction as shown by the molecular evolution of glutamine synthetase, there is plasticity in the fitness of each member during the evolution, hence leading the members of the population to move along a dynamical landscape [12]. On a dynamical landscape, instead of optimization, members of the population attained

the stage of competitive coexistence, leading to their molecular diversification as shown in Figure 4B. In view of an experimentalist, I hereby proposed that the mode of molecular evolution changes due to the interchangeability between the static and dynamic landscape (Figure 5). When the population density is low, interaction among members of the population is also weak, hence, giving a static landscape and the mode of the evolution is on optimization. While evolution pushed its way, population becomes dense allowing members to interact via the environment, the landscape dynamically changed and allow the coexistence of the members with different level of properties, thereby leading to the diversification process even in a single environment.

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