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Characterization of Green Fluorescent Protein Using Aqueous Two-Phase Systems

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The surface net hydrophobicity and local hydrophobicity of green fluorescent protein was evaluated by using the aqueous two-phase partitioning method. The surface net hydrophobicity of GFP allowed it to be classified as a hydrophobic protein although the local hydrophobicity was low. The local hydrophobicity of the GFP was increased by a pH shift to acidic conditions and was maximal at pH 4. The surface net hydrophobicity increased at acidic conditions less than pH 4. The conformational change of GFP at acidic pH is discussed based on the above results.

1. Introduction

Since Shimomura discovered the green fluorescent protein (GFP) [1], the GFP has been utilized as a reporter protein in gene expression and has been as a guiding star for biochemists, biologists, medical scientists and other researchers [2]. It has no external fluorophore and exhibits the fluorescence at the conjugated structure of three amino acids inside a “beer can-like” structure consisted of the β-barrel structure (**Fig.1**) [3]. It can, for example, illuminate growing cancer tumors; show the development of Alzheimer’s disease in the brain or the growth of pathogenic bacteria. In addition, study of the conformational change of the GFP is also of interest because it has a easily-folding nature and also has an interesting β-barrel structure. Although there have been many reports on the kinetic analysis of the GFP refolding [4-6], a study on the surface characteristics of the GFP has not been carried out.

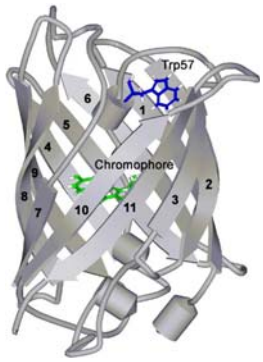

Fig.1 GFP Structure. [3]

In biological systems, the denatured proteins interact with lipid bilayers and with molecular chaperones during folding. Partially denatured proteins as in a molten-globule state have been reported

to be a key conformation in interacting with membranes [7]. It has previously been reported that refolding of denatured bovine carbonic anhydrase was assisted by liposome, probably by weak interactions between the proteins and the liposome, similar to the protein refolding mediated by molecular chaperones [8]. The effectiveness of the use of aqueous two-phase systems (ATPS) for the design of the liposome-assisted refolding process [8-11] has already been reported. It has also been reported that the liposome could affect the GFP expression system in cell-free translation systems [12]. It is important to investigate the surface properties of the GFP according to the previously-reported findings.

The purpose in this study is to characterize the surface properties of GFP under acidic pH conditions. The surface properties of the GFP, such as surface net charge and hydrophobicity and local hydrophobicity, have been quantitatively characterized at various pH values by using the ATPS.

2 Experimental

Green fluorescent protein (GFP) was purchased from Roche Diagonistics. Poly (ethylene glycol) (PEG 1540, 4000, 6000; Mw 1.5 kD, 3kD and 7 kD, respectively) and dextran (Dex) (100~200k; Mw 100-200 kD) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). All other chemicals of analytical grade were purchased from Wako.

The basic composition of the aqueous two-phase systems (the total weight is 5 g) for the partitioning of the cells were 7~13 wt% PEG 1540, 4000, 6000 and 7~13 wt% Dex 100-200k. The ATPSs were prepared by mixing stock solutions of 30 wt% PEG and 30 wt% Dex with the suspension described above. The pH values of these systems were adjusted using a pH-adjusted citrate buffer. The surface properties such as surface net hydrophobicity (*HFS*) and local hydrophobicity (*LH*) of the proteins were analyzed by using the aqueous two-phase partitioning method as was applied for the characterization of protein surfaces [13,14]. When the pH value is then selected at the system pI at lower ionic strength, the following relationship can be obtainable.

$$\ln K = HFS \times HF \quad (1)$$

where the hydrophobic factor (*HF*), which has been defined from the partition coefficient of amino acids [12], is an indicator of the hydrophobicity difference between the two-phase systems and the *HFS* value is defined as the surface net hydrophobicity of the proteins. Further, the local hydrophobicity of proteins under heat stress was also determined as $LH_{Pr} = \Delta \ln K_{Pr} = \ln K_{Pr,Triton} - \ln K_{Pr.0}$, where $K_{Pr,Triton}$, and $K_{Pr,0}$ are the partitioning coefficient of protein in the aqueous two-phase systems with and without 1m M Triton X-405 [13]. The surface charge of the GFP was calculated based on its amino acid sequence [3].

The GFP concentration in the PEG- or Dex-phases was measured from the GFP fluorescence (λ_{ex} = 400nm, λ_{em} = 509nm) [1] or total protein concentration using the BCA method [15].

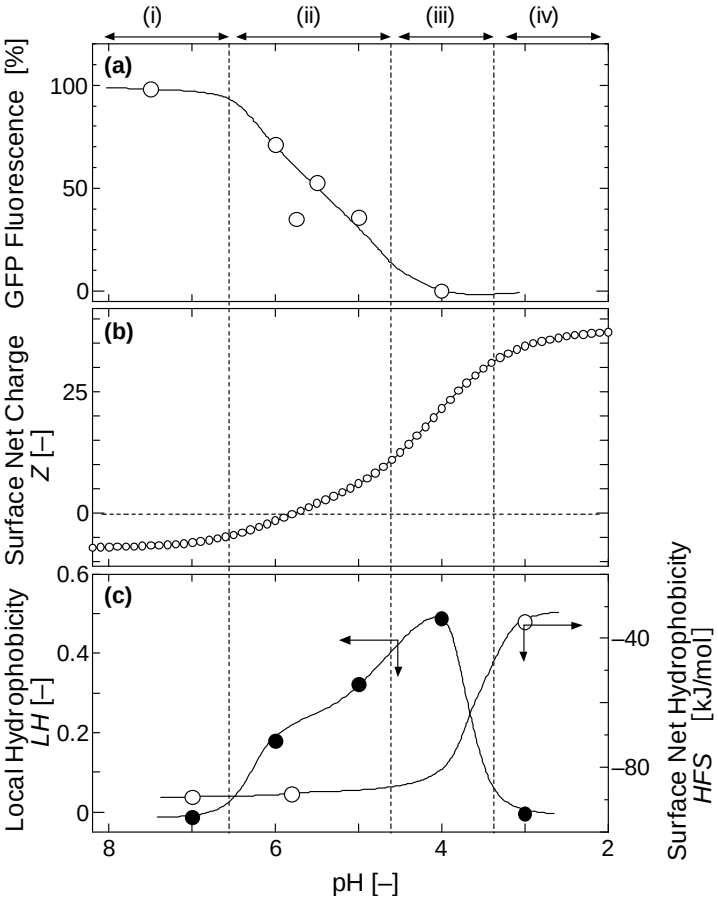

Fig.2 pH Dependence of (a) GFP Fluorescence, (b) Its Surface Net Charge (Calculated) and (c) Surface Properties Determined by Aqueous Two-Phase Partitioning Method

3. Results and Discussion

The conformational change of GFP was characterized at acidic pH by measuring the intrinsic fluorescence and by using the aqueous two-phase partitioning method. **Figure 2** shows the pH dependence of (a) the GFP fluorescence and (b) the surface properties of GFP. As shown in **Fig.2(a)**, the GFP activity did not changed in the pH range between 8.0 to 6.5 but decreased in the pH range between 6.0 and 4.0. It has been reported that the GFP conformation was changed at pH 4-5 [16]. The above results on the GFP fluorescence correspond well with the previous findings. **Figure 2(b)** shows the pH dependence of the surface net charge of the GFP, calculated from its amino acid sequence. Although the GFP surface has a negative charge at pH value below 6, it gradually increased to positive values at acidic pH. The values were increased at pH values below 5.5 and the GFP fluorescence disappeared (**Fig.2(a)**). As shown in **Fig.2(c)**, the surface properties of the GFP (surface net hydrophobicity (*HFS*) and local hydrophobicity (*LH*)) were also determined by using the aqueous two-phase partitioning method [13-14]. The *HFS* values increased inversely in correspondence with the decrease of GFP fluorescence in the pH range from 4 to 3. The *LH* value showed a maximum value at pH 4.0 and decreased at more acidic pH values. In addition, a slight increase in *LH* was also observed at pH 5-6.

In our previous report, two types of hydrophobicities, *HFS* and *LH*, of various proteins have reported to be correlated with the accessible surface area (ASA) and with the fluorescence intensity of the hydrophobic probe ANS, respectively [17]. From the comparison of both values, GFP in its native form was found to have a relatively hydrophobic surface although it has less local hydrophobic sites on the surface. In addition, the surface hydrophobicities of proteins have been systematically characterized under various stress conditions (pH, denaturant, temperature and so on) [18], resulting in the *HFS* values becaming greater for the proteins in the unfolded state and the *LH* values were maximal at the specific stress condition to induce the conformational change to an MG-like state. A general tendency of the variation of the surface properties of GFP upon acidification corresponds well with the previous

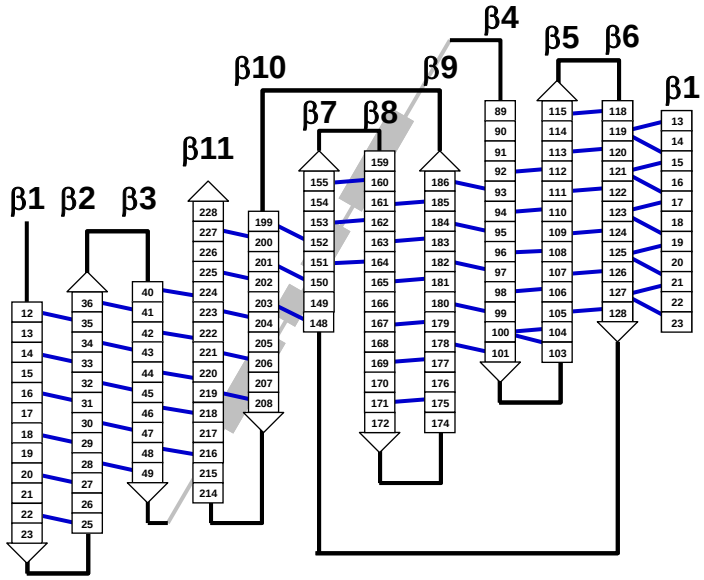

Fig.3 Schematic Illustration of The Structural Domain of GFP (β-Sheet). Blue Lines show Hydrogen Bonds. [20]

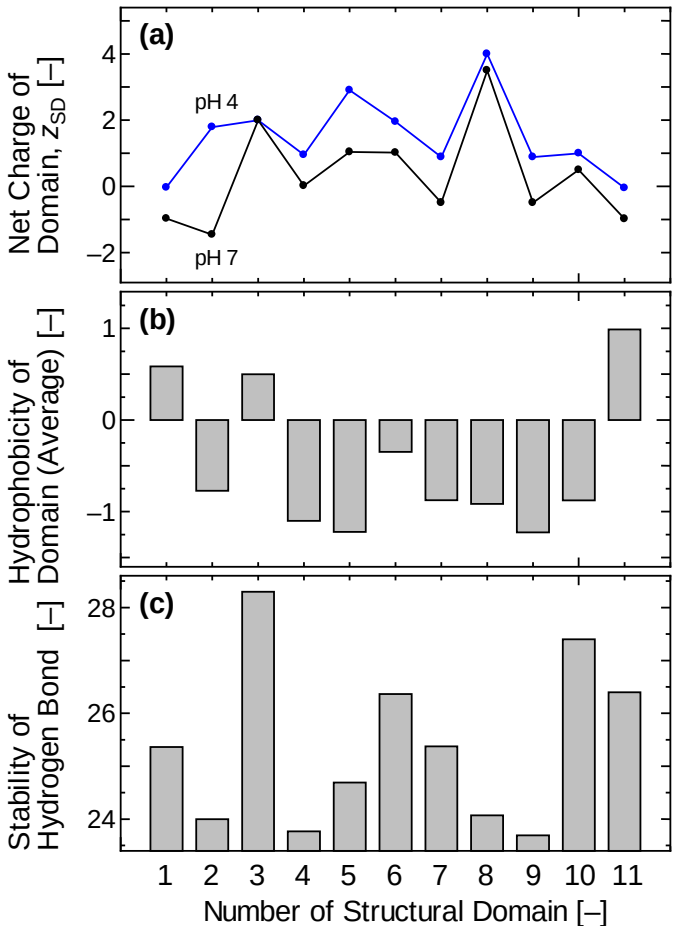

Fig.4 Characteristics of The Structural Domain of GFP (β-Sheet). (a) Net Charge at pH 7 and 4, (b) Hydrophobicity, and (c) Stability of Hydrogen Bond (Extent of Desolvated Hydrogen Bond) [20]. The amino acids between the domains were not included in the above data.

findings on other types of proteins. It has been reported that there could be some possible intermediate during the unfolding of the GFP [19]. According to previous reports, the unfolding of GFP under acidic condition, could be initiated by the destruction of the specific structural domains of the GFP. In order to clarify the situation of the specific domain of the GFP upon its unfolding, the surface characteristics of the structural domain of the GFP (**Fig.3**) was further investigated based on the amino acid sequence data. **Figure 4(a)** shows the net charge of the structural domains at various pH values. It was thus found that a drastic change can be obtained in some structural domains such as 1-2, 4-7, 9 and 11 upon acidification of the GFP solution. The above change of the net charge of the GFP is considered to induce a variation of the electrostatic interactions between the side residues. **Figure 4(b)** and **4(c)**, respectively, show the hydrophobicity based on the amino acid sequence [3] and hydrogen bond stability of the structural domains [20]. In general, the hydrogen bonds of the main chain of the structural domains were unstable (**Fig.4(c)**) in the case of the lower hydrophobicity of the structural domains (**Fig.4(b)**) (such as 2, 4-5, and 7-9). It has been reported that the hydrogen bonds can be stabilized in the hydrophobic environment

[20]. The hydrogen bonds in the main chain of the above mentioned domains (2, 4-5, and 7-9) could easily be destabilized by the variation of the net charge of the structural domains upon tacidification. It has been reported that structural domains 7-10, neighboring to the chromophore, can easily be unfolded upon the unfolding of the GFP [19]. Although further investigation is needed, it is suggesting that increasing local hydrophobicity could be caused by the pH induced conformational change of some structural domains with unstable hydrogen bonds of a relatively hydrophilic nature and with the highly variable charged residues.

The surface properties of GFP were thus characterized by using the aqueous two-phase partitioning method, together with the basic characteristics of the structural domains. It has been reported that the liposome could assist the refolding of the proteins, depending on their surface charge density and local hydrophobicity [9-11]. It has also been reported that the adsorption of the oxidized and fragmented superoxide dismutase on the liposome surface could also be modulated by the above mentioned surface characteristics [21-23]. It could be expected that the above findings could also contribute to the improvement of the protein refolding and also the design of the liposome-based biofunctional materials (i.e. LIPOzyme) [22-24] and the sensing of the stress-response dynamics of the liposome membrane [25].

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