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Short communication

Thermal stability of chemically denatured green fluorescent protein (GFP) A preliminary study

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Abstract

Green fluorescent protein (GFP) is a light emitter in the bioluminescence reaction of the jellyfish *Aequorea victoria*. The protein consist of 238 amino acids and produces green fluorescent light ($\lambda_{max} = 508$ nm), when irradiated with near ultraviolet light. The fluorescence is due to the presence of chromophore consisting of an imidazolone ring, formed by a post-translational modification of the tripeptide –Ser^{65}–Tyr^{66}–Gly^{67}–, which buried into β-barrel.

GFP is extremely compact and heat stable molecule. In this work, we present data for the effect of chemical denaturing agent on the thermal stability of GFP. When denaturing agent is applied, global thermal stability and the melting point of the molecule is decreases, that can be monitored with differential scanning calorimetry. The results indicate, that in 1–6 M range of GuHCl the melting temperature is decreasing continuously from 83 to 38 °C. Interesting finding, that the calculated calorimetric enthalpy decreases with GuHCl concentration up to 3 M (5.6–0.2 kJ mol^{-1}), but at 4 M it jumps to 8.4 and at greater concentration it is falling down to 1.1 kJ mol^{-1}. First phenomena, i.e. the decrease of melting point with increasing GuHCl concentration can be easily explained by the effect of the extended chemical denaturation, when less and less amount of heat required to diminish the remaining hydrogen bonds in β-barrel. The surprising increase of calorimetric enthalpy at 4 M concentration of GuHCl could be the consequence of a dimerization or a formation of stable complex between GFP and denaturing agent as well as a precipitation at an extreme GuHCl concentration. We are planning further experiments to elucidate fluorescent consequence of these processes.

Keywords: GFP; DSC; Thermal unfolding; Chemical denaturation; GuHCl

1. Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is one of the most widely studied and applied proteins in biochemistry and cell biology. GFP converts the blue light (that would otherwise be emitted by the Ca^{2+}-sensitive protein aequorin) into a brilliant green fluorescence [1–3]. GFP and its genetically modified variants are widely used as fluorescent biosensors for protein expression and to study the dynamics and protein–protein interactions in living cells [4,5].

GFP (28 kDa, 238-aa residues) is a barrel-shaped molecule, 24 Å in diameter and 42 Å in length. Outer "layer" of the barrel is composed of 11 antiparalell β sheets. Antiparalell sheets are connected with α-helical stretches. One α-helix extends to the interior of the "β-can" and forms the fluorescent chromophore. Chromophore is composed from three (–Ser^{65}–Tyr^{66}–Gly^{67}) post-translationally modified amino acids [6]. Fluorescent properties of GFP and its genetically modified variants are determined by interaction between these three amino acids and neighboring residues. Extensive digestion of GFP with papain has yielded a hexapeptide (Phe64–Gln69), which contains tripeptide, but this fragment has been found nonfluorescent [7].

Enhanced GFP (EGFP) is a mutant of GFP with 35-fold increase in fluorescence [8–10] This variant has mutations of Ser to Thr at amino acid 65 and Phe to Leu at positions 64 and encoded by gene with optimized human codons [9].

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Thermally denatured GFP can be renatured at the low temperature, so the process is reversible [11,12]. Denaturating agents, such as GuHCl lowers the temperature of denaturation of GFP, which can be monitored by differential scanning calorimetry. Thermally or chemically unfolded polipeptide cannot be an ideal mathematically random chain. There is considerable experimental evidence for local order in proteins denatured by different denaturants [13–15]. It is interesting to check the simultaneous influence of the two denaturing processes and the question of reversibility of the unfolding for GFP because intrinsic viscosity data given at identical temperatures suggest that unfolded chains evoked either heat treatment or GuHCl are very nearly random coils. The free energy associated with both kind of denaturation are very similar at the same temperature and pH [16]. Therefore, in this study we present data for the effect of denaturing agent (GuHCl) on the global thermal stability of GFP.

2. Materials and methods

2.1. Cloning of EGFP

E-GFP clone was purchased from BD Biosciences, Clontech. pEGFP-N1 encodes a red-shifted variant of wild-type GFP [17–19] which has been optimized for brighter fluorescence and higher expression in mammalian cells (excitation maximum = 488 nm; emission maximum = 507 nm). pEGFP-N1 encodes the GFPmut1 variant [6] which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the *EGF*P gene contains more than 190 silent base changes which correspond to human codon-usage preferences. For prokaryotic expression EGFP was cloned into pET-28a vector (Novagen).

2.2. Expression and purification of GFP

EGFP was expressed solubly in *Escherichia coli* (BL21(DE3)pLysS) at temperature 12 °C for 72 h. His_6-tagged proteins were purified on Ni^{2+} NTA columns under native conditions following manufacturer's instructions (Qiagen). Final eluate was dialyzed against EGFP-puffer (100 mM NaCl, 20 mM NaH_2PO_4, 5 mmM mercaptoethanol at pH = 7.5) and further purified on Sephadex G-25 column. Concentrations were determined with Bradford reagent (Sigma) or calculated from extinction at 488 nm.

2.3. Calorimetric measurements

The thermal unfolding of EGFP was monitored by a SETARAM Micro DSC-II calorimeter. All experiments were carried out between 0 and 100 °C with a scanning rate of 0.3 K/min. Conventional Hastelloy batch vessels were used during the denaturation experiments with an average 850 μl sample volume. GFP buffer was used as a reference sample. The sample and reference vessels were equilibrated with a precision of 0.1 mg. It was not necessary to correct for heat capacity between the sample and reference vessels. The calorimetric enthalpy was calculated by the SETARAM two points fitting integrating software.

3. Results and discussion

Since each amino acid influences the free enthalpy of both the folded and unfolded states, insight into denaturated proteins is crucial for understanding protein stability [20]. During unfolding the polypeptide chain becomes less compact, more highly solvated and much more flexible [21]. According to our measurements the native GFP seems to be very heat stable. It has one endotherm with 83 °C melting temperature and 56 J/kg calorimetric enthalpy in 0–100 °C temperature range (Fig. 1). Its thermal denaturation is highly reproducible but the baseline is changing slightly from batch to batch. The transition exhibits strong cooperativity, the system behaves as a single big domain. Applying chemical denaturation by administrating GuHCl in 1–6 M concentration the melting temperature is decreasing continuously from 83 to 38 °C (Table 1). Using Fourier transform infrared spectroscopy Scheying et al. [22] has found a smaller thermal unfolding transition temperature for EGFP mutant (79.5 °C) but they used a smaller scanning rate than we did. It is known from the studies of Sanchez-Ruiz et al. [23] and Vogl et al. [24] that the melting peak temperature is heating rate dependent. It follows a saturation like function and above 0.5 K/min it is roughly scanning rate independent. This was the reason that we used 0.3 K/min which is generally accepted for protein samples. Our calorimetric enthalpy decreases with GuHCl concentration up to 3 M ($5.6 \rightarrow 0.2\,kJ\,mol^{-1}$), but at 4 M it jumps to 8.4 and at greater concentration it is falling down to $1.1\,kJ\,mol^{-1}$ with simultaneous decreasing of melting temperature. The first finding can be explained by the effect of the extended chemical denaturation with increasing GuHCl concentration. This way smaller amount of heat feeding is enough to destroy the remaining structure at lower melting temperature. The unexpected increase of calorimetric enthalpy at 4 M concentration could be the consequence of a structural rearrangement

Table 1
Thermal denaturation parameters (mean ± S.D.) of GFP in the function of GuHCl concentration

GuHCl (cm^3/M)	Melting temperature (T_m, °C)	Calorimetric enthalpy (ΔH, $kJ\,mol^{-1}$)
0	82.8 ± 0.3	5.57 ± 0.12
1	74.5 ± 0.2	1.90 ± 0.06
2	68.7 ± 0.2	0.68 ± 0.02
3	63.2 ± 0.2	0.20 ± 0.01
4	44.6 ± 0.2	8.38 ± 0.16
5	44.0 ± 0.2	7.21 ± 0.12
6	38.3 ± 0.2	1.08 ± 0.04

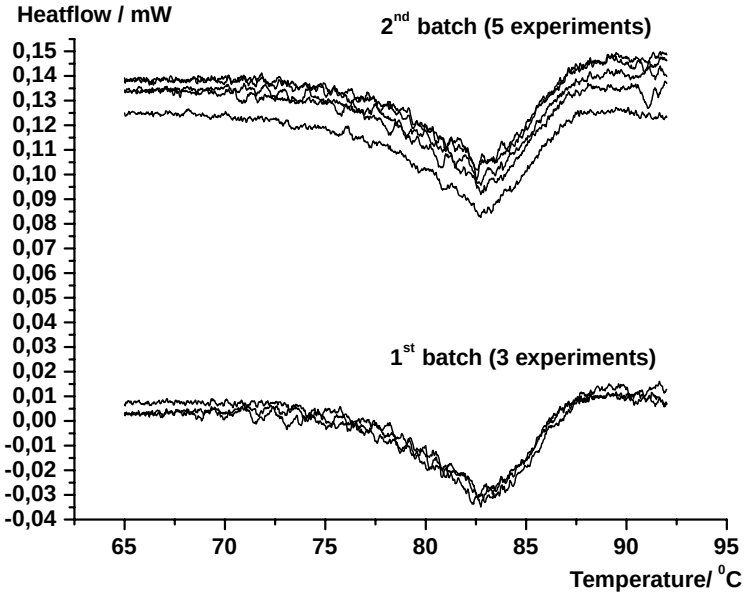

Fig. 1. Reproducibility of thermal denaturation of native GFP (endothermic deflections are directed downwards).

caused by the chemical denaturation. Some observation provide data for the possibility of aggregation above the melting (T_m) temperature [22,25]. In contrast during the whole thermal denaturation range (0–100 °C) we could not detect any sign of aggregation of GFP which is also confirmed by the data of Seifert et al. [26]. Another possible explanation could be a dimerization process, the structure evoked by this way is easily denaturated by the successive chemical and heat treatment by smaller calorimetric enthalpy at lower melting points. It is supported by the observations that whatever structure forms in the unfolded state under a given set of conditions, it is likely to be very transient [20], e.g. bond rotations are slowed up to 100-fold to free amino acids [27] and unfolded proteins undergo rapid hydrogen-exchange reactions [28,29]. Further experiments are needed to look for the fluorescent consequence of these processes to collect all the wanted information to calculate reliable activation energy and free enthalpy data.

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