Biophysical research on the process of adopting the native structure and properties of proteins from GFP family

Abstract

Green Fluorescent Protein (GFP) from jellyfish *Aequorea victoria* is the first discovered protein from Fluorescent Proteins family (FPs), which became widely used fluorescent markers, especially in the visualization of processes in cells. Fluorescent proteins are applied e.g. in the real-time monitoring of molecules behaviour, as reporter proteins, as pH and ion concentration biosensors, as well as, due to Förster Resonance Energy Transfer (FRET), in protein folding research or in monitoring the process of ligand binding. FPs are considered as labels neutral for living organisms.

GFP becomes fluorescent after adopting the native structure, when autocatalytical reaction between three residues (Ser65-Tyr66-Gly67) occurs resulting in formation of chromophore absorbing and emitting in the visible range. Chromophore is a persistent post-translational modification of polypeptide chain, which might affect refolding process in comparison with protein, which has never created chromophore (*de novo* folding). This dissertation presents results of research on GFP mutant with enhanced fluorescence properties (EGFP) comparing *de novo* folding with the refolding process described in literature. For the first time I have proposed the possible model of progress of refolding, *de novo* folding including an aggregation associated and, moreover, the probable interpretation of each phase of the process. Using multiple spectroscopic methods I have discovered that chromophore presence affects state of unfolded protein at pH 1 and the protein folding as well as aggregation. Refolding and *de novo* folding are both multistage, parallel-pathway processes and the main difference is observed at the beginning of folding, including the measurement dead time. Only in refolding some phases are related exclusively to the changes in chromophore fluorescence showing alterations in its environment. Both refolding and *de novo* folding reveal proline isomerization. It takes around 20 min. to acquire 90% of chromophore fluorescence intensity for refolding, while for *de novo* folding this time is extended to around an hour, due to chromophore formation. Aggregation accompanying folding differs in the dynamic of the process and in its efficiency – when it is related to refolding, the nucleation phase is shorter but efficiency is lower in comparison to the results obtained during *de novo* folding. Therefore, it is of significance for the results of research using GFP as fluorescent marker, if the applied protein has the matured chromophore or not.

Additional experiments on aggregation accompanying *de novo* folding, performed using analytical ultracentrifugation, showed that fluorescent is only EGFP monomer with proper protein structure and matured chromophore. However, absorption measurements indicated presence of non-fluorescent aggregated forms and monomers without functional chromophore. Therefore, it is important in experiments using GFP fluorescence as an

indicator to consider the possibility of presence of non-fluorescent protein forms, which may cause misinterpretation of the results received, if neglected.

Investigating the reason for the lack of fluorescence in S65T/G67A-GFP mutant in comparison to S65T-GFP we got involved in 30-year discussion about mechanisms of chromophore formation. Mass spectrometry results indicated that oxidation reaction was suppressed in S65T/G67A-GFP. Thus, for GFP family with S65T mutation the chromophore formation follows the sequence: cyclisation –dehydration – oxidation.

GFP is also suspected to generate or scavenge reactive oxygen species (ROS). To confirm these properties we used Electron Paramagnetic Resonance (EPR) measurements. Experiment results showed that in contrast to some FPs, e.g. KillerRed, EGFP is not capable to generate, but it can scavenge ROS. We indicated that this property might be caused by chromophore damage by free radicals produced under intensive illumination. Therefore, GFP may fulfil photoprotective role, as it is also suggested for FPs from corals. ROS scavenging efficiency for EGFP was comparable to that observed for BSA and papain, which are known for protective function against ROS.

Moreover, I have created new EGFP single-cysteine mutants: C48S-EGFP and C70S-EGFP. Mutations did not alter either absorption or fluorescence properties of chromophore, however, C48S-EGFP is more stable and has shorter chromophore maturation time in comparison to C70S-EGFP. Thus, C48S-EGFP is more suitable for studying the properties of FPs using e.g. spin labels binding exclusively with cysteines and characterising the label's nearest surroundings.