Docking-guided identification of protein hosts for GFP chromophore-like ligands†

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Synthetic analogs of the Green Fluorescent Protein (GFP) chromophore emerge as promising fluorogenic dyes for labeling in living systems. Here, we report the computational identification of protein hosts capable of binding to and enhancing fluorescence of GFP chromophore derivatives. Automated docking of GFP-like chromophores to over 3000 crystal structures of Escherichia coli proteins available in the Protein Data Bank allowed the identification of a set of candidate proteins. Four of these proteins were tested experimentally in vitro for binding with the GFP chromophore and its red-shifted Kaede chromophore-like analogs. Two proteins were found to possess sub-micromolar affinity for some Kaede-like chromophores and activate fluorescence of these fluorogens.

Introduction

The synthetic analogue of the chromophore of green fluorescent protein (GFP) is about three orders of magnitude dimmer in solution than its natural archetype buried within the protein β-barrel. It is established that this drastic decrease in fluorescence quantum yield is a result of the photoinduced isomerisation of its core.1 The ability to fluoresce could be restored by hindering the isomerisation by means of metal ion complexation,2 tight binding to specifically designed RNA aptamers,3 binding to protein hosts,4,5 introduction of chemical conformational lock,6 or aggregation-induced emission in the solid state.7 The fluorescence increase upon binding to protein hosts is of particular interest as it could be used in imaging and other cell biology applications.1,8 However, the only known protein host4 capable of restoring GFP chromophore fluorescence belongs to the albumin family, abundant plasma proteins with unique ability to bind a wide variety of hydrophobic small-molecule ligands.9 This protein was found as a result of high-throughput screening in vitro, laborious experiments that require significant quantities of tested compounds.

An alternative approach – molecular docking – is a potent tool for the identification of interacting pairs of small molecules and protein hosts, and even for the computational design of such pairs from the known binding mode.10 Unfortunately, judging by the vast experimental evidence on the role of the molecular properties of small-molecule ligands in receptor–ligand interaction,11 the GFP chromophore lacks sufficient size, flexibility, and the number of hydrogen-bonding groups to be a good candidate for specific protein binding. Therefore, very accurate docking with full-atom scoring functions and flexible receptor geometry would be necessary to identify protein hosts for the GFP chromophore, increasing computational costs of such a screening. These costs can be lowered if chromophores with auxochromic groups in the core are considered, as these groups increase ligand quality and simplify the search of high-affinity protein–chromophore pairs. But nevertheless, the tendency of docking algorithms to converge towards the lowest interface energy often leaves the specific requirements necessary for the fluorescence recovery out of the equation.

Here, we performed automated docking of GFP-like chromophores to available crystal structures of Escherichia coli proteins and tested selected candidate proteins experimentally.

Experimental section

Cloning, protein expression and purification

Genes corresponding to PDB IDs 3HO2, 1DOS, and 2QRY were PCR-amplified (primers are listed in the ESI† Table S4) from E. coli XL1 Blue genomic DNA and cloned into the pBAD expression vector by self-assembly cloning.12

For protein expression E. coli strain BW25113 was used. Expression was induced by 0.1% arabinose at 25 °C for 16 h. Cells were centrifuged, sonicated in PBS pH 7.4 (10 mM phosphate
buffer, 137 mM NaCl, 2.7 mM KCl) with the PMSF protease inhibitor (Thermo Fisher Scientific), and then purified using TALON metal-affinity resin (Clontech). Finally, the protein was dialyzed against 10 000× volume of PBS pH 7.4 supplied with 5 mM β-mercaptoethanol.

Spectral properties

A Varian Cary 100 UV/VIS Spectrophotometer and a Varian Cary Eclipse Fluorescence spectrophotometer were used to measure absorption and excitation–emission spectra.

Fluorescence quantum yields (QYs) of the 3HO2–A12H protein–fluorogen pair (20 μM protein, 0.5 μM fluorophore, PBS pH 7.4, 23 °C) were determined by direct comparison with EGFP (QY = 0.6). For unbound chromophores, compound 4c from ref. 13 was used as a reference for QY measurements. The solubility of the chromophores was determined by absorption measurements.

Molecular docking and DFT studies

The B3LYP DFT functional has already been successfully applied to related GFP-based systems,14–16 and it was selected for present studies. Ligands were optimized at the B3LYP/def2-SVP level of theory17 using ORCA 3.0.3 software,18 all obtained geometries had no imaginary frequencies. Time-dependent density functional theory (TDDFT) studies, with Tamm–Dancoff approximation, were performed at the PBE0/def2-TZVP (COSMO: H2O) level of theory. The zeroth order regular approximation (ZORA) in conjunction with the corresponding basis set 19 was used for TDDFT calculations to take into account relativistic effects (for A12 and A12H series). RIJ/COSX approximation20 was used in order to significantly speed up geometry optimization, computing of analytical Hessian,21 and TD-DFT studies.22

The ligand and receptor files for docking with rigid protein geometry were prepared using AutoDockTools version 1.5.6. The bounding box for the docking region was calculated automatically using PyMol. Docking was performed using AutoDock Vina as described23 with exhaustiveness set to 20 and the maximum number of collected distinct binding modes set to 20.

Docking with flexible geometry of the ligand-binding pocket was performed using Rosetta software (weekly release 2015.05.57576). The crystal structures, PDB IDs: 1PVS and 3HO2 (the residue A163 was manually changed into the native C) were preimemized using relax application25 with the following flags: -flip_HNQ -no_optH -relaxconstrain_relax_to_start_coords -nstruct 50 -ex1 -ex2 -use_input_sc. The output structures with the lowest total score were used for further calculations. For ligand docking we used the RosettaLigand docking algorithm.26 Three rounds of docking were made for each protein–ligand pair. During the first round, 5000 structures were generated using the transform mover with move_distance = 5 Å and angle = 360°. Fifty structures with the lowest interface delta score among 2000 structures with the best total score were selected for the second round to generate another 5000 structures (100 from each of the selected) decreasing the move_distance to 1 Å and the angle to 45°. The third round of ligand docking was performed using the best 50 structures of the second round and the transform mover with move_distance = 0.2 Å and angle = 5°.

In vitro fluorescence bead assay

Purified proteins (1 mg ml⁻¹ solution) were immobilized on a 1/100 volume of TALON metal-affinity beads, washed with PBS pH 7.4 and placed in 200 μl chambers with cover glass at the bottom. The chromophores were added to a final concentration of 10 μM from EtOH stock solution. A Leica AF6000 fluorescence microscope (Wetzlar, Germany) was used for imaging with GFP (excitation BP470/40, emission BP525/50) and TxRed (excitation BP560/40, emission BP645/75) filter sets.

Results and discussion

Since the high quantum yield of fluorescence of the GFP chromophore is determined mainly by the sterical hindrance of its isomerization, we hypothesized that placing emphasis on the geometrical match between the GFP chromophore and the potential binding pocket within a protein is important. To separate affinity optimization and geometrical matching of the core GFP chromophore, we devised a two-step docking approach. First, we selected potential hosts for the GFP chromophore. Second, we docked a wider library of GFP-like chromophores to these protein hosts.

We applied molecular docking of the GFP chromophore to a number (over 3000) of crystal structures of E. coli proteins available in the Protein Data Bank with resolution better than 2.0 Å and the chain length shorter than 500 amino acid residues. We chose one of the fastest available docking tools24 and performed docking in a blind and automatic manner: ligands were stripped out from PDB files and only the first protein chain was examined. This massive docking analysis allowed us to select top-scoring structures that can putatively bind the GFP-like chromophore (Table S1, ESI†). We then performed molecular docking of larger GFP-like chromophore derivatives – so called Kaede-like chromophores27 – against top 500 structures from previous round of docking. As a result, chromophores A5, A12, A12H, A24, A26, A27, and A28 (ESI† Methods) were selected for further tests. From the candidate protein list, we succeeded in cloning and expression of 4 proteins with a high (Table 1) GFP or Kaede docking rank (here and further named by PDB IDs) 3HO2 (β-ketoacyl-acyl-carrier-protein synthase II), 1DOS (fructose-bisphosphate aldolase), 2QRY (thiamine binding protein), and 1PVS (3-methyladenine glycosylase II).

Purified proteins were immobilized on beads and examined under a fluorescence microscope. However, no fluorescence increase was observed upon incubation with the 100 μM GFP chromophore. At the same time, the addition of chromophores A5, A12, A12H, and A24 resulted in a considerable increase of bead fluorescence (Fig. 1).

Table 1 Docking results. The rank corresponds to the position in the list of proteins, sorted by their Autodock Vina docking score. GFP, Kaede – types of chromophores

<table>
<thead>
<tr>
<th>PDB IDs</th>
<th>GFP rank</th>
<th>GFP score</th>
<th>Kaede rank</th>
<th>Kaede score</th>
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<td>1PVS</td>
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</table>
Next, we studied spectral changes upon mixing of the chosen chromophore–protein pairs in solution. Some of the tested pairs exhibited submicromolar $K_d$ (Fig. 2A). Interestingly, in the case of binding of chromophores A5, A12, and A24 to 3HO2 protein in solution, the fluorescence intensity increase was minor, less than 2-fold, under protein or chromophore saturating conditions (Fig. 2A). Thus, the corresponding signal increase in the bead-based assay can probably be attributed to chromophore accumulation uncoupled from the increase in fluorescence quantum yield. In contrast, A12H and A12 showed a strong fluorescence increase upon binding to 3HO2 and 1PVS in solution (Fig. 2B) demonstrating truly fluorogenic behavior. Indeed, the binding of the chromophore to the protein host resulted in two orders of magnitude increase in fluorescence quantum yield. In some cases (A12 and A12H) in protein complexes we might have observed the emission of the anionic form. Deprotonation of the hydroxyl group in all the compounds resulted in ~80 nm red-shifts of absorption and emission maxima. The process is characterized by the $pK_a$ of 7–8, and it can take place in water buffer solutions with neutral pH.28,29 This suggests that in some cases (A12 and A12H) in protein complexes we might have observed the emission of the anionic form.

In order to further investigate this point, we performed time-dependent density functional theory (TDDFT) calculations on the free chromophores. According to these studies, the main peak in the experimental absorption spectrum of the A12H chromophore (Fig. 2C), at 465 nm, along with the 485 nm excitation peak in the complex with the 3HO2 protein host can be attributed to the S0 → S1 excitations of different anionic species (Fig. S3, ESI†). Similar red-shifting of anionic species in comparison to neutral species was found for all the studied chromophores (see ESI†, Fig. S4–S6).

In order to address the observed strong fluorescence increase in pairs 3HO2/A12H and 1PVS/A12H despite the inconsistencies with ranking in rigid-residue docking, we performed docking analysis with flexible residues and the full-atom Rosetta scoring function. High-resolution docking resulted in highly converged docking poses of the A12H chromophore within a tight binding
Similar to antibody-based Fluorogen Activating Proteins and tags in heterologous expression models, e.g. and their mutant variants can potentially be used as fluorescent bacterial proteins found in this work or analogous protein hosts for the experimental verification of various docking protocols. Second, upon binding to a protein host provides an easy and reliable way of visualizing native endogenous proteins, e.g. provide a way to visualize native endogenous proteins. Finally, this method can be further applied in various fields. First, the fluorescence increase of fluorogenic dyes absorption-shifting tag (Y-FAST).33.34 This work was supported by the Russian Foundation for Basic Research (grant 15-33-20518-mol-a-ved) and by the Molecular and Cell Biology program of Russian Academy of Sciences. Experiments were partially carried out using the equipment provided by IBCH core facility (CKP IBCH).

**References**


**Conclusions**

For the first time we applied molecular docking for the identification of the candidate protein host for binding of GFP chromophore analogs. Two out of four proteins that were cloned and tested in vitro (3HO2, 1PVS) showed fluorogenic behavior and sub-micromolar $K_d$ towards Kaede-like chromophores.

We believe that the present approach could be further applied in various fields. First, the fluorescence increase of fluorogenic dyes upon binding to a protein host provides an easy and reliable way of the experimental verification of various docking protocols. Second, bacterial proteins found in this work or analogous protein hosts and their mutant variants can potentially be used as fluorescent tags in heterologous expression models, e.g., mammalian cells, similar to antibody-based Fluorogen Activating Proteins and the recently developed Yellow Fluorescence-Activating and absorption-Shifting Tag (Y-FAST). Finally, this method can provide a way to visualize native endogenous proteins, e.g., 3HO2 in living cells.