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Ca^{2+}-regulated Photoprotein Obelin as N-terminal Partner in the Fusion Proteins

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Ca^{2+}-regulated photoproteins genetically fused with biospecific polipeptides have been extensively used in intracellular Ca^{2+} measurements and in the development of binding assays. Gene fusions to aequorin have been limited to its N-terminus, since as previous studies indicated the protein loses bioluminescent activity upon modification of its C-terminus. To investigate this in regard to another photoprotein – obelin (OL) the one was elongated at its C-terminus with tyrosine (Y) and then fused with green fluorescent protein Clytia gregaria (cgreGFP) through the flexible 31 aa linker. Both proteins (OL-Y and OL-cgreGFP) were isolated and investigated. The OL-Y was found to form a stable photoprotein comlex, possessing 75 % of WT-OL bioluminescent activity. OL-cgreGFP activity preserves 46 % of WT-OL activity and demonstrates an effective resonance energy transfer, where OL-partner and cgreGFP-partner are energy donor and acceptor respectively. Thus, it was shown that the labels on the base of Ca^{2+}-regulated photoprotein obelin may be obtained by fusing with biospecific polypeptides regardless its termini.

Keywords: obelin, C-end fusing, cgreGFP.

Introduction

Ca^{2+}-regulated photoproteins of marine coelenterates, e.g. aequorin from the jellyfish *Aequorea* (Shimomura et al., 1962) and obelin from the hydroid *Obelia* (Morin et al., 1971) are stable complexes consisting of single-chain apoprotein (about 20 kDa) and pre-oxidyzed substrate – peroxycoelenterazine, tightly but non-covalently immobilized in hydrophobic cavity (Head et al., Liu et al., 2000). The proteins are characterized by high sequence homology and contain three ‘‘EF-hand’’ Ca^{2+}-binding consensus sequences like other Ca^{2+}-binding proteins. The binding of Ca ions initiates conformational changes in a molecule resulting in peroxycoelenterazine decarboxylation and emission of visible light (λ_{max} = 470-490 nm). Some photoprotein cDNAs were cloned and expressed in *E. coli* cells (e.g. Prasher et al., 1985; Inouye et al., 1993; Illarionov et al., 1995), making the recombinant apoproteins available. The apophotoproteins are turned into photoproteins by simple incubation with synthetic coelenterazine at reducing conditions without any folding problems. Due to the high quantum yield

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and low background it is possible to discriminate as small as 10^{-18} mol of photoprotein. That is why the recombinant Ca^{2+}-regulated photoproteins (aequorin and obelin, first of all) are widely used as effective reporters in cells and *in vitro* assays (see for ref. Blinks et al., 1982; Lewis et al., 2000). To become a specific label, photoproteins are conjugated with proper bio-specific molecule chemically or by engineering of genetically fused proteins (chimeras). Many photoproteins cloned for to date (aequorin, obelins, clytin) contain C-end Pro. As was reported for native aequorin, any modifications on the C-terminus proline (deletion, substitution or addition) result in almost total loss of its bioluminescent activity or stability (Nomura et al., 1991; Watkins et al., 1993). Since modification of the N-terminus of aequorin and obelin has no any adverse effect on luminescence activity, the gene fusions had been made only through their N-termini (Zenno et al.; Frank et al., 1996; Waud et al., 2001; Baubet et al., 2000; Gorokhovatsky at al., 2004). At the same time, the functionality of some polypeptides lies in their C-terminal region, as well as the recognition of some antibodies occurs only at the epitope on polypeptide C-terminus. Hence, the development of C-terminus-extended derivatives of obelin would essentially broaden its analytical applications.

The goal of our work was to investigate Ca^{2+}-regulated photoprotein obelin as N-terminal label in the fusion proteins. The main background for the study was the fact that mitrocomin – photoprotein of the hydromedusan *Halistaura (Mitrocoma)*, contains additional tyrosine after C-end proline (Fagan et al., 1993). This is the reason why tyrosine was taken for the shortest prolongation of obelin C-terminus. Then OL-Y was fused with N-terminus of a green fluorescent protein from *Clytia gregaria* (*cgre*GFP). In the paper we describe the construction of expression vectors encoding the proteins of interest, their expression in *E. coli* cells, purification and properties.

Materials and methods

1. Reagents

QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, QIAquick PCR purification kit were from QIAGEN (Germany); phosphatase CIP and T4 ligase were from BioLabs (USA); Pfu-Turbo polymerase, *E. coli* cells strains XL1-Blue and BL21-Gold were from Stratagene (USA).

Coelenterazine was obtained from PJK GmbH (Kleinblittersdorf, Germany). Recombinant obelin of wild type (WT-OL) and *cgre*GFP of high purity were obtained as described in (Illarionov et al., 2000; Markova et al., 2002; 2010).

All other chemicals were from standard sources and were of reagent grade or better.

Oligonucleotides were purchased from Syntol (Russia):

1. 5’-ACTCCATGGCTTCAAAATACG-CAGTTA-3’
2. 5’-TGTCTCGAGCTTAGTAGGGAAC TCCGTTGCC-3’
3. 5’-TACAATACCGGTGCCACCATAG GGAACTCCGTTGCC-3’
4. 5’-CCGGTGGTTCAGGCGGTTCT-GGTTCAG-3’
5. 5’-GAGGTTCAGGCTCTGGTGGC-TCAGGAT-3’
6. 5’-ACCAGAGCCTGAACCTCCTGAA CCAGAACCGCCTGAACCA-3’
7. 5’- CTGGAGGCTCAGGTTCCGGGG-ATCTGGTCA-3’
8. 5’-TATGACCAGATCCACCGGAACC-TGAGCCTCCAGATCCTGAGCC-3’

2. Plasmid construction

The DNA fragment encoding obelin with tyrosine on its C-terminus (OL-Y) was amplified by PCR with specific primers No.1 and No.2

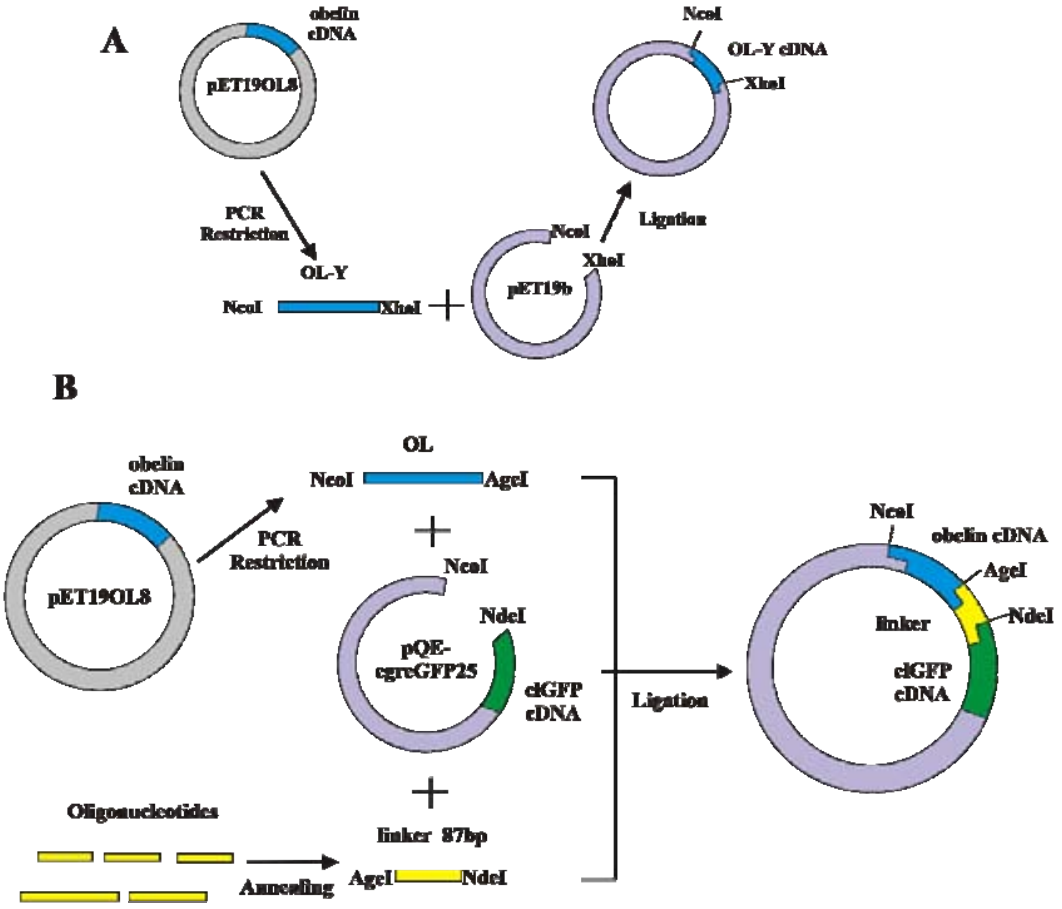

Fig. 1. Engineering of OL-Y (A) and OL-*cgre*GFP (B) proteins

containing NcoI and XhoI sites. The pET19-OL8 expression plasmid carrying the *Obelia longissima* wild-type apo-obelin gene (Markova et al., 2001) was used as a template. The PCR products were cloned into NcoI/XhoI sites of pET19b vector (Fig. 1).

For OL-*cgre*GFP chimera cloning, the DNA fragment encoding obelin with additional amino acids on its C-terminus – tyrosine and two glycines (OL-Age) – was amplified by PCR with specific primers No.1 and No.3 containing NcoI and AgeI sites. The above mentioned pET19-OL8 expression plasmid was used as a template. The PCR products were digested with NcoI/AgeI. The DNA fragments encoding flexible linker for OL-*cgre*GFP chimera were obtained by annealing of five complimentary oligonucleotides (No. 4-8), which were phosphorylated with polynucleotide kinase (Sibenzyme, Russia) beforehand. The annealing was carried out in 10 мМ Tris-HCl, pH 7.5, 2 мМ $MgCl_2$, 50мМ NaCl buffer by heating up to 82°C followed by slow cooling to 23°C. The DNA fragment encoding flexible linker had AgeI sticky end on its 5'-end and NdeI sticky end on its 3'-end. As a vector for OL-*cgre*GFP construction we used dephosphorylated and digested with NdeI and NcoI pQE-cgreGFP25 plasmid carrying the *Clytia gregaria* wild-type GFP gene (Markova et al., 2010) (Fig. 1).

The plasmids harboring the target insertion segments were verified by DNA sequencing. We used *E. coli* BL21Gold cells for expression of OL-Y, and XL1-Blue cells for expression of OL-*cgre*GFP construction.

3. Proteins expression and purification

The transformed *E. coli* cells were cultivated with vigorous shaking at 37ºC in LB medium containing ampicillin (200 mg/l) and induced with 1 mM IPTG when the culture reached an OD_{600} of 0.5–0.6. After addition of IPTG, the cultivation of OL-Y expressed cells was continued for 3 h at the same conditions. OL-*cgre*GFP expressed cells were cultivated for 24 h with vigorous shaking at room temperature and than stored for more 24 h at 8ºC.

In both cases the *E. coli* cells were harvested by centrifugation, the pellet resuspended in 20 mM Tris-HCl pH 7.0 (1:5, w/v), and disrupted by sonication (20 s x 6) at 0 ºC. Then the mixtures were centrifugated. The fusion apo-OL-Y was purified from inclusion bodies and charged with coelenterazine as previously reported for recombinant WT obelins (Illarionov et al., 2000; Markova et al., 2002).

The pellet inclusion bodies of fusion apo-OL-*cgre*GFP was washed with the following solutions: (a) 0.1 M NaCl, 20 mM TrisHCl pH 7.0 (x2); (b) 1 % Triton X-100, 20 mM TrisHCl pH 7.0 (x2); and (c) 5mM $CaCl_2$, 20 mM TrisHCl pH 7.0 (x1) to remove contaminating substances. All the washing procedures were performed with centrifugation. The final pellet was resuspended in 20 mM TrisHCl pH 7.0, contained 6 M urea, kept at 4ºC for 2 h with stirring and then centrifuged. Supernatant (the solution of chlorine color, illuminating under UV-irradiation) was diluted 5-fold with 20 mM TrisHCl pH 7.0, contained 10 mM DTT, 5 mM EDTA and coelenterazine (1.1 molar excess to protein) and kept at 4ºC overnight. Then the solution was concentrated and the recombinant protein was separated from the other components by gel-filtration on D-Salt Column (Pierce, USA) equilibrated and eluted with 20 mM TrisHCl pH 7.0, 5 mM EDTA.

4. Protein analysis

The electrophoresis was performed according to Laemmli, using a 12.5 % polyacrylamide gel. Protein molecular weight calibration mixture (Amersham Bioscience, USA): phosphorylase *b* (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

Protein concentration was measured with the BioRad DC protein assay kit.

5. Bioluminescence measurement

The bioluminescence intensity was measured with a BLM 8801 photometer (SCTB ‘‘Nauka’’, Russia) by rapid injection of 0.2 ml of 100 mM $CaCl_2$, 100 mM Tris–HCl, pH 8.8, into the photometer cell containing 0.5 ml of 5 mM EDTA, 100 mM TrisHCl, pH 8.8, and the protein aliquot.

6. Spectral measurements

The absorption spectra were obtained with an UVIKON 943 Double Beam UV/VIS spectrophotometer (Kontron Instruments, Italy). The bioluminescence and fluorescence spectra were measured with an AMINCO spectrofluorimeter (Thermo Spectronic, USA). The emission spectra were corrected with the computer program supplied with the instrument. All spectral measurements were carried out at room temperature.

The bioluminescence spectra were measured in 1 mM EDTA, 50mM Bis-Tris propane buffer pH 7.0. The bioluminescence was initiated by injection of $CaCl_2$ solution in the same buffer. The concentration of free Ca^{2+} was around 0.5 mM in order to provide an approximately constant light level during the spectral scans. The calcium concentration was estimated with the MAXICHELATOR program.

Results and discussion

The obelin C-end extension was performed with minimal fragment – one amino acid (tyrosin), and also with the whole protein approximating obelin molar mass – green fluorescent protein of *Clytia gregaria* (cgreGFP) through 31 aa flexible linker of almost 110 Å (Fig. 2). The linker contains amino acids lacking large bulky hydrophobic side chain: $GGT(GGS)_2GS(GGSGS)_3GGSGH$, that provides its flexibility. Obelin molecule is a compact globule with a diameter of 50 Å (pdb code 1EL4), *cgre*GFP is a barrel with a height of 45 Å (pdb code 1HPW). As a result, the linker length and flexibility allow independent orientation of fused molecules. Simultaneously, it can provide the energy transfer according to BRET mechanism, where the photoprotein moiety is a donor and GFP – acceptor (Ward, 1979). The scheme of fusion proteins engineering is presented in the Fig. 1. The final construction OL-Y was obtained by ligation of OL-Y and vector pET19b, both carrying NcoI/XhoI restriction sites. The final construction OL-*cgre*GFP was obtained by ligation of three DNA fragments – OL (NcoI/AgeI), annealed linker (AgeI/NdeI) and vector pQE*cgre*GFP25 (NcoI/NdeI). The *E. coli* cells transformed with corresponding plasmids were used to express the proteins of interest.

SDS-PAGE analyses indicate that the expression level of both proteins – apo-OL-Y and apo-OL-*cgre*GFP is rather high and most of the proteins are accumulated in inclusion bodies (Fig. 3). To produce a highly-purified OL-Y we applied the method developed for WT obelin. The method was as follows: apoprotein (inclusion bodies pellet dissolved in 6 M urea) was purified chromatographically under denaturating conditions (Fig. 3A, lane 5), charged with coelenterazine (overnight) and then additionally purified on Mono Q-column. This chromatography step allowed separation of obelin from apoprotein that has not been charged with coelenterazine. The yield of activated OL-Y was 68 %.The final product was homogeneous according to SDS-PAGE (Fig. 3A, lane 6).

The apo-OL-*cgre*GFP inclusion bodies after thorough washing were dissolved in 6M urea solution. The sample was diluted (1/10, v/v) with 20 mM TrisHCl pH 7.0, 10 mM DTT, 5 mM EDTA, incubated with coelenterazine at 8 ºC overnight and purified by gel-filtration. The final protein was of 80-90 % purity (Fig. 3B,

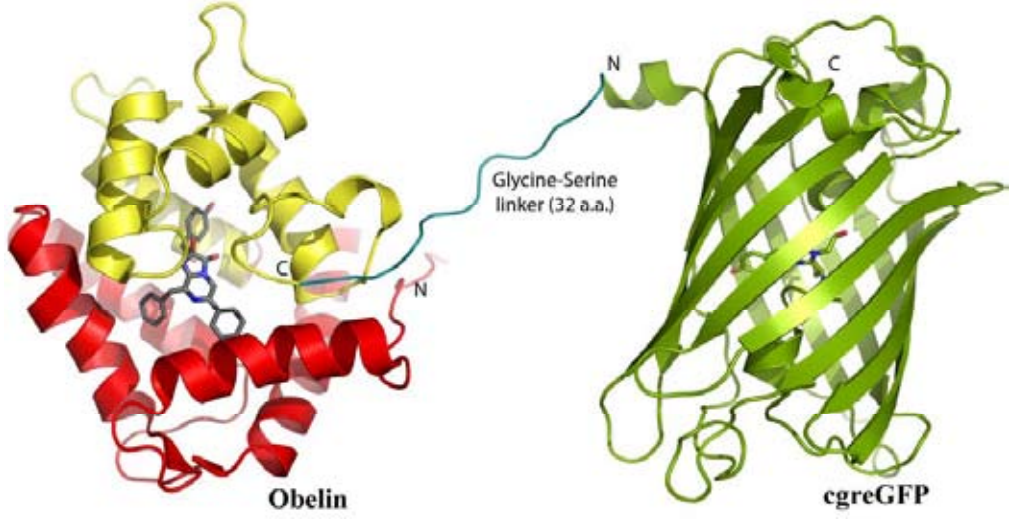

Fig. 2. Schematic view of OL-cgreGFP fusion protein. Peroxycoelenterazine and GFP chromophore are displayed by stick models in the center of molecules

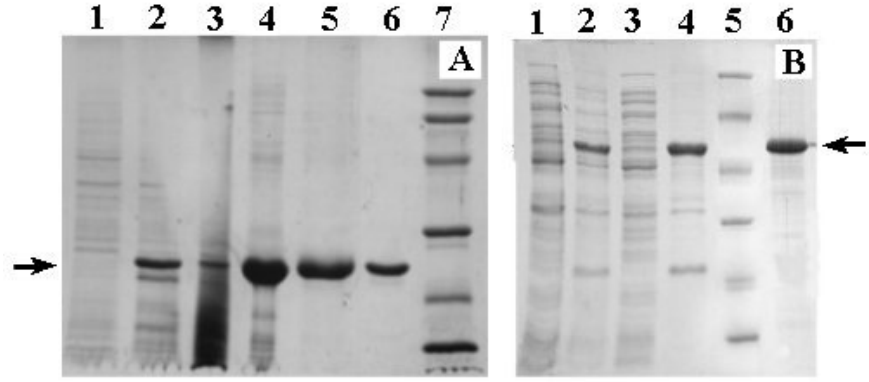

Fig. 3. SDS-PAGE analysis of OL-Y (panel A) and OL-cgreGFP (panel B) at purification. Lanes 1 and 2, whole-cell lysates before and after IPTG induction respectively. Lane 3, cytosolic proteins. Lane 4, 6M urea extracts of inclusion bodies. Lane 5 (A), apo-OL-Y after purification on DEAE-Sepharose FF column. Lane 6 (A), OL-Y after purification on Mono Q column. Lane 6 (B), final OL-cgreGFP solution. Lanes 7 (A) and 5 (B) – standard proteins (see Materials and Methods). Arrows show recombinant proteins bands

lane 6) and is a polypeptide with Mr about 50 kDa that is in good agreement with the molecular weight calculated from amino acid sequence of the designed protein – 50.9 kDa. Unfortunately, we failed to separate chromatographically the charged and uncharged OL-*cgre*GFP due to their high unspecific sorption on MonoQ column. The protein solution was of chlorine color and illuminated under UV-irradiation.

Of special interest was the process of the OL-Y apoprotein activation (formation of the complex apoprotein-peroxycoelenterazine). Fig. 4 presents the kinetics of OL-Y activation in comparison with WT apoobelin. Note, the reaction of chimera activation is almost by an order slower and reaches the plateau in 12-14 h. Thus, apoobelin C-end prolongation with one amino acid does not prevent it to form an active photoprotein complex, but decelerates the process.

Bioluminescent properties of chimeric proteins as opposed to those of WT obelin are summarized in the Table. One can see that the addition of one amino acid to obelin C-end had no dramatic effect on bioluminescent activity which retained 75 % of the WT obelin activity. In the case of OL-*cgre*GFP, the lower activity can be attributed to the lack of chromatographic separation stage after coelenterazine activation, so the sample may be the mixture of charged and uncharged proteins.

The level of Ca^{2+}-independent bioluminescence (due to spontaneous photoprotein decomposition), normalized to protein (specific $I_{Ca\text{-}ind}$) may serve as the indicator of stability of the apoprotein-peroxycoelenterazine complex. According to this parameter, OL-Y is 4 times less stable than WT obelin. At the same time, Ca^{2+}-independent bioluminescence is million times lower, than Ca-triggered bioluminescence (for WT obelin the ratio of Ca^{2+}-independent to Ca^{2+}-triggered luminescence equals 7 log units).

There are no differences between the absorption spectra of OL-Y and WT obelin: both display maxima at 278 nm (polypeptide absorption) and at 460 nm (absorption of peroxycoelenterazine, immobilized into protein cavity) (Fig. 5A). The *cgre*GFP spectrum (Fig. 5B) displays maxima at 278 nm and 485 nm, with the 278 nm/445 nm absorption ratio 1.6. It should be noted that this ratio reflects the level of *cgre*GFP maturation (spontaneous fluorophore formation inside the molecule). The fusion protein OL-*cgre*GFP spectrum presents

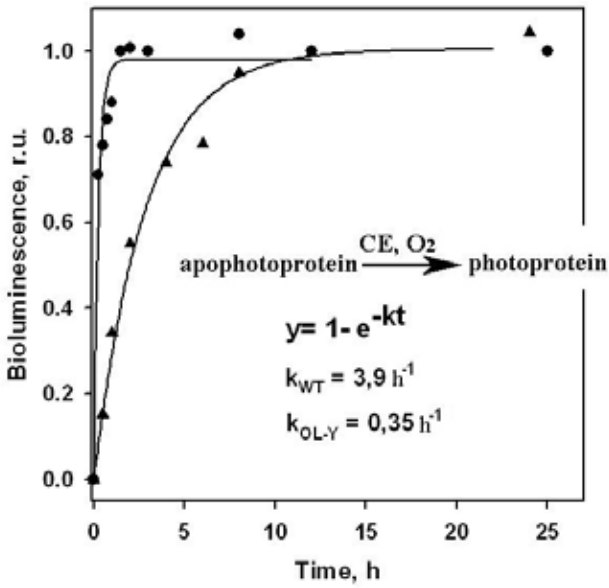

Fig. 4. Activating kinetics of WT obelin (●) and OL-Y (▲). Axis Y shows bioluminescence signal ratio I_t/I_{max}, where I_{max} – the bioluminescent signal after 24 h of activation. The lines are fits according to an exponential growth model

Table. Bioluminescent properties of fusion proteins as opposed to those of WT obelin

Protein	Decay rate, s^{-1}	Bioluminescence yield[a] (%)	Bioluminescence λmax/shoulder (nm)	Specific $I_{Ca\text{-}ind}$ (r.u./mg)	$I_{Ca\text{-}ind}/I_{Ca}$ [b] x 10^{-7}
WT-OL	6.3	100	485/400	1.8	1.1
OL-Y	5.8	75	485/400	7.2	12
OL-*cgre*GFP	4.8	46	500/485/400	not detected[c]	not detected

[a]Total bioluminescence emission relative to WT obelin
[b] $I_{Ca\text{-}ind}/I_{Ca}$ ratio of Ca^{2+}-independent to Ca^2-triggered bioluminescence
[c]The protein obtained was of 80-90 % purity

the superposition of WT obelin and *cgre*GFP spectra with maxima at 278 nm and 485 (Fig. 5C), with the 278 nm/485 nm absorption ratio 0.18. Since these two proteins in fusion are spaced with 31 aa linker and hardly affect each other we may consider them as equimolar solution of obelin and *cgre*GFP. The spectrum of equimolar mixture of OL-Y and maturated *cgre*GFP is presented in the same Fig. 5C (dashed line) and it shows that the 278 nm/485 nm absorption ratio amounts to 0.56. Thus, in chimera, the maturity of *cgre*GFP moiety constitutes only 38 %. The result is not surprising since the fusion protein was isolated from inclusion bodies. For aequorin GFP, Cubitt and co-authors (1995) reported that fluorophore results from autocatalytic cyclization of the polypeptide backbone between residues Ser^{65} and Gly^{67} and oxidation of the α-β bond of Tyr^{66}. In *cgre*GFP the residues are Ser^{68}, Tyr^{69} and Gly^{70} (Markova et al., 2010). In our case the high expression level of recombinant protein causes its "packing" into insoluble inclusion bodies (Fig. 3). As a consequence, insolubility prevents post-translational fluorophore maturation of the major part of the expressed *cgre*GFP module.

The OL-Y Ca^{2+}-triggered bioluminescence spectrum (Fig. 6A, line 1) is broad and like WT obelin bioluminescence spectrum displays

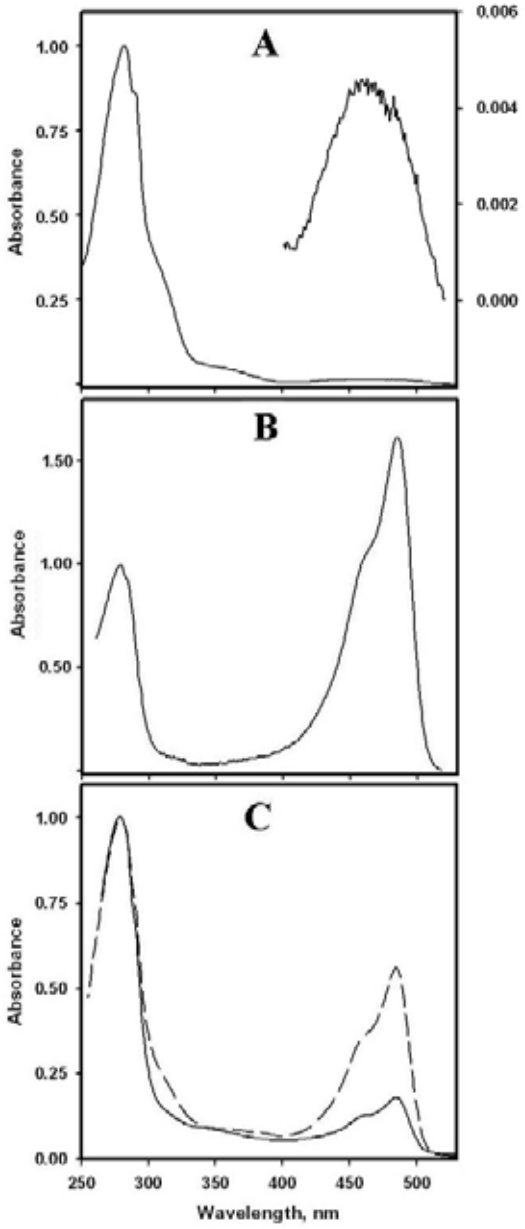

Fig. 5. Normalized absorption spectra of Ob-Y (A), *cgre*GFP (B), and OL-*cgre*GFP (C). Dashed line – the absorption spectrum of 1:1 molar mixture of *cgre*GFP:Ob-Y

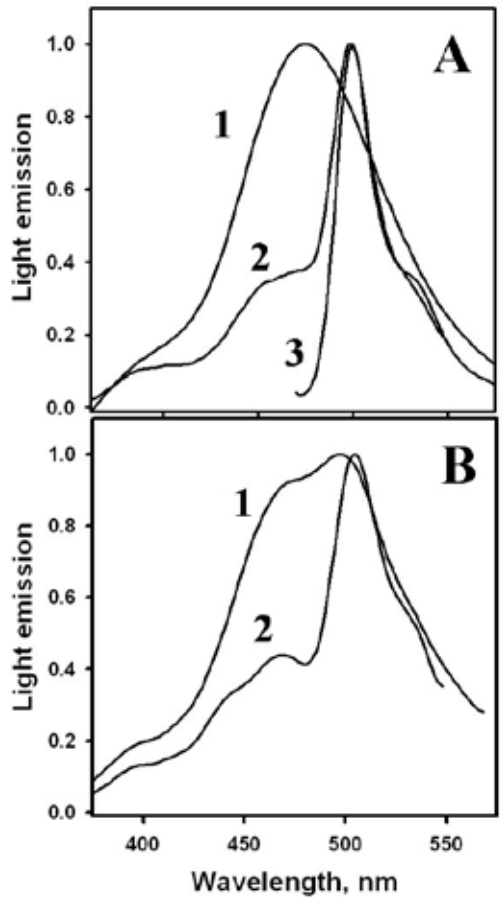

Fig. 6. Panel A: Normalized bioluminescence spectra of OL-Y (line 1), OL-*cgre*GFP (line 2, 2.9 μM solution), and fluorescence spectrum of *cgre*GFP (line 3, λ_{ex}=465 nm); Panel B: Normalized bioluminescence spectra of equimolar mixture of WT-OL and *cgre*GFP in 2.4 μM solution(line 1) and 24 μM solution (line 2)

a peak at 485 nm with a shoulder at 390 nm. The chimera OL-cgreGFP Ca^{2+}-triggered bioluminescence spectrum (Fig. 6A, line 2) has the maximum at 500 nm that is virtually identical to the fluorescence spectrum of cgreGFP (Fig. 6A, line 3), and shoulders at 485 nm and 390 nm (belonging to obelin bioluminescence spectrum). The observed green bioluminescence of OL-*cgre*GFP occurs due to reradiation of obelin module (energy donor) to fluorophore of *cgre*GFP module (energy acceptor) according to BRET mechanism (Ward, 1979). The ratio of luminescence intensities at wavelengths of the acceptor and donor emission maxima (I_{500}/I_{480}, in our case) correlates with BRET efficiency and account for 2.6 (protein concentration in the experiment was 2.9 μM). The efficiency of the process is known to depend on overlapping of donor luminescence and acceptor absorption spectra and on the distance between the proteins. As one can see obelin bioluminescence spectrum (Fig. 6A, line 1) totally overlaps *cgre*GFP absorption spectrum (Fig. 5B). The linker between modules holds them near each other at a distance of almost 110 Å (calculated as a sum of 31 distances between neighboring α-carbon atoms in polypeptide chain). Actually the linker flexibility provides any mutual orientations of donor and acceptor, and, as a consequence, the inter-modules distance may be much shorter and approach 100 Å required for effective BRET.

For comparison, we carried out the energy transfer experiments in solution of equimolar mixtures WT-OL and maturated *cgre*GFP (Fig. 6B). At 2.4 μM proteins concentration (Fig. 6B, line 1) the ratio I_{500}/I_{480} equals 1.1, so the energy transfer efficiency is essentially lower than observed for chimera OL-*cgre*GFP 2.9 μM solution. Meanwhile, the close to chimera ratio I_{500}/I_{480} of 2.4 was observed in a 24 μM mixture of OL and *cgre*GFP (Fig. 6B, line 2), the concentration when the trivial energy transfer takes place.

Thus, it was found that obelin extended with tyrosine at C-end (OL-Y) forms an active and stable photoprotein complex, not too much differing from WT obelin. Fusing with the *cgre*GFP gives the chimera of moderate bioluminescence activity demonstrating effective energy transfer to GFP. The results clearly show: i) there is a principle possibility to construct active obelin derivatives fused through C-end with the other polipeptides, and, ii) the bioluminescent properties of final chimeras will substantially depend on the polipeptides' nature.

It should be noted that similar results were obtained by S. Deo and co-workers (2001) at studying cysteine-free aequorin mutant (more stable that WT-aequorin) fused through C-end with octa- and pentapeptide. The authors found that contrary to WT aequorin, the cysteine-free mutant retains practically all of its bioluminescent activity after extending the C-terminal region.

The gene-fusing technique has become an increasingly useful tool in biomedical research. Fusion proteins are constructed, when it is necessary: to increase cellular stability and biological activity of functional proteins, to increase solubility of a protein expressed in bacterial cells or to facilitate its purification, to select and produce antibodies etc. The construction of bifunctional molecules containing bio-specific and reporter modules creates effective markers to be applied in both *in vivo* and *in vitro* assays. Ca^{2+}-regulated photoproteins seem to be promising reporter modules providing high sensitivity of the assay under development. The perspective to obtain obelin labels by its fusing with bio-specific polipeptides regardless to its termini likely considerably broadens the application of the photoprotein as an effective analytical tool.

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Ca^{2+}-регулируемый фотопротеин обелин как N-концевой партнер при конструировании слитых белков

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Ca^{2+}-регулируемые фотопротеины, генетически слитые с биоспецифическими полипептидами, широко используются как репортеры для внутриклеточного мониторинга Ca^{2+}, а также в анализе in vitro. Ранее было показано, что модификации фотопротеина акворина по С-концу приводят к потере его биолюминесцентной активности, поэтому химерные белки получали только присоединением полипептида к его N-концу. В представленной работе исследуется возможность получения слитых белков по С-концу другого фотопротеина – обелина. При этом вначале обелин (OL) генетически удлинили на одну аминокислоту – тирозин (Y), а затем получили обелин, слитый через длинный гибкий линкер (31 аминокислота) с зеленым флуоресцентным белком медузы Clytia gregaria (cgreGFP). Оба белка (OL-Y и OL-cgreGFP) были выделены, их основные свойства изучены. Показано, что OL-Y образует устойчивый фотопротеиновый

комплекс, биолюминесцентная активность которого составляет 75 % активности обелина дикого типа (WT-OL). Активность OL-cgreGFP составляет 46 % от активности WT-OL. При запуске биолюминесцентной реакции ионами кальция наблюдается эффективный резонансный перенос энергии, в котором обелиновая часть химерного белка является донором, а cgreGFP – акцептором энергии. Таким образом установлено, что репортерные молекулы на основе Ca^{2+}-регулируемого фотопротеина обелина могут быть получены генетическим слиянием с биоспецифическими полипептидами по его С-концу.

Ключевые слова: обелин, генетическое удлинение по С-концу, cgreGFP.