ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 1, pp. 77-83. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 1, pp. 91-99.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-183, December 10, 2006.

Engineering, Expression, Purification, and Physicochemical Characterization of a Chimeric Protein, Full-Length Cytochrome b_5–Green Fluorescence Protein ($HMWb_5$–EGFP)

A. V. Yantsevich, I. N. Harnostai, O. P. Lukashevich, A. A. Gilep, and S. A. Usanov*

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Kuprevicha 5, 220141 Minsk, Belarus; fax: 375 (172) 637-274; E-mail: usanov@iboch.bas-net.by

Received July 10, 2006
Revision received September 11, 2006

Abstract—In this article we report on construction of expression vector, heterologous expression in *Escherichia coli*, isolation, purification, and physicochemical characterization of an artificial chimeric protein $HMWb_5$–EGFP consisting of full-length cytochrome b_5 ($HMWb_5$) and green fluorescence protein (EGFP) from *Aequorea*. Optimization of expression conditions yielded an expression level up to 1500 nmol of chimeric protein per liter of culture. Recombinant chimeric protein $HMWb_5$–EGFP was purified from cell membranes by using metal-affinity chromatography. It possesses physicochemical, spectral, and fluorescence properties of cytochrome b_5 and EGFP indicating independent character of protein folding in frames of the chimera. It is shown that there is a fluorescent resonance energy transfer in $HMWb_5$–EGFP between the fluorophore of EGFP and heme of cytochrome b_5, and the distance between chromophores in the chimeric protein is approximately 67.3 Å. The chimeric protein was shown to exist as a monomer in aqueous solution in the presence of detergents. The data indicate that the $HMWb_5$–EGFP designed in the present work is a very promising model for modern biosensors and an instrument to study protein–protein interactions.

DOI: 10.1134/S0006297907010099

Key words: microsomal cytochrome b_5, green fluorescence protein (EGFP) from *Aequorea*, expression in *Escherichia coli*, affinity chromatography, purification

Cytochrome b_5 is a membrane-bound heme protein that consists of two domains [1, 2]. The N-terminal hydrophilic domain with molecular weight 12 kD contains heme and preserves the spectral properties of the full-length cytochrome b_5. The C-terminal domain of cytochrome b_5 with molecular weight 4 kD is nonpolar and serves to interact with membrane [3]. Cytochrome b_5 participates in the desaturase reaction of fatty acid oxidation [4], reduction of methemoglobin in erythrocytes [5], hydroxylation of N-acetylneuraminic acid [6], as well as in monooxygenase reactions catalyzed by microsomal cytochromes P450s [7-10]. Cytochrome b_5 exerts a stimulating effect on a number of important reactions catalyzed by cytochrome P450, including detoxification of xenobiotics and biosynthesis of steroid hormones, prostaglandins, leukotrienes, bile acids, and vitamins of the D3 group. However, the molecular mechanism of modulation of cytochrome P450-dependent reactions by cytochrome b_5 is still unclear. Therefore, it is of great importance to understand the intrinsic mechanism of interaction of cytochrome b_5 with cytochrome P450 and NADPH-cytochrome P450 reductase.

The hydrophobic domain of cytochrome b_5 plays a critical role in functional interaction with cytochrome P450 [11, 12]. However, the tertiary structure of full-length cytochrome b_5, the location of the hydrophobic domain relative to hydrophilic one and its functional role are still unknown.

Until recently, the main method used to study protein–protein interactions between cytochrome b_5 and cytochromes P450s has been the registration of changes in spectra of cytochrome P450 during complex formation and experimental determination of enzyme activity [13-15]. However, fluorescently labeled (dansyl chloride) cytochrome b_5 was efficiently used to analyze its interac-

Abbreviations: $HMWb_5$–EGFP) high molecular weight cytochrome b_5 and enhanced green fluorescent protein; IPTG) isopropyl β-D-thiogalactopyranoside.

* To whom correspondence should be addressed.

tion with lipid vesicles and bilayer [16]. Selectively labeled cytochrome b_5 was also effectively used to study its interaction with cytochrome P450cam [17, 18], since in this case the protein interaction does not cause changes in the absorption spectra.

Recently, fluorescence proteins from *Aequorea* have been wide used in studies of protein translocation in cells, analysis of protein–protein interactions, and selective identification of the domains responsible for interaction [19-21]. In the present work, to create a prototype of modern biosensors, we engineered an artificial chimeric protein based on full-length cytochrome b_5 (134 amino acid residues) and one of the mutant forms of the green fluorescence protein (EGFP, 238 amino acid residues) to study protein–protein interactions between cytochrome b_5 and cytochrome P450, as well as to study conformational changes in cytochrome b_5 as a result of its interaction with partner proteins.

EGFP has some advantages for use as a fluorescent marker compared to other forms of fluorescent protein (photostability, high quantum yield, large molar extinction coefficient, temperature stability) [22-25]. In the present work we constructed an expression vector for heterologous expression of chimeric protein in *Escherichia coli*, purified it using metal-affinity chromatography, studied its physicochemical properties, and analyzed some of its conformational characteristics. We also describe prospects for using the chimeric protein in analysis of protein–protein interactions and behavior of the protein in phospholipid bilayers.

MATERIALS AND METHODS

In the present work we used the following reagents and chemicals: restriction enzymes and other molecular biology reagents from New England Biolabs (USA), tryptone, peptone, and yeast extract from Difco (USA), and other chemicals from Sigma (USA).

Engineering of genetic construct pCWori+HMWb$_5$–EGFP. The gene of green fluorescence protein in plasmid pEGFP-C1 (Clontech Laboratories) was amplified by using primers 5′-GTCGTCGACATGGTGAGCAAGGGCGAGGAG-3′ and 5′-ACCGTCGACTGCAGAATTCGAAGC-3′ to insert restriction site *Sal*I, and then cloned into vector pCWori+HMWb$_5$ [26]. The resulting genetic construct (Fig. 1) contained the full-length gene of rat liver microsomal cytochrome b_5 and the gene coding green fluorescence protein connected with the sequence -Ser-Thr-, which is introduced during insertion of the *Sal*I site.

Thus, in the chimeric protein (hereafter designated as HMWb$_5$–EGFP, high molecular weight cytochrome b_5–enhanced green fluorescent protein) the N-terminal sequence is constituted by cytochrome b_5, while the C-terminal sequence is that of EGFP. Moreover, the C-terminal sequence of the gene coding the chimeric protein contains the sequence coding the cluster of six histidine residues (6-His cluster) that allows purifying the recombinant protein by using metal-affinity chromatography.

Heterologous expression and purification of the chimeric protein. Plasmid DNA pCWori+HMWb$_5$–EGFP was used to transform *E. coli* DH5α cells. Optimization of the expression conditions for HMWb$_5$–EGFP revealed that its expression at temperatures higher than 24°C results in formation of insoluble aggregates (inclusion bodies) from which purification of the chimeric protein in non-

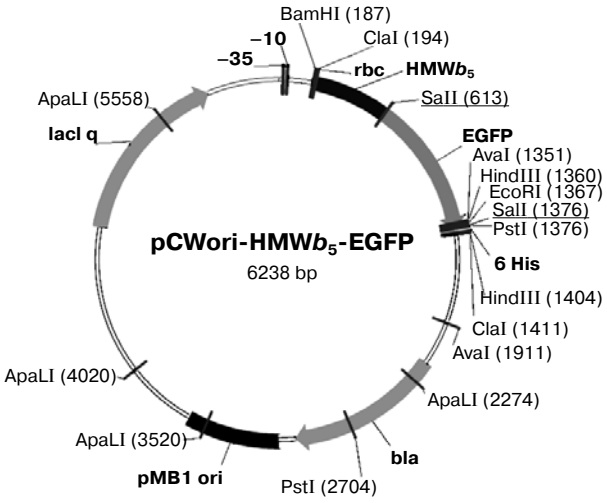

Fig. 1. Scheme of the construct of the expression plasmid pCWori-HMWb_5–EGFP.

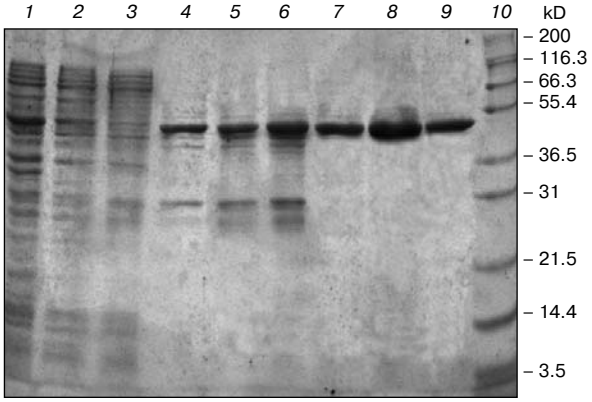

Fig. 2. SDS-PAGE of chimeric protein HMWb$_5$–EGFP at different steps during its purification: *1*) cell lysate after protein expression; *2*) supernatant after solubilization of membrane with sodium cholate and centrifugation; *3*) supernatant after solubilization of membrane, centrifugation, and application on the column with Ni-IDA-agarose 6B; *4-6*) fractions eluted from the Ni-IDA-agarose 6B; *7-9*) eluted fractions after chromatography on DEAE-cellulose; *10*) protein standard (molecular weight of the protein standard is shown on the right).

denaturing conditions proved to be impossible. To express native HMWb$_5$–EGFP, the optimal conditions proved to be the following: 22°C; 140 rpm; 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG); incubation time 24 h from the moment of induction of expression.

Overnight culture of *E. coli* DH5α cells in Luria–Bertani medium (5 ml) was added to 500 ml of Terrific Broth medium, and the culture was incubated for 5 h at 37°C, 180 rpm (to reach optical density 0.8 at 600 nm). Then the temperature was decreased to 22°C, frequency of mixing was decrease to 140 rpm, and the culture was further incubated for 24 h. The cells were collected by centrifugation (3000*g*) and suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.3 M NaCl, 0.1% sodium cholate (w/v), and 20% glycerol (v/v) (5 ml/g of wet cells).

To prepare membranes, cells were disrupted by ultrasonic treatment using a UZDN-2T ultrasonic disintegrator (22 kHz). Membranes were solubilized by adding drops of 10% sodium cholate to final concentration 1% and intensive mixing, and then the sample was further mixed at 4°C during 1 h. Non-solubilized particles were removed by centrifugation (10,000*g*), and the supernatant was applied to a column containing Ni-IDA-Sepharose 6B. The column was washed with five column volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 30 mM imidazole, 0.3 M NaCl, 0.1% sodium cholate, and 20% glycerol. The protein was eluted with 50 mM Tris-HCl buffer, pH 7.4, containing 250 mM imidazole, 0.3 M NaCl, 0.1% sodium cholate, and 20% glycerol. Eluted fractions were analyzed for content of the chimeric protein using SDS-PAGE in 12% gel [27] (Fig. 2), pooled, diluted with buffer to NaCl concentration 0.14 M, and applied to the column with DEAE-cellulose. The chimeric protein was eluted with 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M NaCl, 0.1% sodium cholate, and 20% glycerol.

Spectroscopy. Protein absorbance spectra were recorded using a Specord M40 spectrophotometer (Karl Zeiss, Germany). Luminescence spectra were recorded using a Solar CM2230 spectrofluorimeter (Belarus). All measurements were carried out at 25°C.

Determination of the efficiency of singlet–singlet energy transfer. The efficiency of singlet–singlet energy transfer was determined by direct measurement of the lifetime of the excited singlet state, since this method is free from the effect of trivial processes that imitate energy transfer [28]. The efficiency of energy transfer is calculated according to the formula:

$$E = 1 - \frac{\tau_{D.A}}{\tau_D} , \qquad (1)$$

where E is efficiency of singlet–singlet energy transfer; $\tau_{D.A}$ is fluorescence lifetime for HMWb$_5$–EGFP; τ_D is fluorescence lifetime for EGFP.

Calculation of intramolecular distances. The distance between fluorophore EGFP and heme of cytochrome b_5 was determined according to the Forster equation:

$$R = \sqrt[6]{\frac{R_0^6}{E} - R_0^6} , \qquad (2)$$

where R is the distance between fluorophore EGFP and the heme of cytochrome b_5; R_0 is characteristic distance of energy transfer (Forster distance); E is the efficiency of singlet–singlet energy transfer.

The characteristic distance of energy transfer was calculated by the following method:

$$R_0 = 0.2108(J\kappa^2\Phi_D^0 n^{-4})^{-\frac{1}{6}} , \qquad (3)$$

where J is the overlap integral of the emission spectrum of EGFP and absorption spectrum of cytochrome b_5; κ^2 is orientation factor; Φ_D^0 is quantum yield of fluorescence of EGFP in the absence of cytochrome b_5; n is index of refraction of the medium separating fluorophore and chromophore (donor and acceptor proteins).

The orientation factor was taken to be equal to 2/3 since, as earlier suggested, both proteins possess degrees of freedom relative to each other, i.e. undergo rotation relative to the sequence connecting the two protein globules. As a rule, the error in that parameter does not result in serious error in determination of the distance between chromophores. Relative quantum yield of fluorescence was determined using fluorescein in 0.1 M sodium hydroxide as a standard (quantum yield 0.93). As the refractive index of the medium, we used the value 1.39, typical for protein water solutions.

The overlap integral of emission spectrum of EGFP and absorption spectrum of cytochrome b_5 was calculated according to the formula:

$$\int_0^\infty I_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda , \qquad (4)$$

where $I_D(\lambda)$ is intensity of donor fluorescence fixed in such a way that $\int_0^\infty I_D(\lambda)d\lambda = 1$; $\varepsilon_A(\lambda)$ is coefficient of molar extinction for cytochrome b_5 at wavelength λ (nm) expressed in dm^3·mol^{-1}·cm^{-1}; λ is wavelength, nm.

The coefficients of molar extinction were calculated from the absorption spectra of cytochrome b_5.

Determination of fluorescence anisotropy. Steady state fluorescence anisotropy of EGFP and HMWb$_5$–EGFP were calculated from the fluorescence spectra obtained at four different positions of excitation polarizer and emission polarizer (λ_{ex} = 490 nm, λ_{em} = 510-514 nm). The data of five measurements were averaged. Protein concentration in 50 mM sodium phosphate buffer, pH 7.4, containing 0.1% sodium cholate was 200 nM. All measurements were carried out at 25°C.

The OriginPro 7.5 (OriginLab Corporation) program was used for calculation and presentation of data.

RESULTS AND DISCUSSION

The use of EGFP (the size of which is 1.5 greater than that of the studied protein, cytochrome b_5) as a fluorescent probe is explained by a number of advantages of this fluorescent protein: the studied protein is not affected by the relatively severe conditions used for protein modification with the label and the protein surface and conformation is not dramatically changed, which is extremely important when studying protein–protein interactions. On one hand, cytochrome b_5 is one of the best partners for constructing and expression of chimeric proteins, it has a chromophore group, and it facilitates folding of co-expressed proteins [29, 30]. For example, the construction of a chimeric protein based on the bacterial cytochrome b_{562} and green fluorescence protein to model the processes taking part in photosynthetic light collecting complexes has been described [24]. Under the conditions described in this paper, the chimeric construct is expressed in *E. coli* much less effectively, there is formation of inclusion bodies, and the final chimeric protein contains together with holo-cytochrome b_{562} significant amount of apo-form of the heme protein, which makes it necessary to separate these forms via an additional step of ion-exchange chromatography.

Recently, we succeeded in using cytochrome b_5 as a leading protein to construct and express in bacteria chimeric protein consisting of cytochrome b_5 and NADPH-cytochrome P450 reductase [29, 30], in which not only spectral, but also electron-transfer properties of both proteins are retained. The linker connecting globules of two proteins plays an important role in engineering of chimeric proteins; this role may be played by the hydrophobic fragment of cytochrome b_5. In the present work, we tried to apply a similar approach to construct a unique artificial chimeric protein consisting of full-length cytochrome b_5 and EGFP.

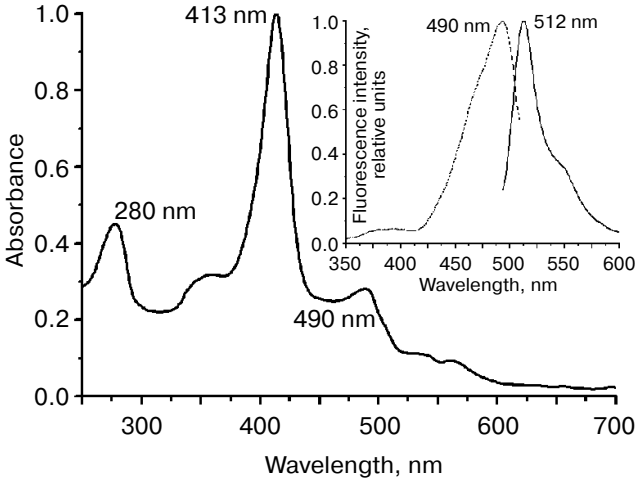

Fig. 3. Absorption spectrum of $HMWb_5$–EGFP (10 mM sodium phosphate buffer, pH 7.4, 0.1% sodium cholate; normalized). Inset: fluorescence excitation (dashed line) and emission spectra (solid line) of $HMWb_5$–EGFP in the visible region (10 mM sodium phosphate buffer, pH 7.4, 0.1% sodium cholate; spectra are normalized).

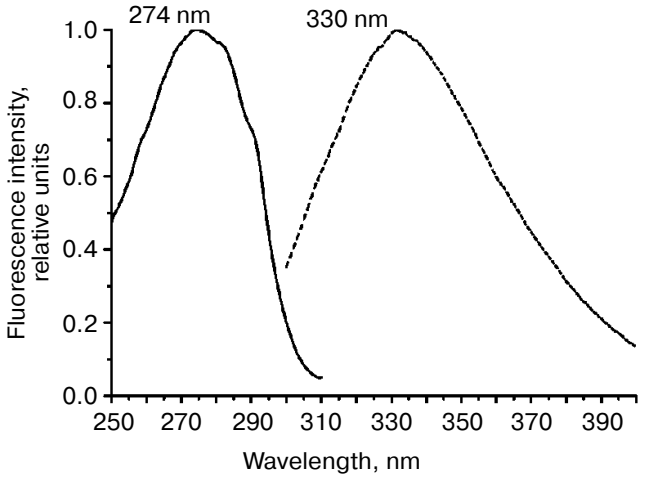

Fig. 4. Fluorescence excitation (solid line) and emission spectra (dashed line) of $HMWb_5$–EGFP in the ultraviolet region (10 mM sodium phosphate buffer, pH 7.4, 0.1% sodium cholate; spectra are normalized).

Optimization of conditions for heterologous expression of chimeric protein (time, temperature, concentration of IPTG) yielded expression level of the chimeric protein up to 1500 nmol per liter of culture (12-15 g of wet cells). The distinctive feature of this expression system is that together with formation of the correctly folded chimeric protein, the bacterial cell contained some amount of non-folded chimeric protein, the amount of which significantly depended on the incubation temperature during expression.

The insertion of additional histidine residues into the C-terminal sequence of the chimeric protein allowed us to use metal-affinity chromatography, which significantly simplifies the purification procedure practically to one step. This method of purification of $HMWb_5$–EGFP produced homogeneous preparations of the chimeric protein with yield up to 300 nmol from 1 liter of culture. An additional step of ion-exchange chromatography on DEAE-Sepharose 6B was included due to the presence in the fractions obtained after metal-affinity chromatography of free EGFP, which appeared to be formed due to proteolytic modification of $HMWb_5$–EGFP.

The absorption spectrum of highly purified $HMWb_5$–EGFP shown in Fig. 3 represents the sum of the absorption spectra of individual proteins (EGFP and $HMWb_5$) in the chimeric protein. As follows from Fig. 3, the absolute absorption spectrum of the chimeric protein is very similar to that of oxidized microsomal cytochrome b_5 [31] and has an absorption maximum in the Soret

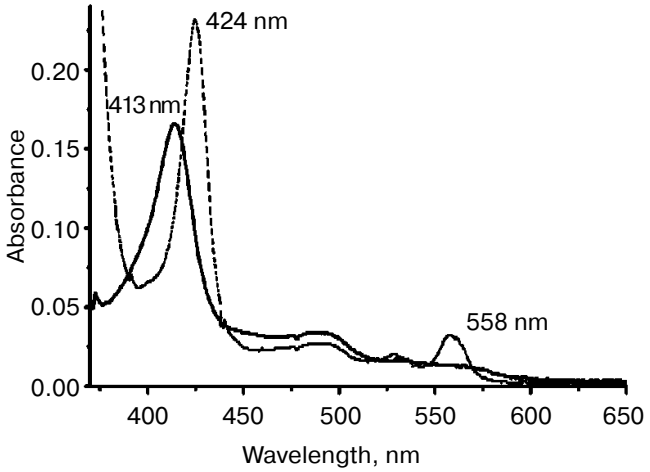

Fig. 5. Absorption spectra of oxidized (solid line) and sodium dithionite-reduced (dashed line) forms of HMWb$_5$–EGFP (10 mM sodium phosphate buffer, pH 7.4, 0.1% sodium cholate, protein concentration 1 μM).

region at 413 nm as well as two small peaks in the long wavelength region of the spectrum. Also, one can see in the spectrum of the chimeric protein the components characteristic for green fluorescence protein (maximum at 490 nm). These data indicate the absence of dramatic changes in conformation of cytochrome b_5 in the chimeric protein induced by EGFP and confirm our conclusion on correct independent folding of the individual protein components of the chimeric protein.

The maxima and the shape of fluorescence spectra of EGFP in HMWb$_5$–EGFP (Fig. 3) are also practically unchanged. There are some changes in kinetic parameters of fluorescence of EGFP. The fluorescence spectra of HMWb$_5$–EGFP in the ultraviolet region (Fig. 4) have an evident maximum of excitation (characteristic for the excitation spectra of tyrosine fluorescence with maximum at 274 nm) and maximum emission (characteristic for fluorescence spectra of tryptophan with maximum at 330 nm), which is explained by non-radiative energy transfer of tyrosine excitation to tryptophan. Fluorescence of the chimeric protein in the UV region is mainly connected with tryptophan residues that are located mostly in the hydrophobic domain of HMWb$_5$. The maximum in the emission spectrum at 330 nm indicates that tryptophan residues are in a hydrophobic environment (the same maximum is characteristic also for cytochrome b_5). Based on these results, we conclude that conformation of HMWb$_5$ within HMWb$_5$–EGFP does not undergo significant changes, and the EGFP globule does not induce steric hindrances in the position of the hydrophobic domain of HMWb$_5$.

After isolation and purification of HMWb$_5$–EGFP, the HMWb$_5$ is in the oxidized state with maximum of absorption in the Soret region at 413 nm, although originally cytochrome b_5 in the chimeric protein is expressed in bacteria in the reduced state (results of difference spectroscopy of recombinant *E. coli* cells expressing HMWb$_5$–EGFP). Chemical reduction of cytochrome b_5 with sodium dithionite shifts the absorption maximum to 424 nm (Fig. 5), confirming the retention of the main functional characteristics of the protein.

The kinetics of fluorescence decay for HMWb$_5$–EGFP and EGFP are shown in Fig. 6. The time of fluorescence quenching for HMWb$_5$–EGFP is 2.62 nsec, while for EGFP it is 2.89 nsec, which indicates singlet–singlet energy transfer. The overlapping of the absorption spectrum of cytochrome b_5 and fluorescence emission of EGFP is presented in Fig. 7. The efficiency of

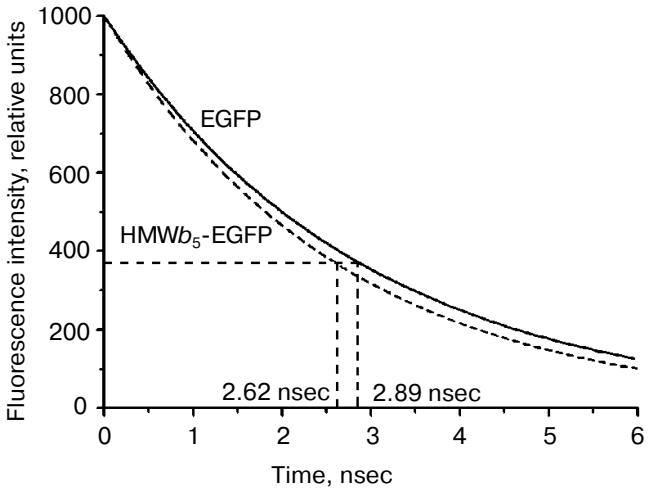

Fig. 6. Kinetics of fluorescence decay for EGFP and HMWb$_5$–EGFP (10 mM sodium phosphate buffer, 0.1% sodium cholate).

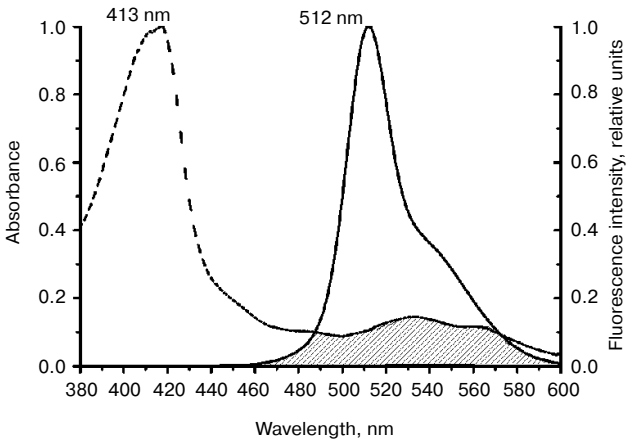

Fig. 7. Overlapping (filled area, spectra are normalized) of emission spectrum of EGFP (solid line) and absorption spectrum of cytochrome b_5 (dashed line).

Calculated distance between chromophores in $HMWb_5$–EGFP

κ^2	Φ_D^0	n	$J \cdot 10^{14}$, $M^{-1} \cdot cm^{-1} \cdot nm^4$	R_0, Å	$\tau_{D.A}$, nsec	τ_D, nsec	E	R, Å
2/3	0.6	1.39	10.3	46.1	2.62	2.89	0.093	67.3

the energy transfer calculated according to Eq. (1) is 0.0934.

The characteristic distance of energy transfer (Forster distance) calculated according to Eq. (4) is 46.1 Å. This distance is very similar to that reported in [24] for the cytochrome b_{562}–green fluorescence protein couple. The dependence of efficiency of energy transfer for $HMWb_5$–EGFP from the distance between chromophores indicates the possibility to precisely determine the distances between heme and fluorophore under conformational changes from 21 to 72 Å ($R_0 = 1 \pm 0.55$). The results of such calculations are presented in the table.

The distance between heme of cytochrome b_5 and fluorophore of EGFP determined according to Eq. (2) is 67.3 Å. By using the tertiary structure of green fluorescence protein (1gfl) and the hydrophilic domain of cytochrome b_5 (1aw3) and the WebLab Viewer Pro3.7 program, we calculated the following parameters: distance between Ala1 and Tyr66 in EGFP (26 Å) and distance between iron atom of heme of cytochrome b_5 and Leu94 in EGFP (16 Å) (Fig. 8).

There is an amino acid sequence (about 40 amino acids) between residue Leu94 of cytochrome b_5 and Ala1 of EGFP. Taking into account the fact that isoelectric point of both proteins is at acidic pH, and at pH 7.4 both proteins forming the chimeric protein carry net negative charge, the globules of EGFP and cytochrome b_5 should be at maximal distance each from other (the result of electrostatic repulsion). We suggest that the secondary structure of the hydrophobic domain of cytochrome b_5 is mostly right-handed α-helix with pitch of about 1.5 Å. Taking into account the parameters of this type of secondary structure, we can calculate the length of the hydrophobic domain, which in this case is 60 Å. Based on these data, we can conclude that the hydrophobic domain of cytochrome b_5 is located not strictly normal to the surface of cytochrome b_5 globule, but in some different way. The available data, however, do not reveal the character of mutual location of the protein globule in the chimeric protein.

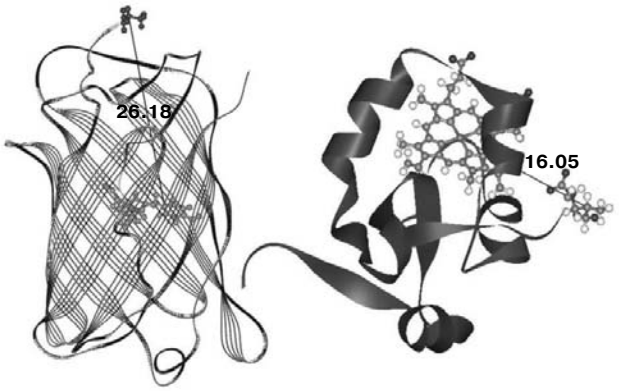

Fig. 8. Tertiary structures of EGFP and the hydrophilic domain of cytochrome b_5. Distances between Ala1 and Tyr66 (chromophore) in EGFP and the iron of heme and Leu94 in the hydrophobic domain of cytochrome b_5 are indicated.

To analyze the form and aggregation state of the chimeric protein in solution, we measured steady state anisotropy of emission of free EGFP and EGFP in $HMWb_5$–EGFP. From Perrin's equation for the steady state polarization, it is easy to calculate that for a 1.5-fold increase in the volume of a molecule the size of EGFP (i.e. for the volume of cytochrome b_5) and shape of the molecule close to globular, the steady state anisotropy will increase by 0.06. The steady state anisotropy of fluorescence determined for free EGFP was 0.3375, while in $HMWb_5$–EGFP it was 0.35, i.e. the difference is 0.0125. In the case when the protein globules are in more tight contact, we might expect more significant changes. These results indicate that the increase in fluorescence anisotropy is the result of acquiring by the chimeric protein molecule of dumb-bell shape, where the spacer between the two protein globules is the hydrophobic domain of cytochrome b_5. Moreover, the data of fluorescence anisotropy indicate that the chimeric protein does not have a tendency to form aggregates or oligomers, since in that case the changes in fluorescence anisotropy should be more than 0.06.

Thus, in the present work we constructed the model artificial chimeric protein $HMWb_5$–EGFP consisting of cytochrome b_5 and green fluorescence protein. The idea of engineering such a complex chimeric protein was to create a polyfunctional module construct consisting of a functional protein, cytochrome b_5, which participates in multiple electron transfer reactions, and the fluorescent marker protein EGFP, which from the changes in fluorescent properties can be used to follow the state of the basic component. Taking into account the fact that the heme of cytochrome b_5 is a powerful quencher of fluorescence, manipulation of the heme group (removal or binding of heme) might be used to establish $HMWb_5$–EGFP as a prototype biosensor.

Optimization of the conditions of heterologous expression of the chimeric protein in *E. coli* cells allowed

us to express in bacteria functional protein, the spectral properties of which are the sum of the optical properties of the constituent proteins (Fig. 3). A characteristic feature of the model chimeric protein is the ability of the two unrelated proteins in the chimera to undergo independent folding in the fused protein. Moreover, spectral properties and physicochemical characteristics of the individual proteins (cytochrome b_5 and EGFP) are retained in the chimeric protein, and cytochrome b_5 retains its ability for enzymatic (in bacteria) and chemical (sodium dithionite) reduction (Fig. 5). This indicates that in the chimeric fusion protein cytochrome b_5 and EGFP possesses a definite degree of freedom enough for correct folding of the molecule and interaction with partner proteins. Hence, the membrane-binding domain of cytochrome b_5 may perform the function of the spacer group giving the relative degree of mobility to the hydrophilic domain of cytochrome b_5. However, it should be noted that the hydrophobic domain of cytochrome b_5 in HMWb$_5$–EGFP does not significantly change its environment as compared to the native molecule.

The distinctive feature of engineered HMWb$_5$–EGFP is its ability for singlet–singlet energy transfer in the fusion complex from the EGFP fluorophore to the heme of cytochrome b_5. The distance between chromophores calculated by using Forster equation is 67.3 Å (table), which from our point of view reasonably reflects the location of chromophores in the tertiary structure of the chimeric protein. At the same time, in the chimeric protein there is no dramatic effect of EGFP and HMWb$_5$ globules on each other, and the chimeric protein molecule appears to have a dumb-bell shape.

Finally, an important finding is that the engineered chimeric protein under conditions of experiment exists in solution as monomer that does not tend to form oligomers, which is of a great importance in studies of protein–protein interactions.

Thus, in the present work we engineered a model artificial chimeric protein and described the promise of using this chimeric protein to create new generation biosensors and to study protein–protein interactions. Such studies of HMWb$_5$–EGFP with different forms of cytochrome P450 are now underway.

REFERENCES

1. Vergeres, G., and Waskell, L. (1995) *Biochimie*, **77**, 604-620.
2. Schenkman, J. B., and Jansson, I. (2003) *Pharmacol. Ther.*, **97**, 139-152.
3. Vergeres, G., Ramsden, J., and Waskell, L. (1995) *J. Biol. Chem.*, **270**, 3414-3422.
4. Kozutsumi, Y., Kawano, T., Yamakawa, T., and Suzuki, A. (1990) *J. Biochem.*, **108**, 704-706.
5. Hultquist, D. E., and Passon, P. G. (1971) *Nat. New Biol.*, **229**, 252-254.
6. Takematsu, H., Kawano, T., Koyama, S., Kozutsumi, Y., Suzuki, A., and Kawasaki, T. (1994) *J. Biochem.*, **115**, 381-386.
7. Hildebrandt, A., and Estabrook, R. W. (1971) *Arch. Biochem. Biophys.*, **143**, 66-79.
8. Hlavica, P. (1984) *Arch. Biochem. Biophys.*, **228**, 600-608.
9. Porter, T. D. (2002) *J. Biochem. Mol. Toxicol.*, **16**, 311-316.
10. Naffin-Olivos, J. L., and Auchus, R. J. (2006) *Biochemistry*, **45**, 755-762.
11. Chiang, J. Y. L. (1981) *Arch. Biochem. Biophys.*, **211**, 662-673.
12. Bendzko, P., Usanov, S. A., Pfeil, W., and Ruckpaul, K. (1982) *Acta Biol. Med. Germ.*, **41**, 1-8.
13. Tamburini, P. P., and Gibson, G. G. (1983) *J. Biol. Chem.*, **258**, 13444-13452.
14. Schenkman, J. B., and Jansson, I. (2006) *Meth. Mol. Biol.*, **320**, 11-18.
15. Szczesna-Skorupa, E., and Kemper, B. (2001) *J. Biol. Chem.*, **276**, 45009-45014.
16. Pugh, E. L., Kates, M., Szabo, A. G., and Krajcarski, D. T. (1989) *Biochim. Biophys. Acta*, **985**, 255-265.
17. Stayton, P., Fisher, M. T., and Sligar, S. G. (1988) *J. Biol. Chem.*, **263**, 13544-13548.
18. Stayton, P., Poulos, T. L., and Sligar, S. G. (1989) *Biochemistry*, **28**, 8201-8205.
19. Tsien, R. Y. (1998) *Ann. Rev. Biochem.*, **67**, 509-544.
20. Heim, R. (1999) *Meth. Enzymol.*, **302**, 408-423.
21. Miyawaki, A., and Tsien, R. Y. (2000) *Meth. Enzymol.*, **327**, 472-500.
22. Miyawaki, A., and Tsien, R. Y. (2000) *Meth. Enzymol.*, **327**, 472-500.
23. Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) *Science*, **273**, 1392-1395.
24. Takeda, S., Kamiya, N., Arai, R., and Nagamune, T. (2001) *Biochem. Biophys. Res. Commun.*, **289**, 299-304.
25. Nordeen, S. K., Housley, P. R., Wan, Y., and Day, R. N. (2001) *Meth. Mol. Biol.*, **176**, 179-199.
26. Gilep, A. A., Estabrook, R. W., and Usanov, S. A. (2003) *Biochemistry* (Moscow), **68**, 86-98.
27. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
28. Stryer, L., and Haugland, R. (1987) *Proc. Natl. Acad. Sci. USA*, **98**, 719-725.
29. Gilep, A. A., Guryev, O. L., Usanov, S. A., and Estabrook, R. W. (2001) *Arch. Biochem. Biophys.*, **390**, 215-221.
30. Gilep, A. A., Guryev, O. L., Usanov, S. A., and Estabrook, R. W. (2001) *Arch. Biochem. Biophys.*, **390**, 222-234.
31. Chudaev, M. V., Gilep, A. A., and Usanov, S. A. (2001) *Biochemistry* (Moscow), **66**, 677-681.