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Role of Conservative Residue Cys158 in the Formation of an Active Photoprotein Complex of Obelin

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Abstract—Using site directed mutagenesis, the conservative residue Cys158 of recombinant apoobelin was substituted for serine (C158S, S-mutant) or alanine (C158A, A-mutant). These point mutations resulted in significant changes in the apoobelin structure accompanied by slowing of photoprotein complex formation, decrease of its stability, and changing of its bioluminescence characteristics. The enzymatic properties of the photoprotein decreased in the series: wild-type protein > S-mutant > A-mutant. This is consistent with rank of nucleophilicity SH > OH > CH_3 of cysteine, serine, and alanine side chain functional groups, respectively. Possible mechanisms of the involvement of the apoobelin Cys158 SH-group in the formation of the enzyme–substrate complex are considered.

Key words: photoproteins, obelin, apoobelin mutants, bioluminescence

Understanding of the relationship between structure of proteins and their specialized functions has basic importance. Studies of bioluminescence may help to understand the particular role of proteins in mechanisms of conversion of the energy of chemical bonds into visible light. Ca^{2+}-activated photoproteins from marine coelenterates are suitable objects for such studies [1].

In the absence of calcium ions, Ca^{2+}-activated photoproteins form a stable enzyme–substrate complex consisting of a low molecular mass (~20 kD) apoprotein, a substrate (coelenterazine), and oxygen [2]. Photoproteins belong to a family of EF-hand proteins; the structure of their calcium-binding sites is similar to that of calmodulin, troponin C, myosin light chain, and parvalbumins [3, 4]. Coordination of calcium ions in EF-centers of the photoprotein causes conformational changes of the protein; this causes a bioluminescence reaction that follows the dioxetane mechanism [2]. The reaction results in formation of an oxidized product (coelenteramide), CO_2, and a quantum of light. After removal of the reaction products, the photoprotein can be regenerated (charged) in the presence of chelators (EDTA, EGTA), synthetic coelenterazine, an SH-reducing agent (dithiothreitol or 2-mercaptoethanol), and oxygen [5]. However, the mechanisms of the formation of the active photoprotein complex and the bioluminescent reaction catalyzed by Ca^{2+}-activated photoprotein complex still require further investigation. An approach to the elucidation of these mechanisms is the identification of particular amino acid residues and segments of the protein molecule responsible for the formation of the active photoprotein complex: apoprotein–coelenterazine–oxygen.

Recombinant Ca^{2+}-activated photoprotein obelin is a suitable object for such studies [6]. The apoobelin molecule is a single polypeptide chain of 195 amino acid residues; it contains a hydrophobic site for substrate binding and three EF-hand structures for Ca^{2+} binding [7]. Recent selective chemical modification revealed that only one of five cysteine residues of apoobelin is essential for coordination of the substrate on the apoprotein during the formation of the enzyme–substrate complex [8]. Modification of only one SH-group resulted in almost total inability of apoobelin to form the photoprotein complex [8]. However, it remains unclear which cysteine residue is involved.

In the present study, we investigated the formation of an active photoprotein complex by recombinant apoobelin and its two mutants with substitutions of highly conservative cysteine 158 for serine (C158S, S-mutant) or alanine (C158A, A-mutant).

MATERIALS AND METHODS

The following chemicals were used in the study: synthetic coelenterazine from Molecular Probes Inc.

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(USA); ampicillin, Tris (Tris-(hydroxymethyl)-aminomethane), urea, and SDS from Sigma Chemical Co. (USA); peptone and yeast extract from Gibco BRL (England); dithiothreitol from Aldrich Chemical Co. (USA); Bio-Gel P-4 and reagents for electrophoresis (acrylamide, bis-acrylamide, TEMED, ammonium persulfate) from BioRad (USA); EDTA (sodium salt), bovine serum albumin, Coomassie Brilliant Blue G-250 from Serva (Germany); isopropyl-β-D-thiogalactopyranoside (IPTG) from SibEnzym (Novosibirsk, Russia).

IPTG-Inducible strains BL21 of *Escherichia coli* were used for apoobelin production. Site directed mutagenesis was carried out using a modification of a procedure described previously [9]. The main scheme of mutagenesis included two stages of amplification using two flanking primers: 5′-GACTGCAGTTAGGGAACTC-CGTTGCCATA-3′ (A) and 5′-GATCTAGAATAAAG-GAAATCGATA-3′ (B) and one primer carrying the required nucleotide substitution: 5′-TTCGACATTCT-GATTTGGACAACAG (C158A) or 5′-TTCGACAT-GCTGATTTGGACAACAG (C158S).

DNA pOL103 was used as the template [6]. The first stage of five cycles was carried out using primer A and one of the mutagenic primers, then flanking primer B was added to the mixture and the second stage included ten cycles of amplification. The amplification product was purified by gel electrophoresis in 1% agarose [10], treated with restriction endonucleases *Xba*I and *Pst*I, and cloned into vector pT7-7 [11] pretreated with the same restrictases. Resultant mutations and intactness of the remaining part of the apoobelin gene were confirmed by sequence analysis using chemical hydrolysis [12].

Cell cultivation, harvesting and preparation of biomass, and isolation of apoproteins were carried out using previously developed protocols [6].

Purity of resulting apoprotein preparations was evaluated by electrophoresis in 12.5% polyacrylamide gels in the presence of 0.1% SDS as described by Laemmli [13]. An electrophoresis protein kit from Pharmacia Biotech (USA) was used as the standard.

Protein content was assayed by the microbiuret method [14] using bovine serum albumin as the standard. Before protein determination, the preparations were desalted by gel filtration on a Bio-Gel P-4 column.

For activation of apoproteins by synthetic substrate (conversion of apophotoproteins into active photoproteins), apoprotein preparations were incubated in 20 mM Tris-HCl buffer, pH 7.0, containing 15 μM coelenterazine, 10 mM EDTA, and 10 mM dithiothreitol (5 μl aliquots were mixed with 100 μl buffer) at 4°C.

Stability of the resulting photoproteins was evaluated by their residual luminescent activity during storage in the same buffer at 4 and 20°C. Bioluminescent activity of photoproteins was measured using a BLM 8801 luminometer (SKTB Nauka, Krasnoyarsk, Russia) calibrated by a Hastings–Weber calibration standard [15]. One luminescent unit corresponds to 10^7 photons per second. The reaction mixture included 500 μl of 100 mM Tris-HCl buffer, pH 8.8, containing 10 mM EDTA and 5 μl active photoprotein. The reaction was initiated by injection of 200 μl 100 mM Tris-HCl buffer, pH 8.8, containing 100 mM $CaCl_2$ into the reaction mixture. Luminescent signal was recorded using an LKB recorder (model 2210) (Sweden).

Quantum yield of the bioluminescent reaction was calculated as described previously [16]. The molecular mass of recombinant apoobelin of 22.2 kD was used for calculations [7].

RESULTS AND DISCUSSION

Among five cysteine residues of recombinant apoobelin, we chose Cys158 because it is a highly conservative residue that is found in all Ca^{2+}-activated photoproteins that have been cloned (aequorin, clytin, mitrocomin, and obelin) [7, 17]. The choice of amino acid substitution was guided by the reduced nucleophilicity of side chain functional groups in the order SH > OH > CH_3 (cysteine, serine, and alanine, respectively).

All apoproteins isolated from *E. coli* cells were purified to electrophoretic homogeneity (Fig. 1).

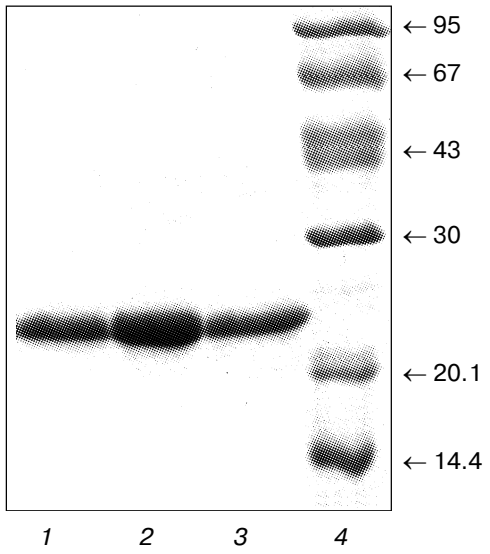

Fig. 1. SDS-PAGE of preparations of apoobelin and its mutants obtained after purification from *E. coli* cells: *1*) apoobelin; *2*) S-mutant; *3*) A-mutant; *4*) marker proteins (and their molecular masses in kD): phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin.

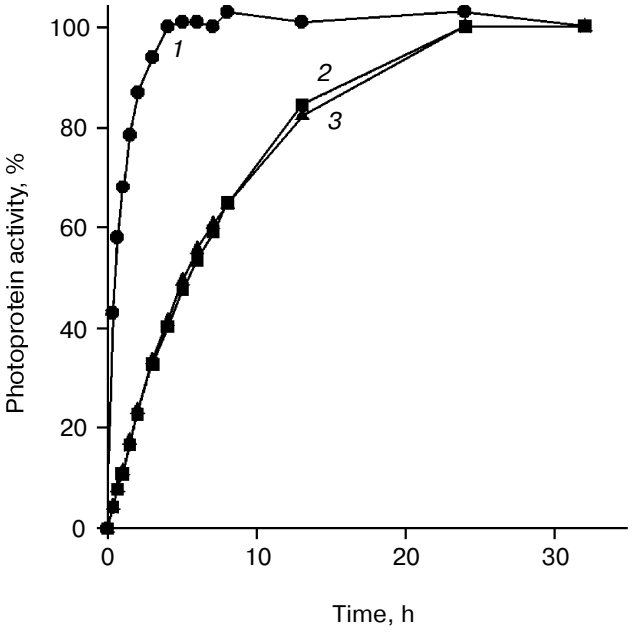

Fig. 2. Kinetics of activation of wild-type apoobelin (*1*) and its S- (*2*) and A-mutants (*3*) by synthetic substrate.

Figure 2 shows the kinetics of apoprotein activation by synthetic coelenterazine. Complete conversion of wild-type apoobelin into active photoprotein complex occurs within the first four hours of incubation. This is consistent with a previous report on the activation of recombinant apoobelin [18]. In contrast to the wild-type protein, maximal manifestation of photoprotein activity of the S- and A-mutants required much longer incubation, the maximal luminescent reaction of these mutants being developed only after 24 h incubation with the substrate. The activation kinetics was the same for both mutants.

Because the activation kinetics of apoobelin and its mutants by synthetic substrate differed in the time required for maximal luminescent reaction (Fig. 2), the luminescence characteristics of all activated photoproteins were determined after 24 h incubation. The table shows that substitution of Cys158 in recombinant apoobelin for the other amino acid residues reduced the luminescence of the obelin mutants. The luminescence parameters decreased in the order: wild-type protein > S-mutant > A-mutant (cysteine > serine > alanine, respectively). This is consistent with the nucleophilic properties of the side chain functional groups of the corresponding amino acid residues.

Luminescence characteristics of wild-type recombinant obelin and its mutants after activation of the apoproteins by synthetic coelenterazine

Parameter	Wild-type obelin	S-Mutant	A-Mutant
Rate constant of luminescence decay (k), sec^{-1}	6.6	1.8	0.9
Specific activity, quanta per mg protein	$9.53 \cdot 10^{15}$	$6.67 \cdot 10^{15}$	$3.55 \cdot 10^{15}$
Quantum yield of luminescent reaction	0.35	0.25	0.13
log (L/L_{max})	–6.13	–5.29	–5.08

Note: L is a value of photoprotein basal luminescence (calcium-independent luminescence at $[Ca^{2+}]_i < 10^{-8}$ M), L_{max} is a value of maximal light response obtained at the same content of the photoprotein in the presence of saturating calcium concentrations ($[Ca^{2+}] \geq 10^{-2}$ M).

Because the activation kinetics of apoobelin and its mutants by synthetic substrate differed in the time required for maximal luminescent reaction (Fig. 2), the luminescence characteristics of all activated photoproteins were determined after 24 h incubation. The table shows that substitution of Cys158 in recombinant apoobelin for the other amino acid residues reduced the luminescence of the obelin mutants. The luminescence parameters decreased in the order: wild-type protein > S-mutant > A-mutant (cysteine > serine > alanine, respectively). This is consistent with the nucleophilic properties of the side chain functional groups of the corresponding amino acid residues.

Stability is an important characteristic of the photoprotein complex, and therefore we investigated the stability of obelin and its mutant forms at various temperatures (Figs. 3 and 4). Wild-type recombinant obelin retained its activity during prolonged incubation at 4°C (up to 10 days). Similar results were previously obtained for wild-type obelin [19]. The A- and S-mutants were much less stable during storage at this temperature (Fig. 3), and after 10 days the remaining activity was only 50 and 40% of their initial activities, respectively. Increasing the storage temperature to 20°C caused a sharp decrease in activity of the obelin mutants (Fig. 4). During the first three days of storage at this temperature, the remaining activity was less than 5-10% of the initial values. After 10 day storage at 20°C the remaining activity of these mutants was only 0.1-0.2% of the initial values. Under these conditions inactivation of wild-type recombinant obelin was only ~30% (Fig. 4). The latter is consistent with the reported stability of wild-type obelin [19]. It should be noted that long-term storage of these proteins did not result in their proteolysis.

The data suggest that substitution of the highly conservative residue Cys158 in recombinant apoobelin results in significant changes in the structure of the protein molecule. This slows formation of the photoprotein complex, decreases its stability, and changes the bioluminescence characteristics. Thus, good evidence exists that the SH-group of Cys158 is important for the formation of the active photoprotein. Consider several possibilities of the involvement of SH-group in this process.

First, the SH-group might be directly involved in substrate coordination on the apoprotein molecule via the formation of a hydrogen bond with OH-groups of the side benzene rings of coelenterazine; the possibility of the formation of such hydrogen bonds (O–H···S) is discussed in the literature [20]. However, it seems unlikely that an SH-

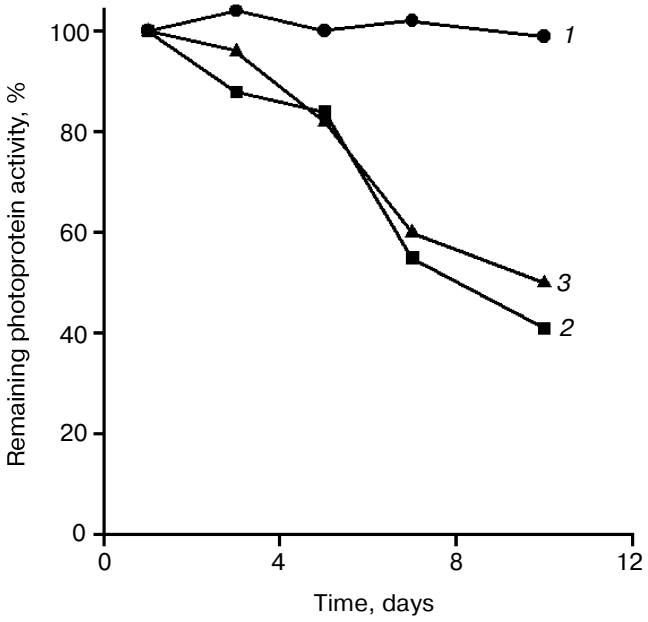

Fig. 3. Stability of wild-type obelin (*1*) and its S- (*2*) and A-mutants (*3*) during storage at 4°C.

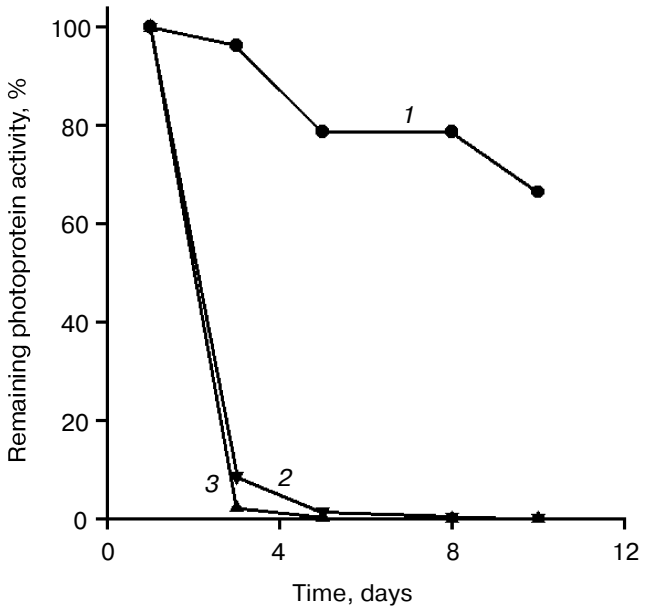

Fig. 4. Stability of wild-type obelin (*1*) and its S- (*2*) and A-mutants (*3*) during storage at 20°C.

group is directly involved in substrate binding because the substitution of cysteine by other amino acids did not abolish the formation of the photoprotein complex.

The second possibility suggests the involvement of the SH-group in the formation of an intramolecular disulfide (S–S) bond in apoobelin which shields the hydrophobic region (putative substrate binding site). During substrate activation of apoobelin, when SH-reducing reagents (dithiothreitol and 2-mercaptoethanol) are added to the reaction mixture, the disulfide bond is cleaved, and this allows substrate binding at the active site. However, computer modeling of the apoobelin structure based on its amino acid sequence excludes the possibility of intramolecular disulfide bond formation [21].

In our view, a third variant suggesting the involvement of the SH-group in the formation of an essential intramolecular hydrogen bond (O–H···S or N–H···S) is most probable. This hydrogen bonding maintains stable native conformation of the apoobelin molecule (especially the active site conformation), thus facilitating coordination of the substrate.

Consider this variant in detail. Calcium-activated photoproteins are characterized by a low basal level of luminescence which does not depend on the presence of low concentrations of free Ca^{2+} in the medium ($[Ca^{2+}]_i < 10^{-8}$ M) [2, 6]. This phenomenon has been called "calcium-independent luminescence" [2]. This property may reflect some features of the structural organization of the active photoprotein complex, especially possible fluctuations of the photoprotein molecule. The basal luminescence is very low. The values of the base-ten logarithm $\log(L/L_{max})$ (where L is the basal signal value and L_{max} is the value of the maximal bioluminescent signal obtained at the same photoprotein content in the presence of saturating calcium concentrations $[Ca^{2+}] \geq 10^{-2}$ M) are within the interval from −6 to −7. If the SH-group of Cys158 (involved into intramolecular hydrogen bonding) is important for the formation of stable native conformation in the apoprotein, the substitution of this amino acid residue would lead to destabilization of the protein molecule. One probable consequence of such substitution is an increase in the basal calcium-independent luminescence of the mutant.

To test this hypothesis, we investigated the basal luminescence of native recombinant obelin and its mutants. Substrate activation of the apoproteins was carried out in the luminometer cuvette with continuous registration of the luminescent signal. The content of each apoprotein in the cuvette was 3.25 μg. The luminescence was monitored during the incubation for 4 h. The experiments showed that the basal luminescence of obelin reaches a plateau during the first hour and then remains unchanged for the next 3 h. The basal luminescence of the S- and A-mutants reached a plateau during the first two hours of the incubation and remained at the same level during the next 2 h. However, the maximal level of the basal luminescence of the mutants was 6-13 times higher than that of the wild-type recombinant obelin.

Values of $\log(L/L_{max})$ calculated using basal luminescence values and specific activity of the photoproteins increased in the order: wild-type obelin < S-mutant < A-mutant (table). These data strongly suggest that the decrease in specific activity and quantum yield in the order obelin > S-mutant > A-mutant may be explained by an increase in calcium-independent luminescence, which was observed in the same order. It seems likely that the increase in the basal luminescence in mutants is the consequence of the increase in the lability of the photoprotein complex. However, the decrease of the rate constant of luminescent reaction decline cannot be attributed to spontaneous (calcium-independent) fading of the obelin mutants. The most probable explanation of the reduced rate constant is the decrease of the reaction rate of intermediate stage(s) of coelenterazine oxidation, which represents a multistage process (as shown in the case of aequorin, e.g., [2]).

Thus, the data obtained in the present study suggest that SH-group of highly conservative Cys158 of recombinant apoobelin is important for the formation of its stable native conformation, which allows the formation of the stable enzyme–substrate complex. Good evidence exists that the SH-group of Cys158 is involved in intramolecular hydrogen bond formation (O–H···S or N–H···S). Substitution of this amino acid residue for serine or alanine destabilizes the photoprotein complex. This is accompanied by an increase in calcium-independent luminescence of the mutants and decrease in their Ca^{2+}-dependent bioluminescent parameters. Impairment of the enzymatic properties of apoobelin mutants depends on the nucleophilicity of the side chains of the amino acids replacing Cys158, which decreases in the order SH > OH > CH_3 (cysteine, serine, alanine). It is possible that the conservative position of this cysteine (Cys158) in the apoobelin molecule is the result of evolutionary selection in the group of Ca^{2+}-activated photoproteins of marine coelenterates. This should be taken into consideration during construction of obelin mutants by gene engineering methods.

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