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REVIEW

Excited Flavin and Pterin Coenzyme Molecules in Evolution

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Abstract—Excited flavin and pterin molecules are active in intermolecular energy transfer and in photocatalysis of redox reactions resulting in conservation of free energy. Flavin-containing pigments produced in models of the prebiotic environment are capable of converting photon energy into the energy of phosphoanhydride bonds of ATP. However, during evolution photochemical reactions involving excited FMN or FAD molecules failed to become participants of bioenergy transfer systems, but they appear in enzymes responsible for repair of UV-damaged DNA (DNA photolyases) and also in receptors of blue and UV-A light regulating vital functions of organisms. The families of these photoproteins (DNA-photolyases and cryptochromes, LOV-domain- and BLUF-domain-containing proteins) are different in the structure and in mechanisms of the photoprocesses. The excited flavin molecules are involved in photochemical processes in reaction centers of these photoproteins. In DNA photolyases and cryptochromes the excitation energy on the reaction center flavin is supplied from an antenna molecule that is bound with the same polypeptide. The role of antenna is played by MTHF or by 8-HDF in some DNA photolyases, i.e. also by molecules with known coenzyme functions in biocatalysis. Differences in the structure of chromophore-binding domains suggest an independent origin of the photoprotein families. The analysis of structure and properties of coenzyme molecules reveals some specific features that were significant in evolution for their being selected as chromophores in these proteins.

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Key words: evolution, photoreceptors, photocatalysis, excited states, selection of chromophores, coenzymes, flavins, pterins, folates, DNA photolyases, cryptochromes, phototropins, LOV domain, BLUF domain, modeling of prebiotic processes, photophosphorylation

Photoreceptor proteins having as chromophores (i.e. photon sensors) molecules known in biochemistry as coenzymes of dark biocatalysis have been discovered relatively recently. These proteins absorb short wavelength visible and ultraviolet (UV-A) light and transform it into signals regulating metabolism and ontogenesis of plants and fungi, motility of some lower eukaryotes, and also correcting circadian rhythms in various organisms. Studies of these phenomena, called blue light responses, were started even in the XIX century: Charles Darwin was the author of the first publication reporting the role of blue light in plant phototropism [1].

Based on the resemblance between the spectra of physiological responses and the light absorption spectra of flavins, the involvement of these compounds in light reception has been supposed from the 1950s (Fig. 1). For a long time attempts to isolate photoreceptors were unsuccessful [2]. Only in the 1990s new approaches for gene isolation and expression opened the way for directly analyzing flavoprotein receptors and functions of chromophores within them [3-7]. It should be noted that not long before it was shown that excited molecules of flavins and of some other cofactors can be involved in the other phenomenon – photorepair of UV-caused damages in DNA structure under the influence of the enzyme DNA photolyase (EC 4.1.99.3) [8].

Thus, there are two groups of photoproteins in which excited coenzyme molecules can function. In photoenzymes (DNA photolyases) a cofactor localized in the active center of the protein is chemically active only in the excited state, i.e. its excitation is necessary for the catalytic act. In other proteins, photoreceptors of regulatory processes, the light excites a chromophore bound in the photosensor domain, and this leads to activation of another (effector) domain of the same protein and initi-

Abbreviations: ETC, electron transport chain; 8-HDF, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; MTHF, 5,10-methenyltetrahydrofolate.

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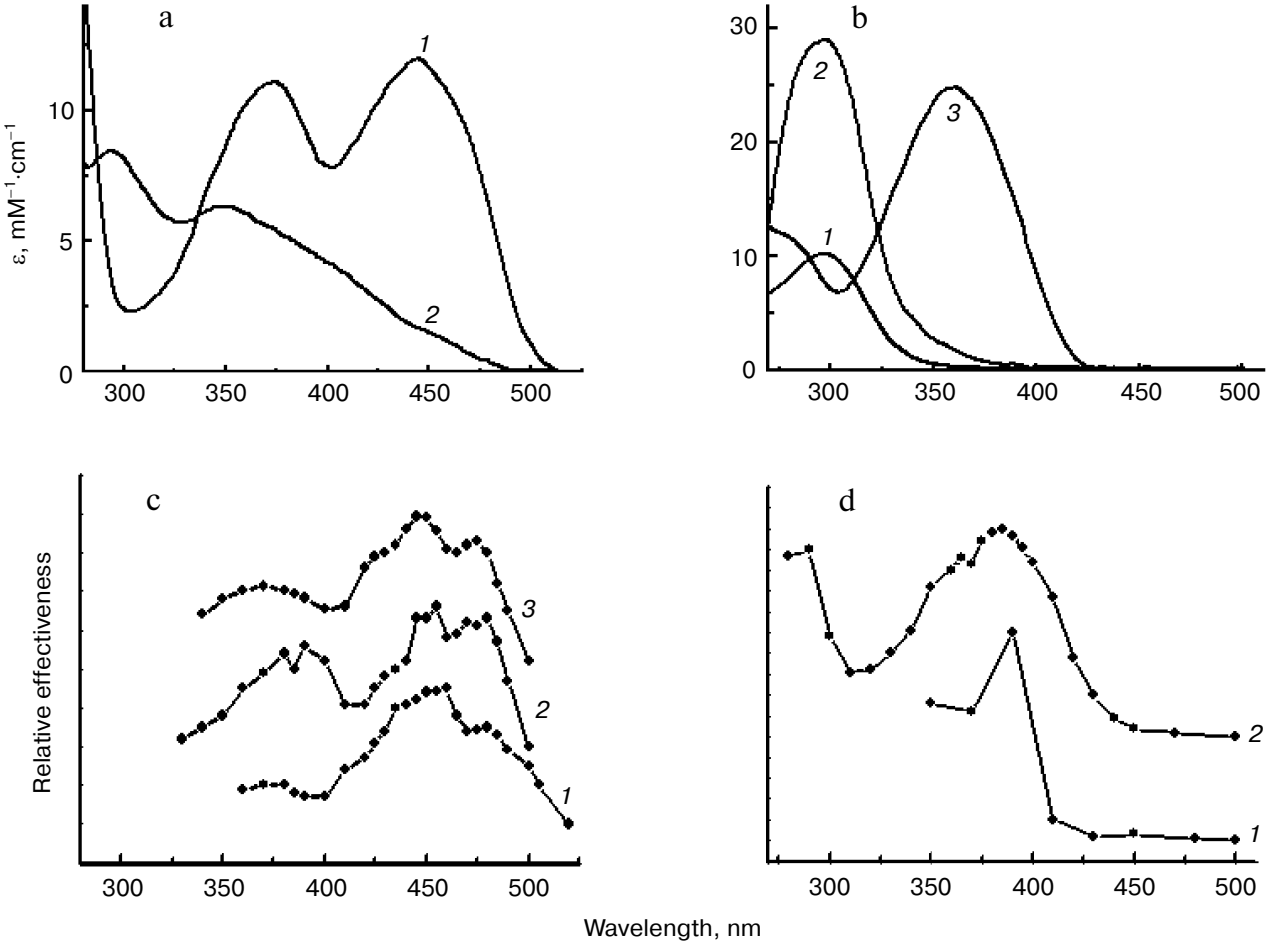

Fig. 1. Light absorption spectra of flavins and pterins and spectra of physiological responses to blue light and near ultraviolet. a) Absorption spectra of riboflavin solutions (pH 7.0): *1*) oxidized form; *2*) dihydro-form. b) Absorption spectra of aqueous solutions of tetrahydropterin coenzymes: *1*) tetrahydrobiopterin (pH 7.0); *2*) tetrahydrofolic acid (pH 7.0); *3*) 5,10-methenyltetrahydrofolate (pH 3.0). c) Spectra of physiological responses of organisms to blue light: *1*) shift of the circadian rhythm of *Drosophila pseudoobscura* pupae emergence [167]; *2*) phototropism of macrosporangiophores of the fungus *Phycomyces blakesleeanus* [168]; *3*) phototropism of oat coleoptiles [169]. d) Spectra of physiological responses of organisms to near ultraviolet: *1*) physiological photodegradation of *Drosophila*'s cryptochrome [170]; *2*) photoreactivation of DNA (photocatalytic activity of DNA photolyase of *Saccharomyces cerevisiae*) [171].

ates a cascade of signal transduction, which includes protein kinases, molecules of secondary messengers, and mechanisms of gene expression. The signal is increased rather efficiently, and some processes involving photoproteins can occur in response to a very weak flow of photons. For example, macrosporangiophores of the fungus *Phycomyces blakesleeanus* display phototropism at radiation power of only 10^{-9} W/m^2 [9].

Derivatives of isoalloxazine (2,4-dioxo-7,8-dimethylbenzo-[g]-pteridine) flavin cofactors of oxidoreductases, FMN and FAD, are identified as chromophores of photoproteins, as well as 7,8-didemethyl-8-hydroxy-5-deazariboflavin (8-HDF), which has the N5 atom of isoalloxazine substituted by carbon. This compound serves as a structural element of the redox cofactor F_{420} involved in some microorganisms in two-electron transfer reactions that in other organisms are performed by nicotinamide coenzymes [10-13]. A derivative of pterin (2-amino-4-hydroxypteridine) — 5,10-methenyltetrahydrofolate (MTHF), which is known as a participant of metabolism of one-carbon residues, also serves as a chromophore (Fig. 2).

But why did coenzymes become photon sensors, i.e. were they introduced into an alien molecular physiological niche occupied by specialized pigments? And another question is also reasonable: can photochemically active coenzyme molecules be involved in biological utilization of solar energy? This review considers these questions, and the discussion is focused on the features of flavin and pterin, which have been shown to be active in photoproteins and also in models mimicking prebiotic processes with involvement of these compounds. Structural–functional features and phylogenesis of photoproteins themselves are considered in detail in other references [14-22].

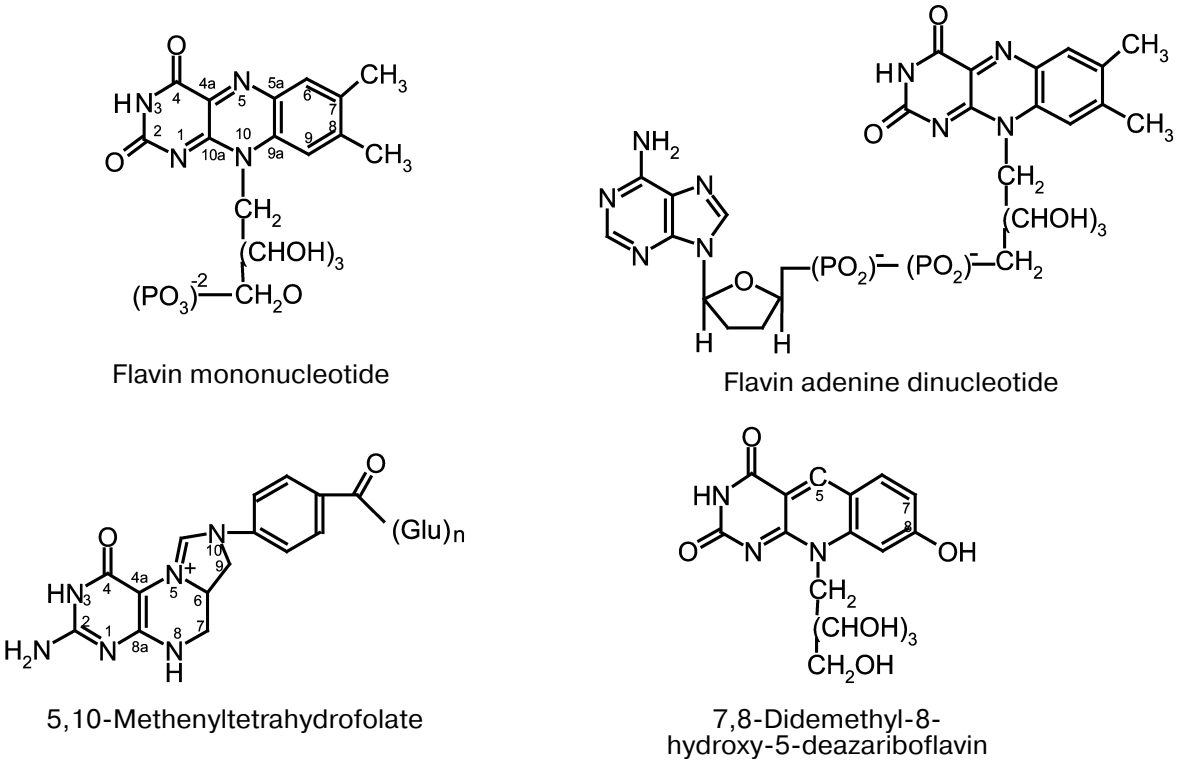

Fig. 2. Formulas of coenzymes that function as chromophores in photoenzymes and photoreceptors of regulatory processes.

EVOLUTIONARY ANTIQUITY OF COENZYMES

Coenzymes are of interest as participants of evolution because of the inability of the cell metabolic system to function without them. Therefore, coenzymes are supposed to have functioned even in the early stages of formation of metabolism and now to be some metabolic relicts [23, 24]. The structural resemblance with nucleotides suggested that coenzymes could function in a hypothetical "RNA world" where genetic and biocatalytic functions of organisms were completely satisfied by nucleotide compounds without the involvement of proteins [25-28].

According to modern concepts, the Earth's crust was formed about 4.5 billion years ago and the age of the oldest sedimentary rocks containing fossil microorganisms is close to 3.5 billion years (fossilized relics are not preserved because of metamorphization of more ancient rocks). These microfossils are morphologically similar to the contemporary cyanobacteria and therefore are supposed to be a result of the evolution of more primitive organisms that started about a billion years earlier [29-32]. Comparative analysis of the secondary structure of proteins indicates that the age of flavodoxins (and consequently of flavins) corresponds to that of the oldest organisms of the Earth and can be 3.2-4.5 billion years old [33].

Coenzyme heterocycles including pteridines and benzopteridines can be synthesized on modeling the conditions that existed on the prebiotic Earth [34-37]. Thus, heating to 150-200°C of anhydrous amino acid mixtures in the absence of oxygen results in production of conjugates of isoalloxazines and pteridines with amino acid polymers (flavoproteinoids) [35-37], which aggregate in aqueous medium resulting in phase-separated structures, the so-called "Fox microspheres" [38]. Under enzyme-free conditions of the abiotic environment and also under the influence of polyribonucleotide catalysis plausibly functioning during the early stages of evolution just coenzymes themselves could be generated, including quasi-nucleotide molecules NAD^+ and FAD [39-43].

PHOTOPHYSICAL AND PHOTOCHEMICAL FEATURES OF FLAVINS AND PTERINS

Features of flavins and pterins as participants of photoprocesses are determined, first of all, by systems of conjugated double bonds of isoalloxazine and pteridine. The binding by the heterocycle of electrons and protons and also connecting side substituents determine the distribution of electron density and manifestation of these features. These features are also influenced by the ionic

composition of the medium and the polarity of the solvent [44-46]. Absorption spectra of flavins (FMN or riboflavin) in aqueous solution contain light absorption bands at 446, 375, 265, and 220 nm. The long wavelength maximum in the absorption spectrum of deazariboflavin is located at 420 nm. Non-conjugated (i.e. containing small substituents) pterins, e.g. biopterin, have absorption maxima at 275 and 340 nm. Absorption maxima of folic acid are at 282 and 350 nm. The binding of coenzyme chromophores with an apoprotein is accompanied by a bathochromic shift of absorption by 20-50 nm [47].

Connecting an electron to the isoalloxazine heterocycle results in formation of a free radical (semiquinone) flavin molecule, and due adding another electron it becomes a dihydro-form (Fig. 3). Redox transformations of pterins are accompanied by production of free radical states and also of intermediate isomeric dihydro-forms, including quinonoid forms. As discriminated from flavins, dihydro-forms of pterins (including folates) can be reduced further into tetrahydro-forms. The reduction of flavins affects the boundary region at the interface between the pyrimidine and pyrazine rings (-N1-C8a-C4a-N5-), whereas in pterins electrons are joined to atoms of the outer side of the pyrazine cycle (-N8-C7-C6-N5-). The breaking of double bonds of the heterocycle shifts the absorption maxima of flavins and pterins to shorter wavelengths. Thus, dihydroflavins, as discriminated from oxidized forms, absorb virtually no visible light [44]. A similar hypsochromic shift also occurs on reduction of pterins including folates. In MTHF this shift is compensated by the additional presence of an imidazoline cycle, which enlarges the system of conjugated bonds, and the absorption maximum of this molecule is 365 nm [48].

Depending on the acidity of the medium, each redox form of flavins and pterins can exist either in the neutral or ionized (anionic or cationic) state, which influences the spectral and photochemical properties of the molecule (Fig. 3).

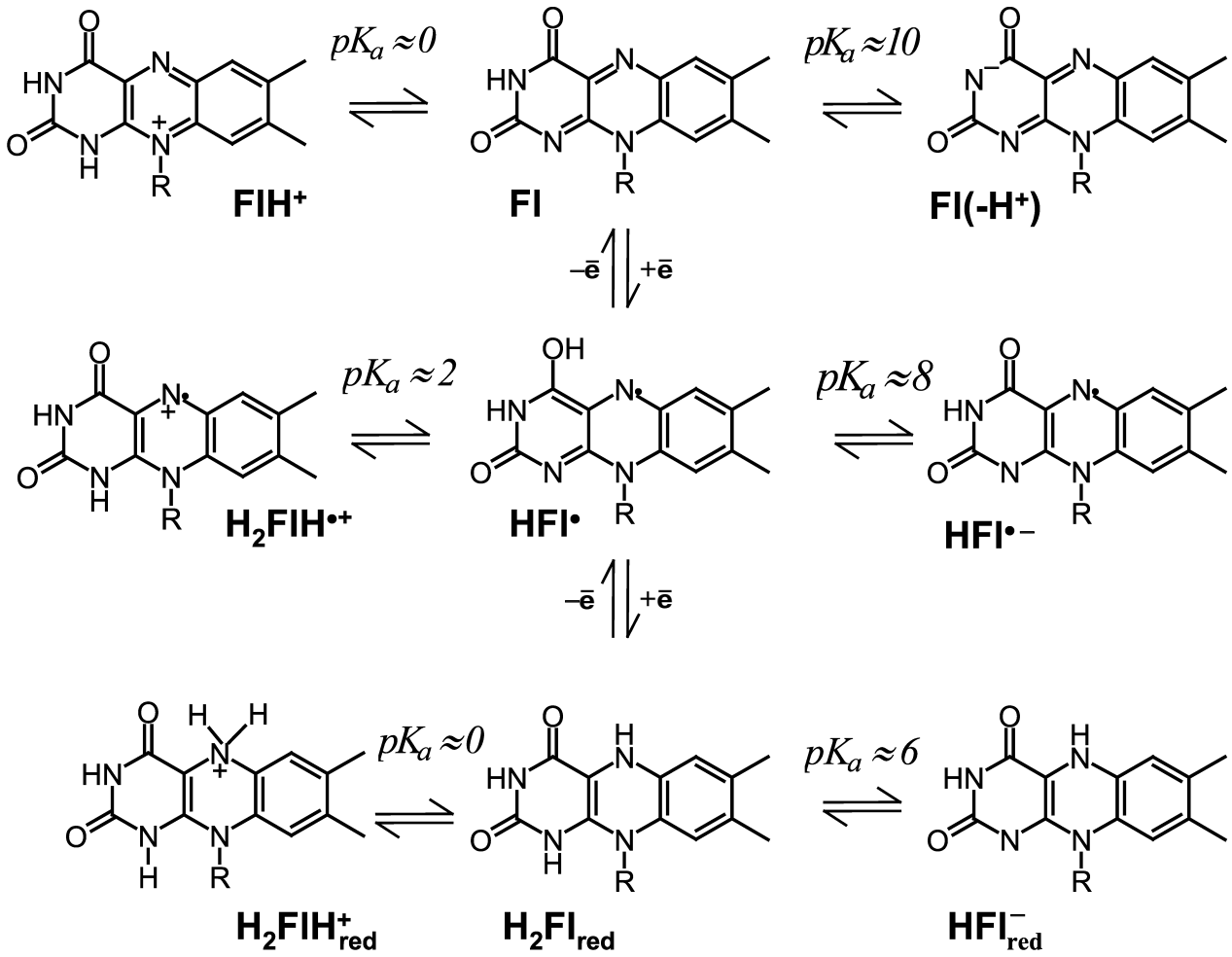

Fig. 3. Different redox and protonated states of flavins.

Molar absorption coefficients of flavin in the long wavelength maxima are close to 10^4 $M^{-1}\cdot cm^{-1}$, and for the majority of pterins they are $(5\text{-}7)\cdot 10^3$ $M^{-1}\cdot cm^{-1}$. These values are significantly lower than the values for chlorophylls, linear tetrapyrroles, or all-*trans*-retinal, which are close to $5\cdot 10^4$ $M^{-1}\cdot cm^{-1}$ [49].

Singlet-excited flavin molecules (riboflavin, FMN) and non-conjugated pterins are characterized by intense fluorescence (λ_{max} = 520-530 and 420-450 nm, F_f = 0.2-0.3 and 0.4-0.6, respectively) and by effective conversion into the triplet state. The $F_{S1\rightarrow T1}$ value reaches 0.6-0.7 for flavins and about 0.1-0.2 for pterins. Lifetimes of the triplet states (τ_{T1}) are, respectively, ~0.2 and ~1.0 sec at 77K and at room temperature these values are in range of tens to hundreds of microseconds [44, 50-52].

The manifestation of excited heterocycle features depends on the spatial proximity of another heterocycle, including the intramolecular stacking of bases within the same molecule. Thus, base stacking in an FAD molecule results in a partial dissipation by adenine of the isoalloxazine excitation energy and decreases the intensity of fluorescence and photochemical activity of FAD compared to FMN and riboflavin [44-46]. The weak fluorescence of folic acid also seems to be caused by stacking of the pterin heterocycle with the benzene ring of *p*-aminobenzoyl glutamate [53].

In DNA photolyases and cryptochromes the flavins and pterins participate in energy transfer by the Förster resonance dipole–dipole mechanism: energy transfer of the singlet-excited antenna chromophore (MTHF or 8-HDF) onto flavin of the reaction center results in its chemical activation. The energy migration of triplet-excited flavins and pterins onto molecular oxygen generates singlet oxygen ($^1O_2$) with 70% quantum yield for riboflavin and in the range of 10-30% for pterins [54-56].

The conversion of flavin (Fl) into an excited state (*Fl) increases its electrophilicity, and it becomes a very strong oxidizer. For the FMN-H$^{\cdot}$/FMN pair, the E'_o value is –0.31 V [57], whereas for FMN-H$^{\cdot}$/*FMN it is +1.85 V. The addition of an electron (more strictly, a hydrogen atom) produces the free radical (semiquinonic) form HFl$^{\cdot}$. In pterins not only an oxidized molecule can participate in photoreduction, but also a dihydro-form, which is converted into a tetrahydro-form. As in the case of flavins, pterins are photoreduced with production of free radicals [57-61]. In the presence of oxygen, free radicals of flavins (and of pterins) are sources of reactive oxygen species (ROS), which influence the mechanism and rate of the photochemical process [44, 62-64].

Flavin semiquinone HFl$^{\cdot}$ is oxidized into the initial form of Fl, or a disproportioning of two HFl$^{\cdot}$ molecules results in production of dihydroflavin H_2Fl_{red} and the oxidized form of F1. The covalent binding of the oxidized substrate residue to the N5 atom of isoalloxazine with production of a photoadduct is another pathway of HFl$^{\cdot}$ conversion [44].

Oxidation by an excited flavin (or pterin) of a donor having redox potential (E'_o) more positive than that of the unexcited pigment results in accumulation of free energy in the product. Such a product can be either a reduced flavin (pterin) molecule, or energy can be accumulated by the reduced acceptor during the photocatalysis by these molecules of its transfer from donor onto the acceptor. Thus, oxidative decarboxylation of the donor ethylenediamine tetraacetate (E'_o ~ +0.40 V) by excited flavin produces reduced forms of flavin (E'_o for the pair FMN-H_2/FMN is –0.21 V, and for the pair FMN-H$^{\cdot}$/FMN it is –0.31 V). In the presence of electron acceptor Fe^{3+}-cytochrome *c*, it changes to the reduced state (Fe^{2+}) with the corresponding value of E'_o ~ +0.25 V. Photocatalysis is characteristic of both biological flavins and abiogenic isoalloxazine pigments [63, 65].

In the 1970-1980s, i.e. before the discovery of coenzyme-binding photoproteins, the reduction of cytochrome by excited flavin was considered to be a probable physiological mechanism of blue light reception, and many efforts were focused on detecting in the cell of cytochromes involved in the act of photoreception. The main attention was given to *b*-type cytochromes, which are present in the cell membrane, and also to the cytochrome-containing electron transport chain (ETC) of eukaryotic nitrate reductases (EC 1.6.6.1) catalyzing the reduction of nitrate ion into nitrite coupled to the oxidation of NADH or NADPH [2, 66].

In the cells of plants, algae, and higher fungi, an enzyme of the ETC contains apoprotein-bound FAD, *b*-type cytochrome heme, and a molybdenum atom coordinated by a molecule of a specific molybdopterin. Upon excitation, the FAD molecule within the enzyme becomes chemically active, and this can manifest itself in a redox interaction with exogenous cytochrome *c* and (under certain conditions) also with the molybdenum-containing cofactor [67]. A hypothesis about a photoreceptor functioning as nitrate reductase [68] was not confirmed experimentally [69-71], and the discovery of true photoreceptor proteins virtually reduced to zero the interest in studies on electron exchange between excited flavin and cytochromes. Now such photosystems can be attractive for the possibility of their being used for photoinjection of electrons into redox proteins [72-75].

COENZYMES AS POSSIBLE PARTICIPANTS OF EVOLUTIONARILY PRIMITIVE PROCESSES OF ENERGY CONVERSION

Modeling of an abiogenic converter of light energy. Isoalloxazines and pteridines able to photocatalyze reactions leading to storage of free energy can be generated in an abiotic medium, and this allows us to consider them as

probable participants of ancient energy-converting systems. These compounds actively absorb photons in the region of the spectrum that was prevalent in solar radiation not filtered through the ozone screen of the atmosphere (λ_{max} ~ 500 nm). The formation of this screen displaced this maximum to longer wavelengths, and now at sea level it corresponds to 630-650 nm [76].

The involvement of these compounds in the synthesis of ATP is especially interesting because the availability of ATP is believed to be the most important prerequisite for development of metabolism [31, 77, 78]. We showed that 5′-AMP molecules and also a system providing for its photophosphorylation into ATP could be produced as a result of thermolysis of a mixture of amino and carbonic acids in an oxygen-free medium. ADP is photophosphorylated by orthophosphate in the presence of flavoproteins, which are conjugates of amino acid polymers and isoalloxazines (and also of pteridines) generated during thermocondensation of amino acid mixtures. In aqueous medium these conjugates aggregate into microspheres [36]. Illumination of suspension of these structures by light in the blue or UV-AB region in the presence of ADP and orthophosphate produces ATP [65, 79, 80]. This system is efficient under conditions of reoxidation of the photochemically active pigment with involvement of O_2, its reactive species, and also of oxygen-free acceptors, e.g. (in experiments) Fe^{3+}-cytochrome *c*. Under conditions of regeneration of the pigment by oxidizers up to 30% of ADP molecules were converted into ATP, whereas removal of the oxidizer decreased the yield to 6-8%.

The yield of the phosphorylation product decreased to 6-7% on using AMP instead of ADP as a substrate. A similar specificity was observed in another model of photophosphorylation when ultraviolet acted directly on nucleoside phosphates absorbed on a mineral surface, without involvement of organic sensitizers. Therefore, this specificity seems to be due to features of AMP and ADP as they are. Even in the middle of the XX century Albert Szent-Gyorgyi had noted that in ADP a spatially admissible interaction between the terminal phosphoryl group and adenine heterocycle results in neutralizing the negative charge of this group by nitrogen atoms of adenine and in a simplified addition of another orthophosphate residue. But the length of the phosphoryl "tail" in the AMP molecule is insufficient for such an interaction [81, 82].

The flavoproteinoid model does not have known analogs in organisms. Its mechanism seems to be like the mechanism proposed for the riboflavin-sensitized photophosphorylation of ADP in aqueous solution when the interaction of free radical flavin molecules with ADP leads to production of the radical form ADP$^{\bullet}$ and then to phosphorylation of this radical by orthophosphate [83, 84]. As compared to flavin solution, the pigment-containing polymeric matrix of microspheres was favorable for more effective phosphorylation. Thus, abiogenic amines could be involved in conversion of the light energy into energy-rich ATP phosphates.

Could flavin photocatalysis compete with photosynthesis? In the 1950s A. A. Krasnovsky attracted attention to photocatalytic reactions of coenzymes as probable evolutionary precursors of photosynthesis, and H. Gaffron emphasized the role of flavins in these processes [85-87]. Model studies have shown that coenzymes (or their abiogenic analogs) sensitize conservation of the light energy into chemical bonds including energy-rich bonds of ATP. Thus, it is reasonable to ask whether these processes could act as converters of light energy in the biosphere, or in other words, whether they could compete with photosynthesis in the evolution.

The light absorption maximum of flavins (including abiogenic pigments) is close to 450 nm. The modern photosynthetic pigments utilize energetically less advantageous photons in the red region, and even the infrared region in some bacteria, and the long wavelength light absorption maximums by chlorophylls are in the region of 670-700 nm [49]. The energy of one einstein, i.e. mole of photons, for these maxima is, respectively, 260 and 170 kJ/mol.

Theoretically, to produce the phosphoanhydride bond in ATP during photosynthetic phosphorylation two photons are required [88], and in the flavoproteinoid model for this (again in the ideal case) only one photon is needed. The energy of production ($\Delta G'$) of the phosphoanhydride bond in ATP is 33.4 kJ/mol and, thus, considering different energetic charge of photons in different regions of the spectrum, efficiencies of using the absorbed photon energy by the flavoproteinoid and photosynthetic systems are comparable: their values are, respectively, 13 and 10% (table). But it should be remembered that photosynthesis also has another pathway of energy conservation which is at least threefold more efficient – generation of NADPH molecules (or NADH in some prokaryotic photosynthesizing organisms). It is not known if flavins can photoreduce nicotinamides.

For evaluation of the competitiveness of the flavin model relative to photosynthesis, it is important to take into account its essentially lower absorption of photons relative to chlorophyll (the molar absorption coefficients in the long wavelength maxima are, respectively, about $1 \cdot 10^4$ and $5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$). This could promote chlorophyll-mediated photosynthesis to become a converter of solar energy in the biosphere. It is also important that chlorophyll, especially combined with satellite pigments, can use photons of virtually the whole visible spectrum, whereas flavins can absorb light only with wavelengths below 520 nm. Nevertheless, flavin catalysis was claimed by evolution. It occupied the functional niche of photoproteins responsible for the organism's adaptation to the environment. These proteins are different in both structure and mechanism of the photoprocess.

Efficiency of photosynthetic phosphorylation and of phosphorylation sensitized by flavoproteinoid microspheres

Parameters of the process	Flavoproteinoid model	Photosynthesis
Limits of the spectral region in physiological range, nm	320-520	400-800
Molar absorption coefficient (ε) for long-wavelength maxima of light absorption by chromophores, $M^{-1}\cdot cm^{-1}$	~$1\cdot10^4$	~$5\cdot10^4$
Energy of mole of quanta, kJ/einstein	260	175
Theoretical yield of P–O–P bonds per photon	1	0.5
Efficiency of using the photon energy in synthesis of energy-rich phosphate, %	13	10

Note: The following values are taken as long-wavelength maxima: 680 nm for chlorophylls (chlorophyll *a*) and 450 nm for flavins.

PHOTOPROTEINS WITH COENZYMES – PHOTON SENSORS

DNA photolyases and cryptochromes. Under the influence of light in the range of 300-500 nm, DNA photolyases catalyze breaking C–C cross-links between pyrimidines in cyclobutane pyrimidine dimers and in (6-4)-pyrimidine-pyrimidone photoproducts arising upon the illumination of DNA by far (200-300 nm) ultraviolet that affects the template functions of DNA [8, 18]. Each defect can be eliminated only by a specialized enzyme, either by CPD photolyase (cyclobutane pyrimidine dimer photolyase) or by (6-4)-photolyase.

The term "cryptochrome" was proposed (even before the discovery of special proteins [89]) for receptors of the short wavelength (blue) region of the spectrum controlling physiological and morphogenetic processes. Just this term was given to the blue light receptor first discovered. Its structure occurred to be like that of DNA photolyases, and the term was attached to photoreceptors homologous to these enzymes [3, 15]. The inability of cryptochromes in photorepair of DNA was thought to be the most important difference between them and DNA photolyases, but this differentiation is rather conventional because cryptochromes were found (the so-called cry-DASH) that could cleaving cyclobutane dimers, but only in single-strand and not in double-strand DNA unlike canonic DNA photolyases [90]. Cryptochromes, which were initially discovered as photoregulators of plant ontogenesis, were shown to be also involved in the photocorrection of circadian rhythms including those in humans [15, 17, 91-94]. The signal conversion by a cryptochrome is associated with light-induced changes in protein conformation resulting in its activation either as a proteinase or as a transcription regulator [95-97]. During recent years these photoreceptors have attracted attention also as components of the mechanisms involved in the control of birds' and insects' orientation in magnetic fields [98-100].

The apoproteins of DNA photolyases and cryptochromes are monomeric polypeptides consisting of 500-750 amino acid residues (a.a.) (Fig. 4). Every polypeptide is noncovalently bound with two chromophores. $FADH^-$ (the anionic form of FAD-H_2) is an immediate participant of the catalytic act in photolyases, whereas in cryptochromes this role seems to be played by the oxidized form of FAD [101]. The other chromophore serves as an antenna from which the excitation energy migrates by the dipole–dipole resonance mechanism onto a catalytic flavin. The higher efficiency of photon absorption by the antenna molecule in DNA photolyases than by the reduced flavin increases tenfold the rate of catalysis. In the majority of photolyases and also in cryptochromes MTHF acts as a photoantenna. In some prokaryotes 8-HDF acts as an antenna of photolyases [8, 14, 16, 18, 20]. DNA photolyases are also found in which light is absorbed by the second flavin molecule [102].

Two domains are revealed in the DNA photolyase structure reconstructed based on results of amino acid sequencing and X-ray crystallographic analysis. On the N-terminus of the polypeptide there is the α/β domain (in canonical DNA photolyase from *E. coli* these are residues 1-131) and on the C-terminus there is a rather conservative 4α-helical domain (204-471 a.a.) with which the flavin molecule is bound (Fig. 5a). The domains are connected by an interdomain loop (132-203 a.a.) wrapped around the α/β domain [18, 19]. The flavin cofactor is located in the depth of the α-helical domain and contacts in it with 14 a.a., most of which are highly conservative [18]. The antenna MTHF is located in the interdomain slit and a glutamate residue is partially above the protein globule surface. The chromophore contacts with 12 a.a. (mainly phylogenetically nonconservative), of which Cys292 (in photolyase of *E. coli*) interacting with the positively charged imidazoline cycle MTHF and Lys293 interacting with its glutamyl residue are especially important. In photolyases containing 8-HDF, its molecule is inserted into the interdomain slit

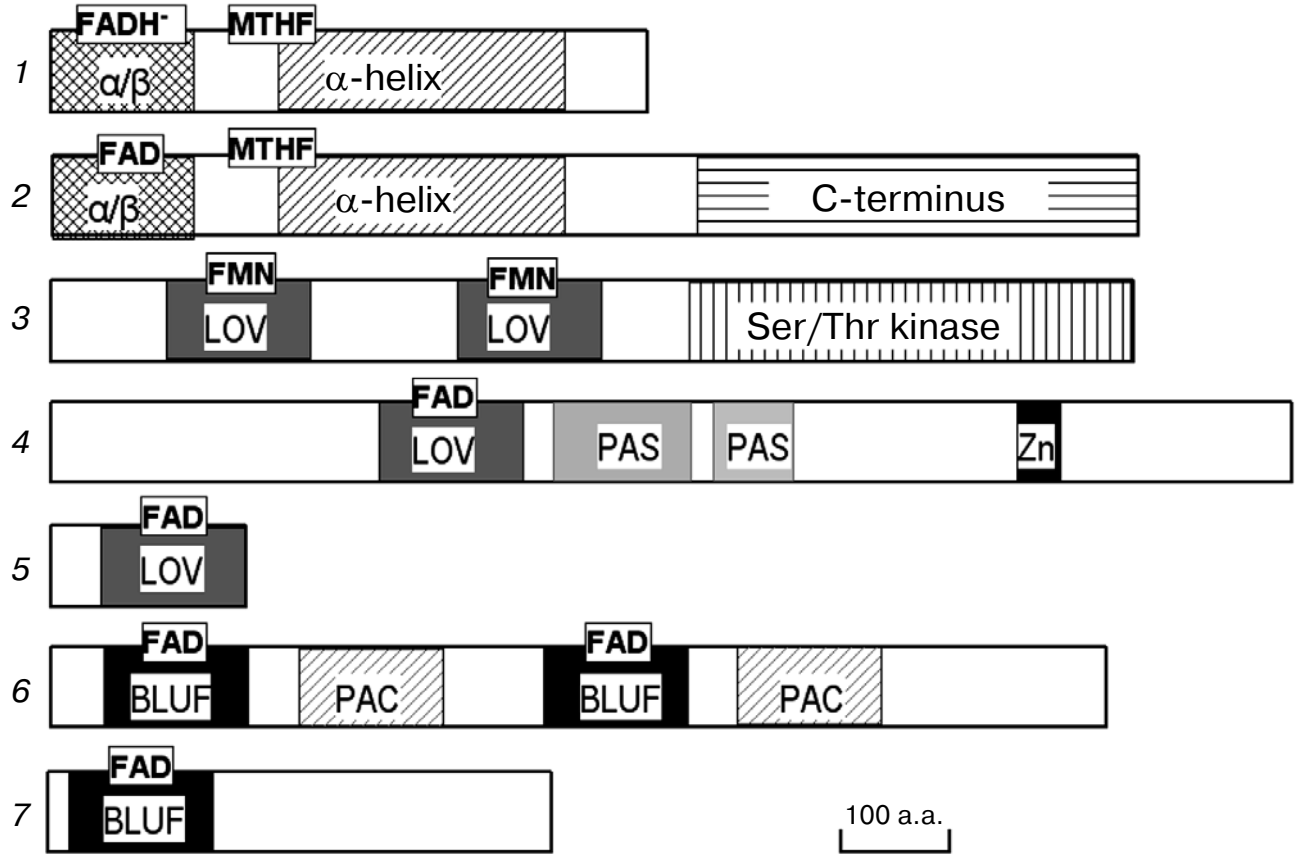

Fig. 4. Domain organization and chromophore-binding regions in photoproteins: *1*) DNA photolyase of *Escherichia coli*; *2*) cryptochrome cry-1 of *Arabidopsis thaliana*; *3*) LOV domain-containing protein (phototropin) nph-1 of *A. thaliana*; *4*) LOV domain-containing protein WC-1, which is a photosensitive component of the photoreceptor White Collar Complex (WCC) of *Neurospora crassa*; Zn, zinc finger; *5*) short LOV-containing photosensitive protein VVD of *N. crassa*; *6*) BLUF domain-containing photoactivated adenylyl cyclase of *Euglena gracilis* (PAC are regions with adenylyl cyclase activity); *7*) AppA is a light-dependent antirepressor of transcription of photosynthetic proteins of *Rhodobacter sphaeroides*.

deeper than MTHF, but the distance from both antenna types to the catalytic flavin is nearly the same (about 17 Å). MTHF is oriented nearly perpendicularly to the plane of the flavin dipole, and therefore its efficiency in energy transfer between chromophores is only 70% as compared to nearly 100% for the deazaflavin antenna oriented parallel to flavin [18].

The binding site of a damaged region of DNA is a positively charged groove on the surface of the helical domain with a pit in its central part corresponding in size to the Pyr<>Pyr dimer and with a flavin chromophore in the "bottom" [103]. Some amino acid residues forming this site (especially Trp277 in DNA photolyase of *E. coli* or its equivalents in enzymes of other organisms) are absent in cryptochromes. Moreover, polypeptides of cryptochromes contain on the C-terminus (after a.a. 500-550) a variable sequence that undergoes conformational changes during phototransduction.

Photoreceptor proteins containing LOV domains. These domains function in proteins as sensors of photons or of oxygen concentration or of voltage (thus, their name is the acronym of Light, Oxygen, Voltage) [104, 105]. LOV domains are a version of PAS domains (the term is formed from the first letters of the gene names (*per, arnt, sim*) in the protein products of which the domains were first detected) distributed in receptor and regulatory proteins of prokaryotes and eukaryotes [106]. They consist of 100-120 a.a. and include a characteristic highly conservative motif GXNCRFLQ and alternating α and β regions ($\beta_A\beta_B\alpha_C\alpha_D\alpha_E\alpha_F\beta_G\beta_H\beta_I$) (Fig. 5b) [107]. The large group of LOV domain-containing proteins, in addition to their own phototropins, i.e. light receptors of plant phototropism, also includes other photoregulators of ontogenesis of plants and algae, as well as proteins of heterotrophic eubacteria [104-114].

Both LOV and PAS domains are also present in photoreceptors regulating ontogenesis and adaptation in fungi. These studies were started by discovery in *Neurospora crassa* cells of a heterodimeric White Collar Complex (WCC), which controls physiological, morpho-

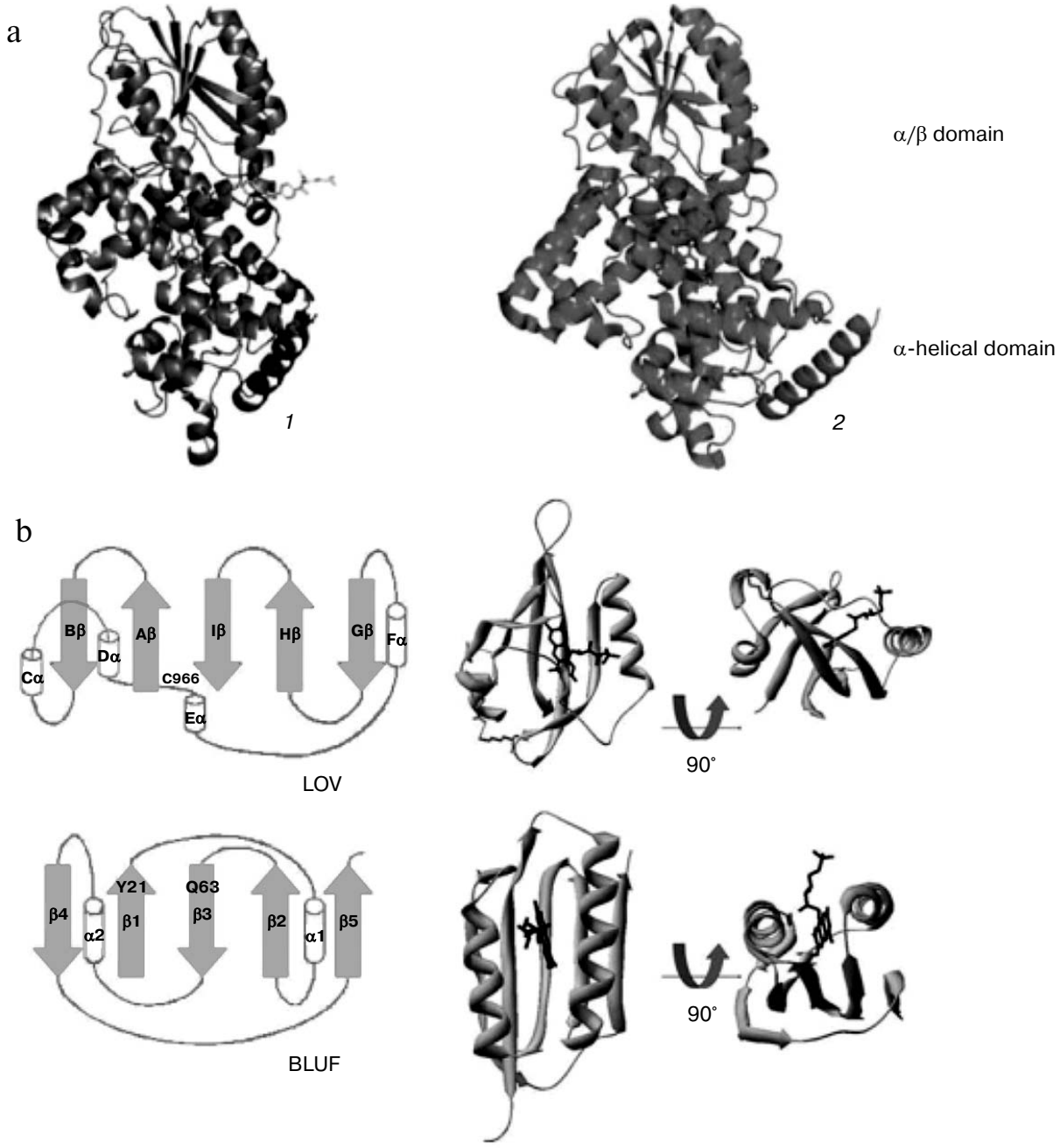

Fig. 5. Scheme of domain organization (a) of DNA photolyases and cryptochromes: *1*) DNA photolyase of *E. coli*; *2*) cryptochrome CRY-1 of *A. thaliana* (after [20]). b) Ribbon diagram of LOV and BLUF domains (after [22]).

genetic, and chronobiological reactions of this organism to light [4, 5, 115, 116]. The complex consists of products of the genes *white collar-1* and *white collar-2*, and they both contain PAS domains involved in the interaction of polypeptides, but only WC-1 contains a LOV domain, which functions in a complex with FAD as a photon sensor [117]. Along with the photoreceptor WCC, another light-sensitive flavoprotein, the product of the *vivid* gene, participates in light-dependent resetting of the circadian clock controlling conidiogenesis. This short 187-a.a. protein is a nearly pure LOV domain *per se* [118]. Other fungi also have photoproteins containing LOV domain, e.g. the product of the *mad A* gene, controlling reactions of *Phycomyces blakesleeanus* to light [119, 120].

Regulatory functions of proteins containing LOV domains in different organisms are different. Phototropins are serine–threonine or histidine kinase protein kinases activated only upon excitation by light of the flavin photosensor in the LOV domain [107]. As discriminated from them, effector domains of some fungal pho-

toreceptors contain the so-called zinc fingers, and this suggests their functioning as transcription factors interacting with GATA-sequences of the gene promoter regions [116-121]. Despite variations in the primary structure, LOV domain maintains the same plan of the spatial organization in proteins of various biological origin and do not lose activity even upon "transplantation" from one photoreceptor protein of this group into another (e.g. from the *A. thaliana* phototropin into WCC of the *Neurospora*), which is accompanied by replacement of the FAD chromophore by FMN [122].

Proteins containing BLUF domains. These domains found in proteins of photosynthesizing and heterotrophic prokaryotes and in lower eukaryotes (euglenoids) are denoted in capital letters: Blue Light sensing Using FAD. These short domains (100-110 a.a.) in complex with FAD form a photon sensor [6, 7, 19, 123-125]. They differ from LOV domains in specific features of secondary structure ($\beta_1\alpha_1\beta_2\alpha_2\beta_4\beta_5\alpha_3$ instead of $\beta_A\beta_B\alpha_C\alpha_D\alpha_E\alpha_F\beta_G\beta_H\beta_I$) (Fig. 5b) [22]. Excitation of the chromophores bound with these domains can activate in different proteins effector domains, which trigger various cascades of photosignal transformation. Thus, in purple bacterium *Rhodobacter sphaeroides* cells the BLUF domain functions in the protein AppA, which is an antirepressor of genes of the photosynthetic apparatus, i.e. this domain regulates gene expression. In other proteins excitation of BLUF domain leads to changes in the cAMP level: in *Euglena gracilis* cells these domains are components of a light-dependent adenylyl cyclase [124], and in *Klebsiella pneumoniae* cells they control the activity of cyclic nucleotide phosphodiesterase [125]. Some data indicate that activation of the effector domain is a result of mechanical changes in the polypeptide chain structure photoinduced by the BLUF domain [124, 125].

Photocycles of flavin chromophores in proteins. Although functioning of all photoproteins under consideration is associated with electron transfer reactions induced by excitation of flavin, the photochemical mechanism is specific in each group. In the case of DNA photolyases the chromophore photoexcitation is a prerequisite for catalysis (the binding of the enzyme with the damaged region of DNA does not require light) [8, 20, 126]. During the catalytic act a singlet-excited molecule $^1FADH^-$ donates an electron required for breaking C–C bonds in the cyclobutane dimer or in the 6-4-product and transforms into the free radical state $FADH^{\cdot}$ (Fig. 6). Upon recovery of the normal structure the electron comes back onto the active center flavin and regenerates it into the initial state of $FADH^-$. In cryptochromes a singlet-

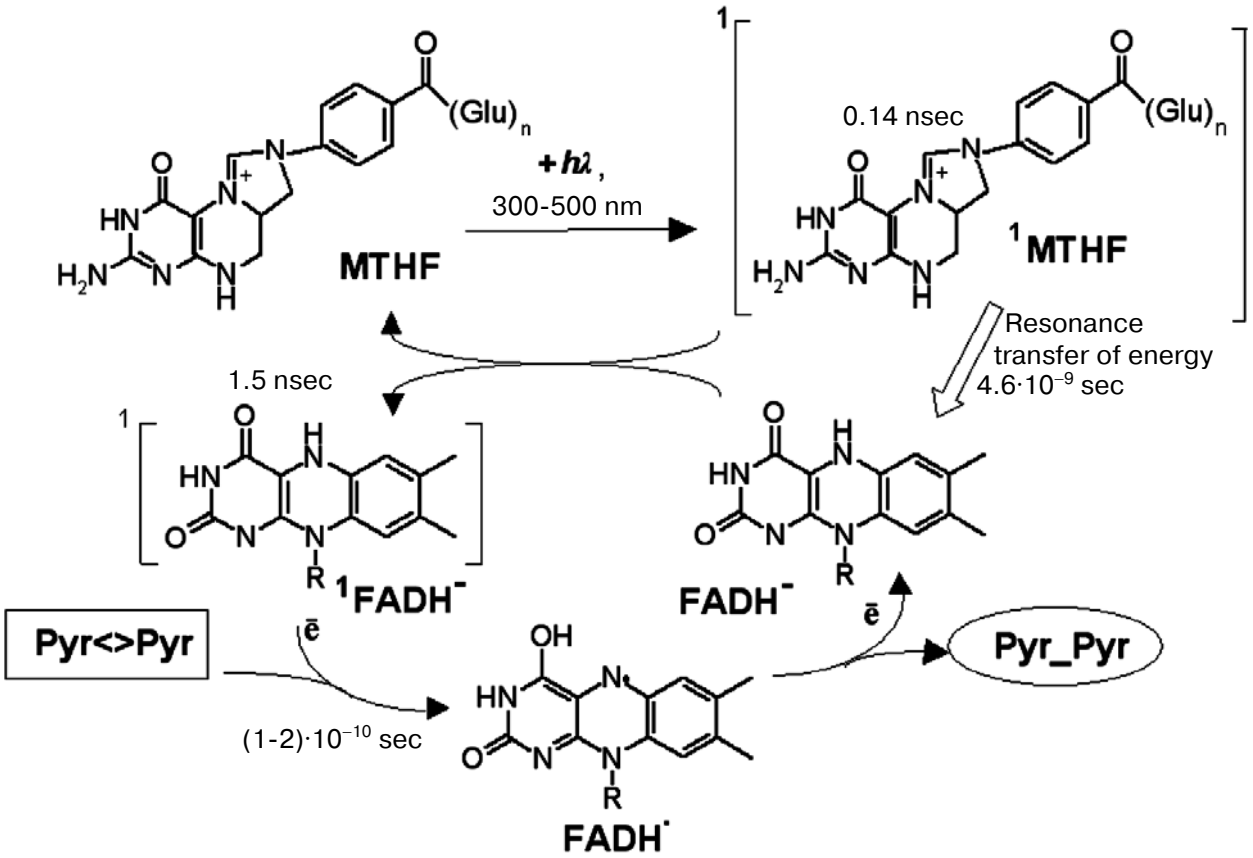

Fig. 6. Photocycle of flavin in DNA photolyase of *E. coli*.

excited flavin is active, and in proteins of different origin different redox states of FAD seem to function (from FAD to $FADH^-$). The change in the flavin reduction level during the photocycle is also characteristic for cryptochromes. As differentiated from DNA photolyases specified by the cyclic electron transfer (after reparation of the damaged structure, e.g. of the Pyr<>Pyr dimer, the electron from $FADH^-$ comes back onto the same flavin molecule), the photocycle of cryptochromes requires an external source of electrons, and tyrosine and tryptophane residues in the apoprotein are likely to be such sources [127, 128]. The photocycle of cryptochromes is accompanied by generation of free radical forms of flavin, including a radical pair consisting of FAD and a tryptophane residue. This radical pair is thought to be also involved in cryptochrome functioning as a magnetoreceptor [98, 129]. The photoregulatory function of cryptochromes in the organism seems to be realized differently in different proteins of this class [111].

Because reduced flavins poorly absorb light, the excitation of $FADH^-$ *in vivo* occurs as a result of the resonance energy transfer from an antenna chromophore with higher molar extinction coefficient than $FADH^-$, i.e. by MTHF, 8-HDF, or by an oxidized flavin [18, 19]. The antenna function imparts a pronounced UV-A component in the spectra of some cryptochrome-controlled photophysiological processes, and this suggests that the folate antenna can be a source of the excitation [131-133].

Studies on the photochemical process in BLUF domains (mainly in AppA and also in the domain Slr 1694 of the cyanobacterium *Synechocystis* PCC 6803) have led to conclusion that the photocycle in the BLUF domain is based on reduction of the singlet-excited molecule with a successive production of free radical forms different in proton binding, i.e. of anionic and neutral flavin radicals. Electrons and protons of amino acid residues of the BLUF domain are also involved in the process, e.g. the residues Y8 and Q50 in Slr 1094 (Fig. 7a). The transfer of electrons from tyrosine and glutamine residues onto the flavin radical is accompanied by protonation of the N5 atom of isoalloxazine. The resulting reorganization of the hydrogen bond system and also a reversible isomerization of the glutamine residue lead to changes in the domain structure that are significant for generating a signal, the development of which can be associated with changes in the quaternary structure of the protein. The efficiency of generation and the involvement of triplet molecules are low [136-138].

As differentiated from BLUF domains and the family of DNA photolyases and cryptochromes, photoprocesses in LOV domains are based on the activity of the triplet state of flavin (Fig. 7b). The excitation of FMN in LOV domains of plant phototropins generates a metastable thiol adduct consisting of the C(4a) atom of isoalloxazine and a cysteinyl residue of the LOV domain (in the first crystallized LOV domain from Phy-LOV2 phytochrome-phototropin photoreceptor of the fern *Adiantum capillus-veneris* it is the Cys966 residue). The two-electron photoreduction is accompanied by generation of free radical intermediates [22, 109, 138-144]. As in the case of BLUF domains, a domain with the bound chromophore slowly (within seconds to minutes) relaxes into the initial state [105, 145].

The detection of a photocycle realizing the energy of a triplet-excited pigment is interesting from the stand-

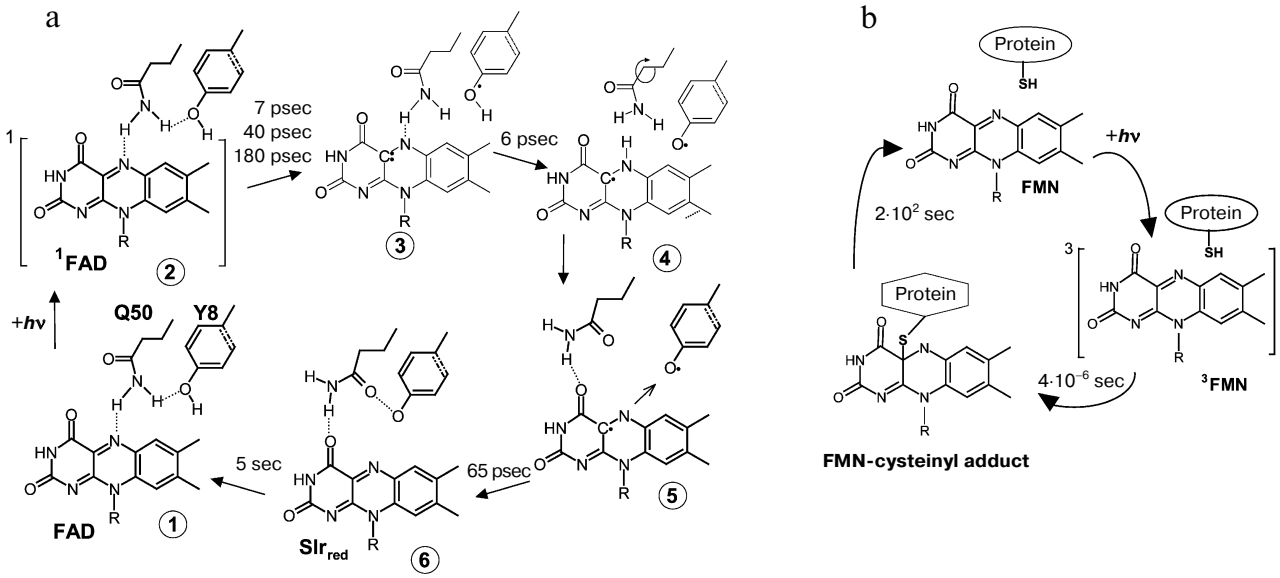

Fig. 7. Flavin photocycles in BLUF domain (Slr-BLUF domain of *Synechocystis*) [134, 135] and in LOV domains [105].

point of evolution. The longer lifetimes of triplets than of excited singlets allow them to participate in chemical reactions in unorganized media, e.g. in solutions. But in contemporary organisms the dominant position belongs to processes with involvement of short-living singlet-excited molecules (first of all, these processes are chlorophyll-mediated photosynthesis and reception of photons by retinal-binding proteins), which are functioning only with ordered spatial distribution of participating molecules on protein and membrane matrices.

PROBLEMS OF EVOLUTION OF PHOTOPROTEINS WITH COENZYME CHROMOPHORES

Evolutionary analysis of apoproteins. Photoproteins with coenzyme chromophores are found in representatives of all kingdoms of the organic world. These proteins and also their chromophores are water soluble molecules. As a rule, their polypeptide chains are not longer than 1000-1100 a.a. Hybrid proteins, which in addition to the LOV domain have another photosensitive domain capable of binding phytochromobilin (e.g. a photoreceptor *phy-3* from the fern *Adiantum capillus-veneris* [105]), are longer.

DNA photolyases and cryptochromes function as monomers. Proteins containing LOV and BLUF domains display a tendency for dimerization, and both this and the reverse process are associated with the regulatory functions of the protein. Thus, in the WCC complex of the *Neurospora* a photoreceptor polypeptide WC-1 containing the LOV domain is inactive in the absence of the polypeptide WC-2 lacking the LOV domain, i.e. it is photochemically inactive but, similarly to WC-1, it contains a PAS domain determining the production of an active dimer [115, 116].

The diversity of primary structures, including those of the chromophore-binding domains, suggests the repeated appearance in metabolism of flavin photocatalysis during evolution. Studies of their relation with evolution of other proteins are fragmentary. Thus, a remote relation is found between DNA photolyases and other nucleotide-binding proteins, including aminoacyl-tRNA synthetases (class I) and, possibly, with some electron-transporting flavoproteins [10, 146]. The discovery in heterotrophic eubacteria of polypeptides with LOV domains homologous to phototropins [19, 21, 22] was important for searching for ancient prototypes of proteins containing a photochemically active flavin.

Groups within families, e.g. DNA photolyases of microbial and animal origin, are rather far from one another except for the sequence of about 150 a.a. on the C-terminus that includes the FAD-binding domain [18]. Based on sequencing data, it was suggested that cryptochromes functioning in animals and plants should be results of convergent evolution – their ancestors were different groups of DNA photolyases [18, 20]. The primary structures of LOV domain-containing proteins of plants and fungi are rather different except for certain strictly fixed conservative residues.

Some secondary structure motifs typical for evolutionarily relict proteins are present in photoproteins. Thus, an elongated region consisting of five parallel β-structures in the α/β domain of DNA photolyases has analogs in flavodoxins. The $\beta_1\alpha_1\beta_2\beta_3\beta_2\beta_4\beta_5$ sequence found in the BLUF domain is also found in ferredoxin [22].

Search for selective characteristics of coenzymes of the photosensor role in proteins. To analyze photoprotein evolution it is important to know what structural and functional features promoted the bifunctionality of FMN, FAD, 8-HDF, and MTHF, i.e. their involvement in "dark" biocatalysis and in light reception. Why has evolution chosen just these molecules for participation in photoprocess, and might other coenzymes be shown to function as photosensors?

Let us analyze only those compounds that are structurally similar to nucleotides and function as coenzymes in biocatalysis. The group under consideration includes oxidized and reduced forms of flavin, nicotinamide, and pterin coenzymes (including single-carbon derivatives of tetrahydrofolic acid), purine and pyrimidine ribonucleoside phosphates. Moreover, the group includes thiamine pyrophosphate and pyridoxal phosphate and also pterins, which lack coenzyme functions. Let us sketch molar extinction coefficients of these compounds in the long-wavelength absorption maxima (ε_{max}) as a function of this maximum wavelength (λ_{max}) (Fig. 8a). To exclude compounds that are unlikely to participate, we have introduced two limitations (in the plot they are indicated by dotted lines). We exclude compounds with the λ_{max} values lower than the physiological reception region of the spectrum (below 330 nm) and establish the lower limit of the ε_{max} value allowing the molecule to serve as an effective photosensor. This limit is between the ε_{max} value for oxidized flavins functioning as photosensors and its value for dihydroflavins [147, 148], which depend on migration onto them of the excitation energy from the antenna molecule of MTHF or of 8-HDF. The introduction of these limitations eliminates virtually all compounds but the earlier found in proteins FMN, FAD, MTHF, and 8-HDF (except the metabolically less active sepiapterin). The revealed regularity indicates that other coenzymes are unlikely to function in photoproteins whose activity strictly depends on excitation of the chromophore group.

Structural features of MTHF as selective characteristics. The significance of structural features of the coenzyme molecule for its functioning as a photosensor can be illustrated using the example of the structure of MTHF, which acts as a coenzyme in metabolism of single-carbon groups and also as an antenna pigment of DNA photolyases and cryptochromes. MTHF can function as an

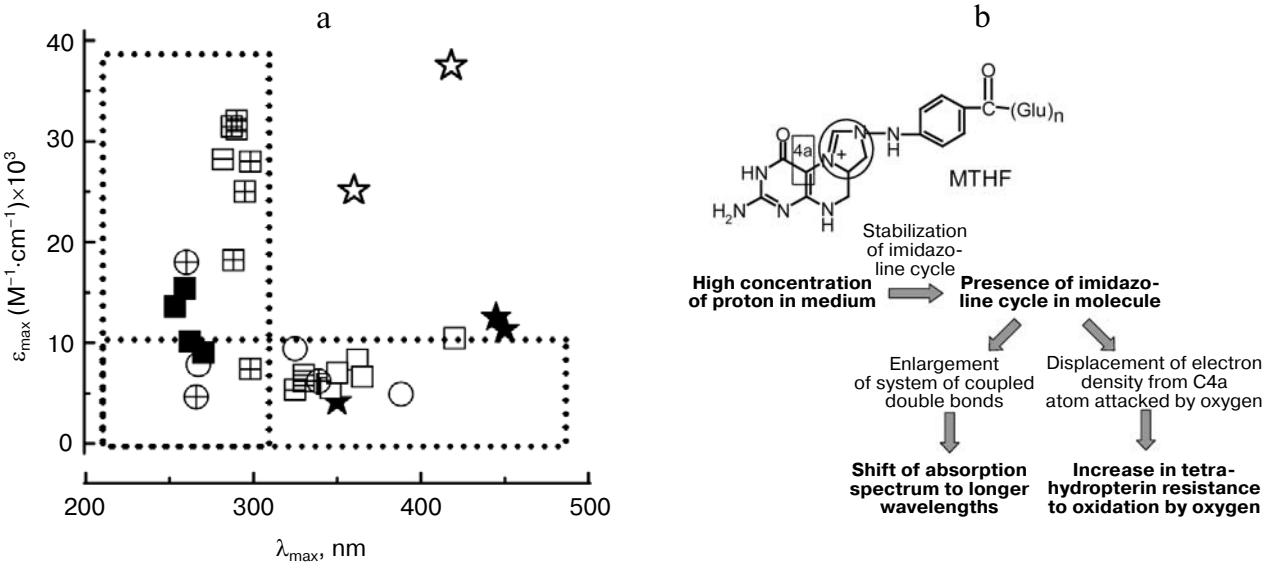

Fig. 8. Selective characteristics of coenzymes and related molecules for the role of chromophores in photoproteins. a) Value of molar extinction coefficient (ε_{max}) as a function of absorption maximum wavelength (λ_{max}) of coenzymes and structurally similar biomolecules. Values of long-wavelength absorption maxima are plotted for flavin coenzymes and their reduced forms (closed asterisk), MTHF and 8-HDF (open asterisk), purine and pyrimidine ribonucleotides (closed square), nicotinamide coenzymes and their reduced forms (open circle with a cross), pterins (open squares), dihydro-forms of pterins and folates (open square with a horizontal line), monocarbon derivatives of tetrahydrofolic acid (except MTHF) (open square with a cross), coenzyme derivatives of thiamine and pyridoxine (open circle). The dotted frames show zones of wavelengths and molar absorption coefficients that prevent functioning of physiological light receptors (see text). b) Structural features of MTHF molecule determining its photochemical properties. The dotted frames show imidazoline cycle and the C_{4a} atom of the pterin heterocycle.

antenna due to the combination of high ability to absorb photons ($\varepsilon_{max} = 2.5\cdot10^4$ $M^{-1}\cdot cm^{-1}$) in the UV-A region ($\lambda_{max} = 360$ nm and $\lambda_{max} = 390$ nm within a photoreceptor), i.e. in the physiologically perceptible region of the spectrum with resistance to oxidation and photolysis [149, 150]. These features distinguish MTHF from the majority of coenzymes including other tetrahydrofolates. First of all, these features are due to the presence of the imidazoline heterocycle (Fig. 8). The presence of the imidazoline ring supplements the system of coupled double bonds of the tetrahydropteridine cycle, which shifts the absorption maximum of the molecule by 50-60 nm to longer wavelengths relative to other tetrahydrofolates. Moreover, the displacement of electron density from the C4a atom (the site of interaction of tetrahydropteridine with oxygen) to the positively charged atom N5 of the imidazoline cycle increases the resistance of MTHF against oxidation. In turn, the imidazoline cycle integrity depends on the proton concentration in the medium: at solution pH higher than 3.0 it degrades, and the molecule loses this unique combination of the features. And a high concentration of proton supports the resistance of the tetrahydropteridine heterocycle to oxidation and photolysis [149, 150]. Thus, the features necessary for participation in photoreception are provided for by the complex of interrelated specific structural features of the molecule.

Considering the evolution of coenzyme catalysis, we can only postulate the significance of photoreactions with involvement of flavins and other coenzymes for formation of a primitive metabolism and also notice the similarity of abiogenic reactions with processes in contemporary proteins. Until now it was not possible to determine a precursor of any photoprotein family. Such an ancient group of flavoproteins as flavodoxins are of little value for such studies because flavins in their molecules are chemically less active, and their activity is not associated with physiological functions [151, 152].

Involvement in photoreception is not the only example of metabolic bifunctionality of coenzyme molecules. Remember only the role of nucleoside phosphates, i.e. substrates of RNA biosynthesis, in the enzyme-mediated transfer of various chemical groups. This bifunctionality seems to be commanded by economical expenditure by the cell of resources that are objects of competition between different physiological processes [153-155]. Because of such a competition the creation of a pathway for biosynthesis of a specialized chromophore can be less advantageous than using of a not ideal but already available chemical material.

Features of excited molecules of isoalloxazines and pteridines are now claimed by evolution of technologies. An intense fluorescence has attracted the attention to these compounds as markers for studies on genes and

protein products of gene expression. Thus, purine analog synthetic pteridines are incorporated for this purpose into DNA sequences [156-158]. The fluorescent complex of FMN with LOV domain is proposed as a more advantageous label than GFP (green fluorescent protein) [159]. The photochemical activity of coenzymes is also used in technology. Thus, the photoreduction of the folic acid pterin heterocycle became a basis for a new approach for synthesis of the coenzyme 5-formyl tetrahydrofolate, which as calcium folinate is used in the treatment of oncologic diseases [160]. And, finally, the combination of flavin (or pterin) with a high potential electron donor is used as a light-dependent injector of electron into redox proteins, e.g. in the ETC of multiheme cytochromes, in projects directed to creation of molecular machines [161-166].

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