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Key Residues for the Light Regulation of the Blue Light-Activated Adenylyl Cyclase from *Beggiatoa* sp.

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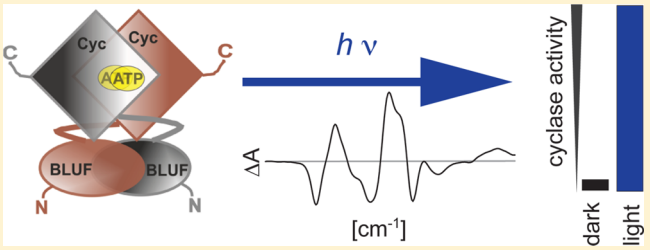
Supporting Information

ABSTRACT: Photoactivated adenylyl cyclases are powerful tools for optogenetics and for investigating signal transduction mechanisms in biological photoreceptors. Because of its large increase in enzyme activity in the light, the BLUF (blue light sensor using flavin adenine dinucleotide)-activated adenylyl cyclase (bPAC) from *Beggiatoa* sp. is a highly attractive model system for studying BLUF domain signaling. In this report, we studied the influence of site-directed mutations within the BLUF domain on the light regulation of the cyclase domain and determined key elements for signal transduction and color tuning. Photoactivation of the cyclase domain is accomplished via strand β5 of the BLUF domain and involves the formation of helical structures in the cyclase domain as assigned by vibrational spectroscopy. In agreement with earlier studies, we observed severely impaired signaling in mutations directly on strand β5 as well as in mutations affecting the hydrogen bond network around the flavin. Moreover, we identified a bPAC mutant with red-shifted absorbance and a decreased dark activity that is highly valuable for long-term optogenetic experiments. Additionally, we discovered a mutant that forms a stable neutral flavin semiquinone radical in the BLUF domain and surprisingly exhibits an inversion of light activation.



The widespread application of optogenetics has stimulated the demand for photoregulated enzymes to modulate signaling messengers like cyclic nucleotides or inositides.[1,2] Besides the synthetic rhodopsin-based GPCR (Optoβ_2XR) and the GαS-coupled opsin from the jellyfish *Carybdea rastonii* being used in only a few cases, the *Euglena gracilis* photoactivated adenylyl cyclase (euPAC) has been widely used to specifically modulate intracellular cAMP levels.[1,3−5] Recently, we introduced a 350-amino acid short PAC from the sulfide-oxidizing bacterium *Beggiatoa* sp. as an alternative cAMP-manipulating tool.[6,7] bPAC encompasses an N-terminal BLUF (blue light sensor using flavin adenine dinucleotide) and a C-terminal class IIIb adenylyl cyclase. A similar design was recently reported for a LOV (light−oxygen−voltage) domain-regulated AC.[8] The catalytic active site of these cyclases is formed by a homodimer of two cyclase domains (Figure 1A). Functional expression of bPAC was demonstrated in *Escherichia coli*, *Xenopus laevis* oocytes, *winstar* rat neurons, and *Drosophila melanogaster* (see below). Compared to euPAC, bPAC features a reduced basal activity in the dark, a >100-fold increased activity in the light, and a significantly smaller size, which is advantageous for delivery and expression in heterologous systems. So far, bPAC has been successfully utilized to study cAMP-dependent signaling in various cell types and whole animals. Elevation of the cellular cAMP level increased antibiotic susceptibility and the level of biofilm formation in *E. coli*.[9] cAMP-related behavioral responses were studied using bPAC even in whole animals like zebrafish.[10] The combination with conventional pharmaceutical cAMP stimulants allowed the resolution of the physiologically different functions of the cAMP effectors PKA and EPAC in the *Drosophila* renal system.[11] Furthermore, the approach was expanded to a symbiotic parasite−host system to control parasite or host cAMP levels independently. Specific expression in *Toxoplasma gondii* revealed that cAMP affects host cell invasion, stage-specific expression, and parasite differentiation.[12]

Besides the optogenetic application of photoregulated enzymes, these modularly composed photoreceptors are perfect objects for studying general principles of sensor/effector domain communication and intramolecular signal transduction. BLUF domains (Figure 1B) regulate various effector domains, but most of their signal transduction mechanisms are poorly understood.[13] Although their photoinduced reactions have been studied in detail, the controversial discussion about the nature of the signaling state, which is characterized by a mere hydrogen bond rearrangement around the flavin, persists.[14] Moreover, especially the difficulty in preparing full-length photoreceptors in high yields and with high quality, which also have a large dynamic range of light-dependent activity, has limited the study of BLUF interdomain signaling so far.

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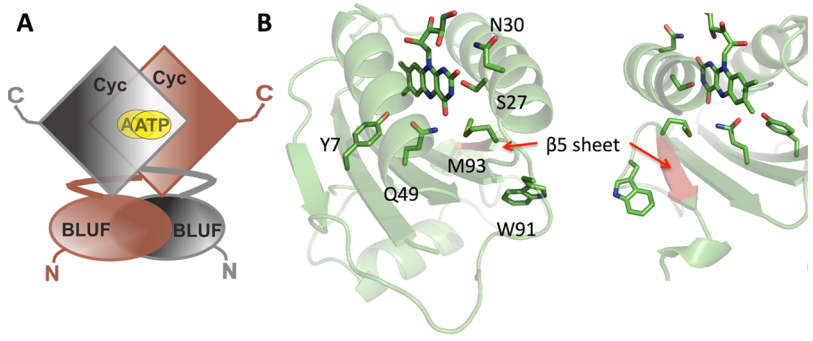

Figure 1. (A) Suggested domain organization of blue light-activated adenylyl cyclase based on the pH-activated mycobacterial cyclase Rv1264. (B) Homology model of the bPAC BLUF domain based on the X-ray structure of Tll0078. The structure on the right is rotated to illustrate the position of sheet β5 (red).

Theoretical calculations and structural analyses of AppA, BlrP1, YcgF, and Slr1694 generally suggest that light-induced movements of the β4−β5 loop and sheet β5 are transferred to C-terminal cap helices α3 and α4 (Figure 1B).[15−19] In this regard, the functional contribution of two residues in the signal transduction process has been discussed controversially since the first X-ray structures of BLUF domains were determined.[20−25] In the crystal structures, both the conserved methionine (M93 in bPAC) and the semiconserved tryptophan (W91 in bPAC) are present in an inward and outward orientation. Because of their linkage to the hydrogen bond network surrounding the cofactor and their location in the β4−β5 loop, a signaling residue function was proposed. The first studies described a functional photoswitch of tryptophan in the BLUF protein AppA.[26,27] Recent investigations indicate only minor light-induced movements.[28,29] Moreover, methionine but not tryptophan was shown to be essential for the light-regulated interaction of Slr1694 (PixD) with its downstream partner and implicates a minor role of tryptophan for signal transduction in this case.[19,30] Besides their putative involvement in signal transduction processes, tryptophan mutations are known to affect the stability of the signaling state and thereby the speed of dark recovery. While the replacement of tryptophan with alanine in all so far investigated BLUF domains led to an acceleration of the recovery kinetics, the substitution of tryptophan with phenylalanine induced opposite effects in different BLUF domains: the W104F mutation in AppA resulted in an ∼150-fold faster decay rate,[31] and the corresponding replacement in Slr1694 (W91F) slowed the reaction by a factor of ∼20.[32]

Besides these rather large-scale structural changes, essential residues like the conserved tyrosine and the glutamine residues participate in light-induced proton-coupled electron transfer to the cofactor and are crucial for the formation of the signaling state.[23,33] Other less conserved residues in BLUF domains tune the cofactor environment and define its sensitivity to light.[34]

The availability of recombinant full-length protein with strong light-modulated enzyme activity makes bPAC an attractive model object for studying signal transduction in BLUF photoreceptors. Signal generation in the BLUF sensor can be monitored conveniently by UV−vis or vibrational spectroscopy and correlated to biochemically determined enzyme activity. Here, we studied the effect of several key residues within the BLUF domain and its effect on AC activity. We selected six specific amino acid residues for site-directed mutation, on the basis of their previously attributed photochemical, kinetic, and spectral relevance. In detail, we aimed to obtain bPAC variants with accelerated and reduced BLUF recovery kinetics to modulate the dynamic range of bPAC as well as variants with modified absorbance spectra to allow for a red-shifted stimulation. Furthermore, we investigated photochemically inactive mutants to address their influence on the dark state of bPAC.

EXPERIMENTAL PROCEDURES

Molecular Biology and Protein Purification. The expression construct for the *E. coli* codon-optomized full-length bPAC without SUMO fusion was cloned in frame behind the N-terminal His_6 tag of pET28a(+) using restriction sites *Nde*I and *Hin*dIII (kindly provided by W. Gärtner, MPI Mühlheim, Mühlheim, Germany). The expression construct for the bPAC BLUF domain (residues 1−124) was amplified from the same gene using oligonucleotides 5′-GGTATTGGATCC-CATATGATGAAACGCCTGGTGTATATTAGC-3′ and 5′-TTTTTTAAGCTTCTATTTTTCCAGCACACGATGGCT-CTGG-3′ and ligated in frame behind the N-terminal His_6 tag of pET28a(+) using the same restriction sites. The expression plasmid for the bPAC cyclase domain, henceforth named pASK43p-cyc, was constructed by PCR amplification of the *E. coli* codon-optimized bPAC gene from residue 97 to 350, using oligonucleotides 5′-AAAAGAATTCAACCTGAACGAAAAC-AGC-3′ and 5′-GTCTATTCTCGAGTGCGGCCGCAAGC-3′. The PCR product was cloned in frame behind the N-terminal His_6 tag of pASK43p (IBA, Göttingen, Germany) using restriction enzymes *Eco*RI and *Xho*I.

Point mutations were introduced into the previously described pET-SUMO vector encoding the *E. coli* codon-optimized bPAC gene[6] by site-directed mutagenesis (QuickChange II XL Kit, Stratagene), according to the supplier's instructions. Recombinant bPAC and variants were expressed and purified as described previously.[6] For the preparation of FTIR samples, constructs without the SUMO fusion protein were expressed from the pET28a(+) constructs in strain BL21-IS, which additionally harbors a plasmid expressing GroES (kindly provided by I. Schlichting, MPI for biomedical research, Heidelberg, Germany). Expression of pASK43p-cyc was induced with 200 μg/mL anhydrotetracycline and purified in the same way. Unless stated otherwise, all proteins were kept in aqueous buffer containing 50 mM sodium phosphate (pH 7.5), 300 mM sodium chloride, 0.1 mM phenylmethanesulfonyl fluoride, and 5 mM β-mercaptoethanol. Protein concentrations were determined via UV−vis absorption of the flavin S_1 absorption band (ε_{450} = 11500 M^{-1} cm^{-1}) or the calculated absorption coefficient of aromatic side chains (using ε_{280} values

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of 5500 and 1490 M^{-1} cm^{-1} for W and Y, respectively) for the cyclase domain alone.

***In Vitro* Enzyme Assay.** Enzyme assays were performed at 21 °C (Eppendorf Thermomix) under dim red light in black vessels (Rotilabo, Roth). The assay mixture contained 50 mM sodium phosphate (pH 8), 2 mM magnesium chloride, 10% glycerol, and 100 μM ATP (Fermentas). The reaction was started by addition of 0.19 μM protein. Aliquots were either kept in the dark or irradiated with blue light (LED Luxeon Star O, 456 nm, effective intensity of 1.7 mW cm^{-2}) for 3 min and inactivated by being heated to 95 °C for 10 min. Light-induced heating of the sample was prevented using a heat filter (KG 3, 2 mm, Schott, Mainz, Germany) between the vessel and light-emitting diode (LED). Under these conditions, the temperature remained constant (K-type probe, STE-KB-0.5-250, Rössel Messtechnik, Werne, Germany) inside the reaction vessel. Before nucleotide analysis, precipitates were removed by centrifugation at 16000*g* and 4 °C and filtered through a 0.2 μm nylon filter (Acrodisc 13 mm, Life Sciences). For conditions that included a higher rate of substrate turnover, 5-fold more protein was added to the mixture.

Nucleotide Determination. Nucleotide analysis from *in vitro* enzyme assays was performed using high-performance liquid chromatography (HPLC) (Smartline Autosampler 3600, WellChrom Degasser K-5004, WellChrom Solvent Organizer K-1500, WellChrom HPLC-pump K-1001, WellChrom spectrophotometer K-2600, Knauer, Germany). Nucleotides were separated in an isocratic manner on a C18 reversed-phase column (Supelcosil LC-18-T, 150 mm × 4.6 mm, 3 μm pore size, Supelco) in 100 mM potassium phosphate, 4 mM tetrabutylammonium chloride, and 10% (v/v) methanol (pH 5.9) and detected at 253 nm.

UV−Vis Spectroscopy. Dark-adapted spectra, transient absorption spectra, and single-wavelength kinetics of recombinant bPAC and variants were recorded under aerobic conditions at a flash photolysis setup or in a Cary300bio UV−vis spectrometer (Varian, Palo Alto, CA) as described previously.[6]

Fluorescence Spectroscopy. Fluorescence lifetime measurements were taken with second-harmonic light pulses of a mode-locked titanium sapphire laser system (Hurricane from Spectra-Physics, pulse duration $Dt_P \approx 3$ ps, wavelength $I_P = 400$ nm) and a microchannel plate photomultiplier (Hamamatsu type R1564U-01) connected to a fast digital oscilloscope (LeCroy type 9362). The time resolution of the detection system was approximately 500 ps.

FTIR Difference Spectroscopy. Protein samples, provided in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 2.5 mM EDTA, 100 mM NaCl, 2 mM DTE, and 10% glycerin, were concentrated to an OD_{441} of ∼70−100; ∼2−5 μL was placed between two CaF_2 plates without a spacer, and the plates were sealed with silicon grease for tightness. A dehydrated sample was prepared using a gentle stream of N_2 applied to the sample on one CaF_2 window before the cell was closed and sealed. FTIR spectra between 1800 and 1000 cm^{-1} were recorded using a Bruker IFS66s spectrometer with 3 cm^{-1} resolution. Light-minus-dark difference spectra were generated by recording 100 scans of the background without application of blue light and 100 scans with application of blue light (LED Luxeon, 1 W, 456 nm, effective intensity of 20 mW cm^{-2}). To estimate the experimental drift during the measurement, 100 scans of the background and 100 scans of the sample were recorded without application of blue light to generate a dark-minus-dark difference spectrum. The results of 10 experiments of each light-minus-dark and dark-minus-dark spectra were averaged, and the resulting data sets were subtracted to correct for experimental drift.

RESULTS

Photoactivation of bPAC Induces Structural Changes in the Cyclase Domain. So far, no molecular structure of bPAC is available to describe the interaction of the BLUF sensor and cyclase effector domain in detail. Here, we investigated the structural changes induced by light using light-minus-dark FTIR difference spectroscopy. The bPAC BLUF domain lacking the cyclase domain and the full-length protein were compared to describe local structural changes in the BLUF domain and induced changes in the cyclase domain (Figure 2). To minimize nonfunctional protein absorption in the infrared, we used bPAC samples lacking the SUMO fusion. The full-length protein and BLUF domain are prepared as monodisperse proteins in dimeric form (Figure S1 of the Supporting Information). Dark recovery of the BLUF domain alone is similar to that of the full-length protein (Figure S2 of the Supporting Information). All FTIR difference spectra feature the characteristic carbonyl signature at 1710(−)/1694(+) cm^{-1}, which is mainly attributed to the newly formed hydrogen bond to the C4═O carbonyl of the flavin from the conserved glutamine in the light-adapted state.[26,35−39] In the secondary structure region between 1700 and 1600 cm^{-1}, clear differences are observed between the full-length protein and BLUF domain alone (Figure 2B). The overall shape of the difference spectrum of the bPAC BLUF domain (Figure 2, black) is similar to that of a corresponding spectrum of the AppA BLUF domain.[26] The signal around 1623(+) cm^{-1} most likely corresponds to the 1632(+) cm^{-1} signal in AppA that was assigned to strand β5, which harbors the crucial methionine residue and an important, semiconserved tryptophan residue.[20,21,24,26] In the steady state FTIR difference spectrum of AppA-W104F, this signal is lost but might still occur transiently

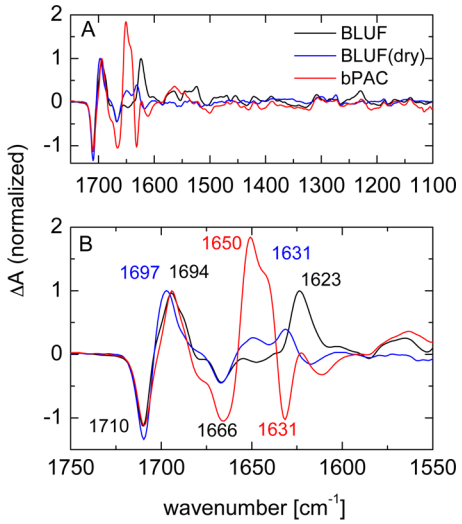

Figure 2. Light-minus-dark FTIR difference spectra of the bPAC BLUF domain without the cyclase domain under hydrated (black) and partly dehydrated (blue) conditions and the full-length bPAC protein under hydrated conditions (red): (A) overview from 1750 to 1100 cm^{-1} and (B) close-up of the amide I region for illustration of changes in secondary structure.

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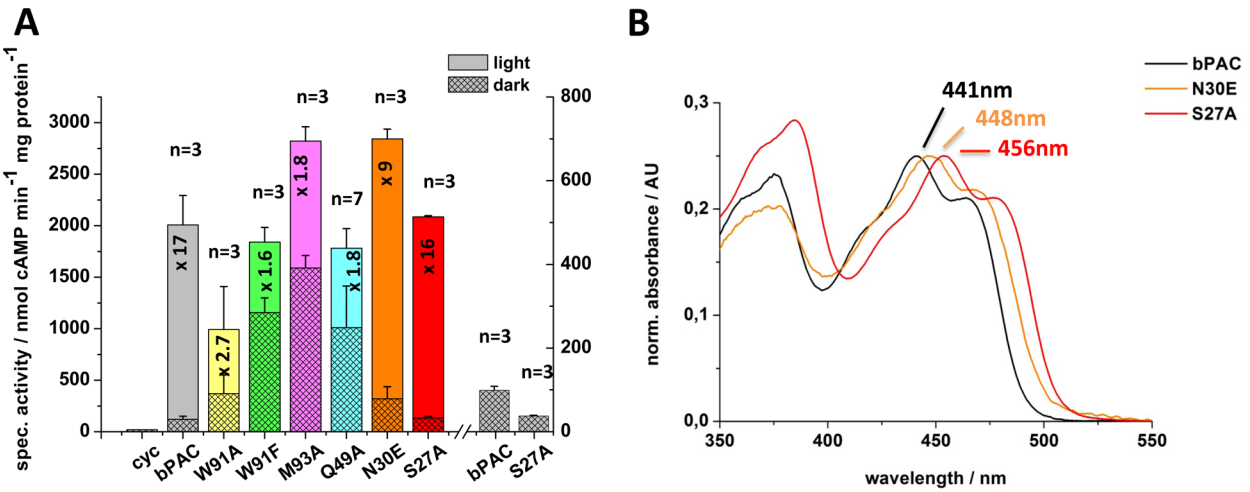

Figure 3. (A) Light-regulated adenylyl cyclase activity of bPAC and variants as well as the bPAC cyclase without the BLUF domain (cyc). (B) Absorption properties of dark-adapted bPAC and dark-adapted selected mutants.

with a lifetime of ~100 μs.[40] Because of W104's importance for signaling in AppA, a central role was attributed to strand β5, accordingly. In bPAC BLUF, the corresponding signal is suppressed by removing the water from the sample and the resulting spectrum closely resembles the spectrum of AppA-W104F. In a dried sample (Figure 1, blue curve), the hydrogen bond switch around the flavin is maintained as indicated by the C4═O signature, but peripheral secondary structures are "frozen" out as previously observed for LOV domains.[41] This further suggests that the signal observed here can indeed be assigned to strand β5 as observed in AppA. It should be noted, however, that in AppA the positive β-sheet signal at 1631 cm^{-1} is accompanied by a probably associated negative signal at 1619 cm^{-1}. In the BLUF domain of bPAC, no corresponding negative signal is observed.

In the full-length protein (Figure 2, red curve), the β5 signal is clearly present but partly compensated by a neighboring signal at 1631(−) cm^{-1}, which also can be attributed to β-sheet-related structural changes, most likely in the cyclase domain.[42] Furthermore, a large additional signal is observed in the frequency domain of α-helical amide I vibrations around 1650(+) cm^{-1}. The broad signal features a low-frequency shoulder and shows similarities to a negative feature previously observed in LOV domains at this frequency, which was assigned to unfolding of the C-terminal α-helical linker.[41,43,44] Here, the signal is positive and is therefore attributed to an increase in helical content in the cyclase domain upon illumination. At 1666 cm^{-1}, a negative signal is observed in all samples, which may be attributed to changes in turn structures overlapping with α-helical changes.[45] 3^{10}-helices are also known to absorb in this region but have not been observed previously in BLUF domain structures.[45] The signal is most pronounced in the full-length protein.

Regulation of the Cyclase Domain. So far, it is not known whether the BLUF domain inhibits the cyclase domain in the dark or activates it in the light. We expressed the isolated cyclase of bPAC (residues 97−350) without the BLUF domain. Purification of the cyclase construct gave only a low yield and a low purity [~60%, according to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (not shown)] compared to those of the full-length protein (~80%). Its activity of 18 nmol of cAMP min^{-1} mg^{-1} is in the range of full-length bPAC's dark activity (Figure 3 and Table S1 of the Supporting Information). Therefore, the bPAC cyclase in absence of the sensor modules is considered inactive. Unless the BLUF domain is generally required for the functional formation of the cyclase dimer, this means that the BLUF domains do not inhibit the cyclase domain in the dark but, instead, reconfigure the cyclase into an active state upon photoactivation.

Residues Affecting Signal Transduction and Dark Recovery. Earlier findings demonstrated that the recovery of the dark-adapted state directly correlates to the decay of the enzymatic activity.[6] Note that the lifetime of the light-adapted state of the wild type (WT) provided here (28 s) is longer than that previously reported (12.5 s), which most likely is due to the presence of β-mercaptoethanol here. Similarly, the specific cyclase activity is increased under these conditions compared to the results of our previous publication.[6] With the aim of obtaining faster and slower photocycling bPAC variants and thus altered light-modulated enzyme activities, bPAC-W91A and bPAC-W91F were produced. Additionally, we introduced the L61F mutation into bPAC inspired by the so far fastest (<1 s) photocycling BLUF mutant, L66F, described for BlrB.[46] Unfortunately, this construct was not soluble in our hands and could not be analyzed here. To address the role of methionine in signal transduction in bPAC, we additionally produced bPAC-M93A.

bPAC-W91A and bPAC-W91F form the BLUF-typical red-shifted signaling state under illumination, albeit with a red shift slightly reduced from 12 nm (WT) to 9 and 10 nm, respectively. Light- and dark-adapted absorption spectra are almost identical to those of the wild type (not shown). Similar to Slr1694, the W91A mutation accelerates the dark recovery by a factor of ~3 (10 s vs 28 s), whereas the dark recovery of bPAC-W91F is slowed by a factor of ~4 (120 s vs 28 s) (Figure S2 of the Supporting Information). In addition to the altered stability of the light-adapted state, also the dark state of bPAC is drastically affected, especially by the W91F substitution as observed by an increased dark activity of the enzyme (Figure 3A and Table S1 of the Supporting Information). A similar effect is produced by the M93A mutation, which also shows a significant increase in dark activity. In this case, however, the stability of the light-adapted state (23 s) is similar to that of the WT (28 s), as well as the enzyme activity in the light. Mutants

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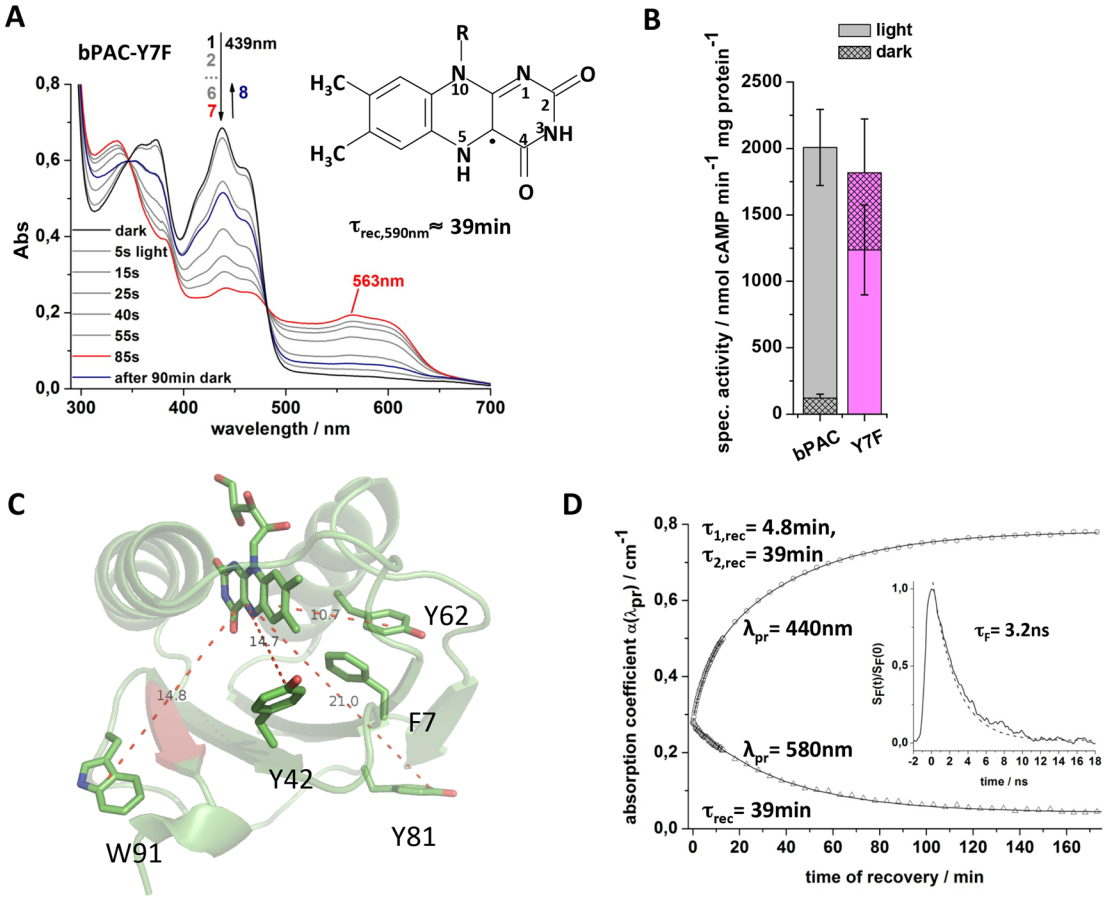

Figure 4. (A) Light-induced formation of a neutral flavin semiquinone in bPAC-Y7F excited at 455 nm with a light intensity of 0.18 W cm^{-2} for the indicated duration. (B) The cyclase activity of bPAC-Y7F is increased in the dark and reduced in the light. (C) Putative electron donors in the BLUF domain and their distance from the flavin N5 position. (D) Recovery of the semiquinone to the oxidized flavin visualized by the absorption coefficient development after excitation light switch-off at 440 and 580 nm. The inset shows a fluorescence signal trace with the indicated fluorescence lifetime τ_F of unexposed bPAC-Y7F obtained by sample excitation at 400 nm with a 3 ps laser pulse.

M93A, W91A, and W91F show a reduced light-induced increase of only ~1.5−2.7 and are therefore considered severely impaired in terms of their light regulation ability. The lower activity of W91A in the light state may be due to its faster recovery to the dark state, while M93A and W91F are considered locked in a pseudolit state.

Color-Tuned bPAC Variants. Excitation of photoreceptors with light of the red spectral region is highly desirable in optogenetic experiments because of its greater depth of penetration in tissue and its lower energy. Because of the chemical nature of the cofactor, color tuning in flavoproteins is not as straightforward as it is, for example, in rhodopsins or phytochromes.[47,48] The most pronounced red shift found in a BLUF photoreceptor so far is a 20 nm shift of the S_1 absorption maximum (460 nm) in the photoactivated adenylyl cyclase ngPAC2 from *Naegleria gruberi*.[49] To induce a comparable red shift in bPAC, we modified two residues, S27 and N30 (Figure 1B). S27 is analogous to Slr1694-S28, which has been previously shown to red shift the absorption by 16 nm.[32,50] N30 on the other hand is unusually replaced with a glutamate residue in ngPAC2. Both residues, S27 and N30, are within hydrogen bonding distance of the C2═O carbonyl of the flavin and were changed to alanine and glutamate, respectively. The resulting variants bPAC-S27A and bPAC-N30E both retain WT-like light-modulated enzyme activities (Figure 3A and Table S1 of the Supporting Information). Spectroscopic analysis reveals WT-like photocycle behavior [τ values of 19 s (S27A) and 22 s (N30E) (Figure S2 of the Supporting Information)], and the absorption spectra of dark-adapted bPAC-S27A and bPAC-N30E are, as expected, red-shifted by 15 nm (456 nm vs 441 nm) and 7 nm (448 nm vs 441 nm), respectively. Additionally, under assay conditions that favor a higher substrate turnover rate, an up to 40% reduced dark activity becomes obvious in bPAC-S27A. The observed low dark activity is especially interesting for optogenetic applications that require long-term expression.

Photocycle Inactive Variants with Light-Modulated Enzyme Activity. bPAC variants that are unable to form the red-shifted signaling state are expected to be light insensitive and putatively inactive enzymes. Recent studies of other BLUF domains, however, showed that such photoinactive variants may not necessarily reflect the WT dark state. Moreover, the possibility that spectroscopically silent transitions that affect the sensor−effector interaction are possible in these mutants cannot be completely ruled out. To address the correlation of signaling state formation and adenylyl cyclase activity in bPAC, we replaced the conserved Q49 with alanine and the conserved Y7 with phenylalanine (Figure 1B). Mutations of these residues are known to abolish signaling state formation in BLUF domains.[23,32,51,52] The absorption spectra of both Q49A and Y7F are slightly shifted compared to that of the WT (443 and 439 nm, respectively). UV−vis spectroscopic measurements

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confirm the inability of both mutants to generate the red-shifted intermediate (not shown). Surprisingly, under continuous illumination, a species with an absorption peaking at 563 nm and a shoulder at 598 nm that is typical for a neutral flavin semiquinone radical is formed in bPAC-Y7F (Figure 4A).[53,54] Isosbestic points at 482 and 346 nm indicate the predominant existence of oxidized flavin and neutral semiquinone radical. Experiments with the isolated BLUF domain containing the Y7F mutation show that the ability to form semiquinone is an intrinsic feature of this particular BLUF domain (not shown). The radical species decays with a time constant of ∼39 min (Figure 4D). Detailed UV−vis and fluorescence spectroscopic analyses show a biexponential dark recovery at 440 nm of the dark oxidized state. In contrast, the decay of the semiquinone absorption at 590 nm is monoexponential. A detailed report about the photochemistry of bPAC-Y7F will be published elsewhere. After 6 h in the dark, the absorption at 440 nm and fluoresence reach only ∼70 and ∼50%, respectively, of the initial level indicative of irreversible processes. In contrast to the light-induced formation of semiquinone intermediates in the WT photocycle, the semiquinone here is most likely formed from the flavin triplet state. The fluorescence lifetime is in the range of 3 ns (Figure 4D), similar to those of LOV domains, leaving ample time for intersystem crossing to the triplet state. Because the primary electron donor Y7 is absent in this mutant, the electron is most likely provided from other nearby tyrosine or tryptophan residues (Figure 4C). According to a homology model of the bPAC BLUF domain, the closest candidate with a distance of ∼11 Å is Y62. It is not completely clear how to assess the possibility of electron transfer (ET) from W91, because this residue may adopt either a distal (Figure 4C) or proximal (not shown) conformation with respect to the flavin. Competitive electron transfer from the corresponding residue was observed for the photoactivation of AppA.[31,55] In Slr1694, this nonproductive deactivation of the flavin by W91 was excluded because of a predominantly distal orientation (∼14 Å).[32] In the corresponding proximal configuration, the distance is expected to be ∼4 Å in Slr1694, which would allow for efficient ET from this residue. The latter is, however, not observed spectroscopically. Because the fluorescence lifetime of bPAC-Y7F is in the range of a few nanoseconds (Figure 4D), a flavin−tryptophan distance characteristic of a distal location is expected. It should be noted that structural data on BLUF domains especially vary in the conformation of this particular residue. Therefore, we cannot accurately address the real orientation of W91 in bPAC on the basis of the homology model provided here. It should also be noted that the formation of a stable semiquinone radical at room temperature as observed here has so far not been reported in other BLUF domains. Continuous illumination causes temporary accumulation of anionic and neutral semiquinone species in some BLUF domains with low quantum yields (∼0.01%) and lifetimes in the range of a few minutes.[56] Cryotrapped anionic flavin radicals have been described for the corresponding Tll0078-Y8F variant below 80 K.[57]

Both bPAC-Y7F and bPAC-Q49A feature a dark activity significantly higher than that of the WT, like the W91F and M93A mutants (Figures 3A and 4B and Table S1 of the Supporting Information), and are therefore classified as pseudolit state variants. Although (classical) signaling state formation is inhibited, both proteins still respond to blue light with a change in enzyme activity. While bPAC-Q49A reacts with an ∼1.8-fold increase in cyclase activity, the Y7F substitution surprisingly causes a decrease in activity in the light of 30% (Figure 4B).

DISCUSSION

Because no molecular structures of bPAC are available, the overall domain organization of BLUF and cyclase domains is unknown. In a dimer of class III adenylyl cyclase, secondary structure elements are arranged in an antiparallel orientation. Accordingly, different spatial domain organizations are conceivable. In analogy to the architecture of the soluble pH-regulated adenylyl cyclase Rv1264, BLUF and cyclase domains most likely share an intramolecular interface in bPAC (Figure 1A).[42] This could indicate a cooperative mode of sensor activation, which, however, has not been investigated so far. Illumination of the BLUF domains of bPAC leads to secondary structural changes in the cyclase domain as observed by FTIR difference spectroscopy. The helical content of the cyclase domain is increased in the light-adapted state, which might reflect reorganization of the cyclase domain itself or a helical regulatory element, similar to the mycobacterial pH-regulated AC.[42] Cyclase domains generally have a high helical content in their active site.[42] Furthermore, two so-called helical switches, which undergo helical to random coil transformation, have been previously identified in the pH-regulated cyclase mentioned above. One of the switches lies directly in the linker region between the N-terminal pH-sensing domain and the catalytic domain and the other directly in the catalytic domain. The latter changes from random coils into a helical structure, while the former switch is transformed from a helical structure to random coils. This might be partly reflected by the neighboring negative signal centered at 1666 cm^{-1}, which is in a characteristic frequency region for turn structures with minor contributions of α-helical amide I vibrations.[45] A similar yet weaker signal is also present in the BLUF domain alone, which most likely reflects similar structural changes in the BLUF core, as the signal is also present for the dehydrated sample. In the full-length protein, it is, however, also likely that structural rearrangements occur in the C-terminal cap helices (α3 and α4) as previously suggested.[15,58,59] In general, a mechanism similar to that in the pH-activated mycobacterial cyclase might be the case in bPAC, but molecular structural information about neither the active nor the inactive state of bPAC is available for a detailed molecular interpretation. On the basis of our observations, we propose that the photoactivation in bPAC involves structural propagation through strand β5 to the cyclase domain. There, a major transformation from random coils to helical structure takes place, most likely associated with the formation of the functional catalytic active site.

Structural propagation from the BLUF domain to the cyclase via strand β5 is expected to involve the conserved methionine residue that is crucial for signal transduction. The nature of the immediate flavin environment appears to be not necessarily involved to a large degree. The W91 and M93 mutants that are still able to form the hydrogen bond switched state around the flavin promote an active state of the cyclase domain already in the dark. Because the light activation as witnessed by the smaller relative increase in enzyme activity in the light is severely impaired, the communication between the BLUF domain core and the cyclase domain might be even considered decoupled from the BLUF domain photochemistry in agreement with earlier findings for Slr1694 and AppA.[19,27,30]

Similar to nuclear magnetic resonance (NMR) observations and protein−protein interaction studies on Slr1694,[19] our

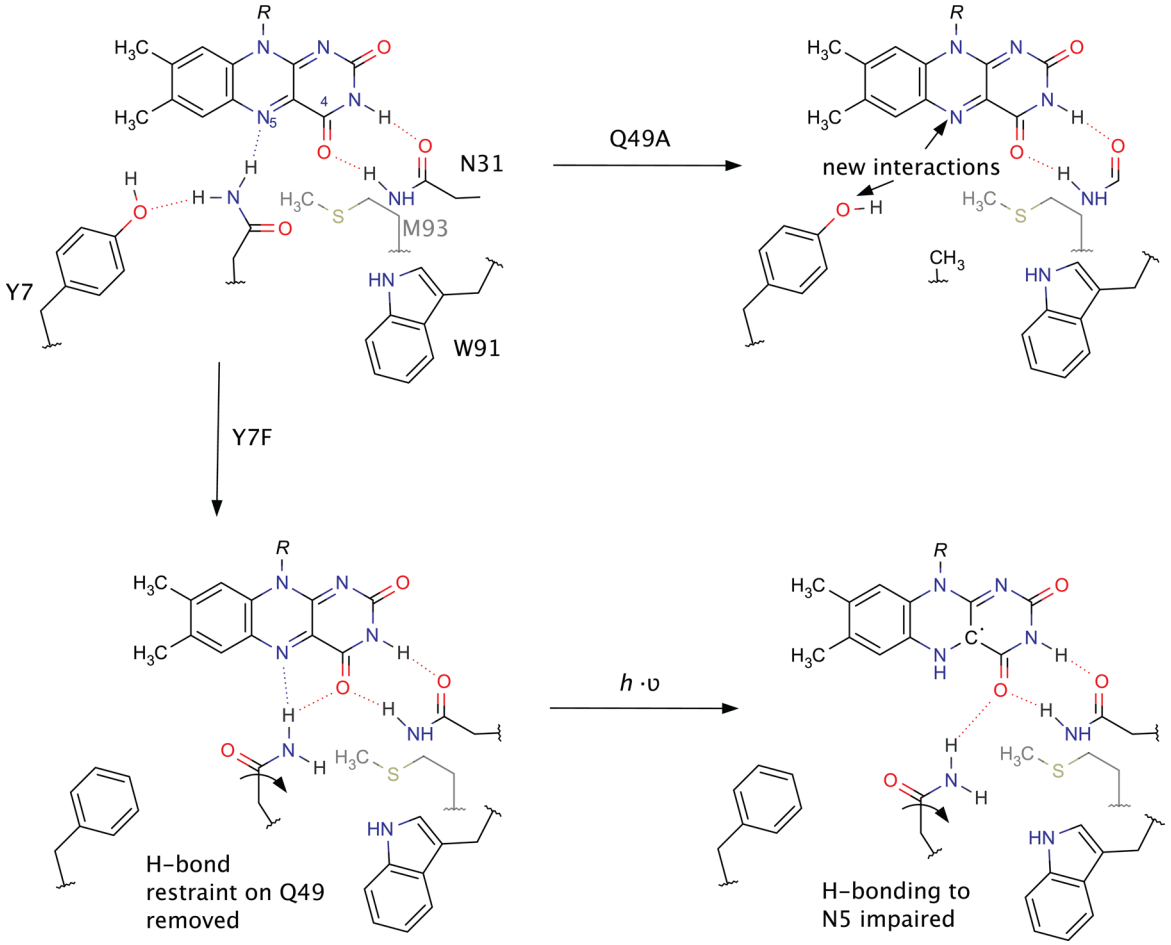

Figure 5. Proposed effects on the flavin binding pocket induced by removal of the conserved glutamine (Q49, top right), removal of the hydroxyl group of Y7 (Y7F, bottom left), and formation of a neutral flavin semiquinone radical in Y7F (bottom right).

results show that mutants not directly situated on or close to strand β5 may also influence its structural properties and accordingly signal transduction. The Y8F mutant of Slr1694 shows a β5 region significantly more flexible than that of the WT as observed by NMR.[19] In combination with protein−protein interaction experiments, this conformation was assigned to a pseudolit state, which has properties of the light state but has impaired light activation in line with our observations about bPAC-Y7F.[19] Accordingly, the corresponding tyrosine mutations in Slr1694 and euPAC lead to a high dark activity, as well.[19,60] It is conceivable that the perturbation of the hydrogen bond network in the flavin binding pocket upon removal of the phenolic hydroxyl group creates a configuration similar to the light-adapted state, which is propagated to the effector domains (see below). A similar effect may be achieved by removal of the conserved glutamine residue. The here observed permanent 3 nm red shift in bPAC-Q49A could even be an indicator of such a pseudolit state in analogy to conclusions drawn from investigations of AppA-Q63E.[17] NMR-based structural analysis revealed a conformation of this photoinactive mutant similar to the light-adapted state of the wild type. The 3 nm red shift of this mutant was assigned to stronger hydrogen bonding of the glutamate to Y21 and the flavin C4═O carbonyl and to a weaker interaction with flavin N5, an effect that is also observed in quantum chemical calculations on different configurations of the flavin binding pocket in WT AppA.[61]

The variability in dark activity induced by the mutants described here indicates that the interaction between the BLUF sensor and the cyclase effector domain is delicately determined by the overall structural properties of the BLUF domain. Besides being possibly induced by an altered flexibility of strand β5, the pseudolit states might be due to an altered linker conformation, a change in BLUF−cyclase interaction, or a perturbation of dimer association.

Surprisingly, mutations that appear to completely abolish the classical BLUF photochemistry (Q49A and Y7F) still show light-induced modulation of the cyclase domain. Because of their elevated dark activity, both mutants can be classified as pseudolit configurations. Both mutations directly affect the local hydrogen bond network around the flavin, which is the most likely explanation for this behavior (Figure 5). Replacement of Q49 with alanine removes the central component of this network and thereby opens various possibilities for its reconfiguration. Not only the flavin but also Y7 that was previously hydrogen bonded to Q49 is expected to form new interactions. In regard of the observations made for the W91 and M93 mutations, it is also likely that either W91 or M93 adopts a position proximal to the hydrogen bond network around the flavin and even forms a hydrogen bond directly to Q49, as previously suggested for AppA.[20,27] Upon removal of Q49, the residue is released and available for new interactions. A similar scenario is conceivable for the Y7F mutant. The removal of the hydrogen bond to Q49 (Figure 5, bottom left)

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eliminates a restraint for the orientation of Q49 and may lead to a reconfiguration of the H-bond network into a light-adapted state-like conformation.

The question of how these mutants accomplish light-induced signaling remains. From spectroscopic studies, we know that the removal of the conserved glutamine still allows for the formation of transient flavin radical intermediates similar to those observed in WT BLUF domains (J. Mehlhorn, Fudim, and T. Mathes, unpublished observation). Although their lifetime (tens of nanoseconds) is short in terms of large-scale protein dynamics, the altered electrostatic and chemical properties of the flavin radical are expected to induce a local reconfiguration of the protein around the flavin, which may suffice to be propagated further within the protein. Because of the relatively short lifetime of these radical states, the signaling efficiency is expected to be low as potentially reflected by the minor light versus dark difference in cyclase activity observed here. This, however, also poses the general question of whether the radical intermediates alone are in principle sufficient to drive signal transduction in BLUF domains.

In the Y7F mutant, in contrast, a flavin semiquinone radical stable for tens of minutes is formed by light-induced electron transfer from a nearby tryptophan or tyrosine residue. The changed electrostatic properties compared to those of the oxidized flavin are expected to have a strong effect on the flavin binding pocket (Figure 5, bottom right). Besides a different charge distribution,[62] the flavin also receives a proton at N5 in this form. In the dark-adapted state, N5 is expected to be hydrogen bonded by Q49, a bond that is most likely abolished in the radical state. New interactions can now be formed by Q49 with nearby residues that in the end induce structural changes in the cyclase domain obviously leading to a reduced cyclase activity. A clear molecular assignment of the structural changes induced by the formation of the radical is not possible at present. It should also be noted in this regard that a direct correlation between radical formation and the increase in activity in this case is not provided by our studies and requires further investigation. In any case, in this particular construct, the light regulation by the BLUF photoreceptor is inverted by a single mutation. A similar yet more pronounced effect of a single mutation in the sensory module has only recently been observed for the LOV domain-regulated histidine kinase YF1.[63]

Another goal of this study was to provide color-tuned variants of bPAC that allow for a more red-shifted and selective excitation in optogenetic experiments. Color tuning of flavins by the protein environment is very limited and by far less effective as with retinal or tetrapyrroles. By comparison of the primary structure and previous spectroscopic and theoretical investigations of various BLUF proteins, two residues that can be used to shift the absorbance in BLUF domains were identified. N30 and S27 are partially conserved residues, and their side chains are located within hydrogen bonding distance of the flavin C2=O group (Figure 1B). Experimental and theoretical results suggest that the missing hydroxyl group at position 27 is responsible for the absorption red shift observed in several BLUF domains.[32,50,64,65] According to quantum mechanical calculations on the AppA BLUF domain, the hydroxyl group of the homologous residue to S27 particularly contributes to the delocalization and stabilization of the HOMO of both the dark state and the red-shifted intermediate. Consequently, the removal of this favorable interaction leads to an energetic elevation of the HOMO, explaining the observed red shift in bPAC-S27A.[50] In analogy, the introduction of the negatively charged glutamate in the vicinity of flavin N1 by the N30E mutation would preferably destabilize the ground state and produce a similar effect. Residues in this position usually have a minor influence on the recovery kinetics, but they affect the selectivity of the BLUF domain for charged flavins and influence the electron transfer rates and signaling.[34] Mutations of S27 and N30 to alanine and glutamate in bPAC, respectively, lead to shifts of 15 and 7 nm, respectively. Taken together, both mutations may possibly account for a 20 nm red shift observed in nPAC2 from *N. gruberi*.[49] Both mutated proteins feature light-modulated cyclase activities similar to that of the WT and can therefore be applied in optogenetics in the same way, whereas only bPAC-S27A exhibits a significantly reduced dark activity. The reason for this phenotype is not completely clear at the moment, but the removal of a hydrogen bond interaction with the flavin via the S27A mutation may result in a stronger coordination by residues like Q49 and thus lead to a stabilization of the dark-adapted state. In behavioral optogenetic experiments with animals, it is crucial to work with a minimal amount of dark activity, which may lead to cumulative effects during long-time expression and render the experiment at hand strongly biased. bPAC-S27A therefore represents an optimized tool for studying cAMP signaling pathways in such optogenetic approaches.

■ CONCLUSIONS

The communication between sensor and effector domains of bPAC is delicately facilitated by the BLUF domain and most likely takes place via strand β5 to induce the formation of helical structures in the full-length protein. Specific mutations in the BLUF domain strongly influence the BLUF−AC interaction and lead to pseudolit states with impaired light modulation. Interestingly, also mutations that abolish the classical BLUF photochemistry exhibit (impaired) light modulation. The formation of a stable neutral semiquinone radical (in Y7F) in the light surprisingly leads to a decrease in cyclase activity and reflects an inversion of BLUF-modulated enzyme activity. Additionally, we discovered a red-shifted bPAC (S27A) with reduced dark activity that represents a very promising new optogenetic tool for long-term studies.

■ ASSOCIATED CONTENT

Supporting Information

Additional information about the biochemical properties of the FTIR samples as well as dark recovery kinetics and enzyme activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

PAC, photoactivated adenylyl cyclase; cAMP, cyclic adenosine monophosphate; FTIR, Fourier transform infrared; FAD, flavin adenine dinucleotide; PCR, polymerase chain reaction.

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