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ORIGINAL PAPER

NTP-binding properties of the blue-light receptor YtvA and effects of the E105L mutation

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Abstract YtvA is a blue-light-sensing protein from *Bacillus subtilis* related to plant phototropins. It carries a LOV (light, oxygen and voltage) domain, binding FMN (flavin mononucleotide) as chromophore, and a STAS (sulphate transporters and antisigma-factor antagonists) domain with poorly characterized function. We have recently shown that YtvA binds triphosphate nucleotides (NTP) and highlighted a structural similarity between the STAS domain and small GTP-binding proteins. In this work we further investigated the NTP-binding properties of YtvA, employing a fluorescent derivative of GTP (GTP_{TR}) and mutagenesis experiments. The main results are as follows: (a) competition experiments indicate that the affinity of YtvA for GTP is much higher than that for GDP and GMP. (b) Blue-light-induced structural changes are transmitted from the LOV core to the NTP-binding cavity, establishing a possible intraprotein signal-transduction pathway. (c) A mutation in the central β-scaffold of the LOV core, E105L, impairs the light-driven spectroscopic changes of bound GTP_{TR}. This result is supported by circular dichroism data, in that YtvA-E105L does not show the light-induced conformational change in the turn fraction that characterizes YtvA, implying that E105 is functionally important. (d) In the structural model of the LOV-STAS complex, based on docking algorithms, the interface includes the Iβ–Hβ loop on the LOV core, as well as parts of the central β-scaffold. E105 is predicted to interact with the LOV-STAS linker region, suggested to play a role in phototropin signaling.

Keywords LOV domain · STAS domain · Fluorescence · Modeling

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Introduction

The discovery of the ''LOV paradigm'' from the group of Briggs (Christie et al. 1999; Salomon et al. 2000) represents a turning point in the field of photosensory biology. Phototropin (phot) proteins are responsible for a variety of blue-light-driven effects in plants, aiming at maximizing the efficiency of the photosynthesis process, meanwhile minimizing overexposure damages (Briggs and Christie 2002). The LOV (light, oxygen and voltage) domains (LOV1 and LOV2) of phot are readily expressed as autonomously folding protein domains (ca. 110 aa) and have been extensively characterized during the last few years. In the dark, phot-LOV domains bind non-covalently the FMN (flavin mononucleotide) in its oxidized form and absorb maximally at about 450 nm (LOV_{447}) (Christie et al. 1999; Salomon et al. 2000). Blue-light illumination triggers a photocycle with the formation of a blue-shifted, metastable FMN-cysteine C(4a) thiol adduct (LOV_{390}) (Salomon et al. 2000, 2001), via the μs decay of the FMN triplet state (Kottke et al. 2003; Losi et al. 2004a; Swartz et al. 2001). LOV_{390} slowly reverts to LOV_{447} in the dark on the second-to-minute time-scale (depending on the specific system) (Kasahara et al. 2002). LOV domains belong to the PAS (PerArntSim) superfamily and the

structure retains the typical PAS fold, with a central anti-parallel five-stranded β-scaffold, flanked by connecting helices (Crosson and Moffat 2001, 2002; Fedorov et al. 2003). In the following, the secondary structure elements are named in alphabetical order, followed by greek alpha or greek beta to denote helices and strands, respectively: from the N- to C- terminus of the LOV core: Aβ, Bβ, Cα, Dα, Eα, Fα, Gβ Hβ, and Iβ. The reactive cysteine is located within the Dα–Eα loop.

The LOV photochemical paradigm has apparently been conserved among distant phyla and LOV proteins are widely found in prokaryotes (Crosson et al. 2003; Losi 2006). Phot-like photochemical reactions have been demonstrated for *Bacillus subtilis* YtvA (Losi et al. 2002), *Pseudomonas putida* SB2-LOV (Krauss et al. 2005) and a LOV-HK (histidine-kinase) protein from *Caulobacter crescentus* (Losi 2004). Genes coding for LOV proteins are up to now detectable in about 15% of the sequenced prokaryotic genomes, making them the most spread blue-light photosensors in microorganisms (Losi 2006). Although information on the in vivo function of bacterial LOV proteins is still sparse, recent works reported that YtvA acts as a positive regulator of the general stress transcription factor σB in *B. subtilis* (Akbar et al. 2001), specifically in the environmental branch of the complex pathway leading to σB activation (Gaidenko et al. 2006), in a clearly shown blue-light regulated way (Avila-Perez et al. 2006). Consistently, cysteine 62 that forms the adduct with FMN during YtvA photocycle (Losi et al. 2002), is essential for YtvA to exert its positive regulation on σB during environmental stress (Gaidenko et al. 2006). These data indicate that YtvA is indeed a blue-light photoreceptor in *B. subtilis*, although light is not the sole environmental input that can be sensed by this protein (Gaidenko et al. 2006). These recent studies allow regarding YtvA as a real flavin-based blue-light photoreceptor in *B. subtilis*, not only a blue-light sensitive protein. Furthermore it has been suggested that YtvA might be involved in light-control of sporulation, via cross talks of the stress response and sporulation pathways (Avila-Perez et al. 2006). Indeed, the alternative transcription factor σE regulates during sporulation the expression of more than 253 genes in the mother cell (Eichenberger et al. 2003), and in cells overexpressing σE, YtvA is upregulated [supplementary material in Eichenberger et al. (2003)]. In addition, YtvA is down-regulated in cells overexpressing SPOIIID, a transcriptional regulator involved in the synthesis of spore coat proteins [supplementary materials in Eichenberger et al. (2004)], supporting a still putative regulatory function of YtvA within the sporulation pathway.

STAS is thought to be the effector domain of YtvA, although little is known of its molecular functionality and we cannot exclude that it is actually a second sensing domain that acts synergistically with the light-sensitive LOV core. We have recently shown that the STAS domain confers to YtvA the ability to bind guanosine triphosphate (GTP) and adenosine triphosphate (ATP), meanwhile highlighting a topological similarity between the STAS domain and small GTP-binding proteins (Buttani et al. 2006). A general role of STAS domains as NTP-binding units (N = nucleoside) had been anticipated (Aravind and Koonin 2000) and GTP binding was demonstrated for SPOIIAA, a small STAS protein involved in *B. subtilis* sporulation (Najafi et al. 1996). Interestingly, activation of the stress and sporulation pathways in *B. subtilis* are related to a drastic drop of ATP and GTP (Piggot and Hilbert 2004; Zhang and Haldenwang 2005), pointing to a functional role for the in vitro observed NTP-binding properties of YtvA.

In this work we further analyze the nucleotide-binding properties of YtvA, by employing a red-light fluorescent derivative of GTP (BODIPY® TR-GTP, referred to as GTP_{TR}) (McEwen et al. 2001). Competition experiments employing non-fluorescent guanosine derivatives were carried out in order to assess the relative affinity of YtvA towards GTP, GDP and GMP. Besides, we investigated the effects of the E105L mutation (Losi et al. 2005) on the GTP-binding properties of YtvA. E105 is a charged amino acid, unique to YtvA-like LOV proteins and is localized on the Hβ strand, within the central β-sheet of the LOV core (Losi et al. 2005), a molecular surface suggested to be involved in intraprotein interactions (Buttani et al. 2007) and signal transduction in LOV proteins (Harper et al. 2003, 2004). We also present a low-resolution structural model of truncated YtvA, based on docking simulations between the LOV and STAS domains and in agreement with the experimental data.

Experimental

His-tagged YtvA and YtvA-E105L were expressed in *E. coli* (BL21) (Stratagene, Amsterdam, The Netherlands) using IPTG (BioMol, Hamburg, Germany)-induction and employing the pET28a plasmid (Novagen-Merck, Darmstadt, Germany), as described (Losi et al. 2002, 2003, 2005). The proteins were purified by affinity chromatography on Talon (Qiagen, Hilden, Germany) and finally concentrated in Na-phosphate buffer 10 mM, NaCl 10 mM, pH = 8. Protein purity was routinely checked by gel electrophoresis. The flavin:protein ratio was 1:1 in the samples employed here, as judged from the UV/VIS absorbance ratio, that was ≤ 4, meaning that no apoprotein is present (Losi et al. 2005). Guanosine-5′-triphosphate, BODIPY® TR 2′-(or-3′)-O-(N-(2-aminoethyl) urethane), trisodium salt (GTP_{TR}), was purchased from Molecular Probe (Eugene,

OR, USA); Guanosine-5′-[γ-thio]triphosphate tetrasodium salt (GTP), Guanosine-5′-[γ-thio] diphosphate trilithium salt (GDP) and Guanosine 5′-monophosphate disodium salt hydrate were from Sigma-Aldrich (Milano, Italy). Absorbance spectra were recorded with a Jasco 7850 UV/V is spectrophotometer. Steady state fluorescence emission spectra were recorded with a Perkin–Elmer LS-50 spectrofluorimeter, at 20°C. GTP_{TR} was excited at 590 nm with an excitation path length of 0.4 cm. The spectra were divided for the fraction of absorbed energy, $(1–10^{-Abs})$ where Abs = absorbance at the excitation wavelength, λ_{ex} = 590 nm, in order to obtain a signal that is proportional to the quantum yield of fluorescence, Φ_F. Unless otherwise stated, binding experiments were carried out with YtvA in its blue-light activated state (i.e., FMN covalently bound to Cys 62), achieved by illuminating the sample with a blue-light emitting Led-Lenser® V8 lamp (470 nm, Zweibrüder Optoelectronics, Solingen, Germany) as previously described (Buttani et al. 2006). The fluorescence spectra were always recorded upon excitation at 590 nm (where solely GTP_{TR} absorbs and no energy transfer can occur from or to other fluorophores). The dissociation constant (K_D) between YtvA and GTP_{TR} and the maximum fluorescence enhancement (ΔF_{max}) was evaluated by fitting the experimental data with the non-linear equation [rearranged from Eq. 2 in Favilla and Mazzini (1984)]:

$$[P]_{tot} = \frac{K_D + 1 - \frac{\Delta F}{\Delta F_{max}}}{\frac{\Delta F_{max}}{\Delta F} - 1} \quad (1)$$

where $[P]_{tot}$ is the concentration of Ytva and ΔF is the absolute difference value in the normalized fluorescence between GTP_{TR} alone and in the presence of YtvA. An agreeable fitting of the experimental data to Eq. 1 indicates that a 1:1 complex is formed and is equivalent to obtain a linear Scatchard plot, intercepting the *x*-axis in 1 (Favilla and Mazzini 1984). The concentration of GTP_{TR} was kept constant at 1 μM and [YtvA] was varied between 2 and 55 μM.

Circular dichroism spectroscopy and data analysis

Circular dichroism (CD) experiments were carried out using a Jasco J715 spectropolarimeter, calibrated with ammonium d-10-camphorsulphonic acid. The measurements were carried out in the far-UV spectral region (190–250 nm) at a temperature of 20°C and the buffer background was always subtracted. The optical pathway was 0.2 cm. Protein concentration was estimated from the absorption coefficient at 220 nm, $^{YTVA}\varepsilon_{220}$ = 492,800 M^{-1} cm^{-1} (Buttani et al. 2007). The corresponding value for FMN is $^{FMN}\varepsilon_{220}$ = 34,500 M^{-1} cm^{-1}, thus introducing a negligible error. The mean residue ellipticity Θ_{MRW} was calculated from the concentration of residues, *c*, (281 aa/protein for YtvA, including the tag), according to the formula $\Theta_{MRW} = \Theta_{obs}/(10 \times cl)$, where *c* is in mol l^{-1}, l = 0.2 cm, Θ_{obs} is in mdeg and Θ_{MRW} in deg cm^2 $dmol^{-1}$. Typically, the protein concentration was in the μM range and $c = 10^{-6} \times 281 = 2.8 \times 10^{-4}$ mol l^{-1} (Buttani et al. 2007). Prediction of secondary structure composition was performed using the convex constraint analysis (CCA) algorithm (Perczel et al. 1991), as previously described (Buttani et al. 2007).

Docking simulation, evaluation of complexes and model validation

The structural model of the LOV (aa 25–126) and STAS (aa 147–254) domain of YtvA has been previously presented (Buttani et al. 2006; Losi et al. 2002). In order to obtain a model structure of the LOV–STAS interaction complex, the two domains were docked together using the ClusPro Server, including ZDOT 1.0 (Mandell et al. 2001) software and the ZDOCK v.2.3 (Chen et al. 2003). The ClusPro docking algorithm evaluates billions of putative complexes, retaining a preset number with favourable surface complementarities. A filtering method is then applied to this set of structures, selecting those with good electrostatic and desolvation free energies for further clustering. The quality of the ClusPro predicted LOV–STAS complexes was verified by using the protein structure analysis and validation server (SAVS) of the NIH MBI Laboratory for Structural Genomics and Proteomics at the University of California, Los Angeles (UCLA) (http://www.nihserver.mbi.ucla.edu/SAVS/) that implements the programs PROCHECK (Laskowski et al. 1993), WHAT_CHECK (Hooft et al. 1996)(4), ERRAT (Colovos and Yeates 1993), VERIFY_3D (Luthy et al. 1992) and PROVE (Pontius et al. 1996).

Results

GTP_{TR} binding to YtvA and competition with unlabeled nucleotides

The spectral properties of GTP_{TR} have been reported in (Buttani et al. 2006). In phosphate buffer GTP_{TR} absorbs maximally at 590 nm, with emission centered at 620 nm. Upon addition of YtvA (in the blue-light activated state), we observed an enhancement of GTP_{TR} fluorescence and a concomitant red-shift (ca. 13 nm) and hyperchromicity of the absorption spectrum in the visible region (Buttani et al. 2006). Analysis of the experimental data with Eq. 1 gave a

dissociation constant K_D = 38 μM, with a single binding site. This is only true if no apoprotein is present (UV/VIS ratio in the dark state ≤ 4 (Losi et al. 2005), since flavin-free YtvA binds GTP_{TR} with very high affinity. Two binding sites with different affinity were preliminary identified, one of them being most probably on the apo-LOV core (Losi et al., unpublished). Displacement experiments with excess of non-fluorescent GTP (100 μM) resulted in a partial reversal of the absorption red shift and in a diminished fluorescence, indicating a competition for the same binding site as GTP_{TR}. Due to the low-binding constant, the exchange was not complete (Buttani et al. 2006). ATP had a similar effect, albeit with a slower time-course. The displacement experiments confirmed that the nucleotide moiety effectively binds to the protein and that the observed spectroscopic effects are not due to an unspecific binding of the GTP_{TR} BODIPY® moiety.

Conversely, an equivalent excess of guanosine diphosphate (GDP) or guanosine monophosphate (GMP) is not sufficient to displace GTP_{TR} bound to YtvA and under these conditions we could not record any loss of GTP_{TR} fluorescence (Fig. 1) and back shift of the absorption maximum (not shown).

The slight increase in the fluorescence observed upon adding GDP or GMP is due to the partial dark recovery of YtvA (Buttani et al. 2006). These results indicate that the affinity of YtvA for GDP and GMP is much lower than for triphosphate nucleotides.

Effects of the E105L mutation

Glutamate 105 is localized on the Hβ strand, in close vicinity with the conserved W103, which was shown to be a marker of intraprotein interactions in YtvA (Losi et al. 2005) and in phot-LOV2 (Harper et al. 2003). In between, another conserved residue, N104, is hydrogen bonded to O4 on FMN (Crosson and Moffat 2001) and undergoes changes in the lit state of LOV domains (Freddolino et al. 2006). E105 is found in YtvA, YtvA-like family and other bacterial proteins, but is invariably a hydrophobic aa (L, I or M) in phot LOV domains (Losi 2004). For these reasons, E105 could be functionally important in YtvA and represent a specific activation pathway, driven by the formation of the photoproduct.

In the light-adapted state YtvA-E105L binds GTP_{TR} as shown by the increase in fluorescence and the red-shift in the absorption and emission spectra, as in YtvA-WT (wild type) (Fig. 2).

The data obtained by fluorescence titration experiments as described in Buttani et al. (2006) are well fitted with Eq. 1, indicating a 1:1 stoichiometry. The obtained K_D = 35 μM is very similar to the one for YtvA-WT (K_D = 38 μM). Also in YtvA-E105L, GTP_{TR} can be partially displaced by an excess of GTP (not shown).

Despite the sharp similarities in the binding properties, GTP_{TR}-loaded YtvA-WT and YtvA-E105L show significant differences when they switch between the dark- ($YtvA_{450}$) and light-activated state ($YtvA_{390}$). GTP_{TR} bound to YtvA undergoes slight but well reproducible light-driven spectroscopic changes (Fig. 1, 3) In $YtvA_{450}$-WT, the absorption maximum further shifts to the red and the fluorescence slightly increases (Buttani et al. 2006). These changes are fully reversible and indicate that the light-driven conformational changes, centered on the LOV domain, are transmitted to the GTP_{TR}-binding cavity on the STAS domain. For YtvA-E105, upon reversion to the dark-

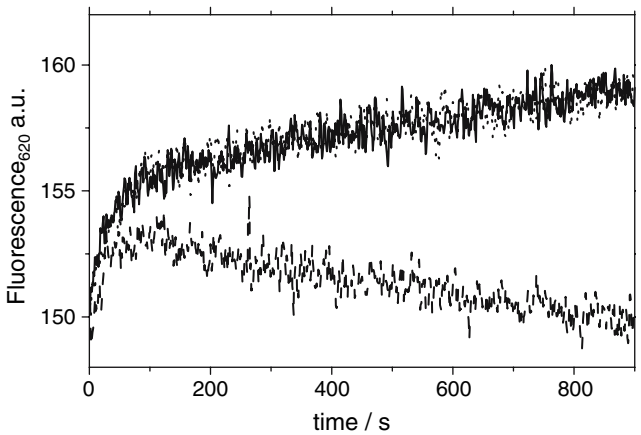

Fig. 1 Time course of fluorescence for GTP_{TR} bound to YtvA upon addition of GTP (*dashed line*), GDP (*dotted line*) and GMP (*full line*). For the experiments, 1 μM GTP_{TR} was incubated overnight with 44 μM YtvA. 100 μM of non-labeled nucleotides were then added, immediately before starting to record the fluorescence change at 620 nm (excitation at 590 nm)

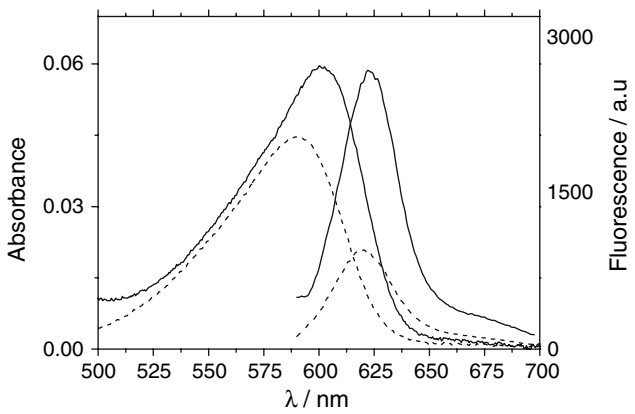

Fig. 2 Visible absorption (*left*) and emission spectra (*right*) for GTP_{TR} in phosphate buffer (*dotted line*) and in the presence of 55 μM YtvA-E105L (*full line*). The emission spectra were recorded upon 590-nm excitation. The output was divided by $1–10^{-Abs}$, in order to normalize for the difference in absorbance [see ''Experimental'' and Buttani et al. (2006)]

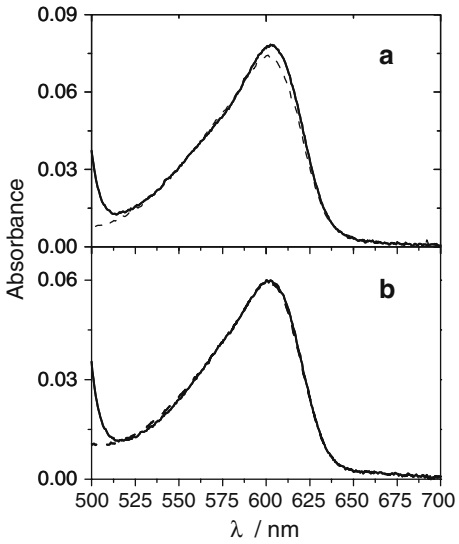

Fig. 3 Absorption spectra of YtvA-bound GTP_{TR} in the visible spectral range in the dark- (*full lines*) and the light- (*dashed line*) adapted state for **a** YtvA-WT and **b** YtvA-E105L. Conditions were as in Fig. 1. The E105L substitution impairs the spectroscopic changes of bound GTP_{TR} in switching from $YtvA_{450}$ (*dark*) to $YtvA_{390}$ (*light*)

adapted state, no further red shift in the absorption spectrum or of the fluorescence intensity are detected. This suggests that the transmission of light-driven conformational changes from the LOV core to the STAS domain is impaired (Fig. 3).

In agreement with this result, a careful inspection of the circular dichroism spectra (Buttani et al. 2007) and of a larger number of investigated samples than previously reported (Losi et al. 2005) indicates that in YtvA-E105L a light-driven change in the turn fractions, characteristic of full length YtvA, is not observed (Table 1), a feature particularly evident in the light–dark difference spectra (Fig. 4).

The conformational changes observed in YtvA-E105L more closely resemble those in the isolated LOV domain (Buttani et al. 2007) and their transmission to the rest of protein is impaired or occurs via a different pathway. This confirms the observations made with GTP_{TR}.

Structural modeling of the LOV-STAS interaction complex in YtvA

Despite its stability as a full-length protein in solution, it was not possible up to now to obtain a crystal structure of YtvA, potentially due to an intrinsic conformational heterogeneity (Buttani et al. 2007). Therefore, we have tried to obtain structural information by means of molecular modeling and domain–domain docking simulation. The structure of Ytva-LOV exhibits the characteristic PAS fold, a pocket including the helix-turn-helix

Table 1 Results of the CCA analysis on CD spectra

Secondary Structure	YtvA-E105L (%)[a] (281 aa)[b]		N-LOV (%)[a] (147 aa)[b]		YtvA (%)[a] (281 aa)[b]	
	Dark	Light	Dark	Light	Dark	Light
I. α-Helix	34.7 ± 6.1 (98 ± 17)	35.6 ± 5.2 (100 ± 15)	24.9 ± 2.4 (37 ± 4)	27.3 ± 0.9 (40 ± 1)	30.9 ± 4.7 (87 ± 13)	32.0 ± 6.5 (90 ± 18)
II. RC	22.5 ± 0.5 (63 ± 1)	22.6 ± 1.7 (63 ± 4)	24.9 ± 0.9 (37 ± 1)	23.6 ± 0.9 (35 ± 1)	22.4 ± 1.0 (63 ± 3)	21.7 ± 3.5 (61 ± 10)
III. β-turns/others	17.0 ± 2.9 (48 ± 8)	17.6 ± 0.4 (49 ± 1)	27.5 ± 1.0 (40 ± 1)	28.3 ± 1.5 (42 ± 2)	16.8 ± 3.6 (47 ± 10)	18.7 ± 3.5 (52 ± 10)
IV. $\beta_{twisted}/\beta_{Par.}$	6.7 ± 2.8 (19 ± 8)	9.3 ± 2.8 (26 ± 8)	12.1 ± 1.6 (18 ± 2)	15.9 ± 0.9 (23 ± 1)	9.9 ± 4.1 (28 ± 11)	11.0 ± 3.7 (31 ± 10)
V. $\beta_{Antipar}$	18.9 ± 4.0 (53 ± 11)	14.9 ± 4.0 (43 ± 11)	10.6 ± 3.4 (15 ± 5)	4.9 ± 2.1 (7 ± 3)	19.9 ± 5.6 (56 ± 16)	16.6 ± 5.8 (47 ± 16)
$\langle\sum_{i=1}^{n}[y_i - f(\lambda)]^2\rangle^c$	5.2 ± 2.5	4.9 ± 3.1	2.2 ± 1.8	2.1 ± 0.7	6.2 ± 2.8	5.2 ± 2.3

[a] The statistical error is the standard deviation and is obtained from three sets of measurements on three different preparations for YtvA-E105L, four sets of measurements on two different preparations for N-LOV (LOV core + N-terminal cap), eleven sets of measurements, and nine different preparations for YtvA. Data for N-LOV and YtvA are taken from Buttani et al. (2007)

[b] The number of aa (amino acids) is given in parenthesis, below the percentage, together with the statistical error

[c] Average squared error, where y_i = experimental curve, $f(\lambda)$ = fitting curve See Buttani et al. (2007) for details

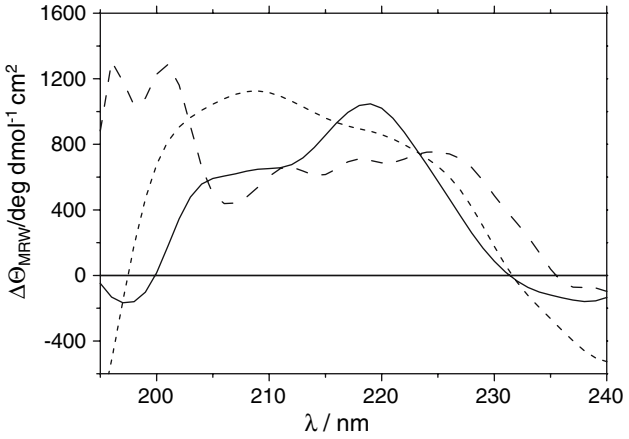

Fig. 4 Light–dark circular dichroism spectra, displayed as difference in the mean residue ellipticity, $\Delta\Theta_{MRW}$, for YtvA-E105L (*full line*) compared with YtvA-WT (*dashed line*) and YtvA-LOV (*dotted line*) (the latter two spectra were taken from ref. (Buttani et al. 2007) for comparison). In YtvA-WT, the positive band at $\lambda > 230$ nm has been assigned to a variation in the turns fraction occurring upon formation of the photoproduct $YtvA_{390}$ (Buttani et al. 2007)

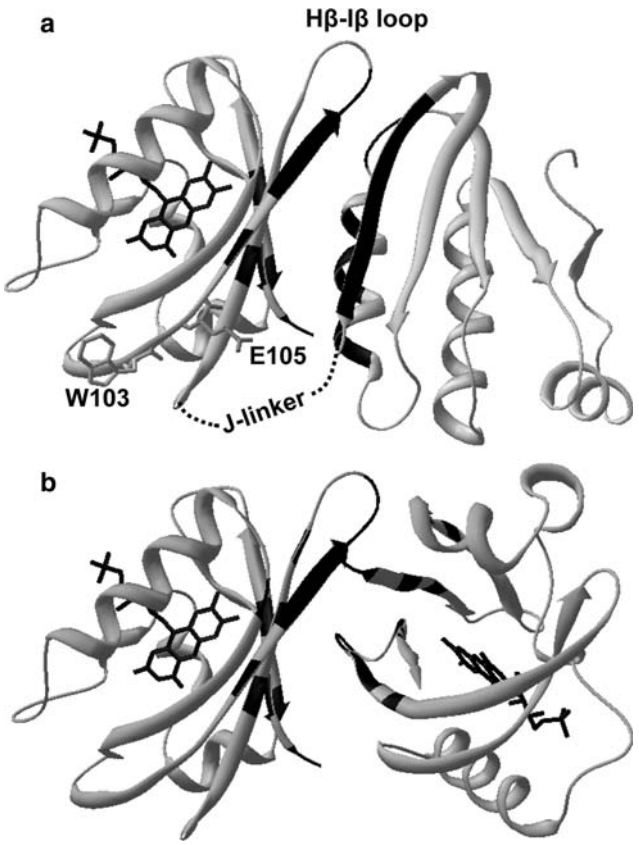

Fig. 5 **a** Model of the LOV–STAS interaction complex in YtvA as compared with the (**b**) LOV–LOV dimer (Buttani et al. 2007). In *black* regions at the contact interface (residues within 4 Å). In both cases the interface comprises residues in the central β-scaffold of the LOV core, namely belonging to Aβ, Bβ, Hβ, Iβ and the Hβ–Iβ loop (indicated in **a**). On the STAS domain ^{S}Aβ, ^{S}Cα and the ^{S}Cα–^{S}Dβ loop participate in the LOV–STAS interaction. The J-linker, joining the LOV and the STAS domains, is schematically drawn to indicate its proximity to E105

Cα–Eα (with the intervening Dα) and the helical connector Fα as walls, and a five-stranded antiparallel β-scaffold as floor (Aβ, Bβ and Gβ, Hβ, Iβ) (Losi et al. 2002). The extended β-scaffold constitutes a sort of pavement for the FMN chromophore, by forming a hydrophobic pocket around the dimethyl benzyl ring and surrounding the isoalloxazine ring by providing polar and apolar interactions. The STAS domain is characterized by an extended β-sheet that includes four parallel β-strands (^{S}Bβ, ^{S}Dβ, ^{S}Fβ, ^{S}Hβ, the superscript ''S'' denotes the STAS domain), with intervening α-helices (^{S}Cβ, ^{S}Eβ, ^{S}Gβ). The first strand, ^{S}Aβ, is antiparallel to the remaining four (Buttani et al. 2006).

In the modeled LOV–STAS complex best ranked by ClusPro (cluster 1), the C-terminal end of the LOV core is too far away from the N-terminus of the STAS domain to be linked with the 20 aa long linker; therefore, it was not taken into consideration. The second-best-ranked model complex (cluster 2) is shown in Fig. 5, together with the model for the LOV–LOV dimer that we have recently reported (Buttani et al. 2007). The Verify-3D score (Luthy et al. 1992) (>93) and the Errat-quality factor for cluster 2 (Colovos and Yeates 1993) (90.1) were quite high, both indicative of a reasonable and well-resolved model-structure.

The model complex proposed in Fig. 5 is in agreement with several observations: (a) the two domains are favourably oriented with their terminal ends to be joined with the linker; (b) from previous works we know that the isolated LOV domain of YtvA forms a dimmer, where the interface is mostly built via the central β-scaffold, and that the same surface is involved in LOV–STAS interactions (Buttani et al. 2007). Accordingly, in cluster 2, the LOV core offers the same interacting surface as within the LOV–LOV dimer; (c) E105 is not part of the LOV–STAS interface; rather it is likely to interact with the linker, that is rich in positively charged lysine residues, and by this pathway it facilitates the transmission of conformational changes to the STAS domain. The same is true for W103, a marker of intraprotein interactions in YtvA (Losi et al. 2004b), most probably also showing a direct contact with the linker (Losi et al. 2005); (d) the LOV–STAS interface includes the Hβ–Iβ loop and part of the adjacent Hβ, Iβ strands on the LOV core. These structural elements have been recently suggested to be part of the activation pathway of phot-LOV2 (Freddolino et al. 2006). Furthermore, two conserved residues that form hydrogen bonds with C4 on FMN, N104 and Q123, are localized on Hβ and Iβ, respectively, and are likely to undergo changes upon formation of the photoadduct (Crosson and Moffat 2002;

Fedorov et al. 2003). This point will be further discussed (See ''Discussion'').

We could not model with sufficient confidence the remaining parts of the protein, namely the N-terminal cap (aa 1–24), the J-linker (aa 127–145) and the C-terminal end (aa 255–261). It has been derived from circular dichroism data that the N-cap is largely helical (Buttani et al. 2007), but we have no hint about its orientation. Also for the J-linker we cannot state from circular dichroism data (Buttani et al. 2007) with certainty that it is helical like in phot-LOV2 (Harper et al. 2003). Preliminary data on an extended YtvA-LOV construct show that the J-linker is unordered in the dark state and does not prevent LOV–LOV dimerization (Losi et al., unpublished), in sharp contrast with phot-LOV2 (Harper et al. 2003).

Discussion

The binding of GTP and ATP to YtvA is in line with the suggested idea that STAS domains may have a general NTP-binding role (Aravind and Koonin 2000). To our knowledge, this has up to now only been demonstrated for the SPOIIA protein (Najafi et al. 1996) and for YtvA (Buttani et al. 2006), and it may be representative of a novel NTP-binding fold. It has been suggested that the conserved ^{S}Dβ–^{S}Eα loop that in STAS domains hosts the DxxG motif is involved in γ-phosphate binding, as in P-loop GTPases (Leipe et al. 2002), and the β-sheet scaffold could accommodate the rest of the NTP molecule (Aravind and Koonin 2000). This would resemble lipid binding by Sec14 domains, which have the same structural fold but show no detectable sequence similarity to the STAS domains (Aravind et al. 1999). The Sec14 family groups substrate carriers or transfer proteins, e.g., guanine nucleotide exchange factors (Schmidt and Hall 2002), phosphatidylinositol/phosphatidylcholine transfer proteins (Sha et al. 1998) and alpha-tocopherol transfer proteins (Meier et al. 2003). A similar binding configuration between GTP and YtvA-STAS would explain the lack of a P-loop pattern, in which the ligand would be deeply embedded within the cavity, formed by the parallel β-scaffold and the two helical segments ^{S}Cα and ^{S}Eα.

The spectroscopic changes undergone by bound GTP_{TR} upon YtvA light–dark conversion, suggest that NTP binding has a functional significance, related to the role of YtvA as a photoreceptor during activation of the general stress factor σB and, possibly, during sporulation (Avila-Perez et al. 2006). Indeed it has been observed that in *B. subtilis* both responses are related to a drastic drop of ATP and GTP (Piggot and Hilbert 2004; Zhang and Haldenwang 2005). It can be imagined that the STAS domain of YtvA is not an effector/catalytic domain, as previously suggested, but rather a sensing unit for the level of NTPs within the cell. This would configure YtvA as a double sensor, for light and metabolic state, somehow reminiscent of the blue-light and redox state sensor Appa in *Rhodobacter sphaeroides* (Braatsch et al. 2002). In agreement with this suggestion, still largely to be verified, we have up to now no hints that YtvA has a GTPase activity. Prolonged incubation and dark-light cycling of YtvA does not alter the affinity of GTP_{TR} that is not blocked on the γ-phosphate and therefore could be hydrolyzed. The displacement experiments show nevertheless that the affinity for GDP is much lower than for GTP, so that, in case of hydrolysis of the γ-phosphate, GDP_{TR} should be released. Nevertheless, a weak hydrolytic activity as observed for SPOIIAA (Najafi et al. 1996) cannot be excluded. It is possible that an essential cofactor is missing: it is well known that in many ATPase*s* and GTPase*s* Mg^{2+} is required for the hydrolytic activity (Leipe et al. 2002), but in YtvA even very low concentrations of this cation induce extensive precipitation, preventing any titration experiments (not shown).

The results obtained with YtvA-E105L, for which the light-driven effects on bound GTP_{TR} are negligible, point to a critical role of this residue in transmitting the conformational changes from the light-sensitive LOV core to the STAS domain, a result confirmed by circular dichroism experiments (Figs. 3, 4; Table 1). In future experiments, this mutation might help to define a physiological role for the NTP-binding activity of YtvA and its link with light sensing. It has been proven that, in the absence of other stress factors, Cys 62 is required for YtvA to exert its positive regulatory role on σB, but it is not required for the positive effect of YtvA overexpression in the presence of salt stress (Gaidenko et al. 2006). Furthermore, transcriptional analysis of the *ytvA* structural gene indicated that it provides the entry point for at least one additional environmental input, mediated by the Spx global regulator of disulfide stress (Gaidenko et al. 2006). The effects of the E105L substitution and of the C62A/E105L double mutation on the complex physiological responses mediated by YtvA should help to understand the relevance of the NTP-binding properties of the protein with respect to its light-sensing role.

Previous spectroscopic data (Losi et al. 2005) and the LOV–STAS model complex presented here, indicate that E105 does not interact directly with the STAS domain, but rather with the interdomain J-linker region. This is similar to the configuration in the NMR-derived structural model of phot-LOV2, where the initial portion of Hβ containing W491 (corresponding to W103 in YtvA) and L493 (E105 in YtvA) interact directly with the initial part of the J-linker (Harper et al. 2003). The J-linker in phot-LOV2 is partially helical in the dark state and undergoes light-driven

unfolding (Harper et al. 2003), a process thought to be involved in regulation of the kinase activity (Harper et al. 2004). This feature is not observed in YtvA (Buttani et al. 2007; Losi et al. 2005), although we have provided evidence here that the linker region might be functionally important, via interactions with the Hβ strand of the LOV core. Future mutagenesis experiments will help in functional mapping the residues important in intraprotein communication in YtvA.

The LOV–STAS complex modeled here agrees well with previous data, indicating that in the full-length protein a LOV-domain mediated dimerization is prevented from the presence of the STAS domain. Dimerization is in fact still observed in an extended LOV construct that comprises the N-terminal cap (Buttani et al. 2007), and it is also not prevented by the presence of the J-linker (Losi et al., unpublished). In general, the relevance of LOV–LOV dimerization in LOV proteins has been poorly investigated, although dimerization is considered a key feature in PAS-mediated sensing/regulation (Taylor and Zhulin 1999; Gilles-Gonzalez and Gonzalez 2005), and LOV1 might be responsible for phototropin dimerization (Salomon et al. 2004). We note also that LOV–LOV dimerization and its competition with intraprotein interactions might provide a functional explanation for the presence of two LOV domains in plant phot [see Buttani et al. (2007) for a detailed discussion].

According to the model in Fig. 5, the interaction surface on the LOV core comprises the Hβ–Iβ loop, a region suggested to be important in activation of phot-LOV2 (Freddolino et al. 2006). The two main pathways for LOV domains activation highlighted by means of molecular dynamics simulations (Freddolino et al. 2006), appear therefore to be both represented in YtvA: (a) conformational changes within Hβ on the LOV core, most probably driven by the light-induced movement of N104 (a LOV1-like pathway) (Freddolino et al. 2006), could be transmitted to the STAS domain via the linker region; (b) the Hβ–Iβ loop and adjacent regions within the β-scaffold might interact directly with the STAS domain, thereby being affected by conformational changes triggered by the movement of Q123, another conserved residue hydrogen bonded to O4 on FMN and located on Iβ. This would represent a LOV2-like pathway (Freddolino et al. 2006).

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