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ABSTRACT

Photosystem II (PSII) catalyzes the light-induced water oxidation leading to the generation of dioxygen indispensable for sustaining aerobic life on Earth. The PSII reaction center is composed of D1 and D2 proteins encoded by the *psbA* and *psbD* genes, respectively. In cyanobacteria, different *psbA* genes are present in the genome. The thermophilic cyanobacterium *Thermosynechococcus elongatus* contains 3 *psbA* genes, *psbA1, psbA2 and psbA3* and a new *c*-type heme protein, Tll0287, was found to be expressed in a strain expressing the *psbA2* gene only, but the structure and function of Tll0287 are unknown. Here we solved the crystal structure of Tll0287 at a 2.0 Å resolution. The overall structure of Tll0287 was found to be similar to some kinases and sensor proteins with a per-arnt-sim (PAS)-like domain, rather than to other *c*-type cytochromes. The 5th and 6th axial ligands for the heme were Cys and His, instead of the His/Met or His/His ligand pairs observed for most of the *c*-type hemes. The redox potential, $E_{1/2}$, of Tll0287 was -255 ± 20 mV *versus* normal hydrogen electrode at pH values above 7.5. Below this pH value, the $E_{1/2}$ increased by ≈ 57 mV/pH unit at 15°C, suggesting the involvement of a protonatable group with a pK_{red} = 7.2 ± 0.3. Possible functions of Tll0287 as a redox sensor under micro-aerobic conditions or a cytochrome subunit of an H_2S-oxidising system, are discussed in view of the environmental conditions in which *psbA2* is expressed as well as phylogenetic analysis, structural and sequence homologies.

Photosystem II (PSII) catalyzes the first photochemical reaction in oxygenic photosynthesis, leading to the splitting of water into electrons, protons and dioxygen. In this way, PSII sustains oxygenic life on Earth by providing oxygen and converting light energy into biologically useful chemical energy. The central part of PSII is largely conserved from

prokaryotic cyanobacteria to eukaryotic higher plants, and a typical PSII core from cyanobacteria consists of 17 trans-membrane subunits and 3 peripheral, membrane-extrinsic subunits, with an overall molecular mass of ≈ 350 kDa for a PSII monomer. PSII exists in a dimeric form, and the structure of cyanobacterial PSII dimer has been solved up to 1.9 Å resolution (1, 2). The core of PSII is formed by two subunits D1 and D2, which bind all the cofactors involved in the PSII electron transfer chain, and are surrounded by two large chlorophyll-protein subunits CP47 and CP43 (3-5).

The D1 protein is encoded by the *psbA* gene which exists in a single copy in higher plants but in multiple forms in cyanobacteria (6). The isoforms of the D1 protein encoded by these different *psbA* genes in cyanobacteria have been reported to be differently expressed in response to various environmental conditions such as light intensity (7-9), the presence of UV-B (10, 11), chilling stress (12), reductive stress (13), and low O_2 (14) or micro-aerobic conditions (15, 16).

In the thermophilic cyanobacterium *Thermosynechococcus elongatus* 3 homologous genes, *psbA1*, *psbA2* and *psbA3*, are present in the genome (17). The PsbA1, PsbA2 and PsbA3 D1 proteins encoded by the *psbA1*, *psbA2* and *psbA3* genes, are expressed under different environmental conditions (15, 16, 18, 19). Out of the 344 amino acid residues, 31 differ between PsbA1 and PsbA2, 21 differ between PsbA1 and PsbA3, and 27 differ between PsbA2 and PsbA3 (see 20, 21 for reviews).

In *T. elongatus*, some of the properties of PSII were found to be different depending on the *psbA* gene product (20, 21). For example, while the O_2-evolving activity was found to be similar in PsbA1- and PsbA2-containing PSII (PsbA1-PSII and PsbA2-PSII) (22), the PsbA3-containing PSII (PsbA3-PSII) had a higher O_2 evolution activity (23, 24). The redox potential of pheophytin, Q_A and Q_B, and the binding properties of some herbicides, were also reported to be different in PsbA1-PSII and PsbA3-PSII (25, 26). On the other hand, the differences between PsbA2-PSII and PsbA1/A3-PSII are less documented. The structures of PsbA2-PSII and PsbA3-PSII have not yet been reported. However, a previous work showed that the geometry of the phenol group of TyrZ and its environment, likely the hydrogen bond between TyrZ and D1/His190, is modified in PsbA2-PSII in comparison with PsbA1/A3-PSII (22, 24).

Interestingly, a new electron paramagnetic resonance (EPR) signal was detected in whole cells expressing the *psbA2* gene only (27). This EPR signal was identified to be originated from a hemoprotein which is the product of *tll0287*, a hypothetical gene encoding a ~19 kDa protein (consisting of 191 residues) with an unknown function (27). Homologous genes to *tll0287* are also found in several cyanobacteria (see Discussion section). The amino acid sequence of the Tll0287 protein has a CXXCH motif characteristic for *c*-type cytochromes. Four different N-terminal sequences were detected for the mature Tll0287, suggesting multiple processing sites. In the present work, we purified the Tll0287 protein, solved its crystal structure, determined its midpoint potential, and investigated its cellular localization by immunoblot analysis. Possible functions of Tll0287 are discussed based on the sequence and structural homologies with other known proteins.

RESULTS

Expression and location of the Tll0287 protein—The previous study has detected the novel EPR signal ascribed to Tll0287 in both soluble and membrane fractions from the *psbA2*-expressing cells, but neither from the *psbA1*- nor *psbA3*-expressing cells (27). In order to confirm the different expression of Tll0287 in these three different strains, we used an anti-Tll0287 antibody. Fig. 1a shows a SDS-PAGE analysis of the purified Tll0287 protein used in this study, and Fig. 1c shows an immunoblot analysis with the anti-Tll0287 antibody in the whole cell and thylakoid fractions from WT (expressing mainly the *psbA1* gene), WT*2 (expressing the *psbA2* gene only) and WT*3 (expressing the *psbA3* gene only) strains. The amount of proteins loaded in each lane of Fig. 1c are shown in Fig. 1b, where exactly the same amount of the samples were loaded in each lane as in Fig. 1c. The results in Fig. 1c clearly shows that Tll0287 is expressed in the WT*2 strain, but little or not at all in the WT and WT*3 strains. In the immunoblots of the purified Tll0287 protein and various

subcellular fractions from the WT*2 strain, weak bands at twice and four times the molecular mass of Tll0287 were detected, which may correspond to non-native oligomeric form of Tll0287 that would be present even under SDS-PAGE conditions in a very low amounts. It should be noted that in lane 2 (labeled 'Tll' of Fig. 1b), the CBB-stained Tll0287 is barely visible, which is required to ensure that the immunoblot in Fig. 1c does not become too dense.

Tll0287 was also detected in a crude-PSII fraction (PSII purified with LDAO) from the WT*2 strain, but not in purified PSII after extensive purification procedures involving two different detergents (LDAO and β-DDM) (Fig. 1c). The previous result has suggested that Tll0287 is co-purified with a His-tagged PSII (27); however, the PSII used in the previous study was obtained with a simple step of purification employing a mild detergent-solubilization and nickel affinity column. The present results therefore suggest that Tll0287 is likely not bound to PSII specifically. However, it should be noted that cyt *c*-550, an extrinsic protein associated with PSII rather strongly, can also be dissociated from PsbA2-PSII, but not from PsbA1-PSII and PsbA3-PSII, during a size exclusion chromatography performed under high pressure (results not shown), implying that it is sometimes difficult to conclude whether the binding of non-membrane proteins is real or not based on the co-purification only.

Although previous studies (27) have suggested the possible presence of Tll0287 at the lumenal side of the thylakoid membrane, its location remained to be confirmed. Therefore, the thylakoid fraction from the *psbA2*-expressing strain was treated with the protease thermolysin, together with the purified Tll0287 as a control, and the protease-treated samples were probed by immunoblot analysis (Fig. 2). The amount of the samples loaded in each lane for the immunoblot analysis was shown in Fig. 2a, where again the band of purified Tll0287 was barely visible by CBB-staining. While the purified Tll0287 is largely digested by thermolysin, it was poorly digested by the protease in the thylakoid fraction (Fig. 2b). An exactly same situation was found for the PsbO protein (Fig. 2c), which has a well-established lumenal location. The CBB-stained gel (Fig. 2b) showed that many bands were weakened in the thermolysin-treated thylakoid membrane fraction as well as the purified PSII, indicating the effective digestion by the protease. These results suggest that at least part of Tll0287 is located in the lumen of the thylakoid, or periplasm if we consider that there may be some contamination of cytoplasmic membranes in the thylakoid membrane fraction used in the present study.

Determination of the midpoint potential of Tll0287—The redox titration of Tll0287 is shown in Fig. 3. Fig. 3a and 3b show the absorption spectra recorded in the oxidative way and reductive way, respectively. The presence of well-defined isosbestic points in the two panels shows that the protein was fully stable in these experimental conditions. Fig. 3c shows a Nernst plot for an oxidation (blue points) and a reduction (red points) titration performed at pH 8.5. The continuous black line is a global fit with n = 1 and $E_{1/2}$' = -255 ± 20 mV *vs* NHE. Finally, Fig. 3d shows the pH dependence of the $E_{1/2}$' determined as above at different pH values (the data points are from electrochemical experiments performed on Tll0287 from 3 batches). The continuous line is the result of a fitting procedure with the following equation: $E_{1/2}' = E_0' - 57 \log((K_{ox} + [H^+]) / (K_{red} + [H^+]))$ where E_0' is the midpoint redox potential of the fully protonated form and K_{red} and K_{ox} are the ionization constants of the protonatable group when the iron is reduced and oxidized, respectively (28). The E_0'value was found to be -255 ± 20 mV *vs* NHE at pH values above pK_{red} = 7.2 and the pK_{ox} value was ≤ 5.5.

Structure determination—Tll0287 was purified from the pool of non-membrane proteins and crystals of the isolated protein were grown at 20°C for a few days. The crystals appeared as red needles with a maximum size of 0.4 mm x 0.02 mm x 0.02 mm. The crystals belong to the space group $P4_22_12$ with unit cell dimensions $a = b$ = 101.2 Å, c = 33.1 Å (Table 1). Phase information was obtained by the single-wavelength anomalous diffraction (SAD) method using anomalous diffraction from the Fe ion of the heme, and the model was refined to a resolution of 2.0 Å with an R_{factor} and R_{free} of 0.1765 and 0.2336, respectively.

The structure of Tll0287 is shown in Fig. 4a. It contains residues 26-186 (the full length of the protein is 191 residues), a heme-*c*, a

tentatively assigned chloride ion, and 84 water molecules. The secondary structure of the domain was defined as follows: α-helices, α1: 28-49, α2: 68-82, α3: 103-114, α4: 139-146, α5: 153-158; β-strands, β1: 85-89, β2: 120-125, β3: 128-136 and β4: 172-181. The helix α2 is antiparallel to the N-terminal end of helix α1. The β-strands are arranged in an antiparallel fashion and surrounded by three α-helices α1, α2 and α3.

Fig. 4b shows the surface charge distribution of Tll0287. The periphery of the heme ligand is relatively positive (Fig. 4c), and a concave pocket was found between the four β-strands bundle and α3 (Fig. 4b), which is also apparent in the overall structure of Tll0287. Fig. 4d shows that Tll0287 indeed exhibits a large hydrophobic surface as predicted from the properties observed during its purification (27).

MALDI-TOF/MS analysis of the Tll0287 protein before crystallization detected peaks at *m/z* values of 18,633, 18,997, 19,162, 19,858 and 20,382 (Table 2). The peak at *m/z* 19,162 Da is equal to the molecular mass of the Tll0287 peptide with 24 residues truncated at the N-terminal region (*Δ*24) and with the heme bound (27). The other fragments may correspond to Tll0287 truncated at 11 or 12, 17 and 26. Because the previous study showed that the *Δ*24 fraction likely dominates the heme-bound form of Tll0287 (27), our heme-containing structure likely corresponds to the *Δ*24-truncated protein. This is also in agreement with the sequence analysis by SignalP which suggested that this protein possesses a signal peptide that cleaves between 24 and 25 residues. The mass analysis, however, did not give information regarding the relative ratio of other fragments in the purified Tll0287 protein solution.

Heme in the Tll0287 protein—The 5th and 6th heme iron axial ligands are Cys68 and His145 (Fig. 4c); this is remarkably different from most of the *c*-type cytochromes in which His/Met or His/His axial ligands are found. It also appears that the Cys68 axial ligand is close to Arg45 and may be stabilized by it, whereas the His145 axial ligand is supported by the carbonyl group of Ala164 and is also close to Arg94 and Arg173.

Expression level of the Tll0287 protein in the dark—As mentioned in the Introduction section, the expression level of *psbA2* was reported to increase under micro-aerobic conditions (14-16) and, as discussed below, in *Synechocystis sp.* PCC6803 it has been shown that *sll5034* (analogous to *tll0287*) was transcribed at a higher level in the dark, an environment possibly comparable to micro-aerobic conditions (29**).** We have therefore compared the level of expression of Tll0287 between the whole cells of WT, which mainly express *psbA1*, with those of WT*2 cells, under different light conditions. Fig. 5 shows the EPR spectra recorded from WT and WT*2 cells cultivated under either normal light conditions (*i.e.* 120 μE m^{-2} s^{-1}) or after a further dark period of 3 to 4 hours. The magnetic field region shown allows us to compare the relative amplitude of 3 resonance signals, namely, the Fe^{3+} superoxide dismutase signal labeled with an asterisk between 1300 and 2000 gauss (30), the g_z resonance of the soluble fraction of cyt *c*-550 labeled with an asterisk between 2200 and 2300 gauss (31), and the g_z resonance of Tll0287 labeled with an asterisk at around 2800 gauss (27). The large signal from Mn^{2+} present in the cells precluded the detection of resonances other than cytochromes. Data in Fig. 5 clearly shows that, while the g_z resonance signal of Tll0287 was virtually not detected in the WT cells grown either under normal light conditions (spectrum *a*) or after a further dark period (spectrum *b*), the level of expression of Tll0287 was approximately doubled upon a short dark period in the WT*2 cells when compared to other EPR detectable proteins (spectra *c* and *d*).

DISCUSSION

Location, structure and physical chemical properties of Tll0287—The present results confirmed that the Tll0287 protein is highly expressed in a strain expressing *psbA2*, but not in the WT (expressing predominantly *psbA1*), and WT*3 (expressing only *psbA3*) strains. Furthermore, it is shown that this protein is likely located in the thylakoid lumen and/or in the periplasm based on the protease digestion results. This location is in agreement with sequence analysis by SignalP which suggested that this protein possesses a cleavage site between residues 24 and 25 (not shown). The lumenal or periplasmic location is also consistent with the structural analysis showing that this protein has a globally similar structure

to the PhoQ-DcuS-CitA (PDC) domain of some proteins involved in signal transduction with a periplasmic PAS fold instead of a cytoplasmic one (see below). However, we cannot exclude the possibility that another fraction of the Tll0287 protein may be located in the cytoplasm. This would be consistent with the results of MS analysis showing that the N-terminal of the purified protein had several N-termini (27) consistent with different cleavage sites. Thus, it is possible that the protein has a dual location and possibly multiple functions. Interestingly, three other possible cleavage sites at the N-terminal region are also predicted by SignalP, but with a lower probability. These predicted sites correspond to the other N-terminal sequences found for Tll0287 although in these cases probably no heme is incorporated (27). Further studies are required to understand these observations.

The crystal structure of Tll0287 showed that the heme has a His/Cys ligation pattern, which is distinctly different from the typical His/Met or His/His ligand pairs for most of the *c*-type cytochromes. Such *c*-type heme with His/Cys axial ligation generally have a lower midpoint potential, typically below - 400 mV, than other cytochromes with either a His/His or a His/Met axial coordination, *e.g.* (32-36). Here, the midpoint potential of Tll0287 was determined to be -255 ± 20 mV *vs* NHE at pH 8.5 and to be pH-dependent involving a protonatable group with a p*K* at 7.2 ± 0.3 in the reduced state. The nature of this group is under investigation by using vibrational spectroscopy.

The His/Cys ligation pattern for the heme in Tll0287 is the second one found in cyanobacteria. The first one is PsbV2 (37), a *c*-type heme protein similar to PsbV (*i.e.* the cyt *c*-550), but with unknown function, although it has been reported that this protein may partially support the photoautrophic growth of *Synechocystis* 6803 in place of the PsbV protein (38). Fig. 6a shows the superimposition of the structure of Tll0287 with that of PsbV2. Surprisingly, although the heme structures can be superimposed with a rmsd (root-mean square deviation) of 0.8 Å, the overall structures of the two proteins are remarkably different. Because the structure of PsbV2 belongs to the general folding of *c*-type cytochromes and is rather similar to other *c*-type cytochromes (37), the present results indicate that the structure of Tll0287 is different from those of typical *c*-type cytochromes.

The ligand pair of His/Cys is different from the His/Tyr ligand pair previously erroneously predicted from the EPR spectrum (27). Indeed, using the formalism developed in (39) (see also 40 for a review), the crystal field parameters such as the rhombicity (ν/Δ) and the tetragonality (Δ/λ) can be calculated from the g_z, g_y and g_x values of the EPR signal. These structural parameters put Tll0287 in a domain close to the His/TyrO$^-$ domain (see Fig. S1). From the EPR data available in the literature, the rhombicity and tetragonality parameters can now be calculated for at least 10 hemoproteins with a His/Cys axial ligation. With the exception of the *b*-type heme, the cystathionine β-synthase (41) and ATP-dependent potassium channels (42), the *c*-type heme PsbV2 (37), DsrJ (32), NaxLS (33), triheme in *R. sulfidophilum* (35), SoxAX (34), cyt *c6* M58C (43), RcoM 2 (44) and cyt *c* T58C/L79G/M80X mutant (45) are very close to each other and at the interface of three other domains already identified (Fig. S1). In contrast, Tll0287 is slightly outside of this small domain. This at least shows that such a graph can only be indicative mainly when it concerns with hemoproteins having a structure largely different from the others as mentioned above. Construction of a cyt *c*-550 mutant with His/Cys axial ligation has been reported in *T. elongatus* (46). However, cyt *c*-550 purified from this mutated strain had the same EPR signals and redox potential as that of the WT cyt *c*-550, which strongly suggests that the construction failed in obtaining a His/Cys cyt *c*-550 mutant.

Sequence and structure similarity—Because the structure of Tll0287 is unique in that it does not resemble those of the typical *c*-type cytochromes, we searched for possible similar structures with the DALI server (47). The results (Table S1) showed that the structure of Tll0287 is similar to that of a sensor domain contained in some kinases or sensor proteins possibly involved in cellular signal transduction, such as the sporulation kinase *d* domain from *Bacillus subtilis* (KinD, PDBID: 4JGO) (48) (Fig. 6b) and a methyl-accepting chemotaxis protein (Mcp, PDBID: 3C8C) (Fig 6c), even though they do not contain a heme. These proteins have a PAS-like domain, especially a

PhoQ-DcuS-CitA (PDC) domain as extracellular sensors (49), although the rmsd values between the overall structures of Tll0287 and these proteins were approximately 2.2 Å and 3.2 Å, respectively. Fig. 7 shows the results of multiple structural alignments by the DALI server (47). It is clear that although the primary sequences of Tll0287 and other proteins are not similar, the secondary structures appear to exhibit significant similarities. On the basis of the comparisons of the secondary (Fig. 7) and three-dimensional structures (Fig. 6), the structure of Tll0287 can be divided into three domains: a PAS-like domain (Ala26-Ala49, Val67-Gln138 and Val169-Ala181), a heme binding site (Ala139-Arg168), and a loop domain (Ser50-Ala66) that separates the PAS-like and heme-binding domains.

PAS domains were discovered as the large regions of *per*, *arnt* and *sim* genes in *Drosophila* (50, 51) that are related to circadian rhythmicity (52), and are signaling modules that monitor changes in the redox potential, light, oxygen level, small ligands, or overall energy level of the cell (53). So far, proteins with the PAS domain are found as transcription factors in clock genes (54), ion channels such as HERG (55) and sensor proteins such as kinases (53). The PAS domains are composed of a single antiparallel, five-stranded β-sheet with the strand order of 2-(1)-5-4-3 (some proteins have only four-stranded β-sheet without the first strand, such as the sensor domain of histidine kinase DcuS (PDB ID: 3BY8)) (48) and several α-helices (56). Although the PAS domains have a conserved three-dimensional fold, these domains share little primary sequence identity (57). The homologous structure of Tll0287 revealed in this study is consistent with these features, and suggests that Tll0287 also contains the PAS-like domain.

A subclass of the PAS domain is the PDC domain, which is a general feature of periplasmic PAS domains (58) and may bind multiple ligands (59). The periplasmic PAS domains typically contain three N-terminal helices, a central helical linker region and a C-terminal α-helix, which are separated by two and three β-strands respectively, forming a 3α-2β-1/2α-3β-1α arrangement. The secondary structure of the *Δ*24-truncated Tll0287 revealed by our present structural analysis was 2α-1β-1α-2β-2α-1β (Fig. 4a, Fig. 7). Considering the lack of the N-terminal region and the possible insertion of the α-helix in the heme binding site, it seems that Tll0287 is similar to the structure of the PDC domain, namely, the periplasmic PAS domain, rather than the cytoplasmic PAS domain which has a 1α-2β-4α-3β folding pattern (58).

Previous studies have suggested that the sensor domain of KinD has an extra ligand between the α-helix and β-strands, and the ligand binding site may accommodate small molecules related to metabolism such as pyruvate or propionate (48). In the electron density map of Tll0287, we indeed found an extra density at the similar position between the α-helix and β-strands (Fig. 8), which is in the concave pocket shown in Fig. 4. It seems that the extra density is surrounded by the guanidine group of Arg87, the carboxyl group of Asp105 and Thr179.

In order to identify the possible ligand corresponding to the extra density, we used the Arp/wARP program (60) for prediction of the extra ligand, which suggested that the extra ligand may be 2-oxoglutaric acid, although its abundance may be low in the thylakoid lumen. On the other hand, MALDI-TOF/MS measurements of the Tll0287 sample after trypsin-digestion showed extra masses of 617 Da, 74 Da and 118 Da (data not shown). The former two correspond to the masses of heme *c* and chlorine, respectively, whereas the 118 Da mass has no corresponding molecules and may therefore be derived from the extra ligand. This mass is similar to those of homoserine, valeric acid such as norvaline, or betaine which was used as an additive in the purification buffer. Although our current results do not allow the identification of the extra ligand, they suggest that Tll0287 has a PDC-like domain with an extra ligand.

The PAS-like domain found in the Tll0287 structure suggests that this protein may have a function in signal transduction, or in response to some environmental factors. In relation to this, it is interesting to note that the expression level of *psbA2* is reported to increase under micro-aerobic conditions (14-16), and the EPR data in Fig. 5 show that under conditions in which O_2 was not produced the expression level of Tll0287 increased in the WT*2 strain, at least transiently.

A second approach searching for structural

similarities was done by using the Phyre[2] server (61), which revealed a good structural similarity with GSU0935, a methyl-accepting chemotaxis protein from *Geobacter sulfurreducens* (PDB 3B42) (62) (Fig. 6d). This *b*-type heme protein also shows a PAS-type fold but exhibits axial ligations (His-water and His-Met) different from that of Tll0287 with a high-spin configuration in the oxidized state. The overexpressed non-membrane part of GSU0935 was crystallized as a dimer (62). Interestingly, as shown in Fig. 6d, the heme part of one monomer of GSU0935 fits very well with the heme part of Tll0287, and the two α-helices of the opposite monomer fit very well with two of the α-helices, α1 and α2 of Tll0287 albeit that they do not have counterparts in the first monomer. Such similarities may suggest that, *in vivo*, Tll0287 is present as a dimer with a structure similar to the dimer of GSU0935.

These structurally similar proteins have a trans-membrane region and active site in the C-terminus after the PAS domain (47, 62), whereas Tll0287 has no region after PAS domain. Instead, Tll0287 has a heme *c* inserted. It is possible that the heme *c* may sense some environmental conditions such as the oxygen level or redox state of the cell, which may therefore induce structural changes of the heme. This structural change may be transmitted and/or enlarged through the PAS-like domain.

Tll0287-homologs, genomic context and phylogeny—Homology sequences of Tll0287 were searched with BLAST for cyanobacteria as well as non-cyanobacterial prokaryotes. Genes with substantial sequence homologies (BLAST search expectation values exceeding e-50), in particular with the C-terminal CXXCH motif, were detected in several cyanobacteria but also outside the cyanobacterial phylum (Fig. S2). While some of these non-cyanobacterial genes coded for larger, two-domain proteins that likely correspond to two-component sensors, others encoded monotopic cytochromes with sizes similar to that of Tll0287 (see phylogenetic tree in Fig. S3A). In order to glean insight into their functions we compared the genomic context of all these Tll0287- and Tll0287-like genes. The following observations emerge: (a) No common features are obvious between cyanobacterial and non-cyanobacterial cases. (b) In cyanobacteria, Tll0287-homologous genes occur almost exclusively in close genomic association with a gene coding for sulphide:quinone-oxidoreductase (SQR). The only exception to this rule is *Arthrospira platensis* in which the Tll0287-homologue and the SQR genes are distantly positioned in the genome. It is noteworthy that the majority of these SQR-genes are annotated as NDH-2 (type-2 NADH-dehydrogenase) but the phylogenetic analysis (Fig. S3B) clearly shows that they all cluster together with *bona fide* SQR from *Oscillatoria limnetica* (63) and not with NDH-2. Remarkably, the tree of SQR-sequences retrieved from the cyanobacterial clade falls into two distinct sub-clades, one contains species in which SQR but not Tll0287-homologous genes are present while in the second sub-clade, SQR-genes co-occur with Tll0287-homologs. Two distinct scenarios can be proposed based on this pattern of co-occurrence: (i) The sulphide dehydrogenase in the clade where "SQR" and Tll0287-homologs co-occur is actually a sulphide:cytochrome-oxidoreductase rather than a sulphide:quinone-oxidoreductase and the Tll0287-gene product would in fact correspond to the cytochrome subunit of an H_2S-oxidising enzyme, reminiscent of so-called flavocytochrome *c*-sulphide dehydrogenase (FCSD) observed in a number of prokaryotes (64); (ii) The association of Tll0287-homologous genes to sulphide metabolism is more indirect and potentially even related to some kind of sensory mechanism (it could be noted here that several of the hemo-proteins with His-Cys axial ligation are involved in the sulphide metabolism). We presently consider the first possibility as less likely since (a) no co-purification of Tll0287 and SQR has been observed so far and, (b) the sequence motif involved in forming the quinone-binding pocket in SQR (GQMTEE in *T. elegans*, deduced from the 3D structure of *Aquifex aeolicus* SQR, pdb-entry 3HYW) is highly conserved in both sub-clades arguing for true SQR-type functional characteristics of all enzymes in both clades. However, in *Synechocystis sp.* PCC6803 it has been shown that *sll5034* (analogous to *tll0287*) and the SQR (coded by *sll5036*) belong to the same operon, and are indeed co-transcribed with a higher level of transcription in the dark, possibly equivalent to micro-aerobic conditions (29). Such a higher level of expression of Tll0287 in the dark was

also observed in the WT*2 strain (Fig. 5), suggesting a role of this protein in sensing the environmental conditions. The actual functional significance of Tll0287-homologous genes in cyanobacteria therefore remains enigmatic, but an involvement in H_2S-oxidation as studied in *Oscillatoria limnetica* (63) represents an intriguing lead. In this context, it is noteworthy that, with the sole exception of *Pleurocapsa minor*, we only found Tll0287 homologs in members of the *Oscillatoriaceae*.

EXPERIMENTAL PROCEDURES

Thermosynechococcus elongatus strain, cultivation of cells and purification of the Tll0287 protein—The *T. elongatus* strain used in this work was the WT*2 strain in which a His-tag was attached in the C-terminus of CP43, and both *psbA1* and *psbA3* were deleted so that only *psbA2* is expressed for the D1 subunit (22, 27). The WT*3 strain that expresses the *psbA3* gene only was made as described in (23). The cells were grown in 1-L cultures of DTN medium with a CO_2-enriched atmosphere at 45°C under continuous light (Grolux, Sylvania). The cells were collected by centrifugation (Beckman) with a JA10 rotor. and were suspended in a buffer containing 100 mM NaCl, 10 mM Tris pH 8.5 and 0.03% β-dodecyl maltoside (β-DDM), 1 mM benzamidine, 1 mM ε-aminocaproic acid and DNase I, and disrupted by a French press. The Tll0287 protein was purified from the soluble proteins upon the breakage of the cells, *i.e.* after the removal of the membrane fraction by centrifugation based on the procedures described previously (27) with some modifications. The supernatant was concentrated and then loaded onto a gel filtration column (Sephadex 75, HiLoad 26/60). The buffer used for this column contained 100 mM NaCl, 10 mM Tris pH 8.5 and 0.03% β-DDM, and the flow-rate was 1 ml/min. The fraction of eluate containing Tll0287 (which was judged based on the approximate molecular masses eluted from the column and the reddish color of the heme together with the measurement of the absorption spectra taken every 4 ml) was collected and further purified with a Mono Q column (GE-Healthcare) at pH 8.5 with a NaCl gradient in the presence of 0.03% β-DDM and a flow rate of 0.5 ml/min. Finally, the fraction eluted from the Mono Q column was diluted 3-5 folds and purified by a Mono S column (GE-Healthcare) at pH 8.5 with a NaCl gradient in the presence of 0.03% β-DDM and a flow rate of 0.5 ml/min. In all cases, the fraction containing Tll0287 was easily followed by the color of the heme. Under the conditions used, Tll0287 was eluted from the Mono Q and Mono S column with a gradient of 100-150 mM NaCl but other proteins which are either more tightly or more loosely bound to the resins eluted differently from the two columns which allowed us to separate them efficiently. It should be noted that: 1) the detergent was added in all of the purification steps to prevent almost irreversible binding of the protein to the resins and to hydrophobic surfaces due to the high hydrophobic character of Tll0287; and 2) hydrophobic resins like phenyl-sepharose cannot be used because the protein binds almost irreversibly to such resins. Tll0287 was then concentrated using a Millipore Amicon Ultra-15 centrifugal filter devices with a cutoff value of 10 kDa. By comparing the relative contents of Tll0287 and soluble Cyt *c*-550 (*i.e.* the fraction of Cyt *c*-550 not bound to PSII easily identifiable by its EPR signal different from the PSII bound fraction) with EPR in whole cells before breakage and the amounts of Tll0287 and cyt *c*-550 purified in parallel from the same batch of the cells, the protocol described above allows the purification of a very large proportion of Tll0287 present in the cells.

Crystallization and X-ray data collection—Initial crystallization conditions were screened with an automatic dispensing machine (Mosquito, TTP Labtech) with over 800 commercially available screening conditions at a concentration of 2 mg protein/ml. One of these conditions composed of Na_2HPO_4, K_2HPO_4, $(NH_4)_2HPO_4$ and Tris (pH 8.5) produced small crystals with a red color of Tll0287. These small crystals were used as seeds to grow larger crystals under the same condition for 2 weeks at 20°C. The crystals obtained were needle-type, and they were cryo-protected using a solution containing 0.7 M Na_2HPO_4, 0.7 M K_2HPO_4, 0.1 M $(NH_4)_2HPO_4$ and 0.1 M Tris (pH 8.6) in either the presence or absence of 25% (w/v) glycerol in a stepwise fashion, and flash-frozen under liquid nitrogen gas. X-ray diffraction data were collected at beamlines BL41XU and BL44XU of SPring-8 at a wavelength of 0.900 Å for a native data set, and at 1.730 Å to measure the Fe anomalous signal

for single wavelength anomalous dispersion (SAD) phasing at 100 K.

Structure determination—Diffraction data were indexed, integrated and scaled by XDS and XSCALE (67). The initial phases were obtained using SHELX C/D/E (68). The iron site identified was provided to the program SHARP/autoSHARP (69, 70) for density modification and automatic model building, which results in a model having 155 residues. The rest of the model was manually built with the program COOT (71, 72) and structural refinement was performed with the program Phenix (73). Ramachandran plot was calculated with the MolProbity (74). The statistics of data collection and structural refinement were summarized in Table 1. For prediction of the extra ligand in the Tll0287 protein, the software Arp/wARP (60) was used. The mF_O-DF_C map and 2mF_O-DF_C map without introducing any ligands were calculated using FFT in the CCP4 suite (75).

Homology search—Search of sequences homologous to Tll0287 was carried out with BLAST (76) and search of the three-dimensional structures similar to Tll0287 was performed by the DALI Server (47). Superposition and root mean square deviation (rmsd) calculation were performed by the LSQ Superpose function of Coot (71, 72) at the heme *c* ligand for comparison with that of PsbV2, and the SSM Superpose function (77) for comparison with Per-Arnt-Sim (PAS)-like domain proteins. Protein structure homology was also investigated with the Phyre^2 server (61).

MALDI-TOF mass analysis—In order to determine the molecular size of Tll0287 the sample was analyzed before the crystallization with MALDI-TOF/MS in linear mode (Voyager-DE PRO MALDI-TOF mass spectrometer; Applied Biosystems) as described previously (27). For identification of possible extra mass, the Tll0287 protein was digested with trypsin (Promega), and the polypeptides produced were analyzed with MALDI-TOF/MS in reflector mode after reduction and alkylation (78).

Prediction of the signal peptide and transmembrane helices—Possible peptide signal cleavage sites were predicted by the SignalP 4.1 Server (79), with the organism group set as Gram-negative bacteria. For prediction of the transmembrane helices, the HMMTOP server was used (80, 81).

Immunoblot analysis—Polyclonal antibodies against Tll0287 were obtained after several injections of purified Tll0287 (see Fig. S5) into rabbits (Service de Pharmacologie et Immunoanalyse, iBiTec-S, CEA Saclay).

Amersham ELC Prime Western Blotting Detection Reagents were used for immunoblot analysis (GE Healthcare). For the experiment in Fig. 1, whole cell samples were prepared by breaking the cell walls with lysozyme-treatment as described previously (82, 83), and the resulted fraction was loaded onto SDS-polyacrylamide gel directly without separation of the soluble and membrane fractions. Thylakoid fractions and purified PsbA2-PSII core complexes were purified from the WT*2 strain as described previously (82, 84). For comparison, the Tll0287 protein purified from the WT*2 strain, whole cells and thylakoid fractions of wild type expressing the *psbA1* gene predominately (WT), and those of a WT*3 (27) strain expressing the *psbA3* gene only were compared.

For determining the cellular location of Tll0287, thylakoid fraction of WT*2 were digested with a protease thermolysin (Sigma-Aldrich) at 5 μg protease/ml for 6 hours at 25ºC, followed by detection with immunoblotting to determine the Tll0287 location. For comparison, the purified Tll0287 and PSII cores purified from the WT*2 strain was digested in the same way, and the PsbO protein was detected by immunoblotting with an anbi-PsbO antibody from Agrisera (Sweden).

Electrochemistry—The redox potential of Tll0287 was determined with the same samples as that used for crystallization. Details of the thin-layer (6 μm) electrochemical cell used for the experiments are given in (85) except that the diamond windows were replaced by CaF_2 windows of 1 mm thickness. The working gold grid purchased from Euromip (France) was surface-modified by dipping it for 10 min into a 5 mM pyrimide-3-carboxyaldehyde thiosemicarbazone (PATS-3, Lancaster) solution heated to 80−90°C, followed by careful washing of the gold grid with ultra-pure water. The following redox mediators (each at 40 μM final concentration) were used to accelerate the electrochemical reaction: methyl viologen (E_m = -440 mV *vs* normal hydrogen electrode, NHE),

benzyl viologen (E_m = -360 mV *vs* NHE), antraquinone 2-sulfonate (E_m = -225 mV *vs* NHE), 2 hydroxy 1-4 naphtoquinone (E_m = -128 mV *vs* NHE), 2,5 dihydroxy-p-benzoquinone (E_m = -62 mV *vs* NHE), duroquinone (-8 mV *vs* NHE), phenazine ethosulfate (55 mV *vs* NHE).

Equilibrium redox titrations were performed by applying potentials to the working gold grid from -100 mV to -500 mV *vs* NHE with 20 mV intervals using a potentiostat (PRGE, Tacussel Electronique). For each potential step the sample was allowed to equilibrate for 5 minutes. Spectra from 400 to 650 nm were recorded using a modified Cary 14 spectrophotometer equipped with a Xenon light source and a UV enhanced silicon detector. Data was analyzed by calculating the integrated Soret absorption (422 to 457 nm) obtained from the difference of the spectrum recorded at a given potential minus the spectrum of fully oxidized Tll0287 obtained at -100 mV *vs* NHE. The electrochemical cell was thermostated at 15°C with a water circulation system and the sample compartment was purged with dry air.

Buffers at different pH values contained 200 mM NaCl, 0.03 % β-DDM and 50 mM of buffer MES (pH 5.5, 6.0, 6.5), Tricine (pH 7.5), Tris (pH 8.5) or CAPS (pH 10.5). An aliquot of 9 µl of the Tll0287 sample solution adjusted to the buffer solution described above was mixed with 1 µl of mediator-buffer solution and dropped on the working gold grid. The path-length of the closed electrochemical cell was adjusted to about 6 µm by adjusting the Amide I/H_2O absorption of the sample to about 0.8 using an FTIR-spectrophotometer (Bruker Tensor 27).

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Author contributions: JRS and AB conceived and coordinated the study. TM crystallized the protein; TM and MS collected the X-ray diffraction data and analyzed the structure. AN and TM performed western blot analysis, TK prepared the samples from the WT*2 strain, and MS performed MS analysis. TLL and AB purified the protein; RH performed the redox analysis. WN performed the phylogenetic analysis, and TM performed structural homology analysis. TM, MS, WN, AB and JRS wrote the manuscript, and all the authors reviewed the results and approved the final version of the manuscript.

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Footnotes

Data deposition: The coordinates for the structure of Tll0287 have been deposited in the Protein Data Bank with the accession number 5B82.

The abbreviations used are: CAPS, N-cyclohexyl-3-aminopropanesulfonic acid; β-DDM, *n*–dodecyl–*β*–maltoside; LDAO, *N*, *N*-dimethyldodecylamine *N*-oxide; MALDI-TOF/MS, matrix-assisted laser desorption/ionization-time of flight mass spectroscopy; MES, 2–(*N*–morpholino) ethanesulfonic acid; MW, molecular weight; NHE, normal hydrogen electrode; Q_A, primary quinone electron acceptor; Q_B, secondary quinone electron acceptor; PDC and PAS domains, PhoQ-DcuS-CitA and Per-Arnt-Sim domains; PSII, Photosystem II; Tris, Tris(hydroxymethyl)aminomethane; TyrZ, redox-active tyrosine on the D1 subunit (D1-Y161) in photosystem II. FCSD, flavocytochrome *c*-sulphide dehydrogenase; SQR, sulphide:quinone-oxidoreductase.

FIGURE LEGENDS

FIGURE 1. Immunoblot analysis on the expression and location of Tll0287

a. SDS-PAGE analysis with a 16% acrylamide gel of the purified Tll0287 protein (lane 2) used in this study. The molecular mass markers are shown in lane 1, with their molecular masses (kDa) indicated. **b.** CBB-stained gel of the samples used for immunoblot analysis shown in panel **c**. The samples shown are the purified Tll0287 protein (Tll), whole cell and thylakoid fractions from the wild type (WT, predominately expressing *psbA1*), WT*2 (expressing *psbA2* only), and WT*3 (expressing *psbA3* only) strains, and crude-PSII (LDAO-PSII), PSII core complex purified from the *psbA*$_2$-expressing strain (WT*2). The amount of proteins loaded for each lane is equivalent to 2 μg chlorophylls for the whole cell fractions and thylakoid membranes, 1 μg chlorophylls for LDAO-PSII, 0.5μg for purified PSII, and 0.16 mg protein for the Tll0287 protein. "M" stands for molecular marker with the molecular masses indicated (kDa). **c.** Immunoblot analysis with an anti-Tll0287 antibody for same samples shown in panel **b**.

FIGURE 2. Digestion of the Tll0287 protein with a protease thermolysin

a. CBB-stained gel of the samples used for immunoblot analysis shown in Panels **b** and **c**. The samples used are the purified Tll0287 protein, thylakoid membrane fraction from the WT*2 strain (A2-Thy, expressing *psbA2* only), and purified PSII from WT*2 (A2-PSII). The amount of the samples loaded on each lane was the same as that in Fig. 1b. **b.** Immunoblot analysis of the Tll0287

protein in the purified Tll0287 sample and thylakoid membrane fraction (A2-Thy) from the WT*2 strain with an anti-Tll0287 antibody, either before (labeled '–') or after digestion of the samples with 5 μg/ml thermolysin for 6 hours (labeled '+TH'). **c.** Immunoblot analysis of the PsbO protein in the purified PSII and thylakoid membrane fraction from the WT*2 strain with an anti-PsbO antibody, before (–) or after digestion of the samples with thermolysin (+TH) at the same conditions as for Tll0287.

FIGURE 3. Electrochemistry on Tll0287
Panel **a** and Panel **b** show the absorption spectra recorded at the indicated potential during the oxidative and reductive processes, respectively. Panel **c** shows a Nernst plot for an oxidation (blue points) and a reduction (red points) performed at pH 8.5 and T = 15°C. The continuous black line is a global fit with *n* fixed to 1 and $E_{1/2}$' = - 255 mV *vs* NHE. Panel **d** shows the pH dependence of the $E_{1/2}$' determined as above at different pH values. The continuous line is the result of a fitting procedure with the following equation: $E_{1/2}' = E_0' - 57 \log((K_{ox} + [H^+]) / (K_{red} + [H^+]))$ where E_0' is the midpoint redox potential of the fully protonated form and K_{red} and K_{ox} are the ionization constants of the protonatable group when the iron is reduced and oxidized, respectively. The pK_{red} was found to be 7.2.

FIGURE 4. Crystal structure of the Tll0287 protein
a. Overall structure of the Tll0287 protein. The PAS-like domain composed by 3 α-helices and 4 β-strands was depicted in green, the heme and its binding part were depicted in red and yellow respectively, and the loop region was depicted in orange. **b.** Surface charge distribution of the Tll0287 protein. Blue and red colors represent negative and positive areas, respectively. The area surrounding the heme is largely positive. The black arrow indicates a concave pocket. **c.** Ligand environment of the heme *c* in Tll0287. The 5th and 6th heme iron axial ligands, Cys68 and His145 and the residues around them are depicted in stick models. **d.** Hydrophobicity characters of the surface of Tll0287. The structure of Tll0287 was depicted in a ball-model in the same direction (left-side) as that in Fig. 3a and 3b, and in a direction rotated 180^o (right-side). Blue represents positively charged residues (R, K, H), red represents negatively charged residues (D, E), white are hydrophilic residues, and orange are hydrophobic residues.

FIGURE 5. Expression level of Tll0287 under dark conditions.
Spectrum a, WT whole cells cultivated for 2 days under normal light conditions. Spectrum b, WT whole cells after a further dark period of 3 to 4 hours. Spectrum c, WT*2 whole cells cultivated for 2 days under normal light conditions. Spectrum d, WT*2 whole cells after a further dark period of 3 to 4 hours. For sake of comparison, the spectra were approximately scaled by using both the Fe^{3+}SOD signals (labeled by '*' between 1300 and 2000 gausses) and the g_z signal of cyt-c550 (labeled by '*' between 2200 and 2300 gausses). The signal from Tll0287 is located around 2800 gauss. Instrument settings: modulation amplitude, 25 G; microwave power, 5 mW; Temperature, 20 K; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz.

FIGURE 6. Structural comparison of the Tll0287 protein with other proteins
a. Superposition of Tll0287 and PsbV2 from *T. elongatus* (PDB ID: 4LJI) (37). The colors for the Tll0287 protein and its heme are green and red, respectively, and those for the PsbV2 protein are as follows: heme, yellow; others, orange. **b.** Superposition of Tll0287 and the sporulation kinase D sensor domain from *Bacillus subtilis* (blue, A chain of PDB 4JGO; pink, the ligand of this protein, pyruvate) (48). **c.** Superimposition of Tll0287 and a methyl-accepting chemotaxis protein (Mcp) (purple, A chain of PDB 3C8C; cyan, the ligand of this protein, alanine). **d**. Superimposition of Tll0287 and the dimer of the non-membrane part of GSU0935 (PDB 3B42) (62).

FIGURE 7. Structural alignment of the Tll0287 protein with 11 homologous proteins
Eleven representative proteins are selected from Table S1 that show structural similarities with Tll0287, excluding their similar molecules. Inserted segment relative to the Tll0287 protein structure

are hidden. The upper part shows the alignment of the amino acid sequence, and the lower part shows the secondary structure assignments performed by DSSP (65, 66). H: helix; E: strand; L: coil.

FIGURE 8. A possible extra ligand in Tll0287
a. The residues Arg87, Asp105 and Thr179 surrounding the concave pocket (shown in Fig. 4b) are depicted in stick models. **b.** Electron density map of a possible ligand corresponding to the ligand binding site in the structure of the sporulation kinase D sensor domain (48)**.** The blue and red mesh represents mF_O-DF_C omit map (positive and negative, respectively) contoured at 3.5 σ distributions, and the grey mesh represents 2mF_O-DF_C map contoured at 1.0 σ distributions.

Table 1. Data collection and structural refinement statistics.

Data collection	Native	Fe-peak
Wavelength	0.900	1.730
Space group	$P4_22_12$	$P4_22_12$
Unit cell	a = b = 101.2 Å, c = 33.1 Å,	a = b = 100.9 Å, c = 33.1 Å,
	α = β = γ = 90.00°	α = β = γ = 90.00°
Resolution (Å)	20-2.00 (2.13-2.00) [a]	20-3.20 (3.39-3.20)
Unique reflections	12,169 (2,047)	5,410 (856)
Redundancy	12.7 (12.9)	25.0 (13.8)
Completeness	100.0 (100.0)	99.6 (97.5)
R_{merge} [b]	10.2 (95.5)	8.8 (21.9)
I/σ(I)	19.1 (2.8)	36.5 (10.9)
R-means [c] (%)	10.6 (99.4)	9.0 (22.7)
CC(1/2) [d]	99.9 (96.8)	99.9 (99.0)
Structural refinement		
R_{factor}	0.1765	
R_{free}	0.2336	
Number of atoms	1,422	
Proteins	1,294	
Ligands	44	
Water	84	
Number of residues	161	
R.m.s.d. (bond)	0.007	
R.m.s.d. (angle)	1.030	
Average B-factor	36.1	
Ramachandran plot [e]		
Favored (%)	99.4	
Allowed (%)	0.6	
Outliers (%)	0.0	

[a] Statistics for the highest resolution shell are shown in parentheses.

[b] $\mathbf{Rmerge} = \sum_{\boldsymbol{hkl}}\sum_{i}|I_i(\boldsymbol{hkl}) - \langle I(hkl)\rangle| \Big/ \sum_{\boldsymbol{hkl}}\sum_{i} I_i(\boldsymbol{hkl})$, where $\mathbf{I}_i(hkl)$ is the intensity of the reflection and $\langle \mathbf{I}(hkl)\rangle$ is the mean intensity of a group of equivalent reflection.

[c] *R*-factor independent of the redundancy.

[d] Percentage of correlation between intensities from two random half-datasets.

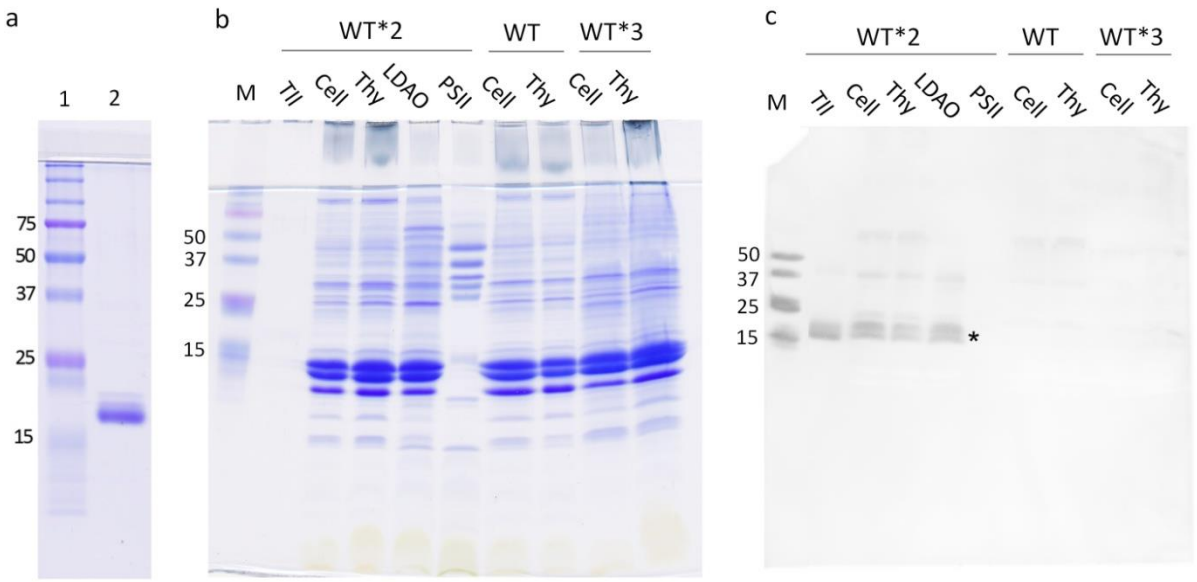
[e] Calculated with the MolProbity (35).

Table 2. Experimentally determined and calculated molecular masses of Tll0287 by MALDI-TOF/MS.

Experimentally measured mass $[M + H]^+$ in Da	Calculated mass $[M + H]^+$ in Da		Processing site	N-terminal sequence
	- Heme	+ Heme[a]		
20382	19,838.16	20,456.37	11/12 (*Δ*11)	ASLWI
20382	19,767.08	20,351.28	12/13 (*Δ*12)	SLWIQ[a]
19858	19,139.35	19,757.56	17/18 (*Δ*17)	GSPAP
18633, 19162	18,545.68	19,163.89	24/25 (*Δ*24)	SANPE
18997	18,387.52	18,969.03	26/27 (*Δ*26)	NPEEL[a]

a) N-terminal sequence found from MALDI-TOF/TOF analyses with the Edman procedure in previous research (27).

Figure 1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2
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37
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WT
WT*3
M
Tll
Cell
Thy
LDAO
PSII
Cell
Thy
Cell
Thy
50
37
25
15
c
WT*2
WT
WT*3
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PSII
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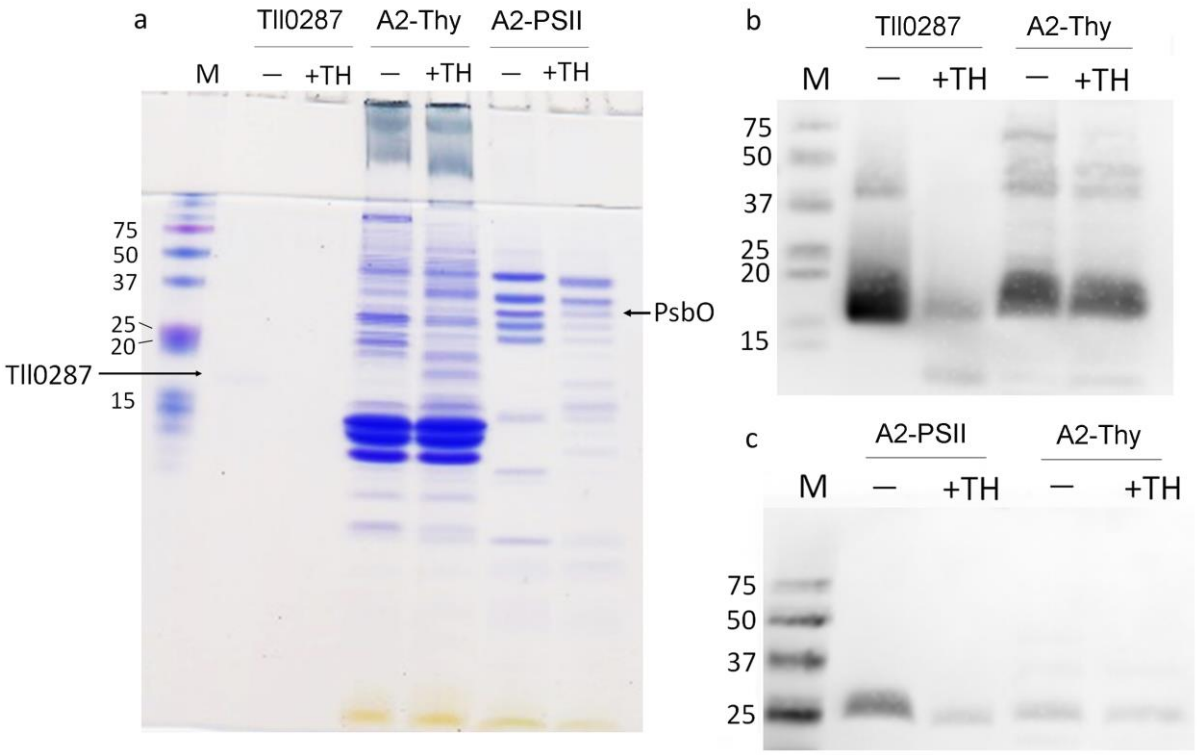
Thy
Cell
Thy
50
37
25
15
*

Figure 2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Tll0287
A2-Thy
A2-PSII
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—
+TH
—
+TH
—
+TH
75
50
37
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20
15
Tll0287
PsbO
b
Tll0287
A2-Thy
M
—
+TH
—
+TH
75
50
37
25
20
15
c
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A2-Thy
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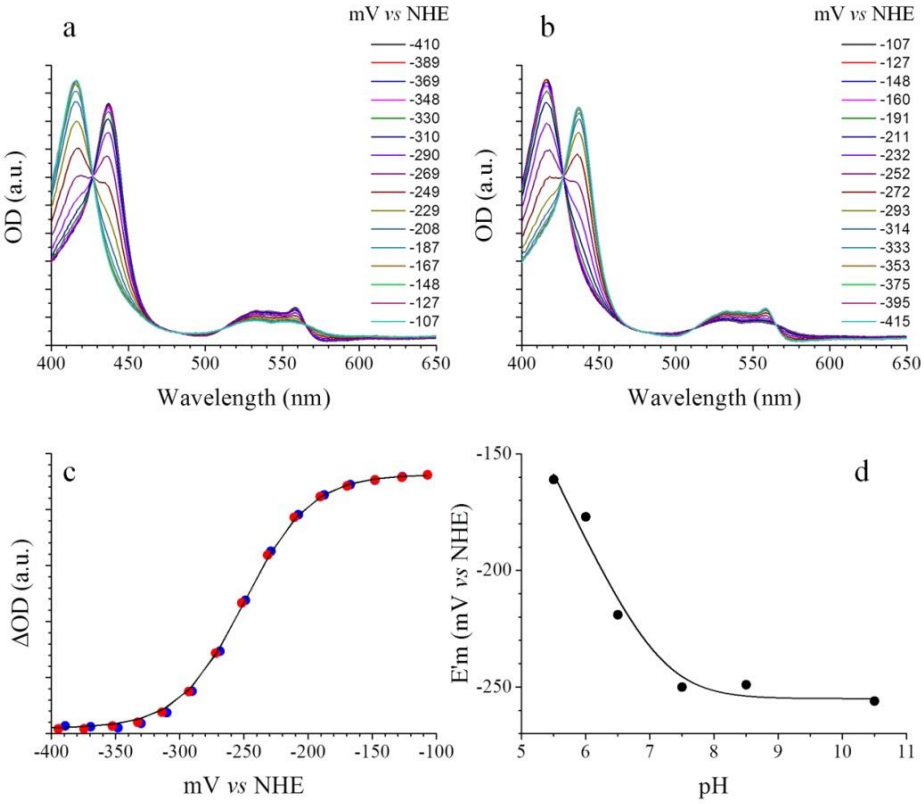
—
+TH
—
+TH
75
50
37
25

Figure 3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-369
-348
-330
-310
-290
-269
-249
-229
-208
-187
-167
-148
-127
-107
OD (a.u.)
Wavelength (nm)
b
mV vs NHE
-107
-127
-148
-160
-191
-211
-232
-252
-272
-293
-314
-333
-353
-375
-395
-415
OD (a.u.)
Wavelength (nm)
c
ΔOD (a.u.)
mV vs NHE
d
E'm (mV vs NHE)
pH

Figure 4

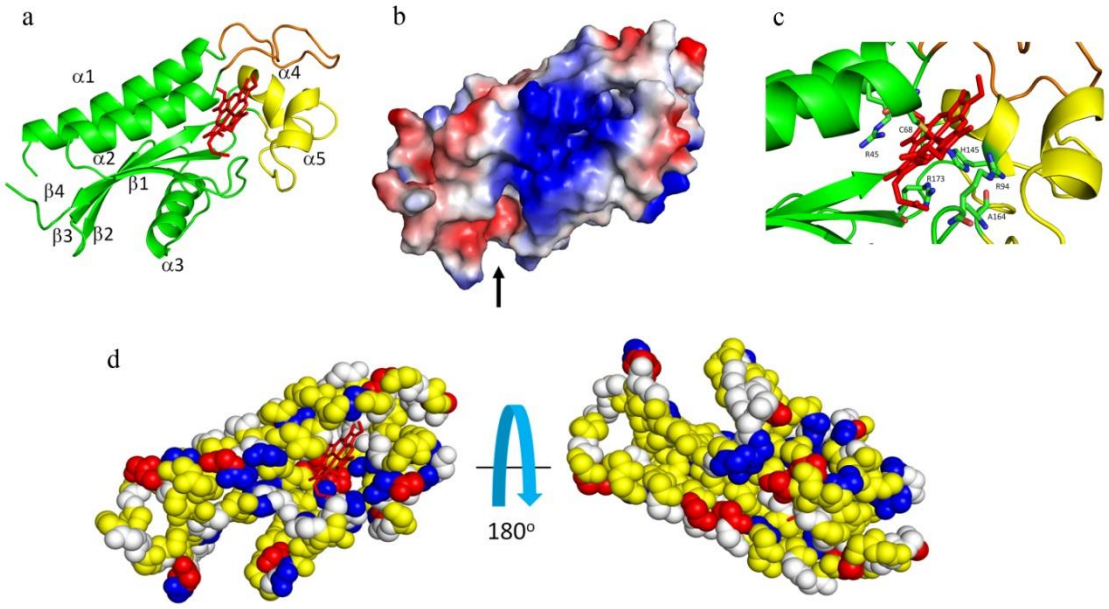

Figure 5

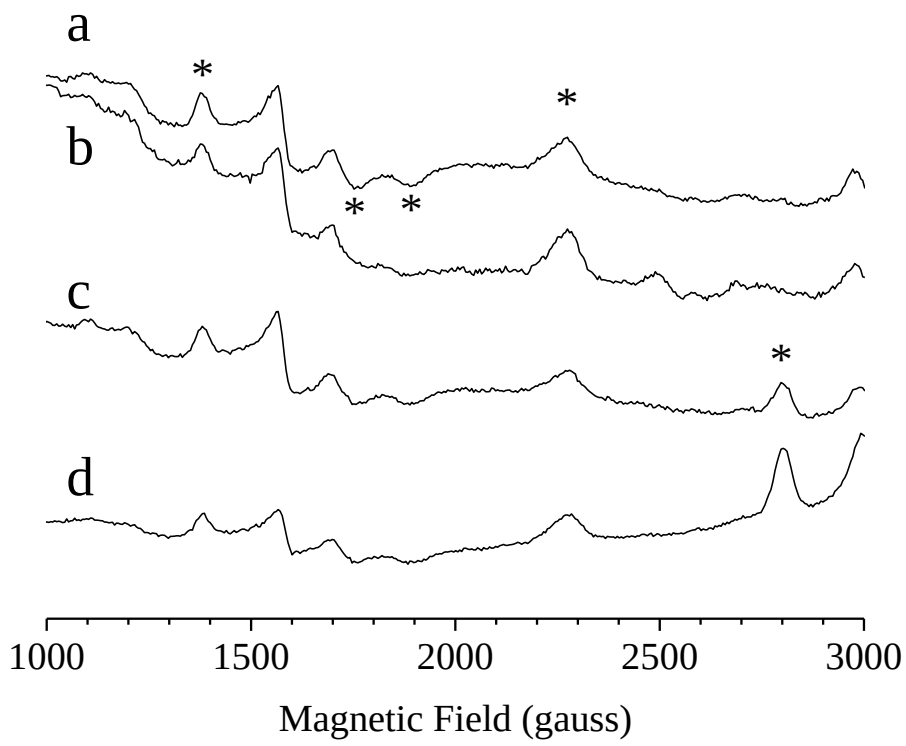

Figure 6

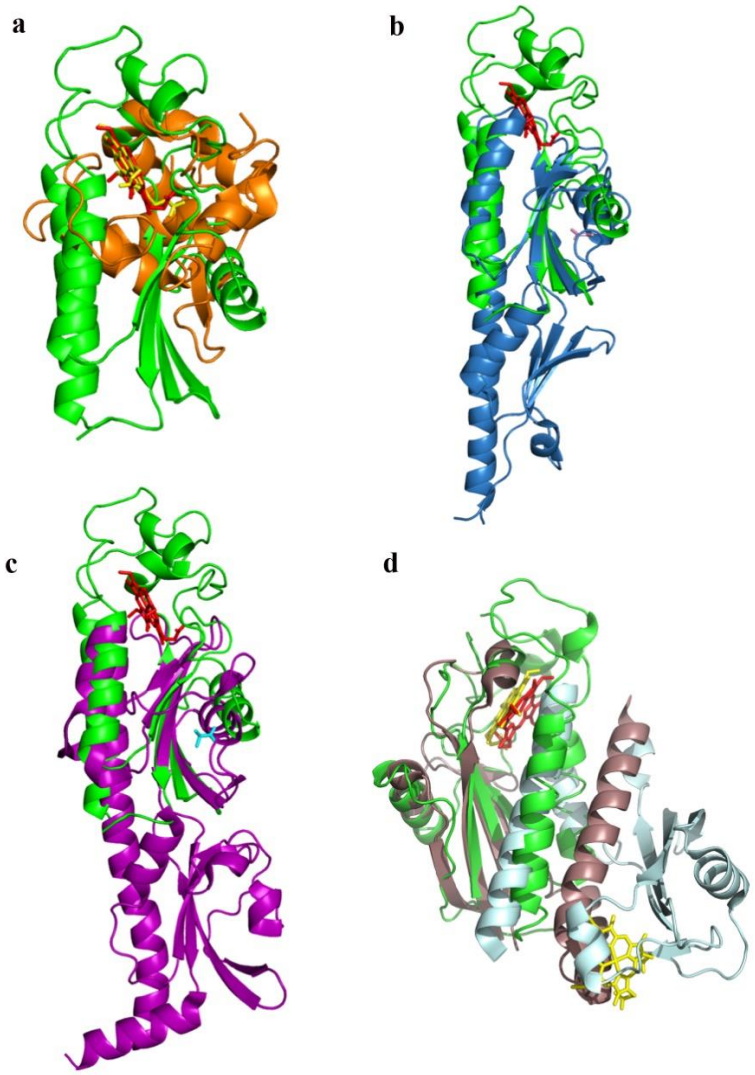

Figure 7

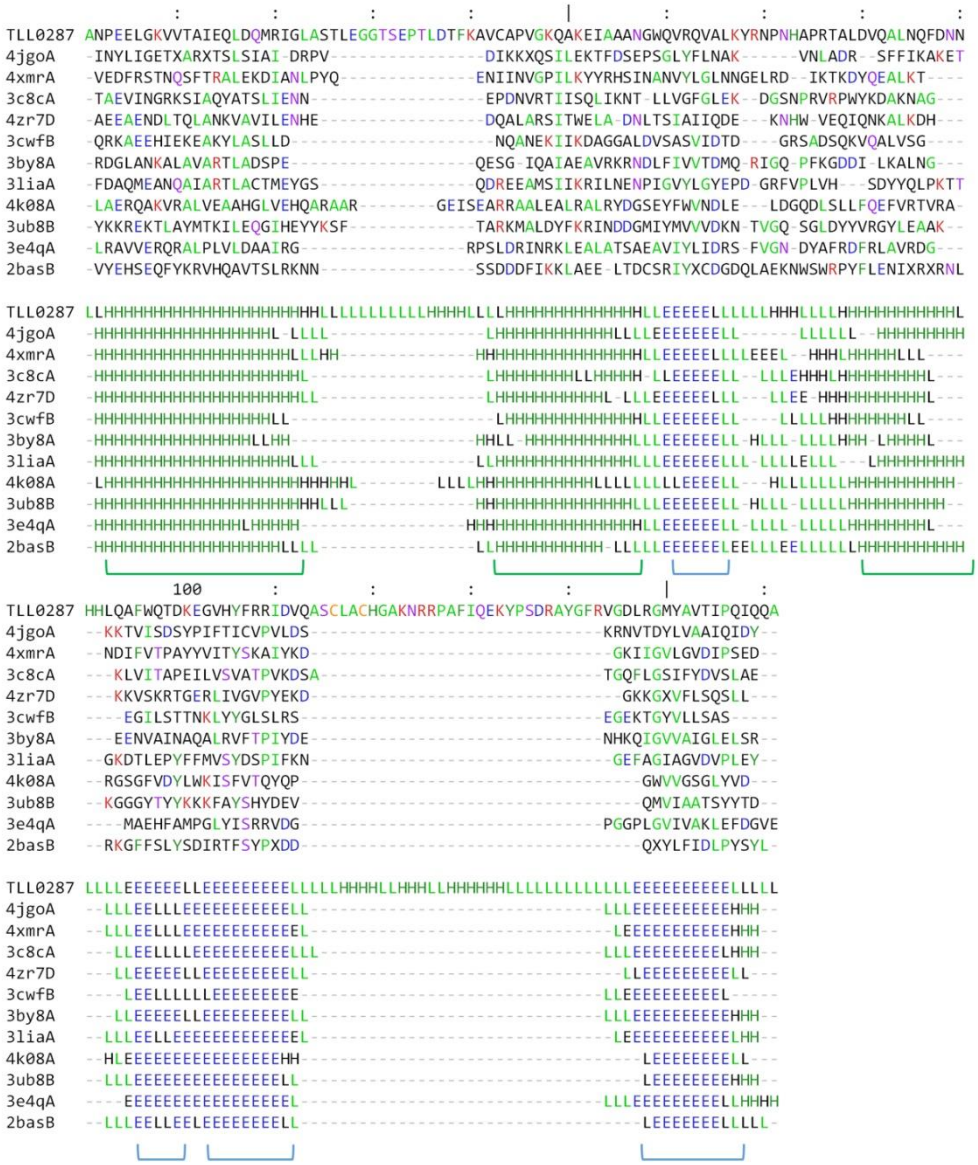

Figure 8