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Module fusion in an A-type flavoprotein from the cyanobacterium *Synechocystis* condenses a multiple-component pathway in a single polypeptide chain

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Abstract

The A-type flavoproteins (ATF) are modular proteins involved in multi-component electron transfer pathways, having oxygen reductase activity. They are complex flavoproteins containing two distinct structural domains, one having an FMN in a flavodoxin-like fold and the other a binuclear iron centre within a metallo-β-lactamase-like fold. Here, we report the purification and characterisation of a recombinant ATF from the cyanobacterium *Synechoystis* sp. PCC 6803, which has the unique feature of comprising an additional third domain with similarities towards flavin:NAD(P)H reductases. The latter was expressed independently as a truncated protein form and found to be capable of receiving electrons from NADH as well as to indiscriminately bind either one FAD or one FMN with equivalent affinities. Further kinetic studies have shown that the intact ATF is an NADH:oxygen oxidoreductase, with the catalytic ability to fully reduce oxygen to water. Thus, this constitutes an example on how structural modules found within partner proteins from an electron transfer pathway can be combined in a single polypeptide chain achieving identical catalytic activities.

Keywords: A-type flavoproteins; Diiron; Oxygen reductase

The A-type flavoproteins (ATF) are a family of soluble enzymes, with dioxygen reductase activity, so far exclusively found in anaerobes and facultative aerobes. The family was originally defined solely on the basis of amino acid sequence comparisons [1], but recent biochemical and structural data obtained for some of its members allowed a significant progress towards the overall understanding of the family [2–6]. The ATF are modular proteins, having a flavodoxin and a metallo-β-lactamase domains as core modules [5]. Whereas the former is closely related to canonical flavodoxins, containing one FMN, the second is quite distinct from the metallo-β-lactamases, as the catalytic zinc centre found in these proteins has been replaced by a diiron site, and the substrate binding groove was eliminated [5]. This accounts for the observed catalytic activity of these proteins, which are able to reduce molecular oxygen to water [2,4,7].

The best-studied ATF is the rubredoxin:oxygen oxidoreductase (ROO) from the sulphate reducing bacterium *Desulfovibrio gigas*. This protein was found to be the terminal element of a three-component electron transfer pathway that operates when this anaerobe is exposed to oxygen [7–9]. In these conditions, polyglucose is consumed and the electrons from NADH produced in the early steps of glycolysis are transferred through an NADH oxidoreductase to a type I rubredoxin, which finally donates electrons to ROO (Scheme 1). This enzyme fully reduces dioxygen to water, thus, scavenging it. The crystal structure of ROO was recently determined which allowed the identification of the family composing structural modules [5].

Whereas most of the composing members of this family solely consist of the two ATF core modules,

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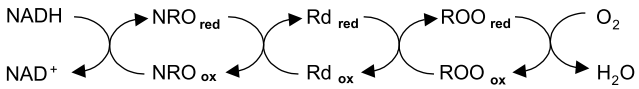
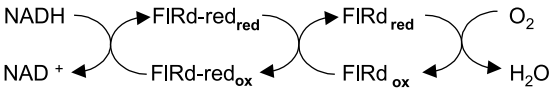


Scheme 1.



Scheme 2.

inspection of amino acid sequence alignments shows that there are two exceptions in which additional modules are present in the same polypeptide chain. One case is found in *Escherichia coli*, whose ATF core is fused to a rubredoxin domain at the C-terminus side [1,4]. This protein was named Flavorubredoxin (FlRd) and its characterisation showed that it contains a rubredoxin FeS centre, apart from the FMN and diiron sites [4]. This is particularly interesting considering that *D. gigas* ROO was shown to receive electrons directly from a rubredoxin, which is encoded in the same operon as ROO [2,5,10]. Thus, the three-component pathway observed in the sulphate reducer has been condensed into a two-component system operating in *E. coli*, involving a NADH:flavorubredoxin oxidoreductase (FlRd-red) and a flavorubredoxin [4]. Interestingly, these two proteins form a putative dicistronic unit (Scheme 2).

The other case, which is even more extreme, is observed in the cyanobacteria *Synechocystis* and *Anabaena*. Analysis of the genomes of these organisms shows that multiple copies of distinct ATFs are encoded: four in *Synechocystis* [1,11] and six in *Anabaena* [12]. Analysis of these sequences establishes that there is an additional NAD(P)H:flavin oxidoreductase module fused at the C-terminus side of the ATF core region. This analysis suggests that these cyanobacterial proteins may be able to receive electrons directly from NAD(P)H, transferring them directly to the catalytic diiron site, thus, bypassing rubredoxin. If these proteins are capable of mediating electron transfer from NAD(P)H to dioxygen this would indicate a complete condensation of the above-mentioned multi-component pathways into a single protein. This hypothesis was tested by studying a recombinant ATF from *Synechocystis* PCC. 6803, which is here reported.

Materials and methods

Amplification, cloning, and expression. The gene coding for *Synechocystis* ATF, *Ss*ATF573, was amplified from cosmid cs0236 DNA from the GenBank of *Synechocystis* strain PCC 6803 [11] using primers Syne1, 5′-GGAGTACCATATGTTCACTA-3′ (*Nde*I site underlined), and Syne2, 5′-CCTTGAAAGCTTTGCGGT-3′ (*Hin*dIII site underlined). The amplified DNA was cut with *Nde*I and *Hin*dIII and cloned into plasmid pUC28 [13]. After sequence verification, the gene was recloned into expression vector pET24a (Novagen) and named pME2336 and the protein was overexpressed in BL21(DE3) cells at 30 °C upon 10 h induction with 25 μM IPTG at an OD^{600} of ca. 0.3. The gene coding for the C-terminal part of *Ss*ATF573, *Ss*ΔATF171, was amplified from the same source using primers Syne4, 5′-CTTGGCTCATATGCTCATCC-3′ (*Nde*I site underlined) and Syne2. The amplified DNA was cut, cloned, sequenced, and recloned in pET24a (to give plasmid pME2470) and *Ss*ΔATF171 was overexpressed as described above. Cells were grown as previously described [1,4].

Protein purification. Cell disruption was performed in a French Press cell at 7000 psi, followed by separation of the soluble extract from membranes through 17-h ultracentrifugation at 100,000*g*, at 5 °C. Purification of *Ss*ATF573 was performed using a HiLoad Pharmacia system. For this protein, the soluble extract was dialysed against 10 mM Tris–HCl, pH 7.6 (buffer A), and subsequently applied to a 34-ml Q-Sepharose column previously equilibrated with buffer A. The protein eluted at ~450 mM NaCl, was concentrated on a Diaflo cell using a YM10 cut-off membrane, and then applied to a Superdex S-75 gel filtration column, equilibrated and eluted with buffer A plus 150 mM NaCl, at 0.75 ml/min. The truncated *Ss*ΔATF171 protein was purified in two steps using 3.5 ml custom-packed Econo-Pac columns (15 × 20 mm) equilibrated in 20 mM potassium phosphate buffer at pH 6 (buffer B). *Ss*ΔATF171 was collected in the flowthrough of a Q-Sepharose Fast Flow column and subsequently applied on an SP-Sepharose column which was eluted with a step gradient of potassium chloride in buffer B. *Ss*ΔATF171 eluted with ca. 200 mM KCl in buffer B. Protein purity was evaluated by SDS–gel electrophoresis [14] and pure protein was divided in aliquots and stored at −70 °C.

Spectroscopic methods. Ultraviolet/Visible spectra and kinetic assays were recorded in thermostatised spectrophotometers, equipped with cell stirring systems (Shimadzu UV 1603 and Shimadzu Multispec 1501 diode-array).

Biochemical methods. Protein purity along the purification steps was assayed with SDS–PAGE mini-gels as described [14], using a molecular mass standards kit (Pharmacia) ranging from 14.4 to 97 kDa. Protein concentration was determined with a BCA (2-Bicinchoninic Acid) Protein Assay Kit (Pierce) and by the Bradford [15] method.

Kinetic assays. NAD(P)H oxidation was followed at 340 nm in 10 mM Tris–HCl, pH 7.6 ($\varepsilon^{340}_{NAD(P)H} = 6200\,M^{-1}\,cm^{-1}$) and ferricyanide reduction was followed at 420 nm ($\varepsilon^{420}_{Ferricyanide} = 1020\,M^{-1}\,cm^{-1}$). NAD(P)H:cytochrome *c* oxidoreductase assays were performed as previously described [16], except that free flavins were absent from the reaction mixture. For oxygen reduction/consumption assays, a YSI-Micro cell Clark-type oxygen electrode was used, thermostatized at 37 and 40 °C for assays with NAD(P)H and ascorbate as electron donors, respectively. Catalase and superoxide dismutase (Sigma) were used to determine if water was the final product of oxygen reduction.

Cofactor analysis and reconstitutions. The iron content of *Ss*ATF573 was determined by the 2,4,6-tripyridil-1,3,5-triazine method [17]. Flavins were quantified spectrometrically ($\varepsilon_{ave} = 12{,}000\,M^{-1}\,cm^{-1}$) after extraction with TCA (10%), followed by centrifugation and supernatant neutralisation with 1 M NH_4CH_3OO, pH 7.0. Flavin HPLC analysis was performed as in [18]. Flavins were reconstituted either by overnight incubation at 4 °C with equimolar amounts of free FAD and FMN followed by dialysis against 10 mM Tris–HCl, pH 7.6, or by titration of the apoprotein as in [19]. Small aliquots of 30 μM *Ss*ΔATF171 apoprotein were added to 3 μM free flavin (FAD or FMN) in 100 mM KPi, 0.3 mM EDTA, pH 7.0, in a spectrophotometer cell and the UV–visible spectrum was recorded after each addition. Spectral changes were followed at 512 nm, after observation of differential spectra, and the data were analysed using MATLAB (Mathworks, South Natick, MA, USA).

Sequence analysis tools. Searches for similar proteins in databases were performed using the BLAST, TBLASTN, and PSI-BLAST

algorithms) using mainly NCBI-BLAST. The search for conserved domains was done using the CD-browser at NCBI. The 3DPSSM [20] fold recognition server (http://www.bmm.icnet.uk/~3dpssm/) carried out sequence threading. Multiple alignments were performed using CLUSTAL X [21] version 1.8.

Results and discussion

Proteomic and genomic analysis

As elucidated from the crystal structure of *D. gigas* ROO, the metallo-β-lactamase and flavodoxin-like domains constitute the core architecture of A-type flavoproteins and are present among all known members of this family. Inspection of sequence databanks and genomic data shows that the cyanobacteria *Synechocystis* sp. PCC 6803 [11] and *Anabaena* sp. PCC 7120 [12] contains, respectively, four and six distinct copies of ATF genes. Further, these proteins contain a C-terminal extension of about 170 amino acid residues, suggesting the presence of an additional structural module. In fact, analysis of the 10 sequences of the cyanobacterial ATFs, using a database of conserved domains, confirmed that they harbour three distinct domains: a metallo-β-lactamase, a flavodoxin, and an additional flavin reductase-like domain (Fig. 1).

An amino acid sequence comparison was carried out between the *Synechocystis* and *Anabaena* proteins and *D. gigas* ROO, the structural prototype of the family (Fig. 2). The cyanobacterial ATFs are very similar between themselves in all their extension, having amino acid identities ranging from 31% to 66% and similarities from 52% to 83%. A direct comparison of the ATF core region (typically between residue 1 and 400) towards *D. gigas* ROO shows a lower degree of conservation, with ~20% identity and ~40% similarity. This does not reflect any major structural difference between these proteins, as threading analysis performed on the cyanobacterial ATFs shows that they all have a high probability of assuming the overall ROO fold. Nevertheless, analysis of the sequence alignment shows that there are some subtle differences in respect to cofactor binding residues.

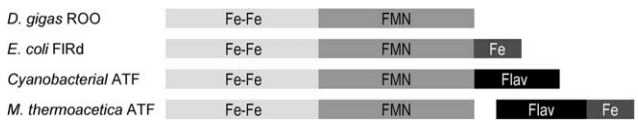


Fig. 1. Structural modules present in several A-type flavoproteins. Boxes represent structural domains and the text inside them the redox cofactors present. Light-grey box, metallo-β-lactamase domain; medium-grey box, flavodoxin domain; dark-grey box, rubredoxin domain; black box, NAD(P)H:flavin oxidoreductase domain. Fe–Fe, binuclear iron site; FMN, flavin adenine mononucleotide; Rd, rubredoxin-type FeS centre; Flav, Flavin, either FMN or FAD in the case of *Synehocystis* ATF573 protein (see text).

Concerning the di-iron site binding residues, half of the cyanobacterial ATFs do not have all of the ligands of the *D. gigas* motif H^{79}–X–E^{81}–X–D^{83}–X_{62}–H^{146}–X_{18}–D^{165}–X_{60}–H^{226} conserved (Fig. 2, marked with *), thus, suggesting that a modified ligation set exists or that those two proteins simply lack the centre. Interestingly, somehow conservative substitutions are observed: H^{81}–X–(N/S)^{83}–X–N^{85}–X_{64}–R^{149}–X_{19}–K^{168}–X_{57}–H^{225} (*Ss*AT F594 numbering). As for the flavodoxin domain, the set of ligands that has been identified in ROO as being involved in contacts with the FMN molecule are, in overall, also found on the cyanobacterial ATFs. Two FMN stabilising regions can be proposed: one involved in contacts with the phosphate group (FMN 1 in Fig. 2) and the other with the isoalloxazine ring and ribityl chain (FMN 2 in Fig. 2). Two other residues that are also in close contact to the FMN group and which are present among the compared ATFs are an aromatic residue in the first position of the partly conserved WPD motif and a strictly conserved proline (Fig. 2, marked with #).

Features of the NAD(P)H:flavin oxidoreductase domain

The presence of the putative NAD(P)H:flavin oxidoreductase domain is uniquely found among the *Synechocystis* and *Anabaena* ATF proteins. This domain is similar to enzymes known to be flavin reductases, ferric reductases as well as various oxidoreductase and monooxygenase components (Pfam entry PF01613). The high molecular weight rubredoxin (Hrb) from *Moorella thermoacetica* [22] is also an interesting case in which a C-terminal NAD(P)H:flavin oxidoreductase domain is found fused to an N-terminal rubredoxin domain (Fig. 1). This protein, which exhibits ca. 30% identity and 45% similarity towards the C-terminal domain of the cyanobacterial ATFs, has the particular feature of being the putative redox partner of *Moorella thermoacetica* ATF, both being encoded in the same operon [22] (Scheme 3).

Altogether, these findings suggest that this NAD(P)H:flavin oxidoreductase domain may be capable of receiving electrons from reduced dinucleotides and transferring them either to a rubredoxin centre or to an FMN moiety. A detailed analysis of the NA D(P)H:flavin oxidoreductase domain was undertaken to identify putative flavin and NAD(P)H binding regions. A fold recognition study performed by the 3D-PSSM server indicated a strong similarity towards the tertiary structure of the *Archaeoglobus fulgidus* ferric reductase [23] (an expectancy value of 5.6×10^{-13} was determined, corresponding to 95% certainty on the fold identification). Based on this similarity, which in terms of amino acid sequence corresponds to 24% identity, a molecular model was built for the *Synechocystis Ss*ATF573 protein from residue 419 to position 573 using the ferric re-

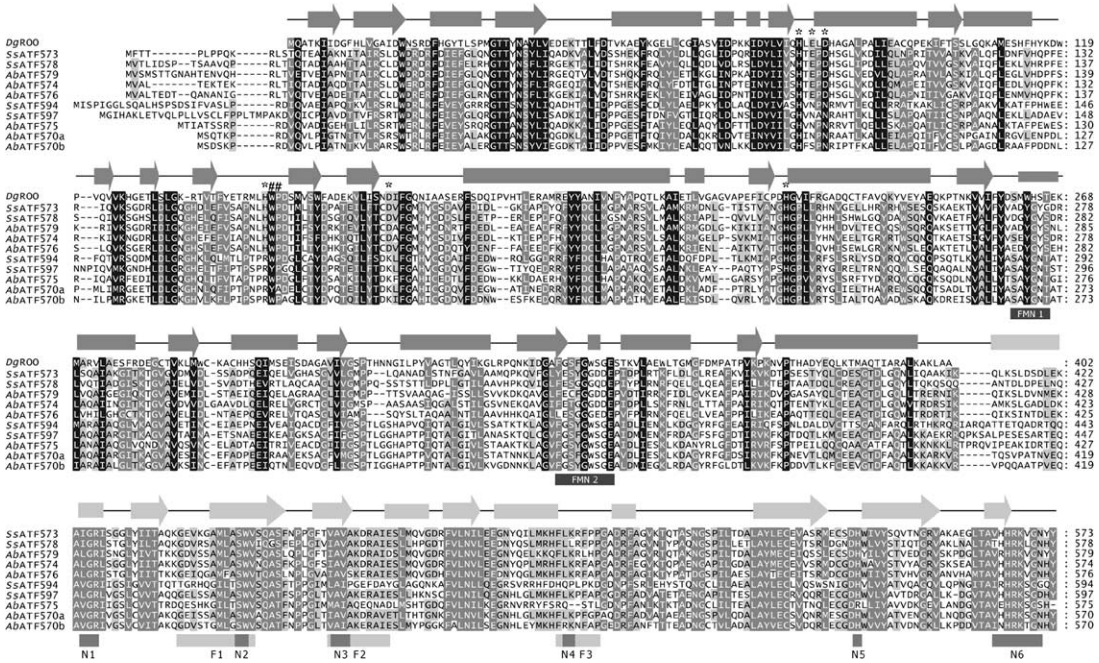
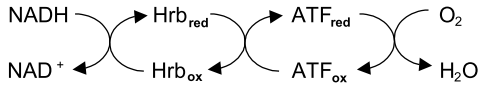


Fig. 2. Amino acid sequence alignment of cyanobacterial A-type flavoproteins. The ATF from *Synechocystis* PCC. 6803, *Anabaena* sp. PCC 7120, and *D. gigas* ROO were aligned using Clustal X: *Ss*ATF573 (S75748), *Ss*ATF578 (S74578), *Ss*ATF594 (S74576), *Ss*ATF597 (S76209), *Ab*ATF579 (NP_484222.1), *Ab*ATF574 (NP_487935.1), *Ab*ATF576 (NP_488486.1), *Ab*ATF575 (NP_488484.1), *Ab*ATF570a (NP_487931.1), *Ab*ATF570b (NP_484221.1), and D_gROO (Q9F0J6). The residues involved in binding of the binuclear iron site in *D. gigas* ROO are marked with an asterisk (*), whereas the regions involved in contacts with the FMN are labelled as FMN1 and FMN2 and marked with a cardinal (#). The secondary structure of the C-terminus domain of cyanobacterial ATF was predicted using PSIPRED. Arrows denote β-sheets and cylinders α-helices. The three regions proposed to bind flavin are marked in boxes labelled F1–F3 and the five regions putatively involved in stabilising NAD(P)H are marked in boxes labelled N1–N5.

ductase coordinates (PBD entry: lios). At this point it is important to stress that the residues involved in FMN and NAD(P)H binding in the *A. fulgidus* ferric reductase are not conserved in the sequences of its homologues [23], as most of the interactions with these cofactors are made through main chain nitrogen or oxygen atoms. Thus, the regions putatively involved in cofactor binding (marked on Fig. 2) were identified by mapping the residues that are at less than 6 Å from the cofactor. Interestingly, in most cases, these regions correspond to highly conserved areas. Nevertheless, an exact description of ligands will require a crystallographically determined structure.

Biochemical studies

To study the role of the NAD(P)H:flavin oxidoreductase domain in the cyanobacterial ATF proteins, *Ss*ATF573 was selected as a target. A truncated form of this protein, consisting on a 171 amino acid long peptide comprising the NAD(P)H:flavin oxidoreductase domain, was also designed. Both proteins were overexpressed in *E. coli* BL21(DE3)Gold cells and purified to homogeneity, as assayed by 12% SDS–PAGE (Fig. 3, inset). The predicted molecular masses for the proteins based on their sequence are slightly different from the values calculated from gel electrophoresis. The intact



Scheme 3.

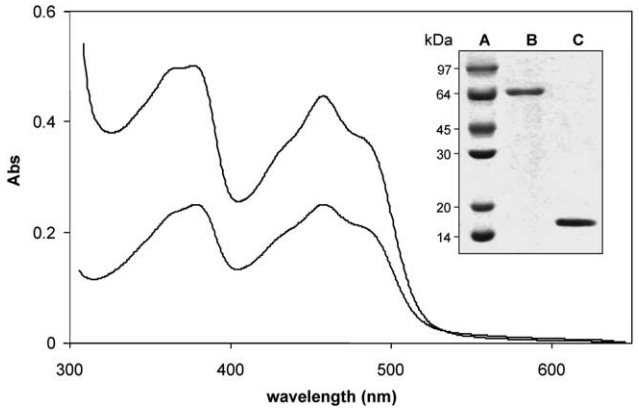


Fig. 3. UV–visible spectra of as-purified proteins. Proteins are in Tris/HCl buffer, pH 7.6. Trace a: *Ss*ATF573 (100 μM); trace b: *Ss*ΔATF171 (25 μM). *Inset*: SDS–PAGE analysis of purified proteins. Lane A, molecular mass markers (Pharmacia); lane B, *Ss*ATF573; lane C, *Ss*ΔATF171.

*Ss*ATF573 is a 63.5-kDa protein, whereas the truncated form *Ss*ΔATF171 has 18.4 kDa, although the apparent masses are 70 and 16 kDa, respectively. The UV/visible spectra of both proteins (Fig. 3) show features typical of flavoproteins, having two major bands with maxima at 378 and 458 nm.

Cofactor analysis

The selected *Synechocystis* ATF is a complex flavoprotein, containing multiple redox cofactors. The as-purified intact *Ss*ATF573 was found to contain an amount of flavin substoichiometric in respect to the expected 2 moles of flavin per molecule, a situation which is commonly observed among flavoproteins. The truncated *Ss*ΔATF171 form contains 0.8 mol of flavin per mol of protein. To identify and quantify the relative flavin content, an acid extraction was performed and the extracts were resolved by HPLC using free FAD and FMN as standards. The intact *Ss*ATF573 was found to contain both FMN and FAD in a 3:1 ratio. On the other hand, the *Ss*ΔATF171 form contains equimolar amounts of FMN and FAD. To confirm this, spectrophotometric titrations were done, where apoprotein was added to free flavin solutions and a spectrum was collected after each addition, as in [19]. Absorbance changes at 512 nm were plotted against apoprotein/flavin ratio (data not shown) and a clear intercept is detected at 1 mol of apoprotein per mol of flavin. The latter result indicates that the NAD(P)H:flavin oxidoreductase domain randomly binds 1 mol of FAD or FMN. The correct interpretation of the flavin ratio observed for the intact protein lies on an established feature of ATF proteins, which is the fact that the FMN which is found in the flavodoxin fold is very poorly labile. Thus, the FMN/FAD ratio of 3 is explainable, assuming that the flavodoxin site is fully loaded, whereas the flavin binding site of the NAD(P)H:flavin oxidoreductase domain either contains FAD or FMN.

Considering that in *Ss*ATF573 there is a conservation of the motif that accounts for binding of a binuclear iron site in ATF proteins (see above), the amount of iron present on the purified sample was determined. Pure *Ss*ATF573 contains approximately 2 moles of iron per protein (1.9 mol Fe/mol of protein), thus, indicating that a bimetallic iron site is present in this protein. Iron was found to be absent from the *Ss*ΔATF171 protein.

Kinetic properties

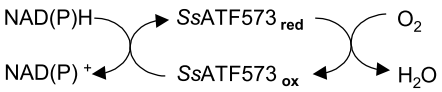
Considering the presence of the NAD(P)H:flavin oxidoreductase domain in the *Synechocystis Ss*ATF573 protein, the ability of this protein to be directly reduced by NADH was tested. An in vitro assay was performed, following the NADH:cytochrome *c* oxidoreductase activity. The measured rate in turnover number units ($24\ min^{-1}$, at 25 °C) is very similar to the one determined for the *E. coli* 4-hydroxyphenylacetate 3-monooxygenase reductase component ($33.4\ min^{-1}$, at 25 °C, calculated from [16]). This protein also belongs to the above-mentioned family of proteins containing a NAD(P)H:flavin oxidoreductase domain [16] and has a canonical NAD(P)H:flavin oxidoreductase activity. As determined from NAD(P)H:ferricyanide oxidoreductase assays, the *Ss*ATF573 protein displays a greater affinity for NADH ($K_M = 22.4\,\mu M$, 25 °C) than for NADPH ($K_M = 130\,\mu M$, 25 °C) (data not shown).

Having established that the *Ss*ATF573 protein can receive electrons from reduced nucleotides, its ability to catalyse the reduction of dioxygen to water, a catalytic activity present in all ATF proteins so far studied, remained to be tested. Assays performed on a Clark-type oxygen electrode showed that in the presence of NADH *Ss*ATF573 consumes oxygen at a rate of $0.38 \pm 0.05\ min^{-1}$ and at a rate of $0.20 \pm 0.06\ min^{-1}$ in the presence of NADPH. Further, as the addition of catalase and superoxide dismutase at the end of the assay had no effect on the observed rate, thus, it can be concluded that oxygen is completely reduced to water and that no partially reduced oxygen species are formed during catalysis.

Altogether, these experiments show that the *Synechocystis Ss*ATF573 is capable of linking NADH oxidation to oxygen reduction, an activity that requires three proteins in *D. gigas* [2,7] and two in *E. coli* [4]. To compare the oxygen reductase activities of the different A-type flavoproteins, which all have distinct electron donors, a polarographic assay was outlined in which ascorbate was used as electron donor for the ATF proteins. The caveat of this assay is that the rates obtained are underestimated with respect to the physiological ones as ascorbate is an unspecific reductant. However, the great advantage is that it provides a comparable measure of the oxygen reductase activity, without generating partially reduced oxygen species. In fact, with this assay, all ATFs tested have identical oxygen reduction rates of approximately $0.1\ min^{-1}$.

Conclusion

The A-type flavoproteins are modular multi-redox cofactor containing flavoproteins [1]. Here, we reported the characterisation of one of these proteins from *Synechocystis* sp. PCC 6803, a representative member of the cyanobacterial-type ATFs. Unlike all other so far known members of the ATF family, these proteins contain a NADH:flavin oxidoreductase module additionally to the two other core metallo-β-lactamase and flavodoxin structural domains. The studied protein was found to have NADH:oxygen oxidoreductase activity, thus, showing that a single polypeptide chain condenses



Scheme 4.

a multiple-component pathway as observed in other organisms [7] (Scheme 4).

This constitutes an excellent example of module fusion of structurally independent units in a single protein. Future studies aimed at comparing the inter- and intra-protein electron transfer kinetics of ATF proteins will eventually provide insights allowing to establish what is the extent of the role played by functional factors on driving protein evolution by module arrangement. The presence of these proteins in all of the so far sequenced obligate and facultative anaerobes genomes, co-related to the biochemical data available for some of the members of the ATF family, suggests that these proteins may have a role in the response to transient oxidative stress conditions. Furthermore, it was recently suggested that the enzyme from *E. coli* may be capable of acting as an NO reductase [24]. This activity remains to be proven among the purified proteins but, if confirmed, it will strongly enhance the important physiological function of A-type flavoproteins.

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