AppA, a Redox Regulator of Photosystem Formation in Rhodobacter sphaeroides 2.4.1, Is a Flavoprotein

IDENTIFICATION OF A NOVEL FAD BINDING DOMAIN*

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The AppA protein is required for increased photosystem gene expression upon transition of the facultatively photoheterotrophic bacterium Rhodobacter sphaeroides 2.4.1 from aerobic to anaerobic photosynthetic conditions. AppA shows no obvious similarity to proteins with established function. Genetic evidence suggests that its effect is exerted through modulation of the activity of the repressor PpsR, which controls expression of multiple photosystem genes. To gain insight into the nature of AppA involvement in redox-dependent photosystem gene expression, the appA gene was overexpressed in Escherichia coli. AppA was produced as insoluble inclusion bodies. The purified inclusion bodies were found to contain FAD. By overexpressing various deletion derivatives, we were able to localize the region of AppA sufficient for FAD binding to approximately 120 amino-terminal residues. To assess the role of FAD binding in AppA function, we constructed an AppA derivative lacking the entire FAD binding domain. Surprisingly, this derivative complemented the AppA null mutant undergoing transition from aerobic to anaerobic photosynthetic growth conditions almost to the same extent as the full-length AppA protein. When the sequence of the amino-terminal portion of AppA was examined, it was shown not to contain any known flavin binding motifs. However, two open reading frames of unknown function, showing significant similarity to the amino terminus of AppA, were identified, i.e. Synechocystis sp. Sr1694 and E. coli F403. The latter gene was amplified and overexpressed in E. coli, and the partially purified F403 protein was found to contain FAD as a cofactor. We have therefore concluded that the amino terminus of AppA represents a novel FAD binding domain present in a small group of bacterial proteins. The binding of FAD by AppA may be the first clue as to how this regulatory protein is involved in redox-regulated reactions.

*Rhodobacter sphaeroides* is a facultative phototrophic proteobacterium of the α3 subdivision and is known for its remarkable metabolic versatility. Under aerobic conditions, *R. sphaeroides* derives energy by aerobic respiration. When oxygen tensions decrease below certain threshold levels, dramatic changes in the cellular physiology occur. An intracytoplasmic membrane system develops that houses various components of the photosystem whose function is to capture photons and to convert light energy into electron flow (1, 2). The transition from aerobic to an anaerobic light environment requires, among other things, a significant increase in expression of the photosystem genes, i.e. genes encoding structural and assembly proteins of the light harvesting and reaction center complexes as well as genes encoding enzymes for bacteriochlorophyll and carotenoid biosynthesis. Under highly aerobic conditions, these genes are expressed at low basal levels. Several transcription factors coordinate the regulation of photosystem gene expression (reviewed in Refs. 3 and 4). Some of these factors are specific to photosystem gene expression, e.g. a redox-sensitive transcriptional repressor PpsR (5–7), whereas others have a broader range of targets comprised of photosystem as well as nonphotosystem genes, e.g. the PrrBA two-component activation system (8, 9) and the anaerobic activator, FnrL (10).

The AppA protein has been identified as yet another critical component required for activation of photosystem gene expression (11, 12). An *R. sphaeroides* 2.4.1 AppA null mutant is impaired in its transition from aerobic to anaerobic photosynthetic growth because of defects in the production of both top pigments and structural protein components of the photosystem. In contrast to the transcriptional factors mentioned above, the primary sequence of AppA gives no clue as to its possible role in photosystem gene expression. AppA contains no DNA binding motif(s) and shows no obvious similarity to proteins with known function. Using a variety of molecular genetic approaches, we have established that AppA exerts its regulatory effect independently of the FnrL and PrrBA regulatory pathways, but it does appear to affect PpsR repressor activity (7). At present, neither the nature of the redox sensitivity of PpsR (13, 14) nor the mechanism of the AppA-PpsR interactions is known. AppA could function as a hypothetical redox-sensing partner directly interacting with PpsR. Alternatively, it could be involved in modulation of a critical redox carrier, which in turn interacts with or is a cofactor of the PpsR repressor. Until now, we have remained unaware of what features, if any, allow AppA to participate in redox-dependent processes.

In this work, we present the first biochemical characterization of AppA, which has revealed that this protein is likely to contain several redox-responsive cofactors. We have identified one of these compounds as FAD. We have located the FAD binding domain within AppA and tested the significance of this domain for protein function. We have also uncovered, as a result of this study, a small group of bacterial flavoproteins showing similarity to the FAD binding domain of AppA.

MATERIALS AND METHODS

Media, Growth Conditions, Bacterial Strains, Bacteriological Techniques—*Escherichia coli* strains were grown at 37 °C (unless specified otherwise) in LB medium (15) supplemented, when required, with the following antibiotics: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml.

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R. sphaeroides strains were grown at 30 °C in Sistrom's medium A (1), supplemented, when required, with tetracycline, 1 μg/ml. An aero-
bio-to-anerobic transition was performed as follows. Liquid cultures (5
ml) of the AppA null strain, APP11 (12), carrying the appropriate
plasmid(s) in trans, were grown aerobically in culture tubes until late
exponential phase, the culture was then diluted 1:50 with fresh medium
at 4–6 °C and transferred to 30 °C to proceed through Microcon-3
(308x526) columns (Ami-

clecular Genetics. All recombinant DNA constructs described here were verified by DNA
sequencing.

Sequence Analysis—Sequence analyses were performed using BLAST+ BEAUTY, WU-BLAST+ BEAUTY, ClustalW 1.7 (multiple
alignment) programs, available at the Baylor College of Medicine
Sequence Analysis. Analysis of protein secondary structures was per-
formed using the conserved secondary structure prediction developed
by James Cuff and available as a part of the Jpred package.2

Construction of appA-overexpression Plasmids—The 1.6-kilobase
pair XhoI-AgeI fragment from plasmid p484-Nco5 (12), carrying the
appA gene, was first cloned into the multiple cloning site of vector
LITMUS28 (NEB Biolabs), digested with XhoI and AgeI, to produce
plasmid pLapp. The XhoI [513] site is positioned 4 codons downstream
of the translational start of AppA; the AgeI site is positioned 270 base
pairs downstream of the appA stop codon. The number in brackets
corresponds to the sequence coordinates deposited in GenBank L42555.

To construct a translational fusion of AppA to the amino terminus of
LacZα (LacZ ⋅ AppA) expressed from LITMUS28, the LITMUS28 plasmid was digested with BglII and
EcoRI (GST-AppA), the 1.6-kilobase pair
from pLapp with
pGEX-2TK (Ami-

clecular Sciences) using 5% Na2HPO4 as a developing solvent (20). 50% meth-

overexpression of expression were used according to recommendations of the manufac-
turer (Amersham Pharmacia Biotech). Proteins were separated by
SDS-PAGE (18). Because full-length AppA, expressed from either
pGapN (as a GST-AppA fusion) or pLappA, was always found in the insoluble fraction, various modifications of the standard protocol were
taken to achieve production of soluble AppA in a soluble form. These modi-

the ampicillin resistance (88 °C, 5 s; 42 °C, 15 s; 72 °C, 10 min). The 1.3-kilobase pair
PCR fragment corresponding to f403 was gel-purified, digested with StulI, and cloned into the Smal site of vector pGEX-2TK to produce plasmid pGORF403. The latter plasmid expresses the GST-F403 translational fusion under control of P_tac.

Overexpression of AppA Protein Derivatives and Inclusion Body Pu-
erification—The plasmids overexpressing various portions of AppA were
maintained in E. coli DH5α. Standard protocols for regulation of
expression were used according to recommendations of the manufac-
turer (Amersham Pharmacia Biotech). Proteins were separated by
SDS-PAGE (18). Because full-length AppA, expressed from either
pGapN (as a GST-AppA fusion) or pLappA, was always found in the insoluble fraction, various modifications of the standard protocol were
attempted to achieve production of soluble AppA. The first modifications
included lower growth temperatures (12 to 37 °C), lower IPTG
concentration (0.05 to 1.0 mM), variable times of induction (1 to 2 h for
low temperature-growing cultures), alternative E. coli hosts in place of
DH5α, e.g., JM109, HB101, solubilization of cell extracts with 0.1% Triton X-100 or sodium lauryl sarcosyl, addition of 1–5 mM dithio-
retiol, etc. These modifications did not result in an increased production of
soluble AppA. The AppA protein was also successfully produced by complete
lysates of E. coli grown with IPTG (500 μM) at 20 °C, or without
IPTG, at 37 °C. The AppA protein was purified by affinity chromatography according to recommenda-
tions of the manufacturer (Amersham Pharmacia Biotech).

Identification of the Flavin Cofactors Associated with AppA and
F403—The flavin enriched inclusion bodies were extracted with acetone/concentrated HCl (1000: 1.3 v/v) and pelleted by centrifugation, and
the supernatant was subjected to spectral analysis. Flavins were
extracted from the yellow-pigmented protein preparations by heating at
95 °C for 15 min in PBS buffer. The released flavins were separated from the insoluble fraction by centrifugation followed by ultrafiltration
through Microcon-10 filtrations in 0.25 ml of distilled water, as described (19).

Spectroscopy—Spectral analyses were performed using a Shimadzu
UV-1601PC diode array spectrophotometer and 1-cm quartz cuvettes. A
Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co.)
was used to measure the absorbance of anaerobically grown R. sphaeroides cultures

The stoichiometry of binding of FAD to purified AppA was estimated

2 http://circinus.ebi.ac.uk:8081
as follows. Exogenous FAD was incubated with the equimolar amount (38.4 μM) of the purified amino-terminal fragment of AppA (150-μl reaction volume) for 30 min at room temperature. The mixture was diluted with 250 μl of PBS, loaded into a Microcon-3 concentrator and centrifuged at 14,000 × g for 25 min. The concentrations of FAD were determined in the filtrate and retentate, based upon absorption at 450 nm. The amount of bound FAD (first estimate) was calculated based upon the amounts of FAD in the original protein sample, filtrate, and retentate. The retentate (200 μl) was further diluted with 4 volumes of PBS and concentrated as above-described, and the amount of bound FAD was calculated (second estimate). The average of the first and the second estimates from each experiment was taken.

**RESULTS**

Overexpression of the Full-Length AppA Protein in E. coli—To gain insight into the biochemical properties of AppA, we intended to purify this protein by using an E. coli overexpression system. For this purpose, the appA gene was cloned into the overexpression vector pGEX-2TK to produce plasmid pGapN (Fig. 1). The latter construct encodes the GST fused to the fifth amino acid residue of the AppA protein, expression of the fusion being controlled by Ptac. The GST::AppA fusion protein was overproduced in E. coli strain DH5α following induction by IPTG (Fig. 2, lane 1). Employing the standard protocol resulted in the production of insoluble protein (not shown). Numerous attempts to obtain the protein in a soluble form were undertaken (see “Materials and Methods”); however the GST::AppA fusion was always produced in insoluble form, i.e. as inclusion bodies. To test whether the GST tag adversely affected solubility, the AppA protein was translationally fused to the amino-terminal residues of LacZ and overexpressed under the control of P<sub>lac</sub> (Fig. 1). The GST::AppA fusion protein was overproduced in E. coli strain DH5α carrying various overexpression plasmids were separated by SDS-PAGE and Coumassie-stained. Lane 1, plasmid pGapN (full-length GST::AppA); lane 2, pGapR; lane 3, pGapRs; lane 4, pGapS; lane 5, pGapB; lane 6, pGapStu; lane 7, pGapSty; lane 8, pGapNae; lane 9, vector pGEX-2TK; lane 10, molecular mass standards. Numbers at the right correspond to molecular mass (in kDa).

**Fig. 1.** Schematic presentation of the GST::AppA and LacZ::AppA fusion proteins. FAD binding domain of AppA is vertically striped in those proteins that are capable of FAD binding; the GST tag is marked with triangles; the LacZ' fragment is horizontally striped. The numbers correspond to the residues of the AppA protein.

**Fig. 2.** Expression of the GST-AppA derivatives. Crude extracts of E. coli strain DH5α carrying various overexpression plasmids were separated by SDS-PAGE and Coumassie-stained. Lane 1, plasmid pGapN (full-length GST::AppA); lane 2, pGapR; lane 3, pGapRs; lane 4, pGapS; lane 5, pGapB; lane 6, pGapStu; lane 7, pGapSty; lane 8, pGapNae; lane 9, vector pGEX-2TK (GST tag); lane 10, molecular mass standards. Numbers at the right correspond to molecular mass (in kDa).

**Fig. 3.** A, AppA-enriched inclusion bodies. Lane 1, partially purified inclusion bodies from DH5α(pLappA), separated by SDS-PAGE and stained with Coumassie Blue; lane 2, molecular mass standards. Numbers at the right correspond to the molecular mass (in kDa). B, spectrum of the acidic acetone extract of AppA-enriched inclusion bodies.
The nature of the flavin cofactor was determined by TLC of the flavin extracted from the heat-denatured protein. The extracted flavin had a mobility identical to that of FAD (data not shown). FAD content in the purified amino-terminal fragment of AppA was 0.58 ± 0.09 mol/mol of protein. Because repetitive resin washes resulted in visible loss of yellow pigment, we determined the stoichiometry of FAD binding using exogenously added FAD. At saturating concentration of FAD, 1 mol of the amino-terminal domain of AppA was found to bind 0.89 ± 0.10 mol of FAD, suggesting that 1 mol of the amino-terminal fragment of AppA is capable of binding 1 mol of FAD.

Role of the FAD Binding Domain of AppA Following an Aerobic-to-Anaerobic Transition—To gain insight into the role of the FAD binding on AppA function, we constructed an in-frame deletion (residues 5 to 190) derivative of AppA, which lacked the entire FAD binding domain (Fig. 5A, plasmid p484-Nco5Δ). The truncated protein was expressed from the intact upstream regulatory region of appA to enable a direct comparison with the plasmid containing the full-length appA.

Plasmid p484-Nco5Δ was introduced into the AppA null mutant, APP11. The resulting strain as well as strains carrying in trans the full-length appA gene in plasmid p484-Nco5 or the vector pRK415 alone (Fig. 5A) were tested for their ability to transit from aerobic to anaerobic photosynthetic conditions. Strain APP11(pRK415) has an extensive lag phase before resuming growth under anaerobic photosynthetic conditions, and its subsequent growth is very slow (Fig. 5B, trace 3), in line with our earlier observations (7). In contrast, strain APP11(p484-Nco5) performed the aerobic-to-anaerobic transition at least as efficiently as the wild type 2.4.1 (Fig. 5B, trace 1). Surprisingly, the transition of strain APP11(p484-Nco5Δ) was only slightly impaired as compared with that of APP11(p484-Nco5) (Fig. 5B, trace 2). This suggested that the function of AppA in the transition of R. sphaeroides from aerobic to anaerobic photosynthetic conditions is generally preserved despite the absence of the entire FAD binding domain. Therefore FAD binding does not appear to be essential for AppA function, at least under these experimental conditions. It should be mentioned that the appA gene derivatives were present in 4 to 6 copies/cell. Therefore, an increased abundance of these derivatives over the physiological concentration of AppA could serve to have masked the extent of the impairment resulting from the absence of the FAD binding domain in the deletion derivative (see also “Discussion”).

A Novel Flavin Binding Domain—Searching the data bases for proteins similar to the FAD binding domain of AppA, i.e. residues 5–124, revealed two entries present in the sequences of the genomes of E. coli and Synechocystis sp., i.e. F403 and Sr1694, respectively (Fig. 6). The sequence identity over the 94-residue fragment between AppA and Sr1694 is 39%, between AppA and F403 is 33%, and between F403 and Sr1694 is 30%. By using similarity search programs based upon the multiple alignment present in Fig. 6, we could not identify any other members of this protein group.

The three proteins, AppA, F403, and Sr1694, differ in size, i.e. 450, 403, and 150 residues, respectively. Sequence similarities among these proteins are clearly limited to their amino-terminal regions, i.e. residues 16–108 of AppA, residues 1–94 of F403, and residues 1–95 of Sr1694 (Fig. 6). Both the differences in size and confinement of the similarity to only the amino-terminal portion of AppA implied that AppA, F403, and Sr1694 perform different functions. We suggest that the region of similarity among these three proteins represents a novel flavin binding domain. This domain does not contain an obvious pattern of similarity to known flavoproteins; however, cer-
tain features common to flavin binding domains can be deduced based upon multiple alignment (see “Discussion”).

The secondary structure of the flavin binding domain was determined by several prediction algorithms based upon the multiple alignment shown in Fig. 6. According to the derived consensus, the FAD binding domain contains predominantly α-helices and only one evident β-sheet.

**Amplification, Overexpression, and Flavin Binding of E. coli F403**—We set about to test the hypothesis that F403 and Srl1694, which show similarity to the FAD binding domain of AppA, could be involved in flavin binding. For this, we chose the protein with the least similarity to AppA, i.e. E. coli F403. The f403 gene was PCR-amplified and cloned into pGEX-2TK to generate the GST::f403 translational gene fusion (plasmid pGORF403). The GST::F403 fusion protein was overproduced in strain DH5α(pGORF403) (Fig. 7A, lanes 1 and 2). Similar to cells overproducing AppA, cells overproducing F403 appeared yellow following the course of induction. The GST::F403 fusion migrated as a 71-kDa protein, i.e. in good agreement with the calculated value. The GST::F403 protein was produced under these conditions in soluble form (Fig. 7A, lane 3). It was absorbed to glutathione-Sepharose and washed, and the F403 portion was released from the GST tag by thrombin digestion. The resulting 48-kDa F403 protein appeared to be at least 50% pure on SDS-PAGE (Fig. 7A, lane 4). The nature of the two major contaminating proteins was not investigated further because they did not interfere with the analysis of F403.

The spectrum of the F403 protein preparation was typical of
a flavoprotein (Fig. 7B). The flavin moiety was extracted from the heat-denatured protein and identified by TLC as FAD (data not shown). Therefore, similar to AppA, F403 is a FAD binding protein. Although the ability of *Synechocystis* sp. Srl1694 to bind flavin has not been tested experimentally, we believe that it would be likely to do so, because the amino terminus of Srl1694 shares more similarity to the FAD binding domain of AppA than does the amino terminus of F403.

**DISCUSSION**

The AppA protein plays a critical, albeit as yet biochemically undefined, role in the ability of the facultative phototroph *R. sphaeroides* 2.4.1 to transit from aerobic to anaerobic photosynthetic conditions (12) (Fig. 5B). It is also required for anaerobic photosynthetic growth *per se*; however, it is dispensable for anaerobic growth in the dark with Me₆SO as a terminal electron acceptor, suggesting a specific involvement of AppA in photosynthesis-related processes. The impairment imposed by the absence of AppA lies, at least in part, in inefficient expression of photosystem genes (12). We have presented genetic evidence that AppA can affect photosystem gene expression through modification of the repressor PpsR, which coordinates expression of multiple photosystem genes (7). However, it is unclear at this time whether this is the only, or for that matter the main, function of the AppA protein. Because the primary structure of AppA has no obvious similarity to proteins with known function, we were unaware of what features of AppA allow it to affect PpsR activity.

Numerous attempts to overproduce and purify AppA in soluble form from *E. coli* have been undertaken; however, the full-length AppA protein was always present as yellow-colored inclusion bodies. The yellow pigment associated with AppA was extracted from 

Flavoproteins with similar flavin binding domains are usually grouped into families. When employing the FAD binding domain of AppA, we found two homologues in the public data bases. An approximately 94-residue-long region of *E. coli* F403 and *Synechocystis* sp. Srl1694 were significantly similar to the FAD binding domain of AppA. To prove that flavin binding is not unique to AppA but common to this group of proteins, we amplified, overexpressed, and partially purified F403. As anticipated, this protein was found to noncovalently bind FAD.

In general, structural components required for binding flavins are poorly defined. However, in many flavoprotein families, a characteristic signature for dinucleotide binding is readily apparent, *i.e.* GXXG, where X is any amino acid (22). The sequence surrounding the conserved glycine 52 of AppA is reminiscent of this signature, *i.e.* ARAQLTG, with two alanine residues in place of the glycines and an extended distance between the residues (Fig. 6). The corresponding sequence of Srl1694 differs from the dinucleotide binding motif by only one residue, *i.e.* a conserved glycine-to-alanine substitution, ANGITG. However, in the sequence of F403, only one glycine residue is present. The secondary structure prediction places the putative dinucleotide binding site between the α-helix and β-sheet, which would be consistent with its proposed role. Tyrosine residues are often found to interact with the isoaflaxazine ring of FAD. Perhaps the best candidate for such a role would be the conserved tyrosine 21 of AppA (Fig. 6). The local sequence surrounding tyrosine 21, YRS, is conserved in AppA, Srl1694, and F403. Similar residues, albeit in a different order, RXYS, surround the conserved tyrosine in the isoaflaxazine ring binding sites in a number of flavoproteins (23).

It is possible that the residues that lie outside of the minimal FAD binding domain of AppA can participate in interactions with FAD. However, as few as 94 to 120 residues of AppA may be sufficient for stable flavin binding. In light of the unorthodox structure and compactness of this FAD binding domain, it would seem fair to suggest that its structural analysis could extend our understanding of the means by which proteins bind flavins.

It is worth mentioning that the newly identified FAD binding domain must be of a relatively ancient origin. Because it is present in members of both the Proteobacteria and Cyanobacteria, it may have arisen before the divergence of these two bacterial branches, albeit horizontal gene transfer can not be ruled out. It is somewhat surprising that this domain is found only once in the genomes of both *E. coli* and *Synechocystis* sp., whereas many other known flavin binding domains occur more frequently. Furthermore, we were unable to find homologues of this domain in other bacterial, archaeal, or eukaryotic species whose complete genome sequences are presently available. Whether the rare occurrence of this domain is associated with any peculiarity of function or origin has yet to be determined.

We believe that FAD binding is the only feature common to the three proteins. In each protein, the FAD binding domain is positioned at the amino terminus. The carboxyl-terminal portion of F403, *i.e.* the portion downstream of residue 95, shares the most noticeable similarity with the proteins belonging to the YJCC/YEGA/YHDA/YHJK family, *e.g.* *E. coli* YJCC (SwissProtein P32701). Representatives of this protein family are abundant among various bacterial species. However, to our knowledge, the function of this family of proteins has not yet been elucidated. It will be interesting to determine whether any of the other members of this family bind flavin(s) or if this is a feature unique to *E. coli* F403.

We could not find similarity of the carboxyl terminus of *Synechocystis* sp. Srl1694 to any of the proteins in the data bases. Srl1694 is annotated, apparently after AppA, as an activator of photopigment and *puc* expression. Given the limits in similarity to, specifically, the FAD binding domain of AppA...
and the absence of a puc operon in Synechocystis sp., such annotation would seem no longer appropriate.

As far as the function of AppA is concerned, our finding that AppA contains FAD provides the first clue as to how AppA could be involved in the redox-dependent regulation of photosystem gene expression. Previously, we showed that AppA exerts its effect through the repressor PpsR, which controls expression of multiple photosystem genes (7). One can envision several possibilities as to the mode of AppA-PpsR interaction. AppA could directly interact with PpsR, thus making the DNA binding ability of PpsR dependent on the changes in redox state of AppA. This scenario would be similar to the recently described NifL-NifA system of Azotobacter vinelandii, where NifL is an FAD-binding protein that directly binds the DNA binding response regulator of nitrogen fixation genes, NifA (24). Alternatively, AppA could affect PpsR indirectly, e.g. by modulating the redox state of a possible cofactor (if any) associated with PpsR or an additional redox carrier interacting with PpsR. These possibilities will be explored in the future.

We constructed a derivative of AppA lacking the entire FAD binding domain, and this derivative was able to complement the lesion present in the AppA null mutant upon a gradual transition from aerobic to anaerobic photosynthetic growth. At this point, it is unclear why deletion of the FAD binding domain resulted in only a minor effect upon its function under these conditions. One obvious possibility is the gene dosage effect, i.e. presence of the truncated appA gene in several copies could partially mask the absence of the intact AppA. Other explanations are possible and are currently under investigation.

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