Azotobacter vinelandii NADPH:Ferredoxin Reductase Cloning, Sequencing, and Overexpression*

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Azotobacter vinelandii ferredoxin I (AvFdI) controls the expression of another protein that was originally designated Protein X. Recently we reported that Protein X is a NADPH-specific flavoprotein that binds specifically to FdI (Isas, J. M., and Burgess, B. K. (1994) J. Biol. Chem. 269, 19404–19409). The gene encoding this protein has now been cloned and sequenced. Protein X is 33% identical and has an overall 53% similarity with the fpr gene product from Escherichia coli that encodes NADPH:ferredoxin reductase. On the basis of this similarity and the similarity of the physical properties of the two proteins, we now designate Protein X as A. vinelandii NADPH:ferredoxin reductase and its gene as the fpr gene. The protein has been overexpressed in its native background in A. vinelandii by using the broad host range multicopy plasmid, pKT230. In addition to being regulated by FdI, the fpr gene product is overexpressed when A. vinelandii is grown under N₂-fixing conditions even though the fpr gene is not preceded by a nif specific promoter. By analogy to what is known about fpr expression in E. coli, we propose that FdI may exert its regulatory effect on fpr by interacting with the SoxR region.

Azotobacter vinelandii ferredoxin I (AvFdI) is a small protein, Mᵣ = 12,700 that has been characterized extensively by x-ray crystallography (1–5) and by detailed spectroscopic (6–9) and electrochemical (10) studies. The protein contains two types of [Fe-S] clusters: one [3Fe-4S]¹/² cluster and one [4Fe-4S]¹/² cluster with pH 7.8 reduction potentials of −425 mV and −647 mV, respectively. Sequence comparisons show that AvFdI is a member of a closely related class of 7Fe ferredoxins found in a variety of organisms (10). Given the enormous amount of information that is available concerning these proteins, it is surprising that their specific cellular functions have not as yet been determined.

One approach that was taken to this problem involved the disruption of the A. vinelandii fdxA gene that encodes FdxI (11). The resulting A. vinelandii strain, designated LM100, had no obvious phenotype with respect to cell growth (11). However, two-dimensional gel electrophoresis analysis of LM100 revealed that there was another small acidic protein that was dramatically overproduced in the FdI⁻ strain when compared to the wild-type. This protein was given the trivial name Protein X (11, 12). The observation that the synthesis of Protein X was "repressed" by FdI further led to the proposal that FdI might be a novel DNA binding repressor protein (13). Thus, FdI appears to have both an electron transfer and a regulatory function. In order to gain insight into both of these functions, we previously reported the purification and characterization of Protein X (14). It was shown to be a Mᵣ — 29,000, NADPH-specific flavoprotein whose physical properties and NH₂-terminal amino acid sequence showed striking similarity with the NADPH:ferredoxin reductase from Escherichia coli (14). Protein X was further shown to bind FdI specifically, suggesting that the two proteins were likely to be redox partners in vivo (14).

Here we report the cloning and sequencing of the A. vinelandii gene encoding Protein X and the overexpression of the protein product.

EXPERIMENTAL PROCEDURES

Materials—AvFdI was purified and crystallized (2, 8) and A. vinelandii NADPH:ferredoxin reductase was purified (14) as described previously. Polyclonal antibodies against both proteins were raised at Behyl Laboratories, Montgomery, TX. The restriction enzymes MboI and SfuI, T₄ DNA ligase, T₄ poly

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1 The abbreviations used are: kb, kilobase(s); bp, base pair(s); FdI, flavodoxin; FdI, A. vinelandii ferredoxin I.
exposed on Kodak XAR film overnight. From the primary screening, 10 plaques were selected and treated to a second round of screening. The secondary screening resulted in 10 pure plaques which were stored in 1 ml of SM buffer (15) and 20 μl of chloroform.

Construction of Subclones—λ phage DNA containing the A. vinelandii fpr gene was purified from the 10 positive plaques using published methods (16). The DNA was digested with Sall, subjected to agarose gel electrophoresis, transferred onto nitrocellulose filters, and hybridized to oligonucleotide 1. A 1.5-kb Sall fragment containing the fpr gene was identified and subsequently gel-purified from agarose gels using the GeneClean method. This Sall fragment was then subcloned into Sall-digested pBluescriptII SK(−) using T4 DNA ligase and transformed into competent DH5α E. coli cells (15). The transformants were screened for ampicillin resistance and blue-white selection. Recombinant DNA was purified using the Wizard Miniprep kit.

DNA Sequence Determination and Southern Blot Analysis—The A. vinelandii fpr gene was sequenced in both directions from the 1.5-kb insert that was subcloned into two versions of pBluescript II, SK(−) (pS-XF1.5Sal) and KS(+) (pK-XF1.5Sal). Both sequences were in agreement. Nested deletions were constructed in both vectors using the Erase-a-Base kit. Each of the clones was sequenced twice with universal or reverse primers from the Pharmacia Auto-Read sequencing kit. The resulting reaction products were resolved and processed on an Automated Laser Fluorescence (Pharmacia) sequencer. For Southern analysis, A. vinelandii strain OP chromosomal DNA was isolated, digested to completion with restriction endonucleases, subjected to agarose gel electrophoresis, transferred to nylon filters, and hybridized (15) to a 1.2-kb Xhol 32P-labeled fragment containing the entire fpr coding region.

Construction of a NADPH:Ferredoxin Reductase Overexpression Strain—The fpr gene was subcloned into a broad host-range multicopy plasmid pKT230 which was used previously to successfully overexpress Fdi (17). For this study, pS-XF1.5Sal (Fig. 1) and pKT230 (17) were both digested with Xhol and then the two fragments were ligated together and transformed into E. coli strain DH5αF′ (15) which was screened for kanamycin sensitivity and streptomycin resistance. The subcloned DNA was then isolated by using Promega's Wizard Miniprep kit, and the orientation of the A. vinelandii DNA insert was determined by restriction analysis. The pKT230 derivatives with the gene in the two orientations were designated pKTFL.2 and pKTR1.2. These plasmids were then introduced into the A. vinelandii wild-type trans-strain using electroporation as described by BTX Inc. Following electroporation, the A. vinelandii cells were recovered for 5 h in 1 ml of Burk's media supplemented with 36 mM ammonium acetate at 30 °C with shaking at 200 rpm. The cells were then plated on selective Burks's media minus ammonium acetate supplemented with 0.1 μg/ml streptomycin.

RESULTS AND DISCUSSION

Cloning and Subcloning of the A. vinelandii NADPH:Ferredoxin Reductase Gene—We recently published the NH2-terminal amino acid sequence of a NADP+/NADPH-specific flavoprotein that is overexpressed in Fdi− strains of A. vinelandii (14). We selected a region of this amino acid sequence for the synthesis of the corresponding oligonucleotide probe. The probe (5′-CACCACTGGAACGA(CT)ACCCT(GC)TTCTCCTTCAAG ACCACCACCTAACC-3′), a 47-mer (degenerate in 3 places), was based on residues 13–28 of the protein sequence (14) and the known codon bias of this G-C-rich organism (18). This oligonucleotide, end-labeled with γ-32P, was used to screen a λ library of Sall-digested A. vinelandii DNA. Following initial screening of 10,000 plaques, 10 positive plaques were selected. Purified DNA was digested with Sall, and a 1.5-kb fragment which hybridized to the oligonucleotide was selected. This fragment was then subcloned into Bluescript as described under "Experimental Procedures." Fig. 1 is a restriction map of the A. vinelandii DNA portion of the resulting plasmid designated pS-XF1.5Sal.

DNA Sequence Analysis—The complete nucleotide sequence of the A. vinelandii DNA fragment contained in pS-XF1.5Sal (Fig. 1) was determined by making unidirectional deletions of pS-XF1.5Sal and pK-XF1.5Sal and subsequently sequencing selected deletion clones. The sequencing strategy is shown in Fig. 2. A total of 1505 bp were sequenced, 776 bp of which make up the gene of interest with 454 bp 5′ and 275 bp 3′. The nucleotide sequence of the coding strand, which has been given the Genome Sequence Data base accession number L36319, is

**Fig. 1. Construction of pS-XF1.5Sal.** Plasmid pS-XF1.5Sal is an A. vinelandii DNA-Bluescript II hybrid which contains approximately 1.5 kb of A. vinelandii genomic DNA including the fpr gene. Its construction is described in the text. Restriction enzymes used were: S, Sall; X, Xhol; A, Asml; N, Ncol; E, EcoRV; BglII; and P, PstI.

**Fig. 2. Sequencing strategy used to establish the nucleotide sequence of the fpr gene.** Details are as described in the text.
were found upstream of the start site for the stem loop structure, denoted by ( ).

The plant ferredoxin NADP reductases, which catalyze the reduction of cytochrome f to yield NADPH, are designated FNR and have an NH2-terminal extension that serves as a targeting sequence for localizing these proteins to the chloroplasts. The bacterial NADPH:ferredoxin reductases are missing the NH2-terminal extension and are designated FPR because the FNR designation had already been used for an unrelated protein in E. coli (19).

As shown in Table I, the A. vinelandii ferredoxin reductase protein falls into the bacterial class.
with respect to the regulation of cytochrome codon (Fig. 3). This situation is reminiscent of the situation binding site found within 400 bp upstream of the found immediately upstream of the A. vinelandii (27) previously reported that in under N2-fixing conditions. It is possible that the 29,000-dalton membrane-bound electron transport protein was up-regulated under N2-fixing conditions. It is interestingtonotethatKlugkist et al. (27) previously reported that in A. vinelandii a NADPH-specific 29,000-dalton membrane-bound electron transport protein was up-regulated under N2-fixing conditions. It is possible that their membrane-bound protein and the soluble protein reported here are the same, and that the reported differences in solubility arise from the different methods used to rupture the cells.

In spite of obvious up-regulation of the NADPH:ferredoxin reductase under N2-fixing conditions (Fig. 7), the restriction fragments shown in Fig. 1, and the sequence shown in Fig. 3, which includes the fpr gene that codes for A. vinelandii NADPH:ferredoxin reductase, are not found in the 50 kb of A. vinelandii DNA from the nif region that has been mapped to date. All nif genes so far sequenced from Azotobacter and Klebsiella contain a conserved nif promoter sequence and a nif binding site (e.g. Ref. 21). This nif promoter sequence is not found immediately upstream of the fpr gene nor is a nif binding site found within 400 bp upstream of the fpr initiation codon (Fig. 3). This situation is reminiscent of the situation with respect to the regulation of cytochrome d in A. vinelandii

Because A. vinelandii has multiple copies of some genes (21), we examined whether the fpr gene was also reiterat on the wild-type A. vinelandii chromosome. To accomplish this, Southern analysis was performed using a 1,2-kilobase XhoI restriction enzyme fragment containing the fpr gene as a probe of A. vinelandii genomic DNA digested with various restriction enzymes. As shown in Fig. 6, in each case where the enzyme used did not cut within the gene, the fpr probe hybridized to only a single restriction enzyme fragment in the respective Southern analysis. These results show that there is only a single fpr encoding sequence on the A. vinelandii chromosome.

Relationship to N2 Fixation—When A. vinelandii is grown under N2-fixing conditions, the enzyme nitrogenase receives electrons directly from reduced flavodoxin (Fld), however, the mechanism of Fld reduction in this organism has not been established (22, 23). Although E. coli does not fix nitrogen, it does contain a Fld which is used as an electron donor in anaerobic metabolism (24–26). In that organism, the fpr gene product (NADPH:ferredoxin reductase) appears to serve as the immediate electron donor to Fld (19, 26), suggesting that in A. vinelandii the fpr gene product might similarly serve to mediate electron transfer between NADPH and Fld. In A. vinelandii, however, the reduced Fld could then be used to support N2 reduction by nitrogenase. In support of that idea, we observed during the purification of A. vinelandii NADPH:ferredoxin reductase (14) and by Western blot analysis (Fig. 7) that the protein was up-regulated when the cells were grown under N2-fixing conditions. It is interesting to note that Klugkist et al. (27) previously reported that in A. vinelandii a NADPH-specific 29,000-dalton membrane-bound electron transport protein was up-regulated under N2-fixing conditions. It is possible that their membrane-bound protein and the soluble protein reported here are the same, and that the reported differences in solubility arise from the different methods used to rupture the cells.

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FIG. 5. Conserved regions that characterize the ferredoxin reductase family. The regions are numbered according to Ref. 22 with the fingerprint regions for the A. vinelandii and E. coli proteins aligned in lines 1 and 2. The consensus sequence for the whole family is shown in line 3 with capital letters representing residues that are always conserved and two stacked uppercase letters indicating positions where only two residues are allowed. The first and second sequence bind the isoalloxazine and phosphate groups of FAD, respectively. FMN-containing proteins have a different sequence in the second region. The third, fourth, and fifth regions are involved in NADP+ binding. NAD+ proteins have a different sequence in the fourth region. Where structures are available, the aromatic residue in the sixth region is stacked against the flavin (23).

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Fig. 6. A. vinelandii contains only one copy of the NADPH:ferredoxin reductase gene. Southern analysis of A. vinelandii genomic DNA digested with the enzymes shown and probed with a DNA fragment containing the fpr gene. For EcoRV, there is a single site within the fpr coding region. For all other enzymes there are no sites within the fpr coding region.

Fig. 6. A. vinelandii contains only one copy of the NADPH:ferredoxin reductase gene. Southern analysis of A. vinelandii genomic DNA digested with the enzymes shown and probed with a DNA fragment containing the fpr gene. For EcoRV, there is a single site within the fpr coding region. For all other enzymes there are no sites within the fpr coding region.

Fig. 7. NADPH:ferredoxin reductase is up-regulated when cells are grown under N2-fixing conditions. Western blot analysis showing reaction with anti-A. vinelandii NADPH:ferredoxin reductase antibodies after separation of cell-free extracts by SDS-polyacrylamide gel electrophoresis. A. vinelandii strain LM100 is shown grown on NH4 (a) and under N2-fixing conditions (b).
Cytochrome d is involved in maintaining the low intracellular O2 concentrations that are required for N2 fixation. The gene encoding cytochrome d is not located in the nif region of the chromosome, and it is not preceded by a nif-specific promoter nor nifA binding sequence. However, like the fpr gene (Fig. 7), the cytochrome d gene is up-regulated when the cells are grown under N2-fixing conditions (28).

Further analysis of the regions upstream of the fpr gene shows that two putative ρ70 type E. coli consensus promoter elements are present (Fig. 3). The data in Fig. 3 also show an excellent ribosome binding site (29) for A. vinelandii NADPH:ferredoxin reductase 12 bases upstream from the initiation codon. Downstream of the coding region there is a predicted stem-loop structure denoted by the arrows (Fig. 3) which probably acts as a ρ-independent transcription termination signal (30). Thus, it is unlikely that additional genes are in the same operon downstream of fpr.

Overexpression of A. vinelandii NADPH:ferredoxin reductase in A. vinelandii using the broad host range multicopy plasmid pKT230. Western blot analysis showing reaction with anti-A. vinelandii NADPH:ferredoxin reductase antibodies after separation of cell-free extracts by SDS-polyacrylamide gel electrophoresis. A. vinelandii strains are: a, wild-type trans; b, trans/pKTR1.2; and c, trans/pKTF1.2. All were grown under N2-fixing conditions.

Fig. 8 shows the SDS-gel electrophoresis separation of cell-free extracts of wild-type A. vinelandii compared to the overproduction strains trans/pKTR1.2 and trans/pKTF1.2. Clearly there has been an overproduction of the NADPH:ferredoxin reductase in both trans/pKTR1.2 and trans/pKTF1.2. The fpr gene product overexpressed in A. vinelandii is indistinguishable in terms of activity and crystallization properties from the protein purified from A. vinelandii strain LM100. However, the amount of protein is much greater for the strain that has the gene in the same orientation as the kanamycin promoter on the vector. This observation is in contrast to the result obtained for the overexpression of Fdi using the same pKT230 system (17). In that case, the protein was being maximally expressed from its own promoter such that the levels of overexpression were the same regardless of the orientation of the insert. The data in Fig. 8 show that something is preventing the maximal expression of the NADPH:ferredoxin reductase from its own promoter in trans/pKTR1.2. These data therefore suggest that unlike the situation with respect to Fdi, expression from the fpr promoter may be tightly regulated, by a factor(s) whose concentration is limited.

Function and Regulation of the fpr Gene Product in E. coli and A. vinelandii—Investigators working on three very different problems have now converged on the same protein. First, the fpr gene product from E. coli was identified as a component of the anaerobic ribonucleotide reductase system (19). In that context, the NADPH:ferredoxin reductase mediates electron transfer from NADPH to Fld which then serves as a specific electron donor for the activation of anaerobic ribonucleotide reductase (24) and other enzymes involved in anaerobic metabolism (e.g. pyruvate formate-lyase (25, 26)). Although A. vinelandii is an obligate aerobe, while fixing N2 it appears to maintain a very low internal O2 concentration in order to protect the O2-sensitive enzyme nitorgenase (e.g. Ref. 28). Thus, the fpr gene product could serve a similar anaerobic enzyme activation function by reducing Fld in A. vinelandii. In A. vinelandii, however, Fld has an additional function as the immediate electron donor to nitorgenase, and, consequently, it is up-regulated under N2-fixing conditions (22, 23). Our observation that the fpr product is also up-regulated under N2-fixing conditions (Fig. 7) suggests that in A. vinelandii the NADPH:ferredoxin reductase reported here may also serve as an electron donor to Fld in support of N2 reduction.

Second, in our work, we encountered the fpr gene product while working on AvFdI. In that context, we have shown that the NADPH:ferredoxin reductase binds specifically to AvFdI and that it can mediate electron transfer between NADPH and Fdi (14). In E. coli, the fpr gene product also mediates electron transfer between NADPH and ferredoxin (26). However, it should be noted that the only ferredoxin that has so far been isolated from E. coli is a 2Fe-2S protein (32) that is very different from the 7Fe-containing AvFdI (1–5). Given the extent of similarity of the E. coli and A. vinelandii fpr gene products throughout their entire length (Fig. 4), the specificity of the A. vinelandii fpr product for Fdl (14), and the known multiplicity of different types of ferredoxins in a number of organisms, it may be that one natural redox partner of NADPH:ferredoxin reductase in E. coli is an as yet unrecognized Fdl-type ferredoxin.

The final way in which this protein was identified is through studies of oxygen toxicity in E. coli. In that case, mutants sensitive to methyl viologen, a superoxide radical propagator, were isolated, and the gene involved was cloned (33) and shown to be identical with the E. coli fpr gene (19). Subsequently, Fridovich and co-workers (34) showed that the gene product, NADPH:ferredoxin reductase, was part of an O2 protection system that was controlled by the SoxRS regulon. SoxR is proposed to be an [FeS]-protein (35). In response to superoxide it is converted into an activator of the soxS gene, and SoxS then activates the transcription of a number of other proteins including the fpr gene product. Fridovich and co-workers (34) proposed that reduced ferredoxins or flavodoxins might control the system by reducing SoxR which would then turn off the activation. Thus, it is proposed that when SoxR is oxidized, the soxS gene is activated causing NADPH:ferredoxin reductase to be overexpressed. It then reduces flavodoxin and ferredoxin, which in turn reduce SoxR, which then deactivates the SoxRS
It is important to note that in *A. vinelandii* the fpr gene product initially came to our attention because it was overexpressed in strains that do not synthesize FdI (11, 12). It is not overexpressed in strains that do not synthesize Fld (12). Further studies will therefore be directed at determining how FdI exerts its regulatory effect on fpr and examining the possibility that it might act through a SoxRS-like regulon in *A. vinelandii*.

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