Identification of a Palindromic Sequence That Is Responsible for the Up-regulation of NAPDH-Ferredoxin Reductase in a Ferredoxin I Deletion Strain of Azotobacter vinelandii*

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Azotobacter vinelandii ferredoxin I (AvFdI) is one member of a class of 7Fe ferredoxins found in a variety of organisms that are all capable of aerobic growth. Disruption of the fdxA gene, which encodes AvFdI, leads to overexpression of its redox partner, NADPH-ferredoxin reductase (FPR). In this study the mechanism of FdI-mediated regulation of FPR was investigated. Northern analysis has shown that regulation is at the level of fpr transcription, the start site for transcription has been identified, and it is preceded by a canonical σ70-type bacterial promoter. Gel mobility shift assays show that there is a putative regulatory protein in A. vinelandii that binds specifically upstream of the −35 region. That protein is not AvFdI. A palindromic sequence was identified as a putative binding site, and randomization of that sequence completely eliminates binding of the putative regulatory protein. A luciferase reporter gene was placed under control of the A. vine-landii fpr promoter and introduced into wild type and FdI− strains of A. vinelandii. Luciferase activity was enhanced 7-fold in the FdI− mutant relative to the wild type. Alteration of the palindromic sequence reduced the luciferase levels in the FdI− strain to those of the wild type, demonstrating that FdI regulates FPR through the palindrome and that the reaction is an activation rather than a repression. The identified palindrome is ~50% identical to the SoxS binding site up-stream of Escherichia coli fpr, suggesting that A. vinelandii may have a SoxS-like regulatory system and that the function of FdI might be to specifically inacti-vate that system.

Azotobacter vinelandii ferredoxin I (AvFdI) is a small protein (Mr = 12,700) that contains two different types of [Fe-S] clusters: one [3Fe-4S]1+/2 cluster and one [4Fe-4S]2+/3+ cluster. Sequence comparisons show that AvFdI is a member of a closely related class of 7Fe ferredoxins found in a variety of organisms that share the ability to grow aerobically (1). Despite the fact that AvFdI has been extensively characterized by x-ray crystallography (2–6) and by detailed spectroscopic (7–10) and biochemical (1) studies, the specific cellular function(s) of the protein has yet to be determined. The initial approach to that problem involved the construction of a strain of A. vinelandii, designated LM100, that had a disruption of the fdxA gene that encodes FdI (11).

Although the FdI− strain LM100 had no obvious phenotype with respect to growth, two-dimensional gel analysis revealed that there was another small acidic protein that was dramat-ically increased in abundance in LM100 relative to the wild type A. vinelandii strain (11). In part this observation led Thomson to propose that FdI might be a novel DNA-binding repressor protein (12). Recently a major advance in the field of [Fe-S] proteins has come with the recognition that a growing number of [Fe-S] proteins (e.g. iron regulatory protein (13, 14), endonuclease III (15), Mut Y (16), Pat B (17), the FNR protein (18), and Sox R (19)) bind DNA or RNA and that many of them regulate gene expression. To determine whether AvFdI is a member of this class of proteins, the acidic protein that is overexpressed in the FdI− strain LM100 was purified and characterized (20), and the gene encoding the protein was cloned and sequenced (21). The protein was shown to be a Mr −29,000 NADPH-ferredoxin reductase that was designated FPR because its physical properties and amino acid sequence showed striking similarity to the FPR from Escherichia coli (21, 22). The A. vinelandii FPR was further shown to bind very specifically to AvFdI, suggesting that the two proteins were likely to be redox partners in vivo (20).

Here we report the identification of the A. vinelandii fpr promoter and a promoter element that is responsive to fdxA deletion, leading to the overexpression of FPR in the FdI− strain LM100.

EXPERIMENTAL PROCEDURES

Materials—A. vinelandii FdI was purified (9), and FdI strain LM100 was constructed (11) as described elsewhere. All chemicals were purchased from Sigma, Fisher, or Bio-Rad. All enzymes were purchased from Promega, New England Biolabs, or Life Technologies, Inc. Radio-isotopes were purchased from New England Nuclear (DuPont NEN) or Amersham Corp., and film was purchased from Eastman Kodak Co. The Luciferase assay system and Primer Extension system were purchased from Promega. The Sequenase kit was purchased from U. S. Biochemical Corp.. Oligonucleotides were purchased from Midland Certified Reagent Company. All chemicals used were of reagent or molecular biology grade.

Strains and Plasmids—The E. coli strains DH1 (F’ gyrA96 relA1 endA1 thi-1 hsdR17 supE44 λ− C600 (thr-1 leuB6 lacI tetA21 supE44 λ−) were used as host cells for plasmids. The A. vine-landii strains used were OP wild type and LM100 (fdxA:Km−) (11). All E. coli strains were grown on Luria-Bertani medium at 37 °C with 300 rpm agitation. All A. vinelandii strains were grown in 100 ml of Burk’s medium, at 30 ºC and 200 rpm agitation. All plasmids were introduced into E. coli using standard procedures; all plasmids were introduced into A. vinelandii using electroporation as described elsewhere (21). The luciferase fusion reporter plasmid pXluc9 was constructed by sub-cloning a 400-bp PCR derived fragment containing the fpr promoter into the BamHI-PstI sites of pBIIKS+, the sequence was confirmed, and then the KpnI-HindIII fragment was subcloned into pSP-luc+NF (Promega) to create a translational fusion. The vector pKTXlac7 was constructed by subcloning the 1.9-kb XhoI liberated luciferase fusion

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1 The abbreviations used are: AvFdI, A. vinelandii ferredoxin I; FdI, ferredoxin I; bp, base pair(s); PCR, polymerase chain reaction; kb, kilobase; FPR, NADPH-ferredoxin reductase.
construct from pXLuc9 into the broad range host vector pKT230 (23). The vector pXLM7 was constructed by the same strategy as for pXLuc9, except the PCR fragment was mutagenized using the following oligonucleotides and methods described in PCR Protocols (24). Palmitut 1 (taagtaatttacccggacctgtcagcggtctcgttaaatgattcactctgcg) and pal-clone. Nucleotide sequence of the coding strand is shown on the same oligonucleotide and purified vector DNA containing the entire Procedures." The DNA sequencing ladder was generated using the identified transcriptional start site indicated by the arrow

transcript is noted by an cated approximately equal amounts of intact rRNAs in both lanes. The "Experimental Procedures." The ethidium bromide-stained gel indi-
equal quantities of whole cell RNA and probed as described under DNA standards are indicated on the right

FIG. 1. Northern blot detection of fpr transcripts in AvOP (wild type) and FdI strain. The positions and sizes of denatured DNA standards are indicated on the right. Both lanes were loaded with equal quantities of whole cell RNA and probed as described under "Experimental Procedures." The ethidium bromide-stained gel indicated approximately equal amounts of intact RNAs in both lanes. The transcript is noted by an arrow on the left and is approximately 900 nucleotides in length.

FIG. 2. Mappings of the 5′ end of the fpr transcript by primer extension. Radiolabeled primer (30-mer) was extended on whole cell RNA isolated from FdI strain LM100 (lane 1) and wild type strain AvOP (lane 2) and electrophoresed as described under "Experimental Procedures." The DNA sequencing ladder was generated using the same oligonucleotide and purified vector DNA containing the entire fpr clone. Nucleotide sequence of the coding strand is shown on the left with the identified transcriptional start site indicated by the arrow.

RESULTS AND DISCUSSION

FPR Is Regulated at the mRNA Level—In previous studies we have shown that FPR protein levels are greatly increased in FdI strain LM100 relative to the wild type A. vinelandii strain (11, 20, 21, 28). To determine if the increase of FPR was reflected in the fpr mRNA, total cell RNA was isolated from

FIG. 3. Sequence and restriction sites of the fpr promoter. The −10 and −35 promoter elements are boxed, deviations from the consensus are indicated in bold type. The transcriptional start adenosine is indicated by underlined bold type and arrow. The ribosomal binding site is underlined, and the translational start site is also indicated by underlined bold type. The identified palindrome is indicated by convergent arrows.
wild type *A. vinelandii* and from the FdI− strain LM100, and Northern analyses were performed using a 1.2-kb DNA fragment that included the entire *fpr* open reading frame as a probe. Two conclusions can be drawn from these data, which are shown in Fig. 1. First, the levels of *fpr* mRNA are greatly increased in the FdI− strain LM100, indicating that the *fpr* gene is not regulated solely at the level of translation and/or protein stabilization. Rather, the increase in FPR appears to be due to regulation at the level of the *fpr* transcript. Second, only one major transcript was observed, which was approximately 900 nucleotides in length from both wild type *A. vinelandii* and from the FdI− strain LM100 (Fig. 1). A message of this length is only consistent with a monocistronic message originating at a promoter proximal to the open reading frame.

Identification of the Transcriptional Start Site for the *A. vinelandii* *fpr* Gene—To identify the specific start site for transcription, a series of primer extension experiments were carried out. A 30-bp oligonucleotide primer that was complementary to the region of the *fpr* mRNA overlapping the region encoding the first seven amino acids of FPR was used. The radiolabeled primer was annealed to an *A. vinelandii* total RNA preparation and extended using avian myeloblastosis virus reverse transcriptase. To determine the exact size of the product of that reaction the same oligonucleotide primer was used to sequence a DNA template containing the entire *fpr* clone (21) including 450 bp upstream of the *fpr* coding region. As shown in Fig. 2 those data clearly identified the start site for transcription as the adenosine noted in Fig. 3. It should also be noted that the same transcriptional start site was identified for both wild type and FdI−LM100 strains of *A. vinelandii*, indicating that the same promoter is initiating transcription in both cases. Inspection of the sequence immediately upstream of this start site revealed a canonical ς70-type promoter (Fig. 3). The identified ς70 promoter has the typical 7 bp spacing between the +1 adenosine start site and the Pribnow box, which has only a single deviation from the consensus at a highly variant position (noted in bold type in Fig. 3). The spacing between the Pribnow box and the −35 hexamer is the typical 17 bp; the −35 consensus also has only a single deviation from the consensus. It should be noted that the identification of the nearly ideal ς70 promoter confirms our earlier conclusion (21) that FPR is encoded by a non-nif gene.

**A Putative Regulatory Protein Binds Upstream of the *fpr* Promoter, and It Is Not FdI**—If, as proposed by Thomson (12), FPR expression is being directly repressed in the wild type *A. vinelandii* strain by FdI, then FdI should bind within or proximal to the promoter shown in Fig. 3. If instead FPR expression is being regulated by another protein in the FdI− strain LM100, then that regulatory protein should show evidence of binding the promoter region. To determine if any protein present in *A. vinelandii* binds specifically to the DNA proximal to the promoter, we undertook a series of gel mobility shift experiments using *A. vinelandii* extracts. As shown in Fig. 4, we tested a number of overlapping DNA fragments derived from our original clone, which extended 450 bp upstream of the *fpr* coding region (21). These fragments were radioisotope labeled and incubated with extracts from the FdI−LM100 strain of *A. vinelandii* before running them out on a 4.5% nondenaturing gel. In Fig. 4 the free DNA probe migrates fastest and is the lowest band in each lane. The upper bands represent probes where the migration has been retarded due to the association of protein(s) with the DNA. Several fragments very clearly show a significant mobility shift, indicating that some protein(s) present in *A. vinelandii* extracts binds very tightly to the DNA probe. Further analysis of these data (Fig. 4) localized the specific binding to a 34-bp region of DNA just upstream of the −35 consensus of the promoter (Fig. 5).

To confirm the importance of this region and to examine this region further, we performed the gel shift experiment using a 60-bp *Alu* to *MseI* fragment of DNA that extends from the 5′

**Fig. 4.** Gel mobility shifts with fragments of the *fpr* promoter and upstream region. All fragments were prepared and incubated with *A. vinelandii* extracts as described under “Experimental Procedures,” the approximate size in base pairs is indicated above the respective lanes. The band migrating furthest in each lane is the free DNA probe. Only intensely shifted bands are scored as positive. The free probe and probe-protein complex are indicated for the 47-bp probe.

**Fig. 5.** Gel shift fragment map and data summary diagram. A, the entire *fpr* clone and partial restriction map. The 220- and 330-bp fragments were initially tested for gel mobility shifts, and only the 330-bp fragment showed strong gel mobility shifting. B, an enlarged map of the 330-bp fragment and subfragments. Each fragment is denoted with its length in base pairs. On the right is noted the behavior of the fragments in mobility shift assays (Figs. 4 and 6). Vertical dotted lines indicate the region of DNA that was included in all fragments that shifted and excluded in all fragments that failed to shift.
concentrations of purified FdI. In separate experiments DNA for the shift is present in LM100, and no shift is seen with high density; purified FdI was also used. As shown in Fig. 6 both purified probes (lane 4), with FdI strain LM100 crude extract (lane 2), with FdI strain LM100 crude extract (lane 3), or 250 nmol of pure FdI (lane 4).

In order to determine the effect of randomizing the palindrome on protein binding, two 160-bp gel purified probes (XhoI to Msel) wild type (wt) palindrome (lanes 1, 2, 1', and 2') and altered palindrome (lanes 3, 4, 3', and 4') were incubated in the absence of cell extracts (lanes 1, 1', 3, and 3'), in the presence of wild type AvOP crude extract (lanes 2 and 4), or in the presence of FdI strain LM100 crude extract (lanes 2' and 4'). The major bands designated B and the lighter bands designated A and C are discussed in the text.

End of the −35 hexamer, upstream (Fig. 5). Protein extracts were prepared from parallel cultures of wild type A. vinelandii and the FdI strain LM100 and harvested at the same cell density; purified FdI was also used. As shown in Fig. 6 both wild type and LM100 extracts gave strong shifts with the 60-bp probe, confirming that there is a protein present in A. vinelandii that binds to this region. The data in Fig. 6 further show that the protein cannot be FdI because the protein responsible for the shift is present in LM100, and no shift is seen with high concentrations of purified FdI. In separate experiments DNA fragments representing the entire 450-bp upstream region were also tested for binding to purified AvFdI, and no binding was detected in any of those experiments. It should be noted that although experiments of the type shown in Fig. 6 do not give quantitative information about the relative amounts of the DNA-binding proteins present in wild type versus LM100, we always observe strong shifts for extracts from both types of cells.

Inspection of the sequence (Fig. 3) of the DNA just upstream of the −35 consensus (complex B) no longer binds when the palindrome has been mutated, further confirming the binding is not specific for the palindrome.

FdI Regulates FPR Expression Indirectly through the Identified Palindrome—To try to correlate the gel shift results with the overexpression of FPR in LM100, a luciferase reporter gene was placed under the control of the A. vinelandii fpr promoter. This translational fusion was then subcloned into the broad-host-range multicopy plasmid pKT230 (29), which was then introduced into both the wild type and LM100 strains of A. vinelandii. As shown in Fig. 8A, the luciferase activity was

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**Fig. 6.** Gel mobility shift using the 60-bp *Alu*I to *Mse*I DNA fragment as a probe. Gel mobility shift reactions were carried out as described under "Experimental Procedures." The 60-bp probe was incubated without added extract (lane 1), with wild type AvOP crude extract (lane 2), with FdI− strain LM100 crude extract (lane 3), or 250 nmol of pure FdI (lane 4).

**Fig. 7.** Gel mobility shift assays determining the effect of randomizing the palindrome on protein binding. Two 160-bp gel purified probes (XhoI to Msel) wild type (wt) palindrome (lanes 1, 2, 1', and 2') and altered palindrome (lanes 3, 4, 3', and 4') were incubated in the absence of cell extracts (lanes 1, 1', 3, and 3'), in the presence of wild type AvOP crude extract (lanes 2 and 4), or in the presence of FdI− strain LM100 crude extract (lanes 2' and 4'). The major band designated B and the lighter bands designated A and C are discussed in the text.

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**Fig. 8.** A comparison of luciferase activity of AvOP (wild type) and LM100 (FdI−) carrying the luciferase reporter construct pKTlac7. Parallel cultures were grown on Burk's medium with ammonia to an A600 of approximately 0.700 and harvested in log phase as described under "Experimental Procedures." Two aliquots were measured for luciferase activity. B, the same experiment only using the construct pXL7 that has the palindrome randomized.

**Fig. 9.** A comparison of the *fpr* DNA sequences just upstream of the −35 consensus sequence from *E. coli* and *A. vinelandii*. Both −35 sequences are in bold type, the identified Sox S footprint is underlined in the *E. coli* sequence (30), and the *A. vinelandii* palindrome identified in this work is indicated by convergent arrows. The vertical lines denote sequence agreement; 13 of the 30 nucleotides in the SoxS binding site are identical with a 54% identity over the 24-bp palindrome.
enhanced about 7-fold in LM100 relative to the wild type strain, further confirming that the up-regulation of FPR levels in LM100 is due to regulation at the fpr promoter. This construct was then mutated only at the palindrome by randomizing the palindromic sequence. As shown in Fig. 8B the level of luciferase activity in LM100 was reduced to that of the wild type when driven by a promoter with a mutant palindrome. These data demonstrate not only that FdI regulates FPR expression through the identified palindrome but also that the regulation is activation rather than repression. If the gene were regulated by a repressor protein, then disruption of the protein DNA interaction should have led to an increase of luciferase activity in the wild type rather than the observed result that levels of activity declined in LM100.

**Relationship between FPR Regulation in A. vinelandii and E. coli**—Taken together the above data demonstrate that fpr transcription in A. vinelandii is activated in response to deletion of fdxA, that the activation results from interactions at a specific palindrome, and that a putative regulatory protein other than FdI binds to that palindrome. Thus, the proposal that FdI might regulate FPR expression directly by acting as a novel repressor protein (12) is not correct. The similarity between the E. coli FPR and the A. vinelandii FPR proteins led us to compare the sequence of the regulatory palindrome identified here to the region upstream of the fpr promoter in E. coli. The alignment illustrated in Fig. 9 shows ~50% identity between the palindromes identified in this study and the parallel E. coli region. The locations of both regions relative to the promoter are also very similar. In E. coli this region has been defined as a SoxS binding site (30), and SoxS has been shown to activate expression through the identified palindrome but also that the activation is activation rather than repression. If the gene were regulated by a repressor protein, then disruption of the protein DNA interaction should have led to an increase of luciferase activity in the wild type rather than the observed result that levels of activity declined in LM100.

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