Characterization of the Fatty Acid-responsive Transcription Factor FadR

BIOCHEMICAL AND GENETIC ANALYSES OF THE NATIVE CONFORMATION AND FUNCTIONAL DOMAINS*

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In Escherichia coli, fatty acid synthesis and degradation are coordinately controlled at the level of transcription by FadR. FadR represses transcription of at least eight genes required for fatty acid transport and β-oxidation and activates transcription of at least two genes required for unsaturated fatty acid biosynthesis and the gene encoding the transcriptional regulator of the aceBAK operon encoding the glyoxylate shunt enzymes, IclR. FadR-dependent DNA binding and transcriptional activation is prevented by long chain fatty acyl-CoA. In the present work, we provide physical and genetic evidence that FadR exists as a homodimer in solution and in vivo. Native polyacrylamide gel electrophoresis and glycerol gradient ultracentrifugation of the purified protein show that native FadR was a homodimer in solution with an apparent molecular mass of 53.5 and 57.8 kDa, respectively. Dominant negative mutations in fadR were generated by random and site-directed mutagenesis. Each mutation mapped to the amino terminus of the protein (residues 1–66) and resulted in a decrease in DNA binding in vitro. In an effort to separate domains of FadR required for DNA binding, dimerization, and ligand binding, chimeric protein fusions between the DNA binding domain of LexA and different regions of FadR were constructed. One fusion, LexA1–87-FadR102–239, was able to repress the LexA reporter sulA-lacZ, and ß-galactosidase activities were derepressed by fatty acids, suggesting that the fusion protein had determinants both for dimerization and ligand binding. These studies support the conclusion that native FadR exists as a stable homo-dimer in solution and that determinants for DNA binding and acyl-CoA binding are found within the amino terminus and carboxyl terminus, respectively.

In all organisms lipid metabolism is tightly regulated to coordinate the cellular requirements for metabolic energy and to maintain the fluidity of cellular membranes. In Escherichia coli the transcription factor FadR plays a pivotal role in regulating the anabolic and catabolic pathways of fatty acid metabolism. FadR specifically represses the transcription of each of the genes that are essential for fatty acid transport and β-oxidation including fabL, fabD, fabE, fabF, fabG, fabBA, and fabH (1–3). This transcription factor is required for long term stasis survival, partly through the regulation of expression of the uspA gene, which encodes a universal shock protein (4). FadR is also an activator of transcription of the fabAB and fabB genes, which are required for unsaturated fatty acid biosynthesis (1, 3) and of the gene encoding the regulatory protein IclR, which in turn represses the expression of the aceBAK operon encoding the glyoxylate shunt enzymes (5). The growth of E. coli in minimal medium containing long chain fatty acids (C14–C16) results in derepression of the genes negatively controlled by FadR and in a decrease in the expression of the genes activated by FadR (2, 3). It has been demonstrated in vitro that FadR binds to DNA and that this interaction is specifically prevented by long chain fatty acyl-CoA (2, 6, 7).

Previously, we estimated the native molecular mass of FadR to be 29 kDa using gel filtration chromatography, suggesting that this protein is a monomer in solution, since the molecular mass predicted from the DNA sequence is 26,972 Da (2, 8). However, two lines of evidence argue that FadR interacts with its DNA binding sites as a multimer: (i) dyad symmetry in the operator regions of target genes, a feature commonly observed in transcriptional regulators that bind to their respective operators as homomultimers, (9) and (ii) the identification of a potential helix-turn-helix (H-T-H)$^1$ motif (between amino acid residues 34 and 54) in the amino-terminal region of FadR, a motif most commonly observed in homodimeric or homotrameric prokaryotic DNA-binding proteins (8, 9). While many transcriptional regulators may be stable homomultimers in solution, for others dimerization in solution may be required for DNA binding or, in other cases, dimerization may occur on the DNA (10, 11). In the latter two situations, dimerization may be limiting for DNA binding.

In this work, we reassessed the native molecular weight in solution using purified FadR by nondenaturing polyacrylamide gel electrophoresis and glycerol gradient ultracentrifugation to determine whether dimerization may be limiting for DNA binding. The results of these experiments support that dimerization is not limiting for DNA binding. We also identified fadR alleles which exhibited a dominant negative phenotype (fadR$^{-}$$^{-}$). These altered alleles were expected to have mutations within the putative DNA binding domain that would decrease DNA binding but not multimerization (12–14). Four such alleles were identified after hydroxylamine mutagenesis of fadR, and the changes were localized to amino acid residues Ala$^5$, Arg$^{35}$, His$^{65}$, and Gly$^{66}$. Furthermore, alanine scanning mutagenesis within and adjacent to the putative H-T-H motif identified five additional fadR$^{-}$$^{-}$ mutations. The amino acid substitutions in these mutations were localized to Arg$^{35}$, Arg$^{39}$, His$^{65}$, Gly$^{66}$, and Lys$^{67}$, thus confirming the importance of the three amino

$^*$The abbreviations used are: H-T-H, helix-turn-helix; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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acid residues Arg^{28}, His^{65}, and Gly^{66} identified by hydroxylamine mutagenesis. To further distinguish functional domains of FadR, chimeric fusions between the amino-termina DNA binding domain of LexA and different portions of FadR were constructed and analyzed. Our results indicate that a fragment of FadR including amino acid residues 102–239 contains determinants for dimerization and ligand binding.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The E. coli strains used were: JM109 for propagation of pSELECT-3 derived phagemids (15), LS1085 for plasmid isolation (3), LS1155 (fadR::kan) (1) for expression of galactosidase activity in response to plasmid encoded wild-type or mutant FadR (3), LS1154 (fadB::lacZ) to test negative transdominance (3), BL21(DE3) pLyS for controlled expression of FadR (16), and JL1436 to assay β-galactosidase activity in response to plasmid encoded wild-type LexA or chimeric LexA-FadR fusions (17). For biochemical assays cells were routinely grown in Tryptone broth (18). Where necessary to maintain plasmids, antibiotics were added to a final concentration of 100 μg/ml ampicillin, 40 μg/ml kanamycin, or 12.5 μg/ml chloramphenicol. The minimal medium used was medium E (18). Fatty acids were provided at 5 mM in 0.5% Brij-58. Liquid cultures were grown at 37 °C with shaking in a New Brunswick gyratory incubator. Bacterial growth was monitored in a Klett Summerson colorimeter equipped with a blue filter.

Hydroxyamine and Site-directed Mutagenesis—Chemical mutagenesis of wild-type FadR encoded within pCD126 using hydroxylamine was described previously (6). The mutated DNA was transformed into LS1155, and transformants were tested for β-galactosidase activity in response to plasmid encoded wild-type LexA or chimeric LexA-FadR fusions (17). For biochemical assays the cell lysates were routinely grown in Tryptone broth (18). Where necessary to maintain plasmids, antibiotics were added to a final concentration of 100 μg/ml ampicillin, 40 μg/ml kanamycin, or 12.5 μg/ml chloramphenicol. The minimal medium used was medium E (18). Fatty acids were provided at 5 mM in 0.5% Brij-58. Liquid cultures were grown at 37 °C with shaking in a New Brunswick gyratory incubator. Bacterial growth was monitored in a Klett Summerson colorimeter equipped with a blue filter.

Native Molecular Mass of FadR—Native molecular mass of FadR was determined using aqueous two-phase partitioning of the egg yolk by guest on June 24, 2017 http://www.jbc.org/ Downloaded from

Fatty Acid-responsive Transcription Factor FadR

PREPARATION AND ANALYSIS OF WILD-TYPE AND MUTANT FADR PROTEINS—For the overexpression of wild-type and mutant FadR proteins, the T7 system of Studier was used as previously described (2). The wild-type template was single-stranded pCD152. Mutagenic oligonucleotides (21–27 nucleotides in length) carried an alanine codon in place of the target. Mutagenic oligonucleotides were synthesized by primer extension with T4 DNA polymerase.

Restriction enzymes, T4 DNA ligase, chemical mutagenesis, and DNA sequencing were described previously (6). Purified FadR was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a set of 7.5, 10, or 12.5% nondenaturing gels with polyacrylamide concentrations of 7.5, 10, or 12.5%. The distance of protein migration relative to the distance of the marker (i.e. the retardation coefficient, κp) was determined. The negative slopes obtained for each protein were then plotted against the native molecular masses of the standards to produce a linear log/log plot from which the molecular mass of FadR was extrapolated.

Glycerol Gradient Ultracentrifugation—Glycerol density gradient sedimentation assay developed by Martin and Ames (25) was used to define the native molecular mass of FadR. Protein standards (100 μg) and purified FadR (25 μg) were layered on the top of a 5–25% glycerol gradient in 20 mM HEPES, pH 7.9, 100 mM KCl, 4 mM diethiothreitol, and 0.2 mM EDTA, which was spun at 32,000 rpm at 4 °C for 3 h in a SW41 Ti rotor in a Beckman L-60 ultracentrifuge. Following centrifugation, supernatants were removed. Quantification of the protein in each fraction was determined by Bradford (26) assay.

Construction of Chimeric LexA-FadR—A 1-kilobase pair EcoRI-HindIII fragment was subcloned from pJW184 (22) to pSELECT-1 to generate pCD170. Using pCD170 as a template, lexA-specific oligonucleotides (19–24 bases in length) were synthesized to engineer either Ndel or Ncol sites at amino acid 88 to generate pCD171 and pCD172, respectively.

A 1.3-kilobase pair HindIII-EcoRI fragment encoding FadR was subcloned from CD1901 (8) to pSELECT-1 to generate pCD152R. Using pCD152R as a template for fadR-specific oligonucleotides (19–24 bases in length) were synthesized to engineer either Ndel sites at amino acid positions 81, 100, 120, or 167 to generate pCDR307-1, -2, -3, or -4, respectively, or an Nol site at amino acid 140 to generate pCDR307-2. An EcoRI-Ndel fragment from pCD171 was then fused in frame with Ndel-BamHI fragments from pCDR307-1, -2, -3, and -4, and ligated into pUC18 vector to generate pCD205 (LexA^1–87 HM FadR^100–239), pCD208 (LexA^1–87 HM FadR^120–239), pCD209 (LexA^1–87 HM FadR^167–239), and pCD207 (LexA^1–87 HM FadR^140–239), respectively. An EcoRI-Ncol fragment from pCD172 was fused in frame with Ncol-BamHI fragment from pCDR 307-2 and ligated into pUC18 vector to generate pCD206 (LexA^1–87 HM FadR^239–323). The resulting chimera plasmids were sequenced across the fusion juncture to confirm the in-frame fusions. The expression of all the fusion proteins was driven off the isopropyl-1-thio-β-D-galactopyranoside-inducible promoter (IacUV5) (15).

Biochemical Procedures—β-Galactosidase activities in strains LS1154, LS1155, or JL1436 harboring the plasmid of interest were assayed as described by Miller (18).

DNA Sequencing—DNA was sequenced using Sequenase V 2.0 and a reagent kit purchased from U. S. Biochemical Corp. (Cleveland, OH).

Enzymes and Radiochemicals—Restriction enzymes, T4 DNA ligase, DNA polymerase, and Sequenase V 2.0 were purchased from U. S. Biochemical Corp., α-32P-ATP was purchased from DuPont NEN. Fatty acids and molecular mass markers were obtained from Sigma.

RESULTS

Native Molecular Mass of FadR—In previous work the native molecular mass of FadR was estimated to be 29 kDa using gel filtration chromatography which is close to the calculated monomeric molecular weight 26,972 from the DNA sequence (8). However, wild-type predicts the native form of FadR is a multimer because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i) the binding sites for FadR have dyad character because (i)
glycerol gradient ultracentrifugation.

Analysis of purified FadR using nondenaturing polyacrylamide gel electrophoresis estimated the native molecular mass of this protein was 53.5 kDa (Fig. 1A). Using glycerol gradient ultracentrifugation, the native molecular mass was estimated to be 57.8 kDa (Fig. 1B). These data support the conclusion that FadR exists as a stable homodimer in solution. On the basis of these data and because of the dyad character of the binding sites, we propose FadR interacts with DNA as a homodimer.

Dominant Negative Alleles of fadR—We performed hydroxylamine mutagenesis of wild-type fadR encoded within the moderate copy number vector, pACYC177 (6). A pool of mutagenized DNA was transformed into LS1155 (fadR fadB-lacZ) and selected on plates containing ampicillin and X-gal. Colonies that appeared dark blue had high levels of β-galactosidase. Plasmid DNA was isolated from those colonies, and the fadR alleles encoded within the plasmids were then tested for the ability to confer a dominant negative phenotype over wild-type FadR in strain LS1154 (fadB-lacZ). Those mutant alleles induced by treatment of plasmid DNA with hydroxylamine, which resulted in at least a 3-fold increase in β-galactosidase activities in LS1154, were designated fadR<sup>-d</sup> (Table I). Alleles that fulfilled this criterion had one of the following amino acid changes: A9V, R35C, H65Y, G66D, or G66S. Each of the substitutions were localized to the amino terminus of FadR; however, only one residue, Arg<sup>35</sup>, resides within the putative H-T-H motif (8). To further test the importance of other amino acid residues within and adjacent to the predicted H-T-H motif, we constructed a series of fadR alleles carrying substitutions of alanine.

As above, each of these alleles substituted with alanine were tested for repressor function in strain LS1155 (fadR fadB-lacZ) (Fig. 2). The fadR alleles that showed constitutive levels of β-galactosidase were subsequently tested for negative transdominance in strain LS1154 (Table II). Substitution of alanine for Arg<sup>35</sup>, like the substitution with cysteine induced by hydroxylamine mutagenesis, resulted in a 5-fold dominance. Therefore we suggest that this amino acid is critical for DNA binding of FadR to O<sub>B</sub>, the FadR binding site within the fadB promoter. Additional residues that appeared to be important for regulation of fadB included Arg<sup>49</sup>; His<sup>65</sup>, Gly<sup>66</sup>, and Lys<sup>67</sup>, each of which resulted in levels approximately 3-fold higher when substituted with alanine. The fadR alleles with alanine substitutions at Glu<sup>34</sup>, Leu<sup>37</sup>, Arg<sup>45</sup>, Leu<sup>48</sup>, Arg<sup>54</sup>, Trp<sup>60</sup>, Ile<sup>63</sup>, and Thr<sup>69</sup> showed slightly elevated levels (1.4–2-fold) with respect to the control.

Dominant Negative fadR Alleles Have Reduced DNA Binding in Vitro—To test whether or not the fadR<sup>-d</sup> isolates described above, including A9V, R35C, H65Y, G66D, and G66S, were able to bind to DNA, we subcloned the mutant genes into the T7 RNA polymerase expression plasmid, pT7-5, so that FadR was produced as the major protein after induction of T7 RNA polymerase. Fig. 3A represents a Coomassie Blue-stained 15% SDS polyacrylamide gel of FadR and protein standards through a 30–65% glycerol gradient. Each method is detailed under “Experimental Procedures.”

![Fig. 1.](image)

**Fig. 1.** Estimation of the native molecular mass of FadR. A, the retardation coefficient ($K_r$) was determined for each protein after electrophoresis in native polyacrylamide gels and plotted against the molecular mass. B, sedimentation profiles of FadR and protein standards through a 5–25% glycerol gradient. Each method is detailed under “Experimental Procedures.”

**Table I**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amino acid</th>
<th>Codon</th>
<th>β-Galactosidase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Negative dominance&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Substituted</td>
<td>Native</td>
<td>Substituted</td>
</tr>
<tr>
<td>pACYC177</td>
<td>NR&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCD126</td>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRW5</td>
<td>Ala&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Val</td>
<td>GCG</td>
<td>GTG</td>
</tr>
<tr>
<td>pRW9</td>
<td>Gly&lt;sup&gt;66&lt;/sup&gt;</td>
<td>Asp</td>
<td>GGC</td>
<td>GAC</td>
</tr>
<tr>
<td>pRW12</td>
<td>Gly&lt;sup&gt;66&lt;/sup&gt;</td>
<td>Ser</td>
<td>GGC</td>
<td>AGC</td>
</tr>
<tr>
<td>pRW17</td>
<td>His&lt;sup&gt;65&lt;/sup&gt;</td>
<td>Tyr</td>
<td>CAT</td>
<td>TAT</td>
</tr>
<tr>
<td>pRW20</td>
<td>Arg&lt;sup&gt;35&lt;/sup&gt;</td>
<td>Cys</td>
<td>CGT</td>
<td>GTG</td>
</tr>
</tbody>
</table>

<sup>a</sup>The results are the averages of three separate experiments each assayed in triplicate.

<sup>b</sup>Dominance determined by dividing the value obtained for the strain carrying the plasmid encoded mutant allele by same strain carrying the vector pACYC177.

<sup>c</sup>Not relevant, vector control.

<sup>d</sup>Number in parentheses indicates standard error.
FIG. 2. β-Galactosidase activities in transformants of LS1155 (fadR fadB-lacZ). R− indicates values obtained for cells transformed with the vector pACYC177; R+ indicates values obtained for cells transformed with pCD126; amino acid changes are as indicated using the standard one-letter amino acid designation. Values are the average of three experiments each assayed in triplicate. Error bars indicate the S.E. of the experimental mean.

TABLE II

<table>
<thead>
<tr>
<th>Plasmid encoded FadR allele</th>
<th>β-Galactosidase activitya</th>
<th>Negative dominanceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>190 (17)b</td>
<td>1.0</td>
</tr>
<tr>
<td>Wild type</td>
<td>135 (12)</td>
<td>0.7</td>
</tr>
<tr>
<td>E34A</td>
<td>327 (21)</td>
<td>1.7</td>
</tr>
<tr>
<td>R35A</td>
<td>962 (14)</td>
<td>5.1</td>
</tr>
<tr>
<td>L37A</td>
<td>135 (37)</td>
<td>0.7</td>
</tr>
<tr>
<td>V43A</td>
<td>238 (28)</td>
<td>1.2</td>
</tr>
<tr>
<td>R45A</td>
<td>270 (51)</td>
<td>1.4</td>
</tr>
<tr>
<td>L45A</td>
<td>395 (49)</td>
<td>2.1</td>
</tr>
<tr>
<td>R49A</td>
<td>575 (46)</td>
<td>3.0</td>
</tr>
<tr>
<td>R54A</td>
<td>300 (45)</td>
<td>1.6</td>
</tr>
<tr>
<td>W60A</td>
<td>312 (20)</td>
<td>1.6</td>
</tr>
<tr>
<td>I63A</td>
<td>273 (18)</td>
<td>1.4</td>
</tr>
<tr>
<td>H65A</td>
<td>589 (86)</td>
<td>3.1</td>
</tr>
<tr>
<td>G66A</td>
<td>106 (55)</td>
<td>2.7</td>
</tr>
<tr>
<td>K67A</td>
<td>524 (37)</td>
<td>2.8</td>
</tr>
<tr>
<td>T69A</td>
<td>256 (33)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a None indicates values obtained for transformants carrying the vector pACYC177. Wild type indicates transformants carrying pCD126. All other plasmids were pACYC177 derivatives carrying the designated amino acid substitution in fadR.

b The results are the averages of three separate experiments each assayed in triplicate. Within each experiment a single colony after transformation of LS1155 was used as an inoculum to 10 ml of Tryptone broth.

c Dominance determined by dividing the value obtained for transformants of plasmids encoding the relevant fadR allele by that obtained for cells transformed with the vector pACYC177 (None, above).

d Number in parentheses indicates standard error.

SDS-polyacrylamide gel of extracts of the cells after FadR induction. There was no discernible difference in the amount of protein produced in the mutants compared with the wild type. To verify that the major protein band was FadR, antibodies generated against purified FadR were used to probe an immunoblot of a duplicate gel (Fig. 3B). The specificity of the antibody preparation was demonstrated by reacting preimmune antisera or immune antisera against extracts from strain BL21(DE3) carrying pT7-5 (vector) or pCD129 (fadR+) (data not shown).

The concentration of FadR in the crude extracts was estimated to be to be 3 × 10⁻⁶ M by comparing the FadR band in the crude extract with a standard concentration of purified FadR on a Coomassie Blue-stained polyacrylamide gel (protein concentration was compared as band intensity using a Millipore BioImage analysis system). Each of the extracts was used to test binding to fadB DNA using the gel shift assay and a DNA fragment carrying O_r (2). A FadR-specific shift was observed for the crude extract enriched in wild-type FadR over a dilution range of 0.05–0.0005-fold (data not shown). This was estimated to be 2 × 10⁻⁷ to 6 × 10⁻¹³ M FadR. The apparent K_acc for wild-type FadR in extracts of BL21(DE3) carrying pCD129 was 3 × 10⁻⁶ M or essentially that which was determined for the purified protein (2). Fig. 4 is a representative gel shift experiment using extracts of cells harboring the vector pT7-5, pCD129 (wild type), or pCD20 (R35C). Under the same conditions we were not able to detect FadR-specific DNA binding activity to O_B for the fadR⁻ mutants A9V, R35C, H65Y, G66D, or G66S (data not shown).

LexA-FadR Chimeras Distinguish Functional Domains—
The bacterial repressor protein LexA binds to a site within the sula promoter to repress transcription. Binding to DNA and subsequent repression of sula is dependent upon homodimerization of the LexA protein (10). Chimeras between the LexA DNA-binding domain (amino acids 1–87) and segments of proteins such as GAL4, Jun, glucocorticoid hormone receptor, and AraC have been used successfully to identify and analyze functional heterologous dimerization domains (29–33). For the present study, amino acids 1–87 of LexA were fused in frame to FadR. None of the LexA-FadR chimeras were able to repress fadB-lacZ expression, since they were missing the predicted DNA binding domain of FadR (data not shown). In contrast, two chimeras (LexA1–87/FadR83–239 and LexA1–87/FadR102–239) were able to repress the LexA-responsive sulA-lacZ reporter (Table III). The chimera LexA1–87/FadR83–239 repressed at levels comparable to wild-type LexA, while LexA1–87/FadR102–239 had an intermediate effect. Three additional LexA-FadR chimeras which included amino acid residues 122–239, 142–239, and 167–239 of FadR did not repress sulA-lacZ, suggesting that...
either determinants critical for dimerization were missing or that the protein structure had been altered so that it was not capable of binding DNA.

Previous studies found that amino acids in the carboxyl-terminal region of FadR were specifically required for acyl-CoA binding (6). Therefore we tested whether LexA1–87FadR83–239 was capable of binding DNA containing OB.

Repression of sulA-lacZ by LexA-FadR chimeras in the presence and absence of fatty acid

Table III

<table>
<thead>
<tr>
<th>Plasmid encoded LexA-FadR Chimeras</th>
<th>β-Galactosidase Activityb</th>
<th>TBc</th>
<th>TBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA</td>
<td>202</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>FadR</td>
<td>239</td>
<td>4100(100)</td>
<td>NDd</td>
</tr>
<tr>
<td>1 LexA</td>
<td>87</td>
<td>31</td>
<td>25.5</td>
</tr>
<tr>
<td>1 FadR</td>
<td>239</td>
<td>343 (85)</td>
<td>1331 (301)</td>
</tr>
<tr>
<td>1 FadR</td>
<td>103</td>
<td>2486 (373)</td>
<td>ND</td>
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<tr>
<td>1 FadR</td>
<td>122</td>
<td>1946 (486)</td>
<td>ND</td>
</tr>
<tr>
<td>1 FadR</td>
<td>142</td>
<td>1809 (463)</td>
<td>ND</td>
</tr>
<tr>
<td>1 FadR</td>
<td>167</td>
<td>3125 (500)</td>
<td>3258 (328)</td>
</tr>
<tr>
<td>Vector</td>
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</table>

a Filled rectangles represent LexA and hatched rectangles represent FadR. The numbers above the filled rectangles indicate amino acid residues derived from LexA and the numbers below the hatched rectangles indicate amino acid residues from FadR. Transformants of JL1436 (lexA sulA-lacZ) carrying the plasmid encoded the chimeras were assayed for β-galactosidase activity as described under “Experimental Procedures.”

b The values given are the averages of three experiments each assayed in triplicate. (SE) indicates standard error of the mean between experiments.

c TB (Tryptone broth) indicates repressing media and TBO (Tryptone broth containing 5 mM oleate) derepressing media.

d ND, not determined.

DISCUSSION

Most prokaryotic transcriptional regulators which bind DNA through a helix-turn-helix motif do so as homodimers (e.g. LacI and Cro) or homotetramers (e.g. TrpR) (9). The proteins may exist as stable multimers in solution at physiological concentrations. Alternatively, dimerization may be limiting for DNA binding or may occur on the DNA (10, 11). Previous work on purified FadR suggested that the protein was monomeric (2). To understand the function and mechanism of FadR-dependent gene regulation, it was important to investigate the possibility that dimerization was limiting for DNA binding. The results of native gel electrophoresis and glycerol gradient ultracentrifugation studies presented here clearly demonstrate that FadR is a stable dimer in solution. These data support the conclusion that dimerization is therefore not a limiting parameter for DNA binding. Our previous results suggesting FadR was a monomer in solution was based upon the behavior of this protein a gel filtration column. We suspect that the contradictory data result from anomalies of FadR migration when compared with molecular weight standards. Alternatively, FadR may interact with the gel matrix resulting in an anomalous elution pattern and an incorrect molecular weight determination.

FadR regulates a large number of genes required for fatty acid biosynthesis and degradation. FadR DNA binding is inhibited by long chain fatty acyl-CoA thioesters (2, 6, 7). In previous work we used random and site-directed mutagenesis to identify mutations in fadR that led to decreased ligand binding (6). The fadR alleles which showed decreased affinities toward long chain fatty acyl-CoA contained mutations that were mapped to the carboxyl terminus to amino acid residues including Gly216, Glu218, Ser219, Thr223, and Lys228 (6). In an analogous manner, we used random and site-directed mutagenesis in the present studies to probe for mutations that affected DNA binding but not multimerization. FadR was predicted to bind DNA using an H-T-H motif formed by amino acid residues 34–53 (8). By comparison to the LacR family, amino acids in this region of FadR that fulfill the criterion to form this structure include: 1) Gly at position 42, which is critical to form the turn; 2) hydrophobic residues Leu9, Ile41, Val43, and Leu48; and 3) hydrophilic residues Glu74, Arg75, Gly76, Thr74, Arg45, Thr46, Thr47, Arg49, and Glu50, which might provide DNA contacts; and 4) the absence of proline, which would disrupt domain architecture. FadR shares amino acid identities in this region with the GntR family subclass of H-T-H proteins (27). To test for amino acid residues that disrupt DNA binding but retain the ability to multimerize, we used the standard dominant negative test (34, 35). In general, those residues which are expected to be intolerant to substitution by alanine would be those making direct contact with the DNA and those which are required for maintaining the architecture of the protein exclusive of the determinants for multimerization (34, 35). Residues that are not critical for contacting DNA or to maintain the structure of the protein would result in a wild-type phenotype upon substitution of the native amino acid with alanine. Our results show that amino acid residues critical for DNA binding include two residues in the putative H-T-H domain, Arg85 and Arg49, as well as Ala8, His65, Glu66, and Lys67. Yoshida et al. (36) reported the identification of four amino acid residues in Bacillus subtilis GntR that were required for maximal DNA binding and exhibited a dominant negative phenotype including Ser61 Ala66 Glu74 and Arg75. Two of these residues in GntR, Glu74 and Arg75, correspond exactly by position to His65 and Gly66 identified here for FadR based on the alignment of Haydon and Guest (27). Since mutations in both FadR and GntR were localized to homologous residues outside of the H-T-H motif, this extended DNA binding domain may consti-
tute a DNA-binding structural motif characteristic of this family of proteins. Residues Ile\(^{41}\) through Thr\(^{44}\) constitute the predicted turn of the H-T-H motif and therefore are expected to be critical to maintain the architecture of this portion of the DNA binding domain. This region of FadR proved to be relatively tolerant to substitution with alanine. Substitution of alanine for Leu\(^{37}\) and Leu\(^{48}\), in contrast, eliminated repression and were only weakly dominant negative. These residues, therefore, appear to be structurally important, and we further speculate that they may be necessary for maintaining the geometry of the two helices relative to one another as has been demonstrated to be the case for Ala\(^{37}\) and Val\(^{47}\) in the H-T-H region of ACI (9).

Based on the in vivo \(\beta\)-galactosidase activities in strains carrying the sulA-LacZ reporter fusions, it was concluded that two of five chimeras generated between the DNA binding domain of LexA and segments of FadR, LexA\(_{1–87}\)FadR\(_{83–239}\) and LexA\(_{1–87}\)FadR\(_{102–239}\), were functional and able to repress sulA-LacZ. Previous studies with FadR have demonstrated that amino acids 215–230 in the carboxyl-terminal region of FadR are specifically required for acyl-CoA binding (6). Therefore, we expected that the chimeras LexA\(_{1–87}\)FadR\(_{83–239}\) and LexA\(_{1–87}\)FadR\(_{102–239}\) would be inducible by long chain fatty acids (Table III). LexA\(_{1–87}\)FadR\(_{102–239}\) was inducible by long chain fatty acids indicating that it retains both the ability to dimerize and to bind acyl-CoA. However, LexA\(_{1–87}\)FadR\(_{83–239}\) behaved like a super-repressor. The phenotype of this chimera may have resulted from: 1) a decreased affinity for inducer molecule, 2) an increased affinity for operator, or 3) an impaired ability to undergo the appropriate allosteric change required for induction. Since the determinants for DNA binding are attributable to the LexA segment of the chimera, increased affinity for DNA is not likely to be the cause for non-inducibility. Additionally, LexA\(_{1–87}\)FadR\(_{83–239}\) is expected to retain the determinants for inducer binding present in LexA\(_{1–87}\)FadR\(_{102–239}\). We therefore favor the view that the chimera cannot undergo the allosteric transition that must take place upon ligand binding which results in either the inhibition of, or release of, the protein from DNA binding.

FadR is a global regulator of lipid metabolism in \textit{E. coli}, regulating more than 10 genes and operons. The ligands which modulate the activity of FadR are essential intermediates in fatty acid metabolism, long chain fatty acyl-CoAs. Therefore, the molecular mechanisms by which FadR controls gene expression and by which long chain fatty acyl-CoAs control FadR activity are of general interest. In the present work, we have determined that FadR functions as a homodimer and we have provided supportive evidence that the DNA binding domain lies in the amino terminus and that the determinants for dimerization lie within amino acid residues 102–122.

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REFERENCES