Fatty Acyl-CoA Binding Domain of the Transcription Factor FadR
CHARACTERIZATION BY DELETION, AFFINITY LABELING, AND ISOTHERMAL TITRATION CALORIMETRY

(Received for publication, June 11, 1998, and in revised form, September 10, 1998)

Concetta C. DiRusso, Vadim Tsvetnitsky‡, Peter Højrup, and Jens Knudsen‡‡

From the Department of Biochemistry and Molecular Biology, Albany Medical College, Albany, New York 12208 and the Institute of Biochemistry, Institute of Molecular Biology, Odense University, DK-5230 Odense M, Denmark

The Escherichia coli transcription factor FadR regulates genes required for fatty acid biosynthesis and degradation in an opposing manner. It is acting as an activator of biosynthetic genes and a repressor of degradative genes. The DNA binding of FadR to regions within the promoters of responsive genes and operons is inhibited by long chain acyl-CoA thioesters but not free fatty acids or coenzyme A. The acyl-CoA binding domain of FadR was localized by affinity labeling of the full-length protein and an amino-terminal deletion derivative, FadRΔ1–167, with a palmitoyl-CoA analogue, 9-p-azidophenoxy[9-3H]nonanoic acid-CoA ester. Analysis of labeled peptides generated by tryptic digestion of the affinity-labeled proteins identified one peptide common to both the full-length protein and the deletion derivative. The amino-terminal sequence of the labeled peptide was SLALGFTYHK, which corresponds to amino acids 187–195 in FadR. Isothermal titration calorimetry was used to estimate affinity of the wild-type full-length FadR, a His-tagged derivative, and FadRΔ1–167 for acyl-CoA. The binding was characterized by a large negative ΔH°, −16 to −20 kcal mol⁻¹. No binding was detected for the medium chain ligand C8-CoA. Full-length wild-type FadR and His₉-FadR bound oleoyl-CoA and myristoyl-CoA with similar affinities, Kₛ of 45 and 63 nM and 68 and 59 nM, respectively. The Kₛ for palmitoyl-CoA binding was about 5-fold higher despite the fact that palmitoyl-CoA is 50-fold more efficient in inhibiting FadR binding to DNA than myristoyl-CoA. The results indicate that both acyl-CoA chain length and the presence of double bonds in the acyl chain affect FadR ligand binding.

Long chain acyl-coenzyme A thioesters (LCA-CoA) are critical intermediates in cellular metabolism. These activated fatty acids are required for the biosynthesis of higher lipids, the acylation of proteins, and catabolism of fatty acids (1). Acyl-CoA plays a critical role in mitochondrial energy metabolism and in certain pathological responses related to disease. LCA-CoA are important regulatory ligands for a number of enzymes including, for example, acetyl-CoA carboxylase, citrate synthase, glucokinase, the mitochondrial adenine nucleotide translocase, and the uncoupling protein. LCA-CoA have also been implicated as effectors of vesicular transport and fusion. A number of studies have been directed at elucidating a role for acyl-CoA compounds in the regulation of the activity of transcription factors. However, there has been only indirect evidence that acyl-CoA compounds regulate gene activity of eucaryotes at the level of transcription (2, 3).

To date, the Escherichia coli FadR protein is the only transcription factor for which there is substantial and convincing evidence that direct binding of LCA-CoA to the protein prevents DNA binding, transcription activation, and repression (4–8). FadR is a 239-amino acid protein that regulates the transcription of many unlinked genes and operons encoding proteins required for fatty acid synthesis and degradation. Among the genes directly regulated by FadR are those encoding a specific membrane-associated fatty acid transport protein (FadL), acyl-CoA synthetase, all of the enzymes required for the β-oxidation of fatty acids, two enzymes essential for unsaturated fatty acid biosynthesis, and the repressor of the genes encoding the glyoxylate bypass genes, IclR.

The effect of FadR on the level of transcription is caused by its direct binding to DNA in the promoter regions of FadR-responsive genes (5, 6). This binding in vitro is specifically prevented by long chain fatty acyl-CoA esters and not medium chain acyl-CoA esters or fatty acids (5). The protein binds DNA as a dimer (9). Our interests lie in understanding the mechanism by which acyl-CoA controls FadR activity. Previous genetic and biochemical analyses have identified amino acid residues in the carboxyl terminus of FadR that are likely to constitute in part the acyl-CoA (CoA presumably) ligand-binding pocket (8, 9). In the present work, we have further localized the acyl-CoA binding domain by deletion and affinity labeling. Additionally, we have used isothermal titration microcalorimetry to assess acyl-CoA binding to the full-length protein, a His-tagged derivative and an amino-terminal deletion protein. Together these studies contribute to our understanding of FadR-DNA and FadR-acyl-CoA interactions and help to further define the region of FadR involved in forming the acyl-CoA binding pocket.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strains LS1155, fadB lacZ, and LS1348, fadB fabA-lacZ, were used to assess FadR function in vivo as described previously (8). BL21(DE3)/pLysS was used as host for overexpression of FadR proteins (10).

Plasmid pCD129 was used for the production of full-length wild-type FadR as previously detailed (5). Plasmid pCD307-6 encoding His₉-FadR was constructed as follows. An NdeI site was generated at the initiating methionine codon of the wild-type FadR gene by site directed mutagenesis of pCD152 to generate pCD306-6 using the Altered Sites System of Promega as described previously (8). An NdeI–BamHI fragment from pCD306-6 containing the complete coding sequence of FadR was cloned into the T7 RNA polymerase-responsive expression plasmid pET15b (Novagen) such that the coding sequence of FadR was fused in frame at the amino terminus to the His tag in the vector to generate pCD307-6 encoding His₉-FadR. Plasmid pCD307-3 encoding FadRΔ1–167 was

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Odense University, Institute of Biochemistry, Campusvej 55, DK-5230 Odense M, Denmark. Tel.: 45-6557-2454; Fax: 45-6557-2467; E-mail: jkk@biochem.ou.dk; WWW: http://www.ou.dk.

‡‡ Complementing functions of FadR and His₉-FadR bound oleoyl-CoA and myristoyl-CoA with similar affinities, Kₛ of 45 and 63 nM and 68 and 59 nM, respectively. The Kₛ for palmitoyl-CoA binding was about 5-fold higher despite the fact that palmitoyl-CoA is 50-fold more efficient in inhibiting FadR binding to DNA than myristoyl-CoA. The results indicate that both acyl-CoA chain length and the presence of double bonds in the acyl chain affect FadR ligand binding.

* The abbreviations used are: LCA-CoA, long chain acyl-coenzyme A thioesters; HPLC, high pressure liquid chromatography; ES-MS, electrospray-mass spectrometry; [9-3H]APNA-CoA, 9-p-azidophenoxy[9-3H]monanoyl-CoA; ITC, isothermal titration calorimetry.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
constructed in a similar manner, but in this case an NcoI site was constructed in pCD152 at Met168 in wild-type FadR to generate pCD306–3. The NcoI–BamHI fragment encoding amino acid residues 168–239 was subcloned from pCD306–3 into the expression plasmid pET3a to generate pCD307–3. All constructions were verified by restriction analysis in primers and DNA sequencing using primers T7- and FadR-specific oligonucleotides as primers.

**Protein Overexpression and Purification—**Wild type FadR, its truncated mutant FadRΔ1–167, and full-length soluble His6-FadR was overexpressed in *E. coli* BL21 (DE3)/pLyS S harboring the appropriate plasmid using T7 polymerase expression system. Cultures (4 liters) were grown in an Amersham Pharmacia Biotech automa tic pH control at 37 °C in the previously described medium (11) containing ampicillin (100 μg/ml) and chloramphenicol (15 μg/ml) until A600 was about 4. T7 RNA polymerase was induced by the addition of isopropyl thio-β-D-galactopyranoside to 0.4 mM. Growth continued for 3–4 h, and cells were harvested by centrifugation.

For isolation of full-length, native FadR encoded within pCD129, bacterial cells were disrupted in a French press in 300 ml of 20 mM Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA (TDE buffer), and the homogenate was centrifuged at 70,000 × g for 20 min. The FadR protein was subsequently recovered from inclusion bodies as follows. The pellet was dissolved in the same buffer containing 6M urea, and then was removed by dialysis against TDE buffer containing 20% (v/v) glycerol. Protein was concentrated by ultrafiltration using a Centriprep 10 concentrator and stored at −80 °C.

**Functional Characterization of His6-FadR—**The binding of purified His6-FadR to the fadB promoter in vitro was tested using protein-DNA gel shift assays. A 159-base pair fragment containing the fadB promoter was amplified by thermocycling using the oligonucleotides BFW (5′-GTTTCTCTGCAGGCTTGTCT-3′) and BRE (5′-AGTCACGTTACAGGTTGTCAG-3′) as primers and DNA sequencing using primers T7- and FadR-specific oligonucleotides as primers.

**Synthesis of 9-p-azidoephenoxy[9-3H]nonanoic Acid (9-HAPNA-CoA)—**Oleic acid (4 mmol) was dissolved in 40 ml of dioxane, and then 4 ml of 1 N NaOH and 3.2 ml of 2% OsO4 were added. To this mixture, NaIO4 (8 g) was added slowly over 5–10 min with stirring, which was continued for 18 h. The solution was then removed by evaporation and the residue dissolved in water, made alkaline with 2 M Na2CO3, and extracted three times with petroleum spirits (40–60 °C). The aqueous phase was acidified with 2 M HCl and extracted three times with petroleum spirits (40–60 °C)/diethyl ether (1.1) and purified on a silica gel column. The yield was 121 mg (21%). The 9-oxononanoic acid was dissolved in 8 ml of absolute ethanol titrated to pH 12 with 2 M NaOH and reduced with NaBH4 in two steps. NaBH4 (1.9 g) was dissolved in 1 ml of absolute ethanol and mixed with 750 μCi of [3H]NaBH4 (specific activity 20–40 Ci/mmol, Amersham, UK) dissolved in 1 ml of ethanol. This solution was added slowly over 30 min to the 9-oxononanoic acid solution under continuous stirring. The stirring was continued for 90 min, an additional 5.9 mg of NaN3H2 was added, and stirring continued for another 3 h. The solution was removed with a stream of nitrogen; 10 ml of water was added; the solution was acidified with HCl; and the 9-hydroxy[9-3H]nonanoic acid was extracted with diethyl ether. The ether phase was dried over MgSO4, and then was removed by distillation, and the 9-hydroxy[9-3H]nonanoic acid methyl ester was obtained. The following synthetic steps were carried out under dim light. 9-Azidophenol (111 mg) was dissolved in 1 ml of ethanol, 44 mg of sodium methoxide was added, and then the solvent was evaporated in a vacuum centrifuge overnight. The dry residue was dissolved in 1.5 ml of dry dichloromethane and dried with 1 M HCl, and the 9-azidophenol was methylated with an excess of diazomethane and the methyl ester was purified by thin layer chromatography. The yield was 132 mg of 9-azidophenol[9-3H]nonanoic acid methyl ester. All the following synthetic steps were carried out under dim light. 9-Azidophenol (111 mg) was dissolved in 1 ml of ethanol, 44 mg of sodium methoxide was added, and then the solvent was evaporated in a vacuum centrifuge overnight. The dry residue was dissolved in 1.5 ml of dry dichloromethane, and the solution was evaporated with nitrogen. The dry residue was dissolved in 7 ml of NaOH, and the methyl ester was extracted with diethyl ether. The yield was 77 mg. The 9-p-azidoephenoxy[9-3H]nonanoic acid methyl ester was dissolved in 3 ml of ethanol and hydrolyzed with 1 ml of 1 M HCl for 6 h at room temperature, 2 ml of water was added, and the free acid was extracted with diethyl ether. The ether phase was dried with MgSO4, and the solvent was evaporated with nitrogen, and the product was redissolved in petroleum spirits. Purity was evaluated by thin layer chromatography on a silica gel column developed with 1 M HCl, the product was purified and concentrated on a silica gel column. The yield was 132 mg of 9-azidoephenoxy[9-3H]nonanoic acid methyl ester. The reaction was allowed to run for 5 h at room temperature with stirring. The product 9-p-azidoephenoxy[9-3H]nonanoic acid methyl ester was purified on a silica gel column. The fractions containing the product were pooled, and the solvent was evaporated with nitrogen. The dry residue was dissolved in 7 ml of NaOH, and the methyl ester was extracted with diethyl ether. The yield was 77 mg. The 9-p-azidoephenoxy[9-3H]nonanoic acid methyl ester was dissolved in 3 ml of ethanol and hydrolyzed with 1 ml of 1 M HCl for 6 h at room temperature, 2 ml of water was added, and the free acid was extracted with diethyl ether. The ether phase was dried with MgSO4, and the solvent was evaporated with nitrogen, and the product was redissolved in petroleum spirits.
FadR Bind Long Chain Acyl-CoA Esters with High Affinity

was incubated for 3–5 min on ice with [9-3H]APNA-CoA (873 Ci/M) at a molar ratio of 1:1.25 in a final volume of 100 μl in TBE buffer (pH 8.0) followed by UV illumination (300 nm) for 30 s. For competition experiments, a 5-fold excess of palmitoyl-CoA over photoaffinity ligand was included in the reaction. Ten microliters of the reaction mixture were subjected to acrylamide gel electrophoresis. The gel was stained with Cooomassie Blue, soaked in Amplitag reagent, dried, and fluorographed.

For peptide mapping, radioabeled protein was HPLC-purified from nonreacted [9-3H]APNA-CoA on a Dynasphere column (8 × 60 mm) with a 2-propanol linear gradient. This column material was found to provide superior recovery of both protein and very hydrophobic peptides. Label incorporation efficiency was estimated to be 15%. Photoaffinity-labeled protein was dissolved in 50 mM Tris (pH 7.4), 6 mM urea, and digested with trypsin (2%, w/v) after dilution to bring urea concentration to ~0.5 M. Peptides were resolved on a Dynasphere column pre-equilibrated with 0.1% trifluoroacetic acid and developed with a linear gradient of acetonitrile containing 0.08% trifluoroacetic acid (0–70% in 40 min). Poorly resolved peaks containing most of the radioactivity were rechromatographed on the same column using a shallower gradient, and individual peptides were collected and sequenced.

\( \text{ITC—Calorimetric measurements were carried out using an OMEGA titration microcalorimeter from MicroCal, Inc. (Northampton, MA). This instrument has been described in detail by Wiseman et al. (14). A 10-μl protein volume was filled with water containing 0.2% sodium azide. The calorimeter was electrically calibrated at each temperature. All solutions used for the experiments were thoroughly degassed by stirring under vacuum. If necessary, protein solutions were spun for several minutes in a bench top centrifuge to remove any visible particies. The concentration of the protein was estimated spectrophotometrically at 280 nm using } \epsilon = 33806 \text{ M}^{-1} \text{ cm}^{-1} \text{ for FadR and His}_{6}\text{-FadR and 12210 M}^{-1} \text{ cm}^{-1} \text{ for FadR1–167 and was approximately 0.03% protein. Protein under study in appropriate buffer was placed in the sample cell, and a ligand (dissolved in the same buffer as the protein) was driven into the injection syringe, which was then mounted into a stepper motor for delivery into the sample cell. The syringe with stirrer paddle was rotated at 400 rpm during the experiment to assure immediate mixing. Experiments were performed at a temperature of 27 or 31 °C. The concentration of the ligand, about 0.5 mM, was chosen to ensure full saturation well before final injection. Appropriate blank runs were conducted and subtracted from the corresponding data. The peaks of the thermograms obtained in this manner were integrated using the ORIGIN software supplied with the instrument. A nonlinear regression fitting to the isotherm was done using the CALREG (version 3.9) program (15). The fitting procedure yields the binding constant of the ligand } K_a \text{, the heat of binding } H \text{, and the concentration of the binding sites (stoichiometry) } N. \)

Mass Spectroscopy—Electrospray mass spectra of the proteins were recorded on a Vestec instrument (Vestec Corp., Houston, TX). Buffers and salts were removed from the purified proteins by HPLC. The sample in aqueous buffer was loaded onto a 60 × 8 mm D纳斯phere column then stepwise eluted with a mixture of 90% acetonitrile, 10% water, and 0.08% trifluoroacetic acid. The sample was dried and brought to a final concentration of ~20 pmol/μl with methanol/water (1:1, v/v) containing 1% acetic acid. The sample was introduced into the mass spectrometer by infusion with a syringe pump with a flow rate of 0.3 μl/min. Spectra were acquired in the positive ion mode at 10 s/scan and mass window of m/z 600–1500 using Teknivent Vector 2 data system. The molecular mass of the protein was calculated by weighted averaging as described by Mann et al. (16). The spectrometer was independently calibrated using myoglobin.

Determination of Amino Acid Sequences—Purified proteins and radioabeled peptides were sequenced on a Knauer 910 pulsed liquid sequencer with chemicals and program as recommended by the manufacturer. Samples of 20 μl of the amino acid phenylthiohydantoin derivatives were used for amino acid identification on a Knauer on-line HPLC 64 using a 250 × 4.6 mm Lichrosphere 100 C-18 (5-mm particle size) column and a gradient of acetonitrile in 50 mM sodium acetate buffer, pH 5.2, as described by the manufacturer.

Materials—All protein purification was done on an FPLC system (Amersham Pharmacia Biotech) with columns supplied by the manufacturer. Samples of 20 μl of the amino acid phenylthiohydantoin derivatives were used for amino acid identification on a Knauer on-line HPLC 64 using a 250 × 4.6 mm Lichrosphere 100 C-18 (5-mm particle size) column and a gradient of acetonitrile in 50 mM sodium acetate buffer, pH 5.2, as described by the manufacturer.

\( \text{RESULTS} \)

Affinity Labeling of FadR and FadR1–167—In previous work, we identified by random and site-directed mutagenesis a region in FadR including amino acids 216–228 of the 239-amino acid protein that was specifically required for acyl-CoA binding (8). This led us to hypothesize that the acyl-CoA binding domain was located toward the carboxyl terminus of the protein and that the acyl-CoA binding domain might be structurally and functionally separable from the amino-terminal DNA binding domain of FadR (9, 18). Therefore, to further localize and analyze the acyl-CoA binding domain, we have constructed pCD307–3, which overexpresses a protein made up of amino acids 168–239. Amino acid 168 was chosen, since it is an internal methionine that would make a convenient site for translational initiation (18). The protein encoded within pCD307–3, called FadRΔ1–167, has been purified to apparent homogeneity as evidenced by SDS-polyacylamide gel electrophoresis and ES-MS (data not shown). Dimersization of a portion of the purified FadRΔ1–167 protein was observed when the protein was analyzed by ES-MS, and dimerization could be prevented by dithiothreitol treatment. Partial N-acetylation was also suggested by ES-MS. Both full-length FadR and FadRΔ1–167 were photoaffinity labeled under identical conditions with [9-3H]APNA-CoA. [9-3H]APNA-CoA was previously shown to mimic palmitoyl-CoA in binding to acyl-CoA-binding protein, and the labeled peptides identified in that study were later shown by NMR to indeed be involved in acyl-CoA binding (19). In the present work, we are able to show that [9-3H]APNA-CoA can photoaffinity-label FadR, and this cross-linking was prevented by an excess of palmitoyl-CoA (Fig. 1). No labeling of the protein was detected when it was incubated with [9-3H]APNA-CoA without subsequent illumination. Similar labeling experiments in the presence of delipidated bovine serum albumin (which binds acyl-CoA with a } K_a \text{ of ~0.5 μM}) showed that FadR could be labeled in the presence of a 2-fold excess of bovine serum albumin and therefore that FadR competes effectively with bovine serum albumin for the [9-3H]APNA-CoA (data not shown).
The affinity label is at the underlined containing radioactive label were sequenced. Peptide sequences identified in radioactivity-containing peaks are in FadR primary sequence. Methionine 168 is identified by an transcription in response to long chain fatty acids

in vivo by mass spectrometry and amino acid sequencing. Determined to be identical to T19 peptide from full-length FadR (also called superrepressors) are in boldface type (9, 22).

To obviate the problems associated with full-length native FadR and to facilitate purification of large quantities of protein, we constructed an amino-terminal His-tagged derivative of the full-length protein called His$_\text{S}_{\text{FadR}}$. The His$_\text{S}_{\text{FadR}}$-FadR construct encoded in pCD307-6 was determined to be functional by comparison with native FadR in vivo with regard to (i) repression of the fadB gene by assaying $\beta$-galactosidase activities of a fadR fadB-lacZ strain, LS1155, and (ii) activation of the fabA gene by assaying $\beta$-galactosidase activities of a fadR fabA-lacZ strain, LS1348 (Table I). When T7RNA polymerase encoded within pG1-2 was coexpressed with pCD307-6 encoding His$_\text{S}_{\text{FadR}}$, $\beta$-galactosidase activities of LS1155 were very low and comparable with those seen for cells carrying pCD129 encoding wild type FadR. In contrast, LS1155 cells carrying pG1-2 alone or together with the His tag expression vector pET15b had high levels of $\beta$-galactosidase, indicating that expression of fabB-lacZ was constitutive. Like the native protein, the His$_\text{S}_{\text{FadR}}$-FadR also was inactivated by inclusion of oleate in the growth media resulting in elevated $\beta$-galactosidase activities. When $\beta$-galactosidase activities were evaluated in LS1348 transformed with the same set of plasmids, the opposite pattern was observed. $\beta$-Galactosidase activities in cells transformed with pG1-2 and pCD307-6 were higher than the activities observed for cells carrying pG1-2 alone. This indicated that His$_\text{S}_{\text{FadR}}$ was functional with regards to activation of expression of the fabA promoter. FadR-DNA binding in vitro of His$_\text{S}_{\text{FadR}}$ was assessed using electrophoretic mobility shift assays and was equivalent to purified native FadR as previously reported (Fig. 5) (5). Therefore, we concluded that His$_\text{S}_{\text{FadR}}$ was equivalent to native FadR in its ability to bind to DNA to repress or activate target promoters.

Affinity of Full-length and FadR$_\Delta 1-167$ for Acyl-CoA Compounds Estimated by Isothermal Titration Microcalorimetry—We compared the binding affinities of full-length native FadR, His$_\text{S}_{\text{FadR}}$, and FadR$_\Delta 1-167$ for acyl-CoA using isothermal titration microcalorimetry, which allows simultaneous measurement of $K_d$, enthalpy, and free energy of any heat-evolving reaction and the stoichiometry of binding (Fig. 6 and Tables II and III). The limited availability of the amount of native, full-length FadR precluded detailed microcalorimetry studies of its binding to a large number of different acyl-CoA esters. The quantity of native FadR was sufficient to perform a limited number of ITC binding studies (Table II). These experiments indicated that FadR has high affinity for myristoyl-CoA and oleoyl-CoA. The binding affinity of S-ether palmitoyl-CoA was not significantly different from that of natural palmitoyl-CoA ligand estimated for His$_\text{S}_{\text{FadR}}$ or
FadRΔ1–167. For each of the three FadR proteins studied, the stoichiometry of ligand binding for palmitoyl-CoA and oleoyl-CoA was determined to be approximately one acyl-CoA per FadR monomer.

The availability of large quantities of the His$_6$-tagged FadR and FadRΔ1–167 made determination of binding and thermodynamic parameters to acyl-CoA esters by ITC possible (Tables II and III). The results of acyl-CoA ester binding to either of these FadR derivatives showed clear preference toward long chain length compounds (C14- to C18-CoA) and no binding to the medium chain ligand (C8-CoA) as expected (i) from studies examining the chain length dependence of fad gene induction (20), (ii) from in vitro studies using protein-DNA electrophoretic mobility shift assays (5), and (iii) fluorescence analysis of FadR-acyl-CoA binding (8).

FadRΔ1–167 had about a 4-fold reduced affinity for myristoyl-CoA and oleoyl-CoA by comparison with the full-length protein (Table II). Estimates of FadRΔ1–167-palmitoyl-CoA binding were approximately equivalent to His$_6$-FadR. However, the apparent affinity of FadRΔ1–167 followed the same pattern as the full-length native FadR and His$_6$-FadR: no binding of C8-CoA and high affinity for long chain acyl-CoA. Importantly, all long chain ligands studied bound with high affinity to both His$_6$-fusion and truncated FadR, supporting the view that the acyl-CoA binding site of this protein is largely localized in its C-terminal part.

**DISCUSSION**

The Acyl-CoA Binding Domain of FadR—Two lines of evidence led us to suggest that FadR has a separable acyl-CoA binding domain located in the C-terminal portion of the protein. In the first set of experiments, we selected and characterized noninducible mutations in the FadR gene, which were required for binding of long chain acyl-CoA esters (8). Each of these mutations was localized to a single amino acid change. One altered protein carrying the change Ser$^{219}$ to Asn (FadRS219N) was purified and shown by DNA gel retardation assay to have a reduced affinity for oleoyl-CoA. FadRS219N retained the ability to bind DNA and to repress or activate transcription in the presence of oleoyl-CoA. Alanine substitution of amino acid residues 215–230 identified Gly$^{216}$ and Trp$^{223}$ as also required specifically for induction. This region of FadR shares amino acid identities and similarities with the
Biochemistry

In vivo characterization of His<sub>6</sub>-FadR

| Plasmid<sup>a</sup> | Relevant plasmid-encoded proteins | Growth medium<sup>b</sup> | β-Galactosidase activities<sup>c</sup> |
|-------------------|----------------------------------|--------------------------|-----------------------------------|
| pCD129            | FadR                             | T7 RNA polymerase        | fadR fadB-lacZ: 74                 |
| pCD307-6          | His<sub>6</sub>-FadR              | T7 RNA polymerase        | fadR fabA-lacZ: 5150              |
| pGP1-2            | T7 RNA polymerase                 | TBO                      | fadR fadB-lacZ: 888                |
| pGP1-2/pET15b     | T7 RNA polymerase                 | TB                       | fadR fabA-lacZ: NDND              |
| pGP1-2/pCD307-6   | T7 RNA polymerase/His<sub>6</sub>-FadR | TBO                      | fadR fadB-lacZ: 1284              |
|                   |                                  | TB                       | fadR fabA-lacZ: 1136              |
|                   |                                  | TBO                      | fadR fadB-lacZ: 1326              |
|                   |                                  | TB                       | fadR fabA-lacZ: NDND              |
|                   |                                  | TBO                      | fadR fadB-lacZ: 53                |
|                   |                                  | TB                       | fadR fabA-lacZ: 4972              |
|                   |                                  | TBO                      | fadR fadB-lacZ: 710               |
|                   |                                  | TB                       | fadR fabA-lacZ: NDND              |

<sup>a</sup> The host strains for these experiments were LS1155 or LS1348 transformed with the plasmids indicated. These included pGP1–2, encoding T7 RNA polymerase; pCD129, which encodes wild-type FadR under the control of the native FadR promoter; and pET15b, which is the vector used in the construction of pCD307–6. The last encodes His<sub>6</sub>-FadR under the control of the T7 RNA polymerase-responsive promoter.

<sup>b</sup> The growth medium was tryptone broth (TB) or tryptone broth containing 1 mM oleate (TBO).

<sup>c</sup> β-Galactosidase activities are given in nmol min<sup>-1</sup> mg<sup>-1</sup> protein. Each value is the average of triplicate samples from one representative experiment.

<sup>d</sup> ND, not determined.

![DNA-protein gel retardation assay to estimate His<sub>6</sub>-FadR binding to OB](#)

**Fig. 5.** DNA-protein gel retardation assay to estimate His<sub>6</sub>-FadR binding to OB. Reaction mixture contained 1 × 10<sup>-12</sup> M 32P-labeled DNA and His<sub>6</sub>-FadR at the concentrations indicated.

![ITC thermoprofile of His<sub>6</sub>-FadR titrated with palmitoyl-CoA](#)

**Fig. 6.** ITC thermoprofile of His<sub>6</sub>-FadR titrated with palmitoyl-CoA. Top, the raw heat signal; bottom, the integrated total heat.

---

FadR binds long chain acyl-CoA esters with high affinity

The host strains for these experiments were LS1155 or LS1348 transformed with the plasmids indicated. These included pGP1–2, encoding T7 RNA polymerase; pCD129, which encodes wild-type FadR under the control of the native FadR promoter; and pET15b, which is the vector used in the construction of pCD307–6. The last encodes His<sub>6</sub>-FadR under the control of the T7 RNA polymerase-responsive promoter.

The growth medium was tryptone broth (TB) or tryptone broth containing 1 mM oleate (TBO).

β-Galactosidase activities are given in nmol min<sup>-1</sup> mg<sup>-1</sup> protein. Each value is the average of triplicate samples from one representative experiment.

ND, not determined.

---

LexA<sub>1–87</sub>-FadR<sub>102–239</sub> retained the LexA DNA binding activity and was inducible by long chain fatty acids, indicating that the fusion contained the long chain acyl-CoA binding domain of FadR.

In an effort to further delineate the acyl-CoA binding domain to facilitate structural analyses, we constructed a deletion derivative of FadR, FadRΔ1–167, which removes the amino-terminal DNA binding domain but retains the ability to specifically bind long chain acyl-CoA (9, 18). In the present study, we have demonstrated that purified FadRΔ1–167 has similar specificity for acyl-CoA as full-length native or His<sub>6</sub>-tagged FadR and can be affinity-labeled with [9-<sup>3</sup>H]APNA-CoA. These data support our hypothesis that the determinants for acyl-CoA binding reside in the C terminus of the protein included within amino acids 168–239.

Amino Acid Residues 187–195 Are Likely to Be in Contact with the Acyl-Chain of the Ligand—Both full-length FadR and FadRΔ1–167 could be photo-cross-linked to [9-<sup>3</sup>H]APNA-CoA. The labeling reaction was blocked by an excess of palmitoyl-CoA (C16:0-CoA), indicating that FadR binds photoreactive ligand in a specific manner (Fig. 1). Proteolytic digestion of the radiolabeled full-length native protein followed by HPLC separation of the resulting peptides yielded a poorly resolved peak containing most of the eluted radioactivity (Fig. 3A). Rechromatography (Fig. 3B) of the pooled fractions from the major radioactivity peak in Fig. 3A identified three peptides, which were T19 (amino acids 187–195), T3 (amino acids 25–35), and T4 (amino acids 36–45). Each of these peptides could conceivably be involved in formation of the fatty acid binding part of the ligand-binding pocket. However, we suggest that only T19 was specifically labeled for two reasons. First, when the deletion derivative FadRΔ1–167 was photolabeled, only one peptide identical to T19 was isolated from tryptic digestion of the cross-linked protein mutant (Fig. 4B). Second, in recent work, we have demonstrated that fusions between the DNA binding domain of the bacterial repressor LexA and amino acid resi-
dues 102–239 of FadR result in a protein that has the DNA binding specificity of LexA but is derepressed by long chain acyl-CoA, a function associated only with FadR (9). Rechromatography (Fig. 3C) of the second pool of fractions from Fig. 3A identified peptides T8 and T23. T8 includes amino acid residues within the DNA binding domain of FadR, and T23 consists of the C-terminal sequence of FadR. These peptides were labeled to a lesser extent than T19, and their contribution to acyl-CoA binding is unclear. We suggest that the T8 peptide may have been a coeluting contaminant in the poorly resolved peak after HPLC. The T23 peptide, in contrast, was retarded in the chromatogram from its theoretical calculated elution time and is therefore likely to be the labeled peptide in this mixture. These data suggest that in the native protein structure, this C-terminal peptide may be adjacent to the acyl-CoA binding pocket. Future detailed structural analysis will determine whether or not amino acids within T8 and T23 peptides actually contact acyl-CoA.

One additional line of evidence that also suggests the acyl-CoA-binding domain that is localized in the C-terminal part of FadR has been obtained by treatment of the protein with vinylpiridine, which blocks free cysteine SH groups. Binding of palmitoyl-CoA to vinylpiridine-treated FadR1–167, which contains single a cysteine residue corresponding to amino acid 200 in FadR (Cys200) was abolished, as measured by ITC. One plausible explanation of this phenomenon is that either the single free thiol group is important for the ligand binding or simple steric hindrance around Cys200 prevented the ligand from interacting with the protein. Using site-directed mutagenesis techniques, Cys200 was substituted with either alanine or aspartate with no apparent affect on FadR activity or inducibility by long chain acyl-CoA as evaluated in vivo using a fadB-lacZ reporter (22). Therefore, the free thiol in Cys200 does not appear to be making a critical contact with the ligand, and steric hindrance is the most probable cause of inhibition of palmitoyl-CoA binding by vinylpiridine.

**Acyl-CoA Binding Affinity and Specificity Determined by Isothermal Titration Microcalorimetry**—Titration microcalorimetry is a unique method for simultaneous determination of enthalpy of reaction, \( K_D \), and stoichiometry, from which free energy and entropy can then be calculated. The specific interactions that are involved in protein-ligand interactions include hydrogen bonding, electrostatic interactions, hydrophobic bonding, and proton ionization. Therefore, the computed parameters reflect a sum of those processes when more than one is contributing to the protein-ligand interaction. We observed in the experiments reported here that titration of either His\(_{167}\)-FadR protein or FadR1–167 with different acyl-CoA esters caused some precipitation, suggesting protein aggregation. Thus, calculated values should be taken as apparent that, despite all limitations, are nevertheless useful for comparing data obtained for related proteins.

Using ITC, we determined, as anticipated, that FadR, His\(_{167}\)-FadR, and FadR1–167 all exhibited high binding affinity for long chain fatty acyl-CoA esters, while no binding to medium chain ligand C8-CoA was detected (Tables II and III). FadR bound myristoyl-CoA and oleoyl-CoA with the same affinity as His\(_{167}\)-FadR, indicating that the two proteins have similar binding affinity and that His\(_{167}\)-FadR is suitable for binding studies. However, we obtained universally higher \( K_D \) values for the truncated protein FadR1–167 as compared with the full-length proteins (Table I),\(^7\) supporting the idea either that the native conformation of the truncated derivative differs somewhat from the full-length protein or that regions of the protein required for affinity are missing in the truncated protein. An exception from this universal observation is palmitoyl-CoA binding. The \( K_D \) values obtained for palmitoyl-CoA binding were generally 5–6-fold higher than the \( K_D \) values obtained from binding of myristoyl-CoA and oleoyl-CoA and 1.7-fold higher than binding of palmitoyl-CoA to FadR1–167. There are some striking differences in specificity noted by comparison with previous estimates of FadR-acyl-CoA interaction (5, 8). Specifically, the fact that C14-CoA binding to His\(_{167}\)-FadR is stronger than that of C16-CoA. This result was unexpected, since, when potencies of these two ligands to dissociate DNA-FadR complex are compared, palmitoyl-CoA is 50 times more effective than myristoyl-CoA (5). One explanation might be the fact that binding of the shorter acyl-CoA to FadR does not result in the allosteric transition required to prevent DNA binding. Alternatively, it is possible that affinity of FadR alone for any given acyl-CoA may differ from that of the FadR-DNA complex. The large increase in \( K_D \) from myristoyl-CoA to palmitoyl-CoA followed by a similar drop in \( K_D \) from palmitoyl-CoA to oleoyl-CoA indicates that the acyl chain length and/or double bonds may play a significant role in inducing conformational changes in FadR tertiary structure upon ligand binding. Our present model is that acyl-CoA binding prevents DNA binding; however, we cannot yet rule out the possibility that binding of acyl-CoA releases FadR from the DNA. The present ITC data may therefore hint that the latter mechanism is more probable. Future experiments will be required to clarify these interpretations.

### REFERENCES

1. Faergeman, N. J., and Knudsen, J. (1997) Biochem. J. **325**, 1–12
2. Choi, J. Y., Studek, J., Hwang, S. Y., and Martin, C. E. (1996) *J. Biol. Chem.* **271**, 3581–3589
3. Kamaryu, T., Parthasarathy, S., and Nama, S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 386–390
4. DiRusso, C. C. (1996) *in Frontiers in Bioactive Lipids* (Vanderhoek, J. Y., ed) pp. 15–22, Plenum Press, New York
5. DiRusso, C. C., Heimert, T. L., and Metzger, A. K. (1992) *J. Biol. Chem.* **267**, 8685–8691
6. DiRusso, C. C., Metzger, A. K., and Heimert, T. L. (1993) *Mol. Microbiol.* **7**, 311–322
7. Henry, M. F., and Cronan, J. E., Jr. (1992) *Cell* **70**, 671–679
8. Raman, N., and DiRusso, C. C. (1995) *J. Biol. Chem.* **270**, 1092–1097
9. Raman, N., Black, P. N., and DiRusso, C. C. (1997) *J. Biol. Chem.* **272**, 30645–30650
10. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 65–89
11. Mandrup, S., Højrup, P., Kristiansen, K., and Knudsen, J. (1991) *Biochem. J.* **276**, 817–823
12. Christie, W. W. (1982) *Lipid Analysis 2nd Ed.*, pp. 54–55, Pergamon Press, New York
13. Rosendal, J., Erthberg, P., and Knudsen, J. (1993) *Biochem. J.* **304**, 321–326
14. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. (1989) *Anal. Biochem.* **179**, 131–137
15. Sigurskjold, B. W., Berland, C. R., and Svensson, B. (1994) *Biochemistry* **33**, 7847–7852

---

\(^7\) J. Knudsen and C. C. DiRusso, unpublished results.
FadR Bind Long Chain Acyl-CoA Esters with High Affinity

16. Mann, M., Meng, C. K., and Fenn, J. B. (1989) *Anal. Chem.* 61, 1702–1708
17. Sánchez, M., Nicolls, D. G., and Brindley, D. N. (1973) *Biochem. J.* 170, 565–574
18. DiRusso, C. C. (1988) *Nucleic Acids Res.* 16, 7995–8009
19. Kragelund, B. B., Andersen, K. V., Madsen, J. C., Knudsen, J., and Poulsen, F. M. (1993) *J. Mol. Biol.* 230, 1260–7719
20. Overath, P., Pauli, G., and Schairer, H. U. (1968) *Eur. J. Biochem.* 7, 559–574
21. Morton, T. A., Runquist, J. A., Ragsdale, S. W., Shanmugasundaram, T., Wood, H. G., and Ljungdahl, L. G. (1991) *J. Biol. Chem.* 266, 23824–23828
22. Raman, N. (1996) *Studies on Acyl-CoA Binding to the Transcription Factor FadR*, Ph.D. dissertation, University of Tennessee, Memphis
