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A Novel Role of Malonyl-ACP in Lipid Homeostasis[†,‡]

Mariano A. Martinez,[§] María-Eugenia Zaballa,[§] Francis Schaeffer,[∥] Marco Bellinzoni,[∥] Daniela Albanesi,[§] Gustavo E. Schujman,[§] Alejandro J. Vila,[§] Pedro M. Alzari,[∥] and Diego de Mendoza*[,§]

[§]*Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina, and* [∥]*Institut Pasteur, Unité de Biochimie Structurale et URA 2185 CNRS, 75724 Paris, France*

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ABSTRACT: The FapR protein of *Bacillus subtilis* has been shown to play an important role in membrane lipid homeostasis. FapR acts as a repressor of many genes involved in fatty acid and phospholipid metabolism (the *fap* regulon). FapR binding to DNA is antagonized by malonyl-CoA, and thus FapR acts as a sensor of the status of fatty acid biosynthesis. However, malonyl-CoA is utilized for fatty acid synthesis only following its conversion to malonyl-ACP, which plays a central role in the initiation and elongation cycles carried out by the type II fatty acid synthase. Using *in vitro* transcription studies and isothermal titration calorimetry, we show here that malonyl-ACP binds FapR, disrupting the repressor−operator complex with an affinity similar to that of its precursor malonyl-CoA. NMR experiments reveal that there is no protein−protein recognition between ACP and FapR. These findings are consistent with the crystal structure of malonyl-ACP, which shows that the malonyl-phosphopantetheine moiety protrudes away from the protein core and thus can act as an effector ligand. Therefore, FapR regulates the expression of the *fap* regulon in response to the composition of the malonyl-phosphopantetheine pool. This mechanism ensures that fatty acid biosynthesis in *B. subtilis* is finely regulated at the transcriptional level by sensing the concentrations of the two first intermediates (malonyl-CoA and malonyl-ACP) in order to balance the production of membrane phospholipids.

Fatty acid biosynthesis is a vital facet of bacterial physiology that is carried out by a set of discrete enzymes collectively known as the type II fatty acid synthase system (FAS-II)[1] (*1*). Fatty acid synthesis is an energetically expensive process, and the rate of fatty acid production is tightly regulated to ensure that the supply of membrane phospholipids exactly matches the demand. For instance, the expression of FAS-II genes in Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* is tightly regulated by a transcription factor, FapR, that functions as a global transcriptional repressor of several genes involved in lipid synthesis (the *fap* regulon), with the notable exception of acetyl-CoA carboxylase (ACC) (*2*). Binding of FapR to its DNA targets is specifically inhibited by malonyl-CoA (*3*), an essential intermediate of fatty acid synthesis in all living cells, the cellular pool of which provides a mechanism for sensing the status of fatty acid biosynthesis and for adjusting the *fap* regulon accordingly. Recent crystallographic studies of the effector binding domain of FapR, in the free form and in complex with malonyl-CoA, have provided the first structural insights into the ligand-induced transcriptional regulation of fatty acid and phospholipid synthesis (*3*). FapR is a homodimeric protein that displays the typical "hot-dog" fold characteristic of the thioesterase enzyme family. Binding of malonyl-CoA promotes a conformational rearrangement of the protein that impairs formation of the repressor−operator complex. Structure-based mutations that disrupt the FapR−malonyl-CoA interaction prevent DNA binding regulation and result in a lethal phenotype in *B. subtilis*, suggesting this homeostatic signaling pathway as a promising target for novel chemotherapeutic agents against Gram-positive pathogens (*3*).

During the elongation cycle of fatty acids, the malonate group is made available to the FAS-II system by the conversion of malonyl-CoA to malonyl-ACP by malonyl-CoA:ACP transacylase, the product of the *fabD* gene (*4*). Both malonyl-CoA and malonyl-ACP share the malonyl-phosphopantetheine moiety (Figure 1) which is selectively recognized by a binding groove in FapR. This interaction is critical to specifically release FapR from its DNA targets (*3*), suggesting that malonyl-ACP could also be a signal controlling FapR activity. Moreover, ACP is one of the most interactive proteins in biology (*5*−*7*), being able to bind a large number of intracellular proteins in a variety of biochemical pathways (*8*). Therefore, our picture of *fap* transcriptional regulation would become more complex if both ACP and the malonyl-phosphopantetheine moiety could be recognized by the transcription factor. We addressed this question by studying the interaction of malonyl-ACP with FapR by different

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[‡]The final atomic coordinates and structure factors of malonyl-ACP have been deposited in the Protein Data Bank, with accession code 2X2B.

*Corresponding author. Phone: +54 341 4350661. Fax: +54 341 4390465. E-mail: demendoza@ibr.gov.ar.

[1]Abbreviations: ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; FAS, fatty acid synthase system; ITC, isothermal titration calorimetry; CoA, coenzyme A.

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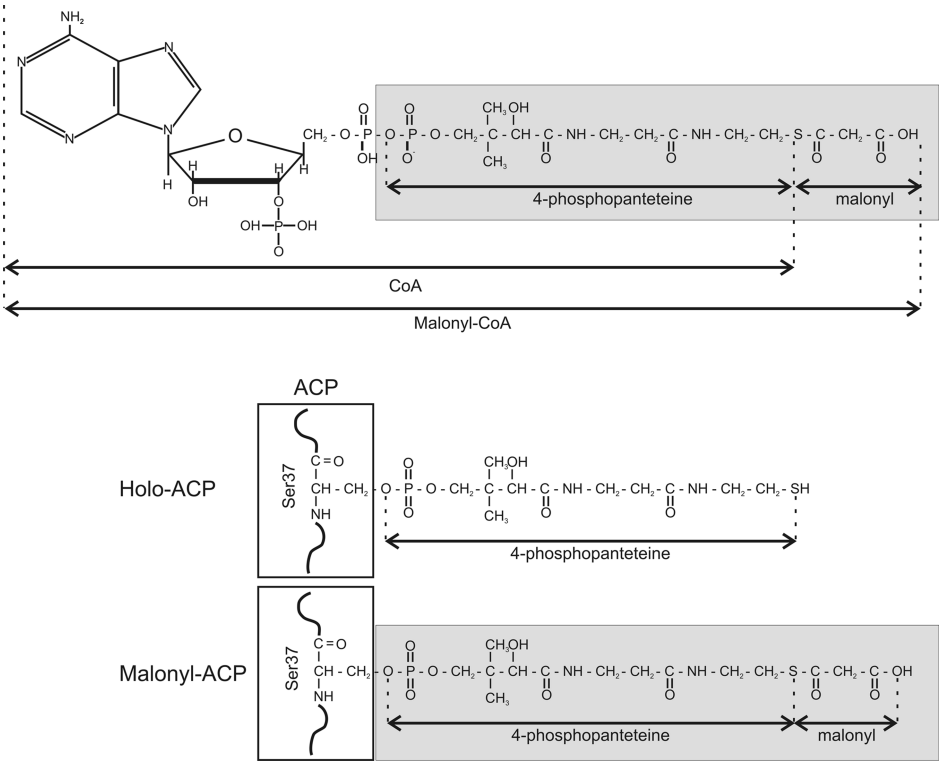

FIGURE 1: Schematic representation of malonyl-CoA, holo-ACP, and malonyl-ACP structures. The gray box points out the structural moiety that is able to interact with FapR and is present in both malonyl-CoA and malonyl-ACP.

experimental approaches. *In vitro* transcription experiments clearly show that FapR-dependent repression of a *fap* promoter is antagonized by malonyl-ACP, which binds FapR with an affinity similar to that of malonyl-CoA (as shown by ITC). Furthermore, the association with the repressor involves essentially the malonyl-phosphopantetheine moiety of malonyl-ACP, since no protein−protein interactions can be detected by NMR. Thus, FapR is a transcriptional regulator that responds to the malonyl-thioester pool available for the initiation of fatty acid biosynthesis and adjusts gene expression to properly balance production of membrane phospholipids.

EXPERIMENTAL PROCEDURES

Materials. Sources of supplies were as follows: Sigma Chemical Co. supplied antibiotics, CoA, and malonyl-CoA; Difco supplied microbiological media; Promega and Invitrogen supplied molecular biology reagents; Perkin-Elmer supplied [α-^{32}P]-uridine 5′-triphophate (3000 Ci/mmol); Qiagen supplied pQE30 vector and Ni^{2+} agarose resin. ^{15}N-Labeled ammonium sulfate (99%) was purchased from Cambridge Isotope Laboratories, Inc. High-quality preparations of the 40 base pair DNA oligonucleotide for isothermal titration calorimetry experiments were purchased HPLC-purified and dialyzed to Eurogentec (Seraing, Belgium). All other chemicals were of reagent grade or better. Protein concentration was determined using the Bradford method with bovine serum albumin as the standard (*9*).

FapR and AcpS were purified as previously described (*2*, *10*).

Plasmids and DNA Manipulation. Plasmids were constructed using standard methods and amplified in *Escherichia coli* DH5α. PCR fragments were amplified from chromosomal DNA of *B. subtilis* strain JH642. Oligonucleotide primers were purchased from Sigma Genosys.

For ACP expression, the *acpP* gene from *B. subtilis* (accession number BG11536) was amplified by PCR using the following primers: ACPBamTEV, 5′-ACTGGATCC*GAGAATCTATATTTTCAAGGC*ATGGCAGACACATAGA-3′ (containing a *Bam*HI restriction site (underlined) and a cleavage site for the protease of tobacco etch virus (TEV) (italicized)), and pQEACPPst, 5′-GCTGTAATAACTGCAGGAATAAAGGGCTAA A-3′ (containing a *Pst*I restriction site, underlined). The resultant 328-bp DNA fragment was purified, digested with *Bam*HI and *Pst*I, and ligated downstream of a histidine tag into the expression plasmid pQE30 digested with the same enzymes. The DNA insert was sequenced to verify the absence of PCR artifacts. The resulting plasmid designated pMM88 was used to transform *E. coli* BL21(DE3) (Novagen) giving rise to strain EMM99.

B. subtilis ACP Sample Preparation. For recombinant ACP purification, strain EMM99 was grown at 37 °C in 1.5 L of LB medium until an A_{600} of 0.6 was reached, and *acpP* expression was induced adding 1 mM IPTG. Following an additional 3 h growth at the same temperature, cells were harvested by centrifugation and frozen. The cell pellet was resuspended in 40 mL of breaking buffer consisting of 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 10% glycerol. Protease inhibitor tablets (Roche) and DNase I (Sigma Chemical Co.) were added to the solution to prevent protease activity and to decrease viscosity of the solution. Cells were lysed by sonication and centrifuged at 15000*g* for 20 min at 4 °C to remove cell debris. The clear supernatant was filtered with a 0.45 μm membrane filter and loaded into a 5 mL HisTrap HP column (GE) previously equilibrated with the same breaking buffer. The column was washed with 10 column volumes of breaking buffer and then with 5 column volumes of the same buffer containing 40 mM imidazole. Finally, ACP was eluted with a buffer containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 10% glycerol, and 500 mM imidazole. Fractions containing ACP were pooled, and to remove the histidine tag, TEV protease was added at a ratio of

1.7 mg of enzyme per 30 mg of ACP and dialyzed overnight against 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 mM DTT at room temperature. The resulting protein mixture was loaded again into a 5 mL HisTrap HP column, and the flow-through containing the cleaved ACP was concentrated with an Amicon membrane (YM3, NMWL 3000) and applied to a HiLoad 16/60 Superdex 75 size exclusion column (GE). The protein was eluted with 20 mM Tris-HCl (pH 7.5), and ACP-containing fractions were pooled, concentrated, and stored in aliquots at −80 °C until required. ACP was obtained as a mixture of apo-ACP and holo-ACP in a ratio of 60:40 as a consequence of the endogenous activity of the AcpS during recombinant expression of ACP in *E. coli*.

For uniform ^{15}N labeling of *B. subtilis* ACP, strain EMM99 was grown at 37 °C in 1.5 L of M9 minimal medium containing (99% ^{15}N) ammonium sulfate until an A_{600} of 0.6 was reached, and *acpP* expression was induced by adding 1 mM IPTG. Following an additional 18 h growth at the same temperature, cells were harvested by centrifugation and frozen. ACP purification was carried out according to the same procedure as described for the nonlabeled protein.

For quantification, the molar coefficient extinction (ε) at 280 nm of ACP and malonyl-ACP was calculated empirically by analytical amino acid content determination of the recombinant proteins.

In Vitro Synthesis of Malonyl-ACP. The recombinant *E. coli* AcpS was used to prepare malonyl-ACP by a modification of a previously described protocol (*10*). A 1 mL reaction mixture containing 1 mM *B. subtilis* ACP, 2 mM malonyl-CoA, 50 mM Tris−acetate (pH 8), 10 mM magnesium acetate, 1 mM DTT, and 4 μM AcpS was incubated at 37 °C for 1 h. AcpS was removed by adding 50 μL of Ni-NTA agarose to the reaction, and the mixture was incubated for 1 h at 4 °C. The resin was then filtered, and the flow-through containing the malonyl-ACP protein was dialyzed for 12 h against 20 mM Tris (pH 6.5) and then concentrated using an Amicon Ultra 15 (5 MWCO). Purity of malonyl-ACP was monitored by SDS−PAGE and enzymatic synthesis by conformationally sensitive gel electrophoresis (13% polyacrylamide, 0.5 M urea) (*11*, *12*) revealing that the sample consisted of a mixture of malonyl-ACP and holo-ACP in a ratio of 60:40. In the experiments where the concentration of malonyl-ACP is stated, it represents the actual concentration adjusted according to the purity of the used preparation. As a control, the remaining content of malonyl-CoA in the preparation was quantified as previously described (*13*), being near 1000-fold lower than malonyl-ACP concentration. Protein samples were stored in aliquots at −80 °C until required.

In Vitro Transcription. *In vitro* transcription assays were performed as previously described (*14*). As template for the reactions, a DNA fragment containing the promoter region P*fapR* (192 bp) was amplified by PCR using the primers ylpC116U (5′-CGGAGGCATGCACGGGGAAATATTAAG-3′) and ylpC116L (5′-TGCTTGGATCCTCTGCTGAAGTAA-TTCC-3′). Recombinant FapR (240 nM final concentration) was incubated in the absence or presence of malonyl-ACP or malonyl-CoA during 10 min at room temperature. Then, purified P*fapR* was added to the reaction mix, giving a final concentration of 16.24 nM, and incubated for another 10 min at room temperature. Transcription was initiated by adding *B. subtilis* RNA polymerase, NTPs, and [α-^{32}P]UTP. The RNA transcripts were precipitated, resuspended in formamide sequencing loading buffer, and subjected to electrophoresis in a 6% polyacrylamide gel containing 7 M urea. Radioactive signals were detected by exposing the gel to a PhosphorImager screen and digitalized with a Storm 840 scanner (GE). Quantification of the corresponding signals was carried out using ImageQuant software (version 5.2).

Isothermal Titration Calorimetry (*ITC*). The association reactions of malonyl-CoA and malonyl-ACP to free FapR and to FapR in complex with a 40 base pair DNA oligonucleotide bearing the sequence of P*fapR* operator were quantified by ITC. Experiments were performed using the high precision VP-ITC titration calorimetric system (MicroCal Inc., MA) and protocols previously described (*15*). Concentrated solutions of FapR, malonyl-CoA, malonyl-ACP, and the DNA oligonucleotide were dissolved in the same buffer preparation (50 mM Tris-HCl, pH 8, 50 mM NaCl, 10 mM $MgCl_2$). The binding enthalpies were obtained by injecting malonyl-CoA or malonyl-ACP molecules into the calorimetric cell containing the free or DNA-bound repressor protein. Solution concentrations were of 0.54 mM malonyl-CoA and 28 μM FapR, and 2.49 mM malonyl-ACP and 47.5 μM FapR, in titrations in Figure 3A, and 1.1 mM malonyl-CoA and 19 μM FapR, and 1.8 mM malonyl-ACP and 35 μM FapR, in titrations in Figure 3B. In Figure 3B, the DNA oligonucleotide was first titrated with FapR (data not shown), and the resulting solution of the repressor−DNA complex was then titrated with malonyl-CoA or malonyl-ACP molecules. Solutions were thoroughly degassed under vacuum, and each titration was performed at 25 °C by one injection of 2 μL followed by 3 μL (Figure 3A) and 5 μL (Figure 3B) injections with 210 s between injections using a 290 rpm rotating syringe. Heat signals were collected with a 2 s filter, corrected for the heats of dilution, and normalized to the amount of compound injected. Normalization and deconvolution of the binding isotherms were carried out using Origin7 (*16*) provided by the manufacturer. As the ACP preparation contained both malonyl-ACP and holo-ACP in a 60:40 ratio, particular care was taken in the quantification of malonyl-ACP binding. Deconvolution of the malonyl-ACP binding isotherm was compared using either a concentration of malonyl-ACP equal to 60% of the ACP concentration, by adjusting the malonyl-ACP concentration to obtain a 1:1 binding stoichiometry (giving a malonyl-ACP concentration equal to 55% of the ACP preparation), or by assuming that malonyl-ACP and holo-ACP molecules both bind to the repressor (see text). All binding isotherm deconvolutions led to identical binding parameters within experimental errors.

NMR Spectroscopy. NMR experiments were carried out on a Bruker Avance II spectrometer operating at 600.13 MHz (^1H frequency), using a triple-resonance (TXI) probehead. Both ACP and FapR samples were prepared in 50 mM Bis-Tris (pH 6.4), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT in 10% D_2O. Protein concentration varied from 0.3 to 0.6 mM. $^1H-^{15}N$-HSQC spectra were recorded on apo- and malonyl-ACP samples to allow a reliable transfer of the resonance assignments (BMRB entry 4989).

Titration of FapR to a mixture of [U-^{15}N]malonyl-ACP and [U-^{15}N]holo-ACP was followed by $^1H-^{15}N$-HSQC-TROSY experiments. The molar ratio FapR:malonyl-ACP was varied from 0 to 3. Data were processed and displayed using Sparky 3 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

Protein Crystallization. Malonyl-ACP was concentrated to 10 mg/mL in 20 mM Tris-HCl, pH 6.5. Initial crystallogenesis screenings were carried out with a nanoliter dispenser robot (Cartesian Technologies) at 18 °C, and promising conditions

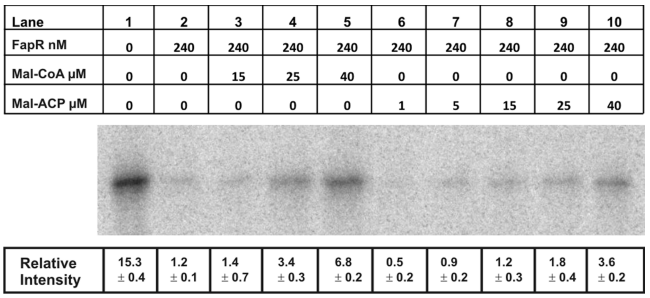

FIGURE 2: Effect of malonyl-CoA and malonyl-ACP on FapR-mediated repression. *In vitro* transcription was performed with a P*fapR* promoter fragment (16 nM) as the template in the presence of FapR (240 nM) and different concentrations of malonyl-CoA or malonyl-ACP (upper panel). The lower panel shows the relative intensity of the radioactivity incorporated in the products of the *in vitro* transcription assay. The values are the mean of three independent experiments, and errors represent the standard deviation of the values obtained.

were further refined by hand using the hanging-drop vapor diffusion method. Malonyl-ACP crystallized in 2 M $(NH_4)_2SO_4$ and 0.1 M citrate−phosphate buffer, pH 4,2. Rhombus-like crystals appeared after 5 days and belonged to the tetragonal space group $I4_122$, with cell dimensions $a = b = 96.9$ Å and $c = 98.8$ Å (Supporting Information Table S1). Single crystals were cryoprotected in mother liquor containing 25% glycerol and flash frozen in liquid N_2 prior to X-ray data collection.

Data Collection, Structure Determination, and Refinement. A single crystal of malonyl-ACP was soaked during 5 min in a cryoprotectant solution containing 50 mM K_2PtCl_4 and then frozen in liquid nitrogen. A highly redundant diffraction data set was collected to 2.7 Å resolution at the ID29 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Data reduction (see Supporting Information Table S1) was carried out with the programs XDS (*17*) and SCALE from the CCP4 suite (*18*). Two Pt binding sites were located with the program SHELXD (*19*) from the anomalous signal, and their parameters were further refined with the program SHARP (*20*). Given the high solvent content of these crystals (80%), the electron density map calculated with solvent-flattened SAD phases at 2.9 Å resolution allowed the manual tracing of the model using the COOT program (*21*). Crystallographic refinement was carried out with the program REFMAC (*22*), alternated with manual rebuilding using COOT. Data collection and refinement statistics are reported in Supporting Information Table S1.

RESULTS

Effect of Malonyl-ACP on in Vitro FapR Repression Activity. In order to examine our hypothesis that malonyl-ACP, in addition to malonyl-CoA, could induce the release of FapR from its DNA binding site, we performed *in vitro* transcription experiments initiating at P*fapR* (see ref *3* and Experimental Procedures). In agreement with the previous report, a transcript of the expected size (93 nt) was obtained in the absence of FapR, whereas P*fapR* transcription was inhibited in reactions containing the repressor (Figure 2). As expected, this repression was relieved by malonyl-CoA at concentrations higher than 25 μM. To test whether malonyl-ACP was also able to induce transcription of P*fapR*, we repeated the *in vitro* transcription experiments in the presence of FapR and malonyl-ACP at concentrations similar to those used with malonyl-CoA. As shown in Figure 2, increasing concentrations of malonyl-ACP gradually induced *in vitro* P*fapR* transcription, confirming that this molecule is also able to interact with FapR promoting its release from the DNA−protein complex. Furthermore, this effect was shown to be specific since apo- and holo-ACP were not able to relieve the inhibitory action of the repressor on P*fapR* transcription (data not shown). The intensity of the bands corresponding to the transcripts was quantified, revealing that the concentrations of malonyl-ACP needed to relieve the repressor activity of FapR over P*fapR* promoter were about 2-fold higher than the concentrations of malonyl-CoA that induce P*fapR in vitro* transcription (Figure 2).

Malonyl-ACP Binds to the Repressor. Given the interdependence between malonyl-ACP binding and FapR repression activity, we needed a method to directly measure malonyl-ACP binding to the transcription factor. To this end, we employed isothermal titration calorimetry (ITC). By using this technique, we have previously shown that malonyl-CoA binds *B. subtilis* FapR with an affinity constant in the micromolar range and a 1:1 stoichiometry (*3*). As shown in Figure 3A, the binding isotherm of malonyl-ACP to FapR is very similar to that observed upon titration of the repressor with malonyl-CoA under identical experimental conditions. Furthermore, the same comparable behavior was also observed when titrating FapR in the FapR−P*fapR* operator DNA complex with either malonyl-CoA or malonyl-ACP (Figure 3B). These results clearly indicate that both molecules, malonyl-CoA and malonyl-ACP, bind the repressor with a similar affinity in the micromolar range (Table 1), suggesting that the common malonyl-phosphopantetheine moiety (Figure 1) plays a central role in the interaction.

Inspection of the thermodynamic parameters (Table 1) reveals that the small differences in the ΔH and ΔS terms compensate each other, giving rise to similar binding affinities. Since employed ACP samples consist of a mixture of malonyl-ACP and holo-ACP, we carried out similar experiments aimed at analyzing possible interactions of pure holo-ACP to free and DNA-bound FapR. The resulting ITC thermograms (Supporting Information Figure S1) revealed a weakly endothermic reaction, with a K_d value for binding of holo-ACP to free FapR ~50-fold higher than that for malonyl-ACP binding. This low affinity precludes a precise determination of the binding stoichiometry or the binding enthalpy at the working reactant concentrations. As a control, neither apo-ACP nor malonate showed any binding to free FapR (Supporting Information Figure S2).

FapR Is Not Recognized by the ACP Protein Framework. *In vitro* transcription experiments reveal that neither apo- nor holo-ACP is able to disrupt the FapR−DNA complex. In order to definitively rule out the possibility of a recognition event between the two proteins, we performed NMR experiments to test whether malonyl-ACP could be recognized by FapR. $^1H-^{15}N$ HSQC NMR spectra are useful to monitor changes in the amide resonances of a protein, being highly sensitive to minor perturbations in their environment and conformation. We then decided to study the possible formation of an interprotein complex by following the NMR features of malonyl-ACP in the presence of FapR.

First, we made use of NMR to check the enzymatic attachment of the malonyl-phosphopantetheine moiety to apo-ACP to obtain malonyl-ACP. As expected, malonyl-phosphopantetheine attachment induces a selective perturbation at the position where the cofactor is bound, Ser37, and the neighboring residues (data not shown). Then, in order to examine a possible protein−protein interaction, [U-^{15}N]malonyl-ACP was titrated with unlabeled

Table 1: Malonyl-CoA and Malonyl-ACP Binding Parameters to Free and P*fapR*-Operator Bound FapR[a]

ligand	FapR	N	K_d (μM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	$\Delta H/\Delta G$ (%)
malonyl-CoA	free	1.0 ± 0.1	11 ± 1	−3.1 ± 0.1	3.8 ± 0.2	45
malonyl-ACP	free	1.0 ± 0.1	24 ± 1	−4.3 ± 0.1	2.0 ± 0.2	68
malonyl-CoA	DNA bound	1.0 ± 0.2	30 ± 1	−3.5 ± 0.1	2.7 ± 0.2	57
malonyl-ACP	DNA bound	1.0 ± 0.2	25 ± 1	−4.7 ± 0.3	1.3 ± 0.3	79

[a]All titrations were performed with the same batch of purified FapR protein. Parameter values are the mean of three (malonyl-CoA) and two (malonyl-ACP) different titrations; errors are variations from the experimental mean. N is the ligand stoichiometry per FapR monomer.

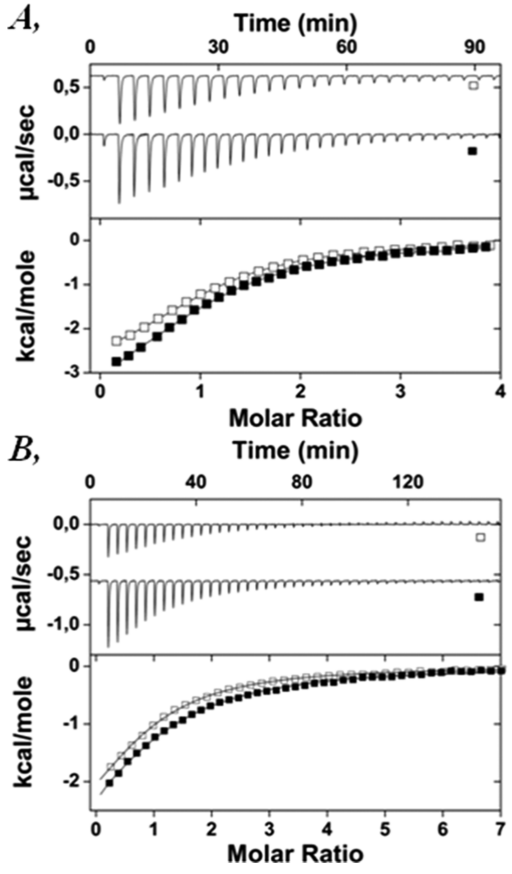

FIGURE 3: Isothermal calorimetric titrations of free and P*fapR* operator bound FapR with malonyl-CoA and malonyl-ACP at 25 °C. (A) Binding to free FapR. The upper panel shows the raw heat signal of the titrations of free FapR with malonyl-CoA (□) and malonyl-ACP (■). The lower panel shows the integrated heats of injections of the above titrations normalized to the ligand concentration (same symbols). (B) Binding to the FapR−DNA complex. Same legend as in (A). In (A) and (B), data are after subtraction of the ligand heat of dilution. In lower panels, the *X*-axis reports the molar ratio of ligand to FapR monomer in the ITC cell. Solid lines in the figure correspond to the best fit of the data using a bimolecular interaction model. In (B), the DNA oligonucleotide was first titrated with FapR, and the repressor−DNA complex formed was then titrated with malonyl-CoA or malonyl-ACP.

FapR up to a malonyl-ACP:FapR ratio of 1:3. A comparison of the spectra of malonyl-ACP along the titration upon different additions of FapR showed neither significant chemical shift perturbations nor any signal broadening (Supporting Information Figure S3). This experiment reveals that there is no protein−protein recognition between malonyl-ACP and FapR. Therefore, the formation of the intermolecular complex must occur by the sole involvement of the malonyl-phosphopantetheine moiety.

Crystal Structure of Malonyl-ACP. The crystal structure of malonyl-ACP was determined using single-wavelength anomalous diffraction methods and refined to 2.7 Å resolution

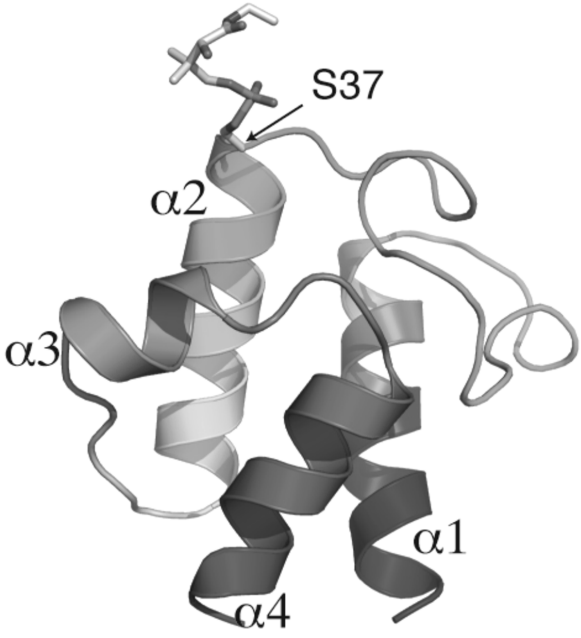

FIGURE 4: Overall structure of malonyl-ACP. Ribbon model of the protein showing the secondary structure elements: α-helix 1−4 and part of the 4-phosphopantetheine moiety attached to Ser37.

(Supporting Information Table S1). The crystal has one protein monomer in the asymmetric unit and a high solvent content, which occupies 80% of the unit cell volume (V_m = 6.7 Å^3/Da). The overall structure (Figure 4) shares the common fold of this protein family, composed of a four-helix bundle defined by helices α1 (residues 2−16), α2 (37−51), α3 (57−62), and α4 (66−75), in agreement with previously reported structures (*23*−*25*).

The malonyl-phosphopantetheine group is covalently attached to the hydroxyl group of Ser37, but only the innermost part of the prosthetic group is well-defined in the electron density map (data not shown), possibly because it is stabilized by intermolecular contacts in the crystal. The rest of the group, including the malonyl moiety, is disordered in the crystal structure. Nevertheless, the structure clearly shows that the prosthetic group protrudes away from the protein into the bulk solvent (Figure 4), suggesting that the malonyl moiety is fully accessible for interactions with putative protein partners. This differs from previous observations of *E. coli* ACP loaded with different acyl chains (*26*), in which the protein was found to sequester the aliphatic chain of the substrate and the β-mercaptoethylamine moiety of the phosphopantetheine group into a central hydrophobic cavity in the core of the four-helix bundle. Although a similar cavity is also observed in *B. subtilis* ACP, it is not occupied by the prosthetic group, possibly due to the high energetic cost of burying the charged carboxylate group of malonate.

These results, taken together with the *in vitro* transcription, calorimetric, and NMR data clearly show (1) that malonyl-ACP

binds both free and DNA-bound FapR eliciting DNA dissociation, as was previously shown to be the case for malonyl-CoA, and (2) the absence of detectable protein−protein interactions between ACP and FapR, indicating that formation of the FapR−malonyl-ACP complex depends solely on the malonyl-phosphopantetheine moiety (as illustrated by the model of the complex shown in Supporting Information Figure S4).

DISCUSSION

FapR is a global regulator of lipid biosynthesis in Gram-positive bacteria, which acts as a transcriptional repressor (*2*). FapR possesses a binding groove able to accommodate the malonyl-phosphopantetheine moiety of malonyl-CoA, thus inducing a conformational rearrangement which leads to dissociation of the DNA−FapR complex, enabling transcription (*3*). Here, we have tested the possible role of malonyl-ACP as an alternative effector of this phenomenon. *In vitro* transcription experiments reveal that malonyl-ACP binding to FapR also induces transcription although at concentrations about 2-fold higher than malonyl-CoA. These results are in good agreement with the K_d values obtained from ITC experiments (Table 1), which indicate that malonyl-ACP binds FapR through the protruding malonyl-phosphopantetheine unit. The crystal structure of malonyl-ACP clearly shows that this moiety sticks out from the protein core, being able to act as a ligand to an interacting protein. The NMR titration experiment reveals that ACP is not recognized by FapR as an interacting partner. Thus, the ubiquitous and promiscuous ACP is not specifically recognized by FapR, which is designed to selectively bind the malonyl-phosphopantetheine unit from either malonyl-CoA or malonyl-ACP.

This work uncovers that malonyl-ACP is a ligand that releases FapR from its DNA-binding sites, adding further insight into the regulation of lipid synthesis in Gram-positive bacteria. The role of malonyl-ACP as inductor of FapR was previously minimized because after conditional inhibition of the *B. subtilis fabD* expression, coding for malonyl-CoA:ACP transacylase, we still observed transcriptional induction of the *fap* regulon by antibiotics that inhibit FAS-II (*3*). However, it seems clear that FabD depletion greatly contributes to increase the intracellular levels of malonyl-CoA, masking the role of malonyl-ACP as a FapR ligand. Although the equilibrium constant of the reaction catalyzed by purified *E. coli* FabD suggests that malonyl-CoA formation is favored (*27*), *in vivo* analysis of the malonyl-ACP pool in this organism showed that the amount of this intermediate available for the FAS-II system is about one-fifth of the total ACP in growing cells (*28*−*30*), being the total content of ACP of approximately 40 μM (*30*, *31*). These measurements translate into an intracellular concentration of malonyl-ACP of approximately 8 μM, whereas the concentration of malonyl-CoA is about 24 μM (*32*). Assuming that similar concentrations are found in *B. subtilis* and given the affinities of malonyl-ACP and malonyl-CoA for FapR, we suggest that the total concentration of malonyl thioesters *in vivo* is enough to regulate FapR but that FapR may not be saturated with these intermediates in growing cells. Indeed, when the concentration of malonyl-thioesters is increased by antibiotics that specifically inhibit fatty acid synthesis, FapR is released from its binding sites leading to a strong transcriptional derepression of the *fap* regulon (*2*, *33*). However, it is worth noting that, depending on the blocked chain elongation step of fatty acid biosynthesis, malonyl-ACP could be converted to short acyl-intermediates or transacylated to malonyl-CoA. Although the relative pool size of malonyl-thioesters accumulated during FAS-II inhibition in Gram-positive bacteria has not yet been quantified, it is possible that malonyl-CoA would be much more abundant than malonyl-ACP in such condition.

The fact that FapR senses the malonyl-thioester pool is an important aspect of the model because it ensures that FapR adjusts gene expression in response not only to the product of the first committed step of the pathway (malonyl-CoA) but also to malonyl-ACP, which is utilized uniquely in the elongation steps in fatty acid synthesis. It should be noted that the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate consumes 94% of ATP used in phospholipid synthesis (*34*). It follows that the control of FapR activity by malonyl-ACP would be energetically efficient since it does not require a higher activity of the ACC complex.

Interestingly, FapR is regulated differently than FabT, another global regulator of fatty acid synthesis identified in the human pathogen *Streptococcus pneumoniae* (*35*). FabT activity is modulated by long-chain acyl-ACPs, which increase its affinity for DNA (*36*). Thus, while the release of FapR from its binding sites is promoted by an increase in the levels of the first intermediates of fatty acid synthesis, the repressive activity of FabT is neutralized by a decrease in the levels of the acyl-ACP end products. This illustrates that Gram-positive bacteria have evolved different structural classes of regulators to globally control the genes required for fatty acid biosynthesis. A complete understanding of how these transcription factors work is clearly relevant to the development of therapeutics because the inactivation of the regulatory network involved in phospholipid synthesis leads to loss of virulence (*37*).

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SUPPORTING INFORMATION AVAILABLE

Additional ITC experiments, $^{1}H-^{15}N$ HSQC NMR spectra, and a table reporting data collection and refinement statistics for malonyl-ACP crystals. This material is available free of charge via the Internet at http://pubs.acs.org.

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