A Mammalian Type I Fatty Acid Synthase Acyl Carrier Protein Domain Does Not Sequester Acyl Chains*

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The syntheses that produce fatty acids in mammals (FASs) are arranged as large multidomain polypeptides. The growing fatty acid chain is bound covalently during chain elongation and reduction to the acyl carrier protein (ACP) domain that is then able to access each catalytic site. In this work we report the high-resolution nuclear magnetic resonance (NMR) solution structure of the isolated rat fatty acid synthase apoACP domain. The final ensemble of NMR structures and backbone 15N relaxation studies show that apoACP adopts a single, well-defined fold. On conversion to the holo form, several chemical shift changes are observed on the ACP for residues surrounding the phosphopantetheine attachment site (as monitored by backbone 1H–15N correlation experiments). However, there are negligible chemical shift changes when the holo form is modified to either the hexanoyl or palmitoyl forms. For further NMR analysis, a 13C,15N-labeled hexanoyl-ACP sample was prepared and full chemical shift assignments completed. Analysis of two-dimensional F2-filtered and three-dimensional 13C-edited nuclear Overhauser effect spectroscopy experiments revealed no detectable NOEs to the acyl chain. These experiments demonstrate that unlike other FAS ACPs studied, this Type I ACP does not sequester a covalently linked acyl moiety, although transient interactions cannot be ruled out. This is an important mechanistic difference between the ACPs from Type I and Type II FASs and may be significant for the modulation and regulation of these important mega-synthases.

Fatty acid biosynthesis in mammals is important not only for energy homeostasis and development but also as a potential target for the treatment of obesity (1) and cancer (2). Type I fatty acid synthases (FASs) 3 that catalyze the biosynthesis of fatty acids in mammals, utilize simple acyl units, bound to the phosphopantetheine arm of a holo acyl carrier protein (ACP) domain, for chain initiation and elongation. The recent elucidation of the low resolution structure of the mammalian FAS by x-ray crystallography (3) has provided key structural and mechanistic insights into this important enzyme. Although the resolution of the crystal is insufficient to discern the backbone and side chains, the electron density has permitted the authors to propose a model based on the structures of individual domains and homologous enzymes. The authors have proposed a “head to head” dimeric model that comprises a central core consisting of the enol-reductase, dehydratase (DH), and ketosynthase with the malonyl transferase and ketoreductase domains being located peripherally. Dimerization occurs through association of the KS domains. Notable absences in the crystal structure are the locations of the peripheral ACP and thioesterase domains, suggesting that the positions of the ACP and thioesterase domains are relatively mobile compared with the core of the FAS.

In comparison, bacterial Type II FASs consist of discrete monofunctional proteins (4). Structural studies of the Type II FAS ACP components are particularly well developed and have revealed the structural basis of acyl chain binding and the phenomenon of conformational switching. The crystal structures of Escherichia coli FAS butyryl (5), hexanoyl-, heptanoyl-, and decanoyl-ACPs (6) and nuclear magnetic resonance (NMR) structures of spinach FAS decanoyl- and stearoyl-ACPs have been reported (7). These structures reveal that during fatty acid biosynthesis, fully saturated acyl chains are sequestered within a central cavity in the ACP formed through conformational changes in the protein. The fatty acid chain is sequestered within the hydrophobic core of the ACP perhaps to protect the thioester moiety from hydrolysis. Binding of the acyl chain also influences the dynamics of the ACPs. Spinach FAS holo-ACP exists in equilibrium between a folded and largely disordered form, however, upon acylation this equilibrium is shifted toward the folded form. At present the physiological role of switching of this, and other ACPs, is unknown (8, 9). It has been suggested that switching confers allosteric regulation of the ACP, whereby its interaction with other enzymes may be modulated and controlled.

Unlike the Type II ACPs, the acyl chain binding and dynamic properties of Type I ACPs are not well understood. We have previously reported the low resolution solution phase structure of the isolated rat FAS apoACP domain (10) and found it to adopt an α helical bundle structure consistent with the Type II ACPs from fatty acid (11) and polyketide synthases (PKSS) (12). Furthermore, it has been shown that the rat FAS ACP can sub-
stitute for the E. coli FAS ACP (13) in vivo and that it is recognized as a substrate by enzymes known to modify Type II ACP's including Streptomyces coelicolor holo-ACP synthase (ACPS) and malonyl-CoA:ACP transacylase in vitro (10).

In this study we report the high-resolution NMR solution structure and $^{15}$N-relaxation properties of the rat FAS apoACP domain. Importantly, we demonstrate that upon acylation of the rat FAS ACP, there is no sequestration of either a hexanoyl or palmitoyl side chain. This may provide the first evidence for a mechanistically distinct role for the Type I ACP domains compared with their Type II homologues.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Rat FAS apoACP was recombinantly expressed from E. coli strain BL21(DE3) carrying the rat FAS ACP (amino acids 2114–2202) in the pET15b expression plasmid as previously described (13). ACP was purified by ammonium sulfate precipitation followed by anion exchange chromatography on a HiLoad 26/10 Q-Sepharose column (GE Healthcare) eluting with a linear gradient of 0–1 M NaCl over 10 column volumes. $^{15}$N-Labeled ACP was obtained by growth and expression in M9 minimal media with $^{15}$N ammonium chloride (1 g/liter) as the sole nitrogen source. $^{15}$N,$^{13}$C-Labeled ACP was obtained by growth and expression in M9 minimal media with [15N]ammonium chloride as the sole nitrogen source and [13C]glucose (2 g/liter) as the sole carbon source.

**NMR of Rat FAS ApoACP Domain**—The protein was dissolved to a final concentration of 1.5 mM in 10% D$_2$O, 90% H$_2$O containing sodium deuterocetate (50 mM, pH 5.5). All NMR spectra were collected on a Varian INOVA 600 MHz spectrometer equipped with Z-pulsed field gradients. For $^{15}$N,$^{13}$C-labeled ACP, $^1$H-$^{15}$N sensitivity enhanced (14) HSQC, HNCA,CB, CBCA(CO)NH, HCCH-TOCSY, $^{13}$C-edited three-dimensional NOEY, and two-dimensional constant time $^1$H-$^{13}$C HSQC data sets were acquired (15). These spectra supplemented the $^{15}$N-edited NOEY-HSQC and TOCSY-HSQC spectra gained previously (10). All spectra were processed and viewed with NMRPipe (16) and CcpN Analysis (17, 18). For the acyl chain binding studies, standard sensitivity enhanced $^1$H-$^{15}$N HSQC spectra were acquired.

**NMR of Rat FAS Hexanoyl-ACP Domain**—A $^{13}$C,$^{15}$N dual acquisition (CN) NOEY experiment (acquired with 150 ms mixing time) and a two-dimensional $^1$F$_2$-filtered NOEY experiment with a 400-ms mixing time (19) were recorded on a 1.5 mM sample of $^{12}$C,$^{14}$N-hexanoyl-$^{13}$C,$^{15}$N-labeled rat FAS apoACP. In this sample both the hexanoyl group and phosphophantetheine side arm are not enriched with $^{13}$C or $^{15}$N.

**Structure Calculation**—Peak and shift lists were assigned manually using standard sequential assignment methods. Peak intensities were measured using the default CcpN Analysis program settings assuming a Gaussian peak shape. Additional backbone torsion angle restraints were obtained from chemical shift data using the TALOS algorithm (20, 21). Structures were calculated using Ambiguous Restraints for Iterative Assignment (ARIA) 1.2 (22) in conjunction with CNS (23) by means of a torsion angle dynamics procedure. ARIA has been developed for the automated assignment of NOEY spectra, where it cycles through a number of rounds of peak assignment and validation (by structure calculation using simulated annealing). At the end of first step the lowest energy structures are used to improve the peak assignment that is used in the next calculation cycle. This process was continued for nine iterations, with each step stricter in relation to accepted assignments, thus decreasing the ambiguity of the data and converging to an assignment that fits the experimental data. Calculation of the rat FAS apoACP domain structure was based on three separate ARIA runs with the resultant constraint list being checked manually at each stage. 200 structures were calculated in the final run with the 30 lowest energy structures selected and water refined. The quality of the structures was checked by PROCHECK (24) and WHATCHECK (25).
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positive ion mode on a QStar XL mass spectrometer (Applied Biosystems).

RESULTS

Structure of Rat FAS ApoACP—$^{15}$N-Labeled and $^{15}$N-$^{13}$C double labeled rat FAS apoACP were overexpressed and purified in a similar fashion to that described previously (10). Routinely, 5–10 mg/liter of $^{15}$N-labeled and 3–5 mg/liter of $^{15}$N-$^{13}$C double labeled rat FAS ACP was obtained. Spin systems were unambiguously assigned using standard multidimensional multinuclear NMR experiments, performed at 25 °C (pH 5.5). NOEs were automatically assigned using the ARIA algorithm (22, 30) and 200 structures were generated in the final calculation. The structures were based on a total of 1700 restraints, of which 330 were long range. Among these, 53 long range NOEs remained ambiguous (for example, the final structure may simultaneously satisfy two or more possible NOE assignments that cannot be discriminated against on the basis of chemical shift). Ambiguous restraints were checked manually along with any peaks rejected by the ARIA algorithm. The 30 lowest energy structures were selected and the structural statistics for this ensemble are summarized in Table 1. All NOEs were checked for consistency and accuracy at the end of the structure generation procedure. Experimental restraints were well satisfied with no NOE violations exceeding 0.3 Å. Over 95% of the angles fell within the most favorable or favorable regions of the Ramachandran plot (31) and the ensemble gave good Z-score values (calculated with WHATCHECK) when compared with other published ACP structures and other NMR structures in general (32).

Fig. 1A shows the final ensemble of 30 rat FAS apoACP structures. The lack of short or long range NOEs in the N- and C-terminal regions resulted in poorly defined segments of structure over residues Gly$^{2114}$ to Arg$^{2120}$ and Ser$^{2187}$ to Asn$^{2202}$. Excluding these residues, the final family of structures had fewer residues in disallowed regions of the Ramachandran plot than previously reported (10) reflecting the improvement obtained with implementing the greater number of restraints and spin diffusion corrections in the structure calculation. The overall fold of the family of conformers was well defined, with a root mean square deviation (r.m.s. deviation) over the backbone and heavy atoms in well defined regions of 0.55 and 0.91 Å, respectively. The ACP forms a helical bundle composed of four $\alpha$-helices, helix I (Asp$^{2121}$ to Ile$^{2129}$), helix II (Val$^{2155}$ to His$^{2165}$), helix III (Ile$^{2171}$ to Gln$^{2176}$) and helix IV (Arg$^{2180}$ to Met$^{2185}$). A small turn was observed between residues 2144 and 2147 with NOEs only detected between residue $i$ and residue $i+2$ residues. Comparison of the closest to average structure (Fig. 1B) with that previously reported (Fig. 1C) showed that although the general fold of the previous structure was retained, the recalculated structure was more compact and better defined by virtue of a greater number of long range NOEs (330 versus 41). The greater compactness was predominantly due to the positions of helix I and III being unambiguously identified.

The Loop Regions—There are 3 principal loop regions observed in the rat FAS ACP. Loop I runs from Leu$^{2130}$ to Ser$^{2143}$, connecting helix I to the small turn between residues

| TABLE 1 |
| --- |
| Structural statistics for the rat FAS ACP ensemble |
| **Rat FAS ACP** |
| No. of distance restraints | 1732 |
| Intraresidue | 695 |
| Sequential | 392 |
| Medium range | 314 |
| Long range | 352 |
| Unambiguous | 1643 |
| Ambiguous | 89 |
| No. of $\phi/\psi$ angles restraints | 38 |
| No. of distance violations $>$0.3 Å | 0 |
| No. of dihedral angle violations $>$5° | 0 |

**Structural quality from Ramachandran plot**

- Most favored regions: 75.3°, 78.3°
- Additionally allowed regions: 20.3°, 17.8°
- Generously allowed regions: 2.5°, 2.4°
- Disallowed regions: 1.9°, 1.5°

**Root mean square deviation (Å)**

- Backbone atoms (secondary structure): 0.55
- Heavy atoms (secondary structure): 0.91
- Backbone atoms (residues 2123–2184): 0.69
- Heavy atoms (residues 2123–2184): 1.12

*All residues.

Residues 2122–2186.

Fig. 1A, ensemble of 30 rat FAS ACP structures. Average structures of both the new (B) and old (C) rat FAS apoACP structures. All structures are shown in the same orientation with helix II to the front left and the phosphopantetheine attachment site Ser$^{218}$ at the bottom of helix II. C and N termini and helices are labeled.
2144 and 2147, and has an r.m.s. deviation of 0.75 Å. The loop has just three exposed charged residues (Arg<sup>2133</sup>, Asp<sup>2134</sup>, and Asp<sup>2144</sup>) and four hydrophobic residues (Ile<sup>2136</sup>, Leu<sup>2135</sup>, Ile<sup>2138</sup>, and Leu<sup>2140</sup>) that pack alongside Leu<sup>2179</sup> to form a miniature hydrophobic core. This core is spatially distinct from the main hydrophobic packing that stabilizes the four helices. Residues 2144–2151 form loop II, comprised of a well structured turn (2144–2147) followed by a short stretch of residues (2148–2150) connecting this to helix II. Finally loop III (residues Asp<sup>2166</sup>–Arg<sup>2172</sup>) shows additional hydrophobic contacts with both helix II, through contact of Val<sup>2168</sup> and the side chain of Arg<sup>2158</sup>, and helix IV, with Leu<sup>2167</sup> and Leu<sup>2169</sup> packing against the hydrophobic Met<sup>2183</sup>.

<sup>15</sup>N Backbone Dynamics—At 500 and 600 MHz, complete<sup>15</sup>N relaxation data could be obtained for 67 and 75 of the 89 residues of rat FAS apoACP, respectively. Missing residues included the N-terminal residue and 2 proline residues. <i>T</i><sub>1</sub>, <i>T</i><sub>2</sub>, and NOE values and associated errors are shown in Fig. 2. <i>A</i>–<i>C</i>, at both 500 and 600 MHz. For N-terminal residues 2115–2121 and the C-terminal residues 2187–2202, large <i>T</i><sub>1</sub> and <i>T</sub><sup>2</sup> values and negative NOE values indicate that they are highly disordered in solution, in agreement with the structural data where few NOEs were observed. Loop I (residues 2130–2143) showed small increases in <i>T</i><sub>1</sub> and <i>T</sub><sup>2</sup> and a reduction in the magnitude of heteronuclear NOEs indicating a degree of disorder. Backbone<sup>15</sup>N nuclei with NOEs smaller than 0.65 are considered to be structured (33) and only residues 2132–2137 in loop I have values below this threshold. Over the remainder of the protein there were fairly uniform <i>T</i><sub>1</sub>, <i>T</sub><sup>2</sup>, and NOE values with the exception of Asp<sup>2150</sup> that displays shorter <i>T</i><sub>2</sub> values than expected, possibly indicating localized conformational exchange.

To further interpret the relaxation data, the internal dynamics of the protein were analyzed using reduced spectral density mapping (34, 35). This approach estimates the form of the spectral density function for each N–H vector at three frequencies, <i>J</i>(0), <i>J</i>(0.87ω<sub>N</sub>), and <i>J</i>(0.87ω<sub>ω</sub>).<sup>4</sup> <i>J</i>(0) is sensitive to motions on a broad range of time scales and essentially smaller <i>J</i>(0) values correlate with broader spectral density profiles and more rapid rotational fluctuations of a backbone N–H vector (36). The <i>J</i>(0) values determined at 500 and 600 MHz are shown in Fig. 2D. <i>J</i>(0) values for the long C-terminal tail are small, ranging from 3.5 ns at the C-terminal of helix IV to below 0.5 ns at the C terminus. The average <i>J</i>(0) values over residues 2123–2131, 2139–2146, 2148–2161, and 2171–2180 were 3.6 ± 0.4 ns at 600 MHz and 3.6 ± 0.3 ns at 500 MHz, respectively. Importantly the values determined at 500 and 600 MHz show no systematic scaling with field that would suggest conformational averaging of the structure on a microsecond-millisecond time scale in this region. The exception to this is residue Asp<sup>2150</sup> that shows an elevated <i>J</i>(0) value of 5.1 ns at 600 MHz and 3.9 at 500 MHz possibly indicating microsecond-millisecond conformational exchange. The values of <i>J</i>(0) over loop I (2130–2143), the latter part of loop II (2148–2150), and loop III (residues 2166–2172) are reduced and non-uniform and reflect the disorder observed in these regions in the structural model.

<i>J</i>(ω<sub>N</sub>) samples the spectral density at 50 and 600 MHz for the 500 and 600 MHz data, respectively, and is sensitive to higher frequency internal motions. The values for <i>J</i>(ω<sub>N</sub>) at both fields are shown in Fig. 2E and these scaled with the field as expected (<i>J</i>(ω<sub>N</sub>) 500 > <i>J</i>(ω<sub>N</sub>) 600). The average <i>J</i>(ω<sub>N</sub>) is 0.41 ± 0.02 ns (600 MHz) and 0.47 ± 0.02 ns (500 MHz). <i>J</i>(ω<sub>N</sub>) values supplement the <i>J</i>(ω<sub>N</sub>) values and reveal more clearly the reduced spectral density, particularly over resi-
dues 50–56 that show a shift in $f(\omega_n)$ to a distribution covering higher frequency motions. Over the helical regions, $f(\omega_n)$ was uniform (helix I, 0.41 ns; helix II, 0.41 ns; helix III, 0.41 ns; helix IV, 0.41 ns).

Acylation of Rat FAS ApoACP—Type II fatty acid and polyketide apoACPs can be phosphopantetheinylated and acylated in a single step using S. coelicolor phosphopantetheinyl transferase (ACPS) and a corresponding acyl-CoA (37). Furthermore, we have shown previously that the same ACPS can be used to convert the Type I rat FAS ACP domain into the active holo form and, like other ACPs, transfer of the phosphopantetheine is not affected by a covalent modification of the phosphopantetheine thiol (10). We have therefore used ACPS in this work to form holo-, hexanoyl-, and palmitoyl-ACP from rat FAS apoACP and -CoA or respective acyl-CoAs. For each preparation, rat FAS apoACP was incubated with a catalytic amount of ACPS and a 5-fold molar excess of the relevant acyl-CoA. The degree of acylation was monitored by electrospray mass spectrometry and found to be complete within 3 h.

Acylation Chain Binding—The $^1$H-^15$N$ HSQC spectrum of rat FAS apoACP has excellent dispersion and was ideal for monitoring structural changes via chemical shift perturbation mapping. The chemical shifts of protein amides are very sensitive to environmental changes and consequently, $^1$H-^15$N$ HSQC NMR experiments were used to probe whether acylation induced any chemical shift changes on the rat FAS ACP. We first prepared $^1$N-labeled rat FAS apoACP and modified separate samples of this to the holo, hexanoyl, and palmitoyl forms using ACPS. A series of $^1$H-^15$N$ HSQC experiments were then collected under identical conditions of 25 °C and a protein concentration of 150 $\mu$M. Fig. 3A shows overlay plots of the resulting spectra of apoACP (black) and the three modified rat FAS ACP spectra (holo, red; hexanoyl, green; palmitoyl, blue). Upon conversion from apo to holo, a number of backbone amide chemical shifts were visibly perturbed. As shown in Fig. 3B, the residue with the greatest chemical shift changes is the phosphopantetheine attachment site, Ser2151, consistent with its covalent modification. Immediately preceding Ser2151, a number of residues in the structured loop, Leu2144, Ala2145, Leu2149, and Asp2150, are perturbed and in addition, residues in helix II (Gly2154, Val2155, Val2157, and Arg2158) and the loop connecting helix II and III (Ile2171 and Glu2173) show very small changes. The degree of chemical shift change was small enough to allow the resonances in the holo form to be assigned by comparison to the apo form (Fig. 3A).

When the HSQC spectrum of rat FAS hexanoyl-ACP was compared with the holo form, however, virtually no chemical shift perturbations were observed. The discernible changes were extremely small. For example, Met2153 showed a weighted average chemical shift perturbation of 0.05. Again, these changes immediately surrounded the phosphopantetheine covalent attachment site. Upon modification of holo- to palmitoyl-ACP an almost identical pattern emerged, with only small
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localized changes along helix II and III when compared with holo-ACP. In both cases, the small size and localization of these chemical shift changes did not indicate any significant interaction of the acyl chain with the protein or substantial structural rearrangement that might be expected if the acyl chain was sequestered (7). The HSQCs indicated the hexanoyl- and palmitoyl-derivatized ACPs remained well folded, with a chemical shift dispersion that was effectively super-imposable with that of holo-ACP. However, unlike apo, holo, or palmitoyl preparations, rat FAS palmitoyl-ACP was not stable in solution at 150 μM and was observed to completely precipitate over ~48 h. Due to a small amount of precipitation prior to data acquisition the sample was more dilute than the other samples recorded in this study and line broadening of several peaks was visible in the HSQC (Fig. 3A). However, there was sufficient time to collect the 1H-15N HSQC (1.5 h). The 1H-15N HSQC confirmed that the protein was correctly folded, suggesting aggregation is being driven by another factor, presumably attributable to the exposure of the long fatty acid chain and the formation of hydrophobic intermolecular interactions with other exposed palmitoyl groups. In conclusion, however, for the majority of residues, the 1H and 15N chemical shifts were identical for rat FAS holo-, hexanoyl-, and palmitoyl-ACP.

Two-dimensional Filtered/Edited NOESY of Hexanoyl-ACP—To determine the true extent of acyl chain sequestration in the rat FAS ACP, a series of NOESY experiments were applied to identify NOEs between the labeled NOEs and protein residues and the unlabeled hexanoylated phosphopantetheine side chain (19). The two-dimensional experiment yields symmetrical cross-peaks (either side of the diagonal) between protons on unlabeled carbons ([1H-12C]-F2 to [1H-13C]-F1 cross peaks), and a set of unsymmetrical ([1H-12C]-F2 to [1H-13C]-F1 NOEs; where F2 and F1 are the directly and indirectly detected dimensions, respectively. Using both a 250- and 400-ms NOESY experiments we could clearly detect the expected pairs of strong symmetrical ([1H-12C]-[1H-13C]-NOEs) arising from proton-proton connectivities within the hexanoyl chain and phosphopantetheine side chain. Fig. 4 shows an expansion from the two-dimensional F2-filtered NOESY experiment where correlations through the hexanoyl moiety are clearly delineated. We were also able to fully assign the phosphopantetheine side chain using the filtered NOESY experiment. Fig. 4 shows the two geminal methyl groups of the phosphopantetheine side chain (numbered 30 and 31 in Fig. 4) and the expected NOEs to adjacent protons (proton 32 and the pair of diastereotopic protons, 28-CH2). However, at both mixing times, no [1H-12C]-F2 → [1H-13C]-F1 NOEs were observed between the phosphopantetheine side chain or acyl group with the protein.

Despite using a concentrated sample of hexanoyl rat FAS ACP it was possible that very weak NOEs may not have been detected. Therefore a sensitive three-dimensional 15N,13C dual acquisition NOESY spectrum was acquired. A complete analysis of this spectrum revealed a network of NOEs that were essentially identical to the apo form. Again, no new NOEs could be detected to the hexanoyl side chain. Finally we doped the sample with free hexanoyl-CoA to compare the chemical shifts of the phosphopantetheine side chain and hexanoyl moiety in the free and covalently linked forms. Fig. 4 shows the phosphopantetheinyl region of hexanoyl-CoA with the assigned chemical shifts shown in free solution (in parentheses) and when covalently bound as hexanoyl-ACP (no parentheses). The hexanoyl portion of the side chain showed a set of indistinguishable chemical shifts between the two forms. The only small chemical shift differences (marked in parentheses in Fig. 4) detected were in the phosphopantetheine side chain. Small changes in the phosphopantetheine side chain are consistent with the small chemical shift changes we observed in the 1H-15N HSQC when the apoACP was modified to the holo form.

DISCUSSION

The Structure of Rat FAS ApoACP Domain—The previously published low resolution structure of the rat FAS apoACP domain was calculated on the basis of a limited number of long
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This problem has been overcome by performing a series of standard three-dimensional NMR experiments on a sample of uniformly $^{15}$N,$^{13}$C-labeled rat FAS apoACP thus allowing resonances from residues forming the protein core to be better resolved.

Amino acid sequence alignment of both Type I and II ACPs reveals that there is little sequence similarity between the rat FAS ACP and Type II ACPs from *E. coli* FAS (34.8% similar, 18.0% identical), *Bacillus subtilis* FAS (30.3% similar, 18.0% identical), or *S. coelicolor* actinorhodin PKS (35.1% similar, 21.3% identical). The identity to the Type I fungal FAS from *Saccharomyces cerevisiae* is lower, at only 14.1% identity. The rat FAS ACP domain (helices I, II, and IV) superimposed on *B. subtilis* ACP (Protein Data Bank code 1HY8) with an r.m.s. deviation of 3.0 Å and *E. coli* ACP (PDB 1T8K) with a r.m.s. deviation of 2.8 Å and the ACP fragment from the *S. cerevisiae* fungal FAS (PDB 2UV8) with r.m.s. deviation of 3.0 Å. All four FAS ACP structures are compared in Fig. 5, *B*–*E*. Although the rat FAS ACP forms the conserved α-helical bundle structure common to all ACPs, there is a slightly lower degree of α-helical content (46% over residues 2114–2192) than other ACPs (*E. coli* FAS ACP 50% (39, 40), *S. cerevisiae* ACP (50%) (41), *B. subtilis* FAS ACP 60% (42), and the actinorhodin PKS ACP 50% (12)). The shorter helices are replaced instead by the longer loop regions in the rat FAS ACP. In the fungal Type I ACP, the higher helical content is attributable to a longer helix I and IV, both of which are 4 residues longer that the rat FAS ACP, but loss of helix III. Helix I may be stabilized by packing against an additional adjacent four-helix scaffolding region in the complete fungal synthase structure. Typically helices I and IV form a rigid scaffold in Type II ACP structures with helices II and III showing a varying degree of increased motility most likely associated with their role as mediators of protein–protein interactions and the portal to the internal hydrophobic cavity. Both helices II and III of the isolated rat FAS ACP are of similar length to those in the Type II and ACPs and appear to fold autonomously (without a need for specific packing interactions from the complete synthase). The dynamics reveal that although shorter, helices I and IV remain rigid and pack efficiently against the core of the ACP structure.

Despite their common basic function, the large r.m.s. deviations and poor sequence conservation between the Type I and II ACPs reflect their specialized roles. Interestingly the helix II glutamic acid residues (Glu$^{2156}$ and Glu$^{2162}$, respectively) are conserved and solvent exposed in rat FAS ACP. These acidic residues serve as the ACP recognition motif in bacterial FASs (43, 44) and their conservation may suggest that the mode of ACP recognition has been preserved through evolution. This is supported by the observations that rat FAS ACP can productively interact with both ACPS and malonyl-CoA:ACP transacylase (10) and partially substitute for *E. coli* ACP *in vivo* (13).
Dynamics and Biological Implications—Globally the NMR data shows the rat FAS apoACP domain to be a single, extremely well ordered conformer despite shorter α-helices and longer loop regions. The dynamics data reveals that although there is an expected increase in flexibility in the loop regions, these are not highly disordered. Loop I (2130–2143) for example is stabilized by formation of a miniature hydrophobic core within the loop and only residues 2132–2137 showed a significant reduction in J(0) and J(ω). The rat FAS apoACP does not show destabilization of helices II and III as has been observed in several Type II ACPs. In the frenocin and oxytetracycline PKS ACPs, conformational exchange on a microsecond-millisecond time scale were observable in helix II of oxytetracycline apoACP (45) and helix III of frenocin holo-ACP (46). Amide protons showed reduced protection factors in helix II of E. coli FAS holo-ACP (47) although more recent dynamics studies showed no significant difference for helix II (8). We observe only a single case of microsecond-millisecond exchange, for Asp2150 adjacent to the Ser2151 phosphopantetheine attachment site. This residue has been found to be essential in other ACPs for recognition by ACPS (48) and the structure of the complex of the B. subtilis FAS ACP and ACPS revealed a salt bridge from the analogous Asp37 residue to Arg14 on the ACPs. An inherent flexibility of this residue may be important for correct alignment with a discrete phosphopantetheiny transferase.

Despite the increased number of NOEs, the structure and dynamics indicate that the protein C-terminal loop is disordered. We have proposed previously that it forms part of the highly mobile ACP-thioesterase inter-domain linker that allows these two modules to move with respect to each other or may only become structured in the context of the complete FAS.

Upon modification to the holo form, there is a weak interaction between the phosphopantetheine chain and the ACP, suggested by small chemical shift changes in the HSQC spectra and small chemical shift changes in the phosphopantetheine side chain. These minor chemical shift changes most likely indicate a change in local environment as the phosphopantetheine chain rapidly samples conformations in close proximity to helix II and III in the fast exchange limit on the NMR time scale. This is consistent with related ACP structure, dynamics, and chemical shift mapping studies where no interaction between the phosphopantetheine chain and ACP has been observed (8, 49, 50). Recent dynamics studies of E. coli FAS apo- and holo-ACPs, for example, have revealed only transient interaction of the 4'-phosphopantetheine chain with the protein (8). Where there may be more significant interaction of the 4'-phosphopantetheine with the ACP, slow conformational averaging of the structure has also been observed (see below).

There was no evidence for slow, two-state exchange within either the apo or holo form of rat FAS ACP and a single set of cross-peaks was observed in the respective 1H,15N HSQC spectra. In contrast, conformationally averaged apo and holo forms have been observed in related peptidyl carrier proteins and Type II FAS ACPs. The “Type I” peptidyl carrier protein from the third module of tyrocidin A synthetase was shown to exist as three distinct conformations, an apo conformation (A), a holo conformation (H), and a third more structured conformation (A/H) in exchange with both apo and holo forms (9). In contrast to the rat FAS apoACP, the peptidyl carrier protein (A) form had a flexible, more open three-dimensional structure accompanied by loss of helix III. Exchange between two conformers has also been observed in the Type II spinach FAS holo-ACP (7), the Type II FAS ACP from Plasmodium falciparum (PfACP) (51) and Mycobacterium tuberculosis, AcpM (49), where this has been attributed to differences with the interaction and solvent exposure of the 4'-phosphopantetheine arm. Our analysis, however, does not rule out a faster two-state exchange of the holo form as observed in E. coli holo-ACP. In the instance a single set of cross-peaks was observed but a two-state model was required to satisfy all of the observed NOEs (52).

Rat FAS ACP Does Not Bind Fatty Acids—Previously it has been noted that rat acyl-ACPs migrate more slowly by native gel electrophoresis than the equivalent malonyl species (13), whereas acylated Type II E. coli ACPs migrated more quickly after an apparent compacting of the overall ACP fold (53). This result is explained by the striking observation that unlike Type II FAS ACPs, the Type I rat FAS ACP does not sequester long hydrophobic chains from aqueous bulk solvent. Comparison of the holo-, hexanoyl-, and palmitoyl-1H-15N HSQC spectra revealed almost identical chemical shift values between the three species. In contrast, related NMR and x-ray studies of spinach and E. coli ACPs have shown extensive chemical shift differences between acylated forms upon burial of the fatty acid chain. In the spinach hexanoyl-ACP NMR structure, the decanoyl fatty acid chain is bound in a hydrophobic cavity of ∼160 Å3 between helices II and III (7). The same cavity increases in size to 228 Å3 to accommodate the longer stearoyl acyl chain. This structural rearrangement to accommodate the extension of the acyl chain from decanoyl to stearoyl results in weighted chemical shift changes of residues in helix III of up to 0.35 ppm. In comparison, over the equivalent region of rat FAS ACP, no chemical shifts greater than 0.01 ppm are observed when the chain length is increased from hexanoyl to palmitoyl. Slabas and co-workers (5) reported the structure of E. coli FAS butyryl-ACP, which has been followed recently with crystal structures of E. coli FAS hexanoyl-, heptanoyl-, and decanoyl-ACP (6). These structures reveal that an expanding cavity on the ACP sequesters the aliphatic chain and the thioester linkage of its substrate into a hydrophobic pocket at the center of the four-helix bundle.

Given the significance of this observation we sought to test conclusively whether the acyl chain made significant contacts with rat FAS ACP. To do this we recorded a series of NOESY experiments to identify interactions between the chain and protein. In particular a F2 filtered NOESY was used to identify NOEs between the unlabeled side chain and the labeled protein. This approach has been used successfully to derive distance constraints for the interaction of the decanoyl and stearoyl chains and the spinach ACP (7). We, however, observed no NOEs between the protein and the side chain using this experiment. Furthermore, standard CN NOESY analysis showed no new peaks that may have arisen from contact with the acyl chain.
chain or changes in NOE patterns that might result from a structural rearrangement of the rat FAS ACP. These NOE results are consistent with our HSQC data and would seem to confirm that the acyl chain is not sequestered by the ACP. The lack of chemical shift changes in the hexanoyl- or palmitoyl-ACP HSQC spectra, lack of observable NOEs, and lack of chemical shift changes in the hexanoyl group suggest at most transient interactions of the acyl groups and the surface of the rat FAS ACP.

A sequence comparison of several Type I and Type II FAS ACPS is shown in Fig. 5A. The Type I ACPs show almost complete conservation of Leu2144, Leu2149 (with the exception of the analogous residue 2149 in mosquito where the bulky hydrophobic methionine is substituted), and a hydrophobic valine or isoleucine at position 2174 that define the packing and structure of the potential entrance to the hydrophobic cavity. Fig. 6, A–D, compares the structure of this region in rat, E. coli, and B. subtilis FAS ACP models. V2174 on helix III packs against either a glycine or serine on helix II, whereas in Type II ACPs the packing is reversed, with the bulky hydrophobe situated at the analogous 2154 position. Val2174 packs against both Leu2144 and Leu2149 in loop II, forming a well packed triad between helices II and III (Fig. 6B). However, whereas the Type II ACPs all show a highly similar loop II structure between Leu2144 and Ser2151, the rat ACP is distinctly different. The principle change appears to be the removal of Leu2149 from the hydrophobic core and its repositioning at the mouth of the cavity between helices II and III and packed against Leu2144. When analyzed using CASTp (54, 55) the rat FAS ACP structure has a cavity of only ~60 Å³, indicating that the rearrangement of loop II and occlusion of the entrance by Leu2149 essentially removes this cleft.

The analogous residues in Type II FAS ACPS, alanines or aromatic groups, are buried well within the core of the protein, drawing the loop away from the entrance to the cavity (Fig. 6, C and D). It is possible that formation of this hydrophobic triad (Leu2144, Leu2149, and Val2174) may prevent the binding of the acyl chain and lock the rat FAS ACP in a “closed” conformation.

One caveat is that our observations are based on the isolated rat ACP domain and the possibility remains that protein-protein interactions within the full synthase may modify its acyl chain binding properties. Recently the Ban group has reported the x-ray crystal structure of fungal FAS from the yeast S. cerevisiae in which the ACP has been locked into position at the KS active site (41). Electron density can be clearly ascribed to the phosphopantetheine moiety that is extended into the KS active site cleft and the ACP structure more closely resembles the expanded Type II decanoyl-ACP with a cavity of ~130 Å³. With analogy to the acyl binding function of the Type II FAS ACPS the authors suggest a “switchblade-type” mechanism in which the acyl chain initially non-covalently interacts with the ACP and then, upon recognition and binding to a domain, is flipped into the active site cleft allowing the chain to reach the catalytic residues. Similarly a 4 Å resolution crystal structure of the same synthase reveals an ACP in an “active” conformation separated from the KS catalytic cysteine by a distance that could be crossed by an extended phosphopantetheine arm (57). It is also suggested that a diffusion controlled process is enabled by flexible interdomain linkers and a swinging phosphopantetheine arm, and this plays a central role in delivering the appropriate ACP-bound substrate to the FAS active sites. Sequence and structural comparison of the fungal and mammalian FAS ACPS...
shows that fungal FAS ACP lacks the conserved hydrophobic triad. Instead there is a lysine in the homologous position to Leu2134 and glutamic acid in position Val2135, both of which are solvent exposed. However, the placement of the loop structure connecting helices I and II closely resembles that of the rat FAS ACP rather than the Type II ACPs (Fig. 6E). Clearly a high resolution acyl-bound fungal ACP structure will reveal whether it possesses the ability to bind acyl chains and shed light on the mechanisms employed in these related synthases.

It has been suggested that a major role of fatty acid burial in Type II FASs is to shield the acyl substrate from degradation by oxidation or hydrolysis. Such a function may not be necessary in a Type I system where the reaction chamber is formed by a series of contiguous active sites that may be partially shielded from solvent (3). Type II ACPs' acyl chain binding may also serve to induce a conformational change in the protein that communicates the nature of the acyl intermediate to the correct enzymes of the synthase. Clearly this argument does not hold for the rat FAS ACP that does not sense its bound intermediate. Complete exposure of the phosphopantetheine chain and bound fatty acid could be important for reaching into each of the catalytic domains without having to unnecessarily withdraw and insert the acyl chain into the ACP between each catalytic step. However, their remains a need for some degree of domain motion for the ACP to reach each catalytic site. Therefore the synapse may employ a "programmed" conformational change between the domains (for instance, through switching of the linker regions).

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