Heterologously expressed acyl carrier protein domain of rat fatty acid synthase functions in *Escherichia coli* fatty acid synthase and *Streptomyces coelicolor* polyketide synthase systems

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Introduction: Fatty acid synthases (FASs) catalyze the *de novo* biosynthesis of long-chain saturated fatty acids by a process common to eubacteria and eukaryotes, using either a set of monofunctional proteins (Type II FAS) or a polypeptide containing several catalytic functions (Type I FAS). To compare the features of a Type I domain with its Type II counterpart we expressed and characterized an acyl carrier protein (ACP) domain of the Type I rat FAS.

Results: An ACP domain of rat FAS was defined that allows expression of a small percentage of active holo-ACP both in *Escherichia coli*, increasing fivefold upon co-expression with an *E. coli* holo-ACP synthase, and in *Streptomyces coelicolor*. The rat ACP domain functions with some components of the *E. coli* FAS, and can replace the actinorhodin polyketide synthase (PKS) ACP in *S. coelicolor*A3(2). Purification of the rat ACP domain from *E. coli* resulted in loss of its functionality. Purified apo-ACP could be converted to its holo-form upon incubation with purified *E. coli* holo-ACP synthase *in vitro*, however, suggesting that the loss of functionality was not due to a conformational change.

Conclusions: Functionality of the recombinant rat ACP was shown in distantly related and diverse enzyme systems, suggesting that Type I and Type II ACPs have a similar conformation. A procedure was described that might permit the production of rat FAS holo-ACP for structural and further biochemical characterization.

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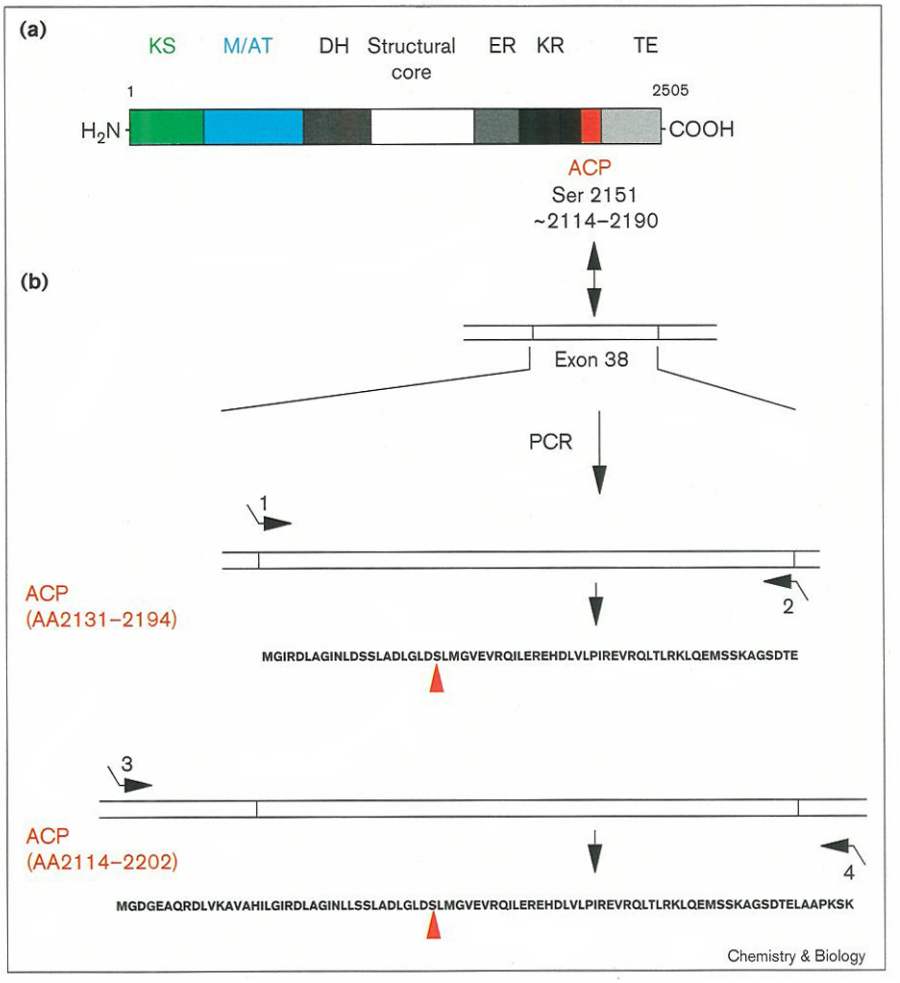
Introduction

The vertebrate fatty acid synthase (FAS, EC 2.3.1.85) catalyzes the *de novo* biosynthesis of long-chain saturated fatty acids from acetyl-CoA, malonyl-CoA and NADPH in an iterative process involving seven catalytic cycles. A single reaction cycle requires seven functions that are integrated into a large polypeptide, each forming a distinct domain within this Type I FAS system [1,2]. In contrast, the Type II FAS, such as that found in *Escherichia coli* and plant plastids, consists of several discrete monofunctional polypeptides, each of which bears reasonable sequence similarity to the corresponding domain of the Type I FAS [3,4]. Thus, the Type I and Type II FASs appear to be structurally and mechanistically related, possibly reflecting an early gene fusion that gave rise to the Type I FAS found in vertebrates. We are interested in exploring the relatedness between the domains of the Type I rat FAS and the corresponding monofunctional polypeptides of the Type II FASs.

The active vertebrate FAS is a dimer with two identical subunits orientated in a head-to-tail fashion to form two catalytic centers, each center being made up of functions from both subunits [1,5,6]. Initiation of fatty acid biosynthesis requires the malonyl/acetyl transferase (M/AT)-catalyzed loading of substrates onto specific sulfhydryl groups in the FAS complex. An acetyl residue from acetyl-CoA is transferred onto the cysteine sulfhydryl group in the active center of the β-ketoacylsynthase (KS), via the acyl carrier protein (ACP), and a malonyl residue is transferred to the sulfhydryl group of the 4′-phosphopantetheine prosthetic group of the ACP. KS then catalyzes a decarboxylative condensation to yield β-ketoacyl-ACP. The resulting β-ketogroup is reduced to a methylene functionality by the action of a ketoreductase (KR), a dehydratase (DH) and an enoylreductase (ER) prior to transfer back to the KS, and condensation with malonyl-ACP. During the reduction sequence, all the intermediates remain bound to the sulfhydryl group of the 4′-phosphopantetheine residue attached to the ACP. Once a chain length of 16 carbon atoms is reached, the thioesterase (TE) releases free palmitic acid from the complex [1,2]. These reactions are basically the same in all organisms regardless of the structural organization of the FAS complex. In *E. coli*, however, there is no TE and acyl chains are directly transferred from acyl-ACP to

Figure 1



Rat fatty acid synthase. **(a)** Linear domain map of rat FAS showing the active site residues. KS, β-ketoacylsynthase; M/AT, malonyl/acetyl transferase; DH, dehydratase; ER, enoylreductase; KR, ketoreductase; ACP, acyl carrier protein; TE, thioesterase. **(b)** Extents of the two ACP constructs. DNA sequences encoding rat ACP were amplified by PCR using pcRFAS601C [54] as the template and primers 1–4 as described in the Materials and methods section. ACP(aa2131–2194) corresponds to exon 38, and ACP(aa2114-2202) corresponds to the extended ACP domain. The red triangles indicate the expected attachment site for the 4′-phosphopantetheine residue.

membrane phospholipids. Furthermore, the *E. coli* FAS system contains more than one protein for some of the functions; for example, there are three known KS enzymes with different specificity for chain length and degree of saturation of the growing carbon chain [4].

Within the Type I FASs, the individual functions are catalyzed by distinct domains, which are thought to be separated by short linker regions of about 15 amino acids [2]. A linear domain map of the vertebrate FAS was first established by isolation of functional fragments after partial proteolysis of FAS [7–10]. The biochemical map was further confirmed after cloning and sequencing of cDNAs and genes for FAS from rat, chicken and man, which allowed comparison to known genes of Type II FASs [11–15]. Biochemical and molecular biological approaches were used to map the domain boundaries within rat FAS [16] (Figure 1a). The catalytic independence of some of the domains of vertebrate FAS was shown by isolation of functional domains after limited proteolysis [9,17] and by heterologous expression, in *E. coli*, of the TE domain [18,19] and, more recently, the M/AT and the DH domains [20,21] as individual proteins.

Functional analysis and sequence comparisons have shown that the Type I and Type II FASs are closely related [2]. It is, therefore, interesting to ask whether an independently expressed Type I domain would retain its partial activity and/or whether it could interact with the components of a Type II system; and whether the three-dimensional structure of such a domain would resemble that of the independent Type II protein. A first step towards this understanding was the definition of a functional domain, its heterologous expression and the functional analysis of the resulting protein.

We investigated the ACP domain of rat FAS. Type II ACPs, such as those found in *E. coli*, are small acidic proteins of about 80–100 amino acids that are well characterised not only biochemically but also at the level of their tertiary structures [22–28]. The active holo-ACPs have a 4′-phosphopantetheine moiety attached to a specific serine

residue in a well-conserved region; this reaction is catalyzed by the holo-ACP synthase [29]. We show here that it is possible to express an ACP domain of rat FAS in *E. coli*, that a small percentage is post-translationally modified, and that the rat holo-ACP is functional within the *E. coli* FAS system. The rat ACP domain was purified after overexpression in *E. coli*, and was post-translationally modified by the overexpressed *E. coli* holo-ACP synthase specific for the FAS ACP *in vitro*.

We have also explored the possibility of expressing the rat ACP in *Streptomyces coelicolor* to assess its functionality within a Type II polyketide synthase (PKS) system. The key reaction in polyketide biosynthesis catalyzed by the PKS is, as in fatty acid biosynthesis, the decarboxylative condensation of carboxylic acids. In contrast to fatty acid biosynthesis, however, the start of a new condensation round does not require reduction of the β-ketogroup. These reactions can be fully or partially omitted in polyketide biosynthesis, a factor contributing to the variety of structures found among polyketides. Characterisation of the enzymes involved in polyketide biosynthesis and the cloning of various PKS genes have revealed that the enzymes not only are functionally similar to those of fatty acid biosynthesis but also have a reasonable degree of structural similarity, and probably share a common evolutionary origin. As with FAS, different structural organisations of the enzyme systems are distinguished: PKSs consisting of a set of monofunctional proteins (Type II), such as those found in *S. coelicolor*; and multifunctional Type I PKSs, which are either of modular construction or the iterative type like vertebrate FAS [30–32]. We show that the rat FAS ACP domain can functionally replace, albeit with low efficiency, the ACP of a Type II PKS in *S. coelicolor*.

Results and discussion

Definition of a domain

The attachment site for the 4′-phosphopantetheine prosthetic group in rat FAS had been identified previously as Ser2151 [12]. This amino acid is encoded within exon 38, which corresponds to amino acids 2132 to 2194, of the rat FAS gene [12]. It has been suggested that multienzyme proteins of vertebrates, such as FAS, might have evolved by recombination within certain introns at or near the boundaries of monofunctional 'progenitor-genes' to give multidomain gene-fusion products [33]. From sequence comparisons with monofunctional (Type II) ACPs and those of other multifunctional FASs, and results from limited proteolytic digestion of rat FAS, it was proposed that the boundaries of the ACP domain lie approximately between amino acids 2114 and 2190, which corresponds, more or less, to a protein fragment encoded by exon 38 with an extension at its amino terminus [16]. Two approaches were taken to define a possible functional domain. First, a protein encoded strictly by exon 38 was expressed. In the second approach, a protein was expressed that corresponds to the fragment released by limited proteolysis of multifunctional FAS and has, compared to the exon 38-encoded protein, an amino-terminal extension of 17 amino acids and an additional eight amino acids at the carboxyl terminus.

To create a plasmid for expression of a protein encoded by exon 38 of rat FAS, a segment of DNA coding for amino acids 2131 to 2194 was constructed as described in the Materials and methods section. This plasmid was designated pET15b–ACP(aa2131–2194). It codes for a protein of 65 amino-acid residues with a calculated M_r of 7233.2 Da, with the start methionine lying 21 amino acids upstream of Ser2151. The calculated higher isoelectric point and the overall net charge show that this protein is much less acidic and less charged than the *E. coli* ACP (Table 1). Attempts to express the rat ACP(aa2131–2194) did not result in induction of any additional protein when compared to the pET15b vector control as judged by SERVA Blue R staining of a 20% native polyacrylamide (PA) gel (Figure 2a, lanes 1 and 2). Further attempts to improve the level of expression by incubation of the cultures for different times after induction, or by inducing at different temperatures, were not successful. Attempts at detection of an active ACP domain using the highly sensitive malonyl–CoA:ACP transacylase assay (see below) did indicate that occasionally some low level of induced ACP product was present, but the level varied from one induction to the next.

The second expression clone, pET15b–ACP(aa2114–2202), was constructed as described in the Materials and methods section. Similar to the clone for the exon 38-encoded protein, the second clone encodes a slightly acidic and charged protein, but contains 90 amino acids with a calculated M_r 9817.2 Da, in which the initial methionine is located 38 amino acids upstream of serine 2151 (Table 1). Expression from this plasmid led to induction of a soluble protein corresponding to approximately 10% of total *E. coli* extract, which could be visualized using 20% native polyacrylamide gel electrophoresis (PAGE; Figure 2a, lane 3). Although the codon usage of the first clone, comprising exon 38 only, was unaltered, seven of the first ten codons agree with an optimal codon usage in *E. coli* (codons at position 5, 7 and 8 are suboptimal [34]), so overall expression

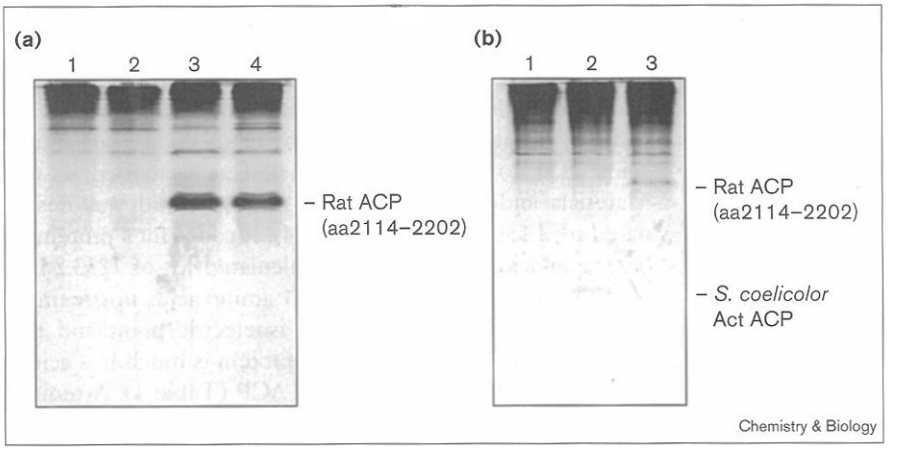
Table 1

Some physical characteristics of the rat ACP domains compared to those of the *E. coli* ACP.

	Rat ACP (aa2131–2194)	Rat ACP (aa2114–2202)	*E. coli* ACP
Amino acids (+ Met)	65	90	78
Molecular mass (Da)	7233.2	9817.2	8639.5
pI	4.59	5.21	3.81
Net charge (pH 7.0)	−4	−3	−14

Calculations were done for proteins with the start methionine present using algorithms from the UWGCG programme [65].

Figure 2



Expression of rat FAS ACP domains. Soluble fractions of each cell extract were analyzed on 20% native polyacrylamide gels. 20 μg of protein was loaded per lane. The gels were stained with SERVA Blue R. **(a)** Expression of rat ACP domain in *E. coli* BL21(DE3) cells transformed with: pET15b, lane 1; pET15b–ACP(aa2131–2194), lane 2; pET15b–ACP(aa2114–2202), lane 3; and pET15b–ACP(aa2114–2202)–*acpS*, lane 4. **(b)** Expression of rat ACP in *S. coelicolor*A3(2) CH999 (lane 1 is CH999 alone) transformed with: pRM5–rat ACP, lane 3; and pRM5, lane 2.

should not be affected. This would suggest that the additional amino acids, especially at the amino terminus, in the 90 amino acid ACP(aa2114–2202) might be important for folding to form a stable protein. Amino-terminal sequencing of the first eight amino acids of ACP(aa2114–2202) showed the expected sequence for approximately one third of the induced protein, the remainder having had the amino-terminal methionine removed (consistent with the predictions of Hirel *et al.* [35]). ACP(aa2114–2202) was used for all further studies.

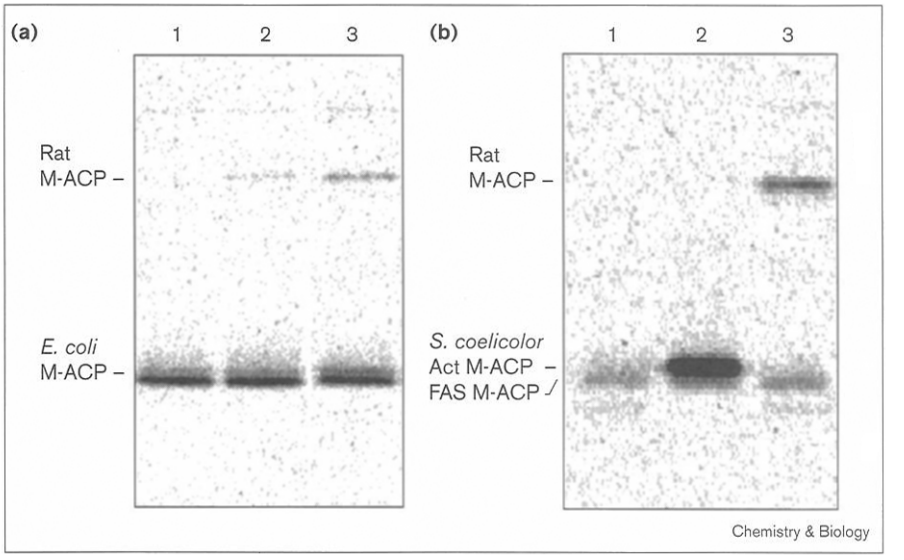
Evidence for post-translational modification of rat ACP(aa2114–2202) upon expression in *E. coli*

Functionality of the rat ACP(aa2114–2202) domain would require correct post-translational modification by addition of 4′-phosphopantetheine, transferred from coenzyme A (CoA) to the inactive apo-ACP to form the active holo-ACP, catalyzed by one of the *E. coli* holo-ACP synthases [29]. Holo-ACP would then be the substrate for the malonyl–CoA:ACP transacylase (MCAT), which transfers the malonyl residue from its CoA ester onto holo-ACP. The MCAT reaction was used to test the extent of phosphopantetheinylation of heterologously expressed rat ACP. Addition of [2-^{14}C]malonyl-CoA to *E. coli* crude cell extract in which the rat ACP was expressed resulted in labeling of an additional protein (Figure 3a, lane 2) in comparison to the reaction with *E. coli* BL21(DE3)/pET15b extract (Figure 3a, lane 1). This labeled band co-migrates with the induced ACP(aa2114–2202), identified by Coomassie Blue staining, under the same gel conditions (data not shown). No further increase in intensity of the rat ^{14}C-malonyl-ACP was obtained by prolonging the assay incubation time, by addition of *E. coli* DH5α crude extract to alter the ratio of MCAT:ACP or by increasing the amount of [2-^{14}C]malonyl-CoA added. The level of the ^{14}C-malonyl-ACP signal was directly proportional to the degree of expression of the rat ACP in the extract, however (data not shown). This suggests that, under the induction conditions used, the availability of rat holo-ACP is a limiting factor in the assay. Assuming that the *E. coli* ACP accounts for up to 0.25% of the total *E. coli* protein [4], and 100% of the *E. coli* ACP is present in its holo-form [36], comparison of the intensities of the labeled bands suggests that about 1% of the total induced rat ACP is in its holo-form. Attempts to increase the proportion of rat holo-ACP by induction at a lower temperature (25°C), or by incubation for an extended time after induction (up to 14 h at 25°C and 37°C), as previously described for some *Streptomyces* ACP [37,38], were unsuccessful.

The *acpS* gene encodes one of three phosphopantetheinyl transferases in *E. coli* required for activation of ACP-like proteins [39]; the gene product, holo-ACP synthase (ACPS), is required for *in vivo* modification of the FAS ACP of *E. coli*. Previous studies had shown that by increasing the level of *acpS* expression in *E. coli*, producing large quantities of PKS ACP, a dramatic increase in the amount of holo-ACP formed was observed [38]. Using a similar method, we engineered the rat FAS expression plasmid pET15b–ACP(aa2114–2202) so that *acpS* was cloned downstream of the rat ACP gene in the same transcriptional unit to create plasmid pET15b–ACP(aa2114–2202)–*acpS*. Results from induction of the plasmid ACP–*acpS* in *E. coli* are shown in Figure 2a, lane 4. The MCAT assay performed with extracts from induced cells of *E. coli* BL21(DE3)–pET15b–ACP(aa2114–2202)–*acpS* showed a fivefold stronger labeling of the ACP adduct (Figure 3a, lane 3). For all further experiments described below, extracts were prepared from cells co-expressing the rat ACP gene and the *E. coli acpS*.

The results from co-expression of rat ACP and *E. coli* ACPS showed that the holo-ACP synthase is capable of transferring the 4′-phosphopantetheine onto the heterologously expressed rat ACP. The increase in the amount of rat holo-ACP generated when co-expressed with the *E. coli acpS* was

Figure 3



Loading of the [2-^{14}C]malonyl residue from its CoA ester onto the rat ACP(aa2114–2202). The panels show autoradiographs of assay incubations after separation using 20% native PAGE and transfer to PVDF membrane. The MCAT assay relies on the presence of endogenous FAS malonyl:CoA–ACP transacylase in the crude cell extract. **(a)** MCAT assay in *E. coli* crude cell extracts prepared from induced cultures of *E. coli* BL21(DE3) transformed with: pET15b, lane 1; pET15b–ACP(aa2114–2202, lane 2; and pET15b–ACP(aa2114–2202)–*acpS*, lane 3. **(b)** MCAT assay in *S. coelicolor*A3(2) CH999 crude cell extracts: CH999 alone, lane 1; CH999/pRM5, lane 2; and CH999/pRM5–rat ACP, lane 3. M-ACP, malonyl-ACP; act, actinorhodin PKS.

not as great as with the PKS ACP [38], however, for which up to 90% holo-form was obtained. A possible reason for the inefficient modification in the *E. coli* is that the rat ACP(aa2114–2202) is a poor substrate for the *E. coli* ACPS. Gehring *et al.* [39] observed a correlation between high net charge of the ACP and efficiency as a substrate for the ACPS protein. The rat ACP(aa2114–2202) has an overall lower net charge, a significantly higher isoelectric point, and only 29% amino-acid identity with the *E. coli* ACP, so it may well be that differences in the conformation of the rat ACP could make it a poor substrate for the ACPS protein. Previously, a *Bacillus subtilis* peptidyl carrier protein (PCP) domain of a multifunctional peptide synthase, which exhibits a similar high isoelectric point and a similar low net charge to the rat ACP(aa2114–2202) (Table 1), was shown to be a better substrate for the *E. coli* EntD protein, the phosphopantetheine transferase specific for modification of the multifunctional EntF protein [40,41] of *E. coli* than for ACPS. One might therefore speculate that the *E. coli* ACPS is also not the best enzyme for modification of rat ACP(aa2114–2202). Reports of the expression in *E. coli* of the complete human FAS as a maltose-binding protein-fusion [42], or the ACP–TE fusion of chicken FAS [18], have shown that the heterologous proteins are 100% and about 60% phosphopantetheinylated, respectively: this might mean that expression of an ACP domain in the context of surrounding domains allows more efficient modification in *E. coli*, either by the *E. coli* ACPS or by one of the other holo-ACP synthases (such as EntD).

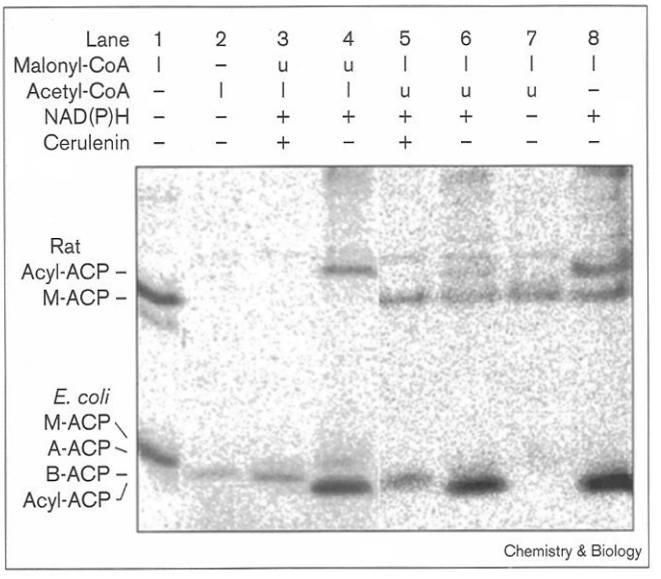
Functionality of the rat ACP in diverse enzyme systems

Functionality within the E. coli *Type II FAS system*

β-Ketoacylsynthase (KS) III of *E. coli* specifically catalyzes the initial condensation of fatty acid biosynthesis, between acetyl-CoA and malonyl-ACP, to form acetoacetyl-ACP, which is subsequently reduced, in three steps, to form butyryl-ACP. KS III can also act as an acetyl-CoA:ACP transacylase, though this is a relatively minor reaction *in vitro* compared with the condensation reaction [43]. KS I and KS II then catalyse further condensations to form long-chain fatty acids [4]. KS I has also been shown — at least *in vitro* — to circumvent the requirement for KS III by decarboxylation of malonyl-ACP to form acetyl-ACP and condensation of acetyl-ACP and malonyl-ACP. Post-Beittenmiller *et al.* [44] described the use of conformationally sensitive PAGE in the presence of 0.5 M urea to separate short-chain length acyl-ACP species. We used this system to explore further possible interactions between the rat FAS ACP and other components of the *E. coli* FAS system. Figure 4 shows the results of various incubations, described below, analyzed by 15% PAGE using 0.5 M urea and autoradiography.

In comparison to the *E. coli* control reactions (no rat ACP expressed, data not shown), additional bands with higher mobilities were obtained in some of the reactions in which the rat ACP was included. The result of an MCAT assay yielding [2-^{14}C]malonyl-ACP is shown in Figure 4, lane 1. Incubating *E. coli* crude extract with [1-^{14}C]acetyl-CoA, results in faint labeling of the *E. coli* ACP (Figure 4, lane 2), which is consistent with previous observations of a low acetyl-CoA:ACP transacylase activity [43]. An even fainter signal, corresponding to rat [1-^{14}C]acetyl-ACP, was only observed when high specific activity [1-^{14}C]acetyl-CoA was used (data not shown). When both substrates ([2-^{14}C]malonyl-CoA and acetyl-CoA) were used together no distinct band corresponding either to rat acetoacetyl-ACP or to *E. coli* acetoacetyl-ACP could be seen (Figure 4, lane 7). This is consistent with the known instability of acetoacetyl-ACP [45]. When acetyl-CoA, malonyl-CoA,

Figure 4



Formation of acyl-ACP species of the rat ACP domain in *E. coli* extracts. The assays were as described in the text, and the reaction products were analyzed by autoradiography after separation on 15% PAGE in the presence of 0.5 M urea and blotting to PVDF membrane. –, substrate, NAD(P)H or inhibitor not added; +, NAD(P)H or inhibitor added; u, addition of unlabeled substrate; l, addition of the ^{14}C-labeled substrate; M-ACP, malonyl-ACP; A-ACP, acetyl-ACP; B-ACP, butyryl-ACP; acyl-ACP, ACP labeled with intermediate-chain to long-chain saturated fatty acids.

NADH and NADPH were all used in the assay, a band of lower mobility was readily visualized, however (Figure 4, lanes 4,6). The altered mobility indicates that a new product had been formed by the action of one or more of the *E. coli* KS with the rat ACP; the presence of reducing agents allowed reduction of the β-ketoacyl-ACP condensation product to form a more stable acyl-ACP intermediate, which is amenable to analysis using native PAGE. To determine with which KS the rat ACP is capable of interacting, the same reaction was performed in the presence of cerulenin. This antibiotic specifically inhibits KS I and KS II (but not KS III) by covalently binding to the sulfhydryl group of the fatty acyl-binding site of the enzyme [3]. When conditions described for lanes 4 and 6 were used, in the presence of cerulenin (lanes 3,5), no rat ACP species was detected. *E. coli* butyryl-ACP was detected, however, indicating that the *E. coli* KS III was functional in this assay, modifying its own *E. coli* ACP but not the rat ACP. Thus, the rat acyl-ACP band seen in the absence of cerulenin (lane 4) is presumably the result of an interaction with KS I or KS II. When incubations were performed using only malonyl-CoA, NADH and NADPH, again the band of lower mobility was observed (Figure 4, lane 8), consistent with previous observations that KS I can decarboxylate malonyl-ACP [3] and initiate fatty acid carbon chain assembly, indicating that at least KS I is interacting with the rat ACP *in vitro*. In all of these reactions, the observed pattern of *E. coli* acyl-ACP species formed is consistent with what was expected [46], and serves as an internal control. It is worth mentioning that the acyl-ACP species generated from the rat ACP migrates more slowly than the malonyl-ACP, whereas for *E. coli* ACP the opposite is true (lane 4).

Several groups have described the use not only of coenzyme A but also of substrate analogs or acyl-CoAs by the *E. coli* holo-ACP synthase (ACPS) [38,39,47]. Using the ACPS reaction described below, it was possible to synthesize rat malonyl-ACP, acetyl-ACP and palmitoyl-ACP. Under the gel conditions used, these acyl-ACP standards co-migrated with the labeled product of the MCAT assay (Figure 4, lane 1), ACAT assay (lane 2) and FAS assay (lane 4) (data not shown); it was not possible to distinguish between different long-chain acyl-ACP, but the co-migration of palmitoyl-ACP and the 'acyl'-ACP synthesized in the FAS assay (lane 4) confirmed that at least butyryl-ACP was formed.

In *E. coli*, free long-chain fatty acids are reintegrated into fatty acid metabolism by transfer onto holo-ACP, catalyzed by the acyl-ACP synthetase [48]. To test the potential for interaction with the rat ACP, acyl-ACP synthetase was incubated with crude cell extract of *E. coli* BL21(DE3)/pET15b–ACP(aa2114–2202)–*acpS* and [1-^{14}C]palmitic acid The reaction products were analyzed by conformationally sensitive PAGE in the presence of 2.5 M urea. Under conditions where the *E. coli* [1-^{14}C]palmitoyl-ACP was readily detected, no rat [1-^{14}C]palmitoyl-ACP could be seen (data not shown). It is interesting to note that in the complete rat FAS (Figure 1), the ACP domain would not normally interact with an acyl-ACP synthetase domain nor a KS III, but would be expected to interact with the equivalent KS I, an MCAT and the reductases. Structural studies on the rat FAS ACP domain might shed light on the features that allow this discrimination in *E. coli*.

Functionality of the rat ACP(aa2114–2202) in a Type II PKS

The Type II PKS of *Streptomyces* spp. consists of, amongst other components, an ACP, a KS and an additional protein called the chain length factor. These three components form the minimal PKS. Polyketides are synthesized by condensation of acetyl and malonyl units to form long-chain products, which, unlike fatty acids, remain unreduced at most or all β-carbons. A more rigorous test for the functionality of the rat ACP domain was its interaction with components of such a PKS system. The *S. coelicolor*A3(2) CH999/pRM5 PKS expression system was developed for the construction and expression of recombinant PKS [49]. The parental pRM5 vector encodes the so-called 'minimal PKS', a ketoreductase, an aromatase and a cyclase [49] which together direct synthesis of the diffusible yellow–brown shunt product aloesaponarin II.

Expression is performed in *S. coelicolor*A3(2) CH999, in which the entire chromosomal actinorhodin (*act*) PKS gene cluster has been deleted.

ACP(aa2114–2202) was cloned in pRM5 precisely replacing the *act* ACP gene (*act*I–ORF3) in a similar manner to that described by Revill *et al.* [50] for the FAS ACP of S. *coelicolor* itself. The resulting plasmid, pRM5–rat ACP, when introduced into *S. coelicolor*A3(2) CH999 produced a small amount of diffusible yellow–brown pigment characteristic of aloesaponarin II (approximately 1% of that seen for the *S. coelicolor* CH999/pRM5 control), whereas *S. coelicolor* CH999 alone did not produce any pigment. The nature of the product synthesized was confirmed by extraction of the pigment and co-migration with standard aloesaponarin II on TLC plates (Figure 5). To confirm that the low amount of pigment production by CH999/pRM5-rat ACP was dependent on the presence of the rat ACP, an inactive pRM5-rat ACP derivative was constructed in which the attachment site for the prosthetic group (Ser2151) of rat ACP had been changed to an alanine residue. No pigment production was directed from this plasmid in CH999 (data not shown). Structural features and/or a low percentage of rat holo-ACP(aa2114–2202) might account for the inefficient replacement of the *act* ACP, but it is nevertheless significant that the rat FAS ACP domain could function in this system.

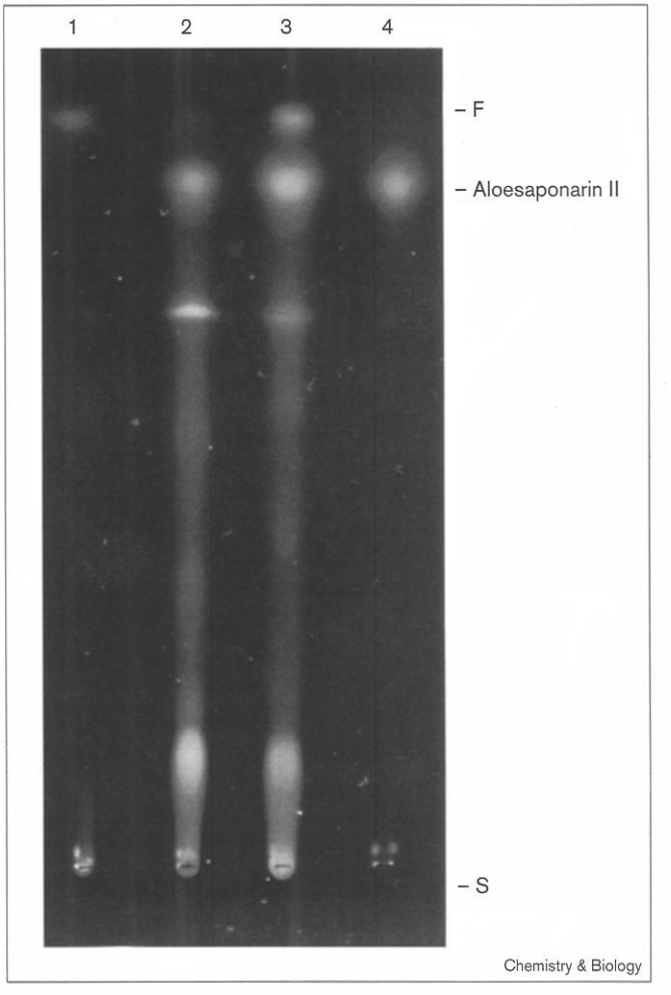
Analysis of the crude protein extract of *S. coelicolor* CH999/pRM5–rat ACP using Coomassie Blue staining of a 20% native polyacrylamide gel showed that the rat ACP domain is expressed at a moderately high level (Figure 2b, lane 3); this could provide an alternative source for the rat ACP domain for future analysis. By way of confirming the activity of the rat ACP domain, *S. coelicolor*/pRM5-rat ACP extracts were analyzed using the MCAT assay (Figure 3b), demonstrating that the rat ACP is post-translationally modified in *S. coelicolor* and interacts with the host's MCAT (lane 3).

Our biochemical characterization of the independently expressed rat ACP domain has shown that rat ACP(aa2114–2202) can interact as a Type II component within structurally and functionally divergent systems, suggesting a conservation in the structural characteristics of a Type I ACP domain and Type II FAS and PKS ACP [23–28].

Purification of the recombinant rat ACP(aa2114–2202) domain from *E. coli*

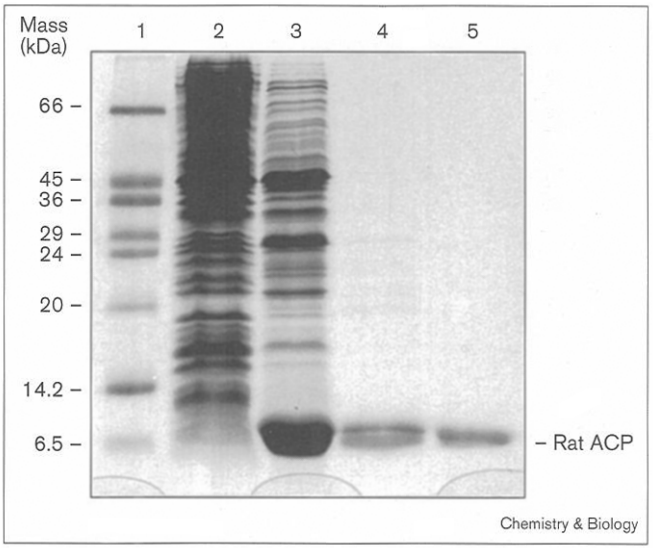
The *E. coli* strain BL21(DE3)/pET15b–ACP(aa2114–2202)–*acpS* was used to produce rat ACP(aa2114–2202). From a 500 ml culture of induced bacteria, 1.9 mg of the protein was purified in three steps, as described in the Materials and methods section, and illustrated in Figure 6. Functionality of the purified rat ACP(aa2114–2202) was tested in the MCAT assay by addition of *E. coli* DH5α cell extract and [2-^{14}C]malonyl-CoA. No labeling of the rat ACP was detected (data not shown). In MCAT assays with fractions obtained after the second purification step only a faint labeling of the rat ACP was detectable, whereas after the first step, a freeze–thaw extract, strong labeling was detectable even after repeated freezing and thawing. Further experiments showed that purification by other methods (such as simple gel filtration of crude extracts, or even just dilution of the crude *E. coli* extract), resulted in loss of the ability to be labeled by the *E. coli* MCAT using the [2-^{14}C]malonyl residue. Addition of bovine serum albumen (BSA), glycerol or ethylene glycol had no stabilizing effect on the rat ACP(aa2114–2202) in diluted *E. coli* extracts.

Figure 5



Thin layer chromatography (TLC) analysis of extracts from *S. coelicolor*A3(2) CH999, lane 1; CH999/pRM5, lane 2; CH999/pRM5–rat ACP, lane 3; authentic aloesaponarin II, lane 4. To detect aloesaponarin II produced by CH999/pRM5–rat ACP a highly concentrated extract was analyzed. PKS proteins encoded by pRM5 also produce other compounds, one of which was identified as 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid [49], and which shows no migration in the solvent system used. S, start; F, front.

Figure 6



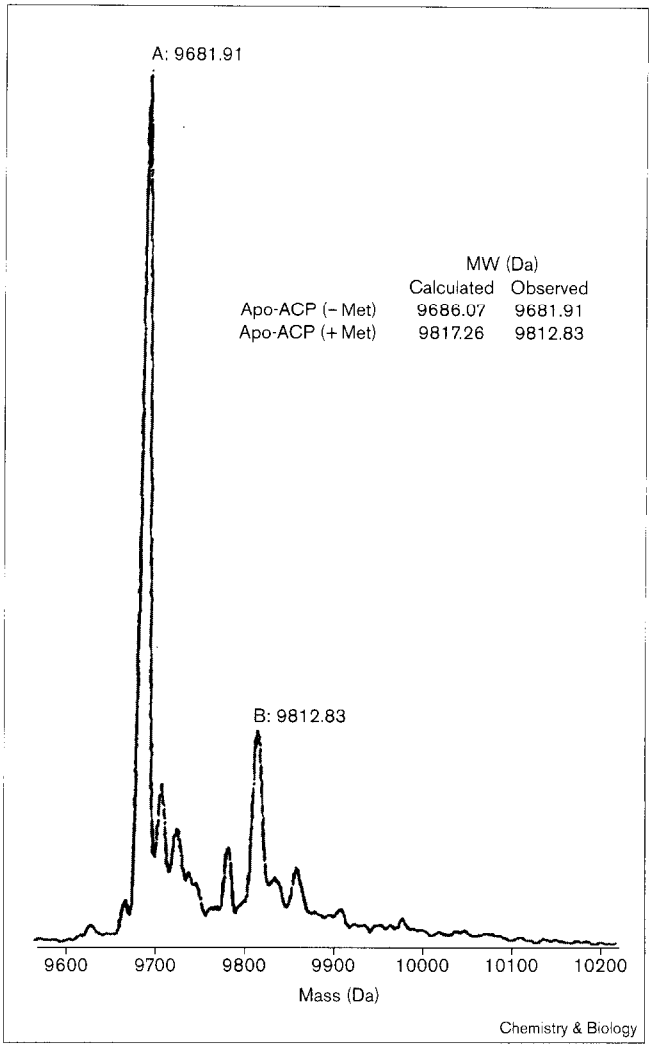
Purification of the heterologously expressed rat ACP domain (amino acids 2114–2202). Samples of the recombinant ACP protein were analyzed by SERVA Blue R staining of a 15% SDS PAGE at different stages of a typical purification procedure. Molecular mass standards, lane 1; extract from cells transformed with pET15b (20 μg), lane 2; freeze–thaw extract of cells transformed with pET15b–ACP(aa2114–2202)–*acpS* (20 μg), lane 3; combined fractions after preparative 20% native PAGE (10 μg), lane 4; combined fractions after anion-exchange chromatography on MonoQ (10 μg), lane 5.

Electrospray mass spectrometry (ESMS) of the purified protein gave a molecular mass of 9682.43 ± 4.20 Da and 9812.32 ± 1.38 Da for a second, minor species (Figure 7). The first molecular mass is in good agreement with the calculated mass of 9686.07 Da for the apo-ACP(aa2114–2202) lacking the amino-terminal methionine, whereas the molecular mass of the second species corresponds to the apo-ACP with the methionine still attached to the amino terminus. Partial removal of the methionine had already been shown to occur, by amino-terminal sequencing of the ACP (see above). As expected, no peak corresponding to a molecular mass of the holo-ACP(aa2114–2202) was detected. Possible explanations for this apparent loss of activity are: spontaneous oxidation of the sulfhydryl group of the 4′-phosphopantetheine prosthetic group; spontaneous elimination of the prosthetic group (both processes are known to occur with *E. coli* ACP at higher pH values and during prolonged incubation at higher temperatures [22]); or a change in the overall conformation of the ACP, which would make an interaction with the *E. coli* MCAT impossible.

In vitro conversion of purified rat apo-ACP into [1-^{14}C]acetyl-holo-ACP using the *E. coli* ACPS

To test whether the purified rat apo-ACP could be charged by the *E. coli* ACPS (therefore still possessing a conformation recognized by the modifying enzyme), the

Figure 7

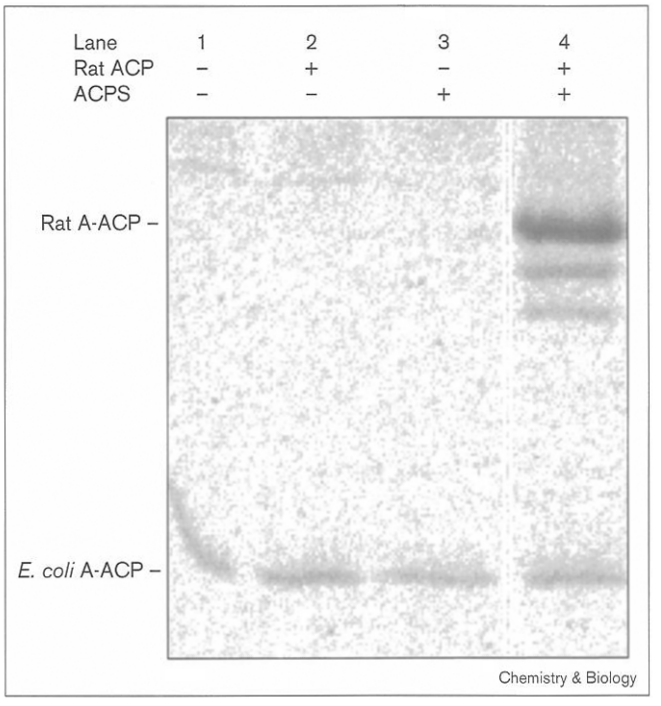


Electrospray mass spectrometry analysis of rat ACP(aa2114–2202).

ACP was incubated with [1-^{14}C]acetyl-CoA and a 30–60% $(NH_4)_2SO_4$ fraction of *E. coli* cell extract in which *acpS* had been overexpressed. A rat [1-^{14}C]acetyl-ACP was formed (Figure 8, lane 4) indicating that ACPS is capable of charging purified rat ACP by transferring the [1-^{14}C]acetyl-4′-phosphopantetheine residue from [1-^{14}C]acetyl-CoA. The fact that rat ACP can be modified by *E. coli* ACPS suggests that loss of functionality during purification was due to dephosphopantetheinylation rather than loss of ACP conformation.

To further optimize the transfer of the [1-^{14}C]acetyl-4′-phosphopantetheine residue onto the rat ACP, highly purified *E. coli* ACPS was used in the *in vitro* reaction. A time course of the reaction showed saturation of ACPS after 30 minutes of incubation at 37°C (data not shown), similar to the reaction with *E. coli* ACP [40]. Rat ACP

Figure 8



In vitro conversion of purified rat apo-ACP into [1-^{14}C]acetyl-holo-ACP upon incubation with *E. coli* holo-ACP synthase (ACPS) and [1-^{14}C]acetyl-CoA. The reaction products were analyzed by autoradiography after separation on 20% native PAGE and blotting on PVDF membrane. (–), *E. coli* extract added without ACPS expressed; (+), *E. coli* extract added with the ACPS expressed; and A-ACP, [1-^{14}C]acetyl-ACP. The lower bands are probably degradation products of [1-^{14}C]acetyl-holo-ACP.

concentrations (protein concentration based on Bradford [51]) higher than 4–8 μM inhibited the ACPS-catalyzed reaction. A similar substrate inhibition was also found for the *E. coli* ACP and the *Streptomyces* granaticin ACP [29,39]. When 1 μM rat ACP and 2.86 μM of the *E. coli* ACPS preparation were used, the molarity of labeled product corresponded to the molarity of ACP used in the assay, which is consistent with 100% conversion into the [1-^{14}C]acetyl-ACP form. This reaction might provide a way to synthesize rat holo-ACP for further analysis of the structural and biochemical characteristics of the rat ACP(aa2114–2202) domain.

Significance

Long-chain fatty acids are synthesized in eubacteria and eukaryotes using fatty acid synthase (FAS) complexes. Type II FASs, as found in bacteria, are composed of a set of monofunctional proteins, whereas the eukaryotic Type I FAS is a single polypeptide that contains several catalytic functions. We have defined an acyl carrier protein (ACP) domain of the multifunctional Type I rat FAS, which can be expressed as a soluble protein, for investigation of its capacity to replace Type II ACPs of fatty acid and polyketide synthases (PKS). Expression in *Escherichia coli* resulted in a small percentage of post-translationally modified holo-ACP, increasing fivefold upon co-expression with the *E. coli* FAS-specific holo-ACP synthase (ACPS). The rat ACP was capable of interacting with some components of the *E. coli* FAS, namely MCAT, KS I and the reductases, to form (at least) butyryl-ACP. It was further shown to functionally replace — though with low efficiency — a Type II ACP from the more distantly related and more diverse enzyme system, the actinorhodin PKS of *Streptomyces coelicolor*. The results suggest that the recombinant rat ACP domain can fold into a conformation similar to the *E. coli* FAS ACP and the *S. coelicolor* actinorhodin PKS ACP. Our results provide some support for the hypothesis that Type I FAS evolved from the Type II monofunctional system by fusion of the corresponding Type II genes. We also describe a three-step purification procedure that resulted in the isolation of inactive rat apo-ACP. The purified rat apo-ACP was activated by overexpressed *E. coli* ACPS using [1-^{14}C]acetyl-4′-phosphopantetheine *in vitro*. Using purified ACPS fraction in the *in vitro* reaction resulted in 100% conversion of the rat ACP into the acetyl-ACP form, which might provide a way to synthesize sufficient rat ACP for further structural and biochemical investigations.

Materials and methods

Bacterial strains and plasmids

E. coli DH5α (supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used as the standard cloning host [52]. Expression of proteins from T7-controlled pET vectors was performed in *E. coli* BL21(DE3) (F$^-$ *ompT hsdS gal* r_B^- m_B^-) [53]. The T7-based expression vector pET15b was obtained from Novagen. The full-length rat FAS plasmid pcRFAS601C was described by Kupfer *et al.* [54]. The plasmid containing the *E. coli acpS* gene was described previously [38]. *E. coli* BL21(DE3)/pDPJ was described by Lambalot and Walsh [29]. *E. coli* ET12567 (*dam*$^-$ *dcm*$^-$ *hsdM*$^-$) was used to isolate unmethylated DNA [55]. *S. coelicolor* A3(2)CH999 (*proA1 argA1* SCP1$^-$ SCP2$^-$ *redE60 Δact*) and plasmid pRM5, directing the expression of the actinorhodin minimal PKS and associated subunits, are described by McDaniel *et al.* [49]. Standard conditions for culture of *Streptomyces* were as described by Hopwood *et al.* [56].

General DNA techniques

Recombinant DNA techniques using *E. coli* were performed as described previously [57]. Plasmid DNA was prepared according to Jones and Schofield [58]. DNA techniques using *S. coelicolor* were performed as described previously [56]. DNA for sequencing was prepared using Qiagen plasmid purification columns (QIAGEN) according to the manufacturer's recommendations. Ampli Taq^R (Perkin Elmer) was used in PCR according to the manufacturer's recommendations. Sequencing reactions were carried out using the Applied Biosystems cycle sequencing PRISM mix with Taq FS enzyme in an Applied Biosystems 373 sequencing machine.

General protein techniques

Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [59] using a Mini-Protean II System (Bio-Rad). Native PAGE using 20% (w/v) total acrylamide with bis-acrylamide at 1.0% of the final volume was performed as described previously [50]. Conformationally sensitive PAGE in the presence of urea was performed according to Post-Beitenmiller *et al.* [44]. Proteins

were visualized by staining with SERVA Blue R (SERVA, Germany). Low range protein standards were obtained from Sigma. Protein concentrations were measured according to Bradford [51]. Amino-terminal amino acid sequence of the ACP was obtained after partial purification, separation on a 15% (w/v) SDS–PAGE and transfer onto ProBlott membrane (Applied Biosystems). Purified proteins were analyzed by electrospray mass spectrometry (ESMS) as described previously [37].

Construction of expression clones encoding the rat ACP domain

The ACP domain was amplified by PCR using pcRFAS601C [54] as a template. Primers used for the construction of pET15b–ACP(aa2131–2194) were: (1) 5′-primer: 5′-*CCCATGAGCTCTGTACCATG*GGCATCCGCGACCTCGCAGG-3′; (2) 3′-primer: 5′-*TTGGATCCTTA*CTCAGTGTCTGAGCCAGCCTTGG-3′. For the construction of pET15b–ACP(aa2114–2202) the following primers were used: (3) 5′-primer: 5′-*GCTGTGGCCATG*GGTGACGGTGAAGCTCAGCGTGATCTGG-3′; (4) 3′-primer: 5′-*GGCCTGCTTCAGGGATCCTTA*ATTCTTGGACTTGGGGGC-3′ (nucleotides deviating from the original gene sequence are italicized). The 5′-primers introduced an ATG codon as well as a *Nco*I restriction site. The 3′-primer introduced a stop codon as well as a *Bam*HI site. In clone pET15b–ACP(aa2114–2202) the first seven codons were changed to synonymous codons present in highly expressed *E. coli* genes [34]. The PCR used the following cycles: 1 × 5 min 94°C, 30 × (2 min 60°C, 1 min 72°C, 1 min 94°C), 1 × (2 min 60°C, 7 min 72°C). PCR products were digested with *Nco*I and *Bam*HI prior to cloning into pET15b. Sequencing was performed with the pET forward and reverse primers.

For co-expression of the rat ACP with the *E. coli acpS* gene the plasmid pET15b–ACP(aa2114–2202) was digested with *Bam*HI and the *Bgl*II fragment from the plasmid containing the *acpS* gene [38] was introduced to create pET15b–ACP(aa2114–2202)–*acpS*. Expression of the rat ACP was performed in *E. coli* BL21(DE3) as described previously [53]. Expression was performed at 37°C for 4 h unless otherwise stated. Because of plasmid instability, the co-expression plasmid, pET15b-ACP(aa2114–2202)–*acpS*, was grown from freshly transformed *E. coli* BL21(DE3) colonies until slightly turbid. Several glycerol stocks were kept at −80°C and directly used to inoculate LB broth with ampicillin for inductions.

Malonyl-CoA:ACP transacylase (MCAT) assay

Induced *E. coli* cell pellets were washed with 50 mM potassium phosphate pH 7.2, 0.5 M NaCl, followed by a wash with 50 mM potassium phosphate pH 7.2. Cell pellets from 10 ml culture were resuspended in 50 mM potassium phosphate pH 7.2, 10% glycerol, 2 mM dithiothreitol (DTT) and broken by sonication in a Soniprep 150 (MSE) at 50% power for four cycles of 6 s. The supernatant fraction was collected by centrifugation and dialysed overnight against 50 mM potassium phosphate pH 7.2, 10% glycerol, 2 mM DTT at 4°C. Protein extracts from *S. coelicolor* CH999 mycelium were obtained by sonication for six cycles of 10 s after growth on solid medium until pigment production was visible for CH999/pRM5. The MCAT assays were modified from Crosby *et al.* [37]; 5–10 μg of total cell protein and 0.86 nmol [2-^{14}C]malonyl-CoA (2.2 GBq/mmol; DuPont NEN) were used. The assay was analyzed by autoradiography, after separating the reaction mixture on a 20% native PAGE and transferring onto a 0.2 μm pore-size polyvinylidene difluoride (PVDF) membrane (Trans-Blot, Bio-Rad), using a type BAS-IIIs imaging plate and BAS 1000 Bioimaging analyzer (Fuji).

Acetoacetyl-ACP synthase assay

E. coli BL21(DE3)/pET15b–ACP(aa2114–2202)–*acpS* was grown and induced as described above. The washed cells were broken by sonication, and a 40–70% $(NH_4)_2SO_4$ cut was prepared (fraction A, which contains both the acetoacetyl-ACP synthase [22] and the rat ACP). Precipitated proteins were stored in aliquots at −80°C, and desalted on a Sephadex G25 column prior to use. The reaction was performed in a final volume of 20 μl modified according to the method described by Jackowski *et al.* [46] with 20 mM potassium phosphate pH 7.2, 2 mM DTT, 50 μg/ml cerulenin (in ethanol), 1 mM NADH, 1 mM NADPH, 125 μM malonyl-CoA (for ^{14}C-labeled substrate with 20 Bq/nmole), 150 μM acetyl-CoA (for ^{14}C-labeled substrate with 154 Bq/nmole) and 50-80 μg of total protein. The reaction was started by addition of the ^{14}C-labeled substrate after preincubation for 10 min at room temperature and further incubation for 10 min at 37°C. The assay was analyzed by 15% (w/v) conformationally sensitive PAGE in the presence of 0.5 M urea, followed by blotting to PVDF membrane and autoradiography as described for the MCAT assay.

Acyl-ACP synthetase assay

The assay was performed using a 40–70% $(NH_4)_2SO_4$ cut prepared as described for the acetoacetyl-ACP synthase assay. The assay conditions were as described by Rock and Cronan [48] using 2.4 kBq [1-^{14}C] palmitic acid (1.2 nmol; DuPont, NEN) per assay and adding 0–0.3 mU acyl-ACP synthetase (Sigma) per assay. The reaction mixture was analyzed by 15% (w/v) conformationally sensitive PAGE in the presence of 2.5 M urea, followed by transfer to PVDF membrane and autoradiography.

Construction of the hybrid rat FAS ACP domain/act PKS expression system and its expression in S. coelicolor

The ACP(aa2114–2202) domain was amplified by PCR using pcRFAS601C [54] as the template. The 5′-primer was designed to introduce (in order): an *Xba*I site, a *Streptomyces* ribosome-binding site, an ATG start codon, and the first eight codons changed to synonymous codons but which are present in highly expressed genes in *Streptomyces* spp. [60]. The sequences were as follows: 5′-*ATAGGTCTAGAACTGGAGAAAGCCATG*CACGGCGACGGCGAGGCCCAGAGGGAT-3′. The 3′-primer was designed to introduce a stop codon and a *Pst*I site as follows: 5′-GCTGGGCCTGCT*GCAGGGAT*GT*TCA*ATTCTTGGAGTTGG-3′ (bases deviating from the original sequences are italicized). The PCR cycles were: 1 × 5 min 94°C, 30 × (2 min 60°C, 1 min 72°C, 1 min 94°C), 1 × (2 min 60°C, 7 min 72°C). The PCR product was digested with *Xba*I and *Pst*I and cloned in pBluescriptSK(+). DNA sequencing confirmed that the amplified gene had the expected sequence. The *Xba*I/*Pst*I rat ACP fragment was cloned in place of the *act* ACP gene in pIJ5639 [61] to create pIJ5639–rat ACP. The *Xba*I/*Eco*RI fragment was isolated and cloned into pRM5 (in place of the original *Xba*I/*Eco*RI fragment; carrying the set of *act* PKS and cyclase genes) to create pRM5–rat ACP (now *act* PKS in which *act* ACP is replaced by rat ACP). For construction of a rat ACP, in which the attachment site for the prosthetic group is mutated from a serine to an alanine residue, the 'Quick Change™ Site-Directed Mutagenesis Kit' (Stratagene) was used according to the manufacturer's recommendations with the following oligonucleotides: 5′-CCTCGGCCTGGACGCGCTCATGGGTGTG-3′ and 5′-CACACCCATGAGCGCGTCCAGGCCGAGG-3′. Prior to introduction into *S. coelicolor* CH999 (the Δ*act* PKS expression host), the plasmid was passaged through *E. coli* ET12567.

Extraction of pigment from S. coelicolor *CH999 colonies*

Aloesaponarin II was isolated according to Bartel *et al.* [62] and analyzed on a thin layer chromatography (TLC) plate with co-migration of standard aloesaponarin II on silica gel TLC plates (Merck) in ethyl acetate/1% HCl.

Purification of recombinant ACP(aa2114–2202)

Cells from 500 ml culture of BL21(DE3)/pET15b–ACP(aa2114–2202)–*acp*S were harvested and washed with 20 mM Tris-HCl pH 7.4, 0.5 M NaCl, followed by a washing with 20 mM Tris-HCl pH 7.4. For extraction of proteins, the freeze–thaw method described by Johnson and Hecht [63] was used with 20 mM Tris-HCl pH 7.4, 2 mM DTT as extraction buffer. DNA from the clear supernatant was precipitated by adding ice-cold 10% streptomycin sulfate to a final concentration of 1%, further stirred for 1 h in ice water and centrifuged. The clear super-

natant was loaded on a 20% (w/v) preparative native PAGE (Prep Cell-491; Bio-Rad) as described by Revill *et al.* [64]. Proteins were eluted using 20 mM Tris-HCl pH 7.4. Fractions containing the rat ACP were pooled and separated from contaminating *E. coli* proteins by anion-exchange chromatography (Mono Q HR5/5; Pharmacia). The ACP was eluted at 0.5 M KCl in a linear salt gradient of 0-1 M KCl. Purification was monitored using 15% SDS–PAGE and the MCAT assay. Alternatively, for the *in vitro* reaction with *E. coli* ACPS, rat ACP was purified by freeze-thaw extraction, ultracentrifugation (60 minutes, 100,000 rpm, 4°C), streptomycin sulfate precipitation and anion-exchange-perfusion chromatography (HQ). This procedure resulted in about 95% pure rat ACP.

Holo-ACP synthase (ACPS) assay

E. coli ACPS was expressed from pDPJ in *E. coli* BL21(DE3) as described previously [29]. A 30–60% $(NH_4)_2SO_4$ cut of the crude extract or purified ACPS (purification as described recently [29]) was used in the ACPS assay, performed according to Cox *et al.* [38] except that reactions were performed in a final volume of 20 µl with 200 µM [1-^{14}C]acetyl-CoA (74 Bq/nmole). The assays were analyzed either by autoradiography as in the MCAT assay or by tricholoroacetic acid precipitation as described previously [37].

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