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New partners of acyl carrier protein detected in *Escherichia coli* by tandem affinity purification

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Abstract **We report the first use of tandem affinity purification (TAP) in a prokaryote to purify native protein complexes, and demonstrate its reliability and power. We purified the acyl carrier protein (ACP) of *Escherichia coli*, a protein involved in a myriad of metabolic pathways. Besides the identification of several known partners of ACP, we rediscovered ACP/MukB and ACP/IscS interactions already detected but previously disregarded as due to contamination. Here, we demonstrate the specificity of these interactions and characterize them. This suggests that ACP is involved in additional previously unsuspected pathways. Furthermore, this study shows how the TAP method can be simply used in prokaryotes such as *E. coli* to identify new partners in protein–protein interactions under physiological conditions and thereby uncover novel protein functions.**

Key words: Acyl carrier protein; Tandem affinity purification; IscS; MukB; Complex purification; *Escherichia coli*

1. Introduction

In the post-genomic era, many people are looking for ways to build systematically and in the most automated way possible protein–protein interaction networks. This would open the way for a chain of predictions: open reading frame function, complex formation, and supramolecular organization of the proteome. Among such methods are two-hybrid studies that provide large-scale interaction data [1,2], but that are often artefactual, especially when performed in heterologous organisms, and restricted to the description of pairs of proteins. Others are developing direct purification of protein complexes on a proteome-wide scale to gain access to the multiple interactions of a particular protein at one time, in the studied organism, and under physiological conditions [3]. This has been rendered feasible by the advent of ultrasensitive mass spectrometric protein identification methods [4]. Naturally, all the techniques developed to build these interaction networks can also be used for specific research, i.e. for answering the questions 'What are the partners of my favorite protein?', 'Can this tell me something about its function?'. In this case, the screening of libraries against one bait in a two-hybrid system or the purification of one recombinant protein would give starting points for specific studies. Concerning prokaryotes, the only large-scale interaction network published so far has been obtained for *Helicobacter pylori* by a two-hybrid method in yeast [5]. Two other large-scale studies have been obtained by purification of protein complexes in yeast, first using the tandem affinity purification (TAP) method [6] and the second using the Flag epitope [7]. The TAP method [8] has also been used for specific studies with great success [9,10] and reports of studies performed in other organisms than yeast are appearing in the literature [11]. However, the principle of the TAP method has not been reported in prokaryotes so far although it is clear that there are no more theoretical difficulties to use it in prokaryotes than in *Saccharomyces cerevisiae*.

We have chosen to use the TAP method to purify complexes containing the acyl carrier protein (ACP) from *Escherichia coli*. ACP is an evolutionarily conserved, very abundant molecule (6×10^4 molecules/cell), involved in a myriad of metabolic pathways in the cell: fatty acid and lipid biosynthesis, lipid A formation, membrane-derived oligosaccharide biosynthesis, acylation of the HlyA toxin, carrier of lipoic acid, etc. [12]. It is composed of 77 residues and is modified post-translationally by addition of a 4′-phosphopantetheine prosthetic group that is covalently attached by phosphodiester linkage to the hydroxyl group of a serine residue, Ser36 in *E. coli*. Acyl chains are linked to ACP by thioester linkage to the unique sulfhydryl group of the prosthetic group [12]. Due to its participation in numerous metabolic pathways, ACP must interact with various proteins. The unusually mobile structure of ACP is best represented as a dynamic equilibrium of two conformers [13], a property that would predispose it to multiple interactions [14], but the nature of the specific recognition between ACP and its many protein partners is not understood.

The choice of ACP to test the TAP method in prokaryotes thus appears very interesting. Using N-terminal and C-terminal fusions of ACP, we were able to identify many interactions in vivo. Beside expected enzymes of the fatty acid synthesis pathway, we identified three proteins, SpoT, IscS, and MukB, whose functions have not been described to date to require ACP. We further showed that the IscS and MukB interactions were specific and required the functional Ser36

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Abbreviations: ACP, acyl carrier protein; TAP, tandem affinity purification; CBP, calmodulin binding peptide; DTT, dithiothreitol; PCR, polymerase chain reaction; PAP, peroxidase–antiperoxidase complex

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residue of ACP. Furthermore, we showed that the interaction between IscS and ACP involved the formation of a disulfide bond between the thiol group of the ACP prosthetic group and the Cys328 residue of IscS, a crucial residue in the active site of IscS.

2. Materials and methods

2.1. Strains, media, and antibodies

W3110 [15] is the wild type strain used for all purifications and co-purification experiments except AZ5372 (mukB::kan) [16] and iscS::kan (L. Loiseau, unpublished) strains. Cells were grown at 37°C on Luria–Bertani (LB) agar plates or in liquid medium [17]. The plasmids were maintained with ampicillin (100 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (30 μg/ml). Anti-MukB [18], anti-IscS [19], and peroxidase–antiperoxidase complex (PAP; Sigma) were used respectively to detect MukB, IscS, and the Prot.A (or TAP) tagged proteins.

2.2. Plasmid constructions

All plasmids used are listed in Table 1. The *NtermTAP* sequence was amplified by polymerase chain reaction (PCR) from plasmid pBS1761 and inserted in pBAD24 using the *Nco*I and *Sal*I sites to give pEB327. The *acpP* sequence was amplified by PCR from colonies of W3110 and inserted in pEB327 using the *Sal*I and *Hin*dIII sites to give pEB293. The *CtermTAP* sequence was obtained from plasmid pBS1479 by *Nco*I/*Hin*dIII digestion, the *acpP* sequence was amplified by PCR from colonies of W3110, digested by *Eco*RI/*Nco*I, and both fragments were inserted in pBAD24 digested by *Eco*RI/*Hin*dIII to give pEB304. The *CBP-acpP* sequence was amplified by PCR from pEB293 and inserted in pBAD24 by *Nco*I/*Hin*dIII to give plasmid pEB540. The *acpP* sequence was amplified from pEB293 and inserted in pBAD-6His-A by *Xho*I/*Hin*dIII to give plasmid pEB539. *iscS* sequence from pIscS digested by *Sal*I/*Xho*I was inserted into pBAD-6His-B digested by *Xho*I to give pEB556. The *Prot.A* sequence was obtained by *Sac*I/*Hin*dIII digestion from plasmid pBS1365, the *acpP* sequence was amplified by PCR from colonies of W3110, digested by *Eco*RI/*Sac*I, and both fragments were inserted in pBAD24 digested by *Eco*RI/*Hin*dIII to give pEB351. Fragment *Hin*dIII/*Eco*RV from pEB351 was transferred in pACYC184 to give pEB370. Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) to obtain pEB578 and pEB586 plasmids (see Table 1).

2.3. TAP method

ACP–TAP and TAP–ACP complexes were purified using the standard yeast TAP procedure [20] adapted to *E. coli*: a 400 ml culture in LB is induced at $OD_{600nm} = 0.8$ during 60 min with 0.001% arabinose. Cells are pelleted, washed in water and then resuspended in 10 ml buffer IPP150-Prot.A (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% NP40). After passage through a French press, the extract is centrifuged for 30 min at 20 000 × *g*. 10 ml of extract is incubated on 200 μl IgG-Sepharose beads (Pharmacia) washed in buffer IPP150-Prot.A for 2 h at 4°C. After three washes of 10 ml with buffer IPP150-Prot.A, the beads are incubated for 2 h at 16°C in 1 ml of rTEV buffer (Gibco) with 100 U of rTEV (Gibco). This 1 ml fraction is then incubated for 60 min on 200 μl of calmodulin beads (Stratagene) washed in IPP150-calmodulin binding buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% NP40, 1 mM Mg-acetate, 2 mM $CaCl_2$). After three washes of 10 ml of IPP150-calmodulin binding buffer, the complex is eluted with five times 200 μl of elution buffer where the 2 mM $CaCl_2$ is replaced by 2 mM EGTA.

2.4. Identification of proteins by mass spectrometry

Protein bands were excised from the gel, reduced, alkylated and digested overnight with trypsin [21]. The proteins were identified by MALDI peptide mapping with a MALDI-TOF spectrometer Voyager DE-RP (PerSeptive).

2.5. Co-purification on cobalt beads or calmodulin beads

The experiments were performed on extracts of W3110 cells transformed with a pBAD6His derivative (ACP and IscS and their mutants) and when required a PACYC184 derivative (ACP-Prot.A). From a 200 ml culture in LB induced for 60 min at 37°C with 0.01% arabinose, an extract was prepared by sonication in 10 ml buffer 1 (20 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.2% NP40, 10 mM imidazole). After centrifugation for 30 min at 20 000 × *g*, glycerol was added to 15% final concentration and the extract was frozen in liquid nitrogen. For each co-purification assay, 1 ml of extract was incubated on 30 μl of Talon beads (Clontech) washed in buffer 1. After 60 min incubation at 4°C, the beads were washed three times in 1 ml of buffer 1, then resuspended in 80 μl of loading buffer, and then heated for 10 min at 96°C.

The same procedure was followed for the co-precipitation tests on calmodulin beads (Stratagene), using the calmodulin binding buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% NP40, 1 mM Mg-acetate, 2 mM $CaCl_2$).

2.6. Miscellaneous

Standard methods were used for DNA manipulations [22]. DNA sequences were determined using the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electrotransfer onto nitrocellulose membranes, and Western blot analysis were performed as previously described [23–25].

3. Results

3.1. Purification of ACP containing complexes by the TAP method

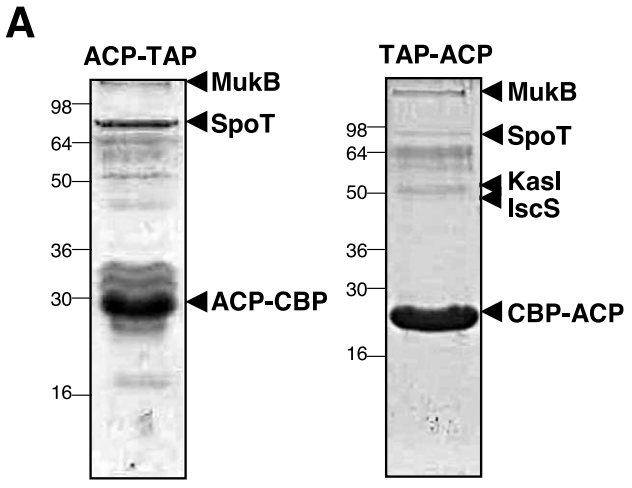
In order to perform the TAP method in *E. coli*, the TAP

Table 1
Plasmids used in this study

Name	Relevant characteristics	Reference
pBAD24	ColE1 replication origin, P_{BAD} promoter	[26]
PBAD-6His-A	ColE1 replication origin, P_{BAD} promoter, 6His	InVitrogen
PBAD-6His-B	ColE1 replication origin, P_{BAD} promoter, 6His	InVitrogen
pACYC184	p15A replication origin, Cm^R	Biolabs
PBS1365	*Prot.A-K.l.URA-Amp*^{R}	[39]
pBS1761	*Amp*^{R}*-K.l.TRP1-GAL1-NtermTAP*	[20]
pBS1479	*CtermTAP-K.l.TRP1-Amp*^{R}	[8]
pIscS	*iscS*	L. Loiseau
PAX851	*mukF mukE mukB*	[40]
pEB327	pBAD24-*NtermTAP*	this work
pEB293	pBAD24-*NtermTAP-ACP*	this work
pEB304	pBAD24-*ACP-CtermTAP*	this work
pEB351	PBAD24-*ACP-Prot.A*	this work
pEB370	PACYC-*ACP-Prot.A*	this work
PEB540	PBAD24-*CBP-ACP*	this work
pEB539	pBAD-*6His-ACP*	this work
pEB578	PBAD-*6His-ACP(S36T)*	this work
pEB556	pBAD-*6His-IscS*	this work
pEB586	PBAD-*6His-IscS(C328S)*	this work

tags (N-terminal or C-terminal tag) were introduced in the pBAD24 plasmid [26]. In this plasmid, genes are under the control of the P_{BAD} promoter of the *araBAD* operon that permits tight control of the expression of the recombinant protein by arabinose. Two constructs were used, with the TAP tag before the N-terminus or after the C-terminus of ACP. Expression of the recombinant proteins was verified using the PAP antibody that binds the Prot.A moiety of the TAP tag (data not shown). TAP purifications were performed following the classical procedure (see Section 2). ACP-associated proteins purified from 400 ml cultures were concentrated, fractionated by SDS–PAGE, and detected by Coomassie blue staining. Fig. 1A shows the typical purification patterns that we obtained. ACP–CBP or CBP–ACP were the major proteins visible in the gels, but many other bands were observed, ranging from 16 to 150 kDa. The various bands were excised from the gels and the proteins were identified by MALDI peptide mapping [21]. The proteins that we identified with certainty in diverse purifications are listed in Fig. 1B.

Among the identified proteins, DnaK and TnaA are proteins that we found several times in purifications of proteins



B

already known and characterized interactions	KasI (43 kDa) : 3-oxoacyl-[acyl-carrier-protein] Synthase I KasII (43 kDa) : 3-oxoacyl-[acyl-carrier-protein] Synthase II
already known but uncharacterized interactions	MukB (170kDa) : Structural Maintenance of Chromosome IscS (45 kDa) : Cysteine Desulfurase
new interaction	SpoT (79 kDa) : ppGpp synthase II
aspecific interactions	DnaK (69 kDa) : Heat Shock Protein 70 kDa Protein TnaA (53 kDa) : Tryptophanase

Fig. 1. Purification of ACP containing complexes by the TAP method. A: ACP–TAP or TAP–ACP (in W3110) were purified from 400 ml cultures in LB, induced for 60 min at 0.001% arabinose. Extracts were done by French press, the TAP protocol was followed (cf. Section 2) and the totality of the purifications was loaded on a single lane of a 12% SDS–PAGE. The gels were stained with Coomassie blue. The proteins spotted at the right of the gels are those identified by mass spectrometry on these very same gels that are shown. Molecular weights are indicated on the left. B: List of all the proteins identified in various ACP–TAP and TAP–ACP purifications by mass spectrometry. The proteins listed were repeatedly and significantly identified by mass spectrometry in independent experiments.

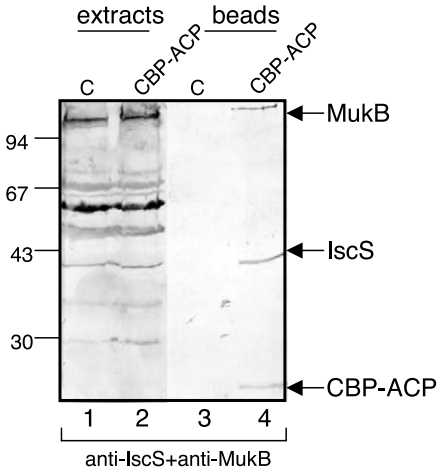


Fig. 2. Confirmation of co-purification of MukB and IscS with ACP. Cultures of W3110 or CBP–ACP/W3110 were induced for 60 min with 0.01% arabinose at $OD_{600nm} = 0.8$ at 37°C. Final OD_{600nm} was approximately 2. Extracts were done in buffer 1 (20 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.2% NP40, 10 mM imidazole) at a concentration of 50 uOD_{600nm}/ml by sonication. 30 μl of calmodulin beads were used for each test with 1 ml of extract. Lanes 1 and 2: extracts; lanes 3 and 4: elution 2 mM EGTA+ 500 mM NaCl; lanes 1 and 3: control W3110; lanes 2 and 4: CBP–ACP/W3110. Samples were analyzed by 10% SDS–PAGE and Western blot with an anti-MukB antibody followed by an anti-IscS antibody.

other than ACP (Bouveret, unpublished results). However, they are not contaminants resulting from unspecific binding to the two columns of the TAP procedure because when we performed the purification with extracts prepared with a strain containing only the pBAD-NtermTAP vector, we did not obtain these contaminants (data not shown). Therefore, this suggests that DnaK and TnaA interact with ACP or other TAP-tagged proteins by a side effect that results from the expression of a recombinant protein, unnatural to the cell, or from its overexpression.

KasI and KasII are two β-ketoacyl-ACP synthases of the elongation cycle of fatty acid synthesis. The interaction of ACP with these enzymes is well known and studied [12]. Their detection in the purification of ACP demonstrates the reliability of the TAP method for the purification of proteins interacting with ACP.

SpoT is a (ppGpp)ase that catalyzes the degradation of ppGpp into GDP. It may also be capable of catalyzing the synthesis of ppGpp [27]. There was no previous indication of a physical link between SpoT and ACP. However, this result is very exciting from a functional point of view, because acyl-ACP, like ppGpp, has been shown to be a key player in regulating the activities of enzymes and expression of genes involved in fatty acid and phospholipid metabolism [28]. Furthermore, it has previously been postulated that a *spoT*-dependent accumulation of ppGpp could be due to an inhibition of SpoT function by fatty acyl-ACP [29].

MukB is a protein belonging to the ubiquitous Structural Maintenance of Chromosomes (SMC) protein family [30]. It is thought to be involved in chromosome partitioning by condensing replicated DNA strands within each cell half [30]. IscS is a pyridoxal phosphate-dependent cysteine desulfurase involved in the synthesis of sulfur-containing biomolecules such as Fe–S clusters, 4-thiouridine, 5-methylamino-methyl-

2-thiouridine, and thiamine [31]. There is to date no indication of a functional link between ACP and MukB and IscS. Nevertheless it has already been shown that ACP co-purifies with each of them [32,33]. In the case of IscS, it was proposed that ACP might carry in vivo the S° produced by the cysteine desulfurase reaction to apoproteins. In the case of MukB, it was suggested that the interaction was not specific and was caused by the abundance and the acidic property of ACP. After these initial studies, these two interactions were not mentioned again. Since in a reverse purification (the purification of ACP described here) we again detected these interactions, we concluded that they were significant and we decided to characterize them further.

3.2. Confirmation of the IscS/ACP and MukB/ACP interactions

To confirm the interactions, we performed co-purification experiments on calmodulin beads using a CBP-tagged ACP recombinant protein. In wild type cells, MukB and IscS were specifically co-precipitated on calmodulin beads when CBP-ACP was present (Fig. 2, lane 4). The antibodies that we used revealed several contaminant bands in the extracts (Fig. 2, lanes 1 and 2). Therefore, we also performed the experiments in the corresponding mutant strains (*iscS*$^-$ or *mukB*$^-$) to demonstrate that the proteins detected in the co-precipitated samples were indeed IscS and MukB (data not shown). It should be noted that the anti-MukB antibody also recognized purified CBP–ACP, suggesting that the MukB purification used to obtain this antibody [18] was contaminated by ACP, confirming the previous report [32] (Fig. 2, lane 4).

3.3. ACP is linked to IscS by a disulfide bond

To study the interaction between ACP and IscS and in order to enrich the extracts with both IscS and ACP proteins at the same time, we performed co-precipitation experiments with a 6His-tagged IscS and a Prot.A-tagged ACP. This permits the purification of 6His–IscS on cobalt beads and the detection of bound ACP–Prot.A by the PAP antibody that recognizes the Prot.A tag.

When the samples were not reduced, a band at 90 kDa was detected by PAP (detection of Prot.A), in the presence of 6His–IscS, which may correspond to a 6His–IscS/ACP–Prot.A complex (Fig. 3A, lane 10). This band disappeared when the sample was reduced with dithiothreitol (DTT) and was replaced by a band at 35 kDa corresponding to ACP–Prot.A (Fig. 3A, lane 12). This suggested that ACP was forming a

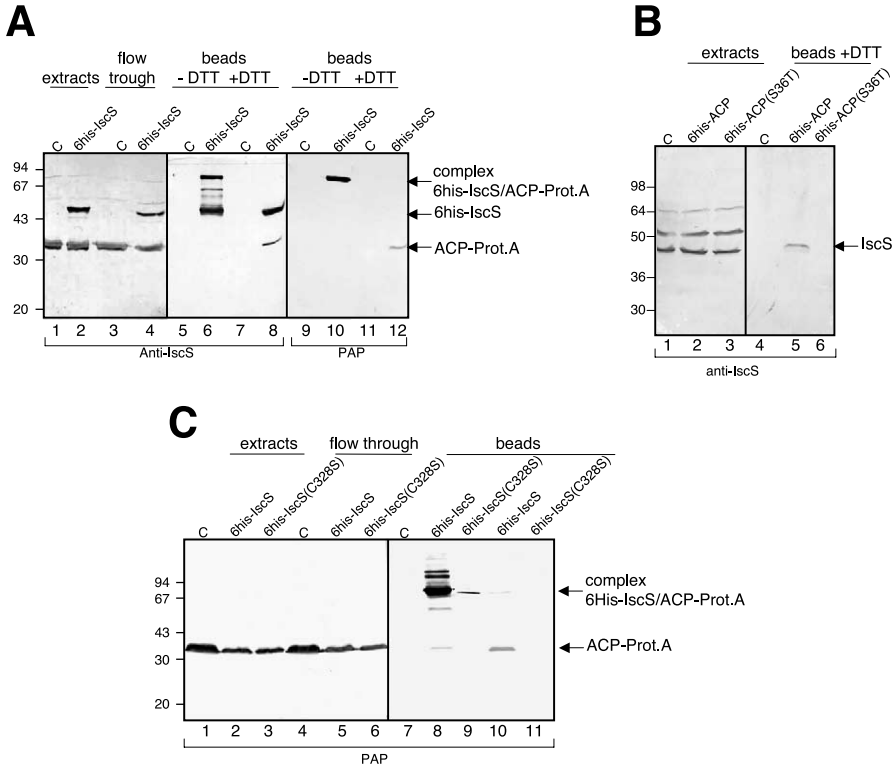


Fig. 3. ACP is linked to IscS by a disulfide bond. Cultures were induced for 60 min with 0.01% arabinose at OD_{600nm} = 0.8 at 37°C. Final OD_{600nm} was approximately 2. Extracts were done in buffer 1 without reducing agent (20 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.2% NP40, 10 mM imidazole) at a concentration of 50 uOD_{600nm}/ml by sonication. 30 μl of cobalt beads were used for each test with 1 ml of extract. Elution was done in Laemmli loading buffer without or with 2 mM DTT. A: Co-precipitation of ACP–Prot.A with 6His–IscS. In this experiment, both ACP–Prot.A and 6His–IscS expressions were induced by arabinose. Lanes 1 and 2: extracts; lanes 3 and 4: flow through; lanes 5,6,9 and 10: beads without DTT; lanes 7,8,11 and 12: beads with 2 mM DTT added after elution; odd lanes: control ACP–Prot.A/W3110; even lanes: 6His–IscS/ACP–Prot.A/W3110. Samples were analyzed by 12% SDS–PAGE and Western blot with an anti-IscS antibody (lanes 1–8) or a PAP antibody detecting the Prot.A tag (lanes 9–12). Using the anti-IscS antibody, ACP–Prot.A was also detected in lanes 1–8 owing to the fixation of any antibodies to the Prot.A tag. B: IscS does not co-precipitate with 6His–ACP(Ser36Thr). Lanes 1–3: extracts; lanes 4–6: beads treated with DTT; lanes 1 and 4: control W3110; lanes 2 and 5: 6His–ACP/W3110; lanes 3 and 6: 6His–ACP(Ser36Thr)/W3110. Samples were analyzed by 10% SDS–PAGE and Western blot with an anti-IscS antibody. C: ACP–Prot.A does not bind to 6His–IscS(Cys328Ser). Lanes 1–3: extracts; lanes 4–6: flow through; lanes 7–9: beads without added DTT; lanes 10–11: beads with 2 mM DTT after elution; lanes 1, 4, and 7: control ACP–Prot.A/W3110; Lanes 2,5,8 and 10: 6His-IscS/ACP-Prot.A/W3110. lanes 3, 6, 9, and 11: 6His–IscS(Cys328Ser)/ACP–Prot.A/W3110. Samples were analyzed by 10% SDS–PAGE and Western blot with PAP antibody.

disulfide bond with IscS through the thiol group present on the phosphopantetheine group. Using this time a 6His-tagged ACP instead of CBP-tagged ACP for convenience, we constructed the Ser36Thr mutant that abolishes the post-translational addition of phosphopantetheine to the ACP group [34]. Endogenous IscS was co-precipitated on cobalt beads with 6His–ACP but not with 6His–ACP(Ser36Thr) (Fig. 3B, lanes 5 and 6), showing that the Ser36 residue was necessary for the interaction. These data show that ACP forms a complex with IscS by a disulfide bond involving the thiol group of the phosphopantetheine group.

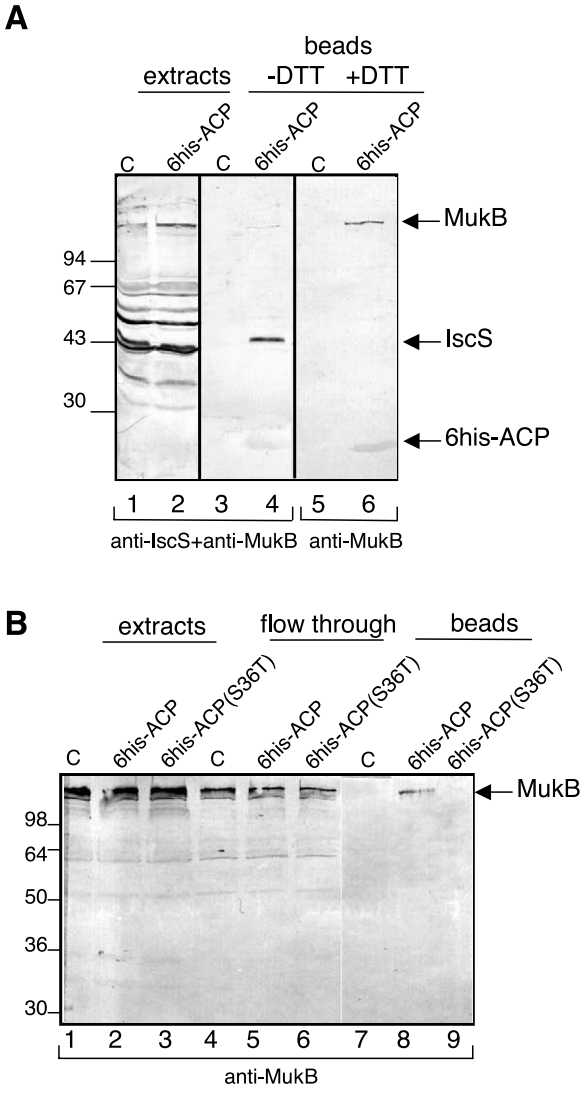


Fig. 4. MukB co-precipitates with 6His–ACP but not with 6His–ACP(Ser36Thr). Cultures were induced for 60 min with 0.01% arabinose at OD_{600nm} = 0.8 at 37°C. Final OD_{600nm} was approximately 2. Extracts were done in buffer 1 (20 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.2% NP40, 10 mM imidazole) at a concentration of 50 uOD_{600nm}/ml by sonication. 30 μl of cobalt beads were used for each test with 1 ml of extract. Elution was performed in Laemmli buffer containing 2 mM DTT. A: MukB co-precipitates with 6His–ACP. Lanes 1 and 2: extracts; lanes 3 and 4: beads, experiment done without DTT; lanes 5 and 6: beads, experiment done with 2 mM DTT; lanes 1, 3, and 5: control W3110; lanes 2, 4, and 6: 6His–ACP/W3110. Samples were analyzed by 10% SDS–PAGE and Western blot with an anti-MukB antibody. Lanes 1–4 were also incubated with an anti-IscS for another purpose. B: MukB does not co-precipitate with 6His–ACP(Ser36Thr). Lanes 1–3: extracts; lanes 4–6: flow through; lanes 7–9: beads with 2 mM DTT; lanes 1, 4, and 7: control W3110; lanes 2, 5, and 8: 6His–ACP/W3110; lanes 3, 6 and 9: 6His–ACP(Ser36Thr)/W3110. Samples were analyzed by 10% SDS–PAGE and Western blot with an anti-MukB antibody.

IscS contains three cysteine residues. To test if the Cys328 residue (which has been shown to be the functional residue of IscS to which the persulfide intermediate binds during the cysteine desulfurase reaction [35]) was also involved in the interaction with ACP, we constructed the 6His–IscS(Cys328-Ser) mutant protein. 6His–IscS(Cys328Ser) no longer interacted with ACP–Prot.A (Fig. 3C). Without DTT, a faint band was detected that may result from a non-specific disulfide bond with one of the two remaining cysteines of IscS (Fig. 3C, lane 9). However, when the samples were reduced with DTT, ACP–Prot.A was clearly detected in co-precipitation with 6His–IscS but not with 6His–IscS(Cys328Ser) (Fig. 3C, lanes 10 and 11).

In conclusion, this series of results demonstrates that ACP interacts with IscS with the formation of a disulfide bond linking the Cys328 of IscS and the sulfhydryl group of the ACP cofactor.

3.4. *The ACP interaction with MukB also requires the Ser36 residue but does not seem to involve a disulfide bond*

In contrast to the IscS/ACP interaction, we were never able to detect a band by Western blot in experiments under non-reducing conditions that could correspond to the MukB/ACP complex (data not shown). This suggests that MukB interacts with ACP without the formation of a disulfide bond. We performed co-precipitation experiments on cobalt beads using 6His–ACP, with DTT present during all the procedure (lysis and incubation on beads) and without DTT. Endogenous MukB interacted better with 6His–ACP in reducing conditions than in oxidative conditions, confirming that the interaction did not involve the formation of a disulfide bond (Fig. 4A, compare lane 4, experiment without DTT and lane 6, experiment with DTT). Using the 6His–ACP(Ser36Thr) construction, we performed co-precipitation experiments on cobalt beads to test if the MukB interaction also depends on the functional Ser36 residue of ACP. MukB was specifically co-precipitated with 6His–ACP but not with 6His–ACP(Ser36Thr) (Fig. 4B, lanes 8 and 9). Therefore, the MukB/ACP interaction is dependent on the presence of the functional Ser36 residue of ACP and is more effective in reducing conditions.

4. Discussion

Using the TAP method, we were able to show that ACP interacts specifically with IscS and MukB. We also detected a new interaction with SpoT but this interaction was not characterized further. IscS and MukB are proteins participating in very different functions, Fe–S cluster formation and chromosome segregation, respectively. Furthermore, no functional link between these proteins and ACP was suspected. Here, we clearly show that these interactions are specific and dependent on the presence of the functional Ser36 residue on ACP.

In the case of IscS, the interaction involves the formation of a disulfide bond between the thiol groups of the phosphopantetheine group on Ser36 of ACP and of the active site residue Cys328 of IscS. Is the disulfide bond really present in vivo in the reducing conditions of the cytoplasm? When 6His–IscS

and ACP–Prot.A were produced in separate extracts and mixed afterward, the formation of a disulfide bond was still taking place but much more slowly and less efficiently whereas the dilution of the extract containing both proteins at the same time did not change the amount of complex formed (data not shown). This shows that the disulfide bond was present as soon as the cells were broken. When the extract containing both proteins was prepared in the presence of the sulfhydryl reagent iodoacetamide, the level of complexed IscS/ACP was slightly reduced (data not shown). The two reactive thiols of ACP and IscS may be in close proximity in vivo in the interaction and, due to their high reactivity, may form a disulfide bond when cells are broken. But another possibility is that some disulfide bond is already formed in vivo and corresponds to the amount of complex that is not affected by iodoacetamide treatment. It is the same situation as what was shown for the interaction of ACP with KasI and KasII [36,37]. In this case, a disulfide bond formed when the cells were lysed and it was suggested that it resulted from the oxidative reaction of the two tightly but non-covalently bound reactive groups of ACP and of the Kas enzymes [37]. Therefore, even if the disulfide bond does not occur in vivo, it reflects a true interaction between the two groups involved, i.e. the ACP thiol and the active site thiol of IscS. The involvement of the active site of IscS suggests that the interaction with ACP is important for IscS function.

One can wonder why no more enzymes known to interact with ACP were identified in the purifications (such as holo-ACP synthase, other enzymes of fatty acid synthesis, HlyC involved in acylation of HlyA). We found only KasI and KasII, two essential enzymes of the elongation cycle of fatty acids. The explanation may be that interactions involving enzymatic reactions are very dynamic and specific for the substrate. They may thus not resist the different steps of the TAP method (two columns, two elutions) and the numerous washes. With the TAP method we certainly isolated more stable interactions, which may be involved in other processes such as regulation of enzymatic activity. Following this thought, the high amount of SpoT found in both types of ACP purifications may reflect a direct regulation of the SpoT-dependent stringent response dependent on the fatty acid availability in the cell that could be sensed by ACP [28]. It will be very interesting to clearly characterize this interaction and to determine the form of ACP in interaction with SpoT to confirm this hypothesis.

In all our experiments, we overexpressed ACP, when we performed co-purification of endogenous IscS or MukB with 6His–ACP or when we performed co-precipitation of ACP–Prot.A with 6His–IscS. It is said that, under these conditions, ACP is found in its apo form, i.e. without its phosphopantetheine group [34]. However, in the case of IscS, we clearly obtained a disulfide bond, demonstrating that at least a fraction of ACP was in its holo form. Similarly, while we have been able to show that the TAP method was as efficient and powerful in a prokaryote such as *E. coli* as it was in yeast, we did not perform the purifications under the ideal conditions: TAP-tagged ACP constructs were under the control of an artificial promoter but here again, co-purification of KasI, KasII, and IscS demonstrated that TAP-tagged ACP constructs were correctly post-translationally modified and functional. Ideally, the gene for the ACP-tagged protein should have been on the chromosome, with the tag sequence at the C-terminus, so that the expression would have been under the control of the natural promoter of ACP. Methods to circumvent the difficulties inherent in *E. coli* to recombine PCR fragments have been developed recently and it would be interesting to use them if a systematic approach is to be considered [38].

In the example presented here, a series of very interesting results were obtained but we certainly recovered a mixture of distinct ACP-containing complexes. We may have an ACP/IscS complex, an ACP/MukB complex, an ACP/KasI complex, etc. without links between them. The next step in the use of this method for a protein as central and important as ACP will be to separate these sub-complexes. To do so, one can use a variant of the TAP method that consists in using a split TAP tag, one half on ACP, the other half on the protein of interest found in the ACP purification [20].

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