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Functional interaction of *Azotobacter vinelandii* cytoplasmic cyclophilin with the biotin carboxylase subunit of acetyl-CoA carboxylase

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ABSTRACT

Cyclophilins (E.C. 5.1.2.8) are protein chaperones with peptidyl-prolyl *cis/trans* isomerase activity (PPIase). In the present study, we demonstrate a physical interaction among *Av*ppiB, encoding the cytoplasmic cyclophilin from the soil nitrogen-fixing bacterium *Azotobacter vinelandii*, and *Av*accC, encoding the biotin carboxylase subunit of acetyl-CoA carboxylase, which catalyzes the committed step in long-chain fatty acid synthesis. A decrease in *Av*ppiB PPIase activity, in the presence of *Av*accC, further confirms the interaction. However, PPIase activity seems not to be essential for these interactions since a PPIase active site mutant of cyclophilin does not abolish the *Av*accC binding. We further show that the presence of cyclophilin largely influences the measured ATP hydrolyzing activity of *Av*accA in a way that is negatively regulated by the PPIase activity. Taken together, our data support a novel role for cyclophilin in regulating biotin carboxylase activity.

1. Introduction

Cyclophilins were originally identified as the intracellular receptors of the immunosuppressive drug cyclosporine while formation of the complex prevents T-cell proliferation via inhibition of the protein phosphatase calcineurin [1–3]. All cyclophilins share a common domain of approximately 109 amino acids, the cyclophilin-like domain, surrounded by domains unique to each member of the family that are associated with subcellular compartmentalization and functional specialization [4]. They are structurally conserved among mammals, plants, insects, fungi, and bacteria and all have PPIase activity, which catalyze *cis/trans* isomerization of proline containing peptide bonds [4] while they can also act as folding helper enzymes [5,6]. According to the conformational switch hypothesis, the function of a protein containing a heterogeneous prolyl bond is regulated by whether this bond is in the *cis* or *trans* conformation [7,8]. Previous studies of various organisms have suggested that cyclophilins are involved in a wide range of cellular processes, including signaling, cell division, transcriptional regulation, and viral replication [9–12]. Nevertheless, the complete biological functions of cyclophilins remain to be explored.

Although there are a few examples of functional characterization of bacterial cyclophilins [13–16], both the spectrum and characteristics of their *in vivo* substrates are largely unknown. To this end, we continue our previous searches for interacting candidate proteins of *Azotobacter vinelandii* cytoplasmic cyclophilin [16,17]. At the present study, we reveal that *Av*ppiB cyclophilin is able to bind *Av*accC, the biotin carboxylase subunit of acetyl-CoA carboxylase. The PPIase active site of *Av*ppiB although is not essential for the binding, since a PPIase active site mutant still binds to *Av*accC, it seems to be involved at the overall interaction given that the measured PPIase activity of *Av*ppiB is lowered by the presence of *Av*accC. Furthermore, the influence of *Av*ppiB is significant considering the measured ATP hydrolyzing activity of *Av*accC, and specific as well since *Av*fkbA2, encoding for an FK506-binding protein (FKBP), had comparatively only little influence on the *Av*accC activity. However, the PPIase activity seems to negatively regulate the observed phenomenon since the influence of *Av*ppiB active site mutant is even higher over the measured ATP hydrolyzing activity of *Av*accC pointing towards a novel role of cyclophilin in biotin carboxylase regulation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli XL-Blue1 strain (Invitrogen) was used for the propagation of recombinant forms of the plasmids pET28a and pCDFDuet-1. *E. coli* strains BL21 (DE3) (Novagen) were used for the expression of recombinant proteins. All *E. coli* strains were grown in LB medium supplemented with kanamycin or streptomycin.

Abbreviations: CoA, coenzyme A; PPIase, peptidyl-prolyl *cis/trans* isomerase.

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2.2. Heterologous co-expression of AvppiB with AvaccC in E. coli and purification of the protein pair

*Av*ppiB was co-expressed with *Av*accC as two separate polypeptides with a $(His)_6$-tag fused at the N-terminus of *Av*ppiB and an S-tag fused at the C-terminus of *Av*accC, using the pCDFDuet-1 vector. The primers used were *Av*ppiB.H.Duet-F: 5′-AAA*GGATCC*AAT CAAGCTGCAAACCAACCACG-3′ with *Av*ppiB.H.Duet-R: 5′-AAA *GCGGCCGC*TTATTCGACGATCTCGGCCTTC-3′ and *Av*accC.S.Duet-F: 5′-GGG*CATATG*CTGGAAAAAGTCCTGATCGCC-3′ with *Av*accC.S.-Duet-R: 5′-AAA*GGTACC*GTGCTTGTCCAGACCCAGTTTC-3 carrying restriction sites for ligation to the pCDFDuet-1 expression vector. The underlined nucleotides at each primer represent *Bam*HI, *Not*I and *Nde*I, *Kpn*I, respectively. The fragments excised from amplified *AvppiB* and *AvaccA* sequences were cloned between the corresponding sites of pCDFDuet-1, resulting in *Av*ppiB.H-*Av*accA.S-pCDFDuet-1. The absence of undesired alterations was checked by nucleotide sequencing. Synthesis of recombinant proteins was initiated by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside when the cultures reached A_{600} of 0.6 and continued cultivation for an additional 4 h at 30 °C. Cells were harvested by centrifugation and were disrupted by sonication in Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole supplemented with 1 mg/ml lysozyme). Cellular lysates were centrifuged and the supernatants were used for protein purification. Recombinant proteins were purified with Ni–NTA chromatography (Ni^{2+}-nitrilotriacetate, Qiagen) according to the manufacturer's instructions. The purity of the purified proteins was analyzed by 15% SDS–PAGE electrophoresis.

2.3. Heterologous expression of AvaccC in E. coli and purification of recombinant protein

The coding sequence of *Av*accC (YP_002797920) was amplified using PCR with *A. vinelandii* genomic DNA as a template and expressed with a $(His)_6$ tag fused at its N-terminus. The primers used were *Av*accC.H.Duet-F: 5′-AAA*GGATCC*GGAAAAAGTCCTGATC GCCAACCG-3′ with *Av*accC.H.Duet-R: 5′-GGG*AAGCTT*GTGCTTGTC-CAGACCCAGTTTCTT-3′ carrying restriction sites for ligation to the pCDFDuet-1 expression vector. The underlined nucleotides at each primer represent *Bam*HI and *Hind*III, respectively. The fragment excised from amplified *AvaccC* sequence was cloned between the corresponding sites of pCDFDuet-1, resulting in *Av*accC.H-pCDFDuet-1. The absence of undesired alterations was checked by nucleotide sequencing. Protein expression and purification was performed as described above. To remove any imidazole and salts in the collected fractions, fractions were dialyzed against 20 mM HEPES at pH 8.0, for 12 h.

2.4. Peptidyl-prolyl cis/trans isomerase enzymatic assay

PPIase activity was tested with a chymotrypsin-coupled PPIase assay [18], which is rate-limited by the *cis*/*trans* isomerisation of the Ala-Pro peptide bond of synthetic Suc-AAPF-pNA (Bachem). The assay mixture contained 50 mM Hepes buffer pH: 8.0 and 100 mM NaCl, 50 μg α-chymotrypsin (dissolved in 1 mM HCl) (Fluka), 25 μM Suc-AAPF-pNA (5 mM stock dissolved in trifluoroethanol supplemented with 0.45 M LiCl) and the appropriate amount of the enzyme. The assay buffer was mixed with α-chymotrypsin and subsequently with the enzyme. The reaction was initiated inside the cuvette with the addition of the peptide and the increase in absorbance at 390 nm was monitored at 4 °C using a HITACHI U-2800 spectrophotometer. For the biotin carboxylase inhibition studies, the appropriate amount of biotin carboxylase was also placed inside the cuvette in addition to the peptide.

2.5. ATP hydrolysis assay

The rate of ATP hydrolysis by biotin carboxylase in the absence of biotin was measured spectrophotometrically [19]. The production of ADP was coupled to pyruvate kinase and lactate dehydrogenase, and the oxidation of NADH was followed at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). Each measurement was carried out in a volume of 0.8 mL in 1 cm path length quartz cuvettes. The reaction mixture contained 10 units of pyruvate kinase, 18 units of lactate dehydrogenase, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 8 mM $MgCl_2$, 3 mM ATP, 15 mM potassium bicarbonate and 100 mM HEPES at pH 8.0.

3. Results and discussion

3.1. AvaccC represents an interacting candidate protein for AvppiB

We have previously demonstrated that *Av*ppiB, a cytoplasmic cyclophilin from *A. vinelandii*, possesses peptidyl-prolyl *cis/trans* isomerase activity against synthetic peptides while it can also act as a chaperone preventing citrate synthase from thermal aggregation [16]. Here, we continue our previous searches among the available experimental protein interaction data provided by IntAct database [20], for interacting candidate proteins [16,17]. Since *A. vinelandii Av*ppiB is 64% identical to *E. coli* ppiB, we hypothesized that the homologues to available *E. coli* prey proteins [21,22] could probably interact with *Av*ppiB as well. One of these prey proteins is *Av*accC, encoding the biotin carboxylase subunit of acetyl-CoA carboxylase, which catalyzes the first step in fatty acid metabolism [23].

In various biotin carboxylase crystal structures described by Thoden et al. [24] and Chou et al. [25], two proline residues of the central ATP-grasp domain, P^{155} and P^{244} according to *E. coli* and *A. vinelandii* numbering, can adopt a *cis* conformation and remain conserved among various bacterial species including *A. vinelandii*. The overall biotin carboxylase structure exists in open and closed conformations depending on the central domain rotation to grasp the ATP [24,25], a signature structural feature of the ATP-grasp superfamily of enzymes [26]. Spontaneous isomerization of peptidyl-prolyl bonds requires free energy and is a slow process, constituting a rate-limiting step in protein folding. Cyclophilins stabilize the *cis/trans* transition state and accelerate isomerization, a process that is considered important not only in protein folding but also during the assembly of multidomain proteins [27]. So, we considered *Av*accC a possible interacting partner for *Av*ppiB and subsequently we investigate this hypothesis.

3.2. AvppiB physically interacts with AvaccC

In order to test whether *Av*accC is an interacting partner for *Av*ppiB, we co-expressed *Av*ppiB with *Av*accC, as two separate polypeptides, with a $(His)_6$-tag fused at the N-terminus of *Av*ppiB and an S-tag fused at the C-terminus of the biotin carboxylase, using the pCDFDuet-1 vector. The soluble co-expressed proteins were purified using Ni-resin affinity chromatography, indicating that the protein pair forms a stable complex (Fig. 1). The existence of both polypeptides was confirmed by SDS–PAGE and immunoblotting with antibodies against the His and S-tags (data not shown). Control experiments were performed where just S-tag fused *Av*accC was not retained by Ni–NTA agarose column.

In order to clarify whether the PPIase activity is necessary for these interactions, we tested if $Av\text{ppiB}_{F99A}$ active site mutant, which retains a considerable part of its chaperone activity but only 1.7% of its peptidyl-prolyl *cis/trans* isomerase catalytic efficiency [16], still interacts with *Av*accC. Yet again, when we independently

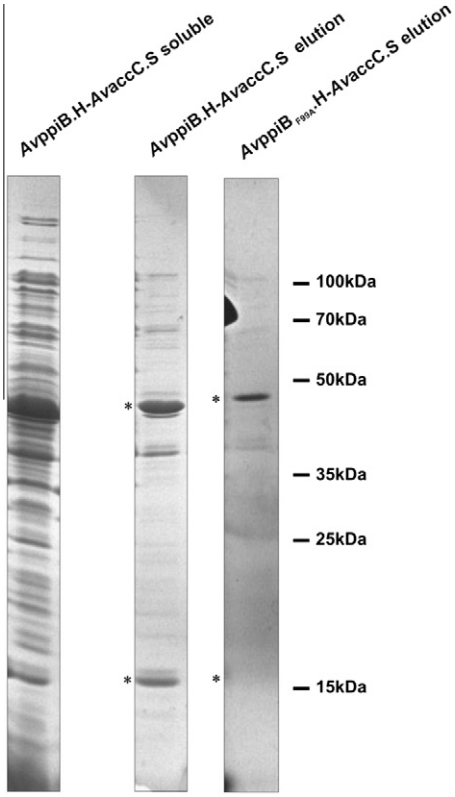

Fig. 1. Molecular interaction between *Av*ppiB or *Av*ppiB$_{F99A}$ and *Ava*ccC. SDS–PAGE analysis of the soluble cytoplasmic fraction from *E. coli* BL21 (DE3) cells over-expressing *Av*ppiB.H-*Ava*ccC.S-pCDFDuet1 (lane 1), the elution fraction after the Ni–NTA purification of the *Av*ppiB.H-*Ava*ccC.S protein complex (lane 2) and the elution fraction after the Ni–NTA purification of the *Av*ppiB$_{F99A}$.H-*Ava*ccC.S protein complex (lane 3).

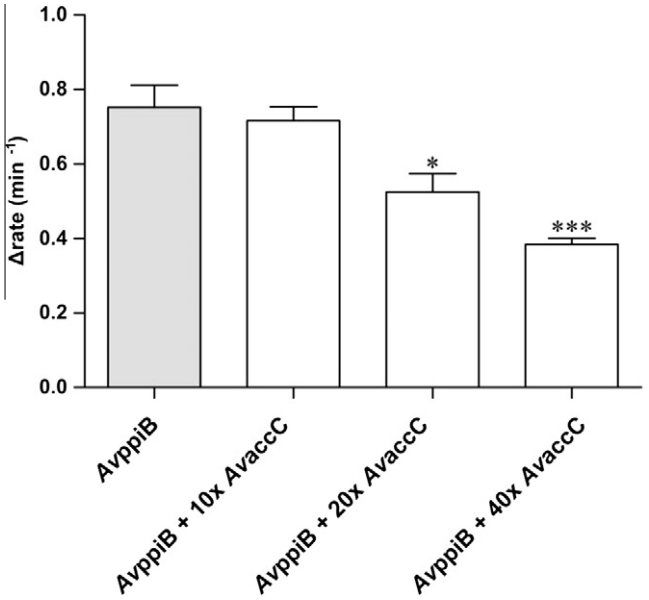

Fig. 2. Effect of *Ava*ccC on PPIase activity of *Av*ppiB. PPIase activity of 60 nM *Av*ppiB in the presence of 600 nM, 1.2 μM *Ava*ccC and 2.4 μM *Ava*ccC. Mean values were obtained from three independent replicates and error bars represent standard errors. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's multiple-comparison test. Asterisks indicate a statistically significant difference ($p < 0.05$).

co-expressed *Av*ppiB$_{F99A}$ with *Ava*ccC, the protein pair formed a stable complex (Fig. 1). This observation indicates that the PPIase activity of *Av*ppiB is not necessary for the formation of the protein complex and probably the remaining chaperone activity of *Av*ppiB is sufficient for the interaction.

3.3. *AvaccC decreases AvppiB activity*

To explore the potential involvement of the PPIase active site on the binding of *Av*ppiB to *Ava*ccC, the inhibitory effect of *Ava*ccC on the PPIase activity of *Av*ppiB was determined. The assay for PPIase activity is based on the conformational specificity of chymotrypsin, which cleaves the 4-nitroanilide moiety from succinyl-Ala-Xaa-Pro-Phe-4-nitroanilides only when the Xaa-Pro peptide bond is in the *trans* conformation. In the presence of PPIases, the Xaa-Pro bond is more rapidly converted to the *trans* conformation, which is readily cleaved by chymotrypsin leading to the formation of the colored product 4-nitroaniline [18]. Kinetic data were obtained in the presence of *Av*ppiB and the difference between the isomerization rate constants k_{obs} and k_o, referring to the catalyzed and uncatalyzed reactions respectively, was determined. As shown in Fig. 2, when we added *Ava*ccC to the reaction containing 60 nM *Av*ppiB, we observed a concentration dependent decrease in activity, up to approximately 51% of the control reaction when *Ava*ccC was added in 40× molar excess over *Av*ppiB, indicating a possible antagonism of *Ava*ccC with the reaction substrates for the PPIase active site.

3.4. *AvppiB stimulates the ATP hydrolysis activity of AvaccC*

The carboxylation of biotin by biotin carboxylase requires the hydrolysis of ATP. However, in the absence of biotin, biotin carboxylase can catalyze the hydrolysis of ATP at a much slower rate, and in a bicarbonate dependent manner [28]. In order to investigate the functional significance of *Av*ppiB and *Ava*ccC association, we measured the ATP hydrolysis activity of *Ava*ccC, in the absence of biotin and in the presence of increasing amounts of *Av*ppiB. When we added *Av*ppiB, we observed a concentration dependent increase in activity, up to approximately fourfold when *Av*ppiB was added in 10× molar excess over *Ava*ccC (Fig. 3). No ATP hydrolysis activity was observed when *Av*ppiB was added to reactions in the absence of *Ava*ccC, indicating that the effect was not due to an ATP hydrolysis activity co-purifying with *Av*ppiB. Furthermore, addition of BSA had no effect on *Ava*ccC measured activity.

The specificity of the observed modulation of ATP hydrolyzing activity of *Ava*ccC by *Av*ppiB was evaluated by replacing *Av*ppiB with *Av*fkbA2. *Av*fkbA2 is an FK-506 binding protein which belongs to the PPIase superfamily and is characterized by PPIase and chaperone activity [29]. When we added *Av*fkbA2 in 10× molar excess, the ATP hydrolyzing activity of *Ava*ccC was very small comparatively to the *Av*ppiB mediated increase and not statistically significant (Fig. 3), indicating the specificity of the interaction.

In an attempt to understand whether the PPIase activity of *Av*ppiB was responsible for the observed increase in ATP hydrolyzing activity of *Ava*ccC, we measured the influence of *Av*ppiB$_{F99A}$ active site mutant on *Ava*ccC activity. We found that the measured increase in *Ava*ccC activity was even higher, approximately eightfold when *Av*ppiB$_{F99A}$ was added in 10× molar excess over *Ava*ccC, than the increase caused by *Av*ppiB (Fig. 3). Since *Av*ppiB$_{F99A}$ retains a considerable part of its chaperone activity, this probably indicates that *Av*ppiB enhances the *Ava*ccC activity through its general chaperone function while the PPIase activity of *Av*ppiB negatively regulates this modulation.

In conclusion, we report here that the cytoplasmic cyclophilin *Av*ppiB from *A. vinelandii* is able to bind *Ava*ccC. The PPIase active site of *Av*ppiB although is not essential for the binding, since *Av*ppiB$_{F99A}$, a PPIase active site mutant, still binds to *Ava*ccC, it seems to be involved or affected by the overall interaction given that the measured PPIase activity of *Av*ppiB is lowered by the presence

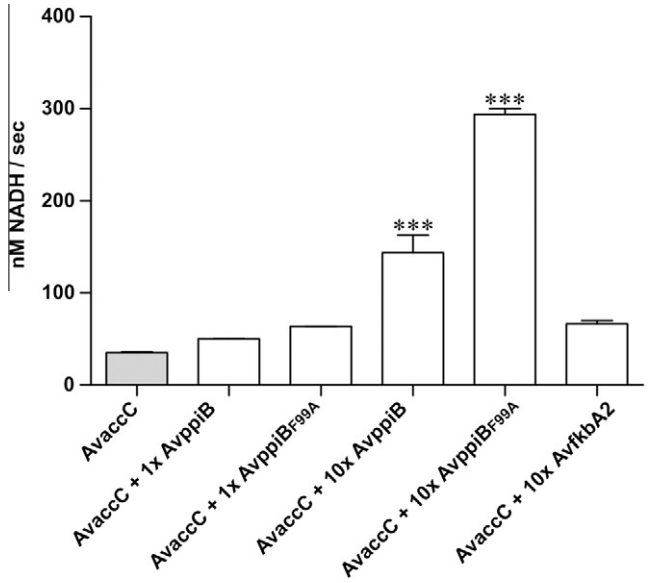

Fig. 3. Effect of *Av*ppiB, *Av*ppiB$_{F99A}$ and *Av*fkbA2 on *Av*accC ATP hydrolyzing activity, in the presence of an ATP regenerating system. Mean values were obtained from three independent replicates and error bars represent standard errors. Statistical comparisons were made using ANOVA followed by Dunnet's Multiple Comparison Test using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA). Asterisks indicate statistically significant differences ($p < 0.05$).

of *Av*accC. Furthermore, the influence of *Av*ppiB is significant considering the measured ATP hydrolyzing activity of *Av*accC and specific as well since *Av*fkbA2 had not significant influence on the *Av*accC activity. However, the observed *Av*ppiB influence is negatively depended on its PPIase activity since *Av*ppiB$_{F99A}$ increases further the measured ATP hydrolyzing activity of *Av*accC. Overall, our data uncover a novel and possibly critical role for cyclophilin in modulating biotin carboxylase activity considering the rate-limiting position of acetyl-CoA carboxylase in fatty acid biosynthesis [30]. Further structural and biochemical analyses are necessary to fully elucidate the mechanism of the interaction.

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