A new topology of ACBP from *Moniliophthora perniciosa*

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**A B S T R A C T**

Acyl-CoA binding protein (ACBP) is a housekeeping protein and is an essential protein in human cell lines and in *Trypanosoma brucei*. The ACBP of *Moniliophthora perniciosa* is composed of 104 amino acids and is possibly a non-classic isoform exclusively from Basidiomycetes. The *M. perniciosa* acbp gene was cloned, and the protein was expressed and purified. Acyl-CoA ester binding was analyzed by isoelectric focusing, native gel electrophoresis and isothermal titration calorimetry. Our results suggest an increasing affinity of ACBP for longer acyl-CoA esters, such as myristoyl-CoA to arachidoyl-CoA, and best fit modeling indicates two binding sites. ACBP undergoes a shift from a monomeric to a dimeric state, as shown by dynamic light scattering, fluorescence anisotropy and native gel electrophoresis in the absence and presence of the ligand. The protein’s structure was determined at 1.6 Å resolution and revealed a new topology for ACBP, containing four α-helices instead of four. α-helices 1, 2, 3 and 4 adopted a bundled arrangement that is unique from the previously determined four-helix folds of ACBP, while α-helices 1, 2, 4 and 5 formed a classical four-helix bundle. A MES molecule was found in the CoA binding site, suggesting that the CoA site could be a target for small compound screening.

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1. Introduction

The basidiomycete *Moniliophthora perniciosa* is the causal agent of witches’ broom disease in the cacao plant and is considered to be the main phytopathogen of the cacao plant, causing a marked decrease in cacao production. Commercial fungicides cannot feasibly be used to control this infection [1]; therefore, efforts to find new drug targets for controlling this plague and methods for producing more resistant plants are of great importance.

Acyl-CoA binding protein (ACBP) is a widely distributed and highly conserved cytosolic protein whose molecular mass is approximately 10 kDa. ACBP has been found in all tested eukaryotes and is expressed in most cells and tissues [2]. ACBP is an essential protein [7] whose function is associated with one or more basic function(s) common to all cells. ACBP is a pivotal player in the trafficking and use of long chain acyl-CoA esters [2]. ACBP is a housekeeping protein and is an essential protein in human cell lines and in *Trypanosoma brucei*. The ACBP of *Moniliophthora perniciosa* is composed of 104 amino acids and is possibly a non-classic isoform exclusively from Basidiomycetes. The *M. perniciosa* acbp gene was cloned, and the protein was expressed and purified. Acyl-CoA ester binding was analyzed by isoelectric focusing, native gel electrophoresis and isothermal titration calorimetry. Our results suggest an increasing affinity of ACBP for longer acyl-CoA esters, such as myristoyl-CoA to arachidoyl-CoA, and best fit modeling indicates two binding sites. ACBP undergoes a shift from a monomeric to a dimeric state, as shown by dynamic light scattering, fluorescence anisotropy and native gel electrophoresis in the absence and presence of the ligand. The protein’s structure was determined at 1.6 Å resolution and revealed a new topology for ACBP, containing four α-helices instead of four. α-helices 1, 2, 3 and 4 adopted a bundled arrangement that is unique from the previously determined four-helix folds of ACBP, while α-helices 1, 2, 4 and 5 formed a classical four-helix bundle. A MES molecule was found in the CoA binding site, suggesting that the CoA site could be a target for small compound screening.

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Crystal structures of ACBP from Plasmodium falciparum [12], cow [12,13], yeast [14], armadillo [15] and human [16] were deposited in the Protein Data Bank (PDB) and classified as four-helix bundles with an up-down-down-up bundle topology with an overhand loop connecting helices 2 and 3. The bundle arrangement of ACBP is unique among proteins with known four-helix folds [6]. In the present study, the acbp gene from *M. perniciosa* was cloned, the protein was characterized, and its structure was resolved. This is the first structure that has been solved in this organism.

2. Materials and methods

2.1. Construction of expression vector

The sequence of the gene encoding ACBP was obtained from cDNA library sequencing of the *M. perniciosa* fruiting body [17] and analysis of contigs from the *M. perniciosa* genome project [18] (www.Ige.ibi.unicamp.br/vassoura) using BLAST [19] and CLUSTALW [20].

First-strand cDNA isolated from a fruiting body was used as a template. A forward primer was designed to include an NdeI restriction site (5′-GGG GTA TAT GTC CAA AGC AAAG-3′), and the reverse primer was designed to include an EcoRI site (5′-GGG GAA TCT AGG CAG CTT CAA TC-3′). PCR was performed using Pfu DNA polymerase (Promega) according to the manufacturer’s instructions, with the following cycling parameters: 5 min denaturation at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C, and a final 10 min elongation at 72 °C. Amplification products of about 330 bp were recovered on a 1% agarose gel. The pT7-blue3 and pET-28a plasmids (Novagen) were purified by mini-preparation from Escherichia coli DH5α transformants. Plasmid pT7-blue3 was digested by EcoRV, and pET-28a was digested by NdeI and EcoRI. The pT7-blue3 and acbp gene were ligated with T4 DNA ligase (New England BioLabs) at 4 °C overnight and transformed into *E. coli* DH5α. Transformants were selected on the chromogenic substrate X-gal. The acbp gene was then digested with NdeI and EcoRI and recovered on a 1% agarose gel. The pET-28a-acbp construct was synthesized by treating with T4 DNA ligase for 2 hours at ambient temperature; *E. coli* DH5α cells transformed with this plasmid were selected on LB agar plates containing kanamycin (50 mg / mL). The pET-28a-acbp product obtained from *E. coli* transformants was confirmed by PCR and restriction mapping and used to transform *E. coli* BL21 (DE3).

The acbp gene sequence of pET-28a-acbp was confirmed by sequencing, which involved 25 cycles of PCR amplification of 10 μl reaction volumes containing about 200 ng plasmid DNA, 10 pmol/μl each of primers T7 promoter and T7 terminator (Novagen), and 4 μl ET Dye Terminator (GE Healthcare). Sequencing reactions were ethanol precipitated, pellets were resuspended in 3.5 μL of loading buffer, 1.5 μL was loaded onto a sequencing gel, and data were collected by an ABI PRISM 377 DNA Sequencer (Perkin Elmer; Applied Biosystems).

2.2. ACBP purification

The pre-culture was grown overnight at 37 °C with agitation, inoculated at a ratio of 1:100, and grown in culture to an OD<sub>600</sub> of 0.6. Expression was induced by the addition of 0.5 mM IPTG and allowed to proceed for 4 h at 37 °C. The cells were centrifuged at 5000×g for 15 min at 4 °C and then suspended in 50 mM sodium phosphate (pH 7.0) buffer plus 300 mM NaCl and 20 mM imidazole. The cells were lysed with a 7-min sonication and centrifuged at 20,000×g for 20 min at 4 °C, and the ACBP in the soluble fraction was purified with a TALON polyhistidine-tag purification resin (Clontech). The column was equilibrated with 10 column volumes, washed with 30 column volumes and eluted with 6 column volumes of buffer containing 200 mM imidazole. The histidine tail was removed with bovine thrombin (Sigma) in 50 mM sodium phosphate at pH 7.2 and 100 mM NaCl. A second purification step was carried out with size-exclusion chromatography with a Superdex 75 HR10/30 using the AKTA Purifier FPLC System (GE/Amersham Pharmacia Biotech, Sweden) in 20 mM Tris at pH 7.2 and 150 mM NaCl. The oligomeric state of the fractions was evaluated by comparison to the retention volume of a standard sample of known molecular weight. The ACBP purification yielded protein of over 95% purity based on 15% SDS PAGE. The size-exclusion chromatography showed that the majority of protein was in monomeric form and another fraction was in dimeric form. The purified protein was concentrated to about 6 mg / mL using an Amicon Ultra centrifugal filter (Millipore) in 20 mM Tris at pH 7.0. Protein concentration was determined by the Bradford method [21].

2.3. Assays to determine affinity for acyl-CoA esters and oligomeric state

A test for ACBP affinity for acyl-CoA esters was performed using isoelectric focusing and isothermal titration calorimetry (ITC). The oligomeric states of the apoprotein and the bound protein were evaluated by fluorescence anisotropy, dynamic light scattering (DLS) and native polyacrylamide gel electrophoresis (Native PAGE).

2.3.1. Isoelectric focusing

The pl of the ACBP at 80 μM in the apo and liganded form with ten-fold excess ligand was determined in an isoelectric focusing gel with a pH range of 3–9 (PhastGel™ IEF 3–9, GE Healthcare Life Sciences, Sweden), using the Pharmacia Phast System (GE Healthcare Life Sciences, Sweden) according to the manufacturer’s protocols. The Broad pl kit pH 3–10 (GE Healthcare Life Sciences, Sweden) was used as a pl marker. The gel was stained with Coomassie Blue.

2.3.2. Isothermal titration calorimetry

All microcalorimetry experiments were carried out in 20 mM Tris, pH 7.0 at 25 °C, using approximately 11- to 18-fold excess ligand. The monoprotein calorimetry solution (13 to 18 μM) was placed in the calorimeter cell with a volume of 1.4173 mL and stirred at 300 rpm. All solutions were thoroughly degassed by stirring under vacuum before use. The sample was titrated with 200–260 μM ligand solution, and the final ligand concentration was determined using a millimolar extinction coefficient of 15.4 at 254 nm. The proportion of ligand and proteins used were similar to those used in previous experiments [22–24]. In total, 30 ligand injections of 8 μL each were added into the calorimeter cell at 130-s intervals from a 300-μL stirring syringe. The reference cell was filled with the same buffer used in the binding assay. All experiments were replicated four times at different protein concentrations, and the best-fit isotherm was used for association constant analysis. Ligand binding to ACBP was analyzed in a VP-ITC microcalorimeter from Microcal Inc. (Northampton, MA). Thermogram data were integrated and fitted to a theoretical titration curve using the ORIGIN software supplied by Microcal Inc. The association constant was calculated with equations for two sets of independent sites:

\[
K_1 = \frac{\theta_1}{(1 - \theta_1)}[X]
\]

\[
K_2 = \frac{\theta_2}{(1 - \theta_2)}[X]
\]

where \(K_1, K_2, \theta\) and \([X]\) are the first binding constant, second binding constant, fraction of sites occupied by ligand X and free concentration of ligand, respectively.

2.3.3. Fluorescence anisotropy

The mean rotational anisotropy of the fluorescent residues of the entire apoprotein and the bound protein were evaluated by fluorescence anisotropy in an ISS K2 Multifrequency Phase Fluorometer.
(Champaign, IL) using a 280-nm excitation wavelength. Intrinsic polarized fluorescence anisotropy assay measurements were done using the samples from microcalorimetry analysis after ligand titration and for the monomeric form used as a reference in the ITC experiments. The dimeric form was obtained from the appropriate size-exclusion chromatography fraction and was used at the same concentration as the monomeric form. Measurements were made in 0.5 mL in a 1-cm path length quartz cuvette. Prior to analysis, the microcalorimetry samples were diluted 10-fold in 20 mM Tris at pH 7.0. Possible free ligand contributions were subtracted from all the protein-ligand sample measurements. The anisotropy was recorded as the mean of 10 measurements across the samples under each condition. All assays were carried out at 25 °C. Values of fluorescence anisotropy \((r)\) were measured according to the equation

\[
(r) = \frac{(I_e - I_h)}{(I_e + 2I_h)}
\]

where \(I_e\) and \(I_h\) are the intensities of emission when the polarizers are oriented parallel or perpendicular to the direction of the excitation polarizer, respectively. Polarization measurement variations were between ±0.005 for each sample.

2.3.4. Dynamic light scattering

DLS was used to determine the hydrodynamic radius \(R_h\) of ACBP at 2.5 μM, in its apo and liganded form using six-fold excess ligand. The \(R_h\) is given by the Stokes–Einstein relation:

\[
R_h = \frac{k_BT}{6\pi\eta D}
\]

where \(k_B\), \(T\), \(\eta\) and \(D\) are the Boltzmann constant, absolute temperature in Kelvin, solvent viscosity and diffusion coefficient, respectively.

DLS measurements were carried out in the experimental buffer of 20 mM Tris at pH 7.0 using a Protein Solutions DynaPro 99 (Protein Solutions, Charlottesville, VA). The samples used were centrifuged at 16,000 × g for 10 min before analysis. The assays were performed at 25 °C with an acquisition time of 2.5 s in a 12 μL cuvette. Thirty acquisitions were averaged in a single measurement, and three sequential measurements were performed for each sample of the apo and liganded form. Dynamics v.5.25.44 software (Protein Solutions Inc.) was used for the data collection and analysis.

2.3.5. Native polyacrylamide gel electrophoresis

Native PAGE analysis of 80 μM ACBP in the apo and liganded form, using ten-fold excess ligand, was performed according to the Laemmli method [25], with some modifications. The electrode buffer was adjusted to pH 8.3, 9.5 and 10. The pHs of the stacking and resolving gels were also changed to pH 9.5 and 10, respectively. The resolving gel contained 10% acrylamide. The stacking and resolving gels, electrode buffer, and gel-loading buffer were prepared without denaturing agents. The gel was stained with Coomassie Blue.

2.4. Crystallization and data collection

ACBP was concentrated to 6 mg/mL in 20 mM Tris pH 7.0 and crystallized by the hanging drop method, using 25% PEG-MME 550, 100 mM MES, pH 6.5, 10 mM ZnSO₄, and three-fold excess palmitoyl-CoA. The drops consisted of 1.5 μL of protein solution plus 1.5 μL of crystallization solution and were equilibrated against 500 μL of the crystallization solution. The crystals grew in about one week. Before dataset collection, the crystals were cryoprotected by soaking for about 30 s in mother liquor with 30% PEG-MME 550. X-ray diffraction data were collected at 100 K. Measurements were made in-house using a Rigaku ULTRAX18 generator using copper radiation (\(\lambda = 1.5418\)) and a Mar345db image plate, over 1° increments in \(\phi\) for a total rotation of 360°. The data were indexed and integrated using the program MOSFLM [26] and scaled using the program SCALA from the CCP4 suite [27].

2.5. Structure solution and refinement

The structure of M. perniciosa ACBP was solved by employing molecular replacement (MR) using the Phaser program [28] and using human ACBP (52% identity) as a search model (PDB ID 2FJ9) [16]. The MR solution was used as an initial model for automatic model building using the ARP/wARP program [29].

Model refinement was carried out initially with REFMAC [30] and Phenix [31] employing TLS parameters in the last stages of refinement. The model-building was performed with COOT [32] using \(\sigma_I^2\)-weighted 2Fo-Fc and Fo-Fc electron density maps. A total of 265 water molecules were included by both COOT and Phenix, and the MES molecule and \(Zn^{2+}\) ion were added using COOT.

In all cases, the behavior of \(R_{free}\) was used as the principal criterion for validating the refinement protocol. The stereochemical quality of the model was evaluated with PROCHECK [33] and MolProbity [34]. The coordinates and structure factors have been deposited in the PDB as 3FP5. Complete statistics for data collection and refinement are summarized in Table 1.

3. Results and discussion

3.1. Gene sequence and ACBP isoforms

Analysis of the acbp gene from the M. perniciosa Genome Project [18] has identified two possible sequences for ACBP. One sequence represents the classical ACBP, encoding a protein with 90 amino acids (isoform 1) [35]. The second sequence represents an isoform with 104 amino acids, which is deposited in GenBank as EEB92840 (referred to in the present study as isoform 2). Various isoforms of ACBP have been found in different organisms, with the exception of fungi; four to six isoforms have been identified in nematode, arthropod and vertebrate species, while mammals have three tissue-specific isoforms [2]. Fig. 1 shows an alignment of M. perniciosa ACBP isoforms with human, bovine, yeast, Theobroma cacao (sequence not deposited), Ricinus communis, Panax ginseng, Zea mays and Sorghum bicolor ACBP sequences. Isoform 2 showed identities of 54% to human, 54% to cow, 47% to isoform 1, 43% to T. cacao, 42% to R. communis, 42% to P.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Full data collection and refinement statistics.</th>
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</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁/2₁/2₁</td>
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<tr>
<td>Unit cell parameters</td>
<td>a, b, c (Å)</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>α, γ</td>
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<tr>
<td>Redundancy</td>
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</tr>
<tr>
<td>Rmax (%)</td>
<td>5.5 (36.80)</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>5.1 (36.8)</td>
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<tr>
<td>Completeness (%)</td>
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</tr>
<tr>
<td>Total reflections</td>
<td>199440 (22624)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>14876 (1825)</td>
</tr>
<tr>
<td>I/σ(I)</td>
<td>34.8 (7.0)</td>
</tr>
<tr>
<td>Refinement parameters</td>
<td>Resolution range (Å)</td>
</tr>
<tr>
<td></td>
<td>Rmax (%)</td>
</tr>
<tr>
<td></td>
<td>Rfree (%)</td>
</tr>
<tr>
<td>Contents of asymmetric unit</td>
<td>Protein atoms</td>
</tr>
<tr>
<td></td>
<td>Water molecules</td>
</tr>
<tr>
<td></td>
<td>Ligand atoms</td>
</tr>
<tr>
<td>RMSD geometry</td>
<td>Bond lengths (Å)</td>
</tr>
<tr>
<td></td>
<td>Bond angles (°)</td>
</tr>
<tr>
<td></td>
<td>Overall B-factor (Å²)</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td>Most favored (%)</td>
</tr>
<tr>
<td></td>
<td>Additional allowed (%)</td>
</tr>
<tr>
<td></td>
<td>Outliers (%)</td>
</tr>
</tbody>
</table>

The values in parentheses refer to statistics in the highest bin.
ginseng, 40% to Z. mays, 38% to S. bicolor, and 34% to yeast variants when aligned with CLUSTALW. These results show that the M. perniciosa ACBPs are more closely related to the human L-ACBP than to the yeast ACBP. However, though this isoform of ACBP is exclusive to fungi, a BLAST search of non-redundant protein sequences showed greater percentage identity with basidiomycete ACBPs, which range from 104 to 110 amino acids in length. BLAST results showed the following percent identities with basidiomycota: Coprinopsis cinerea (XP_001836410), 74%; Laccaria bicolor (XP_001880619), 70%; Cryptococcus neoformans (XP_568458), 63%; Ustilago maydis (XP_759106), 61%; and Malassezia globosa (XP_001729358), 51%. The conserved residues observed in the alignment are involved in the interactions between α-helices 1 (Phe6), 2 (Thy33, Lys37, Gln38, Ala39, Thr40, Val41 and Gly42), 3 (Lys59, Trp60, Asp61, Ala62 and Trp63) and 4 (Ala74 and Tyr78); others residues have been changed but retained their key characteristics (similarity). These conserved residues are important for the maintenance of the characteristic folding of ACBP, and some are also involved in coenzyme A binding. The fact that ACBPs are highly conserved indicates that the search for species-specific ACBP inhibitors may be a difficult task.

3.2. Oligomeric state and binding studies

Analysis of size-exclusion chromatography data showed that about 80% of unliganded M. perniciosa ACBP exists in monomeric form, with the remaining 20% found as dimers (data not shown). Measurements of these fractions using fluorescence anisotropy and a dynamic light scattering technique for rotational diffusion and hydrodynamic radius ($R_H$), respectively, were compared with those from the monomeric fraction bound to various ligands (Fig. 2). The results suggested that M. perniciosa ACBP interacts with ligands and that the equilibrium shifts from the monomeric to the dimeric state principally when bound to long-chain acyl-CoA esters. These data are confirmed by native gel electrophoresis (Fig. 3B), which shows that liganded ACBP assumes dimeric states and preferentially binds long fatty chain acyl-CoA esters. In overview, DLS, anisotropy and native PAGE analysis indicate that monomeric M. perniciosa ACBP binds lauroyl-CoA and myristoyl-CoA preferentially, while the dimeric form binds more strongly to the other, larger acyl-CoA esters. Isoelectric focusing gel analysis (Fig. 3A) indicates that monomeric ACBP and ACBP in the presence of lauroyl-CoA present the same pI (~8.2), while

![Fig. 1. Alignment of ACBP sequences. Alignment of ACBP isoforms 1 and 2 from M. perniciosa with human (PDB: 2FJ9), cow (PDB: 1HB6), yeast (PDB: 1ST7), T. cacao (sequence not deposited), R. communis (GenBank: CAAT02000), P. ginseng (GenBank: BAB85987), S. bicolor (GenBank: EE514605) and Z. mays (GenBank: ACG27448) ACBP sequences.](image1)

![Fig. 2. Oligomeric states of ACBP. (A) The mean rotational diffusions of the entire apoprotein and the bound protein were evaluated by fluorescence anisotropy using the samples from microcalorimetry analysis after ligand titration compared with monomer and dimer forms. (1) Monomeric ACBP. (2) ACBP-lauroyl-CoA. (3) ACBP-myristoyl-CoA. (4) ACBP-palmitoyl-CoA. (5) ACBP-stearoyl-CoA. (6) ACBP-arachidoyl-CoA. (7) ACBP dimer. The rotational diffusion of the bound proteins incline to the dimer form, principally in the presence of larger (longer chain) ligands. (B) Dynamic light scattering measurement of hydrodynamic radius ($R_H$). (1) The monomer form of ACBP, with a calculated $M_r$ of 14.4 kDa. (2) ACBP-Lauroyl-CoA ($M_r$ 15.5 kDa). (3) ACBP-myristoyl-CoA ($M_r$ 17.2 kDa). (4) ACBP-palmitoyl-CoA ($M_r$ 23 kDa). (5) ACBP-stearoyl-CoA ($M_r$ 28.2 kDa). (6) ACBP-arachidoyl-CoA ($M_r$ 24.1 kDa). ACBP bound to palmitoyl-CoA, stearoyl-CoA or arachidoyl-CoA assumes a dimeric form in the presence of ligand, while ACBP bound to lauroyl-CoA and myristoyl-CoA assumes intermediate values for $R_H$, suggesting a mix of monomeric and dimeric forms.](image2)
ACBP has higher affinity for long chain acyl-CoA, suggesting that ACBP binds preferentially to long-chain acyl-CoA esters (Table 2). The association constants themselves, probably because lauroyl-CoA is observed when the protein is liganded with myristoyl-CoA, stearoyl-CoA and arachidoyl-CoA, suggesting that binding occurs [23]. Binding data in accordance with measurements proposed by other authors, where acyl-CoA esters with acyl chains between 14 and 22 carbon atoms in length are favored at the binding site [36]. The monomeric version of ACBP has a pI of around 8.2. This property prevents the measurement of accurate molecular mass by native PAGE, since the buffer used has a pH of 8.3 (at this pH, ACBP charge is near zero; therefore, it did not resolve well in the gel, generating a stain instead of a clear band). The use of the focusing gel experiments (Fig. 3A). Moreover, the analysis of the DLS and anisotropy data indicates that M. perniciosa ACBP bound to lauroyl-CoA is more monomeric than dimeric. Interestingly, the ITC data for lauroyl-CoA-ACBP binding data in two-site or two sequential binding sites models, but cannot fit a one-site model. The K1 for lauroyl-CoA with ACBP is lower than that for other acyl-CoA esters; these data may explain why the liganded compound is not found by an isoelectric focusing gel and a native PAGE. However, if lauroyl-CoA can bind to ACBP, the second association constant is important in determining whether that event will occur, if it is greater than the first constant. The K2 values for M. perniciosa ACBP using K1 from lauroyl-CoA (two sequential binding sites), myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA and arachidoyl-CoA were approximately 109 nM, 57 nM, 55 nM, 2 nM and 0.65 nM, respectively. Comparison of these data with the K2 values from other organisms indicates that M. perniciosa ACBP has greater affinity for arachidoyl-CoA than Trypanosoma cruzi ACBP has for myristoyl-CoA (0.35 nM) [39] or than yeast ACBP has for palmitoyl-CoA (0.55 nM) [23]; these values are minor

Table 2

Association constants of ACBP-acyl-CoA ester binding.

<table>
<thead>
<tr>
<th>Acyl-CoA</th>
<th>2 sites K1 (M⁻¹)</th>
<th>2 sites K₂ (M⁻²)</th>
<th>2 sequential sites K1 (M⁻¹)</th>
<th>2 sequential sites K₂ (M⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauroyl-CoA</td>
<td>(1.48 ± 7.34) × 10⁷</td>
<td>(1.47 ± 3.77) × 10⁷</td>
<td>(9.19 ± 1.70) × 10⁹</td>
<td>(1.89 ± 1.00) × 10⁻⁷</td>
</tr>
<tr>
<td>Myristoyl-CoA</td>
<td>(1.79 ± 0.88) × 10⁷</td>
<td>(1.46 ± 1.40) × 10⁹</td>
<td>(1.54 ± 0.27) × 10¹⁰</td>
<td>(1.45 ± 0.27) × 10⁻⁷</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>(1.85 ± 1.42) × 10⁷</td>
<td>(8.60 ± 7.30) × 10⁹</td>
<td>(7.69 ± 1.30) × 10¹⁰</td>
<td>(1.21 ± 0.86) × 10⁻⁷</td>
</tr>
<tr>
<td>Stearoyl-CoA</td>
<td>(4.21 ± 1.68) × 10⁷</td>
<td>(1.04 ± 0.40) × 10¹⁰</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Arachidoyl-CoA</td>
<td>(1.55 ± 1.34) × 10⁷</td>
<td>(1.56 ± 1.12) × 10¹⁰</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Parameters obtained from isotherms of ACBP-acyl-CoA ester binding. The standard errors were obtained with the ORIGIN program using the best fit of two binding sites and two sequential sites for one protein concentration of each ligand.

Lauroyl-CoA has a standard error greater than K₁ (first association constant) and K₂ (second association constant) for two sites. Data for stearoyl-CoA and arachidoyl-CoA cannot be fitted in the two sequential binding sites model.
compared to the affinity of bovine ACBP for palmitoyl-CoA (4.5 pM) [22]. The minor affinity found for lauroyl-CoA is similar to the $K_d$ value of bovine ACBP and capryloyl-CoA (approximately 277 nM) [24]. These data are consistent with the low affinity for lauroyl-CoA obtained in the isoelectric focusing gel and native PAGE. The $K_d$ values obtained for myristoyl-CoA and palmitoyl-CoA were similar to that found for armadillo ACBP, which has a range of 34 to 75 nM for palmitoyl-CoA [15]. The comparison indicates that *M. perniciosa* ACBP shows as high an affinity for long-chain acyl-CoA esters as does bovine ACBP, while *T. cruzi* ACBP has affinity for short-chain acyl-CoA esters, as previously reported [39]. This result suggests that the *M. perniciosa* ACBP has a greater affinity for long acyl-CoA esters with carbon chains up to C20. This can also be observed in the isothermal data we obtained, where the change from an exothermic to an endothermic isothermal was observed only with arachidoyl-CoA as a ligand, while this change is observed with minor acyl-CoA esters in ITC studies of bovine ACBP [24,37,22].

Analysis of acyl-CoA binding from isoelectric focusing gels and native PAGE showed that ACBP binds lauroyl-CoA weakly, but binds to other acyl-CoA esters with long acyl-CoA chains with greater affinity. The ITC data is in agreement with these data, where the lowest $K_1$ was observed for binding lauroyl-CoA, and increasing values of $K_1$ were observed when binding to longer acyl-CoA chains.

Other than human ACBP, all the proteins studied so far present as monomers in native form or when bound to acyl-CoA esters. The data obtained from DLS and native PAGE indicate that the *M. perniciosa* ACBP behaves as a monomer in its native form, but as a dimer when it is bound with acyl-CoA esters. The mutation of His to Ser in $\alpha$-helix 1 (from human to *M. perniciosa* ACBP) may be responsible for the failure of *M. perniciosa* ACBP to adopt a hexameric state, as does human ACBP. However, bovine ACBP, which contains the same residues as human ACBP, does not form a dimer when bound to acyl-CoA ester. These differing states for the three bound ACBPs can be attributed to structural differences. The *M. perniciosa* ACBP has the longest loop I of all the ACBP structures. Since this loop in human ACBP interacts with the terminus of fatty acids, this is consistent with our finding that the *M. perniciosa* ACBP may preferentially bind acyl-CoA esters with long fatty acids chains up 20 carbons. (Fatty acid chains above 20 carbons were not used in this work.) In order for this to occur, the *M. perniciosa* ACBP must be in dimer form, whereas bovine ACBP can bind shorter fatty acid chains when in monomer form.

3.3. *Moniliophthora perniciosa* ACBP structure description

The three-dimensional structures of ACBP from cow [12,13,40], *P. falciparum* [12], yeast [14], armadillo [15] and human [16] have been solved. All structures present the fold of the peptide backbone as an up–down–down–up four-helix bundle, which is a unique arrangement among the known four-helix folds [6]. The server CATH [41] classifies the topology of ACBP as an acyl-CoA binding protein, and SCOP [42] classifies the ACBP fold as a core of three helices, in a closed up-and-down bundle with a left-handed twist.
Neither described the ACBP fold as a classical four-helix bundle; indeed, the fourth helix has a packing angle of about 47° while the classical definition of a four-helix bundle is restricted to structures containing acute interhelical angles no greater than 40°. From this point of view, the ACBP molecule could not be classified as a classical four-helix bundle.

The *M. perniciosa* ACBP structure presented here crystallizes in the orthorhombic space group P2₁2₁2₁, with one molecule per asymmetric unit. The *M. perniciosa* ACBP structure possesses 104 residues, plus two extra N-terminal residues resulting from his-tag cleavage of the original protein expressed from a pET28a construct. The structure of *M. perniciosa* is similar to that of other ACBPs. However, it contains an extra helix at its C-terminus for a total of five α-helices (helix 1—residues 3–15; helix 2—residues 26–40; helix 3—residues 54–64; helix 4—residues 71–89 and helix 5—residues 92–103). This fifth helix is unique to the *M. perniciosa* ACBP (Figs. 5–6). The packing of helix 5 with helices 1, 2 and 4 forms a classical four-helix bundle (Fig. 7). The packing angles of the helices are shown in Table 3. The packing angle between helices 1 and 4 is 47.13°; as previously described this acute angle could not be considered a classical four-helix bundle. However, the bundle formed between helices 1, 2, 4 and 5 can be classified as a classical four-helix bundle of type D. The presence of the fifth helix in *M. perniciosa* ACBP shows how a packing of five helices could form two distinct four-helix bundles in the same protein. Studies of molecule stability and of the structure of *M. perniciosa* bound to acyl-CoA esters may produce additional information about the function of the fifth α-helix.

Loop I, located between α-helices 1 and 2, is larger (10 residues) than other ACBP loop structures (5 to 7 residues) (Fig. 1). The structure of the *M. perniciosa* ACBP contains two additional residues in the N-terminal region (Ser and His) as a result of the his-tag cleavage of the original protein expressed from a pET28a construct. The additional His participates in an ion–zinc interaction, together with Glu72 and Glu100 of the neighboring molecule. In bovine and human ACBP structures, cadmium and zinc ions are found coordinated by

**Table 3** Helix-packing pairs.

<table>
<thead>
<tr>
<th>Helix packing pair</th>
<th>Global angle</th>
<th>Local angle</th>
<th>Distance (Å)</th>
<th>Covalent Electro H-bond VDW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–2</td>
<td>−139.780</td>
<td>−138.505</td>
<td>12.044</td>
<td>0</td>
</tr>
<tr>
<td>2–3</td>
<td>−60.991</td>
<td>−60.704</td>
<td>8.060</td>
<td>0</td>
</tr>
<tr>
<td>1–4</td>
<td>47.133</td>
<td>46.734</td>
<td>9.012</td>
<td>0</td>
</tr>
<tr>
<td>2–4</td>
<td>−151.343</td>
<td>−150.835</td>
<td>7.184</td>
<td>0</td>
</tr>
<tr>
<td>4–5</td>
<td>−152.654</td>
<td>−150.310</td>
<td>6.074</td>
<td>0</td>
</tr>
</tbody>
</table>

The table shows packing angles and ligations and interactions found between these helices.

Fig. 6. Stereo image of the electron density map for the fifth α-helix. Stereo image of the electron density map 2Fo-Fc contoured at 1σ for the fifth α-helix in the *M. perniciosa* ACBP structure.

Fig. 7. Packing of the α-helices in *M. perniciosa* ACBP. (A) The typical topology of ACBP is an arrangement unique among known four-helix folds, formed by helices 1, 2, 3 and 4. (B) A classical four-helix bundle including helices 1, 2 and 5. Panels A and B were made in the same orientation.
His14 (on α-helix 1) and Glu11 and His15 (on α-helix 1), respectively. The His residue in α-helix 1 is found only in bovine and human ACBP structures, and these are the only structures that have ions bound. Therefore, this residue has an important function in binding ions, and when bound to Zn$^{2+}$, it is responsible for forming the human ACBP-myristoyl-CoA hexamer [16]. However, in the bovine structure, the metal ion is not involved in cluster formation, and this structure is found as a monomer in both the unbound and bound forms. In the M. perniciosa ACBP, the His residue is mutated to a Ser residue, and it is not found in nature. Like the M. perniciosa ACBP, the P. falciparum structure [12] presents an additional His residue at the N-terminus and was found with a nickel ion bound (1HBK).

The binding of molecules from crystallization or even cryoprotection solutions is commonly observed in protein structures. A molecule of the MES used as a buffer in crystallization was found in the M. perniciosa ACBP structure, localized between α-helices 1 and 2 (Fig. 8A). The morpholine ring is piled atop Phe36 on α-helix 2, and the oxygen atom of the ring forms a hydrogen bond with Tyr78 on α-helix 4. The oxygen atoms from the ethanesulfonic group form van der Waals interactions with Gln14 (on α-helix 1), Tyr33, Lys37 (on α-helix 2) and Lys59 (on α-helix 3). The MES molecule is localized in the same orientation as the base adenine from coenzyme-A (Fig. 8B). In order to obtain MES-free crystals, the MES was exchanged for Bis-Tris in crystallization assays in the presence of ligands, but crystals could not be obtained.

ACBP has been identified as a housekeeping protein [7] and was proposed to play a pivotal role in the trafficking and use of long chain acyl-CoA esters [2]. ACBP was confirmed as an essential protein in different cell types [8] and in T. brucei [9]. Moreover, its depletion in S. cerevisiae causes severe alterations in growth, cell structure and metabolism [10,11]. ACBP validation studies in fungi are necessary to confirm whether this protein is essential for these organisms and thus whether ACBP might be a good target for new fungicide molecules. Interestingly, M. perniciosa ACBP bound MES in the traditional binding site of the adenine derived from CoA, suggesting that this region can be utilized in screening for small molecule inhibitors. The search for small molecules able to inhibit the binding between ACBP and acyl-CoA esters will be an important step toward obtaining drugs that repress acyl-CoA ester transport and consequently silence the function of this important protein in fungal pests. Initial candidates might be analogs of purines and pyrimidines.

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