The carboxyltransferase activity of the apicoplast acetyl-CoA carboxylase of *Toxoplasma gondii* is the target of aryloxyphenoxypropionate inhibitors.

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**Running title:** Inhibition of *Toxoplasma gondii* acetyl-CoA carboxylase
Inhibition of growth of the apicomplexan parasite *Toxoplasma gondii* by arylxyphenoxypropionate herbicides has been correlated with the inhibition of its acetyl-CoA carboxylase (ACC) by these compounds. Here, full-length and C-terminal fragments of *T. gondii* apicoplast ACC as well as C-terminal fragments of the cytosolic ACC were expressed in *E. coli*. The recombinant proteins that were soluble showed the expected enzymatic activities. Yeast gene-replacement strains depending for growth on the expressed *T. gondii* ACC were derived by complementation of a yeast *ACC1* null mutation. *In vitro* and *in vivo* tests with arylxyphenoxypropionates showed that the carboxyltransferase domain of the apicoplast *T. gondii* ACC is the target for this class of inhibitors. The cytosolic *T. gondii* ACC is resistant to arylxyphenoxypropionates. Both *T. gondii* isozymes are resistant to cyclohexanediones, another class of inhibitors targeting the ACC of grass plastids.
INTRODUCTION

New treatments for human and animal apicomplexan pathogens are needed. Parasites of this phylum, including Plasmodium, Toxoplasma, Cryptosporidium and Eimeria species cause serious diseases in humans as well as in animals. Identifying candidate drugs either for a stand-alone or combination therapy requires characterization of key metabolic pathways in apicomplexan parasites, including their enzymology and subcellular localization. The basic molecular and cellular mechanisms are likely to be similar in all Apicomplexa. However, a significant divergence of these species with respect to protein structure, physiology and host range point to potential variability in the effectiveness and specificity of inhibitors targeting any particular enzyme. The metabolic pathways located in the apicoplast, a plastid-like organelle found in Apicomplexa (1-3), may have significantly distinct features to allow identification of specific antiparasitic inhibitors harmless to the host (4). Although the function of the apicoplast is not fully understood, it has been suggested to be the site of such pathways as the biosynthesis of isoprenoids, heme, aromatic amino acids and fatty acids (1-5). Apicoplast localization of essential enzymes of the fatty acid biosynthetic pathway, acetyl-CoA carboxylase (ACC) (6) and acyl carrier protein (7) in T. gondii, and acyl carrier protein and β-ketoacyl-ACP synthase III in P. falciparum (7,8), supports the hypothesis that the apicoplast is a site of de novo fatty acid biosynthesis.

ACC catalyzes carboxylation of acetyl-CoA to produce malonyl-CoA (full reaction). The enzyme consists of three major functional domains: the biotin carboxylase (BC) domain, the carboxyltransferase (CT) domain and the biotin carboxyl carrier (BCC) domain containing covalently attached biotin. The first step of the ACC-catalyzed reaction is an ATP-dependent transfer of the carboxyl group from bicarbonate to the biotin residue (first half-reaction). The carboxyl group is then transferred to acetyl-CoA producing malonyl-CoA (second half-reaction). Malonyl-CoA is used for de novo fatty acid biosynthesis as well as in fatty acid elongation.

Our previous study of ACC and its genes in T. gondii (6,9) showed the existence of two isozymes: one located in the apicoplast (ACC1) and the other in the cytosol (ACC2). ACC1 and ACC2 are both of the multi-domain type (all functional domains in one polypeptide) found in the
cytosol of eukaryotes and in plastids of some plants. Genes encoding multi-domain ACC have been found in other Apicomplexa as well (9). We postulated that the apicoplast isozyme is involved in Toxoplasma de novo fatty acid biosynthesis (6). The role of the cytosolic isozyme is not yet clear. The major Toxoplasma ACC activity is sensitive to aryloxyphenoxypropionates (fops) but resistant to cyclohexanediones (dims) (9). These two classes of compounds are herbicides targeting the multi-domain plastid ACC, inhibiting fatty acid biosynthesis in sensitive grasses (10-13). We have localized the herbicide sensitivity determinant to a 400-amino acid fragment of the carboxyltransferase (CT) domain (14) and showed that the second ACC half-reaction is affected (15). We have also shown that a single highly conserved amino acid residue located in this domain is essential for interaction with both classes of herbicide (15).

Enzymes of the fatty acid biosynthetic pathway are promising targets for inhibitors that could be used to treat parasitic diseases. The action of fops and dims on wheat (16), Toxoplasma (9) as well as on yeast gene-replacement strains (14,15,17) indicates that in all these systems, inhibition of ACC activity leads to a complete inhibition of the organism’s growth. On the other hand, these herbicides are not toxic to animal cells, providing a basis for their use in agriculture and potential in medicine. Furthermore, thiolactomycin, an inhibitor of fatty acid elongation, has been shown to prevent the growth of Plasmodium falciparum (7), and triclosan, an inhibitor of enoyl-acyl carrier protein reductase, has been shown to inhibit growth of P. falciparum and T. gondii (18).

In this paper we show that the T. gondii apicoplast ACC is sensitive to fops and that its CT activity is targeted by these inhibitors. We also describe in vitro and in vivo systems to screen for new ACC inhibitors.

**EXPERIMENTAL PROCEDURES**

Various constructs were assembled from cloned cDNA fragments (6) digested with restriction enzymes and ligated appropriately. DNA fragments with engineered restriction sites were obtained using the Expand High-Fidelity PCR system (Roche) to limit the number of PCR-related errors, cloned into the pGEM-T Easy vector (Promega) and sequenced before further use. Convenient restriction sites in the multiple cloning sites (MCS) of the cloning vectors were used.
in some constructions. The pPROEX HT based system (GibcoBRL) was used to express His-tagged proteins in E. coli. The pRS423-based system using the GAL10 promoter and yeast ACC1 leader and 3’-UTR (14,17) was used for ACC expression in yeast. The assembly process was monitored by restriction analysis and sequencing selected regions of each construct. DNA was sequenced at the University of Chicago Cancer Research Center Sequencing Facility. The positions of the cDNA fragments, key restriction sites used in the assembly process, and the structures of the constructs are outlined in Fig.1. Amino acid compositions of the proteins encoded by these constructs are shown in Table 1.

**T. gondii ACC1 and ACC2 cDNA.** ACC cDNA fragments ACC1/Fragment II, ACC1/Fragment III, ACC1/Fragment IV and ACC2/Fragment III were cloned by reverse transcription-PCR into the pGEM-T Easy vector (Promega) as previously described (6). cDNA fragments ACC1/Fragment V and ACC2/Fragment IV were isolated from a *T. gondii* cDNA library in pBluescriptSK (+) vector (Stratagene) as previously described (6). ACC1/Fragment II and ACC1/Fragment III were joined in pGEM-T Easy vector by 3-piece ligation to create ACC1/Fragment II+III: SnaBI-SgrAI fragment of ACC1/Fragment II and SgrAI-NdeI (MCS) fragment of ACC1/Fragment III were inserted between SnaBI and NdeI (MCS) sites of the plasmid containing ACC1/Fragment II. ACC1/Fragment IV and ACC1/Fragment V were joined in the same manner to create ACC1/Fragment IV+V: NotI (MCS)-BssSI fragment of ACC1/Fragment IV and BssSI-BamHI fragment of ACC1/Fragment V were inserted between NotI (MCS) and BamHI sites of the plasmid containing ACC1/Fragment V. ACC2/Fragment III and ACC2/Fragment IV were joined by inserting XmaI-StuI fragment of ACC2/Fragment III with an engineered XmaI site at the 5’-end between XmaI (MCS) and StuI sites of the plasmid containing ACC2/Fragment IV to create ACC2/Fragment III+IV.

**Constructs for expression of T gondii ACCs and their fragments in E. coli.** Construct ACC/fl comprising full-length mature ACC1 was obtained by inserting a NotI (MCS)-RsrII fragment of ACC1/Fragment II+III and an RsrII-AvrII fragment of fragment ACC1/Fragment IV+V into expression vector pPROEX HTa between NotI and XbaI sites. Construct ACC1/bct encoding ~2/3 of the C-terminal part of ACC1 was created by inserting a SnaBI-RsrII fragment of ACC1/Fragment II+III and RsrII-AvrII fragment of ACC1/Fragment IV+V into vector pPROEX HTb between StuI and XbaI sites. Construct ACC1/ct encoding the CT domain of
ACC1 was obtained by inserting NotI (MCS)-AvrII fragment of ACC1/Fragment IV+V into pPROEX HTb vector between NotI and XbaI sites. Shorter construct ACC1/ct1 was created by inserting SacI-BamHI and BamHI-AvrII fragments of ACC1/Fragment V into pPROEX HTc between SacI and XbaI sites. Construct ACC1/ct2 was obtained by inserting Smal-HindIII fragment of construct ACC1/ch3 (described below) into pPROEX HTc between Stul-HindIII sites. Construct ACC1/ct3 was obtained by inserting NcoI-BamHI and BamHI-AvrII fragments of ACC1/Fragment V into pPROEX HTa between NcoI and XbaI sites. ACC2/Fragment III+IV was cut from the vector with SmaI and KpnI (MCS) and inserted into pPROEX HTc between Stul-KpnI sites to create construct ACC2/ct1. A construct shorter at the 5’end, ACC2/ct2, contained a SspI- KpnI (MCS) fragment of ACC2/Fragment III+IV inserted into pPROEX HTc between the Stul-KpnI sites. Construct ACC2/ct3 was created by inserting Stul-KpnI (MCS) fragment of ACC2/Fragment IV into pPROEX HTb cut with appropriate enzymes. Construct ACC2/ct4 was shorter at the 3’end and contained Stul-XhoI fragment of ACC2/Fragment IV cloned into pPROEX HTb.

**Constructs for T. gondii ACC expression in yeast.** The yeast ACC1 3’-UTR with Eco47III and Apal sites engineered by PCR, using construct C100 (14) as template, was cloned between Eco47III and Apal (MCS) sites of ACC1/Fragment V to create an intermediate construct ACC1/Fragment Vyt. Constructs ACC1/ch1, ACC1/ch2, ACC1/ch3, and ACC1/ch4 were created by inserting DNA fragments from construct C100 encoding wheat cytosolic ACC and DNA fragments encoding T. gondii ACC1 from the intermediate ACC1/Fragment Vyt between SacII and Apal sites of yeast vector pRS423. Construct ACC1/ch1 included fragments SacII-AvrII and AvrII-BssHIII from C100, and BssHIII (engineered by PCR)-SgrAI and SgrAI-Apal from ACC1/Fragment Vyt. Construct ACC1/ch2 included fragments SacII-AvrII and AvrII- SphI from C100, and SphI (engineered by PCR)-SgrAI and SgrAI-Apal from ACC1/Fragment Vyt. Construct ACC1/ch3 included fragments SacII-AvrII and AvrII- XmaI (engineered by PCR) from C100, and XmaI (engineered by PCR)-AatII and AatII-Apal from ACC1/Fragment Vyt. Construct ACC1/ch4 was created from ACC1/ch2 by replacing a PmlI-AatII fragment with PmlI-XmaI (engineered by PCR) fragment from C100 and XmaI (engineered by PCR)-AatII from ACC1/Fragment V. To create constructs ACC1/fl1, ACC1/fl2 and ACC1/fl3 NcoI site and a translation start codon were engineered by PCR at three different locations. NcoI-BsrGI fragment
from each of the three PCR-generated DNA fragments, BsrGI fragment of ACC1/fl (described above) and BsrGI-ApaI fragment of the intermediate construct ACC1/Fragment Vyt were inserted between NcoI and ApaI sites of construct C100 (14) to replace the wheat cytosolic ACC ORF encoded by C100 with the apicoplast ACC1 ORF. ACC2/ch2 was created by inserting fragment XbaI-XmaI (both sites engineered) of C100 and XmaI-Eco47III (both engineered) of ACC2/Fragment IV between XbaI-Eco47III sites of ACC1/Fragment Vyt to first create an intermediate construct wheat-ACC2/Fragment IVyt and then inserting a PmlI-ApaI fragment from this intermediate between PmlI-ApaI sites of C100 to replace the 3’-terminal fragment of the wheat cytosolic ACC ORF encoded by C100 with a corresponding fragment of the Toxoplasma ACC2 ORF. Construct ACC2/ch1 was created by replacing a XmaI-ApaI fragment in ACC1/ch3 with a XmaI-XhoI fragment of ACC2/Fragment III+IV and XhoI-ApaI fragment of the intermediate construct wheat-ACC2/Fragment IVyt.

**Protein expression in E. coli, affinity purification, enzymatic activity and inhibition.**
pPROEX HT plasmids carrying various constructs were introduced into E. coli strain DH5α. Liquid cultures started from fresh transformants grown to OD of approximately 0.8 measured at 600 nm were induced by addition of IPTG to a final concentration of 1 mM. Cells were harvested after 4 – 16 hours of culture at room temperature. Protein expression was analyzed by SDS-PAGE using 4-15% gels from BioRad according to the manufacturer’s protocol and staining with Coommasie blue. His-tagged proteins were affinity-purified on a Ni-NTA Superflow resin (Qiagen) according to the manufacturer’s protocol. Poly(Histidine)-tagged protein protease inhibitor cocktail (Sigma) was added before cell lysis. The ACC activity was assayed by measuring incorporation of 14C from bicarbonate to acid-stable malonyl-CoA (at 37 °C) and the reverse CT reaction (at 23 and 37 °C) was assayed by measuring transfer of the carboxyl group from [14C]-malonyl-CoA to biotin ester as described previously (15). The conditions of the reverse CT reaction were such that 10-80% of the substrate was converted within 30-90 minutes. Inhibitors were added as 20-fold concentrated solutions in 10% DMSO in 0.1 M Tris/HCl, pH 8.0. Imidazole from the affinity column buffer had no effect on either enzymatic activity or on the inhibition patterns. Protein biotinylation was analyzed on Western blots using [35S]-streptavidin as described previously (16).
Complementation of the yeast ACC1 null mutation and growth inhibition of yeast gene-replacement strains. These experiments were carried out as described before (14,15,17). S. cerevisiae strain W303D-ACC1ΔLEU2 (relevant genotype: MATa/MATα, leu2-3,112/leu2-3,112 his3-11,15/his311,15 ade2-1/ade2-1 ura3-1/ura3-1 trp1-1/trp1-1 can1-100/can1-100 ACC1/acc1::LEU2) was provided by Dr. S. D. Kohlwein (Technical University Graz, Austria).

Constructs encoding Toxoplasma ACC and wheat/Toxoplasma chimeric ACCs were introduced on replicating plasmids and complementation of the ACC1 null mutation was tested by tetrad analysis. Inhibition by fops and dims was measured in vivo by growing appropriate gene-replacement haploid yeast strains in YPR medium (1% Bacto-yeast extract, 2% Bacto-peptone, 0.1% adenine sulfate, 2% raffinose) supplemented with 0.001% - 2% galactose, in the absence and in the presence of inhibitors added as 100 fold concentrated stock solutions in dimethyl sulfoxide. Growth of the yeast strains was determined by measuring culture turbidity at 600 nm. 1 OD corresponds to approximately 1.3 x 10^7 cells per ml (14). Fungal/yeast protease inhibitor cocktail (Sigma) was added before cell lysis for protein isolation. Total yeast protein for SDS-PAGE was extracted by TCA method.

Inhibitors. Haloxyfop and Sethoxydim were purchased from Crescent Chemicals (Hauppauge, NY). Clodinafop and Cethoxydim (our name for CGA215684) were provided by Novartis (now Syngenta, Research Triangle Park, NC).

RESULTS

An in vitro system to study T. gondii apicoplast ACC using proteins overexpressed in E. coli.

cDNA encoding full length mature (i.e. without the signal/transit peptide) T. gondii plastid ACC1 was assembled from overlapping cDNA fragments cloned by reverse transcriptase-PCR or isolated from a cDNA library (6). These cDNAs were used to prepare a series of constructs for expression in E. coli of full-length T. gondii ACC1 as well as its fragments containing the CT domain (Fig. 1 and Table 1). All of these peptides were fused to a His-tag at their N-termini to permit purification by affinity chromatography. Construct ACC1/fl
encoded all three functional domains of the apicoplast enzyme (ACC1). Mature apicoplast ACC is formed by removal of a signal peptide during transport into the organelle. This signal/transit peptide is located within a 300-amino acid extension located immediately upstream of the conserved BC domain (6). However, the exact location of the processing site is not known. The signal peptide was excluded from ACC1/fl by engineering a fusion with the His-tag at an arbitrary site 30 codons upstream of the first conserved residue of the BC domain (6). Construct ACC1/bct included two domains: BCC and CT. Construct ACC1/ct encoded only the second half of the enzyme and constructs ACC1/ct1-ACC1/ct3 encoded different length C-terminal fragments of ACC1, all including the conserved CT domain.

ACC1/fl protein was expressed in *E. coli* at a high level and approximately half of the protein was soluble. Affinity-purified protein contained multiple shorter polypeptides (Fig. 2) whose presence was not eliminated by adding protease inhibitors during cell lysis. The full-length protein constituted 1.5-4.5% of the total protein and was biotinylated at a level of 0.005-0.025 moles of biotin per mole of the full length ACC1 protein, as estimated by western blot analysis using [35S]-streptavidin to reveal biotinylated peptides (Fig. 2). Some of the smaller peptides were biotinylated as well. ACC1/fl protein had the expected ACC activity (full reaction): carboxylation of acetyl-CoA using bicarbonate and ATP. Its specific activity varied for different preparations from 0.01 to 0.16 nmole of malony-CoA produced per mg of protein per min. This activity was substrate (acetyl-CoA) dependent. The relatively low activity of these preparations can be explained by their purity and by the partial biotinylation. Most of the shorter products did not contain the CT domain (located at the C-terminus of ACC) required for the full reaction. ACC1/fl protein also showed the expected CT activity measured in the reverse ACC second half-reaction: transfer of the carboxyl group from malonyl-CoA to free biotin ester. The specific activity varied for different preparations from 0.4 to 1.2 nmole of malony-CoA converted per mg of protein per min. The reverse CT activity was higher than the full reaction activity because of the higher amount of the enzyme in its unbiotinylated form. Approximately 50% of the malonyl-CoA conversion was due to the enzyme’s activity independent of biotin. Similar biotin-independent CT activity was described previously for *E. coli* ACC subunits (19).

ACC1/ct protein was expressed in *E. coli* at a high level and approximately half of the protein was soluble but the affinity-purified preparation contained multiple shorter polypeptides
Specific activity measured in the reverse reaction varied in different experiments from 1.2 to 4.9 nmole of malony-CoA converted per mg of protein per min. The ACC1/bct protein was also expressed in *E. coli* at a high level but it was insoluble and could not be analyzed further.

ACC1/ct1-ACC1/ct3 proteins were expressed in *E. coli* at a very high level, a significant portion of these proteins was soluble and their affinity-purified preparations contained a single major polypeptide (Fig. 2). A variable fraction of the purified proteins precipitated over time during storage at low temperature or during attempts to concentrate them. The specific activity of these proteins, measured in the reverse CT reaction, varied for different preparations from 0.4 to 23 nmole of malonyl-CoA converted per mg of protein per min, with one preparation of each protein showing activity at the high end of the range and one at the low end of the range. This variability may reflect some properties of these polypeptides, which are also responsible for their spontaneous precipitation. All three proteins, ACC1/ct1-ACC1/ct3, showed a 10-fold higher specific activity when fresh protein preparations were used at 100-fold lower dilution. These maximum specific activities were similar to specific activities of purified *E. coli* CT (19) and purified recombinant CT of pea ACC (20). Such low concentrations of the enzyme were not used for the inhibition studies because of high background variation in the CT assay.

Multiple constructs were prepared in order to overcome potential problems with expressing active *Toxoplasma* ACC1 and its fragments in *E. coli*, such as low levels of expression, low protein solubility, purity and stability, improper folding, incomplete modification, variable enzymatic activity. We encountered all of them. It is important to emphasize that despite these problems, the inhibition patterns could be measured reproducibly and were consistent for different protein fragments and their preparations with different specific activities, for both the full ACC reaction and its second half-reaction.

**Fops inhibit *T. gondii* apicoplast ACC activity *in vitro* but dims do not.**

The effect of two fops, haloxyfop and clodinafop, and one dim, sethoxydim, on activity of the apicoplast ACC (ACC1/fl) was tested at concentrations previously shown to affect ACC
activity in partially purified protein extracts from Toxoplasma tachyzoites (9). Acetyl-CoA dependent activity of ACC1 decreased dramatically when the concentration of haloxyfop or clodinafop increased from 1 µM to 50 µM, with 50% inhibition at approximately 5 µM (Fig. 3). The activity was almost completely inhibited at 50 µM. In contrast, sethoxydim, even at 400 µM, showed no effect on the enzymatic activity. The inhibition profile is similar to that observed previously with crude extracts from whole tachyzoites, suggesting that the fop-sensitive apicoplast ACC is the major source of this enzymatic activity in tachyzoites.

**Carboxyltransferase activity of the apicoplast T. gondii ACC is targeted by fops.**

Previous results on wheat plastid ACC indicated that target sites for both classes of herbicides are located in a 400-amino acid fragment of the CT domain (14,17). However, the T. gondii ACC1 amino acid sequence is very different from that of the ACCs of grasses (9) and its sensitivity to fops and dims also differs considerably ((9) and Fig. 3). Therefore we determined whether fops target the same domain in the Toxoplasma enzyme and if the second ACC half-reaction is also affected. The CT activity (second half-reaction measured in reverse) of the full-length ACC1 (construct ACC1/fl) showed the same haloxyfop inhibition profile (Fig. 4a) as the full ACC activity measured for the same protein (Fig. 3a). However, the CT activity decreased only to 50-60% at 20 µM and the reaction was not inhibited further at higher inhibitor concentrations. In this case, ~50% of the enzyme activity resulted from the biotin-independent decarboxylation of malonyl-CoA, which is not affected by fops (data not shown). Thus, the biotin-dependent part of the CT activity measured is fully sensitive to haloxyfop. Haloxyfop (Fig 3b) and clodinafop (not shown) inhibited the activity of the C-terminal half of the enzyme (ACC1/ct) and sethoxydim had no effect, similar to the whole enzyme (Fig. 4a). The shortest proteins tested, ACC1/ct2 (Fig 3c) and ACC1/ct3 (not shown), were sensitive to haloxyfop and clodinafop over the same concentration range as longer enzyme fragments and insensitive to dims, although sethoxydim at 400 µM concentration decreased activity to 60%. These results narrow the functional CT domain targeted by fops to the C-terminal third of the enzyme. ACC1 C-terminal fragments showed lower biotin-independent CT activity (20-30%) than the full-length enzyme and in all cases the fops inhibited their biotin-dependent activity completely.
T. gondii apicoplast ACC complements a yeast ACC1 null mutation.

Synthetic genes consisting of Toxoplasma ACC1 coding regions fused to the yeast GAL10 promoter (galactose-inducible) were tested for their ability to complement a yeast ACC1 null mutation. Constructs ACC1/fl1 - ACC1/fl3 (Fig. 1 and Table 1) were designed to express full-length mature T. gondii apicoplast ACC in yeast. These constructs differ only by the position of the engineered translation start codon. The signal peptide was excluded from these constructs because it is not needed for ACC activity in yeast but it could misdirect the protein. The translation start codon was placed at three different positions offset by a few amino acids, upstream of the conserved BC domain as described above for the E. coli construct ACC1/fl. All three full-length variants of Toxoplasma apicoplast ACC complemented the yeast mutation and the resulting yeast haploid gene-replacement strains grew as fast as haploid wild-type yeast.

Four synthetic genes (ACC1/ch1-ACC1/ch4, Fig. 1 and Table 1) encoding the CT domain of Toxoplasma apicoplast ACC fused to the appropriate N-terminal part (BC and BCC domains) of the wheat cytosolic ACC (resistant to herbicides) were made. These constructs were prepared in parallel, with the fusion site between proteins from different species offset by 20-50 amino acids, to avoid loss of enzymatic activity caused by local incompatibility of the two sequences. Indeed, this approach has proved very useful since only one of the wheat cytosolic/T. gondii apicoplast chimeras (ACC1/ch3) could replace yeast ACC. The growth of a haploid yeast strain complemented by ACC1/ch3 was poor and the strain was difficult to maintain. After a few passages, the cells died or altered their phenotype with respect to their growth rate and response to fops.

Yeast gene-replacement strains depending on T. gondii apicoplast ACC are sensitive to fops.

We have previously shown that the complementing ACC enzyme confers on the yeast strain its properties with respect to resistance or sensitivity to fops and dims (14,17). Here we show that this strategy can be used for Toxoplasma ACC and can provide a convenient in vivo system to screening chemical libraries for new ACC-targeting inhibitors.

The haploid yeast gene-replacement strains expressing full-length T. gondii apicoplast
ACC (ACC1/fl1-ACC1/fl3) under full induction had doubling times similar to the wild type yeast (Table 1). Full induction of these genes driven by the GAL10 promoter is achieved at high galactose concentration. However, under these conditions no growth inhibition of the gene-replacement strains by fops was observed, even though the in vitro study described above confirmed that the apicoplast ACC is sensitive. A similar phenomenon was observed when herbicide-resistant maize plastid ACC was expressed in yeast gene-replacement strains (15). The most plausible explanation of this observation is that these strains express high levels of ACC activity such that even if a significant portion of the enzyme is inhibited in vivo, there is still enough activity left to support growth of the strains. Such a high expression level is possible if the foreign proteins retain their full enzymatic activity in the yeast biochemical background and escape down regulation imposed on the native ACC.

This suggestion was supported by [35S]-Streptavidin western blot analysis of ACC in the yeast gene-replacement strains. Under full induction (2% galactose), these strains produced 20 times more full-length biotinylated ACC1/fl1 peptide than yeast native ACC produced by the wild-type diploid yeast strain at 0.005% galactose. The growth of the wild-type yeast strain in YPR medium containing 2% raffinose is not significantly reduced at such low galactose concentration. The growth of the gene-replacement strains, however, is reduced significantly at galactose concentrations below 0.01% (Fig. 5). This growth rate reduction is correlated with a 50-fold lower expression of full-length biotinylated ACC1/fl1 protein observed under limited induction (0.005% galactose).

Western blot analysis of ACC1/fl1 expression in the gene-replacement yeast strain, using anti-T. gondii ACC1 antibodies, revealed that the full length protein constituted only ~4% of the total T. gondii ACC1-related polypeptides. Many shorter polypeptides were made, some of which were biotinylated. This is reminiscent of the results of protein expression in E. coli described above. Attempts to purify enzymatically active ACC1/fl1 from those yeast strains were unsuccessful even in the presence of protease inhibitors.

When expression of Toxoplasma ACC1 in yeast is limited by lowering the inducing galactose concentration to a level such that growth of the gene-replacement yeast begins to be inhibited, the strains become sensitive to fops (Fig. 5), consistent with the sensitivity of the native ACC isolated from T. gondii (9) and the Toxoplasma protein expressed in E. coli (this
The galactose concentration sufficient to sustain yeast growth but significantly decreasing the growth rate, in comparison to growth under full galactose induction, varies from experiment to experiment, because growth is very sensitive to the starting conditions (e.g. galactose concentration and its utilization in the pre-culture). In our experiments, this galactose concentration was in the range of 0.003% to 0.01%. After 2-3 days of culture, the growth of strains harboring constructs ACC1/fl1-ACC1/fl3 in the presence of 200 µM haloxyfop stopped at a culture O.D. ~0.6-0.7 while in the absence of inhibitor, growth reached O.D. ~2.5 after 4 days. All three constructs behaved as shown in Fig. 5.

Sethoxydim did not affect growth of ACC1/fl1-ACC1/fl3 gene-replacement strains under any conditions (data not shown). Wild-type yeast and a strain complemented by the resistant wheat cytosolic ACC were not inhibited by any compound tested ((17) and data not shown).

A gene-replacement strain depending on a wheat/Toxoplasma chimeric ACC, ACC1/ch3, was moderately sensitive to fops over a similar concentration range as the purified Toxoplasma enzyme and the corresponding CT fragment. Its inhibition profile was similar to those of the strains depending on the full-length Toxoplasma ACC1 grown under the limiting conditions described above. After 2-3 days of growth on YPRG medium (2% galactose) in the presence of 200 µM inhibitor, the culture density was 4-5 times lower than in the control without inhibitor (e.g. O.D. 0.6 compared to O.D. 3.0 in one experiment, O.D. 1.0 compared to O.D. 4.0 in another). This result shows that the CT domain of *T. gondii* apicoplast ACC, when fused to the N-terminal part of a resistant enzyme (the wheat part of the chimeric ACC originated from the herbicide-resistant wheat cytosolic isozyme), functions *in vivo* and is sufficient to confer sensitivity to the gene-replacement yeast strain. It also provides further proof that the CT domain of Toxoplasma ACC1 carries the fop sensitivity determinant.

*T. gondii* ACC2 is resistant to fops and dims.

A series of four constructs for expression in *E. coli* of different ACC2 fragments containing the CT domain (Table 1) were prepared from previously cloned cDNA fragments (6) and analyzed in the same way as described above. Although ACC2/ct1 and ACC2/ct2 proteins
were expressed at a very low level and ACC2/ct3 and ACC2/ct4 were mostly insoluble, small amounts of ACC2/ct1, ACC2/ct2 and ACC2/ct4 could be purified and tested for enzymatic activity. These protein preparations contained multiple shorter polypeptides. Identity of the full-length ACC2/ct1 and ACC2/ct2 was confirmed by Western blot analysis using anti-*T. gondii* ACC2 antibodies. All these proteins showed the expected CT activity measured in the reverse ACC second half-reaction. The specific activity varied from 0.3 to 1.0 nmole of malony-CoA converted per mg of protein per min with the biotin-independent activity accounting for ~10% of the total. The CT activity of ACC2/ct1 and ACC2/ct2 was resistant to both fops and dims at high concentrations (up to 400 µM). This result supports our conclusion that the properties of the apicoplast ACC, and not the cytosolic enzyme, are responsible for Toxoplasma sensitivity to fops.

Two synthetic genes (ACC2/ch1 and ACC2/ch2, Table 1) were prepared for expression of chimeric wheat cytosolic/Toxoplasma ACC2 proteins in yeast. However, neither of these complemented the yeast ACC1 null mutation.

**DISCUSSION**

Apicomplexan parasites cause serious diseases in humans as well as significant economic losses in animal husbandry. An extensive search for new drugs based on unique apicomplexan physiology, metabolic pathways and structures is needed. New targets can be identified based on whole genome sequencing and metabolic reconstruction. Enzymes in several biochemical pathways have recently been suggested as potential new targets for antiparasitic drugs (1-5,7,9,21,22). ACC is one of the candidates deserving a full exploration in this respect. First, it is already established as a target of two classes of very successful inhibitors (fops and dims): herbicides targeting ACC inhibit fatty acid biosynthesis in grasses leading to death of the susceptible plants (10-13). These herbicides do not kill mammalian cells, which provides the basis for their use in agriculture.

We have shown recently that fops inhibit *T. gondii* growth but do not affect human host cells (9). The parasite growth inhibition correlated well with the *in vitro* inhibition of its ACC activity. Dims had no effect on Toxoplasma growth nor on its ACC, a clear distinction from the
effect of these compounds on grasses. We have also determined that the parasite has two ACC isozymes: ACC1 located in the apicoplast and ACC2 located in the cytosol (6,9), and postulated that ACC1 participating in the apicoplast \textit{de novo} fatty acid synthesis is sensitive to fops and as such is responsible for the growth inhibition exerted by these compounds.

In this work, we tested this hypothesis by \textit{in vitro} and \textit{in vivo} analysis of Toxoplasma ACC1 and ACC2 with respect to their resistance/sensitivity and localization of the sensitivity determinant in the multi-domain enzyme. The \textit{in vitro} analysis relied on purified ACCs and their CT-containing fragments expressed in \textit{E. coli}. Several peptides obtained in this way were soluble and enzymatically active in either the full ACC reaction or in the second half-reaction, or both, depending on their structure. The low specific activity of the affinity-purified his-tagged full-length ACC1 preparations was consistent with their purity and partial biotinylation. Overexpressing a 250-kDa fully-modified eukaryotic protein in \textit{E. coli} was a difficult task. A his-tag rather than biotin was used for affinity purification because the latter method, using commercially available products, were ineffective in our attempts to purify other native multi-domain ACCs (unpublished results). The results of enzymatic tests, using these full-length ACC1 preparations properly reflect the properties of the native protein as indicated by the substrate dependence of their enzymatic activity. In addition, the inhibition patterns were very similar to those measured for protein preparations extracted from \textit{T. gondii} and for CT-fragments made in \textit{E. coli}, which showed both high purity and high substrate-dependent specific activity. Finally, these results were confirmed by \textit{in vivo} assays using yeast gene-replacement strains that depend on the activity of full-length \textit{T. gondii} ACC1 for growth.

The \textit{in vivo} analysis took advantage of the methodology developed earlier for a similar study of plant ACCs (14,15,17). In this approach, the activity of foreign ACCs is tested by complementation of the yeast \textit{ACC1} null mutation and their inhibition is tested by measuring growth of haploid gene-replacement strains, dependent on those foreign ACCs, in the presence of inhibitors. This approach was successfully implemented for Toxoplasma apicoplast ACC, although fop inhibition was detectable only at ACC expression levels limiting yeast growth. A gene-replacement strain with a chimeric wheat cytosolic/Toxoplasma apicoplast ACC grew poorly and was unstable but showed the expected sensitivity to fops.

Sensitivity of the apicoplast Toxoplasma ACC1 to fops was revealed by both methods.
Results of in vitro tests were similar for the full length apicoplast ACC or its fragments containing the CT domain only. On the contrary, the cytosolic isozyme ACC2 was resistant. Both isozymes, ACC1 and ACC2, were resistant to dms. Moreover, the profile of ACC1 inhibition is very similar to that of the crude protein extract isolated from parasites (9) indicating that the apicoplast isozyme contributes the major ACC activity in Toxoplasma tachyzoites. These results, taken together with the sub-cellular localization of the two ACC isozymes (6), support our hypothesis that inhibition of Toxoplasma growth in human fibroblasts by fops is due to inhibition of the apicoplast-localized fatty acid biosynthesis for which ACC1 is a key enzyme.

There is a significant difference in the sensitivity pattern between the wheat (14,17) and T. gondii ((9) and this work) ACCs. Inhibition of the parasite ACC1 requires higher fop concentration and the enzyme is resistant to dms. These differences probably reflect differences in the enzyme structure. The molecular mechanism of fop and dim interaction with the enzyme is not known although we have localized the sensitivity determinant within the wheat plastid ACC to a 400-amino acid region of the CT domain (14). Here we show that the CT domain of the apicoplast Toxoplasma ACC1 is also a target for fops and that this domain is sufficient to confer sensitivity on a chimeric ACC. We suppose that decarboxylation of biotin by the CT activity is affected by fops because the biotin-independent decarboxylation of malonyl-CoA was insensitive to inhibitors. We recently further reported the importance of a single Ile/Leu residue within the 400 amino acid region: all known resistant ACCs including resistant grass biotypes have a Leu residue at this site while sensitive grass enzymes have an Ile residue, and an Ile to Leu mutation confers resistance to previously sensitive enzymes (15). The sensitive Toxoplasma ACC1 has a Leu residue at the site. Interestingly, this residue is still important for the interaction with fops, since its mutation to Ile converts the enzyme to fop-resistant (15).

Studies on the sensitivity of T. gondii ACCs to representative fops and dms carried out to date reveal a good correlation between inhibitor activity against ACC measured using enzyme purified from the parasites or expressed and purified from E. coli, tested in the recombinant yeast system and in living parasites ((9) and this work). The compounds we tested were not specifically designed or selected to affect Toxoplasma. IC₅₀ values for fops were in the range of 5 µM which are too high for their application as drugs. Stronger specific inhibitors are needed. Yeast gene-replacement strains seem especially suitable for a large-scale search, because they
would favor such strong inhibitors whose influence will be much easier to detect. The yeast system worked well for the fops and dims tested so far ((14,17) and this work), and may be expected to work for new inhibitors targeting the CT or any other ACC domain. The *in vitro* system can be used in case the compounds are not taken up by yeast.

Functional studies and the search for inhibitors of ACCs from other Apicomplexa such as *Plasmodium*, *Eimeria* and *Cryptosporidium* can be carried out in the same way. Despite significant similarities between ACCs from different apicomplexan parasites, e.g. their multi-domain character, our comparison of amino acid sequences shows how divergent these sequences are for even the most conserved part of the enzyme, the BC domain (9). For example, the amino acid sequences of *T. gondii* and *P. falciparum* ACC1 are more divergent than the wheat cytosolic and plastid isoforms, which differ dramatically in their sensitivity to fops and dims. In order to design effective and specific drugs, it will therefore be necessary to study the ACCs from each parasite, because each may show different sensitivity to inhibitors. Our studies on ACC strongly support the idea that the other steps of the fatty acid biosynthesis pathway in the apicoplast provide promising targets for therapeutic intervention.

**REFERENCES**


**Footnotes**

**Abbreviations.** ACC, acetyl-CoA carboxylase; ACC1 and ACC2 apicoplast and cytosolic ACC,
respectively; BC, biotin carboxylase; BCC, biotin carboxyl carrier; CT, carboxyltransferase; fop, aryloxyphenoxypropionate; dim, cyclohexanedione.

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Inhibition of *Toxoplasma gondii* acetyl-CoA carboxylase

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**Figure legends**

Fig. 1. (a) Structure of *T. gondii* apicoplast ACC and an outline of the cDNA assembly. (b) Structure of synthetic genes for expression of the ACC and its fragments in *E. coli* and (c) in yeast.

The positions of cDNA fragments and key restriction sites used in the assembly process are shown. Cloning of Fragments II-V and the structure of the apicoplast ACC cDNA has been described previously (6). The His-tag was from vector pPROEX. GAL10 promoter, yeast ACC1 leader and 3′-UTR were as described previously (17). Amino acid composition of proteins encoded by the constructs is shown in Table 1. ACC subunit structure and localization of the herbicide (fops and dims) sensitivity determinant in plastid ACC from grasses were described previously (14). The amino acid site critical for interaction with fops and dims (15) occupied by Ile in plastid ACC of grasses and Leu in *T. gondii* ACC1 is indicated.

Fig. 2. Affinity-purified *T. gondii* ACC1 and its fragments expressed in *E. coli*.

A and B, Coomasie stained gel and autoradiogram of [35S]-Streptavidin western blot of ACC1/fl preparation, respectively. C-F, Coomasie stained gels of ACC1/ct, ACC1/ct1, ACC1/ct2 and ACC1/ct3 preparations, respectively. Protein bands corresponding to the full-length products are indicated.

Fig. 3. Effect of fops and dims on *T. gondii* apicoplast ACC activity (full reaction).

The protein tested was encoded by construct ACC1/fl (Fig. 1 and Table 1). The enzymatic activity in the absence of inhibitor was taken as 100%. Averages from two experiments are shown with errors. Average specific activity of enzyme preparations used in these experiments (100% activity) was 0.08 nmoles of malonyl-CoA produced per mg of protein per min.

Fig. 4. Effect of fops and dims on the carboxyltransferase activity of *T. gondii* apicoplast ACC.

Activity of proteins encoded by construct ACC1/fl (a), ACC1/ct (b) and ACC1/ct2 (c) was tested in the reverse second half-reaction of ACC. These constructs are shown on Fig. 1 and described in Table 1. The enzymatic activity in the absence of inhibitor was taken as 100%. Biotin-independent decarboxylation activity in the absence of inhibitors is shown as a white bar.
Averages from two experiments are shown with errors. ACC1/ct3 gave a similar result (not shown). Average specific activity of ACC1/fl, ACC1/ct and ACC1/ct2 enzyme preparations used in these experiments (100% activity) was 0.8, 2.4 and 6.5 nmoles of malonyl-CoA converted per mg of protein per min, respectively.

**Fig. 5. Inhibition of a yeast gene-replacement strain carrying *T. gondii* apicoplast ACC by haloxyfop under growth limiting conditions.**

Growth of a strain carrying construct ACC1/fl1 (Fig. 1 and Table 1) in the absence and in the presence of 200 µM haloxyfop. Galactose concentration in the growth medium was 0.01%. Under these conditions GAL10-driven ACC expression limited yeast growth (doubling time 14.9±1.5 hr). Strains carrying constructs ACC1/fl2 and ACC1/fl3 (Fig. 1 and Table 1) have similar growth properties (doubling time 9-14 hrs) and haloxyfop sensitivity at limiting galactose concentrations (0.003 and 0.01%).
### Table 1. Amino acid composition and properties of expressed proteins.

<table>
<thead>
<tr>
<th>Construct</th>
<th>N-terminus</th>
<th>wheat ACC</th>
<th>T. gondii ACC</th>
<th>C-terminus</th>
<th>ACC and CT activity</th>
<th>Enzyme properties</th>
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<tbody>
<tr>
<td>Interaction with fops</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Expression in E. coli</td>
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<td>ACC1/fl</td>
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<td>-</td>
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<td>sensitive</td>
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<tr>
<td>ACC1/ct2</td>
<td>33-aa His-tag</td>
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<td>5 aa +</td>
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<td>sensitive</td>
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<td>ACC2/ct1</td>
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<td>7.1±0.2 ¶ sensitive</td>
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<td>3.5±0.2 ¶ resistant/sensitive #</td>
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<td>-</td>
<td>-</td>
<td>2.9±0.2 resistant</td>
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</table>

* amino acid position in T. gondii ACC1 including signal/transit peptide (GenBank AF157612), ‡ amino acid position in T. gondii ACC2, numbering of a partial sequence (GenBank AF330145), § amino acid position in wheat cytosolic ACC (GenBank U10187), † two amino acid residues were added when a restriction site was engineered, # sensitive when ACC expression limited yeast growth (at limiting galactose concentration), ¶ two haploid strains were tested for each construct, + from yeast ACC, & vector encoded, n.d., not determined.
The carboxyltransferase activity of the apicoplast acetyl-CoA carboxylase of toxoplasma gondii is the target of aryloxyphenoxypropionate inhibitors
Joanna Jelenska, Anchalee Sirikhachornkit, Robert Haselkorn and Piotr Gornicki

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