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# Redox Regulation of *Arabidopsis* Mitochondrial Citrate Synthase

**Elisabeth Schmidtmann, Ann-Christine König, Anne Orwat, Dario Leister, Markus Hartl** and **Iris Finkemeier**[1]

Botany, Department I of Biology, Ludwig-Maximilians-University Munich, Grosshaderner Strasse 2, 82152 Planegg-Martinsried, Germany

**ABSTRACT Citrate synthase has a key role in the tricarboxylic (TCA) cycle of mitochondria of all organisms, as it catalyzes the first committed step which is the fusion of a carbon–carbon bond between oxaloacetate and acetyl CoA. The regulation of TCA cycle function is especially important in plants, since mitochondrial activities have to be coordinated with photosynthesis. The posttranslational regulation of TCA cycle activity in plants is thus far almost entirely unexplored. Although several TCA cycle enzymes have been identified as thioredoxin targets *in vitro*, the existence of any thioredoxin-dependent regulation as known for the Calvin cycle, yet remains to be demonstrated. Here we have investigated the redox regulation of the *Arabidopsis* citrate synthase enzyme by site-directed mutagenesis of its six cysteine residues. Our results indicate that oxidation inhibits the enzyme activity by the formation of mixed disulfides, as the partially oxidized citrate synthase enzyme forms large redox-dependent aggregates. Furthermore, we were able to demonstrate that thioredoxin can cleave diverse intra- as well as intermolecular disulfide bridges, which strongly enhances the activity of the enzyme. Activity measurements with the cysteine variants of the enzyme revealed important cysteine residues affecting total enzyme activity as well as the redox sensitivity of the enzyme.**

**Key words: citrate synthase; mitochondria; cysteine residues; redox regulation; thioredoxin; TCA cycle; *Arabidopsis*.**

## INTRODUCTION

The mitochondrial tricarboxylic acid cycle (TCA cycle) plays an important role in energy metabolism of all aerobic organisms. Different carbon fuels are metabolized to acetyl CoA and organic acids in aerobic cells which are subsequently oxidized in the TCA cycle to deliver reduction equivalents for oxidation and ATP production in the mitochondrial electron transport chain. Furthermore, the TCA cycle delivers precursors for other biosynthetic processes such as the synthesis of aspartate. In plants, the regulation of TCA cycle and respiration is more complex than in heterotrophic organisms, as the plant mitochondrial metabolism has to be orchestrated with photosynthetic processes such as carbon assimilation in the Calvin cycle, photorespiration, nitrogen assimilation, and the dissipation of excess energy (Araujo et al., 2012; Schwarzlander and Finkemeier, 2013). The coordination of mitochondrial and chloroplastic functions was previously demonstrated in tomato mutants of different mitochondrial TCA cycle enzymes (Nunes-Nesi et al., 2011). However, not much is known yet about the mechanisms that posttranslationally regulate mitochondrial TCA cycle enzymes, and thus might coordinate the activities of chloroplasts and mitochondria (Nunes-Nesi et al., 2013).

The cellular redox milieu is one of the most important determinants that affect the catalytic activity of many metabolic enzymes by altering the redox state of cysteine residues (Scheibe and Dietz, 2012). In chloroplasts, several metabolic processes, such as the Calvin cycle and starch synthesis, for example, are regulated by NADPH-dependent thioredoxin (TRX) activation through the reduction of inter- or intramolecular disulfide bridges in enzymes (Buchanan, 1984; Schürmann and Buchanan, 2008; Michalska et al., 2009). TRXs are thiol-oxidoreductases and they generally function in redox regulation of diverse cellular processes in most organisms (Meyer et al., 2012). Their catalytic activity is based on two redox-active cysteine residues embedded in a conserved active site (Cys–Gly–Pro–Cys) (König et al., 2012). The redox-active cysteine residues of TRXs are activated by reduction which is usually mediated by NADPH-dependent thioredoxin reductases (NTR). The plant mitochondrion also contains a functional TRX/NTR-system (Laloi et al., 2001), and more than 100 *in vitro* TRX-targets have been identified in plant mitochondria from different species including several enzymes of the TCA cycle such as citrate synthase in

[1] To whom correspondence should be addressed. E-mail i.finkemeier@lmu.de, tel. +49(0)89-2180-74672.



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*Arabidopsis* mitochondria (Balmer et al., 2004; Yoshida et al., 2013). Citrate synthase catalyzes the first committed step in the TCA cycle, which is the condensation of an acetyl group (from acetyl CoA) and oxaloacetate (OAA) to citryl CoA, and requires no co-factor or metal ion for its activity. Citryl CoA is subsequently hydrolyzed by water, and citrate and CoA are released from the enzyme. In contrast to other TCA cycle enzymes, citrate synthase is exclusively localized in mitochondria in green tissues and thus the TCA cycle cannot be bypassed via cytosolic isoforms. Only in tissues with an active glyoxylate cycle are the peroxisomal isoforms of citrate synthase expressed (Pracharoenwattana et al., 2005). This is important in the context that the activity of *Arabidopsis* citrate synthase was previously shown to be inhibited by oxidation (Stevens et al., 1997) and thus cannot be instantly bypassed by the peroxisomal isoforms. Furthermore, alterations in cellular citrate contents were recently demonstrated to cause major changes in the transcriptome (Finkemeier et al., 2013). A controlled regulation of citrate synthase activity could therefore be an important regulatory mechanism for mitochondria-to-nucleus signaling. Hence, the redox regulation of *Arabidopsis* mitochondrial citrate synthase deserves further investigation, and the functional significance of the TRX interaction for its enzymatic function has not been demonstrated so far. In this study, we investigated the redox-dependent regulation of CS4 which is the major mitochondrial isoform of *Arabidopsis* citrate synthase.

## RESULTS

### CS4 Is the Major Mitochondrial Citrate Synthase Isoform in *Arabidopsis*

*Arabidopsis* mitochondria possess two predicted citrate synthase isoforms: CS4 (At2g44350) and CS5 (At3g60100), encoded by two separate nuclear genes. Although CS4 is the main isoform and much more abundant than CS5, both isoforms have previously been identified in *Arabidopsis* mitochondria in different proteomic approaches (Heazlewood et al., 2004; Klodmann et al., 2011). Not only is the protein of CS4 more abundant than CS5, but also the CS4 transcript is present at much higher levels during all growth stages in *Arabidopsis*, while the CS5 transcript is only present at fairly low levels (Genevestigator database; Zimmermann et al., 2004) (Supplemental Figure 1).

### CS4 Contains Six Cysteine Residues of which Cys364 Is the Most Conserved

The mitochondrial citrate synthase sequences from plants and animals are overall highly similar in primary structure. The best characterized enzyme is the porcine citrate synthase (Larson et al., 2009), which shares 63% and 61% sequence identity with *Arabidopsis* CS4 and CS5, respectively (Figure 1), and several sites involved in coenzyme binding, the dimer–dimer interphase, as well as the amino acids of the catalytic triade (His–His–Asp) are conserved between these species (Supplemental Figure 2).

CS4 shares about 85% sequence identity with CS5 on amino acid level and, although both sequences are highly similar, they differ in their number of cysteine residues. While CS4 contains six cysteine residues, CS5 contains only four (Figure 1). The six cysteine residues of CS4 are referred to as Cys108, Cys209, Cys325, Cys365, Cys439, and Cys467 in the following and as depicted in Figure 1. The two cysteine residues, Cys209 and Cys467, which are missing in CS5 are conserved in the protein sequence of citrate synthase of rice (*Oryza sativa*), while Cys209 but not Cys467 is also conserved in the porcine (*Sus scrofa*) but not in the human citrate synthase sequence. While Cys365 seems to be conserved in most organisms, Cys325 and Cys439 are plant-specific. Hence, we selected *Arabidopsis* CS4 for further analysis, since it is the main mitochondrial isoform and it was previously identified as target of the mitochondrial TRX-o1 by TRX-affinity chromatography (Yoshida et al., 2013).

### The Recombinant 6xHis-CS4 Protein Is an Active Citrate Synthase Enzyme

As the function of *Arabidopsis* CS4 is only inferred by sequence homology, we first characterized the enzyme function using a recombinant CS4 protein, which we expressed and purified with an N-terminal 6xHis-tag from *Escherichia coli*. Pettersson et al. (2000) used an N-terminal His-tag for isolation of a related recombinant citrate synthase protein from yeast and showed that it had no inhibitory effect on the enzyme activity. For the basic enzymatic characterization, we purified the recombinant CS4 protein under native conditions and kept a cysteine-reducing environment throughout all purification and dialysis steps (Figure 2A). The purified protein was highly active and showed the typical acetyl CoA- and OAA-dependent citrate synthase enzyme activity (Figure 2B–2D). Maximal citrate synthase activity was observed at 20°C and pH 8, although nearly 50% of the activity was still retained at 35°C, as well as at physiological high (pH 9) or low pH (pH 5) (Figure 2B and 2C). The $K_m$-values of CS4 for acetyl CoA and OAA were determined by keeping one substrate concentration constant (200 μM) and by varying the amount of the other (2.5–120 μM) (Figure 2D). While the $K_m$-values for acetyl CoA and OAA were with 16.5 and 49.4 μM, respectively, in the range of the $K_m$-value observed for pea (31 and 16 μM) and tomato (18 and 19 μM), they were slightly higher than those observed for porcine mitochondrial citrate synthase (5 and 5.9 μM) (Iredale, 1979; Jeffery et al., 1988; Kurz et al., 1995). By comparing the $K_m$-values as well as the specificity constants ($k_{cat}/K_m$) for OAA and acetyl CoA, it becomes clear that CS4 has a higher affinity for acetyl CoA than OAA (Table 1). From pig heart citrate synthase, it is known that, during catalysis, OAA is bound first, which increases the binding constant for acetyl CoA (Johansson and Pettersson, 1977). The turnover number ($K_{cat}$) of CS4 for acetyl CoA (72 $s^{-1}$) and