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(2003) Plant Physiology, 133 (2), pp. 875-884.

Fatty Acid Biosynthesis in Mitochondria of Grasses: Malonyl-Coenzyme A Is Generated by a Mitochondrial-Localized Acetyl-Coenzyme A Carboxylase[1]

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We present biochemical evidence for the occurrence of a 250-kD multifunctional acetyl-coenzyme A carboxylase in barley (*Hordeum vulgare*) mitochondria. Organelles from 6-d-old barley seedlings were purified by differential centrifugation and Percoll density gradient centrifugation. Upon analysis by two-dimensional Blue-native (BN)/SDS-PAGE, an abundant 250-kD protein can be visualized, which runs at 500 kD on the native gel dimension. A similar 500-kD complex is present in etioplasts from barley. The mitochondrial 250-kD protein is biotinylated as indicated by specific reaction with an antibody directed against biotin. Peptide sequence analysis by electrospray ionization tandem mass spectrometry of the 250-kD proteins from both organellar fractions revealed amino acid sequences that are 100% identical to plastidic acetyl-coenzyme A carboxylase from wheat (*Triticum aestivum*). The 500-kD complex was also detected in wheat mitochondria, but is absent in mitochondrial fractions from Arabidopsis. Specific acetyl-coenzyme A carboxylation activity in barley mitochondria is higher than in etioplasts, suggesting an important role of mitochondria in fatty acid biosynthesis. Functional implications are discussed.

Malonyl- coenzyme A (CoA) is the building block of fatty acid biosynthesis. It is formed by carboxylation of acetyl-CoA in an ATP-dependent reaction, which is catalyzed by acetyl-CoA carboxylase (ACC). Consequently, ACC can be considered as the starting enzyme of fatty acid biosynthesis. Because malonyl-CoA concentration is rate limiting in fatty acid biosynthesis (Post-Beitenmiller et al., 1992), ACC also is the major site of regulation of this anabolic pathway. The prerequisite for ACC function is a covalently attached biotin group.

Four different peptide domains were shown to be crucial for carboxylation of acetyl-CoA: the biotin carboxylase, which is responsible for the ATP-dependent carboxylation of biotin resulting in carboxybiotin, the biotin-carboxy carrier protein carrying the prosthetic group biotin linked via the ϵ-amino group of a Lys to the peptide backbone, and two peptides of the carboxyltransferase, which transfer the CO_2 group from carboxybiotin to the final acceptor acetyl-CoA. In bacteria, these four peptides are individual proteins that form a hetero-oligomeric protein complex ("multisubunit" or "prokaryotic-type" ACC). In contrast, eukaryotes typically contain a single subunit ACC combining all four domains within a single 250-kD polypeptide chain ("multifunctional" or "eukaryotic-type" ACC).

In heterotrophic eukaryotes, fatty acids are synthesized in the cytosol, whereas in plants, this pathway mainly occurs in plastids (Ohlrogge et al., 1979). Plastids were found to be capable of forming long-chain fatty acids up to C_{18}, which subsequently can be exported and used for lipid biosynthesis at the endoplasmic reticulum. However, elongation of fatty acids to generate very long fatty acids up to C_{32}, which are abundant in the cuticles of plant cells, occurs in the cytoplasm (Post-Breitenmiller, 1996).

Consequently, plant cells need two acetyl-CoA carboxylases in two different subcellular locations, the cytoplasm and the plastid. Interestingly, the cytoplasmic enzyme is a multifunctional ACC, whereas the plastid enzyme is of the multisubunit form (Kannangara and Stumpf, 1972; Sasaki et al., 1993; Alban et al., 1994; Konishi and Sasaki, 1994; Reverdatto et al., 1999). Hence, ACC structure in plant cells nicely supports the endosymbiont hypothesis for the evolution of eukaryotic cells. Only grasses are an exception because they lack a multisubunit ACC in plastids but contain a multifunctional ACC in two cellular compartments, the cytoplasm and the plastids (Egli et al., 1993; Gornicki and Haselkorn, 1993; Konishi et al., 1996). Another exception is observed in oilseed rape (*Brassica napus*) where plastids were reported to contain a multisubunit and a multifunctional ACC at the same time, and another multifunctional ACC in the cytoplasm (Schulte et al., 1997).

Only 15 years ago, fatty acid biosynthesis was also discovered in mitochondria. Initially, a mitochondrial acyl-carrier protein was identified in fungi, animals, and plants, which is a prerequisite for fatty acid biosynthesis (Brody and Mikolajczyk, 1988; Chuman and Brody, 1989; Runswick et al., 1991; Sackmann et al., 1991; Shintani and Ohlrogge, 1994). Later studies demonstrated the ability of mitochondria to synthesize short-chain fatty acids probably as precursors for lipoic acid biosynthesis (Mikolajczyk and Brody, 1990; Zensen et al., 1992; Jordan and Cronan, 1997; Schneider et al., 1997; Wada et al., 1997; Gueguen et al., 2000). This compound is an important prosthetic group of some mitochondrial enzymes. Synthesis of long fatty acids is only observed to a limited extend, most likely for repair of mitochondrial lipids. Mitochondria contain all enzymes for fatty acid biosynthesis, but curiously ACC, the starting enzyme for fatty acid biosynthesis, has not been found. The absence of a ACC from mitochondria was also reported for plants (at least for dicotyledonous plants; mitochondria from monocotyledonous plants were not investigated with respect to ACC; Baldet et al., 1993; Wada et al., 1997; Gueguen et al., 2000). Therefore, malonate import from the cytoplasm is discussed as being a prerequisite for mitochondrial fatty acid biosynthesis, and enzymes catalyzing the binding of malonate to CoA or acyl-carrier protein have been described for pea (*Pisum sativum*) mitochondria (Gueguen et al., 2000).

Here, we report the identification of a 250-kD multifunctional ACC in mitochondria of etiolated barley (*Hordeum vulgare*) seedlings. Protein complexes from barley were systematically investigated by two-dimensional Blue-native (BN)/SDS-PAGE. In contrast to dicotyledonous plants, mitochondria from barley contain a homodimeric 500-kD complex of two 250-kD subunits, which were identified by mass spectrometry (MS) as multifunctional ACC. In parallel, a homodimeric 500-kD ACC complex was also identified in barley etioplasts. Because the ACC proteins from barley mitochondria and etioplasts have very similar or identical primary structures, they might have to be added to the growing list of so-called "dual-targeting" proteins in plant cells (Peeters and Small, 2001). Interestingly, specific ACC activity was found to be higher in mitochondria than in etioplasts, indicating a quantitatively important contribution of the mitochondrial ACC to fatty acid biosynthesis in etiolated barley seedlings.

RESULTS

### Multisubunit Complexes of Mitochondria and Etioplasts from Barley Seedlings

BN-PAGE is a powerful tool for the separation of membrane-bound and -soluble protein complexes. Combined with SDS-PAGE as a second gel dimension, the procedure allows the resolution of subunits of protein complexes. BN-PAGE originally was developed for the analysis of respiratory protein complexes in fungi and mammals (Schägger and von Jagow, 1991; Schägger, 2001) and later was shown to be very useful for the analysis of mitochondrial and chloroplast protein complexes from dicotyledonous plants (Jänsch et al., 1996; Kügler et al., 1997). We now extend such investigations to mitochondrial and etioplast protein complexes from barley as a representative of monocotyledonous plants.

To minimize contamination of mitochondrial preparations by chloroplast debris, mitochondria were isolated from 6-d-old etiolated barley seedlings. After isolation and purification of organelles by differential centrifugation and Percoll density gradient centrifugation, mitochondrial protein fractions were two-dimensionally separated by BN/SDS-PAGE (Fig. 1A). The multi-subunit complexes I (about 1,000 kD), the HSP60 14-mer (~750 kD), the F_1F_0-ATP synthase (~580 kD), cytochrome c reductase (~480 kD), and the F_1 part of the ATP synthase (~350 kD), which easily becomes detached from the F_0 part during protein solubilization, exhibit very similar subunit compositions as described for mitochondrial protein complexes from dicotyledonous plants (Jänsch et al., 1996; Kruft et al., 2001; Eubel et al., 2003).

For comparison, analogous investigations were also performed with barley etioplasts (Fig. 1B). Four protein complexes are visible on the gels that were identified by their subunit compositions (for comparison, see Kügler et al., 1997) or by MS (Table I): the ~750-kD Rubisco-binding protein complex formed by seven copies of the 60-kD α subunit and seven copies of the 59-kD β subunit, the 530-kD Rubisco complex formed by eight copies of the 53-kD large subunit and eight copies of the 14-kD small subunit, the 260-kD dimeric b_6f complex consisting of cytochrome f (32 kD), cytochrome b_6 (24 kD), the FeS protein (20 kD), and subunit IV (17 kD), and the plastidic F_1-ATP synthase complex. The presence of these protein complexes in etioplasts has been previously reported (Boardmann, 1981; Tabake et al., 1985; Klein and Mullet, 1986, 1987; Musgrove et al., 1987). As expected, photosystems are absent in etioplasts (Takabe et al., 1985).

The BN gels also show that cross-contaminations of mitochondrial and etioplast fractions are not detectable in the limits of the staining procedure and can therefore be considered to be very low. Furthermore, mitochondria and etioplasts remain intact during preparation because membrane-bound (e.g. the respiratory chain complexes of mitochondria or the cytochrome b_6f-complex of etioplasts) and -soluble protein complexes (e.g. the mitochondrial HSP60 complex or the Rubisco complex of etioplasts) are visible on the BN gels (Fig. 1, A and B).
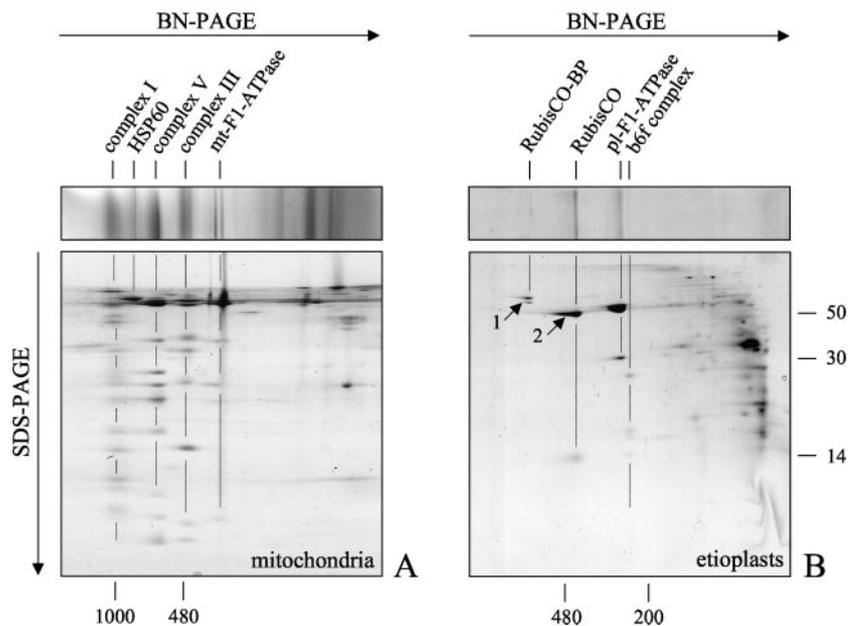
Figure 1. Resolution of mitochondrial and etioplast protein complexes from barley by two-dimensional BN/SDS-PAGE. Stripes of the first gel dimensions are shown above the two-dimensional gels. All gels were stained by Coomassie Blue. The molecular masses of known protein complexes or proteins are given below the two-dimensional gels for the first gel dimensions and on the right side for the second gel dimensions (in kilodaltons). Identities of protein complexes are indicated above the gels. Proteins marked by arrows were identified by MS (Table I).

Detection of a 500-kD Homodimeric Protein Complex in Mitochondrial Fractions

Besides the above described mitochondrial protein complexes, a diffuse protein spot was detectable in the stacking gel of the second gel dimension (data not shown). This spot seemed to represent a very large protein not able to enter the separation gel under the conditions applied. Analysis of mitochondria of dicotyledonous plants by BN/SDS-PAGE did not reveal the presence of a protein of comparable size (Jänsch et al., 1996; Kruft et al., 2001). Therefore, gel conditions were optimized for the separation of very large proteins.

Low-percentage polyacrylamide gels for the second dimension allowed the identification of an abundant protein of about 250 kD that forms part of a 500-kD complex on the BN gel dimension (the complex runs between the cytochrome c reductase complex [480 kD] and the ATP synthase complex [580 kD]; Fig. 2A). No other proteins are visible on the second gel dimension in the same vertical line, indicating that this 500-kD protein complex has a homodimeric structure. Because a diffuse protein spot

was also visible upon analysis of etioplasts by two-dimensional BN/SDS-PAGE, low-percentage polyacrylamide gels were also repeated for these organelles. A protein of comparable migration behavior in both gel dimensions could be visualized (Fig. 2B). However, this protein is of rather low abundance if compared with the corresponding mitochondrial protein.

The Mitochondrial 250-kD Protein Is an ACC

The size of the 250-kD protein as well as its presence in plastids raises the possibility that it represents a multifunctional 250-kD ACC, which is known to form a homodimeric 500-kD protein complex in eukaryotes. To test this hypothesis, mitochondrial fractions from barley were analyzed by immunoblotting using an antibody directed against biotin, the prosthetic group of ACC. This antibody specifically recognizes the 250-kD protein on two-dimensional BN/SDS gels, indicating that the protein seems to represents ACC (Fig. 3, A and B). A 75-kD subunit of methylcrotonyl-CoA carboxylase (MCC), which also

Table I. *Proteins identified by MS*

Spot[a]	Analyzed Peptides[b]	Identity	Accession No.
1	VIAAGANPVQITR	Rubisco Binding subunit, *β* subunit	Q43831 (rye, [*Secale cereale*]).
2	VALEAVCQAR	Rubisco, large subunit	P05698 (barley)
3	TITSNTDTVSEYVSYLVK	Plastidic acetyl-CoA carboxylase	T06161 (wheat, [*Triticum aestivum*])
	WLSYVPANIGGPLPITK	Plastidic acetyl-CoA carboxylase	T06161 (wheat)
	IIEEGPVTVAPR	Plastidic acetyl-CoA carboxylase	T06161 (wheat)
	TDQPIILTGFSALNK	Plastidic acetyl-CoA carboxylase	T06161 (wheat)
4	WLSYVPANIGGPLPITK	Plastidic acetyl-CoA carboxylase	T06161 (wheat)
	GNVLEPQGLIEIK	Plastidic acetyl-CoA carboxylase	T06161 (wheat)

[a] The numbers correspond to those given in Figures 1 and 2.   [b] Peptide sequences were determined by electrospray ionisation tandem mass spectrometry (ESI-MS/MS).

Figure 2. Analysis of mitochondrial and etioplastidic protein complexes from barley by two-dimensional BN/SDS-PAGE using low-percentage polyacrylamide gels for second gel dimensions. Stripes of the first gel dimensions are shown above the two-dimensional gels. Gels were stained with Coomassie Blue. The molecular masses of known protein complexes or proteins are given below and on the right side of the gels (in kilodaltons). Arrows with numbers indicate proteins that were identified by MS (Table I), and arrows with stars point to the large subunit of Rubisco. For abbreviations, see Figure 1.
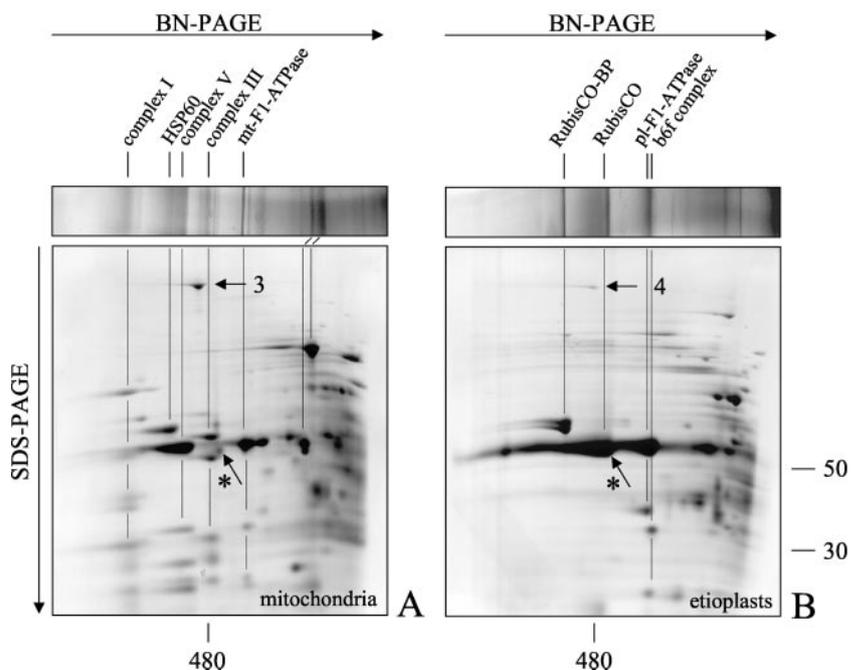
carries a biotin group and which previously was shown to be localized within plant mitochondria (Alban et al., 1993; Anderson et al., 1998), was not recognized by the biotin antibody on our blots of two-dimensional gels. This enzyme also forms part of a protein complex of about 500 kD. However, the protein complex possibly is destabilized under the conditions applied (addition of dodecylmaltoside and Coomassie Blue), and the singular MCC subunits are too small to be resolved on the BN gel dimension. Therefore, the immunoblot experiment was repeated on the basis of one-dimensional SDS-PAGE. The biotin antibody now recognized two proteins of the expected size, a 75-kD protein that most likely represents the biotinylated subunit of MCC and a 250-kD protein representing the putative mitochondrial ACC (Fig. 3, C and D).

For definite identification, the mitochondrial 250-kD protein from barley was cut out of two-dimensional gels, digested with trypsin, and analyzed by ESI-MS/MS. The sequences of four peptides were determined (Table I), which exhibit significant sequence identity to internal stretches of the amino acid sequence of ACC from wheat (*Triticum aestivum*; Fig. 4). While 100% sequence identity is found between the barley mitochondrial peptides and the plastidic form of ACC from wheat, several discrepancies were observed in comparison with the cytosolic version of this protein. Particularly, peptide 1 identifies the here analyzed protein to be homologous to the plastidic version of wheat ACC.

To find out whether the 250-kD protein from barley etioplasts represents the same protein or an additional form of ACC, two peptides of this protein were sequenced. These also exhibit 100% sequence identity to the plastidic form of ACC from wheat (Fig. 4). In addition, one of the two peptide sequences (Fig. 4, peptide 6) is identical with a peptide of the mitochon-
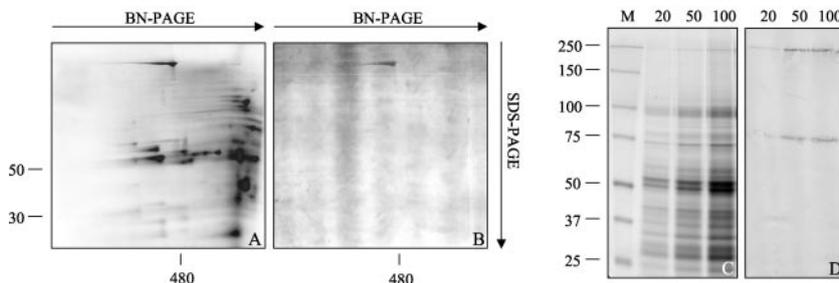
Figure 3. Identification of biotinylated proteins in barley mitochondria. Mitochondrial proteins were separated by two-dimensional BN/SDS-PAGE (A and B) or by one-dimensional SDS-PAGE (C and D) and stained with Coomassie Blue (A and C) or blotted onto nitrocellulose and immunostained with an antibody directed against biotin (B and D). Protein amounts loaded were 0.5 mg for the two-dimensional gels and 20 to 100 μg for the one-dimensional gels. The molecular masses of standard proteins are given to the left of the gels (in kilodaltons). Identities of proteins and protein complexes on the two-dimensional gels are explained in Figure 2.

peptide 1, barley mitochondria	T I T S N T D T V S E Y V S Y L V K		
ACC1 wheat (plastidic)	. . . T I T S N T D T V S E Y V S Y L V K . . .	677	T06161
ACC2-1 wheat (cytosolic)	. . . A S S R S S S V V T D Y V G Y L S K . . .	571	Q41511
ACC2-2 wheat (cytosolic)	. . . A S S R S S S V V T D Y V G Y L S K . . .	574	Q41525
peptide 3, barley mitochondria	I I E E G P V T V A P R		
ACC1 wheat (plastidic)	. . . I I E E G P V T V A P R . . .	407	T06161
ACC2-1 wheat (cytosolic)	. . . I I E E G P I T V A P P . . .	301	Q41511
ACC2-2 wheat (cytosolic)	. . . I I E E G P I T V A P P . . .	304	Q41525
peptide 4, barley mitochondria	T D Q P I I L T G F S A L N K		
ACC1 wheat (plastidic)	. . . T D Q P I I L T G F S A L N K . . .	1828	T06161
ACC2-1 wheat (cytosolic)	. . . L D Q P I I L T G Y S A L N K . . .	1770	Q41511
ACC2-2 wheat (cytosolic)	. . . L D Q P I I L T G Y S A L N K . . .	1774	Q41525
peptide 6, barley etioplasts	G N V L E P Q G L I E I K		
ACC1 wheat (plastidic)	. . . G N V L E P Q G L I E I K . . .	2096	T06161
ACC2-1 wheat (cytosolic)	. . . G N V L E A P G L I E I K . . .	2039	Q41511
ACC2-2 wheat (cytosolic)	. . . G N V L E A P G L I E I K . . .	2042	Q41525
peptide 2, barley mitochondria	W L S Y V P A N I G G P L P I T K		
peptide 5, barley etioplasts	W L S Y V P A N I G G P L P I T K		
ACC1 wheat (plastidic)	. . . W L S Y V P A N I G G P L P I T K . . .	1886	T06161
ACC2-1 wheat (cytosolic)	. . . W L S Y V P P Y V G G P L P I V K . . .	1828	Q41511
ACC2-2 wheat (cytosolic)	. . . W L S Y V P P Y V G G P L P I V K . . .	1832	Q41525

Figure 4. Alignment of the peptide sequences of the 250-kD protein of barley mitochondria and etioplasts (Table I) with internal sequence stretches of ACCs from wheat. Identical amino acids are underlayed with same gray intensities. The numbers of the last amino acids of the internal sequence stretches of the ACCs from wheat are given behind the sequences. Accession numbers of sequences are given to the right.

drial ACC (Fig. 4, peptide 2), and the peptide mass spectra of the 250-kD proteins from the mitochondrial and etioplast fraction did not reveal the presence of differing peptides (data not shown). We conclude that the two proteins have a very similar or an identical primary sequence.

The Identification of the 250-kD ACC in Barley Mitochondria Is Not Due to a Contamination of Mitochondrial Fractions with Other Subcellular Fractions

The occurrence of a very similar or even identical homodimeric 500-kD protein complex in mitochondria and etioplasts from barley cannot be explained by cross-contamination of the etioplast and mitochondrial fractions because both fractions are highly pure as documented by the two-dimensional gels (Fig. 2, A and B): The mitochondrial fraction only contains the protein complexes of the respiratory chain and the HSP60 complex, whereas the etioplast fraction contains the four plastidic protein complexes, which previously were shown on Figure 1B. A very faint contamination of the large subunit of Rubisco can be seen in the mitochondrial fraction (indicated by a star in Fig. 2A). However, this protein is the most abundant plastidic protein and its occurrence in the mitochondrial fraction is very low if compared with the etioplast fraction, whereas the occurrence ACC is much higher in the mitochondrial fraction than in etioplasts. Absolutely no mitochondrial protein complexes are visible in the etioplast fraction.

To verify the purity of our organelle preparations by independent methods, marker enzyme measurements were carried out in mitochondrial fractions and in homogenates of 6-d-old etiolated barley seedlings for Rubisco (marker for plastids), fumarase (marker enzyme for mitochondria), and phospho*enol*pyruvate (PEP) carboxylase (marker enzyme for cytosol). Specific Rubisco activity in the mitochondrial fractions proved to be reduced by more than 97% if compared with total homogenates (fixation of 0.75 vs. 24.2 nmol CO_2 mg^{-1} protein min^{-1}). At the same time, specific fumarase activity was enriched by factor 10 in mitochondrial fractions if compared with whole-seedling homogenates (85 vs. 8 units). PEP carboxylase activity was reduced by 87% in the mitochondrial fractions (fixation of 90.9 vs. 11.8 nmol CO_2 mg^{-1} protein min^{-1}). Thus, we conclude that plastidic contamination of our mitochondrial fraction is very low, while there is some contamination by cytoplasm, which might be due to the lose binding of cytoplasmic enzymes to the surface of mitochondria.

To exclude that the 250-kD ACC of our mitochondrial fractions represents a cytoplasmic contamination, isolated organelles were incubated with thermolysin. As documented in Figure 5, the mitochondrial 250-kD protein is not degraded in the presence of the protease and therefore must be localized inside the organelles. Therefore, we conclude that in barley three subcellular compartments, the cytoplasm, etioplasts, and mitochondria independently contain a 250-kD multifunctional ACC.

Barley Mitochondria Exhibit ACC Activity

To physiologically characterize the mitochondrial ACC from barley, CO_2 uptake of mitochondrial lysates in the presence and absence of known inhibitors for ACC was determined (Fig. 6). Analogous exper-
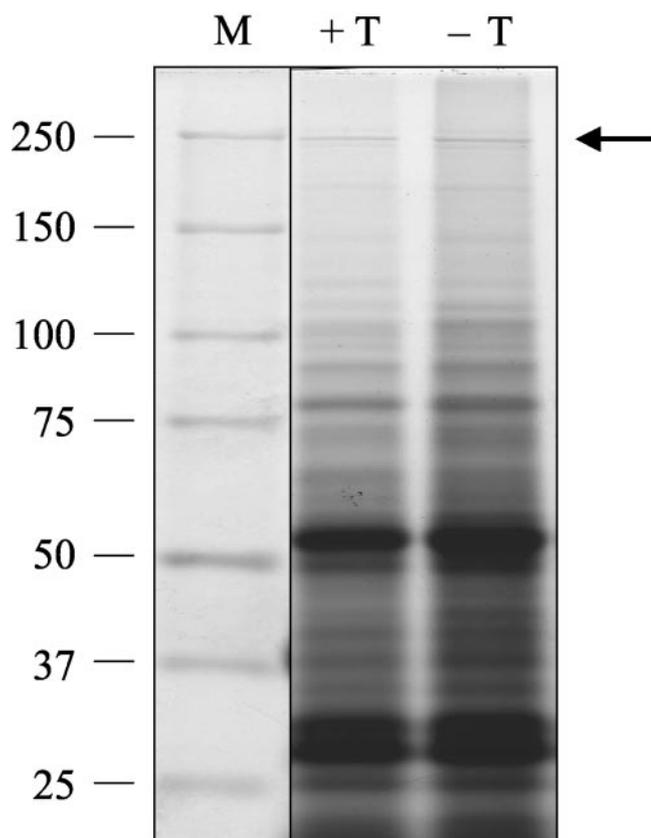
Figure 5. The mitochondrial 250-kD protein is not degraded upon incubation of mitochondria with thermolysin. Isolated mitochondria were treated with or without thermolysin (± T) as described in "Materials and Methods." Subsequently, proteins were separated by one-dimensional SDS-PAGE and were visualized by Coomassie staining. Molecular masses of standard proteins (M) are given to the left of the gel (in kilodaltons). The arrow indicates mitochondrial ACC, which was identified by immunoblotting.

iments were carried out with etioplast lysates. The specific activity of the ACC was higher in the mitochondrial fraction than in the etioplast lysates (450 nmol CO_2 fixation h^{-1} and mg^{-1} protein vs. 150 nmol in etioplasts). Slightly lower activities were found for propionyl-CoA carboxylation in both extracts. Diclofop inhibited ACC activities of both organelle fractions by about 80%, whereas cycloxydim reduced activity by 50%. These results confirm the presence of ACC in both organelles. The generally higher specific activity in mitochondrial fraction is consistent with the higher abundance of this protein in the respective fraction analyzed on the two-dimensional gels (Fig. 2).

The Mitochondrial 250-kD Acetyl CoA Carboxylase Is also Present in Wheat Mitochondria, But Not in Mitochondria from Arabidopsis

To determine whether a mitochondrial ACC is an unique feature of barley mitochondria or a more general feature of grasses, mitochondria were prepared from 7-d-old wheat seedlings and mitochon-

drial proteins were analyzed by two-dimensional BN/SDS-PAGE (Fig. 7). As in barley, a homodimeric 500-kD complex is present in wheat mitochondria. In contrast, no such complex is detectable in mitochondrial fractions from Arabidopsis (Fig. 7). This suggests that the presence of ACC in mitochondria is probably a general feature of Poaceae and possibly monocotyledonous plants.

DISCUSSION

Evidence for a Mitochondrially Localized ACC in Grasses

In contrast to dicotyledonous plants investigated, mitochondria from barley and wheat contain an ACC. The enzyme was discovered in the course of a systematic investigation of protein complexes from barley on two-dimensional BN/SDS-polyacrylamide gels. This experimental approach not only allows us to investigate the subunit composition of protein complexes, but also is a very reliable tool for monitoring the purity of organelle fractions because different organelles contain very different protein complexes with typical subunit patterns (e.g. respiratory chain protein complexes only occur in mitochondria and photosystems or Rubisco only occur in chloroplasts). Thus, this procedure easily allows us to detect plastidic contaminations in mitochondrial fractions and vice versa. Furthermore, BN/SDS-PAGE simultaneously monitors soluble and membrane-bound protein complexes, which documents the intactness of organelles after isolation. As demonstrated in Figures 1 and 2, only minor plastidic impurities were observed in mitochondrial fractions, whereas no mitochondrial subunits of protein complex are detectable in etioplast fractions. Considering these minimal cross-contaminations, the presence of ACC in the mitochondrial fraction of barley certainly cannot be explained as a contamination of the fraction by plastids. In addition, ACC abundance in 6-d-old seedlings was found to be much higher in mitochondria than in etioplasts, which is consistent with higher ACC activities measured in mitochondria. Also, cytoplasmic contaminations of mitochondrial fractions cannot explain the identification of ACC in these organelles because the protein was shown to be resistant to protease treatment of isolated mitochondria.

Further evidence for the localization of a multifunctional ACC in mitochondria comes from an analysis of the rice (*Oryza sativa*) mitochondrial proteome (Heazlewood et al., 2003). In this study, proteins of mitochondria, which were highly purified by two successive Percoll-gradient density centrifugations, were separated by three different procedures: two-dimensional IEF/SDS-PAGE, two-dimensional BN/SDS-PAGE, and a gel-free procedure based on liquid chromatography. A 250-kD ACC did not show up on the gels due to high acrylamide concentrations in the
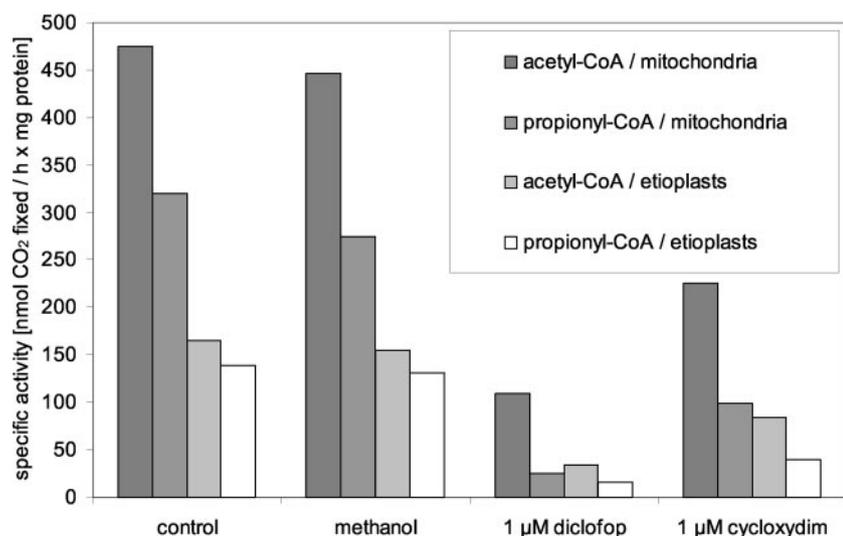
Figure 6. Specific ACC activity in mitochondrial and etioplastidic extracts from barley. ACC activity was taken to be the heat and acid stable incorporation of radioactive hydrogen carbonate in acetyl-CoA or propionyl-CoA. Final concentrations of the inhibitors were 10^{-6} M. For details, see "Material and Methods."

second gel dimension. Furthermore, 250-kD proteins can hardly be focused in pH gradient gels during isoelectric focusing. However, peptides corresponding to a multifunctional ACCs from grasses were identified by MS after protein separations on the basis of liquid chromatography (Heazlewood et al., 2003). Thus, a multifunctional ACC in mitochondria seems also to occur in rice and possibly is a general feature of grasses.

Targeting of ACC in Plant Cells

Based on partial amino acid sequence determination, plastidic and mitochondrial ACC from barley have to be considered to be structurally very similar and possibly even identical proteins. Huang et al. (2002) report that plastidic ACC from barley is the product of a single-copy gene. The mitochondrial/plastidic ACC from barley is possibly a new member

of the group of so-called "dual-targeting" proteins, which include targeting information for transport to more than one subcellular location (Peeters and Small, 2001). Currently, the complete sequences for plastid localized multifunctional ACCs are known for four grasses: maize (*Zea mays*), wheat, black-grass (*Alopecurus myosuroides*), and green foxtail (*Setaria vividis*; Egli et al., 1995; Gornicki et al., 1997; Déle et al., 2002a, 2002b). In comparison with multifunctional cytoplasmic ACCs, the plastidic ACCs have an N-terminal extension of about 100 amino acids, which might function as an organelle-targeting signal. However, this extension has some unusual features when compared with other functionally well-characterized chloroplast transit peptides: it is extraordinary long (about 100 amino acids) and has a very unusual amino acid composition. Consequently, several available software programs for predictions of subcellular protein localizations (TargetP [http://

Figure 7. A mitochondrial ACC is present in wheat mitochondria, but not in mitochondria from Arabidopsis. Mitochondrial protein from Arabidopsis and wheat was resolved by two-dimensional BN/SDS-PAGE using a low-acrylamide gel for the second gel dimension. Both gels were Coomassie stained. Identities of protein complexes are given above, and the sizes of standard proteins are given below and to the right of the gels (in kilodaltons). The arrow marks ACC from wheat.

www.cbs.dtu.dk/services/TargetP/], Psort [http://psort.nibb.ac.jp], or MitoProt [http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter]) do not predict plastidic or mitochondrial localizations of this protein (data not shown). Therefore, targeting mechanisms of the plastidic/mitochondrial ACCs currently is unclear and should be addressed experimentally, e.g. by green fluorescent protein fusions.

Fatty Acid Biosynthesis and Mitochondria

Mitochondria from dicotyledonous plants can synthesize fatty acids starting from malonyl-CoA, which can be generated from malonate (Gueguen et al., 2000). Because a mitochondrial ACC is absent, malonate is postulated to be imported into mitochondria from the cytoplasm. In contrast, complete fatty acid biosynthesis can take place in mitochondria from grasses. Quantitatively, mitochondrial fatty acid biosynthesis in dicotyledonous plants is low if compared with plastidic fatty acid biosynthesis and probably only is necessary for lipoic acid biosynthesis or lipid repair in mitochondria. In contrast, specific ACC activity in etiolated 6-d-old barley seedlings is higher in mitochondria than in etioplasts. Strikingly, abundance of mitochondrial ACC in etiolated barley seedlings corresponds to the abundance of subunits of respiratory chain protein complexes (Fig. 2) that belong to the group of very abundant mitochondrial proteins. Possibly mitochondrial fatty acid biosynthesis in general is quantitatively important in grasses, and mitochondria play a general role in fatty acid biosynthesis for the cell.

The fact that graminicides of the cyclohexan-1,3-dione-type (e.g. cycloxydim) and the aryloxyphenoxypropionic acid type (e.g. diclofop) do not only inhibit plastidic fatty acid biosynthesis in barley, but also the mitochondrial fatty acid biosynthesis (by blocking ACC as shown in this study) opens the question of whether the mode of action of these graminicides is fully understood. In the past, it was assumed that mode of action can be explained by a shortage of fatty acids for general membrane biosynthesis. However, due to an inhibition of more specific products of fatty acid biosynthesis in mitochondria such as lipoic acid, it is possible that special metabolic pathways in mitochondria like acetyl-CoA formation (pyruvate dehydrogenase complex) photorespiration (H-Protein of the Gly decarboxylase complex) or amino acid metabolism (branched-chain-α-keto acid dehydrogenase) are disturbed. All of these biochemical reactions need lipoic acid as prosthetic group and it is clear that a decrease in the activity of these pathways would have drastic effects for plants.

To our knowledge, grasses are the first eukaryotic organisms shown to possess a mitochondrial ACC. In mammals, an multifunctional ACC was shown to be associated with mitochondria (Abu-Elheiga et al., 1995, 2000). However, this enzyme is attached to the outer mitochondrial membrane on the cytoplasmic side and is believed to be involved in the regulation of mitochondrial fatty acid oxidation through the inhibition of carnitine palmitoyltransferase 1 by its product malonyl-CoA. Such a function is highly unlikely for the mitochondrial ACC from grasses because in plant cells, fatty acids are oxidized and degraded in peroxisomes. Furthermore, protease treatment of isolated mitochondria from grasses did not lead to degradation of ACC (Fig. 5).

The presence of an internal ACC in mitochondria from grasses raises several questions. How abundant and active is ACC in plastids and mitochondria in different tissues and developmental stages of grasses? How active and abundant are other enzymes of mitochondrial fatty acid biosynthesis in grasses? Can fatty acids be exported from mitochondria in grasses?

MATERIALS AND METHODS

Cultivation of Plants

Barley (*Hordeum vulgare* cv Alexis) and wheat (*Triticum aestivum*) were grown on peat (TKS 2) covered with a wet tissue at 24°C for 6 (barley) or 7 (wheat) d in the dark. Arabidopsis cell suspensions were cultivated as described by Werhahn et al. (2001).

Preparation of Mitochondria

Starting material for mitochondrial preparations from barley and wheat involved about 200 g of etiolated seedlings. The seedlings were suspended in 1,000 mL of ice-cold "grinding buffer" (0.4 M mannitol, 1 mM EGTA, 25 mM MOPS, 5 mM dithiothreitol [DTT], and 1% [w/v] bovine serum albumin, pH 7.8) and cells were disrupted in a Waring Blender by three periods of 3 s each. All subsequent steps were carried out at 4°C. After filtration through four layers of gaze, mitochondria were enriched by two-step centrifugation: large cell debris were removed by centrifugation at 3,500g for 5 min and afterward, a mitochondrial fraction was sedimented by centrifugation at 18,000g for 30 min. The pellet was resuspended in "resuspension buffer" (0.4 M mannitol, 1 mM EGTA, and 10 mM KH$_2$PO$_4$, pH 7.2) and was layered on top of three-step Percoll gradients (six gradients of 30 mL each containing 10 mL of 14%, 10 mL of 23%, and 6 mL of 45% [all w/v] Percoll in resuspension buffer). After centrifugation for 45 min at 70,000g, mitochondria were collected from the 23%/45% interphase. To remove the Percoll, purified mitochondria were diluted in resuspension buffer and were resedimented three times by centrifugation for 10 min at 18,000g. A typical mitochondrial preparation yields about 100 mg of mitochondria (about 10 mg of mitochondrial protein). Purification of mitochondria from Arabidopsis cell suspension cultures was carried out as described previously (Werhahn et al., 2001).

Preparation of Etioplasts

Etioplasts were isolated from barley seedlings according to a protocol as detailed in Roughan (1987). In brief, about 80 g of seedlings was suspended in 200 mL of ice-cold "isolation buffer" (0.35 M sorbitol, 2 mM HEPES/KOH, pH 7.8, 0.4 mM KCl, and 0.04 mM EDTA) and cells were ruptured using a modified kitchen blender (Kannangara et al., 1977). After filtration through four layers of gaze, etioplasts were enriched by centrifugation through a 40% (w/v) Percoll cushion for 4 min at 2,000g. The etioplast fraction was resuspended in resuspension buffer (0.33 M sorbitol, 2 mM HEPES/KOH, pH 7.8, 0.4 mM KCl, and 0.04 mM EDTA) and were centrifuged for 4 min at 2,000g to remove the Percoll. The resulting pellet was resuspended in a small volume of resuspension buffer. After waiting for about 2 min (starch sedimentation), the supernatant was centrifuged again at 1,800g for 4 min. A typical etioplast preparation yields about 20 mg of organelles.

PAGE and Western Blotting

Two-dimensional BN/SDS-PAGE was carried out as outlined by Schägger and von Jagow (1991) with modifications given in Jänsch et al. (1996). It is important to load freshly prepared organelle samples onto the first dimension gel because some protein complexes got partially lost after freezing and thawing. To visualize ACC, acrylamide concentrations for both gel dimensions were modified in comparison with the original publications: the sample gel of the first gel dimension was 4% (w/v), the separating gel of the first dimension was 4.5% to 12% (w/v), the sample gel for the second gel dimension was 4% (w/v), and the separating gel of the second dimension was 6% (w/v) acrylamide. All gels were stained with colloidal Coomassie (Neuhoff et al., 1990).

One-dimensional SDS-PAGE was carried out as described in Schägger and von Jagow (1987) with the following modification: separating gels had 6% (w/v) polyacrylamide and stacking gels had 4% (w/v) polyacrylamide.

Transfer of proteins onto nitrocellulose membranes was carried out in a semidry blotting apparatus with a discontinuous transfer buffer system (Kyhse-Andersen, 1984) in the presence of 0.01% (w/v) SDS in the cathode buffer to increase the mobility of high-M_r proteins (ACC) during transfer. Biotin-containing proteins were identified using the monoclonal biotin antibody BN-34 (Sigma, St. Louis), and the staining of immunopositive bands was carried out using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Thermolysin Treatment of Isolated Barley Mitochondria

About 20 mg of isolated barley mitochondria (2 mg of mitochondrial protein) was resuspended in 200 μL of "termolysin-resuspension buffer" (0.4 M mannitol, 10 mM Tricine, 1 mM ZnCl$_2$, and 10 mM CaCl$_2$, pH 7.2) and incubated with 10 μg of thermolysin (Sigma) for 30 min at 10°C. Protease activity was monitored by incubation of control proteins with thermolysin. All protease reactions were stopped by adding EDTA (a final concentration of 25 mM) and mitochondria were reisolated by centrifugation through Suc cushions (0.75 mL of a 25% [w/v] Suc solution) at 18,000g for 5 min. Pellets were directly resolved in sample buffer for SDS-PAGE. One-dimensional SDS-PAGE was carried out according to Schägger and von Jagow (1987).

Marker Enzyme Assays

Homogenates of 6-d-old etiolated barley seedlings and isolated barley mitochondria were lysed by treatment with Tween 20 (final concentration of 1%, v/v). The lysates were passed through a PD-10 column (Amersham, Piscataway, NJ) equilibrated in resuspension buffer without EGTA (0.4 M mannitol and 10 mM KH$_2$PO$_4$, pH 7.2) to remove endogenous substrates.

PEP carboxylase as a cytosolic marker was monitored in a coupled assay with malic dehydrogenase (Sigma) according to Wedding and Kline (1994) by measuring the decrease of A_{340} (oxidation of NADH through the reduction of oxalacetate) at 25°C. Final concentrations in this assay were 50 mM HEPES, pH 8, 5 mM NaHCO$_3$, 5 mM MgCl$_2$, 0.2 mM NADH, 6 units malic dehydrogenase, and 2 mM PEP in a total volume of 1 mL. The reaction was measured for 1 min without PEP to determine nonspecific NADH oxidation and afterward was started by the addition of PEP.

Fumarase as mitochondrial marker was measured as described by Hill and Bradshaw (1969) at 25°C. One unit of fumarase was taken to be the amount of enzyme in 3 mL responsible for an increase of 0.001 absorbance units at 250 nm (dehydration of malate to fumarate) in 10 s.

Rubisco as a marker enzyme for plastids was measured as described by Siegel and Lane (1975). Final concentrations in this assay were 200 mM Tris, pH 8, 0.7 mM ribulose-1,5-bisphosphate, 5 mM KHCO$_3$ (with 3,700 Bq ^{14}C), 25 mM MgCl$_2$, 60 μM EDTA, and 2 mM DTT (instead of glutathione) in a total volume of 50 μL. The reaction took place for 10 min at 30°C and was stopped by the addition with 100 μL of 6 M HCl. Finally, assays were heated for 1 h at 95°C to evaporate nonfixed radioactivity. Radioactivity was determined in a liquid scintillation counter. Negative controls without ribulose-1,5-bisphosphate were included to determine nonspecific CO$_2$ fixation.

ACC Activity Assays

Organelles (about 5 mg of protein in 200 μL of resuspension buffer) were diluted in 1.2 mL of assay buffer (500 mM Tricine, pH 8.0, 25 mM MgCl$_2$, and 1.125 mM NaHCO$_3$) and were lysed by the addition of Tween 20 to a final concentration of 0.1% (v/v). After centrifugation at 15,000g for 5 min, the supernatant was purified from endogenous thioesters using PD-10 columns that were equilibrated and eluted with assay buffer. ACC activity assays were carried out in a volume of 50 μL in the presence of 250 mM Tricine, pH 8.0, 12.5 mM MgCl$_2$, 0.46 mM NaHCO$_3$, including 3,700 Bq ^{14}C-hydrogencarbonate (ICN Pharmaceuticals, Costa Mesa, CA), 2 mM ATP, 2 mM DTT, and 16 μL of an organelle fraction. The reaction was started by the addition of 2.5 μL acetyl-CoA- or propionyl-CoA solution (1 mM acetyl-CoA/propionyl-CoA in 10 mM potassium phosphate buffer, pH 3.5) and was stopped after 10 min by adding 50 μL of 6 M HCl. Reaction mixtures were subsequently transferred to scintillation vials and were heated at 90°C for 45 min to evaporate the free radioactive hydrogen carbonate. After heating, scintillation mixture was added and radioactivity was determined in a liquid scintillation counter. The ACC activity directly correlates with the heat- and acid-stable incorporation of ^{14}C-hydrogencarbonate into acetyl-CoA or propionyl-CoA. Negative controls without thioesters were included in each series to determine the nonspecific carboxylation and radiochemical background. Herbicides (diclofop and cycloxydim) were dissolved in methanol and diluted with assay buffer so that the final methanol concentration in the assay was 0.2% (w/v). Protein concentration in the final lysates was determined with a modified Lowry assay as described in Bach et al. (1986).

Protein Identification by ESI-MS/MS

Proteins were identified by ESI-MS/MS as outlined in Kruft et al. (2001). Identified peptide sequences were compared with sequence entries of protein databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Alignments were generated with CLUSTAL-W at the European Bioinformatic Institute (http://www.ebi.ac.uk/clustalw/) and boxed by the SeqVU program (http://oat.bio.indiana.edu:7580/documents/disk0/00/00/01/18/).

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