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Variation of the Polypeptide Composition of Mitochondria Isolated from Different Potato Tissues[1]

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ABSTRACT

The protein contents of mitochondria from different potato (*Solanum tuberosum* L.) tissues (tubers, dark-grown shoots, and green leaves) grown in a greenhouse or *in vitro* were compared by two-dimensional polyacrylamide gel electrophoresis. Two different methods were used: using the method that gave the highest resolution, an average number of 360 polypeptides was revealed on the mitochondrial patterns after silver staining. The mitochondrial protein patterns of etiolated tissues (tubers, dark-grown shoots) are roughly similar but distinct from those of green leaves. The four subunits of the glycine decarboxylase complex (involved in photorespiration) and a few other polypeptides are very abundant in green tissues, compared with nonphotosynthetic tissues. Conversely, some other polypeptides that are abundant in tubers and dark-grown shoots are hardly detectable in green leaf mitochondria. A rabbit antiserum was raised against a 40 kilodalton polypeptide that is among the most characteristic of these nonphotosynthetic tissue-specific polypeptides, and the N-terminal sequence of this polypeptide was determined. No effect of *in vitro* culture was observed on the protein composition of mitochondria isolated from differentiated tissues. However, the protein patterns of callus and cell suspension mitochondria are distinct from those of any differentiated tissues, although their basic pattern is clearly mitochondrial.

In higher plants, little is known about variation in the protein composition of mitochondria from different tissues. This subject is of great interest because mitochondria vary considerably in morphology (2) and display different biochemical and physiological activities in various tissues. For example, it is known that a high capacity to oxidize glycine to serine is restricted to mitochondria from photosynthetic tissues (6). In addition, the alternative cyanide-insensitive pathway is known to vary depending on the tissue and physiological growth conditions.

Some studies on mitochondrial composition in various tissues have been published (7, 16, 22), but they were performed using SDS-PAGE (12), which only detects the most abundant polypeptides. Sahlström and Ericson (22) could separate 35 polypeptide bands on SDS-PAGE of spinach leaf mitochondria. In pea, Rémy *et al.* (19) could separate about 40 polypeptides from whole mitochondria by SDS-PAGE (after Coomassie blue staining) and 90 by two-dimensional PAGE. It is clear that two-dimensional PAGE greatly improves the resolution of mitochondrial polypeptides, especially in the M_r area of 50 to 60,000. In that study of the composition of pea mitochondria isolated from different tissues (19), striking differences were shown between nonphotosynthetic (epicotyls, etiolated leaves) and photosynthetic tissues (green leaves); the most important of these were the quantitative increase of the subunits P, L, T, and H of the glycine decarboxylase complex, and the loss of two polypeptides (35 kD, pI[2] 6.3, and 40 kD, pI 7).

To know if these observations were limited to pea or could be generalized to other plants, we have undertaken a similar study on potato mitochondria: we describe here a comparative study of the mitochondrial composition of various tissues grown in a greenhouse or *in vitro*. Using an improved two-dimensional system that can resolve approximately 360 polypeptides from mitochondria after silver staining, we show that, in addition to major qualitative or quantitative polypeptide changes between photosynthetic and nonphotosynthetic tissues, numerous discrete variations also occur under different physiological conditions. The long-term aim for this study is to characterize the polypeptides that are differentially expressed in green and nongreen tissues.

MATERIALS AND METHODS

Plant Material

Dormant potato tubers (*Solanum tuberosum* L.) cv BF15 were kept in the dark at 16°C. Mitochondria were isolated from these tubers and from the shoots that issued from them at several developmental stages: dormancy, end of dormancy, and during shoot growth.

Some tubers were planted in a greenhouse and mitochondria were isolated from mature green leaves.

Mitochondria from different tissues and organs grown *in*

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[2] Abbreviations: pI, isoelectric point; IEF, isoelectric focusing; Chaps, 3-((3-cholamidopropyl)-dimethylammonio)1-propane-sulfonate; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

vitro (green leaves, microtubers, calli from tubers and cell suspensions from these calli) were also analyzed.

Tissue Culture

Green leaves were harvested from plantlets issued from cuttings and grown on basal medium: Murashige and Skoog minimal organics (14) with Morel's vitamins (13), 0.2 mM Fe EDTA, 2.5% (w/v) sucrose, 0.7% (w/v) agar in a 20°C growth chamber, 16-h photoperiod as described by Quraishi *et al.* (17).

Microtubers were produced by placing nodal cuttings on basal medium with 8% (w/v) sucrose and incubating in the dark at 20°C after growing the plantlets for 3 weeks under 16-h photoperiod and 1 week under 12-h photoperiod.

Calli were produced by placing a tuber fragment on basal medium supplemented with 1 mg L^{-1} indolebutyric acid and 0.5 mg L^{-1} benzyladenine, and subcultured onto fresh medium every 3 weeks (17).

Cell suspensions issued from these calli were grown in the medium described for calli (liquid medium), placed on a rotating shaker in a 25°C growth chamber, 16-h photoperiod, and transferred into fresh medium every 2 weeks (17).

Isolation and Purification of Mitochondria

Mitochondria were isolated from all organs according to Boutry and Briquet (3). Mitochondria from etiolated organs were separated using a 13.5, 20, and 50% Percoll step gradient, and green leaf mitochondria using a 13.5, 24, and 50% Percoll step gradient. Catalase activity was used to estimate contamination of our tuber mitochondria preparations by peroxisomes. It was measured by O_2 production in the presence of 2.5 mM H_2O_2 (15). Contamination by amyloplast membranes was estimated from the carotenoid content (15) of our mitochondria preparations. Carotenoids were extracted in 80% acetone (v/v) and measured at 470 nm using an absorption coefficient of 1980. For green leaf mitochondria, the major contaminants were plastid membranes, which were measured by Chl content (1).

The matrix and membrane fractions were obtained by osmotic shock followed by three freezing-thawing cycles in 50 mM Tris, 1 mM octylglucopyranoside, and centrifugation at 80,000*g* for 30 min. The supernatant was considered as the matrix fraction and the pellet as the membrane fraction.

Electrophoresis

The mitochondrial proteins were precipitated in 80% acetone, pelleted, dried, and resuspended according to Rémy and Ambard-Bretteville (18). Two distinct two-dimensional electrophoresis methods were used. The first one was performed as previously described (18). Briefly, the first dimension IEF rod gels were 10 cm long and 3 mm diameter and the second dimension (SDS-PAGE) was run on 100 × 100 × 1 mm slab gels. This method allows the analysis of samples containing up to 200 μg protein per gel and can be used as a preparative technique for polypeptides of interest. After Coomassie blue staining, the resolution is sufficient to detect variations involving major polypeptides. A higher resolving technique was developed to detect changes in less abundant polypeptides. The IEF was performed in 160 mm long and 1 mm inner diameter glass tubes for 12,100 V h. Gel composition was 4.5% acrylamide, 9.2 M urea, 2% Chaps, and 4% carrier ampholytes (1%, pH 3–10 [LKB]; 3%, pH 5–8 [LKB and Pharmacia]). No more than 20 μg of protein was loaded on these gels. The SDS-PAGE was performed in 12% acrylamide slab gels (140 × 150 mm). No stacking gel was required due to the thinness of the IEF gels. Four slab gels were run simultaneously in a vertical SE 600 gel apparatus from Hoefer. They were silver stained according to Heukeshoven and Dernick (9).

Western Blotting

After electrophoresis, the proteins were transferred onto nitrocellulose membranes and immunoblotted according to Towbin *et al.* (24).

Gel Comparisons

The reported results were obtained after observing at least three gels from different extractions and different runs for each tissue. The comparisons were made by superimposing dried gels. Leaf and tuber mitochondrial patterns were compared with dark-grown shoot patterns; mitochondrial patterns from leaf, microtubers, calli, and cell suspensions grown *in vitro* were compared with mitochondria from green leaves, tubers, and dark-grown shoots, respectively. Maps were drawn for tubers, green leaves, and dark-grown shoots, and were used to note the differences observed.

N-Terminal Sequence

Six two-dimensional gels, each loaded with about 120 μg tuber mitochondrial protein, were run according to the first method described here and the proteins transferred onto 0.45 μm pore size Immobilon-P membranes (Millipore) for 30 min at 15°C in a 10 mM Caps, 10% methanol (v/v) buffer, pH 11. The membranes were stained in a 0.2% Ponceau S, 3% TCA, and 3% sulfosalicylic acid solution.

The spots corresponding to the analyzed protein were excised and placed in the cartridge of the sequencer. Automated Edman degradation of the protein was performed in an Applied Biosystems 470A protein sequencer and its on-line phenylthiohydantoin amino acid analyzer model 120A, according to the manufacturer's instructions.

Antiserum Production

Polyclonal antibodies were raised in rabbits using as antigens the acrylamide-supported protein spots of interest excised from two-dimensional gels. For each immunization, 100 to 150 mg protein emulsified in 0.9% NaCl (w/v) was used. For the first injection, an equal volume of Freund's adjuvant was added to the immunization mixture. Three injections were performed at 15-d intervals and then every month. The rabbits were bled every 6 weeks.

RESULTS AND DISCUSSION

Polypeptide Composition of Mitochondria from Different Potato Organs

On average, 100 polypeptides are revealed on two-dimensional protein patterns of potato mitochondria after Coo-

massie blue staining. The patterns of mitochondria isolated from etiolated tissues, *e.g.* dark-grown shoots (Fig. 1A) and tubers (Fig. 1B) are roughly similar, although each of them has specific spots. The most striking differences are observed between mitochondrial protein patterns of etiolated tissues and photosynthetic tissues (green leaves from plants grown in a greenhouse [Fig. 1C] or *in vitro* [Fig. 1F]). In particular, the abundance of the four subunits P, L, T, and H of the glycine decarboxylase complex (involved in photorespiration) in green leaves (Fig. 1C) contrasts with their low quantities (P) or lack of detection by Coomassie blue staining (L, T, and H) in tuber or etiolated tissue mitochondria. These polypeptides were immunologically identified using rabbit antisera raised against the polypeptides P, L, T, and H from pea (not shown). Conversely, other polypeptides were much less abundant in these leaf mitochondria than in mitochondria isolated from nongreen tissues (shoots or tubers, Fig. 1A and B, respectively). This is particularly obvious for an unidentified 40 kD polypeptide named "w" in Fig. 1, after an immunologically related polypeptide varying in mitochondria from different pea tissues (19).

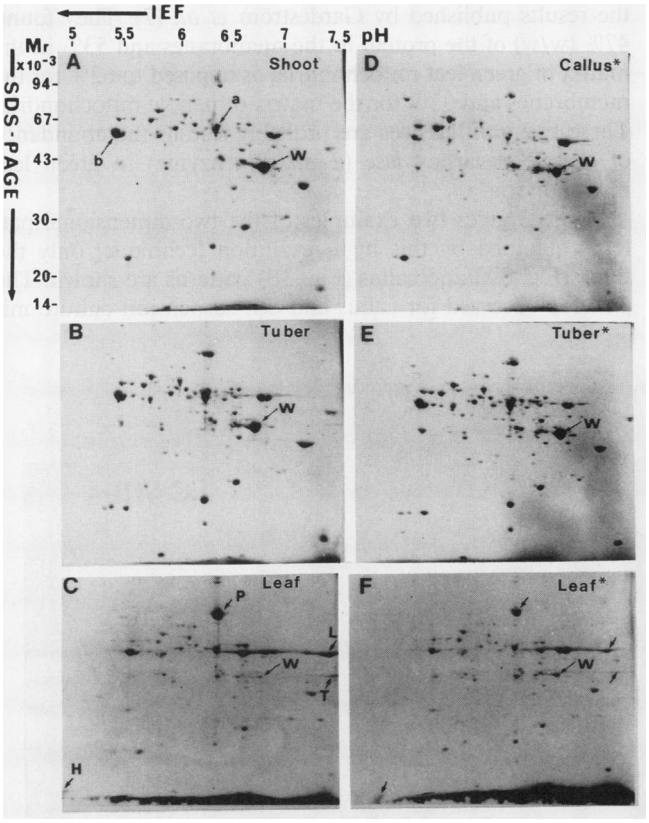

Figure 1. Two-dimensional protein patterns of mitochondria isolated from different potato tissues. A, Dark grown shoots; B, tubers; C, green leaves; D, callus; E, tubers produced *in vitro*; F, green leaves from plantlets grown *in vitro* (* indicates the tissues produced *in vitro*). Two-dimensional PAGE was performed according to the first method described in "Materials and Methods," and the gels were Coomassie blue stained. Input: 150 μg. Some polypeptides of interest are indicated by arrows, such as the subunits α (a) and β (b) of the ATP synthase, the 40 kD polypeptide (w), and the subunits P, L, T, and H of the glycine decarboxylase complex.

Table I. *Variation in the Polypeptides Found in the Two-Dimensional Patterns of Mitochondria Isolated from Different Potato Tissues*

The comparisons were made on at least three silver-stained two-dimensional gels obtained using the second method described in "Material and Methods." Specific spots were found in only one of the analyzed tissues.

	Tuber	Leaf	Callus	Etiolated Shoot
			number	
Tissues grown in a greenhouse	342	373	/[a]	376
Tissues grown *in vitro*	340	382	388	/
Spots found in etiolated shoot and...	282	295	342	376
(Quantitatively unchanged)	(218)	(216)	(211)	(376)
Specific spots	44	59	39	25

[a] Experiment was not done because the material was not available.

The patterns observed for mitochondria isolated from organs grown *in vitro* or in a greenhouse are very similar, as shown for tubers (Fig. 1B) and microtubers *in vitro* (Fig. 1E). Although the leaf mitochondrial patterns from greenhouse or *in vitro* plants are similar, it is worth noting that the 40 kD polypeptide was reproducibly found to be more abundant in mitochondria from plants grown *in vitro*.

Obviously, to be credible, such protein analyses require highly purified mitochondria preparations. If they cannot be avoided, the contaminant polypeptides must be known and taken into account in the analyses. For potato tuber mitochondria, the major possible contaminants were peroxisomes and amyloplast membranes (15). Catalase activity and carotenoid content were used as markers for peroxisomes and amyloplast membranes, respectively. The Percoll-purified mitochondria showed, compared with unpurified fractions, a 30-fold decrease in catalase activity (2 μmol O_2 min^{-1} mg^{-1} protein as opposed to about 60 μmol O_2 min^{-1} mg^{-1} protein). Less than 0.007 μg carotenoids/mg protein was found in Percoll-purified mitochondria. These measurements indicate that the contamination with these organelles is very low. In green leaf mitochondria, the contamination by thylakoid membranes was found to be less than 2 μg Chl/mg protein. The presence of polypeptides of peroxisomal or plastid origins was also checked by two-dimensional analyses of these organelles and deliberate contamination of mitochondrial preparations (not shown). It was concluded that the major polypeptide variations described between mitochondria from different tissues were not due to contaminants.

Variations Revealed by a High Resolution Two-Dimensional Technique

The major results obtained after electrophoresis on bigger gels and silver staining were similar to those described above, but many additional differences were also revealed. Table I gives the results of these comparisons: on average, 360 spots were reproducibly observed on mitochondrial patterns after silver staining. Two hundred eight were found in all tissues

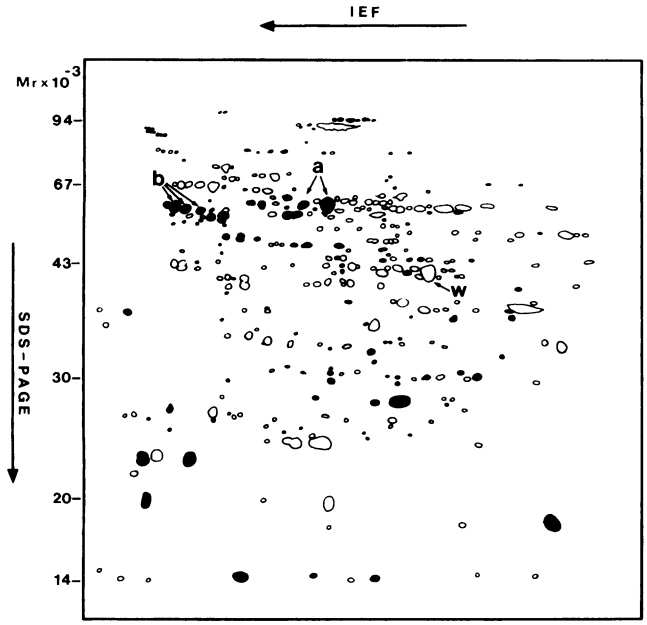

Figure 2. Map of a potato tuber mitochondrial pattern, obtained after silver staining, using the second method described in "Materials and Methods." Membrane and soluble polypeptides are represented by black and open spots, respectively; a and b are the α and β subunits of the ATP synthase, respectively.

(of which 53 were quantitatively unchanged) and presumably represent the basic set of mitochondrial polypeptides. Some polypeptides were found specific to each tissue analyzed (see Table I). Similar observations were made in a comparative study of two-dimensional mitochondrial patterns of different rat organs (25), in which the authors point out the existence of a pool of mitochondrial proteins common to all organs studied, and of some polypeptides specific to each tissue analyzed, suggesting the existence of tissue-specific functions as well as the common mitochondrial function of ATP production.

Figure 2 shows the partitioning of soluble and membrane polypeptides from tuber mitochondria: 77% of the spots revealed after silver staining belong to the soluble fraction and 23% to the membrane fraction. These results are consistent with the few two-dimensional analyses published so far, *i.e.* about two-thirds of the polypeptides are in the matrix and one-third in the membranes of potato tuber (21) and pea (19) mitochondria. These results deal with numbers of different polypeptide species and not with quantities of protein, which are more equally split between membrane and matrix, and probably vary more according to the tissue, as exemplified by the results published by Gardeström *et al.* (7). They found 47% (w/w) of the protein in the membranes and 53% in the matrix of green leaf mitochondria, as opposed to 62% for the membranes and 37% for the matrix of petiole mitochondria. These tissue differences are probably due to the abundance of glycine decarboxylase (a matrix enzyme) in green leaf mitochondria.

Figure 3 gives two examples of the two-dimensional patterns obtained by this high resolution technique: only the tuber (Fig. 3A) and callus (Fig. 3B) patterns are shown. The patterns observed for callus and cell suspension culture mi-

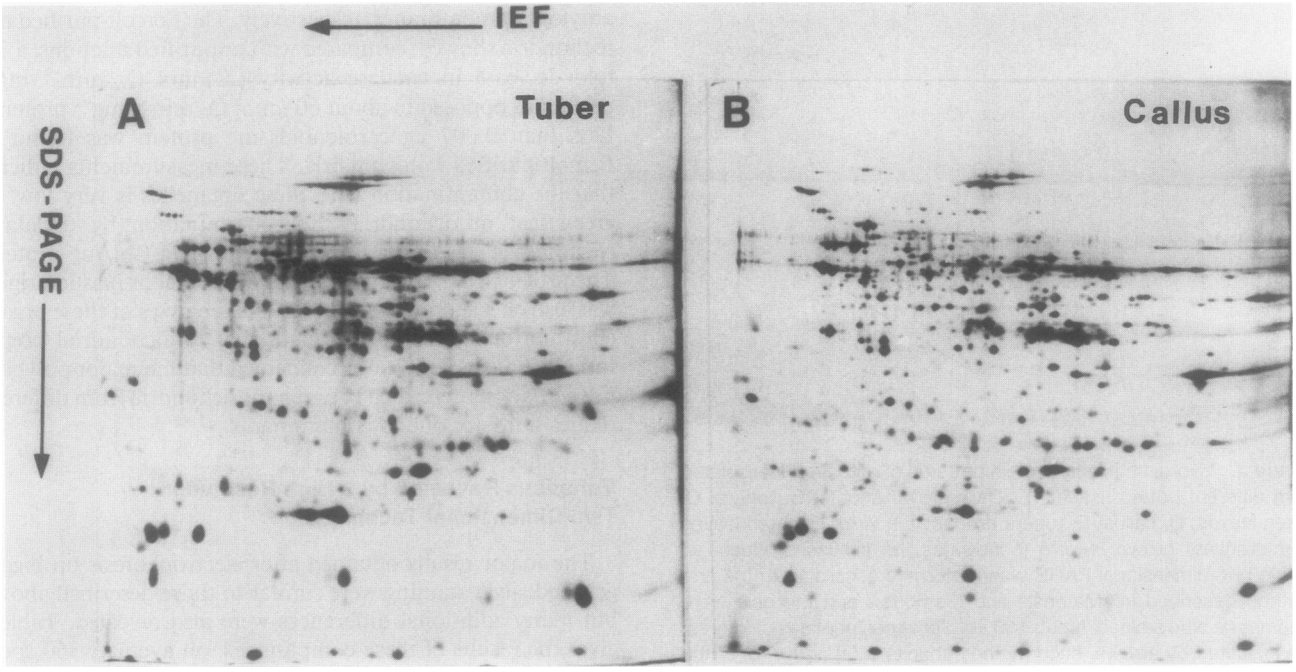

Figure 3. Two-dimensional protein patterns of mitochondria isolated from potato. A, Tubers; B, callus. These patterns were obtained after silver staining, according to the second two-dimensional PAGE method described in "Materials and Methods." Input: 20 μg protein.

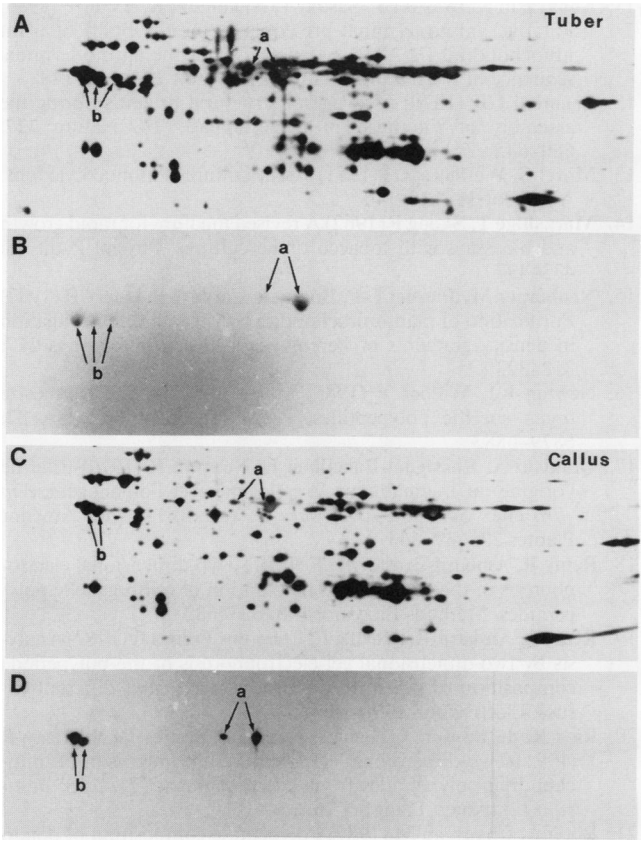

Figure 4. Enlargement of a fraction of the two-dimensional gels shown in Figure 3. A, Tuber mitochondria; C, callus mitochondria, and the corresponding western blots probed with the ATP synthase F_1 antiserum; B, tuber mitochondria; D, callus mitochondria. a and b are the α and β subunits of the ATP synthase, respectively. The β subunit is represented by three spots on tuber patterns (A and B), but only by two spots on callus patterns (C and D). The α subunit is represented by two spots in both cases.

tochondria are almost identical (not shown). More polypeptides are revealed on callus (or cell suspension) patterns than on those of any differentiated tissues (see Table I). A similar observation was reported for wheat callus mitochondria (20) also analyzed by two-dimensional PAGE. In the same way, for *Solanum melongena,* more bands were revealed on the isozymatic patterns of some peroxidases and polyphenoloxidases in callus than in any differentiated tissues (5). These results are in agreement with the hypothesis that the passage from differentiated tissue to callus involves the activation of genes that are silent in the differentiated state. This seems to be true in mitochondria as well.

One-third of the polypeptides have different intensities in callus and etiolated shoots. In particular, the α and β subunits of the ATP synthase complex seem to be in lower abundance in callus mitochondria than in mitochondria from any other analyzed tissues (Fig. 4A and C). These spots have been characterized by western blotting and probing with ATPase F_1 antibody. Only the α and β subunits could be identified with this antibody (Fig. 4B and D). Furthermore, the α subunit, which is mitochondrially encoded, is present on the fluorograms of [^{35}S]methionine-labeled translation products (data not shown). The α subunit is represented as a major spot and a minor spot on two-dimensional gels of tuber and callus mitochondria (Fig. 4A and C). This can be interpreted as a minor charge variance of a single polypeptide (8). It is worth noting that in callus, although the major spot is in lower abundance, the minor spot is bigger than in tuber, suggesting a different regulation in callus. Another interesting observation is that the β subunit is represented as three major spots on two-dimensional gels of mitochondria from tubers (Fig. 4A and B) and other organs, but only two on callus (Fig. 4C and D) and cell suspension mitochondrial patterns. This has been reproducibly observed and only for these two tissues. Boutry and Chua (4) found two distinct nuclear genes encoding the β subunit of ATP synthetase in *Nicotiana plumbaginifolia,* and suggested that other plant species, as supposed for sweet potato (11), contain two or more genes for the β subunit, although yeast only contains one (23). It seems possible that the three spots that are present on our two-dimensional patterns and that react with the ATP F_1 antibody are the products of three distinct ATP β genes in potato, one of them not being expressed in callus or cell suspension mitochondria.

Similar Variation in Other Species

Mitochondria isolated from different plant materials were analysed by two-dimensional electrophoresis, and the quantitative variations between green and nongreen tissue patterns previously observed for potato was confirmed: abundance of the four subunits of the glycine decarboxylase complex and traces of the 40 kD polypeptide in green leaves (seen in pea, tomato, onion, chicory), and the presence in high quantities of the 40 kD polypeptide in etiolated tissues (seen in pea epicotyls [19], etiolated chicory leaves, carrot and sweet potato roots, Jerusalem artichoke tuber, onion bulb, cauliflower inflorescence; our unpublished data). A single polypeptide immunologically equivalent to the 40 kD of potato mitochondria was found in each of these species using the antiserum raised against this 40 kD polypeptide.

Characteristics of the 40 kD Polypeptide

The 40 kD polypeptide (pI 6.8) is a good candidate for a molecular analysis as it is very abundant, tissue specific, and its regulation is opposite to that observed for glycine decarboxylase. A densitometer scan of two-dimensional gels from tuber mitochondria showed that it accounts for approximately 9% of the total mitochondrial protein (not shown). It belongs to the soluble fraction of the mitochondrial proteins, and is probably encoded by a nuclear gene, as it is not synthesized by isolated mitochondria (data not shown).

The 29 amino acid N-terminal sequence obtained from the 40 kD polypeptide was found to be: Leu-Gln-Ala-Ser-Pro-Gly-Pro-Lys-Lys-Ile-Val-Gly-Val-Phe-Tyr-Lys-Ala-Asn-Glu-Tyr-Ala-Glu-Met-Asn-Pro-Asn-Phe-Leu-Gly.

This sequence was used to search the National Biomedical Research Foundation (release No. 26) and Swissprot (release No. 16) protein sequence data bases and the Genbank (release No. 66) and European Molecular Biology Laboratory (release No. 25) nucleic acid sequence data bases using the programs FASTA and TFASTA of the University of Wisconsin Genetics Computer Group software package, but no significant homology was found with any known protein. The fact that this

sequence does not start with a methionine residue suggests that the protein was derived by cleavage of an amino-terminal (pre)sequence that directed the protein to the mitochondrion (10).

In addition, it is interesting to note that despite its abundance in mitochondria, the identity of the 40 kD polypeptide and its role in these organelles are not obvious. The identification and gene characterization of this polypeptide are currently in progress in our laboratory.

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