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Purification and sequencing of cytochrome *b* from potato reveals methionine cleavage of a mitochondriallly encoded protein

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Several mitochondrial genes from a large number of different fungi, mammals and plants have been sequenced but little is known about the corresponding translation products. We have affinity purified cytochrome *c* reductase from potato mitochondria and isolated the mitochondrially encoded cytochrome *b* protein. Amino-terminal sequencing reveals that the polypeptide does not start with a methionine. Comparison of the amino acid sequence with the recently published sequence of the gene encoding the cytochrome *b* apoprotein suggests that the *N*-formylmethionine is removed. This result provides the first evidence for the presence of a deformylase and a methionine aminopeptidase in mitochondria.

Mitochondria; Cytochrome *c* reductase; Cytochrome *b*; Methionine aminopeptidase; *Solanum tuberosum*

1. INTRODUCTION

To assume their proper function many proteins are modified during or after translation. One of the mechanisms of protein modification is the removal of the amino-terminal methionine [1]. It is catalysed by methionine aminopeptidases which have been characterized in *E. coli* [2], *Salmonella* [3,4] and yeast [5–7]. Methionine specific aminopeptidases share similar substrate specificity in pro- and eukaryotes [8,9]. In prokaryotes an additional deformylase is required which makes the N-terminal methionine accessible to the cleavage reaction.

In contrast to prokaryotes, deformylase and methionine amino-peptidase seem to be absent in mitochondria [10,11]. Boissel et al. [8] analysed 1764 published NH_2-terminal sequences of eukaryotic proteins and found, that all proteins which are synthesized in mitochondria, have a N-terminal methionine residue. The only exceptions are the subunits 1 and 2 of cytochrome *c* oxidase (cox 1 and 2) and the subunit 6 of the ATPase from fungi, which carry N-terminal targeting sequences that are cleaved off by a processing protease upon assembly. Meanwhile the NH_2-terminal sequences of probably all mitochondrial encoded proteins from fungi are known and there are no examples of removal of the initiator methionine.

There is also experimental evidence that strongly argues against the presence of deformylase and methionine aminopeptidase in yeast mitochondria. In organello-translation in the presence of [^{14}C]- and [^{3}H]formate led to chloramphenicol sensitive labelling of mitochondrial encoded proteins. The formyl-group is retained on the nascent chains attached to the intramitochondrial ribosomes as well as on complete polypeptides in the mitochondrial membranes [10,12]. Under the same conditions deformylation is readily demonstrable in *E. coli*.

Here we report on the purification and N-terminal sequencing of potato cytochrome *b*, the only mitochondrial encoded subunit of cytochrome *c* reductase. Our data suggest that – in contrast to fungal and mammalian mitochondria – methionine cleavage does occur in plant mitochondria.

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Abbreviations: cox, cytochrome *c* oxidase; ND; NADH dehydrogenase; ATPase, F_0F_1-ATPase/synthase.

2. MATERIALS AND METHODS

Mitochondria from potato tubers were prepared as described previously [13]. Preparation and solubilization of mitochondrial membranes was performed according to Linke and Weiss [14] and membrane proteins were solubilized with 3.3% Triton X-100. The purification of cytochrome *c* reductase is outlined in [15] and is based on the protocol initially introduced by Weiss and Juchs [16]. Purified cytochrome *c* reductase was fractionated in 14% SDS/polyacrylamide gels [17] and either stained with Coomassie blue R250 or blotted onto PVDF membranes. The blotting buffer contained 20 mM Tris-HCl (pH 8.8)/20% methanol/0.05% SDS/0.5 mM DTT and the transfer of proteins was performed for 15 h at 500 mA (BioRad trans-blot cell) to ensure complete transfer of hydrophobic proteins. Polyclonal rabbit antibodies against cytochrome *b* from yeast were a kind gift of Prof. Schatz, Basel. Immunopositive bands were visualized with biotinylated anti-rabbit antibodies, avidin and biotinylated horseradish peroxidase as recommended by the supplier (Vektor Laboratories). For protein sequencing cytochrome *b* was localized immunologically on a narrow strip of the blot and the corresponding area containing about 200 pmol of the polypeptide was cut out and further analysed in an

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Applied Biosystems pulsed-liquid phase sequencer as described elsewhere [18]. Phenylhydantoin amino acids were separated on-line in an Applied Biosystems model 120 A analyzer and identified by manual interpretation of the data.

3. RESULTS

3.1. *Purification of cytochrome* b *from potato mitochondria*

To isolate the cytochrome *b* protein from potato mitochondria cytochrome *c* reductase was purified by affinity chromatography using a cytochrome *c* column [15]. In potato and other plants (Braun and Schmitz, unpublished results) this protein complex consists of ten subunits while the fungal complex comprises nine subunits [19]. Subunit IV of the complex has a slightly variable position on SDS-PAGE depending on the acrylamide percentage of the gel (14%: 35 kDa; 10%: 32 kDa) and the Coomassie stained band has a rather diffuse shape. These characteristics are typical for hydrophobic proteins. Antibodies directed against cytochrome *b* from yeast specifically recognize subunit IV of potato cytochrome *c* reductase [15]. The cytochrome *b* subunit was also identified spectrophotometrically; it has alpha-band maxima at 557 and 562 nm, which are unstable at room temperature due to the noncovalent attachment of the heme group to the protein.

3.2. *Determination of the N-terminal amino acid sequence of cytochrome* b *from potato*

For further analysis cytochrome *b* was blotted onto a PVDF membrane. As under standard blotting conditions most of this hydrophobic protein remained in the gel, different blotting conditions were tested. Elevated levels of SDS (0.05%) in the blotting buffer and high current proved to be essential for effective recovery of the protein from the gel (see Materials and Methods). While all other subunits of cytochrome *c* reductase are stainable with Ponceau S or Amido black after transfer to PVDF membranes, cytochrome *b* does not bind these

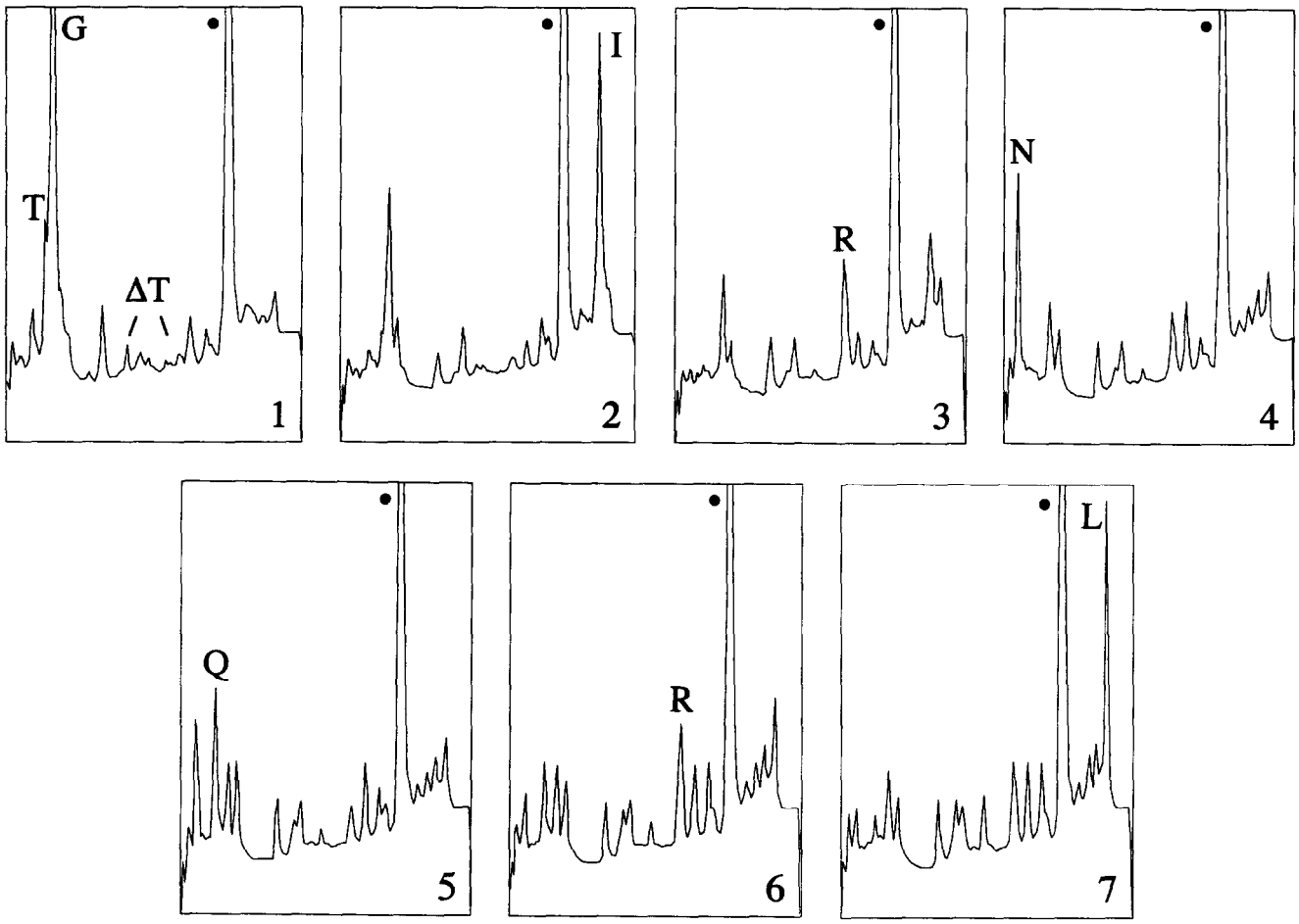

Fig. 1. Determination of the amino acid sequence of cytochrome *b* from potato. HPLC elution profiles of the phenylthiohydantoin amino acid derivates of the first seven degradation cycles are shown. Horizontal axes: time (0–30 min); vertical axes: absorbance. The amino acids are identified by the one letter code. The peaks labelled with a dot correspond to dimethylphenylthiourea. The glycine signal in cycle 1 is due to the presence of this amino acid during SDS/PAGE and appeared in the elution profiles of every subunit of cytochrome *c* reductase upon N-terminal sequencing. Corresponding to the published cDNA sequence for cytochrome *b* from potato there is a signal for threonine in cycle 1. Also the typical phenylthiohydantoin signals for dehydrated threonine (*Δ*T) are visible.

Cycle:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
(1)	*	I	*	N	Q															
(2)	*	I	R	N	Q	R	*	*	L	*	K	Q	*	I						
(3)	T	I	R	N	Q	R	L	S	L	L	K	Q	P	I	S	*	*	L	*	*
(4)	M	T	I	R	N	Q	R	L	S	L	L	K	Q	P	I	S	S	T	L	N
	ATG	ACT	ATA	AGG	AAC	CAA	CGG	CTC	TCT	CTT	CTT	AAA	CAA	CCT	ATA	TCC	TCC	ACA	CTT	AAT ...

Fig. 2. N-Terminal amino acid sequence of cytochrome *b* from potato. Three independent attempts for sequence determination (1), (2) and (3) were made and compared with the amino acid sequence deduced from a corresponding clone (4) [20]. The numbers of cycles of the degradation procedure are indicated above. Degradation cycles in which the amino acid could not be determined are marked with an asterisk.

stains under the conditions applied. Therefore, the filter areas which had bound cytochrome *b* were identified immunologically and subjected to Edman degradation (Fig. 1). The results from three sequencing experiments from different cytochrome *b* preparations are illustrated in Fig. 2. In the first two experiments due to inefficient blotting only small amounts of cytochome *b* could be subjected to the degradation procedure. Therefore just a few amino acids could be detected but in both cases the amino acid released in the second sequencing cycle was isoleucine. This residue is encoded by the third codon of the cytochrome *b* gene from potato [20]. In the third experiment the first amino acid was identified as threonine corresponding to the second codon of the gene (see Fig. 1). The initiator methionine is obviously cleaved off.

For several reasons it is highly unlikely, that the N-terminal methionine is missing due to an artefact, e.g. weak exoproteolytic activity during the purification procedure: (i) sequencing of cytochrome *b* from three independent preparations indicated in all cases the absence of the first amino acid; (ii) no ragged N-termini, which could be the result of chemical degradation or exoproteolytic activity, were observed; (iii) likewise, determination of the N-termini of all nuclear encoded subunits of cytochrome *c* reductase did not indicate degeneration for any protein (Braun et al., 1992; Braun and Schmitz, unpublished results). We therefore conclude that the N-terminal methionine of cytochrome *b* from potato is specifically removed within the mitochondrium by an aminopeptidase.

4. DISCUSSION

Two classes of mitochondrial encoded proteins occur in fungi [11]: a major class with a formylated methionine at the N-terminus and a small class with a transient presequence. Proteins belonging to the first class are blocked for direct sequence analysis by cyclic degradation procedures [21]. A blocked N-terminus was shown for cytochrome *b* from yeast, *Neurospora* and bovine [11,22,23]. Chemical removal of the formyl-group of cytochrome *b* from yeast and bovine revealed methionine as the first residue. Sequences for mitochondrial cytochrome *b* from another 13 organisms were only deduced from the corresponding genes [24]. Besides cytochrome *b* proteins, a formylated methionine was shown to be the N-terminal residue in nearly all other

Potato	M	**T**	I	R	N	Q	R	L	S	L	L	K	Q	P	I	S	S	T	L	N	Q	H	L	I	D	Y ...
Maize	M	**T**	I	R	N	Q	R	F	S	L	L	K	Q	P	I	Y	S	T	L	N	Q	H	L	I	D	Y ...
Wheat	M	**T**	I	R	N	Q	R	F	S	L	L	K	Q	P	I	Y	S	T	L	N	Q	H	L	I	D	Y ...
Yeast							M	**A**	F	R	K	S	N	V	Y	L	S	L	V	N	S	Y	I	I	D	S ...
Neurospora							M	**R**	L	L	K	S	H	P	L	L	K	L	V	N	S	Y	L	I	D	A ...
Chlamydomonas							M	**R**	M	H	N	K	I	Q	L	L	S	V	L	N	T	H	L	V	A	Y ...
Aspergillus							M	**R**	I	L	K	S	H	P	L	L	K	I	V	N	S	Y	I	I	D	S ...
Drosophila					M	**H**	K	P	L	R	N	S	H	P	L	F	K	I	A	N	N	A	L	V	D	L ...
Bovine					M	**T**	N	I	R	K	S	H	P	L	M	K	I	V	N	N	A	F	I	D	L ...

Fig. 3. Comparison of the N-terminal amino acid sequences deduced from clones coding for cytochrom *b* from potato [20], maize [41], wheat [42] yeast [43] *Neurospora* [44] *Chlamydomonas* [45] *Aspergillus* [46] *Drosophila* [47] and bovine [48]. The second amino acid, which mainly determines methionine aminopeptidase accessibility, is written in bold letters.

mitochondrial encoded proteins from yeast, including cox 1 [11], cox 3 [11], the subunits 8 and 9 of ATPase [25,26] and the ribosomal var 1 protein [11]. The ND subunits, which are not present in the yeast mitochondrial genome, proved to start with formylated methionine in *Neurospora* [27]. Also for cox 1, 2, 3, the subunits 6 and 8 of ATPase and subunit 1 of NADH dehydrogenase from bovine N-terminal formyl-methionine was shown [28–31]. Two mitochondrial encoded proteins from *Neurospora* (cox 1 [32] and cox 2 [33]) and two from yeast (cox 2 [34] and subunit 6 of ATPase [35]) were shown to belong to the second class. There is only limited information on N-terminal amino acids of mitochondrial encoded proteins from plants. Subunit 9 and the alpha-subunit of the ATPase start with methionine [36,37] and seem to belong to the first class while cox 2 and subunit 6 of the ATPase like their fungal counterparts are likely to be processed and belong to the second class [38,39]. Cytochrome *b* from potato belongs to a new class of mitochondrial encoded proteins in which only the first methionine is removed. The absence of the initiator residue suggests that plant mitochondria contain a deformylase and a methionine aminopeptidase.

In prokaryotes and eukaryotes, the ability of methionine aminopeptidases to remove the initiator methionine mainly depends on the side chain size (the radius of gyration should be smaller than 0.143 nm) of the second amino acid [9]. Cleavage occurs before Gly, Ala, Pro, Ser, Thr, Val and Cys, but never before His, Gln, Glu, Phe, Met, Lys, Tyr, Trp and Arg. The third residue of the polypeptide may influence these peptidases as well [2,40]. In Fig. 3, N-terminal regions of cytochrome *b* from 9 different organisms are compared. In five organisms, the second amino acid would allow methionine removal according to the rules outlined above (potato, maize, wheat, yeast and bovine). However it does not occur in yeast or bovine as revealed by direct protein sequencing. The only plant mitochondrial proteins which are sequenced so far and belong to the first class (the alpha-subunit and subunit 9 of ATPase) have Arg at position two of translation which is supposed to prevent removal of the initiator residue.

Different suggestions have been made concerning the functional significance of methionine cleavage. As the lifetime of a protein depends on the size of its amino-terminal residue [49], removal of methionine clearly influences protein stability. On the other hand, the rather reactive amino-terminal methionine may also have an impact on the behaviour of a polypeptide during interaction with other polypeptides (e.g. assembly into protein complexes). Finally, methionine cleavage may be important for the recycling of the methionine pool as suggested by Hirel et al. [40].

The plant mitochondrial genome is more than ten times larger than the mammalian one and codes for at least twice as many proteins [50]. Possibly this extraordinary complexity also requires the enzyme machinery for posttranslational modifications, like methionine removal. It will be interesting to see, whether other plant mitochondrial proteins with suitable amino acids at the second position undergo methionine removal and to characterize the enzyme(s) responsible.

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