Synthesis of a dicyclohexylcarbodiimide-binding proteolipid by cucumber (Cucumis sativus L.) mitochondria

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Summary. When isolated cucumber (Cucumis sativus L.) mitochondria were treated with 14C-labelled dicyclohexylcarbodiimide (DCCD), a single polypeptide was predominantly labelled. This polypeptide was soluble in 1-butanol or chloroform : methanol (2 : 1, v/v) and had an apparent molecular mass of approximately 7 kDa; it therefore had the characteristic properties of the DCCD-binding proteolipid subunit of the ATP synthase complexes of mitochondria, chloroplasts, and prokaryotes.

When isolated cucumber mitochondria were allowed to synthesize protein in the presence of [35S]methionine and then extracted with 1-butanol or chloroform : methanol (2 : 1, v/v), a [35S]-labelled proteolipid that migrated more rapidly on SDS-polyacrylamide gels than the proteolipid labelled by [14C]DCCD was solubilized. Treatment of mitochondria with unlabelled DCCD after they had been allowed to synthesize protein, specifically converted some of the [35S]methionine-labelled proteolipid to a form that comigrated with the [14C]DCCD-labelled proteolipid. We therefore conclude that a DCCD-binding proteolipid is synthesized by isolated cucumber mitochondria.

Key words: Dicyclohexylcarbodiimide-binding proteolipid – Plant mitochondrial genes – Organelle protein synthesis

Introduction

The biogenesis of mitochondria requires the cooperation of the nuclear and mitochondrial genomes, and the assembly of polypeptides encoded by both to give functional enzyme complexes (Tzagoloff 1982). One of the intriguing aspects of this cooperation is that the contribution of the two genomes varies between different organisms. The best-documented example of this variation is found in the ATP synthase (oligomycin-sensitive, proton-translocating ATPase) complex. This complex, which has approximately 10 subunits, can be resolved into two portions, F1 and F0. A functional ATP-synthesizing complex can be reconstituted from the F1 and F0 portions and phospholipids. The F1 portion is hydrophilic and has ATPase activity. Its composition is well defined; it has five subunits. The F0 portion is hydrophobic and functions as a proton channel but does not have catalytic activity by itself. Its composition is not entirely clear (Amzel and Pedersen 1983; Tzagoloff 1982). The best-characterized subunit of F0 is the one known as the dicyclohexylcarbodiimide-binding proteolipid subunit because of its two characteristic properties. First, it is soluble in lipid solvents, in particular chloroform : methanol (2 : 1, v/v) and 1-butanol. Second, it covalently binds dicyclohexylcarbodiimide (DCCD) at low DCCD concentrations; this binding causes inhibition of the enzymic activity of the complex and reduces the rate of migration of the proteolipid on SDS-polyacrylamide gels (Cattell et al. 1971; Sebald and Hoppe 1981).

In fungi and metazoans, all five subunits of the F1 portion of ATP synthase are encoded by nuclear genes (Sebald 1977). In the yeast Saccharomyces cerevisiae, the DCCD-binding proteolipid subunit (Hensgens et al. 1979; Macino and Tzagoloff 1979) and another subunit of F0, usually referred to as subunit six (Macino and Tzagoloff 1980), as well as an ATPase-associated polypeptide (Macreadie et al. 1983) are encoded by mitochondrial DNA. In other fungi, such as Neurospora crassa, subunit six is encoded in mitochondrial DNA but the DCCD-binding proteolipid is encoded in nuclear DNA.
DNA and synthesized in the cytosol (the site of synthesis of the ATPase-associated polypeptide has not been defined). Interestingly, in *Neurospora crassa* there is a sequence in mitochondrial DNA that could encode a proteolipid but appears not to be expressed (van den Boogaart et al. 1982). In mammals, such as humans (Anderson et al. 1981), cows (Anderson et al. 1982) and mice (Bibb et al. 1981), and probably in at least some lower metazoans such as sea urchins (Roberts et al. 1983), mitochondrial DNA includes a gene that could encode subunit six but none for the proteolipid.

In higher plants, an additional subunit, the hydrophilic alpha subunit of ATP synthase, is synthesized by mitochondria (Hack and Leaver 1983; Hack et al. 1983). The site of synthesis of the subunits of the FO portion is not known. Preliminary evidence (Leaver et al. 1982) suggests that the proteolipid is synthesized in mitochondria, as in yeast. In this paper we describe experiments that confirm this preliminary evidence.

**Materials and methods**

*Plant material.* Seedlings of cucumber (*Cucumis sativus* L., variety Long Green Ridge) were incubated in water at 4 °C for at least 17 h, then germinated on sterilized cellulose wadding wetted with sterile, distilled water. Trays were kept in continuous darkness with a temperature cycle of 28 °C for 12 h, then 22 °C for 12 h (Becker et al. 1978).

**Preparation of mitochondria.** Mitochondria were isolated from the cotyledons of five-day-old cucumber seedlings by differential centrifugation and isopycnic sucrose density gradient centrifugation as described previously (Leaver et al. 1983). The yield of mitochondrial protein, measured by a modified Lowry protein assay (Miller 1959), was approximately 0.5 mg/g fresh wt.

**In vitro protein synthesis.** Mitochondrial translation products were labelled with \[^{35}S\]methionine as described, with succinate as energy source (Leaver et al. 1983).

**Labelling with \[^{14}C\]DCCD.** Conditions for labelling with \[^{14}C\]DCCD were based on those described by Cattell et al. (1971) and Sebald et al. (1979), but a short treatment at 25 °C was used instead of a prolonged (four to many hours) treatment at 0°-4 °C. Mitochondria (not labelled with \[^{35}S\]methionine) were suspended in 0.01 M Tris-HCl (pH 7.5) and \[^{14}C\]DCCD (54 Ci/mol), dissolved in absolute ethanol at a concentration of 2 mM or 10 mM, was added to the suspension; ratios of DCCD to protein and final DCCD concentrations are given in the figure legends. The mitochondrial suspensions were then diluted by the addition of 0.01 M Tricine-NaOH (pH 7.2), 0.4 M mannitol, 0.001 M EGTA and the mitochondria were collected by centrifugation at 12,000 g~max~ for 3 min. Mitochondria were either used directly for organic solvent extraction or frozen and stored at −80 °C.

**Treatment of mitochondria with unlabelled DCCD.** Mitochondria that had been freshly labelled with \[^{35}S\]methionine were collected by centrifugation and resuspended in 0.01 M Tris-HCl (pH 7.5). Dicyclohexylcarbodiimide, dissolved in absolute ethanol at a concentration of 2 mM, 10 mM or 20 mM, was added to mitochondrial suspensions; ratios of DCCD to protein and final DCCD concentrations are given in the figure legends. The treated mitochondria and a control suspension at a protein concentration of 2 mg/ml in 0.01 M Tris-HCl (pH 7.5), 5% (v/v) ethanol were incubated at 25 °C with shaking for 1 h. The mitochondria were then treated in the same way as mitochondria that were incubated with labelled DCCD.

**Organic solvent extraction.** Mitochondria were suspended in 0.01 M Tris-HCl (pH 7.5) to a concentration equivalent to approximately 10 mg/ml of starting protein (the osmotic shock administered in the DCCD treatment causes loss of matrix protein). 50 volumes of 1-butanol or 25 volumes of chloroform:methanol (2:1, v/v) were added to the mitochondria, giving a single-phase suspension. After being mixed by vortexing, the suspension was incubated overnight on a roller shaker at 4 °C. The suspension was then centrifuged in a horizontal rotor for 5 min at 12,000 g~max~. The pellets were allowed to dry in air. The supernatant was centrifuged again to ensure complete removal of insoluble material.

Extracts were dried in vacuo and the residue was resuspended in 1/10 of the original volume of 1-butanol or 1/5 of the original volume of chloroform:methanol (2:1, v/v). With both solvents, there was a significant quantity of material that did not dissolve, even after incubation overnight at room temperature. Insoluble material remaining after overnight incubation was removed by centrifugation. Five volumes of diethyl ether, chilled to −20 °C, were added to the butanol extracts and 4 volumes of chloroform:methanol extracts. Protein was allowed to precipitate at −20 °C for at least 24 h.

The precipitated protein was collected by centrifugation for 5 min at 12,000 g~max~. The pellet was allowed to dry in air then resuspended in 0.1 ml of 1-butanol or chloroform:methanol. It now appeared to be completely soluble and was therefore collected by evaporation to dryness in vacuo.

**Electrophoresis.** Mitochondria to be analyzed by SDS-polyacrylamide gel electrophoresis were dissolved in sample buffer containing 2% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 0.06 M Tris-HCl (pH 6.8), 0.01% (w/v) bromophenol blue by heating at 95−100 °C for 2 min. Protein extracted with 1-butanol or chloroform:methanol was dispersed in distilled water and dissolved by the addition of an equal volume of 2x sample buffer and incubation at 95−100 °C for approximately 2 min. In both cases, disulfide bonds were reduced, after the samples had cooled, by the addition of 1 M dithiothreitol to a final concentration of 0.05 M. The pellets remaining after organic solvent extraction are difficult to dissolve in sodium dodecyl sulfate, and the polypeptides tend to aggregate. In order to minimize this problem, pellets from 0.2 mg mitochondrial protein were suspended in 0.05 ml 9.5 M urea, 0.01 M sodium carbonate (Horst et al. 1980), in which they gradually dissolved. 0.025 ml 9.5 M urea, 4% (w/v) sodium dodecyl sulfate was then added and the suspensions were incubated at 95−100 °C for 2 min. After they had cooled, 0.025 ml of 0.24 M Tris-HCl (pH 6.8), 0.2 M dithiothreitol, 0.04% (w/v) bromophenol blue was added. Remaining insoluble material was removed by centrifugation.

Protein derived from approximately 0.1 mg of mitochondrial protein was applied to each track of the gels. Gel electrophoresis was carried out using the buffer system of Laemmli (1970) and 12−18% (w/v) acrylamide linear gradient gels. Gels were stained with Brilliant Blue R (C.I. 42660), destained, and dried onto Whatman 3MM filter paper. \[^{35}S\]Methionine-labelled proteins were detected by autoradiography using Dupont Cronex 4 X-ray film.