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Identification of a chaperonin-10 homologue in plant mitochondria

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Abstract

Chaperonin-60 and chaperonin-10 form stable binary complexes in the presence of ATP. This phenomenon has been used as the basis for the identification of a chaperonin-10 homologue in potato (*Solanum tuberosum* L.) mitochondria. ATP-dependent binary complexes formed between chaperonin-60 and chaperonin-10 in potato mitochondrial extracts were isolated by affinity chromatography on a column of immobilised chaperonin-60 antibodies. Partial amino acid sequence analysis of the chaperonin-10 protein indicates that it is related in primary structure to rat mitochondrial chaperonin-10.

Key words: Molecular chaperone; Chaperonin; Plant mitochondrion

1. Introduction

Chaperonin-60 and chaperonin-10 belong to the GroE subclass of chaperonins which are molecular chaperones which assist the correct non-covalent assembly of other polypeptide-containing structures in vivo, but are not components of these assembled structures when they are performing their normal biological functions [1,2].

The two chaperonin proteins have been shown to interact in vivo in the assembly of bacteriophage particles [3] and in vitro in the renaturation of denatured enzymes [4–7].

Chaperonin-10 has been identified in mammalian mitochondria by its ability to substitute for the bacterial protein in the chaperonin-dependent refolding of Rubisco [8]. A similar approach, employing the refolding of ornithine transcarbamoylase as the functional assay, enabled the purification and amino acid sequencing of chaperonin-10 from rat hepatoma cell mitochondria [9].

Association of the bacterial chaperonin-60 and chaperonin-10 to form a stable complex occurs in the presence of MgATP [6,10], a phenomenon also reported for complex formation between bacterial chaperonin-60 and mammalian chaperonin-10 [8]. In this paper we report the identification of a chaperonin-10 homologue in plant mitochondria through its association with chaperonin-60 in the presence of ATP and its subsequent isolation by chaperonin-60 affinity chromatography. This protein is related to rat mitochondrial chaperonin-10 at the level of the primary structure.

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Abbreviations: ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin (fraction V); EGTA, ethyleneglycol bis-(β-aminoaethyl ether) *N,N,N′,N′*-tetraacetic acid; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulphonic acid; PMSF, phanylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SDS, sodium dodecyl sulphate.

2. Materials and Methods

2.1. Chaperonin-60 affinity chromatography column preparation

An immobilised anti-mitochondrial chaperonin-60 antibody column was prepared using the Pierce AminoLink™ system according to the manufacturer's instructions. 18 mg of antibodies against potato mitochondrial chaperonin-60 were used in preparing the column.

2.2. Isolation of mitochondria from potato tubers

Mitochondria were extracted from tubers that were peeled, washed and homogenised. The juice was collected into grinding buffer (0.2 ml/g tissue fresh weight) (grinding buffer; 0.4 M mannitol, 0.5% (w/v) BSA, 5 mM EGTA, 40 mM cysteine, 125 mM MOPS pH 10.0). The homogenised tissue was filtered through 8 layers of muslin followed by centrifugation at $3000 \times g$ for 5 min at 2°C. The supernatant was collected and recentrifuged at $10,000 \times g$ for 15 min at 2°C. The supernatant was discarded, the pellet resuspended in wash buffer (0.4 M mannitol, 0.1% (w/v) BSA, 1 mM EGTA, 5 mM MOPS pH 7.5) and the centrifugation steps repeated.

The pellet obtained from the two rounds of centrifugation was resuspended in wash buffer and layered on to a 20 ml 0.7 M sucrose cushion (0.7 M sucrose, 1% (w/v) BSA, 1 mM EGTA, 10 mM Tricine pH 7.2), followed by centrifugation at $12,000 \times g$ for 30 min. The mitochondrial pellet was resuspended in 0.4 M mannitol, 1 mM EGTA, 10 mM Tricine pH 7.2 followed by centrifugation at $12,000 \times g$ for 10 min and the mitochondrial pellet recovered.

2.3. Extraction of protein from isolated mitochondria

Aliquots (10 mg mitochondrial protein) of potato mitochondria were resuspended to 1 ml in 10 mM $MgCl_2$, 10 mM KCl, 100 mM Tris-HCl pH 7.7 followed by the addition of 2 μl 500 mM PMSF (dissolved in ethanol) and 50 μl 20% (w/v) dodecyl β-D-maltoside. Where required, 2 μl 300 mM ATP was added. Solubilized mitochondrial extracts were incubated on ice for 20 min followed by microcentrifugation at 4°C for 5 minutes.

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2.4. Affinity chromatography

Affinity chromatography was performed according to the instructions of the column manufacturer, using 10 mM $MgCl_2$, 10 mM KCl, 0.2% (w/v) dodecyl β-D-maltoside, 100 mM Tris-HCl pH 7.7 as the column equilibration and wash buffers. Where necessary, ATP was included in the equilibration and wash buffers at 0.6 mM.

2.5 SDS-PAGE

Aliquots of the fractions eluted from the column were fractionated by electrophoresis through 13.5% (w/v) polyacrylamide-SDS gels [11] or 16.5% (w/v) polyacrylamide-SDS gels [12]. Following electrophoresis, gels were silver-stained.

2.6. Reverse-phase HPLC

All samples were run on a Vydac C4 column (Separations Inc.) or an Applied Biosystems Aquapore RP300 (C8) column – 100 × 2 mm, equilibrated in 0.1% (v/v) trifluoroacetic acid/2% (v/v) acetonitrile. Samples were prepared by the addition of an equal volume of 6 M guanidine hydrochloride. A linear gradient of 2–50% (v/v) acetonitrile was applied over 50 min and then a further linear gradient of 50–90% (v/v) acetonitrile over 18 min. The samples were run on a Severn Analytical HPLC system consisting of a gradient controller utilising Flowmaster software, two model SA6410B high pressure pumps and a model SA6504 detector with a microbore flowcell fitted. All detection was performed at E_{215} and data was collected using Waters 'Expert Ease' software from Millipore (UK) Ltd., Watford, UK.

2.7. Proteolytic digestion of chaperonin-10

The peaks of interest from the reverse-phase HPLC protein separation were dried and redissolved in 100 μl 5% (v/v) acetonitrile, 100 mM Tris-HCl pH 8.0 and to this was added 1 μg trypsin (Boehringer, sequencing grade). Samples were incubated at 37°C overnight, in parallel with an enzyme-only control. Complete digestion reactions were fractionated by reversed phase HPLC on an Aquapore RP-300 column.

2.8 Amino acid sequence determination

All samples were run on an Applied Biosystems (ABI) 470A protein sequencer with on-line PTH analyser. Liquid samples were applied to a glass fibre disc pretreated with polybrene to limit sample wash-out and run using the standard 03CPTH program from Applied Biosystems. Data analysis was performed using Waters 'Expert Ease' software.

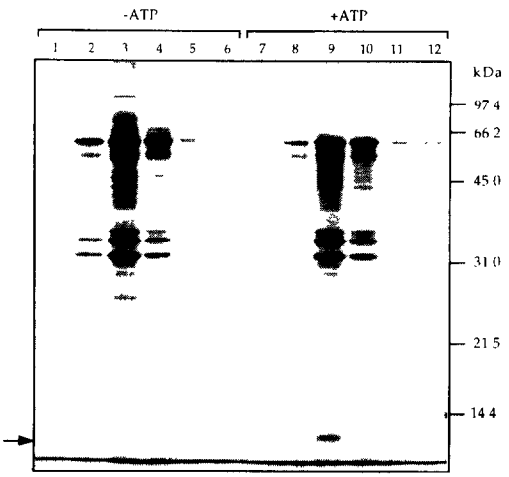

Fig. 1. Chaperonin-60 affinity chromatography in the presence or absence of ATP. Potato mitochondria were solubilised with dodecyl-β-D-maltoside in the presence or absence of ATP, and loaded on to a column of immobilised antibodies against chaperonin-60. Aliquots of the elution fractions were analysed by SDS-PAGE and silver staining. Lanes 1–6, elution fractions 1–6 when the experiment was performed in the absence of ATP; lanes 7–12, elution fractions 1–6 when the experiment was performed in the presence of 0.6 mM ATP. The novel polypeptide which copurifies with chaperonin-60 in the presence of ATP is indicated by an arrow.

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Rat      VLLPEYGGTKV
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Potato   VLLPEYGGTQV
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Fig. 2. Comparison of the amino acid sequence of a trypsin-digestion peptide fragment of potato chaperonin-10 with amino acids 65–80 of rat chaperonin-10.

3. Results and Discussion

Mitochondria were isolated from potato tubers and solubilised with 1% (w/v) dodecyl β-D-maltoside in the presence or absence of 0.6 mM ATP. Mitochondrial extracts were applied to a column of immobilised potato chaperonin-60 antibodies and bound proteins eluted with 0.1 M glycine pH 2.8. In the presence of 0.6 mM ATP, a novel polypeptide is found to copurify with chaperonin-60 (Fig. 1). Fractionation of eluted proteins on a 16.5% (w/v) polyacrylamide-SDS gel [12] indicates that this polypeptide is approximately 8.6 kDa (data not shown), a size consistent with its identity as a chaperonin-10 protein.

Reverse-phase HPLC fractionation of the equivalent affinity chromatography eluates obtained in the presence or absence of ATP led to the purification of the chaperonin-10 polypeptide through identification of the unique peak observed in the elution profile of the proteins isolated in the presence of ATP. Following trypsin-digestion of the purified chaperonin-10 protein and reverse-phase HPLC fractionation of the proteolytic digestion products, amino acid sequence was obtained for one peptide fragment. The sequence of 13 amino acid residues was obtained. This sequence matches that obtained for rat mitochondrial chaperonin-10 [9] in ten out of eleven positions for which sequence data are available for both polypeptides (Fig. 2).

The amino acid sequence confirms that the 8.6 kDa mitochondrial protein which associates with chaperonin-60 in the presence of ATP is a homologue of chaperonin-10.

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