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Chemical synthesis of 10 kDa chaperonin

Biological activity suggests chaperonins do not require other molecular chaperones

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Molecular chaperones are required for the correct folding and assembly of certain other polypeptides. It is not known whether molecular chaperones themselves require other chaperones to become functional. A 97-amino acid chaperone, the chaperonin 10 protein was chemically synthesised so that during synthesis and purification there was no contact of the chaperone with any other protein. The purified, synthetic chaperonin 10 protein formed oligomeric structures spontaneously and was biologically active as a chaperonin. This is the first description of a chemically synthesised chaperonin, and suggests that no other chaperones are required for correct folding, polymerisation and biological activity of this chaperone.

Chaperonin 10; GroES; Heat-shock protein; Chemical synthesis; Protein folding; Rubisco

1. INTRODUCTION

Molecular chaperones, are a family of ubiquitous cellular proteins whose function is to prevent incorrect interactions between the surfaces of other molecules; there is evidence that chaperones are required for the correct folding and assembly of certain other polypeptides [1,2]. This raises the question as to whether molecular chaperones themselves require other chaperones to become functional. In the yeast mitochondrion functional pre-existing chaperone, chaperonin 60 (cpn 60) [1,2] is needed so that new chaperonin 60 can be assembled from imported subunits [3]. In contrast, *Escherichia coli* cpn 60 can self assemble [4] in the presence of Mg-ATP and is stimulated by chaperonin 10 (cpn 10) [5]. So far, the chaperones which have been studied have either been purified from whole cell extracts or have been made by recombinant DNA techniques. In both cases, the opportunity is present for the chaperone protein to transiently interact with other chaperones within the cell during its folding and/or assembly. We have addressed this question by chemically synthesising a chaperone so that during the synthesis and purification there was no contact of the chaperone with any other protein. A 97-amino acid chaperone, the cpn 10 or 10 kDa GroES [5] protein of *E. coli* was synthesised.

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Abbreviations: cpn 10, chaperonin 10; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; cpn 60, chaperonin 60; BSA, bovine serum albumin; TFA, trifluoroacetic acid; HF, hydrogen fluoride; DNP, dinitrophenyl; DMF, dimethylformamide; TFA, trifluoracetic acid; HF, hydrogen fluoride

2. MATERIALS AND METHODS

2.1. *Chemical synthesis of E. coli chaperonin 10*

The synthesis of the polypeptide was carried out in a stepwise fashion using a 430A ABI synthesizer and the chemical scheme based on the combination of N(α)-BOC, benzyl-based side chain-protected amino acids. Other side chain-protecting groups included: tosyl for Arg, dinitrophenyl (DNP) for His, Cl- and Br-carbobenzoxy for Lys and Tyr, respectively. As the solid support we used the phenylacetamidomethyl resin [6] containing 0.34 mmol of Ala. All amino acids were double coupled using the chemical protocols developed by Kent [7] and the efficiency of each coupling step monitored by the quantitative ninhydrin test [8]. The average incorporation thus calculated was 99.35% [9]. After the completion of chain synthesis, the DNP group from the His side chain was removed by treating an aliquot of peptide resin twice with a dimethylformamide (DMF) solution containing 2-mercaptoethanol (20% v/v) and the t-BOC group from the last residue with 100% TFA.

To complete the side chain deprotection and remove the peptide from the resin the low-high HF procedure was used [10]; scavengers utilised were dimethylsulphide, *p*-cresol and *p*-thiocresol (6:0, 8:0.2 ml/0.5 g resin-peptide) for the low HF cleavage and *p*-cresol and *p*-thiocresol (0.8:0.2) during the high HF step.

Separation of the crude, diethylether-precipitated cpn 10 from the peptide-resin mixture proved difficult owing to the poor solubility of the crude polypeptide. However, after several washings with 5% acetic acid and lyophilisation of the combined solutions, 40 mg of crude polypeptide were obtained from 200 mg of resin-peptide material.

For the refolding assay 15 mg of lyophilised material was dissolved in 5 ml of 6 M urea (50 mM Tris buffer, pH 7.5) and dialysed at 4°C for 24 h against 4 liters of the same Tris buffer solution.

After dialysis the solution was tested for its protein content using the Bio-Rad Protein Assay reagent, concentrated by partial lyophilisation to the desired protein concentration (see below) and used without any further purification for the refolding assay, and for the FPLC and SDS-PAGE analysis.

2.2. *Size-exclusion chromatography of synthetic chaperonin 10*

The separation was performed with a Pharmacia Sephacryl S-100 column using a Pharmacia FPLC apparatus. The solvent (100 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.8) was applied at a 2.5 ml/min rate; monitoring was with a UV recorder at 276 nm.

2.3. *SDS-PAGE*

SDS-PAGE analysis was performed on the Pharmacia Phast System with 20% gradient gels.

2.4. *Refolding of recombinant rubisco protein using synthetic* E. coli *cpn 10 and recombinant cpn 10*

Rubisco was denatured in 6 M guanidine-HCl and refolded using the protocol described in [11]. Briefly, reconstitution was initiated by diluting 21 μl denatured rubisco into 1659 μl solution containing 50 mM Tris-HCl buffer, pH 8.0, 3 mM ATP, 7 mM $MgCl_2$, 20 mM glucose, 4 μM cpn 60 and 6 μM cpn 10 at 23°C. At various times the reconstitution reaction was quenched with hexokinase. Reaction mixtures were supplemented with 50 mM [^{14}C]$NaHCO_3$ (300 dpm·nmol^{-1}) and allowed to activate for a further 5 min. Ribulose biphosphate (1 mM) was added and rubisco activity measured as the formation of acid-stable ^{14}C over 20 min.

3. RESULTS AND DISCUSSION

The purified, synthetic cpn 10 formed oligomeric structures which are also found in recombinant GroES [5]. The formation of oligomers was demonstrated by size-exclusion FPLC in non-denaturing buffer. We found that there were peaks at about 200 kDa (numbered 1 in Fig. 1), 90 kDa (peak 2), 30–40 kDa (peak 3) and 15–30 kDa (peak 4). The 90 kDa peak probably corresponds to an 80 kDa oligomer described previously [5]. The 30–40 kDa peak may represent a

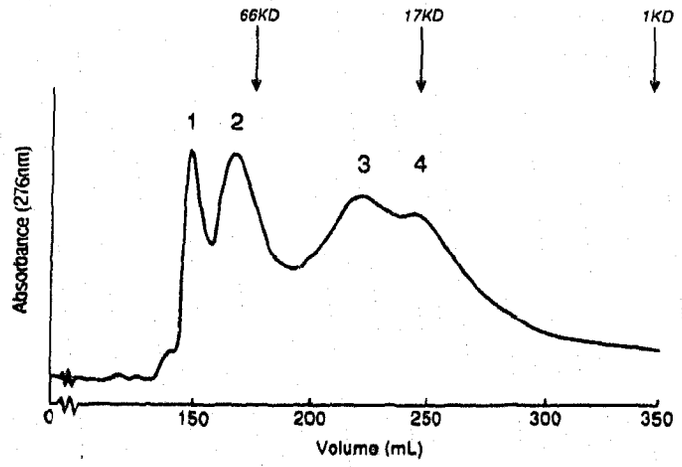

Fig. 1. Size-exclusion chromatography of synthetic chaperonin 10. Peaks 1, 2, 3 and 4 eluted at 150, 170, 225 and 245 ml, respectively. In a calibration run, BSA (mol. wt. 66000) eluted at 175 ml and cytochrome c (mol. wt. 13000) at 250 ml.

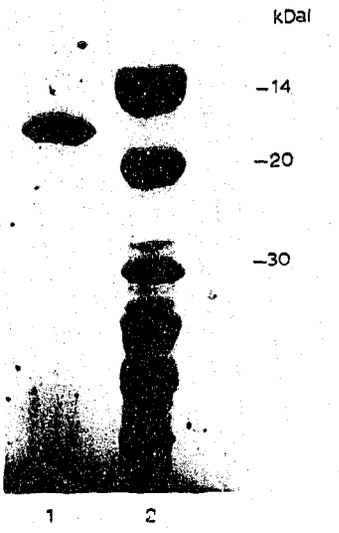

Fig. 2. SDS-PAGE of synthetic cpn 10. (Track 1) Synthetic cpn 10 from dialysis. (Track 2) markers.

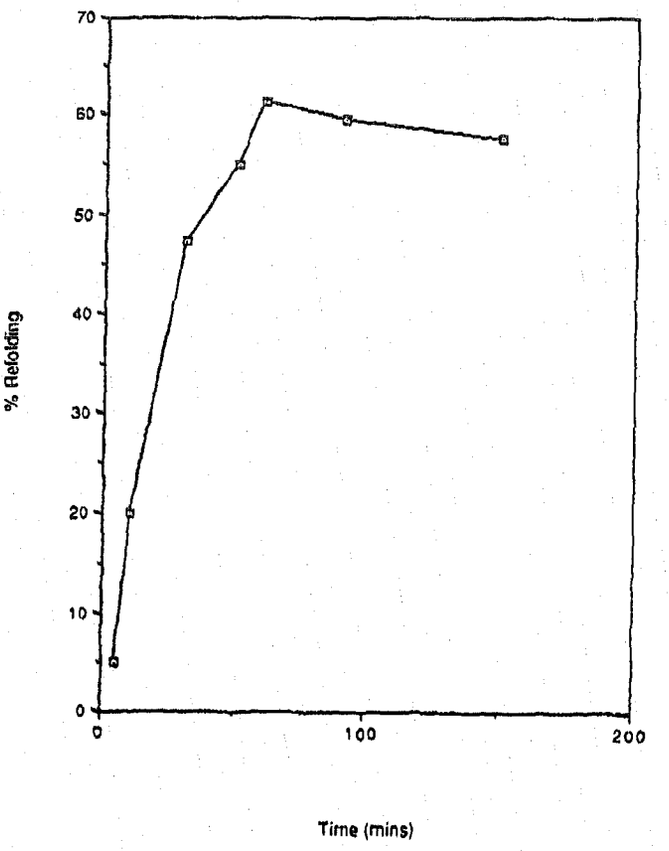

Fig. 3. Refolding of recombinant rubisco protein using whole, unfractionated synthetic cpn 10 and recombinant cpn 60 (rubisco binding protein). Rubisco refolding is expressed as a percentage of the enzymic activity of an equal quantity of non-denatured native enzyme.

dimer or a trimer. On SDS-PAGE (Fig. 2), protein from the 30–40 kDa size-exclusion peak migrated as a single band at about 15 kDa, consistent with the previous observations with recombinant cpn 10 [5]. SDS-PAGE of the 90 kDa and 200 kDa peaks in the size-exclusion chromatography also give a single band at 15 kDa (data not shown).

We then tested the synthetic protein for biological activity as a chaperonin. Recently Goloubinoff and colleagues [11,12] have described an in vitro assay for cpn activity. Denatured recombinant rubisco protein from *Rhodospirillum rubrum* is biologically inactive, but in the presence of recombinant *E. coli* cpn 10 and the associated cpn 60, as well as Mg-ATP and potassium ions, rubisco assembles into a dimer and regains its enzymic activity. We repeated this assay with recombinant reagents kindly supplied to us by A.A. Gatenby and G. Lorimer (Dupont de Nemours & Co., Wilmington, USA) except that we used chemically synthesised rather than recombinant cpn 10. The addition of dialysed, complete synthetic cpn 10 to denatured rubisco in the presence of recombinant cpn 60, Mg-ATP and potassium ions yielded a rubisco which was enzymically active as shown by the fixation of radioactive carbon dioxide (Fig. 3). Denatured rubisco incubated either alone or in the presence of either cpn 60 or cpn 10 was completely inactive in this assay. The synthetic cpn 10 showed comparable specific activity to that published for the recombinant cpn 10 [11]. Denaturation of synthetic cpn 10 with urea, followed by dilution of the urea, also resulted in functionally active cpn 10. To our knowledge this is the first description of the chemical synthesis of a biologically active chaperonin.

These experiments suggest that in the case of the cpn 10, no other chaperones are required for correct folding, polymerisation and biological activity. In other words, unlike rubisco, the cpn 10 does not need chaperones to reconstruct its activity in an in vitro system. Although *E. coli* cpn 60 can self-assemble [4] it is not yet known whether this renatured cpn 60 is active in the rubisco assay. It is unlikely that the addition of cpn 60 in the rubisco assay causes the unfolded cpn 10 to fold up correctly because the cpn 10 which was added was already in oligomeric form. These observations suggest that the cpn 10 molecular chaperone falls into the category of proteins whose folding and assembly into functional structures occurs with sufficient probability to dispense with the services of chaperones [2]. Whether this is true for all chaperones remains to be determined.

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