Affinity Purification, Overexpression, and Characterization of Chaperonin 10 Homologues Synthesized with and without N-terminal Acetylation*  

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Michael T. Ryan‡§, Dean J. Naylor‡§§, Nicholas J. Hoogenraad‡, and Peter B. HojH||  

From the ‡School of Biochemistry, La Trobe University, Bundoora, Victoria 3083 and the §Department of Horticulture, Viticulture and Oenology, University of Adelaide, Waite Campus PMB 1, Glen Osmond, South Australia 5064, Australia

Utilizing the ability of bacterial chaperonin 60 (GroEL) to functionally interact with chaperonin 10 (Cpn10) homologues in an ATP-dependent fashion, we have purified substantial amounts of mammalian, chloroplast, and thermophilic Cpn10 homologues from their natural host. In addition, large amounts of recombinant rat Cpn10 were produced in Escherichia coli and found to be identical to its authentic counterpart except for the lack of N-terminal acetylation. By comparing these two forms of Cpn10, it was found that acetylation does not influence the oligomeric structure of Cpn10 and is not essential for chaperone activity or mitochondrial import in vitro. In contrast, N-terminal acetylation proved crucial in the protection of Cpn10 against degradation by N-ethylmaleimide-sensitive proteases derived from organellar preparations of rat liver. The availability of large amounts of both affinity-purified and recombinant Cpn10 will facilitate not only further characterization of the eukaryotic folding machinery but also further scrutiny of the reported function of Cpn10 as early pregnancy factor.

The partnership between Escherichia coli Cpn10 (GroES) and Cpn60 (GroEL) in the folding of polypeptides has been well documented. GroEL associates with GroES in an ATP-dependent manner and appears to quantize the ATPase activity of GroEL during repeated binding and release of the folding polypeptide substrates (Todd et al., 1994).

The presence of GroES and GroEL homologues has also been established in eukaryotic systems such as fungi, plants, and animals (reviewed by Martinus et al., 1995), but these systems are much less characterized despite some striking differences to their E. coli counterparts. For example, as opposed to the tetradecameric GroEL, mammalian Cpn60 forms a heptamer and has a strict requirement for mammalian Cpn10 (Vitanen et al., 1992). Furthermore, unlike GroEL, the ATPase activity of yeast Cpn60 is not inhibited by Cpn10 (Rospert et al., 1993).

Cpn10 homologues also differ in their oligomeric structure. For example, it was reported that the chloroplast homologue, Cpn21 may exist as a dimer or trimer of identical 21-kDa subunits (Bertsch et al., 1992) as opposed to the likely heptameric structure of the bacterial (Chandrasekhar et al., 1986) and mammalian (Hartman et al., 1992) counterparts.

Perhaps most surprisingly and excitingly, it has recently been concluded that EPF, a secreted substance that exhibits growth factor and immunosuppressive properties, is identical to Cpn10. This surprising link between mammalian Cpn10 and EPF was defined by bioassays in which bacterial Cpn10 (GroES) does not exhibit activity (Cavanagh and Morton, 1994; Quinn et al., 1994). Thus, in order to further elucidate the relationship between Cpn10's role as a chaperone and its suspected role as EPF, there is an urgent need to establish a ready source of mammalian Cpn10. Past purification procedures have, however, proven very cumbersome and will only yield microgram (Cavanagh et al., 1994) to milligram (Hartman et al., 1992) quantities despite the use of large amounts of starting material. Since cDNAs encoding Cpn10 have been cloned (Pilkington and Walker, 1993; Ryan et al., 1994; Monzini et al., 1994), it therefore seems attractive to attempt synthesis of mammalian Cpn10 in a bacterial expression system. Indeed, two recent reports have detailed the synthesis of a soluble His-tagged mouse Cpn10 (Dickson et al., 1994) and a denatured but reconstitutable human Cpn10 in E. coli (Legname et al., 1995). A potential drawback is that bacterially synthesized Cpn10 lacks the N-terminal acetylation unequivocally demonstrated to be present on the rat homologue (Hartman et al., 1992a) and indirectly on the human homologue (Cavanagh and Morton, 1994).

We have now developed a single step affinity procedure for the purification of mg quantities of authentic Cpn10 from a variety of sources and further describe a recombinant expression system which allows the production and purification of several hundred milligrams of active mammalian Cpn10 in 1 day. The availability of these procedures has not only allowed us to further characterize and compare Cpn10 homologues in protein folding reactions but also allowed us to assess the importance of N-terminal acetylation for folding activity, mitochondrial import, and resistance to proteases. While the acetylation is shown not to be crucial for chaperone-mediated folding reactions and import into mitochondria in vitro, it is demonstrated that N-terminal acetylation drastically decreases the susceptibility of Cpn10 to degradation by NEM-sensitive proteases from rat liver organelles. By defining a functional significance for the acetylation event, this questions whether mammalian Cpn10 synthesized in bacteria without this post-translational modification can be used to reliably assess the link between its well established role in protein folding and its suspected role as a secreted growth factor with immunosup-
pressible characteristics (Cavanagh and Morton, 1994; Quin et al., 1994).

MATERIALS AND METHODS
Preparation of GroEL Affinity Matrix
GroEL (650 mg) was purified from lysates of the E. coli strain RCS 677 containing the plasmid pBS559 (6 liters of culture) by Polymin-P precipitation (Hartman et al., 1990; Spath et al., 1990) followed by chromatography on DEAE-Sepharose essentially as described elsewhere (Todd et al., 1993). A slurry (15 ml) of Affi-Gel 15 (Bio-Rad) previously activated according to the manufacturer’s instructions and washed with 0.1 M MOPS-NaOH, pH 7.5, was mixed with purified GroEL (180 mg in 12 ml of 20 mM MOPS-NaOH, pH 8.0). Following gentle agitation for 15 h, about 95% of the GroEL preparation was immobilized, and the matrix was washed with 30 mM ethanolamine HCl, pH 8.0, to quench any remaining reactive sites. Unless otherwise stated these and all subsequent procedures were carried out at 4 °C.

Purification of Cpn10 Homologues
Preparation of Extracts—Extracts from Thermonasoeberochroebrochii were prepared according to Maeda et al. (1992). Spinach chloroplasts were isolated from mature leaves and their stromal fraction prepared according to Hii and Mikkeelsen (1982). Bovine, porcine, and rat mitochondrial extracts were prepared and mitochondrial GrpE purified therefrom by chromatography on a DNAK-affinity column as described previously (Naylor et al., 1995). The flow through from the DNAK column was passed directly onto a 3 x 21 cm Q-Sepharose (Pharmacia) anion-exchange column equilibrated in 25 mM Tris-Cl, pH 8.0, and the unbound fraction depleted of mitochondrial Cpn60 served as the starting material for the affinity purification. In some applications, lysates from crude mitochondrial preparations obtained by differential centrifugation only were used with an identical outcome.

GroEL Affinity Chromatography—All extracts were adjusted to contain 0.1 M Tris-Cl, pH 8.0, 10 mM MgCl2, 10 mM KCl, and 1 mM ATP (Buffer A) before application to the GroEL affinity column (1.6 x 8 cm) previously equilibrated in the same buffer. Following loading of the crude extracts, the column was washed with 10 bed volumes of Buffer A followed by 10 bed volumes of Buffer A containing 1 mM NaCl. Cpn10 was then eluted with 0.1 M Tris-Cl, pH 8.0, and located by SDS-PAGE as described previously (Jarvis et al., 1989). Pooled fractions were concentrated by pressure dialysis using a YM10 filter (Amicon) and stored at -70 °C.

The GroEL column can be used repeatedly and when stored in Buffer A at 4 °C it is stable for at least 1 month.

Separation of 10- and 7-kDa Polypeptides by Reverse-phase HPLC
On several occasions, the affinity-purified, mammalian Cpn10 preparations contained both a 7- and a 10-kDa component. To accomplish separation between these 2 components of the porcine 10- and 7-kDa polypeptides were mixed with an equal volume of 6 M guanidine HCl, 1 mM EDTA, 1 mM Tris-Cl, pH 7.6, before application to a C8 reverse phase HPLC column (4.6 x 250 mm; VYDAC, Phenomenex) equilibrated in 0.1% trifluoroacetic acid and operated at a flow rate of 1 ml/min. Following a 10-min wash with 0.1% trifluoroacetic acid, an elution program consisting of a linear gradient of 0-45% (v/v) CH3CN in 0.1% trifluoroacetic acid (70 min) and an isocratic wash for 10 min was performed. The 10-kDa polypeptide eluted at 45% CH3CN and the 7-kDa polypeptide at 42% CH3CN. Eighty µg of the 10-kDa polypeptide was freeze-dried and denatured in 40 µl of 6 M guanidine HCl, 1 mM EDTA, 5 mM dithiothreitol, 0.1 M Tris-Cl, pH 7.6. An aliquot of this sample (40 µg) was diluted 40-fold into 50 mM Tris-Cl, pH 7.6, and concentrated in a microcentrifuge (ULTRAPIRE-MC, Millipore) to a final volume of 40 µl before use in chaperone mediated folding of porcine mitochondrial malate dehydrogenase.

Glutaraldehyde Cross-linking
Aliquots (25 µg) of bovine Cpn10 in 50 mM MOPS-NaOH, pH 8.0, 3.3 mM MgCl2 were incubated with 0.00-0.05% (v/v) glutaraldehyde (Sigma) in a total volume of 15 µl at 25 °C for 30 min. Reactions were stopped by addition of SDS-PAGE loading dye and the products analyzed using Tris-Tricine SDS-PAGE.

Construction of a Rat Cpn10 Expression Vector
A 391-base pair polymerase chain reaction product was obtained by employing Vent™ DNA polymerase (New England Biolabs), an oligonucleotide corresponding to the first 6 translated amino acids of rat Cpn10 (5'-ATGGCTGGACACGGGTCTTTTT-3'), the KS primer (5'-GGAGTGTCGAGCTTGATCGG-3') of pBluescript (Stratagene), and a rat Cpn10 cDNA in pBluescript (SK- ) as template (Ryan et al., 1994). The product was ligated into the SacI site of the isopropyl-1-thio-β-D-galactopyranoside-inducible pKK223-3 vector (Pharmacia) and transformed into E. coli strain MV1190 (Stratagene). Individual colonies carrying the correct constructs were tested for their ability to direct the synthesis of large amounts of rat Cpn10 but without success. The Cpn10 encoding insert was therefore released from the pKK223-3 vector by digestion with restriction enzymes NcoI and EcoRI and joined into the heat shock-inducible vector pRS550 (obtained from Dr. N. Dixon, Australian National University) previously digested with the same endonucleases. Following transformation of MV1190 cells, individual colonies expressing large amounts of rat Cpn10 were selected and designated pRS560-Cpn10.

Purification of Recombinant Cpn10
An overnight culture (20 ml) of pRS550-Cpn10 grown at 30 °C in Luria Broth containing 50 µg/ml ampicillin was added to 1 liter of Luria broth containing 50 µg/ml ampicillin and shaken at 30 °C until OD600 nm was equal to 0.5. At this time the culture was induced to express Cpn10 by raising the temperature to 42 °C. Following 4 h of growth at this temperature, cells were harvested and lysed by lysozyme treatment (Todd et al., 1993) followed by 10 bed volumes of Buffer A containing 1 M NaCl containing 0.1 M Tris-Cl, pH 8.0, at 4 °C. The unbound fraction represented an essentially pure preparation of Cpn10 (50 mg) which was concentrated and stored at -70 °C.

Production of Anti-Cpn10 Polyclonal Antibodies
Either bovine or recombinant Cpn10 was used as antigen. A final concentration of 0.02% (w/v) glutaraldehyde was used to cross-link Cpn10 (125 µg in 75 µl) as described above. Following addition of 425 µl of 50 mM Tris-Cl, pH 7.6, and 500 µl of Freund’s complete adjuvant (Life Technologies, Inc.), the Cpn10 preparation was injected subcutaneously into a 12-week-old White Leghorn chicken. Two further boosts were made at 21-day intervals utilizing the same conditions but in Freund’s incomplete adjuvant. Eggs containing anti-Cpn10 antibodies at a low titer were obtained 6 days after the final boost.

Anti-Cpn10 antibodies were prepared by precipitation of yolk-lipid using 4% (w/v) PEG 6000 in phosphate-buffered saline essentially as described previously (Gasman et al., 1990). The final precipitate containing the purified antibody mixture was resuspended in phosphate-buffered saline containing 0.05% sodium azide and stored at 4 °C. A dilution of 1:2500 was used for immunoblotting experiments.

Preparation of 125I-Cpn10 Species
Both rat Cpn10 and recombinant Cpn10 were denatured and purified separately using reverse-phase HPLC as above. Twenty µg of either material was radio labeled prior to mitochondrial in vitro import assays as described previously (Jarvis et al., 1995).

Degradation Studies of Cpn10 Species
A rat mitochondrial fraction prepared by differential centrifugation (Griffiths, 1989) was resuspended in 25 mM Tris-Cl, pH 7.6, containing 0.5% Lubrol-PX and lysed by five strokes of a hand-held homogenizer. After ultracentrifugation (100,000 x g for 60 min), the supernatant was layered through a 1.6 x 12 cm CM-Sepharose Phenyl column equilibrated in 25 mM Tris-Cl, pH 7.6, in order to deplete the extract of endogenous Cpn10. This material (100 µg of protein) was added to either authentic rat, or rec-Cpn10 (1 µg), both of which had been previously purified by reverse phase HPLC. The protein mixtures, in a final concentration of 2 mM ATP, 5 mM MgCl2, 20 mM Tris-Cl, pH 7.6 (80 µl), were incubated at 37 °C for various times. In some cases, the solutions were pretreated with apyrase (5 units, Sigma), 1 mM NEM, or 5 mM EDTA prior to incubation. Reactions were stopped by addition of SDS-PAGE loading dye followed by electrophoresis on Tris-Tricine gels and subjected to semi-dry Western transfer (Peralta et al., 1993). Immunoblots were probed with anti-Cpn10 antibodies and horseradish peroxidase-coupled anti-chicken antibodies (Sigma) and detected using ECL™ reagents (Amersham).

Miscellaneous
Chaperone-assisted refolding of chemically denatured porcine mitochondrial malate dehydrogenase was carried out at 36 °C as described.

N-terminal Acetylation of Chaperonin 10
finity Chromatography — and fractions obtained following anion-exchange of the appro-
8) The M of bovine Cpn10 was found to be 10,841.90 ± 0.37 by
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10).
ponent but as opposed to the rat preparations, varying
porcine preparations also contained a prominent 10-kDa com-
1993a). The bovine and the
Molecular weight markers (lane 2) and purified GroEL (lane 1) were also analyzed. B, SDS-PAGE analysis of
finity purified T. brokii (lane 2), chloroplast (lane 4), pore
and bovine (lane 10) Cpn10 homologues. Starting materials (lanes 1, 3, 5, and 8) and fractions obtained following anion-exchange of the appropriate extracts were also analyzed (lanes 6 and 9).

previously by Peralta et al. (1994). Amino acid sequencing and ESI-MS were carried out as described previously (Hartman et al., 1992). Protein concentrations were determined spectrophotometrically assuming an extinction coefficient of 0.4 at 280 nm for a 1 mg/ml solution of pure Cpn10 (Ryan et al., 1994) or by using a Coomassie Brilliant Blue binding assay for protein extracts (Sedmak and Grossberg, 1977). Rat liver nuclei were prepared according to Blobel and Potter (1966) and mitochondria according to Hartman et al. (1992). After centrifugation of the post-mitochondrial supernatant (120,000 g for 1 h), the pellet (microsomes) and the supernatant (cytosol) were retained for immunoblot analysis.

RESULTS
Purification of Authentic Cpn10 Homologues by GroEL Affinity Chromatography — It has previously been established that GroEL and various Cpn10 homologues form a complex in the presence of K+ ions and MgATP, a feature which has been exploited in analytical applications (Chandrasekhar et al., 1986; Lubben et al., 1990; Bertsch et al., 1992; Torres-Ruiz and McFadden, 1992; Rospert et al., 1993; Cavanagh and Morton, 1994). In the present work we exploited this feature on a preparative scale to design a rapid procedure (typical duration 2 days) which yields milligram quantities of purified Cpn10 from five different natural sources (Fig. 1, A and B). For example, while Hartman et al. (1992) employed a very cumbersome multistep procedure over 2 weeks to obtain 2 mg of purified rat Cpn10 from about 600 g of rat liver, a yield of 2 mg was obtained from 100 g of rat liver in this procedure. Electrospray mass spectrometry (Fig. 2, G and H) yielded an M of 10,813.2 ± 0.3 in complete agreement with the primary structure determined previously (Hartman et al., 1993a). The bovine and the porcine preparations also contained a prominent 10-kDa component but as opposed to the rat preparations, varying amounts of an additional component with an apparent molecular mass of 7 kDa was often present (Fig. 1B, lanes 7 and 10). The M of bovine Cpn10 was found to be 10,841.90 ± 0.37 by ESI-MS (Fig. 2, E and F). This is identical to that of human Cpn10 (Cavanagh and Morton, 1994) and to that deduced from its cDNA sequence (Pilkington and Walker, 1993) when corrected for the assumed post-translational acetylation of Ala1 known to exist on rat Cpn10 (Hartman et al., 1993a). ESI-MS of the porcine Cpn10 revealed a major component of M, 10857.61 ± 1.03 suggesting this homologue differs from bovine and human Cpn10 by a single amino acid substitution while the difference to the rat homologue most likely involves two substitutions, one of which is replacement of Gly57 for a Ser residue. These data establish the extreme conservation of the primary structure and post-translational modifications of mammalian Cpn10 homologues.

Purification of the GroES homologue from spinach chloroplasts yielded a 21-kDa protein (2 mg from 1.5 kg of spinach leaves, Fig. 1B, lane 4). A mass for chloroplast Cpn21 was not determined; however, automated Edman degradation (Fig. 3) revealed a single unambiguous N-terminal sequence which is similar to but clearly not identical with that deduced from a cDNA clone encoding spinach Cpn21 (Bertsch et al., 1992). Surprisingly, the N-terminal sequence for the Cpn21 isolated in this study exhibits a greater similarity to a partial pea sequence determined directly by Edman degradation (Bertsch et al., 1992). Given the extensive amino acid conservation of Cpn10 between mammalian species, the amino acid difference between these chloroplast Cpn21 species is unexpected and worthy of further investigation. A much less prominent 10-kDa protein which migrated with mammalian Cpn10 on SDS-PAGE co-purified with the 21-kDa chloroplast species. This may represent a genuine chloroplast Cpn10 homologue or alternatively arise from a minor mitochondrial contamination (Burt and Leaver, 1994). To investigate this proposition further, we attempted to sequence the 10-kDa homologue but without success, most likely due to N-terminal acetylation.

The fifth Cpn10 homologue purified using GroEL affinity chromatography was that from T. brokii. The yield (2 mg from 15 g of cells) was comparable to that previously obtained by laborious standard chromatography (Truscott et al., 1994), and ESI-MS established its identity (M, 10,251.74 ± 2.34).

Affinity Purified Cpn10 Homologues Are Active in Mitochondrial Malate Dehydrogenase Folding Assays — In the presence of GroEL and ATP, all five Cpn10 homologues were able to facilitate the folding of denatured porcine mitochondrial malate dehydrogenase (Fig. 4). As previously found (Hartman et al., 1992), mammalian Cpn10 is as active as GroES with GroEL although some variability between preparations was noted. In particular, the presence of the 7-kDa polypeptide in the porcine Cpn10 preparation correlated with a poor degree of porcine mitochondrial malate dehydrogenase refolding. When these preparations were denatured in guanidinium chloride and subjected to reversed phase HPLC, the 10- and 7-kDa polypeptides were readily separated. The 10-kDa component purified in this way appeared to refold spontaneously and exhibited an enhanced performance in the porcine mitochondrial malate dehydrogenase refolding. When these preparations were denatured in guanidinium chloride and subjected to reversed phase HPLC, the 10- and 7-kDa polypeptides were readily separated. The 10-kDa component purified in this way appeared to refold spontaneously and exhibited an enhanced performance in the porcine mitochondrial malate dehydrogenase refolding. When these preparations were denatured in guanidinium chloride and subjected to reversed phase HPLC, the 10- and 7-kDa polypeptides were readily separated. The 10-kDa component purified in this way appeared to refold spontaneously and exhibited an enhanced performance in the porcine mitochondrial malate dehydrogenase refolding.
FIG. 2. Determination of the molecular mass of Cpn10 homologues by ESI-MS. The left-hand side panel shows the raw m/z spectra, and the right-hand side panels show the corresponding transformations onto a real mass scale of the data for the porcine 7-kDa polypeptide (A and B), the porcine 10-kDa polypeptide (C and D), the bovine 7-kDa/10-kDa polypeptide mix (E and F), and a mixture of affinity purified rat Cpn10 and rec-Cpn10 (G and H).

FIG. 3. Amino acid sequence similarity of chloroplast Cpn21 homologues. The N-terminal amino acid sequence obtained from pea (*Pisum vinosum*; Bertsch et al., 1992) and the translation of the first 72 nucleotides encoding the mature portion of spinach (*Spinacea oleracea* (1)) Cpn21 (Bertsch et al., 1992) were compared to the N-terminal amino acid sequence obtained from affinity-purified chloroplast Cpn21 (*Spinacea oleracea* (2); this study). Residues exhibiting positional identity are shaded gray.
radii of the two molecules were identical in vitro.

N-terminal Acetylation of Chaperonin 10

We recently established that acetylation of Ala\(^1\) stabilizes the N-terminal amphiphilic helix in a synthetic peptide representing residues 1–25 of rat Cpn10 and that the mitochondrial targeting signal resides in these residues (Jarvis et al., 1995). Since mitochondrial targeting efficiency correlates well with helical amphiplicity (von Heijne, 1986), the ability of acetylated and non-acetylated Cpn10 to traverse the mitochondria was investigated (Fig. 9). The qualitative results clearly show that acetylation is not a prerequisite for mitochondrial import in vitro.

Most, if not all cellular compartments including mitochondria contain proteases required for protein turnover (Goldberg and John, 1976). We therefore investigated whether the naturally acetylated rat Cpn10 and non-acetylated rec-Cpn10 differed in their susceptibility to proteolytic degradation when added to lysates of crude mitochondria (Fig. 10A). It was repeatedly found that rec-Cpn10 underwent proteolysis with a half-life of about 1 h whereas rat Cpn10 remained intact over an 8-h period under the same conditions. The presence of endogenous proteases present in the rec-Cpn10 preparation only was ruled out, since both rat Cpn10 and rec-Cpn10 were previously denatured and purified by reverse-phase HPLC. Additionally, it was found that rec-Cpn10 was stable when incubated at 37 °C for a number of hours in the absence of acetylation. To elucidate the importance, if any, of this modification, a Cpn10 expression vector was constructed and transformed into E. coli, a host shown not to perform N-terminal acetylation on proteins which undergo this modification in their natural host (Wang and Lee, 1993). Large amounts of soluble, recombinant Cpn10 (rec-Cpn10) were produced. Owing to its high pI (9.65), rec-Cpn10 was easily separated from the majority of E. coli proteins by passage through an anion-exchange column (Fig. 7). From 5 liters of culture, 250 mg of essentially homogeneous rec-Cpn10 was purified within hours.

Both amino acid sequencing and mass spectrometry revealed that rec-Cpn10 lacked N-terminal acetylation, the only respect in which it differed from rat Cpn10 purified by GroEL-affinity chromatography (Fig. 2, G and H).

N-terminal Acetylation Protects Cpn10 against Proteolysis but Is Not Required for Protein Folding and Mitochondrial Import – Despite the lack of acetylation, rec-Cpn10 was just as efficient as native affinity-purified Cpn10 in the refolding of porcine mitochondrial malate dehydrogenase with GroEL and ATP (Fig. 8), a conclusion that previously had been obtained with a His-tagged mouse Cpn10 and a refolded human Cpn10 expressed in E. coli (Dickson et al., 1994; Legname et al., 1995).

After induction, the E. coli cells were lysed and the supernatant (lane 2) was passed through an anion-exchange column. Initial pass-through fractions containing purified rec-Cpn10 were pooled and concentrated (lane 3). Molecular weight markers (lane 4) are indicated.

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E. coli GroEL, other Cpn60 homologues are less promiscuous with respect to the choice of Cpn10 partners. Thus, only yeast, chloroplast, and mammalian Cpn10 species have so far been identified. With respect to the choice of Cpn10 partners, yeast, and thermophilic bacteria. The procedure has previously been applied to extracts from bacteria (Torres-Ruiz and McFadden, 1992) and spinach chloroplasts, and mammalian Cpn10 species have so far been uncharacterized. Import reactions were stopped by the addition of SDS-PAGE sample buffer and analyzed by PhosphorImager analysis (Molecular Dynamics). proteinase K.

While all these homologues are functionally compatible with E. coli GroEL, other Cpn60 homologues are less promiscuous with respect to the choice of Cpn10 partners. Thus, only yeast, chloroplast, and mammalian Cpn10 species have so far been found to satisfy the requirements of mt-Cpn60 albeit to different degrees (Rospert et al., 1993), whereas little is known about the specific requirements of the chloroplast Cpn60 homologues. In this context the general purification procedure presented here may greatly facilitate studies on structure-function relationships of eukaryotic chaperones.

The availability of large amounts of authentic chaperones allows us to characterize its oligomeric state through glutaraldehyde cross-linking and gel filtration chromatography. The cross-linking of bovine Cpn10 with glutaraldehyde clearly established its heptameric nature, a fact that often had been assumed but not demonstrated by a direct means for any Cpn10 homologue. By contrast, the chloroplast homologue appeared not to constitute a heptamer. Previous work had established that the spinach chloroplast Cpn10 homologue Cpn21 is composed of 21-kDa monomers. Each monomer contains a tandem repeat of "traditional" Cpn10 units, and the native molecular mass for the active chaperone was estimated to be 55 kDa as judged by gel filtration (Bertsch et al., 1992). In this study, gel filtration revealed that Cpn21 co-elutes exactly with the 76-kDa bovine Cpn10 heptamer. This suggests that Cpn21 is trimeric or tetrameric in vitro and therefore does not exhibit the 7-fold symmetry observed for Cpn60 homologues. However, like Bertsch et al. (1992), we also observed a 10-kDa protein which copurified with Cpn21. Due to its low abundance we have not been able to characterize this component in detail. It may either constitute a mitochondrial contaminant (Burt and Leaver, 1994) or alternatively represent a genuine chloroplast Cpn10 homologue. Such a homologue may either form a trimeric or tetrameric unit and therefore does not exhibit the 7-fold symmetry observed for Cpn60 homologues. However, like Bertsch et al. (1992), we also observed a 10-kDa protein which copurified with Cpn21.
(Chandrasekhar et al., 1986). Unfortunately individual Cpn21 monomers were not distinguishable, and the question of the oligomeric state of native chloroplast Cpn21 therefore remains unresolved.

Despite the relative ease with which Cpn10 homologues could be purified from natural sources as described above, it was also decided to express rat Cpn10 in E. coli. Due to the very high expression levels and the high pI of Cpn10 (9.65), several hundred milligrams of pure non-acetylated Cpn10 could be obtained within 1 day. The simultaneous availability of acetylated rat Cpn10 and non-acetylated recombinant Cpn10 allowed us to rigorously test the effect of this modification in three different assay conformation.

First, chaperone-mediated refolding of porcine mitochondrial malate dehydrogenase was tested using the two Cpn10 species and GroEL as a co-chaperone. As established earlier, acetylation was not required for chaperone function (Dickson et al., 1994; Legname et al., 1995) and had no effect on the rate of folding under the in vitro conditions employed here.

Second, since we have previously shown that acetylation stabilizes the amphiphilic N-terminal helix which constitutes the mitochondrial targeting signal of rat Cpn10 (Jarvis et al., 1995), the import of rat Cpn10 and rec-Cpn10 into mitochondria was compared. Although a difference in rate of translocation cannot be ruled out, the results clearly show that both molecules are imported into isolated mitochondria in vitro.

Third, the effect of acetylation on protease susceptibility was also assessed. In this case acetylation had a dramatic effect. While rat Cpn10 showed no sign of proteolytic breakdown, rec-Cpn10 was degraded with a half-life of 1 h. The nature of the protease was not established, but preliminary experiments indicated it may be of lysosomal origin and NEM-sensitive. Given these characteristics and the effect of N-terminal modification, amino-peptidases such as Cathepsin J (Nikawa et al., 1992) are likely to be responsible for the rapid turnover of rec-Cpn10. The importance of acetylation for proteolytic resistance has also been established for a number of neuropeptides (Veber and Freidinger, 1985) and more recently during the development of potent inhibitors of herpes simplex virus ribonucleotide reductases (Paradis et al., 1991). If the proteolytic activity detected in this study resides in an extramitochondrial compartment, what is the significance of this finding? A number of recent reports (Cavanagh and Morton, 1994; Quinn et al., 1994) suggest that Cpn10 is identical to EPF. EPF performs an extramitochondrial function as a growth factor and a peptide hormone which is believed to initiate a cascade of events which ultimately results in an immunosuppressive response of benefit to the developing fetus (Morton et al., 1987; Noonan et al., 1979; Athanasas-Platis et al., 1989, 1991). This unexpected and exiting dual role of Cpn10 is far from understood but the findings in this study clearly indicate that the naturally acetylated Cpn10 would have a much longer half-life and therefore greater biological potential than non-acetylated Cpn10 produced by means of recombinant-DNA technology. With the availability of simple procedures to simultaneously obtain large quantities of both acetylated and non-acetylated Cpn10, these and other pertinent questions concerning the mt-Cpn10/EPF system can now be pursued.

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