In vitro dissociation and self-assembly of three chaperonin 60s: the role of ATP

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Abstract A comparative study has investigated the in vitro dissociation and self-assembly of chaperonin 60 14-mers isolated from *E. coli* (GroEL), yeast mitochondria and pea chloroplasts. In all cases Mg^{2+} inhibits, and low temperature stimulates, the urea-induced dissociation. ATP or ADP in the presence of Mg^{2+} enhance the dissociation of the chaperonins. Re-assembly of the 14-mers from their monomers shows different efficiencies between the three proteins. In all cases, however, self-assembly is stimulated by Mg-adenine nucleotides. Surprisingly, effective self-assembly of GroEL is promoted by 20% glycerol in the absence of ATP. The role of Mg-adenine nucleotides in the dissociation and assembly of the chaperonins is discussed.

Key words: Chaperonin; Cpn60; GroEL; Self-assembly; Mg-ATP; Protein folding

1. Introduction

Chaperonins [1] are a class of ubiquitous sequence-related proteins for which an essential role as molecular chaperones [2] has recently been discovered (see [3–7] for reviews). Highly conserved representatives of the chaperonin 60 (cpn60) subfamily (with subunit molecular masses ofabout 60 kDa) are found in eubacteria, mitochondria and chloroplasts. *E. coli* cpn60, the GroEL protein, exists as a large cylindrical oligomer composed of 14 identical monomeric subunits possessing seven-fold rotational symmetry [8,9]. The mitochondrial and chloroplast cpn60s (mit cpn60 and chl cpn60, respectively) show similar tetradecameric architecture [10,11] but the plant protein is unique in that it consists of two types of homologous but non-identical monomers (called α and β polypeptides) [12]. Interaction of cpn60s with their substrates (unfolded proteins) is ATP-dependent and is modulated by smaller chaperonin proteins (also called 'co-chaperonins'). The latter are less conserved and exhibit monomer molecular masses of either about 10 kDa (cpn10; GroES protein in *E. coli*) [13] or 21 kDa (cpn21 of plant chloroplasts) [14].

Since the tetradecameric cage-like structure [15,16] of the chaperonin 60s is conserved and is thought to be indispensable for its action in assisting the folding of many proteins, it is of special interest to understand in more detail how this unique structure is formed. Self-assembly of GroEL [17] as well as symbionin (a cpn60 of an aphid endosymbiont) [18] from their isolated monomers is promoted by Mg-ATP. However, the precise role of Mg-adenine nucleotides in the formation of the quaternary structures of cpn60s is not clear. Moreover, destabilization of the oligomers in the presence of Mg^{2+} and adenine nucleotides has been reported in several cases [12,19–22]. In this paper data are presented showing that both the dissociation and the self-assembly of cpn60s isolated from three different sources (yeast mitochondria, a eubacterium and plant chloroplasts) are favoured by Mg-adenine nucleotides. This observation means that interaction with the nucleotides affects the structures of the oligomers as well as the corresponding monomeric subunits. The chaperonins from diverse sources show markedly different tendencies to dissociate or self-assemble in vitro. However, the specific effects of Mg-nucleotides (as well as urea, low temperature, or Mg^{2+} without the nucleotides) are similar for the three tested proteins, suggesting that these effects could reflect universal features of all homologous chaperonins. The results throw more light upon the understanding of the role of adenine nucleotides in the structural changes in cpn60 molecules.

2. Materials and methods

Intact GroEL (*E. coli* chaperonin 60) as well as its monomers were isolated as described previously [23]. Detailed procedures used to purify the chaperonin 60 proteins from baker's yeast mitochondria (mit cpn60) and pea chloroplasts (chl cpn60) are available on request. Briefly, mitochondria isolated according to a standard procedure from cells of the yeast *Saccharomyces cerevisiae* bearing a multicopy plasmid YEpHSP60 [24] were disrupted by sonication and the lysate was centrifuged on 10–25% sucrose gradients in an SW28 rotor (Beckman) for 18 h at 28,000 rpm. The mit cpn60 containing fractions (identified with specific antiserum) were then chromatographed on a Mono Q HR5/5 column (Pharmacia) developed with 0–0.5 M NaCl gradient in the buffer containing 20 mM Tris-HCl buffer, pH 8.0 and 5 mM β-mercaptoethanol. Finally, the protein was chromatographed on a Superose 6 HR10/30 column (Pharmacia) in buffer containing 40 mM triethanolamine acetate, pH 7.5, 0.1 M potassium acetate, 0.1 mM EDTA and 1 mM DTT (buffer A), concentrated with Centricon 30 (Amicon) and stored at −20°C.

The chloroplast chaperonin 60 [25] was isolated from the total extract of 10-day-old pea plants (*Pisum sativum*). Crude proteins precipitated at 40–70% saturation of ammonium sulphate were separated on a Q Sepharose column (Pharmacia) with a 0–0.5 M NaCl gradient. The chaperonin-containing fractions (recognised by a specific antibody) were then chromatographed successively on Fractogel-DEAE EMD 650S (Merck) and Mono Q (Pharmacia). Finally, the protein was passed through the sizing column, concentrated and stored as described above for mit cpn60. The preparations of the isolated chaperonin 60 proteins were essentially homogenous as judged by SDS-PAGE, and

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Abbreviations: SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel-electrophoresis; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate).

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showed native molecular masses of about 800 kDa and the typical (tetradecameric) electron microscopic appearance (not shown). The concentrations of the proteins were estimated by UV absorbency measurements assuming the adsorption coefficients (A_{280}, 0.1%, 1 cm) 0.161, 0.136 and 0.267 for GroEL, mit cpn60 and chl cpn60, respectively, calculated from the corresponding amino acid sequences according to the method of Gill and von Hippel [26]. Radioactively labelled [^{3}H]GroEL and [^{3}H]chl cpn60 were prepared by in vitro reductive methylation as in [17].

Chromatography on Superose 6 HR10/30 (Pharmacia) was used as described previously [17,23] to estimate the molecular masses of the intact oligomers as well as for the dissociation and the self-assembly experiments presented in Fig. 3 and Table 1. A Zorbax GF-250 sizing column (Du Pont) connected to an HPLC machine (LKB) was used for the rest of the dissociation experiments at the flow rate 1 ml/min, allowing rapid (within 10 min) separation of the 14-meric and monomeric forms of the cpn60s. The buffer contained 25 mM potassium phosphate, pH 7.2, and 100 mM KCl, and UV absorbency was monitored at 228 nm. Size-exclusion chromatography was used in preference to native PAGE because the results can easily be quantitated, and no increase in the protein concentration (which potentially can cause artefacts) occurs at the start of the separation [17]. Native (non-denaturing) PAGE on a 7.5% gel performed as described in [27] was used whenever the chromatographic approach was not applicable.

A microdialysis-based procedure has been developed to study self-assembly of cpn60s using small amounts of the proteins. Samples (10 μl) containing the chaperonins treated with 4 M urea (20 min, 0°C) were placed into the dialysis cells made from cut micropipette tips (Eppendorf) with the membrane held in place with a silicone tubing band. Each micro cell was then subjected to dialysis (2 h at 23°C) against 1 ml of buffer A containing the additives as indicated. Glycerol (20% v/v) was added to the buffer (except where otherwise indicated) to minimise the osmotic dilution of the samples. At the end of the dialysis EDTA (up to 15 mM) was added to stop the effect of Mg-adenine nucleotides [27], and the samples were analysed by non-denaturing PAGE as described above.

3. Results

3.1. Urea-induced dissociation of chaperonin 60s

Fig. 1 shows dissociation of the 14-mers of the three different chaperonin 60s as a function of urea concentration. In the absence of Mg^{2+} 3.5 M urea at 23°C causes complete (or nearly complete) conversion of the oligomers of mitochondrial cpn60 (Fig. 1A) or GroEL (Fig. 1B) into the monomers. A significantly lower concentration of urea is sufficient for complete dissociation of the chloroplast cpn60 (Fig. 1C). The steepness of the dissociation curves – from about 15% to complete monomerization in as small range of the urea concentration as 1 M or smaller (Fig. 1B,C) – and the absence of any intermediates between the native 14-meric and monomeric forms detected during the chromatographic analysis of the samples (not shown), suggest that the dissociation process is highly co-operative. The notably less steep dissociation profile of mit cpn60 (Fig. 1A) indicates that relatively more stable intermediates can exist in the case of this protein. This observation is in agreement with previously published data showing that an homologous cpn60 from mammal mitochondria can exist in vitro as a stable 7-mer [28].

The addition of 10 mM magnesium acetate to the incubation buffer enhances the resistance of all three tested proteins against the urea-induced dissociation (Fig. 1A–C) suggesting an important role of Mg^{2+} in stabilisation of native structure of the chaperonin 60s. A similar effect was recently reported for GroEL [29]. Earlier we demonstrated that low temperature (0°C) favoured the dissociation of GroEL 14-mers, probably by destabilizing hydrophobic interactions important for the

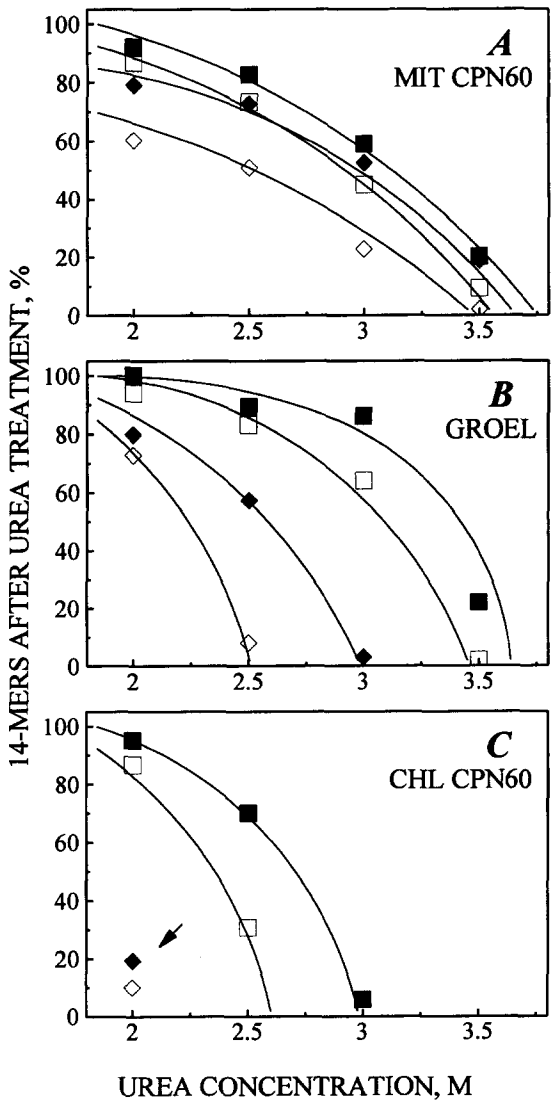

Fig. 1. Urea-induced dissociation of three different chaperonin 60s. Samples (50 μl) containing 1 μg of native mit cpn60 (A), GroEL (B) or chl cpn60 (C) in buffer A (see section 2) were incubated with different concentrations of urea for 10 min at 23°C (□) or 0°C (◇) then chromatographed immediately on a Zorbax GF-250 (Du Pont) column at 23°C in buffer containing 25 mM potassium phosphate (pH 7.0) and 100 mM KCl. The amount of remaining 14-mers was calculated from the UV absorbency (monitored at 228 nm) corresponding to the oligomer peak. The experiments were repeated with 10 mM magnesium acetate added to the incubation buffer either at 23°C (■) or 0°C (♦). An arrow in C indicates 2 M urea treatment at 0°C.

intersubunit contacts or by a possible direct effect of cold on the conformation of the monomers [17,23]. Fig. 1A–C shows that incubation at 0°C enhances the dissociation of all three tested chaperonins. A more pronounced effect of low temperature on the dissociation of GroEL and chl cpn60 compared to that of mit cpn60 correlates with the steepness of their dissociation profiles at 23°C. Similarly to that at 23°C, Mg^{2+} stabilises the native state of all tested chaperonins at 0°C (Fig.1A–C).

3.2. Effects of Mg-adenine nucleotides on the stability of cpn60s

Although a limited ATP-dependent dissociation of GroEL

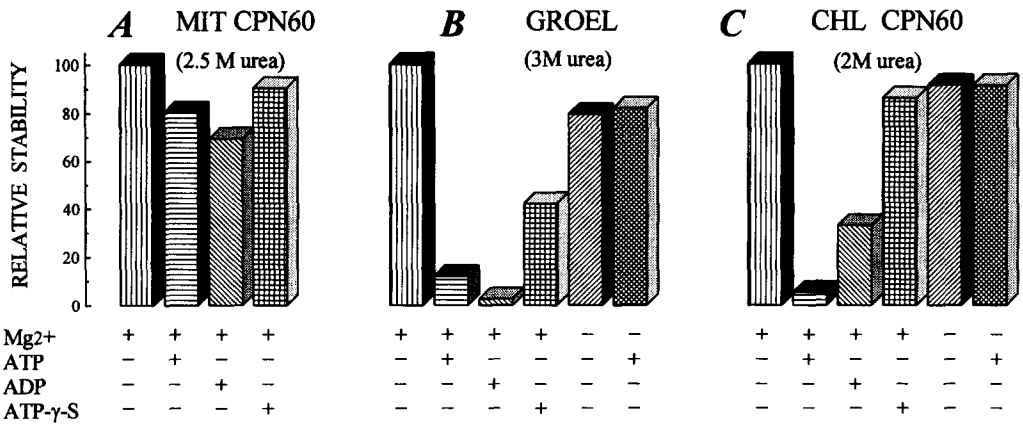

Fig. 2. Destabilization of 14-mers of different chaperonin 60s by Mg-adenine nucleotides. Samples containing native mit cpn60 (A), GroEL (B) or chl cpn60 (C) at 0.025 μM (14-mer) were incubated for 10 min at 23°C in buffer A containing 2.5, 3.0 or 2.0 M urea, respectively. Magnesium acetate (10 mM) and/or different adenine nucleotides (5 mM) were added to the incubation buffer as indicated. The samples were analysed by chromatography on Zorbax GF-250 column (as in Fig. 1) and their relative stabilities (%) were calculated as the amount of 14-mers remaining under particular treatments compared to that in the presence of Mg^{2+} without nucleotides.

has been reported [21], our previous studies showed stability of GroEL 14-mers under the Mg-ATP treatment [17]. On the other hand, a massive dissociation of the cpn60 oligomers caused by Mg-ATP is observed in the stromal extracts of pea chloroplasts [12,19,20]. Recently it has been reported that Mg-adenine nucleotides (ATP or ADP) enhance the dissociation of GroEL 14-mers in the presence of urea [27]. A similar approach was employed in this study to quantitate relative effects of different adenine nucleotides on the three chaperonin proteins. Fig. 2 represents data showing that ATP, ADP or ATP-γ-S in the presence of Mg^{2+} decrease to different extents the resistance of the 14-mers of all tested chaperonins against the urea treatment. (Different concentrations of urea were selected for the three proteins assuming that taken without the nucleotides they would cause dissociation of 5–17% 14-mers, see Fig. 1.) A relatively small effect of the nucleotides is observed in the case of mit cpn60 (Fig. 2A). However, strikingly enhanced dissociation is caused by Mg-ADP and Mg-ATP in the cases of GroEL or chl cpn60 (Fig. 2B,C). Of the three tested nucleotides, ADP is the most powerful agent for mit cpn60 or GroEL, although ATP is much more effective in dissociating the chloroplast protein. In all cases the smallest effects were caused by ATP-γ-S. No effect was caused by ATP if the incubation buffer lacked Mg^{2+} (Fig. 2B,C). Thus, binding of Mg-ADP or Mg-ATP to the chaperonins leads by itself to a specific destabilization of their oligomeric structures. (This effect is opposite to that exhibited by Mg^{2+} in the absence of the nucleotides.) The small effects of Mg-ATP-γ-S compared with those of Mg-ATP indicate that cycles of chaperonin-dependent hydrolysis of ATP might cause further destabilization of the 14-mers. This may be the case of all three tested chaperonins but is particularly evident for chl cpn60, where the difference between the effects of ATP and ATP-γ-S is maximal (Fig. 2C). Interestingly, the higher resistance of mit cpn60 to the effects of the Mg-adenine nucleotide correlates with the less co-operative mode of its dissociation under urea treatment, as well as a smaller effect of low temperature compared to those of GroEL or chl cpn60 (see Fig. 1).

Quantitative effects of 5 mM Mg-ATP on the dissociation of ^{3}H-labelled chl cpn60 were further studied in the absence of urea. Fig. 3 represents data showing that the dissociation rate of chl cpn60 is enhanced by the decrease in the protein concentration within the tested range (0.125–1 μM). This observation is in agreement with earlier published results obtained on chloroplast lysates [19]. A time–course of the dissociation attempted at the initial 14-mer concentration of 0.25 μM (Fig. 3) shows that the process is slow (equilibrium is not reached in 200 min). Incubation at low temperature (0°C) favours the dissociation (Fig. 3, marked with asterisk). Small amounts of monomers (less then 10%) were released upon the prolonged incubation (1000 min) of ^{3}H-labelled GroEL at a 14-mer concentration of 0.25 μM in the presence of 5 mM Mg-ATP both at 23°C and 0°C (not shown).

3.3. *Mg-adenine nucleotide-dependent self-assembly of the cpn60s*

Self-assembly of GroEL 14-mers from the monomers chromatographically purified after the urea treatment is not spontaneous but requires Mg-ATP or (with lower efficiencies) Mg-ADP or Mg-non-hydrolysable ATP analogs [17]. The protein concentration dependence of Mg-ATP-induced dissociation of chl cpn60 (Fig. 3) suggests that the opposite processes of dissociation (first order reaction) and re-assembly of the oligomers (a higher order reaction) compete under these conditions. The effects of Mg^{2+} and the nucleotides on the self-assembly of chaperonin 60s were examined using a dialysis-based approach (see section 2). A small amount of re-assembled oligomers was detected after the attempted dialysis of the urea-dissociated mitochondrial cpn60 at the monomer concentration of 5 μM

Table 1
Effect of 20% glycerol on the self-assembly of GroEL

Mg^{2+}, 10 mM	–	+	+	+	–
ATP, 5 mM	–	–	+	–	–
Glycerol, 20% v/v	–	–	–	+	+
Assembled 14-mers, %	3	12	91	95.5	95

Monomeric GroEL (isolated by Superose 6 chromatography of the 4 M urea-treated protein) was incubated at a concentration of 20 μM in buffer A containing additives as indicated for 2 h at 23°C. The samples were then analysed by chromatography on Superose 6 and the efficiency of self-assembly was estimated from the profiles of UV absorption monitored at 280 nm.

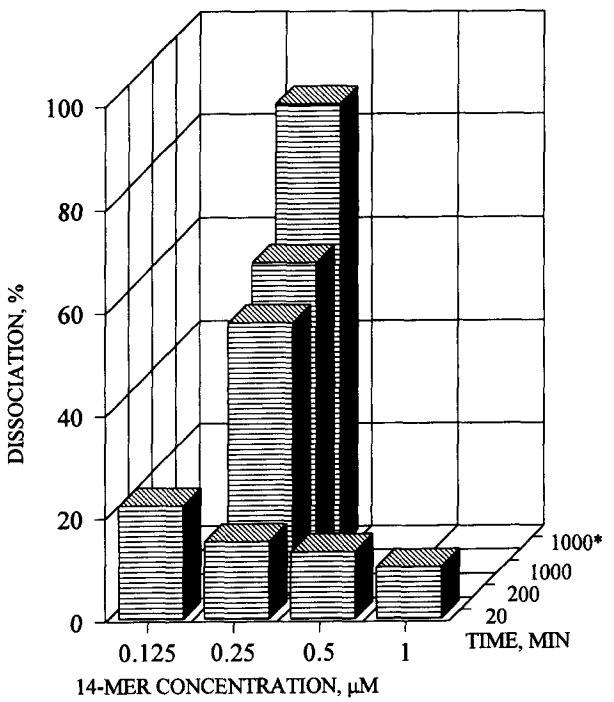

Fig. 3. Mg-ATP dependent dissociation of chl cpn60. Samples containing ^{3}H-labelled chl cpn60 were incubated at 23°C or 0°C (marked with asterisk) in buffer A in the presence of magnesium acetate and ATP (5 mM of each), then analysed by size-exclusion chromatography on Superose 6 (Pharmacia). The amount of dissociated protein was calculated from the comparison of radioactivity found in the monomer peak with the total radioactivity of the sample in each experiment.

against the buffer containing Mg-ATP (not shown). The dialysis of the urea-treated chloroplast protein at a monomer concentration as high as 60 μM against the buffer containing Mg^{2+} and either ATP or ADP resulted in easily detectable levels of self-assembly (Fig. 4). Both Mg-ATP and Mg-ADP were effective, with a higher yield of the 14-mers promoted by the latter. A smaller amount of the re-assembled protein was observed when the monomer concentration was reduced to 30 μM, with no significant 14-mers assembled at 15 μM (not shown).

3.4. *Effect of glycerol on the self-assembly of GroEL*

Surprisingly, essentially complete oligomerization of GroEL was observed after the dialysis of the urea-treated protein against buffer containing 20% glycerol and no nucleotides (not shown). Self-assembly of GroEL 14-mers from the chromatographically purified monomers in the presence of 20% glycerol vs. the Mg-ATP treatment is summarised in Table 1. Either in the presence or absence of Mg^{2+}, glycerol causes an effect which supersedes that of Mg-ATP. The effect seems to be limited to GroEL, since no significant 14-mers assemble under conditions lacking the nucleotides in the case of chl cpn60 (Fig. 4).

4. Discussion

Oligomeric cpn60s are known to be Mg^{2+}-dependent ATPases and *E. coli* GroEL, the best studied member of the chaperonin family, exhibits positive co-operativity in the binding and hydrolysis of ATP [30,31]. It is no surprise, therefore, that the binding of Mg-adenine nucleotides and/or the cycles of ATP hydrolysis can influence the structure of the 14-mers. Reversible ATP hydrolysis-driven transitions between two conformations of the 14-mers have recently been discovered which are believed to be directly connected to the functioning of GroEL [15,27,30,32–35]. Of the two states the 'tense' one (T) is more stable, while the 'relaxed' (R) one possesses higher affinity to the nucleotides and the substrates – non-folded proteins [27]. Since the oligomers of all three proteins tested in this study are destabilized to some extent in the presence of Mg-ATP or Mg-ADP, it is suggested that the nucleotide-dependent transitions between T- and R-like states (from which the latter can more easily dissociate into free monomers) represent a common feature of all homologous chaperonins.

On the other hand, Mg-adenine nucleotides stimulate the in vitro self-assembly of cpn60s from their monomers which are otherwise unable to form 14-mers effectively. The opposite processes of dissociation and assembly thus show the same ligand requirements. A hypothetical two-step mode of oligomerization of various cpn60s in the presence of the nucleotides is shown below.

$$Cpn60_m \xrightleftharpoons{\text{Mg-ATP (ADP)}} Cpn60^*_m \xrightleftharpoons[\text{Mg-ATP (ADP)}]{} Cpn60_{14}$$

The central place in the scheme is occupied by an assembly-competent monomeric state ($cpn60^*_m$). This conformation is a product of an interaction of the monomers ($cpn60_m$) with Mg-ATP and is assumed to be the dominant state of the monomers in vivo. The assembly-competent monomers, in turn, are in equilibrium with the tetradecameric state ($cpn60_{14}$). The proposed role for ATP is thus to facilitate the exchange between the monomeric and tetradecameric states. In contrast to GroEL a significantly higher concentration of the monomers is necessary to shift the equilibrium toward the oligomerization in the case of the chloroplast chaperonin 60. A direct effect of the nucleotides influencing the conformation of monomeric GroEL has recently been reported. Though not capable of oligomerization below 5°C, the nucleotide-stabilised state at 0°C exhibits a Stokes radius similar to that of monomeric GroEL at 23°C, contrary to the unfolded cold-denatured state in the absence of the nucleotides [23]. It is yet not clear what final change in the monomer conformation gives it the ability to form the oligomers.

Unexpectedly, highly efficient self-assembly of GroEL is caused by 20% glycerol in the absence of the nucleotides. Glycerol at concentrations of 10–40% is known to stabilise the native conformation of globular proteins and has been reported to promote self-assembly of monomeric tubulin to form microtubules [36]. A mechanism of the preferential hydration (exclusion of glycerol as poorer solvent from the protein–water interface) has been suggested to explain the effects of glycerol on proteins [37]. Thermodynamically unfavourable, this change in the solvent composition is compensated for by a decrease in the protein–solvent contact area through more tight folding or self-association of the protein molecules. Thus, the glycerol-stimulated self-assembly of GroEL is, evidently, a unidirectional process driven by the stabilisation of the final 14-meric state. A limited stimulation of GroEL self-assembly by magnesium ions (in the absence of the nucleotides) observed at a monomer concentration of 20 μM (Table 1) can also be explained by the fact that the oligomeric state is more stable under these conditions.

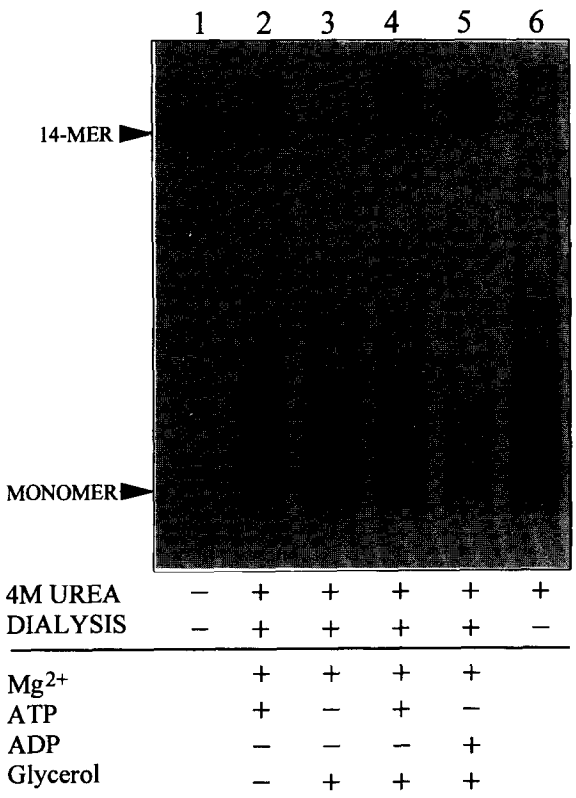

Fig. 4. Mg-adenine nucleotide-dependent self-assembly of chl cpn60. Native chl cpn60 was dissociated (except for sample 1) with 4 M urea (20 min, 0°C) and the aliquots (10 μl) containing the monomers at a concentration of 60 μM were dialysed (except for sample 6) for 2 h at 23°C against 1 ml of buffer A containing 10 mM magnesium acetate and (where indicated) 5 mM ATP or ADP and 20% (v/v) glycerol. The samples were then subjected to non-denaturing PAGE. Samples 1 and 6 (controls) contained equal amounts of native (14-meric) and monomeric chl cpn60, respectively.

Quantitatively the in vitro effects of the particular nucleotides (ADP or ATP-γ-S vs. ATP) on the three tested proteins are different. A much higher efficiency of GroEL self-assembly in the presence of ATP compared to the other nucleotides was earlier interpreted as indicating the existence of an assembly-assisting mechanism based on the hydrolysis of ATP by the assembled 14-mers ('self-chaperoning') [17]. A smaller amount of the oligomers of plant chl cpn60 assembled in the presence of ATP vs. ADP (Fig. 4), correlating with the higher destabilising effect of Mg-ATP on the intact tetradecamers of this protein (Fig. 2). Clearly, the fate of newly synthesised monomers of cpn60s, as well as those released from the dissociating oligomers, can be controlled in vivo by their existing oligomers [17,18,38], co-chaperonins [17,18,27], other molecular chaperones, substrate proteins [27,39], or unknown co-factors. The tetradecameric form, however, must be dominant in vivo not only for GroEL and the mitochondrial cpn60 but also for the particularly unstable plant chl cpn60, taking into consideration the fact that its concentration in the chloroplast stroma is as high as about 10 mg/ml [19,20]. The two types of distinct monomers of the latter protein may play specialised roles in the assembly of the 14-mers [40].

The effects of Mg-adenine nucleotides on both the dissociation and assembly of the chaperonins suggest that their structures in vivo are highly dynamic. One model of GroEL action [39] assumes the possibility of interaction of free monomers with non-folded proteins, followed by the formation of the 7-mers and 14-mers. A chaperone activity of GroEL monomers different from that of the 14-mers has recently been discovered [41]. It is a question for future studies whether the dynamic Mg-ATP-dependent transitions between the monomeric and 14-meric forms can play a particular role in the functioning of the chaperonins.

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