



Reconstitution of Pure Chaperonin Hetero-Oligomer Preparations *in Vitro* by Temperature Modulation

Anna Vitlin Gruber¹, Milena Vugman², Abdussalam Azem² and Celeste E. Weiss^{2*}

¹ Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, Los Angeles, CA, United States, ² Department of Biochemistry and Molecular Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

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*Correspondence:

Celeste E. Weiss
celeste@tauex.tau.ac.il

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Chaperonins are large, essential, oligomers that facilitate protein folding in chloroplasts, mitochondria, and eubacteria. Plant chloroplast chaperonins are comprised of multiple homologous subunits that exhibit unique properties. We previously characterized homogeneous, reconstituted, chloroplast-chaperonin oligomers *in vitro*, each composed of one of three highly homologous beta subunits from *A. thaliana*. In the current work, we describe alpha-type subunits from the same species and investigate their interaction with β subtypes. Neither alpha subunit was capable of forming higher-order oligomers on its own. When combined with β subunits in the presence of Mg-ATP, only the $\alpha 2$ subunit was able to form stable functional hetero-oligomers, which were capable of refolding denatured protein with native chloroplast co-chaperonins. Since β oligomers were able to oligomerize in the absence of α , we sought conditions under which $\alpha\beta$ hetero-oligomers could be produced without contamination of β homo-oligomers. We found that $\beta 2$ subunits are unable to oligomerize at low temperatures and used this property to obtain homogenous preparations of functional $\alpha 2\beta 2$ hetero-oligomers. The results of this study highlight the importance of reaction conditions such as temperature and concentration for the reconstitution of chloroplast chaperonin oligomers *in vitro*.

Keywords: chaperone, chaperonin, chloroplast, *A. thaliana*, oligomer, *in vitro*, temperature

INTRODUCTION

Chaperonins are a subfamily of chaperone proteins found in bacteria and bacteria-derived organelles. In contrast to the well-studied GroEL of *Escherichia coli*, which has one Cpn60 gene product (Johnson et al., 1989), that forms functional homo-oligomers composed of 14 subunits, chloroplasts contain two Cpn60 subtypes, Cpn60 α and Cpn60 β (Musgrove et al., 1987; Martel et al., 1990; Cloney et al., 1992a,b, 1993, 1994; Nishio et al., 1999). These subtypes exhibit ~50% homology to each other, similar to their respective homologies to GroEL, and are each present in two or more paralogous forms in most higher plants (Hemmingsen et al., 1988; Cloney et al., 1994; Hill and Hemmingsen, 2001). These subunits combine to form extremely labile hetero-oligomeric chaperonin species, which dissociate into monomeric form upon dilution, particularly in the presence of ATP (Musgrove et al., 1987; Roy et al., 1988; Lissin, 1995; Viitanen et al., 1998; Dickson et al., 2000; Bonshtien et al., 2009).

Arabidopsis chloroplast contains six Cpn60 homologs: two Cpn60 α subunits and four Cpn60 β subunits (Hill and Hemmingsen, 2001). Unlike Cpn60 β proteins which share a high level of

sequence similarity (Vitlin et al., 2011), significant divergence of primary structure is apparent between the two Cpn60 α paralogs. The two *Arabidopsis* Cpn60 α proteins are similar in length (543 and 541 amino acids) and share 60% identity of peptide sequence (excluding the putative transit peptide). The sequence differences are evenly distributed along the length of the proteins (Hill and Hemmingsen, 2001).

Many species contain orthologs of both Cpn60 α 1 (At5g18820) and Cpn60 α 2 (At2g28000). Several groups characterized knock-out or point mutants of Cpn60 α 2 orthologs, all resulting in severe impairment of plant development (Apuya et al., 2001; Suzuki et al., 2009; Peng et al., 2011; Feiz et al., 2012; Kim et al., 2013; Jiang et al., 2014; Ke et al., 2017). A knockout strain of α 1 was arrested at the globular embryo stage (Ke et al., 2017), while an α 2 knockout was arrested at the heart stage (Apuya et al., 2001). Cpn60 α 1 and Cpn60 α 2 vary greatly in their expression levels. Cpn60 α 2 was shown to be the most highly expressed of the Cpn60 homologs in all tissues and during all developmental stages in comparison with other chaperonins (Weiss et al., 2009). In contrast, Cpn60 α 1 subunit expression is barely detectable at the RNA level (Weiss et al., 2009) although recent studies reported that this protein is highly expressed in the SAM of early seedlings and embryonic cotyledons (Ke et al., 2017).

Several groups have investigated the oligomerization of chloroplast Cpn60 subunits from different plants *in vitro*. Attempts to reconstitute oligomers from purified *P. sativum* α monomers alone were unsuccessful. However, upon addition of β subunits, hetero-oligomers were formed, composed of α and β subunits ($\alpha\beta$ hetero-oligomer) (Dickson et al., 2000). These results were consistent with studies on chaperonins from *Brassica napus* and *C. reinhardtii*, which produced functional $\alpha\beta$ oligomers when over-expressed together in *E. coli* (Cloney et al., 1992a,b; Bai et al., 2015). Similar to GroEL, reconstituted $\alpha\beta$ hetero-oligomers from *P. sativum* could mediate the refolding of denatured substrate when assisted with co-chaperonin from any source: bacteria (GroES), mitochondria (mt-cpn10) or chloroplast (Cpn20) (Dickson et al., 2000), whereas beta homologs were functional *in vitro* with native chloroplast co-chaperonins and with heterologous mt-cpn10 (Dickson et al., 2000; Vitlin et al., 2011).

In this work we used a well-established method for Cpn60 monomer purification and oligomer reconstitution, that was developed in our lab (Vitlin et al., 2011), in order to study both *Arabidopsis* Cpn60 α subunits as monomers, as well as the hetero-oligomers that are formed together with Cpn60 β subunits. We show that the α 2 subunit can form functional oligomers with β subunits, while the α 1 subunit is unable to oligomerize under any conditions that we tested *in vitro*. Since β subunits oligomerize on their own, production of pure $\alpha\beta$ hetero-oligomer is liable to be contaminated by β homo-oligomer. The dependence of reconstitution on temperature and concentration can be manipulated to ensure that the resulting hetero-oligomeric preparations are homogeneous. In this work, we present a method for reconstitution of hetero-oligomers, composed of α 2 and β 2 subunits that are free of contaminating β 2 homo-oligomers.

RESULTS

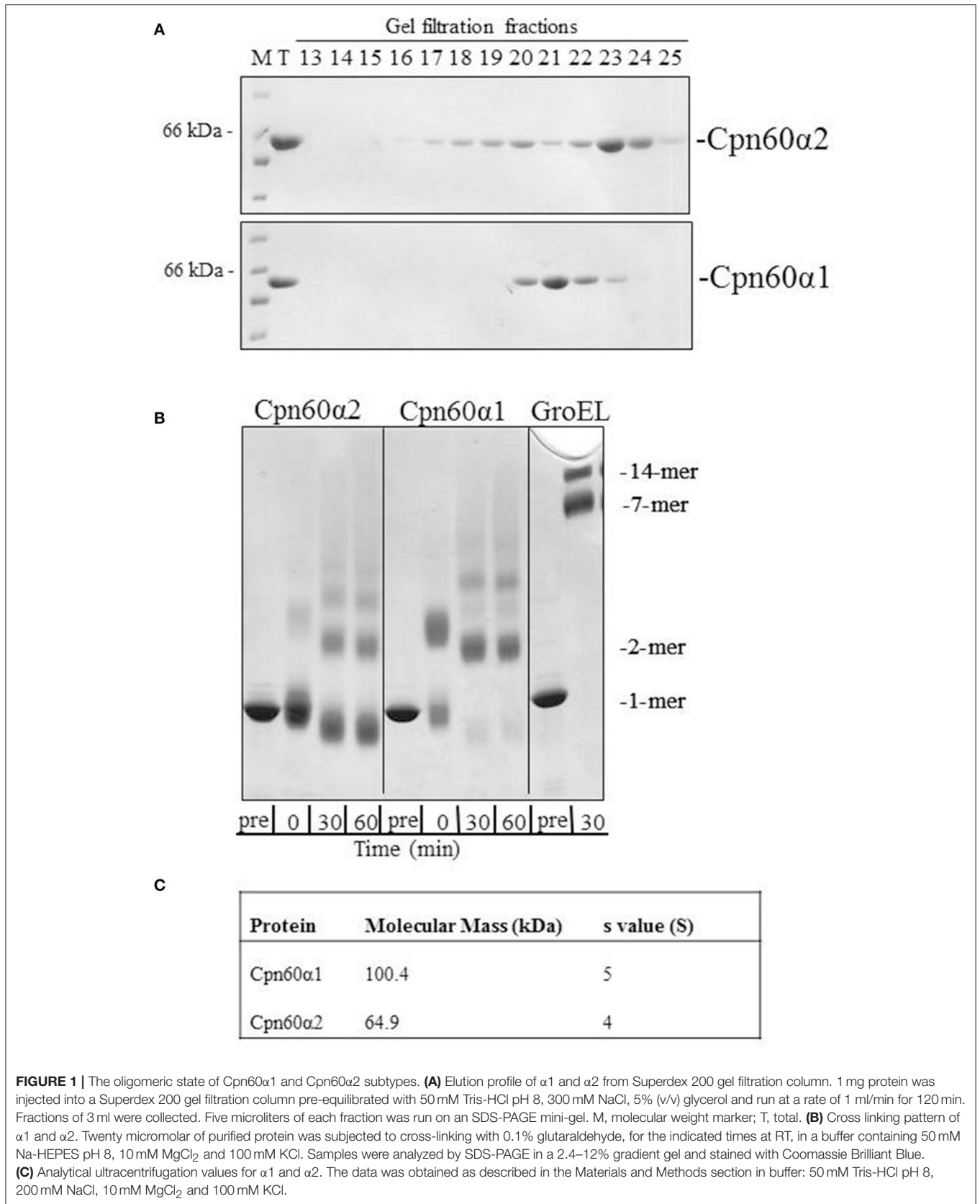
Purification and Structural Characterization of Alpha Subunits

We have cloned and purified both Cpn60 α homologs using a strategy that was developed and described for Cpn60 β subunits (Vitlin et al., 2011). The final step of the purification process hinted at the physico-chemical differences between these two proteins. As can be seen in **Figure 1A** and Figure S1A, α 1 eluted from the gel filtration column earlier than α 2, suggesting that the α 1 form is larger than α 2. In addition, α 1 eluted as a single sharp peak, while the α 2 elution profile displayed several peaks. In order to further investigate these differences, we subjected the proteins to crosslinking with glutaraldehyde, to analyze their oligomeric state. The crosslinking pattern of both subunits exhibited several high molecular-weight bands (**Figure 1B** and Figure S1B). However, one major difference stood out between the α 1 and the α 2 samples: while the main band in the α 2 samples represented the monomeric form, no monomer was observed for α 1, but rather a lower mobility species consistent with that of a dimer.

In order to determine the molecular weight of these proteins, we carried out analytical ultracentrifugation. Two variations of this method, sedimentation velocity and sedimentation equilibrium, were used to analyze the α homologs. Using sedimentation velocity, we found that both subunits were characterized by a single peak, with average sedimentation coefficients of \sim 4S and 5S for α 2 and α 1, respectively. The sedimentation co-efficient of α 2 is similar to the previously published coefficient for GroES (70 kDa), 4S (Seale et al., 1996), indicating that this subunit is mainly monomeric while α 1 is larger and most likely a dimer. Analysis of sedimentation equilibrium (**Figure 1C**) corroborated this observation, with a calculated molecular weight of 100,400 Da for α 1 and of 64,900 Da for α 2. The expected monomer weight for these proteins is \sim 57,000 Dalton. Thus, the results of sedimentation equilibrium indicate that α 1 is best modeled as a dimer and α 2 is primarily monomeric.

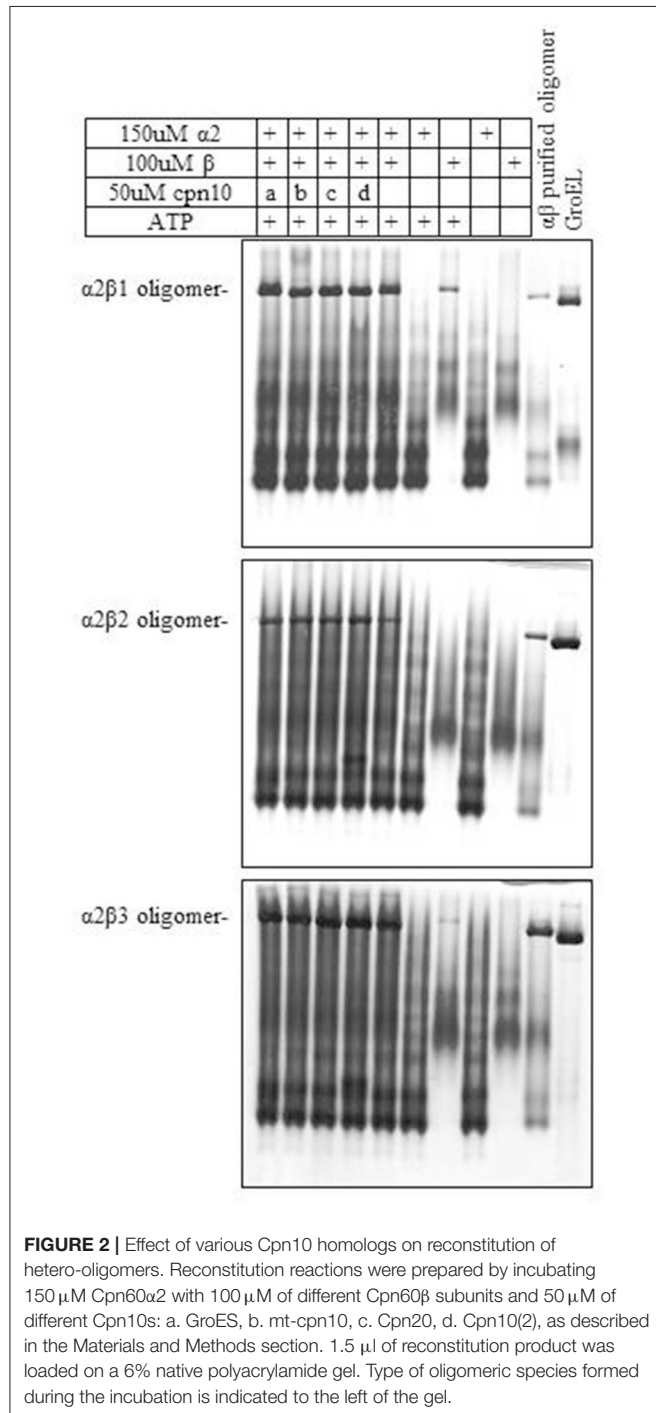
Reconstitution and Functional Characterization of $\alpha\beta$ Hetero-Oligomers

Since their discovery, chaperonin tetradecamers composed of Cpn60 α and Cpn60 β subunits have been considered to be the native form of chaperonin oligomers that are active in chloroplasts (Musgrove et al., 1987), although functional β homo-oligomers were described *in vitro* (Dickson et al., 2000; Vitlin et al., 2011; Bai et al., 2015). To our surprise, attempts to reconstitute hetero-oligomers containing α 1 were not successful. Reconstitution mixtures containing α 1 alone or in combination with any individual β subunit eluted from the gel filtration column as inactive, low molecular weight species (not shown). The fact that no Cpn60 β oligomer was formed in the presence of Cpn60 α 1 was intriguing, since Cpn60 β subunits alone generally tend to form oligomers under the same conditions. This suggests that an interaction is taking place between the monomers but it is not productive in furthering formation of a tetradecamer. A similar phenomenon was described for the α subunit of



Chlamydomonas (Bai et al., 2015), which was incapable of forming mixed oligomers with any individual Cpn60β subunit. Interestingly, in Bai et al. these species composed of one α subunit and one β subunit were still capable of complementing a GroEL deletion strain of *E. coli*.

We next examined the ability of α2 to form mixed oligomers with each of the three β subunits. Initially we followed oligomer formation using native gel electrophoresis. As can be seen in **Figure 2** and Figure S2, α2 does not oligomerize on its own, but is able to form mixed oligomers with β1, β2, or β3 homologs.



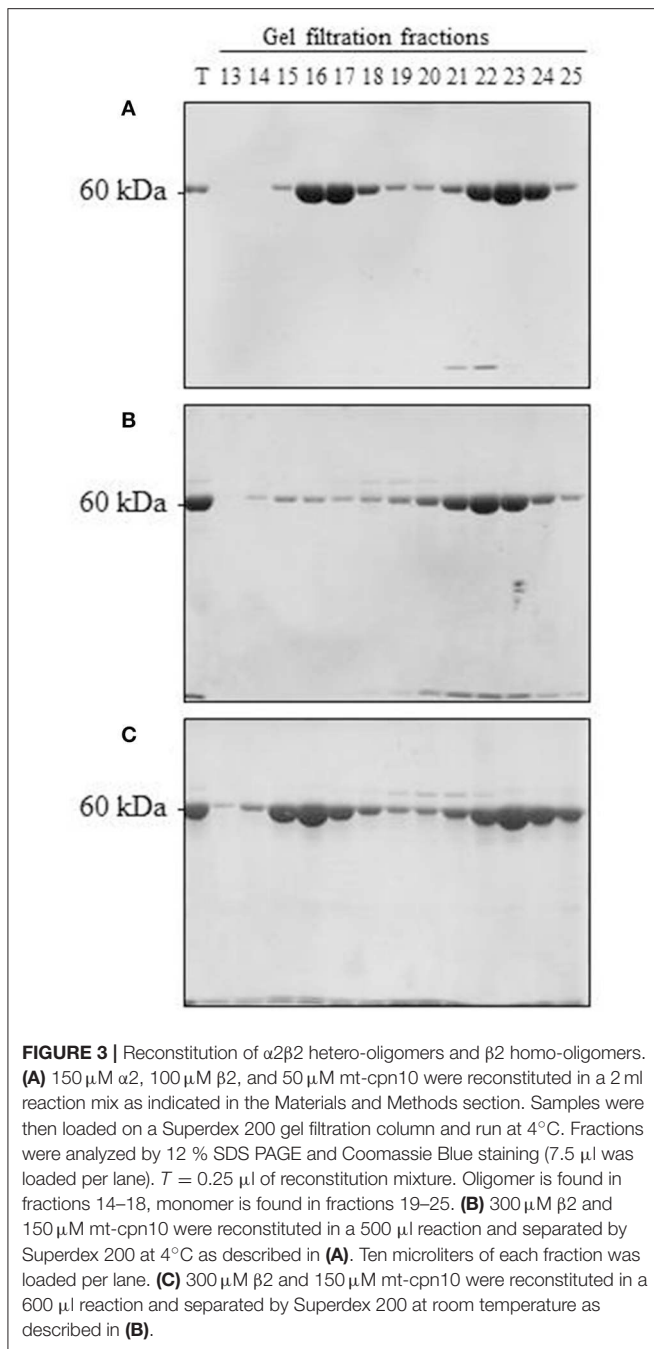
The oligomerization was induced in the presence of Mg²⁺-ATP, however, the presence of different Cpn10s slightly improved the reconstitution efficiency, as was shown previously for Cpn60s from other plant, animal and bacterial sources (Dickson et al., 2000; Bai et al., 2015).

Upon scaling up the oligomerization process, we considered several additional factors. On the one hand, Cpn60α and Cpn60β subunits were shown to be organized in the oligomer in an ~1:1 ratio (Musgrove et al., 1987; Nishio et al., 1999; Dickson et al., 2000). On the other hand, we wanted to ensure that no self-oligomerization of Cpn60β would occur in the reconstitution experiment (when we prepared the mixed Cpn60α2β oligomers). Initially, we were not able to exclude the possibility that a small amount of Cpn60β homo-oligomer was formed during the oligomerization process, together with the hetero-oligomer. The most significant result of this section was the fact that α2β2 hetero-oligomers were found to be stable when separated using gel filtration at 4°C (**Figure 3A** and **Figure S3A**). This is in comparison to β2 homo-oligomers, which dissociate to monomeric form when exposed to the same temperature (**Figure 3B** and **Figure S3B**), yet remain stable at room temperature (**Figure 3C** and **Figure S3C**), as reported in Vitlin et al. (2011). This enabled us to ensure homogeneity of the α2β2 hetero-oligomer preparation. Since α2β2 was the only hetero-oligomer for which we could guarantee a homogeneous preparation, we focused our efforts on α2β2 oligomers and carried out the reconstitution reactions at an excess of Cpn60α2 and at 4°C.

We next examined the chaperonin activity of the α2β2 hetero-oligomers. As demonstrated in **Figure 4** and **Table 1**, the activity of this hetero-oligomer in the presence of chloroplast co-chaperonins [Cpn10(2) and Cpn20] was similar to that of GroEL and reached the maximal yield of ~80%. It can be seen that α2β2 hetero-oligomer was equally functional with both chloroplast co-chaperonins examined and they both had similar effects on the rate ($t_{1/2} = 4-5$ min) as well.

An interesting observation regarding α2β2 hetero-oligomers is the time dependent accumulation of active MDH, observed in the presence of ATP alone without the addition of any co-chaperonin. This is most likely explained by the low stability of the α2β2 oligomer at this concentration, in the presence of destabilizing ATP and the absence of stabilizing co-chaperonin, resulting in dissociation to Cpn60 monomers and release of partly folded MDH, which spontaneously reaches the native folded state as time passes.

We tested the activity of an additional *Arabidopsis* co-chaperonin, Cpn10(1), which was recently characterized (Vitlin Gruber et al., 2014). As can be seen in **Figure 4**, Cpn10(1) alone is not functional with α2β2 hetero-oligomer. This is consistent with the published results with GroEL and α2β3 hetero-oligomer, where Cpn10(1) was shown to be active only as part of hetero-oligomer with Cpn20 (Vitlin Gruber et al., 2014). Although no protein folding activity is observed, Cpn10(1) has some stabilizing effect on the α2β2 oligomeric structure, indicating that an interaction is taking place between chaperonin and co-chaperonin. Cpn10(1) presence in addition to ATP seems to prevent the hetero-oligomer from dissociating to monomers, thus MDH is not released into the solution and spontaneous



folding is not detected as it is in the presence of ATP alone. A similar phenomenon was observed in Bonshtien et al. (2009), where Cpn20 from *Arabidopsis* demonstrated similar binding to β homo-oligomers and $\alpha\beta$ hetero-oligomers from pea, yet was unable to facilitate refolding of substrate protein with the β homo-oligomers.

DISCUSSION

In this study, we have cloned and purified both types of Cpn60 α subunits from *A. thaliana* chloroplast. During the

characterization of these subunits, we showed that $\alpha 1$ forms mainly dimers in solution, while $\alpha 2$ formed several low molecular weight oligomeric forms in solution. Neither of these alpha species showed any ability to refold urea-denatured MDH. Monomeric and dimeric forms of Cpn60 are found in a number of bacterial species. For example, evidence of a low molecular weight Cpn60 protein complex exists in *Mycobacterium tuberculosis*, which crystallizes as a dimer (Qamra et al., 2004; Shahar et al., 2011). However, in contrast to the *Arabidopsis* $\alpha 1$, the protein from *M. tuberculosis* exhibits some protein folding activity *in vitro*, oligomerizes to higher order forms in the presence of ammonium sulfate, KCl and ATP, and can replace GroEL *in vivo*, suggesting that the functional form *in vivo* is an oligomer (Fan et al., 2012). Similarly, in cyanobacteria, the GroEL1 protein seems to form unstable, yet functional tetradecamers, while the GroEL2 protein remains monomeric. Both of these species exhibit a low level of protein-refolding activity, which does not depend upon GroES and ATP (Reviewed in Nakamoto and Kojima, 2017). In general, the chloroplast α and β chaperonin subtypes are both thought to have evolved from bacterial GroEL1.

In all studies of chloroplast chaperonins thus far, homologs of the α subunits were incapable of self-assembly to tetradecamers. The foundation for chaperonin oligomerization was consistently shown to be one or more of the β subunits. For *Chlamydomonas* chaperonins, this ability was determined to lie in residues of the equatorial domain and part of the intermediate domain (Zhang et al., 2016). Likewise, for type II chaperonins, it was demonstrated that only CCT4 and CCT5 out of the eight subunits, were capable of oligomerizing on their own, or facilitating oligomerization of a hetero-oligomer (Sergeeva et al., 2013).

In contrast to $\alpha 1$, $\alpha 2$ monomers easily formed mixed oligomers with all types of β subunits tested. An important achievement of this work was our ability to ensure that reconstituted $\alpha 2\beta 2$ hetero-oligomers were not contaminated by $\beta 2$ homo-oligomers. These pure hetero-tetradecamers were equally and maximally active with authentic *Arabidopsis* chloroplast co-chaperonins: Cpn20 and Cpn10(2). Comparison between the activity pattern of $\alpha 2\beta 2$ hetero-oligomer and the $\beta 2$ homo-oligomer as published in Vitolin et al. (2011), once again assured us that we are dealing with different species with unique patterns of refolding rate and yield. Although Cpn20 could assist both oligomers to reach a maximal yield, Cpn10(2) served as a functional co-chaperonin only with the $\alpha 2\beta 2$ hetero-oligomer and had a very low activity with $\beta 2$ homo-oligomer.

Only a limited number of *in vitro* studies have been carried out on chloroplast chaperonin proteins. Starting with the early studies of Roy et al. (1988), it was consistently demonstrated that oligomerization is a very dynamic process and oligomer stability is highly concentration dependent. For example, pea chaperonin in chloroplast lysate preparations was shown to dissociate in the presence of ATP when the lysate was diluted 15-fold (Roy et al., 1988). Successful reconstitution was in general shown to require relatively high concentrations of the protein. This is consistent with an estimated chloroplast chaperonin concentration of 175 μM protomer (Lorimer, 1996).

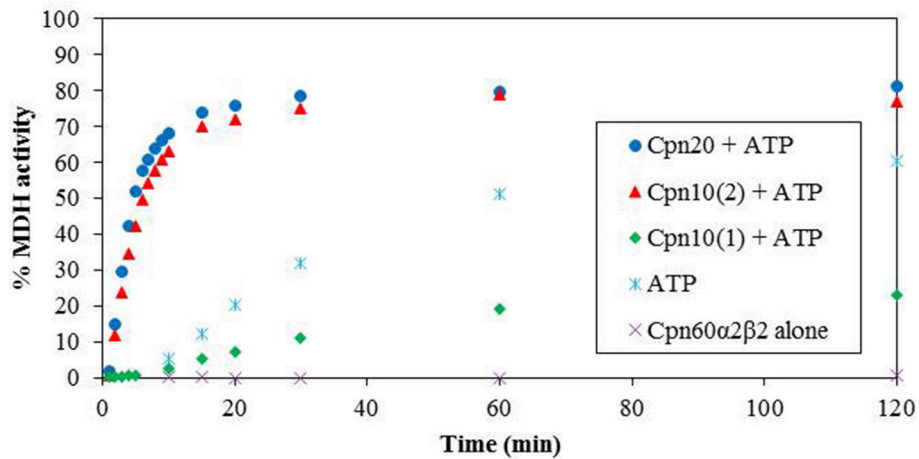


FIGURE 4 | Refolding of denatured MDH by reconstituted $\alpha 2\beta 2$ hetero-oligomers. MDH refolding was carried out by $\alpha 2\beta 2$ hetero-oligomer as described in the Materials and Methods section in 37°C. MDH activity was determined at various time points following the addition of ATP and various Cpn10 homologs. Cpn20: (filled circle), Cpn10(2): (filled triangle), Cpn10(1): (filled diamond), ATP alone: (asterisk), $\alpha 2\beta 2$ hetero-oligomer alone: (multi-sign). One hundred percent was taken as the activity of a sample containing a similar amount of native MDH. Values represent the average of two independent experiments.

TABLE 1 | Rates and yields of MDH refolding by $\alpha\beta$ hetero-oligomers in the presence of chloroplast co-chaperonins*.

		Cpn20	Cpn10(2)
$t_{1/2}$ (min)	$\alpha 2\beta 2$	4	4.5
	GroEL	4.5	5
Final refolding yields (%)	$\alpha 2\beta 2$	79.8	78.8
	GroEL	74.7	74.7

*Data extracted from Figure 4 and average of three experiments with GroEL.

For example, urea-dissociated native pea chloroplast chaperonins were successfully reconstituted at a concentration of 60 μM (Lissin, 1995). Reconstitution of $\alpha\beta$ hetero-oligomers cloned from pea was carried out using 30 μM of each protein (Dickson et al., 2000). While reconstitution of *Arabidopsis* $\beta 1$ and $\beta 3$ homo-oligomer was achieved at over 50 μM protein, $\beta 2$ was able to form oligomers only at concentrations >200 μM (Vitlin et al., 2011), near the estimated *in vivo* concentration. In addition, Bonshtien et al. (2009) showed that ATPase activity of reconstituted pea chaperonins reached a stable rate only at 60 μM monomer, presumably representing the concentration at which equilibrium favored the oligomeric state.

In addition to protein concentration, temperature is another factor that was shown to significantly affect the stability of organellar chaperonins *in vitro*. Dissociation of pea chaperonin in the presence of ATP or urea was potentiated by lower temperatures (Lissin, 1995; Viitanen et al., 1995). Dissociation at cold temperature was used by Dickson et al., to obtain a uniform population of β monomers as starting material for oligomeric reconstitution (Dickson et al., 2000). Our previous results showed that $\beta 2$ oligomers are unable to form at 4°C, although significant oligomerization is observed at 25°C under the same conditions

(Vitlin et al., 2011). This is also consistent with the behavior of mitochondrial chaperonins, which were demonstrated to be highly unstable in the presence of ATP at 4°C, yet were stable at 37°C under the same conditions (Weiss, 1997).

In conclusion, we demonstrate a method for reconstituting pure hetero-oligomeric chaperonin particles *in vitro* that are free from contaminating homo-oligomers. This method takes advantage of the difference in oligomeric stability between $\alpha 2\beta 2$ and $\beta 2$ at 4°C. Our results highlight the complex nature of the chloroplast chaperonin system and emphasize how even the simplest physico-chemical conditions must be taken into account when investigating organellar chaperonins *in vitro*.

MATERIALS AND METHODS

Nomenclature

In this work, we continue with the nomenclature that was established by Hill and Hemmingsen (2001), and which we previously used for *A. thaliana* chloroplast chaperonin subunits (Weiss et al., 2009; Vitlin et al., 2011; Vitlin Gruber et al., 2013a, 2014). It should be noted that different nomenclature is adopted by other groups.

Cpn60 homologs:

- At5g18820 ($\alpha 1$ Cpn60)
- At2g28000 ($\alpha 2$ Cpn60)
- At5g56500 ($\beta 1$ Cpn60)
- At3g13470 ($\beta 2$ Cpn60)
- At1g55490 ($\beta 3$ Cpn60)
- At1g26230 ($\beta 4$ Cpn60)

Cpn10 homologs

- At3g60210 (Cpn10(1))
- At2g44650 (Cpn10(2))
- At5g20720 (Cpn20)

Cloning and Purification of Chaperonin Subunits

Cpn60 α 1 (At5g18820) and Cpn60 α 2 (At2g28000) were cloned between the BamHI–NotI sites of a modified version of pET21d+, which codes for an octa-histidine tag followed by the TEV (Tobacco Etch virus) proteolysis site at the amino terminus of the protein (Opatowsky et al., 2003). The first amino acid of the mature protein was chosen based on presequence predictions (Hill and Hemmingsen, 2001): alanine 33 (α 1) and alanine 46 (α 2). Due to the nature of the cloning, α 1 and α 2 contained an additional glycine-serine at the N-terminus of the protein. The constructs were expressed in *E. coli* Rosetta (Novagen) and purified based on the Cpn60 β purification protocol (Vitlin et al., 2011).

Previously published protocols were used to purify GroES (Bonshtien et al., 2007), Cpn10(1) (Vitlin Gruber et al., 2014), Cpn10(2) (Sharkia et al., 2003), Cpn20 (Bonshtien et al., 2007), mouse mt-cpn10 (Viitanen et al., 1998), Cpn60 β 1/2/3 (Vitlin et al., 2011) and GroEL (Bonshtien et al., 2007).

Reconstitution of $\alpha\beta$ Hetero-Oligomers

The reconstitution protocols were based on Vitlin et al. (2011). In short, the experiments were carried out in 50 mM Tris-HCl pH 8, 0.3 M NaCl, 10 mM MgCl₂, 16 mM KCl, 2 mM dithiothreitol (DTT), 5 mM ATP and different concentrations of Cpn60 and Cpn10 as indicated in the figure legends. The reconstitution mixture was incubated for 5 min at room temperature and then for 1 h at 30°C. For oligomer purification, oligomers and monomers in the reconstitution reaction were separated using a Superdex 200 gel filtration column pre-equilibrated with 50 mM Tris-HCl pH 8, 300 mM NaCl, 5% (v/v) glycerol at 4°C unless stated otherwise. Fractions containing oligomers were pooled, and treated with Ni–NTA-agarose beads in order to remove any traces of his-tagged mt-cpn10 that might have co-purified with the Cpn60. The relevant fractions were concentrated and flash frozen in liquid nitrogen. For

oligomerization tests, reconstitution mixtures were run on native 6% polyacrylamide gels.

Cross-Linking

20 μ M Cpn60 was cross-linked by 0.1% (v/v) glutaraldehyde (GA—Pierce), at room temperature in 50 mM Na-HEPES (Ph = 7.5), 10 mM MgCl₂, 100 mM KCl. The cross-linking reaction was stopped by addition of one-third volume of sample buffer: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% β -mercaptoethanol, 20% glycerol, 1 M urea. Samples were boiled for 5 min prior to electrophoresis in a large 2.4–12% gradient SDS-PAGE.

Analytical Ultracentrifugation

All experiments were carried out as described in Vitlin Gruber et al. (2013b).

In Vitro Refolding of Urea-Denatured MDH

RefoldingA experiments were carried out as described in Vitlin et al. (2011).

AUTHOR CONTRIBUTIONS

AV, MV, CW, and AA: conceived the ideas and designed experiments; AV, MV, and CW: performed experiments; AV, MV, CW, and AA: analyzed data; AV, CW, and AA: contributed toward writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2018.00005/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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