Article

The Hsp60 Protein of Helicobacter Pylori Exhibits Chaperone and ATPase Activities at Elevated Temperatures

Jose A. Mendoza * , **Julian L. Ignacio and Christopher M. Buckley**

Department of Chemistry and Biochemistry, California State University, San Marcos, CA 92096, USA;
ignac010@cougars.csusm.edu (J.L.I.); buckl015@cougars.csusm.edu (C.M.B.)
* Correspondence: jmendoza@csusm.edu

Abstract: The heat-shock protein, Hsp60, is one of the most abundant proteins in *Helicobacter pylori*. Given its sequence homology to the *Escherichia coli* Hsp60 or GroEL, Hsp60 from *H. pylori* would be expected to function as a molecular chaperone in this organism. *H. pylori* is a type of bacteria that grows on the gastric epithelium, where the pH can fluctuate between neutral and 4.5, and the intracellular pH can be as low as 5.0. We previously showed that Hsp60 functions as a chaperone under acidic conditions. However, no reports have been made on the ability of Hsp60 to function as a molecular chaperone under other stressful conditions, such as heat stress or elevated temperatures. We report here that Hsp60 could suppress the heat-induced aggregation of the enzymes rhodanese, malate dehydrogenase, citrate synthase, and lactate dehydrogenase. Moreover, Hsp60 was found to have a potassium and magnesium-dependent ATPase activity that was stimulated at elevated temperatures. Although, Hsp60 was found to bind GTP, the hydrolysis of this nucleotide could not be observed. Our results show that Hsp60 from *H. pylori* can function as a molecular chaperone under conditions of heat stress.

Keywords: Hsp60; molecular chaperone; protein aggregation; heat stress

1. Introduction

Helicobacter pylori is a Gram-negative, microaerophilic bacterium present in the stomach of approximately half of the human population [1]. Chronic infection by this microorganism can, in certain individuals, give rise to gastric and duodenal ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma [2,3]. *H. pylori* survives transient exposure to extreme acid prior to adherence and growth on the gastric epithelium, where the pH can fluctuate between neutral and 4.5, and the intracellular pH can be as low as 5.0 [4,5]. Under neutral and moderately acidic conditions, the heat-shock protein Hsp60 is one of the most abundant proteins in *H. pylori*. [4,6]. Given its sequence homology to the *Escherichia coli* Hsp60 or GroEL [7], Hsp60 from *H. pylori* would be expected to function as a molecular chaperone in this organism. The molecular chaperones are a class of proteins that have been shown to facilitate the folding of nascent polypeptides, activation of other proteins, and protection of native proteins against the effects of heat, and oxidative and acid stress [8–13]. The co-expression of *H. pylori* urease, Hsp60, and Hsp10 in *E. coli* was shown to substantially increase the activity of urease [14], which suggested that urease activity was protected by the heat-shock proteins. Furthermore, we previously demonstrated that Hsp60 from *H. pylori* had a chaperone activity under acidic conditions [15]. No reports, however, have been made on the potential chaperone activity of Hsp60 from *H. pylori* under heat-shock conditions. It is well known that protein denaturation can be induced at elevated temperatures and molecular chaperones, such as Hsp60 may be needed to stabilize them against heat-induced aggregation. This study was performed to determine if Hsp60 could function as a molecular chaperone at elevated temperatures. The enzymes rhodanese, malate dehydrogenase (MDH), citrate synthase (CS), and lactate dehydrogenase (LDH) were used as substrate proteins for Hsp60 given

their propensity to aggregate at elevated temperatures and because they were previously used as protein models to investigate the function of other heat-shock proteins [16–19]. Here, we report that Hsp60 prevented the heat-induced aggregation of these enzymes at elevated temperatures. The aggregation of Hsp60 alone was not observed under these conditions. It is also reported here that Hsp60 was able to hydrolyze ATP in a potassium and magnesium-dependent manner. Furthermore, Hsp60's ATPase activity was found to be highly stimulated at elevated temperatures. Thus, our results show that Hsp60 from *H. pylori* can function as a molecular chaperone with an increased ability to hydrolyze ATP under conditions of heat stress.

2. Materials and Methods

All the reagents used here were of analytical grade. IPTG was purchased from Gold Biotechnology and benzonase nuclease from Novagen. The protease inhibitor cocktail, rhodanese, MDH, CS, and LDH were purchased from Sigma Co. The *H. pylori* Hsp60 gene was synthesized by GenScript (Piscataway, NJ, USA) and inserted into the expression vector pET-22b (+). The resulting plasmid pET-Hsp60 was transformed into competent *E. coli* BL21 (DE3) cells using ampicillin resistance for selection. The expressed protein was purified using His GraviTrap chromatography (GE Healthcare, Chicago, IL, USA) followed by dialysis. Purification of Hsp60 was confirmed by SDS-PAGE [20] and its concentration determined by using a Bradford assay (BioRad, California, CA, USA).

Protein aggregation assay: the aggregation of rhodanese, MDH, CS, and LDH was monitored by using light scattering. Protein (1 μM) samples were incubated in 50 mM Tris-HCl (pH 7.5). Measurement of the light scattering of each enzyme, alone or in the presence of Hsp60 (0.5–2 μM), was made with a Fluoromax-3 spectrophotometer using an excitation wavelength of 350 nm. The light scattered intensity at 90° was recorded at the same wavelength. The appropriate blanks were subtracted.

ATPase activity assay: Hsp60 (1 μM) was incubated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM KCl, 1 mM MgCl$_2$, and 250 μM ATP at 25 °C. Every 5 min, aliquots were removed and the released phosphate was detected using a Malachite Green Assay Kit (Cayman, UK) by measuring the absorbance at 620 nm.

All the experiments were independently performed twice (n = 2), and the results are the average of both. Error bars were used to show the range or variability of the data.

3. Results and Discussion

3.1. Hsp60 Reduces Protein Thermal Aggregation

Given the sequence homology of *H. pylori* Hsp60 to the *E. coli* Hsp60 protein or GroEL [7,21], Hsp60 from *H. pylori* would be expected to function as a molecular chaperone. Here, we tested the ability of Hsp60 from *H. pylori* to prevent the heat-induced aggregation of several heat-sensitive enzymes. Thus, to test the ability of Hsp60 to prevent the aggregation of these enzymes, each enzyme was incubated at 48 °C in the absence or presence of Hsp60, and the samples light scattering was measured after 60 min. As shown in Figure 1, Hsp60 was able to reduce aggregation of the tested enzyme, resulting in decreased scattered light intensities in the case of rhodanese (96.2%), MDH (91.9%), CS (94.2%), and LDH (99.9%). The potential aggregation of Hsp60 alone was monitored by incubating the protein at the same temperature and measuring the sample light scattering with time. No significant changes in light scattering were detected (not shown) that would indicate either Hsp60 aggregation or disassembly by an increase or decrease in the light scattering, respectively.
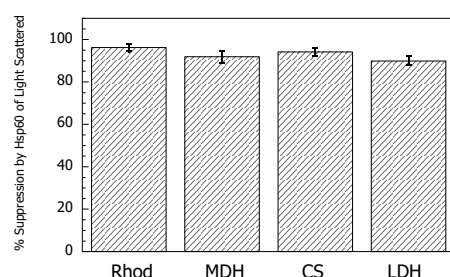
Figure 1. Hsp60 reduced the aggregation of proteins as measured by the reduction in light scattered intensity. Hsp60 (2 μM) was incubated in 50 mM Tris-HCl pH 7.5 at 48 °C for 10 min and then 1 μM of rhodanese or malate dehydrogenase (MDH) or citrate synthase (CS) or lactate dehydrogenase (LDH) was added and the light scattering was recorded after 60 min. n = 2 biologically independent experiments, and the result is the average of both.

Figure 2 shows the time course of the light scattering of MDH (panel A) and CS (panel B) when these enzymes were incubated at 48 °C in the absence or presence of Hsp60. Light scattering was detected to increase within a few minutes after the addition of MDH or CS to the buffer without Hsp60.
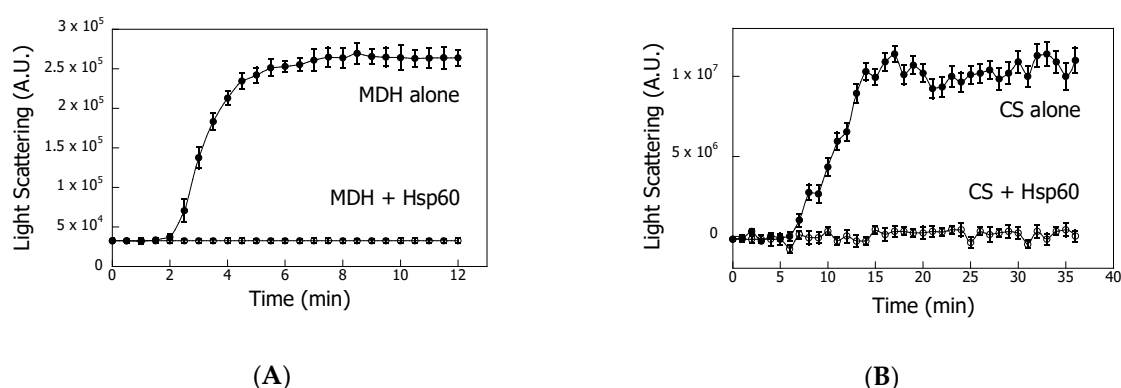

(**A**)   (**B**)

Figure 2. Time course of the light scattering of MDH (**A**) and CS (**B**) without (closed circles) or with Hsp60 (open circles). For samples containing Hsp60, this was incubated at 2 μM in 50 mM Tris-HCl pH 7.5 at 48 °C for 10 min and then MDH (1 μM) or CS (1 μM) was added at t = 0. n = 2 biologically independent experiments, and the result is the average of the two.

As shown in Figure 2 no increase in the intensity of scattered light was observed when MDH or CS were incubated with Hsp60. Thus, these results demonstrate that Hsp60 from *H. pylori* can function as a molecular chaperone at elevated temperature by preventing the heat-induced aggregation of these model proteins. Our findings are consistent with an expected chaperone role for Hsp60 given its high degree of homology to GroEL [7,21]. Given that Hsp60 from *H. pylori* has been reported to exist as a mixture of dimers and tetramers [22], and the tested enzymes were monomeric (rhodanese), dimeric (MDH and CS), and tetrameric (LDH), in these aggregation assays, Hsp60 and enzymes were mixed in a 1:1 tetrameric to monomeric molar ratio.

Figure 3 shows that sub-stoichiometric amounts of Hsp60 did not suppress completely the observed light scattered by rhodanese. The suppression of 42% light scattered by rhodanese was observed with a 1:0.5 tetrameric Hsp60 to rhodanese stoichiometric ratio. As shown in the figure, complete suppression of light scattered by rhodanese in the presence of Hsp60 occurred only with a 1:1 tetrameric Hsp60 to monomeric rhodanese stoichiometric ratio. Our light scattering measurements for Hsp60 could not detect any structural changes at the tested temperatures suggesting that Hsp60 retained its quaternary structure during these experiments. Functional lower oligomers have been reported for the Hsp60 protein from *M. tuberculosis* [23]. The unusual quaternary structure of *H. pylori* Hsp60 raises the question if, under in vivo or certain in vitro conditions, Hsp60 could

undergo oligomerization like the Hsp60 from *M. tuberculosis* into a double ring structure like that of GroEL [24]. Whereas, in the experiments reported here, Hsp60 by itself was an effective chaperone, it has been shown that the folding process of newly synthesized proteins in *E. coli* involves several molecular chaperones [8]. Thus, it remains to be seen whether Hsp60 from *H. pylori* can support the folding of other proteins and interact with other chaperones. Moreover, the nature of the Hsp60-protein complexes detected here and the mechanism of release of the bound proteins remains to be elucidated.
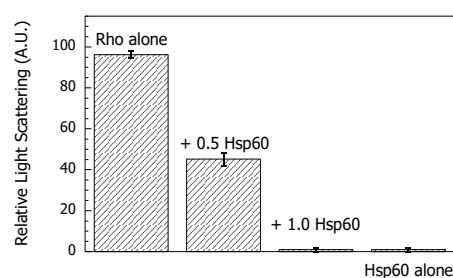

Figure 3. Effect of Hsp60 on the light scattered by rhodanese. Rhodanese (1 μM) was incubated without Hsp60 or with Hsp60 (0.5 μM) or with Hsp60 (1 μM) in Tris-HCl, pH 7.5 at 48 °C and then the light scattering was recorded after 60 min. n = 2 biologically independent experiments, and the result is the average of the two.

3.2. ATPase Activity of Hsp60

Given the ability of *H. pylori* Hsp60 to function as a molecular chaperone, it would be expected to have an ATPase activity like its homologue the *E. coli* Hsp60 protein or GroEL [25]. It has been reported that both potassium and magnesium are required for the ATPase activity of the *E. coli* Hsp60 or GroEL protein [26]. Potassium appeared to increase the affinity of GroEL for ATP, while magnesium is required since the magnesium-bound form of the nucleotide binds GroEL. Thus, the ability of Hsp60 to hydrolyze ATP was tested in the absence or presence of potassium and/or magnesium. The results in Figure 4 show that potassium and magnesium are required by Hsp60 to hydrolyze ATP. We also examined the potential GTPase activity of Hsp60. However, as shown in Figure 4, the hydrolysis of GTP by Hsp60 from *H. pylori* was not observed in the presence of potassium and/or magnesium.
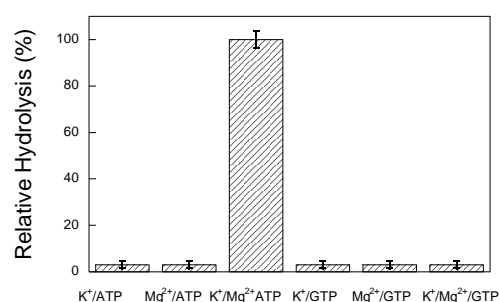

Figure 4. ATP or GTP hydrolysis by Hsp60. Hsp60 (1 μM) was incubated with 50 mM Tris-HCL, pH 7.5 at 25 °C in the presence of 1 mM KCl or 1 mM $MgCl_2$ or both and 250 μM ATP, and in the presence of 1 mM potassium or 1 mM magnesium or both and 250 μM GTP. Then, hydrolysis of ATP or GTP was determined, as described in Materials and Methods. n = 2 biologically independent experiments, and the result is the average of the two.

3.3. Binding of GTP to Hsp60

To determine if GTP was not hydrolyzed by Hsp60 because of the protein's inability to bind the nucleotide, we used the fluorescent nucleotide analog TNP-GTP [27]. An increase in the fluorescence of TNP-GTP, in the presence of a protein, is considered a reliable test for the assessment of the nucleotide-binding capacity of the protein [28]. Figure 5 shows

that Hsp60 had a significant effect on the fluorescence spectrum of TNP-GTP, suggesting that Hsp60 has a GTP-binding site. The significance of the binding of GTP to Hsp60 in the function and structure of the chaperone remains to be determined.
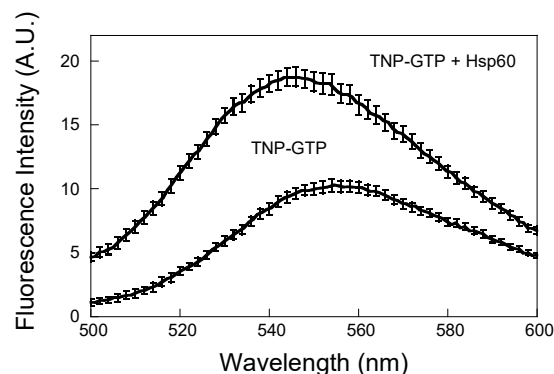

Figure 5. Binding of GTP to Hsp60 analyzed by fluorescence spectroscopy. The fluorescence of TNP-GTP at 50 μM in 600 μL of 50 mM tris buffer, pH 7.8, with and without Hsp60 was measured using an excitation wavelength of 409 nm and its emission recorded from 500 to 600 nm. n = 2 biologically independent experiments, and the result is the average of the two.

3.4. Temperature Dependence of the ATPase Activity of Hsp60

Given that the ATPase of GroEL was previously shown to be stimulated at elevated temperatures [29,30], we investigated the ability of Hsp60 from *H. pylori* to hydrolyze ATP at elevated temperatures. Figure 6 shows the ATPase activities of Hsp60 in the 22–67 °C range in the presence of potassium and magnesium. As shown in the figure, maximum ATPase activity was observed at 62 °C. Thus, it would be expected that the ATPase of Hsp60 from *H. pylori* observed here in that temperature range, would be needed to trigger the release of bound proteins from Hsp60, like in GroEL-mediated protein folding [8]. However, this question awaits further experimentation.
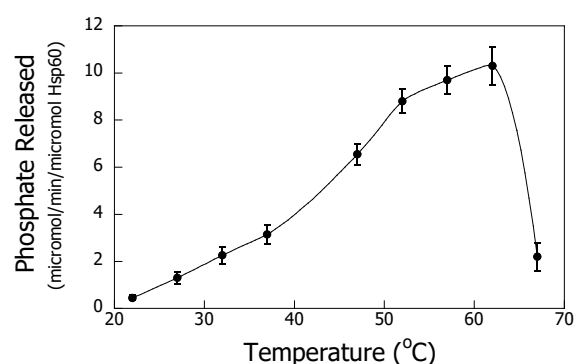

Figure 6. Temperature dependence of the ATPase activity of Hsp60 in the presence of potassium and magnesium in the 22–67 °C range. Hsp60 (1 μM) was incubated at the indicated temperature with 50 mM Tris-HCl, pH 7.5, 1 mM KCl, 1 mM MgCl$_2$, and 250 μM ATP, and the ATPase activity was determined as described in Materials and Methods. n = 2 biologically independent experiments, and the result is the average of the two.

4. Conclusions

Our results demonstrate that Hsp60 from *H. pylori* can function as a molecular chaperone at heat-shock temperatures. Given that that Hsp60 from *H. pylori* contains a significant exposure of hydrophobic surfaces [15] and that these are also present in proteins subjected to elevated temperatures, our results suggest that the interaction between Hsp60 and other proteins undergoing heat-induced denaturation may be mediated by hydrophobic interactions. The ability of Hsp60 to protect a protein exposed to elevated temperatures

is significant in light of the fact that under in vivo conditions of heat shock, like those in which the synthesis of the Hsp60 proteins is stimulated, partially thermally-denatured proteins may actually exist.

References

1. Covacci, A.; Telford, G.; Del Giudice, J.; Parsonnet, J.; Rappuoli, R. Helicobacter pylori virulence and genetic geography. *Science* **1999**, *284*, 1328–1333. [CrossRef] [PubMed]
2. Kusters, J.G.; van Vliet, A.H.; Kuipers, E.J. Pathogenesis of Helicobacter pylori infection. *Clin. Microbiol. Rev.* **2006**, *19*, 449–490. [CrossRef]
3. Ferreira, A.C.; Isomoto, H.; Moriyama, M.; Fujioka, T.; Machado, J.C.; Yamaoka, Y. Helicobacter and Gastric Malignancies. *Helicobacter* **2008**, *13*, 28–34. [CrossRef] [PubMed]
4. Slonczewski, J.L.; McGee, D.J.; Phillips, J.; Kirkpatrick, C.; Mobley, H.L.T. pH-Dependent Protein Profiles of Helicobacter pylori Analyzed by Two-Dimensional Gels. *Helicobacter* **2000**, *5*, 240–247. [CrossRef] [PubMed]
5. Sachs, G.; Weeks, D.L.; Wen, Y.; Marcus, E.A.; Scott, D.R. Acid acclimation by Helicobacter pylori. *Physiology* **2005**, *20*, 429–438. [CrossRef]
6. Jungblut, P.R.; Bumann, D.; Haas, G.; Zimny-Arndt, U.; Holland, P.; Lamer, S.; Siejak, F.; Aebischer, A.; Meyer, T.F. Comparative proteome analysis of Helicobacter pylori. *Mol. Microbiol.* **2000**, *36*, 710–725. [CrossRef] [PubMed]
7. Macchia, G.; Massone, A.; Burroni, D.; Covacci, A.; Censini, S.; Rappuoli, R. The Hsp60 protein of Helicobacter pylori: Structure and immune response in patients with gastroduodenal diseases. *Mol. Microbiol.* **1993**, *9*, 645–652. [CrossRef]
8. Young, J.C.; Agashe, V.R.; Siegers, K.; Hart, F.U. Pathways of chaperone-mediated protein folding in the cytosol. *Nature Reviews Mol. Cell Biol.* **2004**, *5*, 781–791. [CrossRef]
9. Vabulas, R.M.; Raychaudhuri, S.; Hayer-Hartl, M.; Hartl, F.U. Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a004390. [CrossRef]
10. Voth, W.; Jakob, U. Stress-activated chaperones: A first line of defense. *Trends Biochem. Sci.* **2017**, *42*, 899–913. [CrossRef]
11. Muga, A.; Moro, F. Thermal Adaptation of Heat Shock Proteins. *Curr. Protein Pept. Sci.* **2008**, *9*, 552–566. [CrossRef] [PubMed]
12. Kumsta, C.; Jakob, U. Redox-Regulated Chaperones. *Biochemistry* **2009**, *48*, 4666–4676. [CrossRef]
13. Hong, W.; Wu, Y.E.; Fu, X.; Chang, Z. Chaperone-dependent mechanisms for acid resistance in enteric bacteria. *Trends Microbiol.* **2012**, *20*, 328–335. [CrossRef] [PubMed]
14. Suerbaum, S.; Thiberge, J.M.; Kansau, I.; Ferrero, R.L.; Labigne, A. Helicobacter pylori hspA-hspB heat-shock gene cluster: Nucleotide sequence, expression, putative function and immunogenicity. *Mol. Microbiol.* **1994**, *14*, 959–974. [CrossRef] [PubMed]
15. Mendoza, J.A.; Weinberger, K.K.; Swan, M.J. The Hsp60 protein of helicobacter pylori displays chaperone activity under acidic conditions. *Biochem. Biophys. Rep.* **2017**, *9*, 95–99. [CrossRef] [PubMed]
16. Mendoza, J.A.; Lorimer, G.H.; Horowitz, P.M. Chaperonin cpn60 from Escherichia coli protects the mitochondrial enzyme rhodanese against heat inactivation and supports folding at elevated temperatures. *J. Biol. Chem.* **1992**, *267*, 17631–17634. [CrossRef]
17. Hartman, D.J.; Surin, B.P.; Dixon, N.E.; Hoogenraad, N.J.; Høj, P.B. Substoichiometric amounts of the molecular chaperones GroEL and GroES prevent thermal denaturation and aggregation of mammalian mitochondrial malate dehydrogenase in vitro. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2276–2280. [CrossRef]
18. Jakob, U.; Lilie, H.; Meyer, I.; Buchner, J. Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase: Implications for heat shock *in vivo*. *J. Biol. Chem.* **1995**, *270*, 7288–7294. [CrossRef]
19. Delmas, F.; Pierre, F.; Coucheney, F.; Divies, C.; Guzzo, J. Biochemical and physiological studies of the small heat shock protein Lo18 from the lactic acid bacterium *Oenococcus oeni*. *J. Mol. Microbiol. Biotechnol.* **2001**, *3*, 601–610.
20. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef]
21. Brocchieri, L.; Karlin, S. Conservation among HSP60 sequences in relation to structure, function, and evolution. *Protein Sci.* **2000**, *9*, 476–486. [CrossRef] [PubMed]

22. Lin, C.Y.; Huang, Y.S.; Li, C.H.; Hsieh, Y.T.; Tsai, N.M.; He, P.J.; Hsu, W.T.; Yeh, Y.C.; Chiang, F.H.; Wu, M.S.; et al. Characterizing the polymeric status of Helicobacter pylori heat shock protein 60. *Biochem. Biophys. Res. Commun.* **2009**, *388*, 283–289. [CrossRef]

23. Qamra, R.; Srinivas, V.; Mande, S.C. Mycobacterium tuberculosis GroEL homologues unusually exist as lower oligomers and retain the ability to suppress aggregation of substrate proteins. *Mol. Biol.* **2004**, *342*, 605–617. [CrossRef] [PubMed]

24. Braig, K.; Otwinowski, Z.; Hegde, R.; Boisvert, D.C.; Joachimiak, A.; Horwich, A.L.; Sigler, P.B. The crystal structure of the bacterial chaperonin GroEL at 2.8 A. *Nature* **1994**, *371*, 578–586. [CrossRef] [PubMed]

25. Gray, T.E.; Fersht, A.R. Cooperativity in ATP hydrolysis by GroEL is increased by GroES. *FEBS Lett.* **1991**, *292*, 254–258.

26. Todd, M.J.; Viitanen, P.V.; Lorimer, G.H. Hydrolysis of adenosine 5′-triphosphate by *Escherichia coli* GroEL: Effects of GroES and potassium ion. *Biochemistry* **1993**, *32*, 8560–8567. [CrossRef]

27. Hiratsuka, T. Chromophoric and fluorescent analog of GTP, 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene)-GTP, as a spectroscopic probe for the GTP inhibitory site of liver glutamate dehydrogenase. *J. Biol. Chem.* **1985**, *260*, 4784–4790. [CrossRef]

28. Hiratsuka, T. Fluorescent and colored trinitrophenylated analogs of ATP and GTP. *Eur. J. Biochem.* **2003**, *270*, 3479–3485. [CrossRef]

29. Mendoza, J.A.; Warren, T.; Dulin, P. The ATPase activity of chaperonin GroEL is highly stimulated at elevated temperatures. *Biochem. Biophys. Res. Commun.* **1996**, *229*, 271–274. [CrossRef]

30. Melkani, G.C.; Zardeneta, G.; Mendoza, J.A. The ATPase activity of GroEL is supported at high temperatures by divalent cations that stabilize its structure. *Biometals* **2003**, *16*, 479–484. [CrossRef]