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Continuous growth and heat shock of thermoacidophilic Sulfolobus in a triple-stage chemostat for overexpression and isolation of chaperonin

Kurtz Seipel
University of Iowa

CONTINUOUS GROWTH AND HEAT SHOCK OF THERMOACIDOPHILIC *SULFOLOBUS* IN A TRIPLE-STAGE CHEMOSTAT FOR OVEREXPRESSION AND ISOLATION OF CHAPERONIN

by

Kurtz Seipel

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Thesis Supervisor: Professor Tonya L. Peeples

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

Kurtz Seipel

has been approved by the Examining Committee
for the thesis requirement for the Master of Science
degree in Chemical and Biochemical Engineering at the May 2012 graduation.

Thesis Committee: _____
Tonya L. Peeples, Thesis Supervisor

_____
Gary Aurand

_____
Mani Subramanian

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LIST OF ABBREVIATIONS

ABS.....acrylonitrile butadiene styrene, a plastic
Abs### .....optical absorbance,
of indicated wavelength of light in nanometers
ACS.....American Chemical Society
ADH.....alcohol dehydrogenase enzyme
ATP.....adenosine triphosphate,
a biological “energy transfer” molecule
BR .....biomass reservoir
BSA.....bovine serum albumen protein,
often used as a “generic” protein, or as a standard reference
cpn.....chaperonin protein
Da.....Dalton, equal to the unified atomic mass unit,
a common mass unit used in biology
DEAE.....diethylaminoethyl,
a constituent of many chromatography resins
DTT.....dithiothreitol, or Cleland’s reagent,
often used to reduce disulfide bonds in proteins
EDTA.....ethylenediamine tetraacetic acid,
often used to chelate metal ions
epi tube.....Eppendorf tube, a small 1-2 ml plastic capped vessel
G, as in ×G RCF.....gravity, as a unit of force
GR.....growth reactor
GroEL, GroES.....chaperonin and related cofactor, of *E. coli*
HEPES .....(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid),
a common pH buffering agent in biology
HS_1.....heat shock reactor #1
HS_2.....heat shock reactor #2
HSC.....heat shocking chemostat
hsp.....heat shock protein
Hsp###.....a particular heat shock protein or family,
named with its molecular weight, in kDa
ID .....internal diameter
IEC.....ion exchange chromatography
ME.....malic enzyme
Mol Bio.....molecular biology (grade)
MR .....medium reservoir
NAD, NADP.....forms of nicotinamide adenine dinucleotide,
a common biological redox reaction cofactor
NBT/BCIP.....nitroblue tetrazolium, and
5-Bromo-4-chloro-3-indolyl phosphate,
dyes for colorimetric detection of
alkaline phosphatase linked antibodies

OD.....outside diameter
PAGE.....polyacrylamide gel electrophoresis
PES.....polyethersulfone,
a common plastic constituent of filters
PID.....proportional, integral and derivative,
a process control algorithm
PMSF.....phenylmethylsulfonylflouride
PTFE.....polytetrafluoroethylene,
a plastic also known by brand name Teflon
PVDF.....polyvinylidene fluoride, a plastic
RCF.....relative centrifugal force
rRNA.....ribosomal ribonucleic acid
SDS, also as in SDS-PAGE...sodium dodecyl sulfate
SEC.....size exclusion chromatography
Ssocpn.....chaperonin of *S. solfataricus*
TBSTA.....Tris buffered saline with Tween-20 and sodium azide
tris.....tris(hydroxymethyl)aminomethane,
a common pH-buffering agent in biology
USP/FCC.....United States Pharmacopeia Food Chemicals Codex(grade)

CHAPTER 1 BACKGROUND: *SULFOLOBUS* CHAPERONIN IS A HEAT SHOCK PROTEIN AND MOLECULAR CHAPERONE

The thermoacidophilic archaeal genus *Sulfolobus* expresses much chaperonin (cpn) as a heat shock protein (hsp) during above-optimal temperature stress. Acting as molecular chaperone, cpn stabilizes thermally stressed proteins and contributes to increased viability at otherwise lethal temperatures. Cpns are highly conserved proteins (Gupta, 1990) essential for life (archaeal *Haloferax volcanii*, Kapatai et al., 2006; eubacterial *Escherichia coli*, Fayet, Ziegelhoffer, & Georgopoulos, 1989; eukaryal *Caenorhabditis elegans*, Lundin, Srayko, Hyman, & Leroux, 2008), expressed constitutively and nearly ubiquitously throughout the entire tree of life, apparently missing only in the minimal genomes of some genus *Mycoplasma* eubacteria (Glass et al., 2000; Minion et al., 2004). Phylogenetic trees have been constructed to show relationships between cpn of archaea (Large & Lund, 2009; Kagawa et al., 2003) and between representatives of all three domains of life (Kagawa et al., 1995; Ellis, 1996). The properties of ubiquity, high conservation and essentialness make study of this molecule crucial to biology. Cpn is a large, 1 MDa 18-mer forming two stacked rings describing an inner cavity (Braig et al., 1994), access to which is controlled on both ends by ATP-ase driven movements of protrusions functioning as built-in valves (Pereira et al., 2010). Currently, the primary thrust of biological interest in cpn during this genomic age concerns how this molecule assists protein folding and mediates the final stage of information flow from DNA to folded, functional protein.

The cpn of *Sulfolobus* is particularly interesting. The vital physiological importance of this protein in this genus is reflected in its extreme abundance, and in some species, in its overwhelming predominance of hsp expression and consequent accumulation to high levels. The great dependence of this higly successful genus on this

single protein makes it an excellent cpn model for study, the understanding of which is of great consequence to thermophily and proteomics. Moreover, understandings of *Sulfolobus* physiology must accommodate additional roles posited in *Sulfolobus*, roles related to seemingly disparate functions or structures relating to the cytoskeleton (Trent, Kagawa, Yaoi, Olle, & Zaluzec, 1997, Yaoi, Kagawa, & Trent, 1998, Trent, Kagawa, & Yaoi, 1998), RNA metabolism (Ruggero, Ciammaruconi, & Londei, 1998), protein metabolism (Condo, Ruggero, Reinhardt, & Londei, 1998), and membrane functions (Trent et al., 2003). Biotechnological and nanotechnological applications exploiting the unique function and structure of this molecule are developing (Xie et al., 2009; Mitsuzawa et al., 2009).

1.1 *Sulfolobus* is genus of thermoacidophilic, sulfur-oxidizing, coccal archaea

The prokaryotic microorganisms of genus *Sulfolobus* are taxonomically assigned to kingdom crenarchaeota of domain archaea. Domain archaea joins domains eubacteria and prokarya to form a tripartite classification system based upon rRNA sequence, a system which has supplanted the five kingdom system (Woese, Kandler, & Wheelis, 1990; Wheelis, Kandler, & Woese, 1992; Whitman, Pfeifer, Blum, & Klein, 1999). Although archaeal unicellular morphology, single circular chromosome, lack of organelles, and operational mechanisms resemble the eubacteria (Ciaramella, Pisani, & Rossi, 2002), other important life-defining processes such as DNA replication, transcription and translation resemble the eukarya (Forterre, Brochier, & Philippe, 2002). Extremophilic archaea thrive in harsh environments previously thought sterile (Madigan & Marrs, 1997). Extremophilic archaea have changed physiological notions about the limits of life, and have provided highly stable molecules of great interest to the biotechnology industry (Breithaupt, 2001; Egorova & Antranikian, 2005). At one time, all known archaea were extremophilic, but culture-independent ribosomal surveys have

shown archaea to be widespread, and extant in many diverse non-extreme environments (Xu, 2006).

The first organism to be designated *Sulfolobus* was isolated by Brierley in 1966 (Brock, Brock, Belly, & Weiss, 1972). A collection of different isolates from Yellowstone National Park, Italy, Dominica and El Salvador were later named *Sulfolobus acidocaldarius* by Brock et al. (1972) to represent a novel genus widespread in a variety of acid thermal areas, both terrestrial and aquatic. *S. acidocaldarius* was described by Brock as generally spherical cells with frequent lobes, 0.8-1 µm in size, growing optimally at 70-75°C and pH 3-4, capable of heterotrophic growth on yeast extract and a variety of simple organic compounds, and capable of autotrophic growth by oxidizing elemental sulfur.

1.2 Sulfolobus solfataricus cpn and Sulfolobus shibatae cpn

Much of what is known about *Sulfolobus* cpn comes from a long string of publications by Trent (et al.) beginning in 1990 concerning *Sulfolobus* sp. strain B12. This strain was isolated by Yeats, McWilliam, & Zillig (1982) from the sulphurous Beppu Hot Springs at Kyushu, Japan, and named *S. acidocaldarius* strain B12. The strain was later reassigned as a strain of *S. solfataricus* (Reiter et al., 1987; Zillig et al., 1985; Zillig, Schnabel, & Stetter, 1985) before being recognized as representing a novel species, *S. shibatae* (Grogan, Palm, & Zillig, 1990). The cpn of *S. solfataricus* has also received significant attention (Guagliardi, Cerchia, Bartolucci, & Rossi, 1994; Guagliardi, Cerchia, & Rossi, 1995; Guagliardi, Cerchia, & Rossi, 1997; Knapp et al., 1994; Marco et al., 1994; Condo et al., 1998; Ellis et al., 1998; Ruggero et al., 1998; Briganti, Giordano, Londei, & Valle, 2000; Cerchia, Rossi, & Guagliardi, 2000; Valle, Dietler, & Londei, 2001).

S. solfataricus was first isolated by de Rosa, Gambacorta, & Bu'lock (1975) from Pisciarelli Solfatara in Italy and assigned to “form/habitat” group Caldariella as isolates MT3 and MT4. Zillig et al. (1980) later isolated strains DSM 1616, and DSM 1617 from the same solfatara and suggested that all four isolates be reassigned to a new genus, *S. solfataricus*. The strain DSM 1616 designation was afterward concatenated with a P1 designation (König, Skorko, Zillig, & Reiter, 1982), or used interchangeably with that designation, and likewise for DSM 1617 and P2 (Grogan, 1989). The GΘ isolate used by Cerchia et al. (2000) is derived from MT3 (Cannio, Contursi, Rossi, & Bartolucci, 1998).

S. solfataricus strain DSM 1616 P2 has become a model organism for study within the archaea. Similar to other members of its genus, the cocci are motile, exhibiting aerophobic taxis, are photo-induced to produce carotenoid pigment, and are sensitive to some heavy metal ions, some antibiotics, and some organic acids (Grogan, 1989). The complete genome has been sequenced (She et al., 2001), the theoretical proteome investigated by 2D-PAGE (Chong & Wright, 2005), many of its proteins crystallized, and a whole-genome oligonucleotide microarray constructed to investigate transcription (Tachdjian & Kelly, 2006). Methodology for genetic manipulation of the archaea lags behind that for eubacteria, but methods are developing (Wagner et al., 2009; Albers et al., 2006). See Ciaramella et al. (2002) for a review of molecular biology, and Zaparty et al. (2010) for a discussion of laboratory methods.

S. shibatae provided the first archaeal cpn recognized as such (Trent, Nimmesgern, Wall, Hartl, & Horwich, 1991, Ellis, 1992). Cpn is most known for its function as a molecular chaperone, a molecule that mediates protein folding.

1.3 Molecular chaperones assist protein folding

The term molecular chaperone was coined by Laskey, Honda, Mills, & Finch (1978) and applied to *Xenopus laevis* nucleoplasmin to describe its role in preventing

nonspecific ionic interactions between histones and DNA during assembly into nucleosomes. An early and simple definition of a molecular chaperone is a protein that assists another to attain its native conformation. The greatest biological import of the definition is not necessarily in its descriptive application to this or that protein, but in the acknowledgement of the phenomenon of molecular chaperonage, long unexpected per Anfinsen (Pelham, 1986; Ellis, 1987).

Definitions have been refined with details, specifying that chaperones form no part of the final structure, that no covalent interactions be involved, that no steric information be provided, and that processes do not proceed in the absence of chaperones (Ellis, 1993). Definitions have broadened considerably (Hartl, 1996; Hartl & Hayer-Hartl, 2009) to include newly discovered molecules and processes. Whereas older definitions focused on proteinaceous chaperones and substrates, folding and oligomer assembly (Ellis, 2006), more recent definitions include “various cellular components” as chaperones, non-proteinaceous substrates such as metal ions, and delivery of substrates to particular locations (Lund, Large, & Kapatai, 2003).

Molecular chaperones are now recognized as a very large and heterogeneous group with a great diversity of structures, mechanisms, roles, distribution and import. For a survey of molecular chaperones, see Gething (1997) and Morimoto, Tissieres, & Georgopoulos (1994). The functions of these molecules are now observed as integrated with folding-related protein metabolism processes occurring throughout substrate protein’s lifetimes from “cradle to grave” such as transport, “quality control” (Lee & Tsai, 2005) and proteolysis (Vabulas, Raychaudhuri, Hayer-Hartl, & Hartl, 2010). This contrasts with earlier understandings based upon the significant number of molecular chaperones that are hsps.

1.3.1 Folding: linear string of amino acids to 3-dimensional functional protein

Protein folding describes the process whereby a topologically-linear string of L-amino acids adopts a protein's native conformation producing a functional three-dimensional shape able to perform structural and catalytic functions. Folding is the final process in the molecular biology central dogma (Crick, 1970) which describes information transfer from coding DNA to functional protein.

It has been estimated that an astonishing "> 30-50%" of human disease conditions are due to protein misfolding (Naik et al., 2010).Folding is an especially timely biological issue given the wealth of sequence data in this genomic age. After great efforts, much is understood, but the folding process currently cannot be described in detail for any protein (Karplus, 1997; Karplus & Shakhnovich, 1992).

Hydrophobic effects supply much of the driving force in folding as hydrophobic residues are buried in the interior of the protein and the inner core is dehydrated. Leventhal's paradox (1969) points out that given the enormous phase space of potential conformations of even the smallest protein, folding must be somehow "speeded and guided" since a random walk sampling of the phase space would result in extremely long folding times, whereas many proteins fold quickly. Anfinsen's observations of *in vitro* reversible folding became a dogma (Anfinsen, 1973) prescribing that in physiological conditions (temperature, concentration of all molecular species, etc.), a protein's primary sequence alone determines native conformation, a conformation which corresponds to a free energy minimum which is unique, stable, kinetically accessible and global. This can be visualized by the protein folding funnel, a conformation phase potential hypersurface having a central depression corresponding to a Gibbs free energy minimum into which a protein spontaneously descends by folding (Dill & Chan, 1997). Anfinsen's dogma was an extrapolation from experiments with simple proteins in dilute solutions. Anfinsen's dogma does hold for small, single-domain proteins which fold spontaneously and

reversibly, but most multi-domain proteins larger than 10-20 kDa fold on a funnel with a rugged topology, and become kinetically trapped in local minima surrounded by high energy barriers (Csermely, 1999; Pauwels, Van Molle, Tommassen, & Van Gelder, 2007).

The cellular environment has been described as hostile to protein folding (Lin & Rye, 2006). The challenges for proteins with rugged folding funnels is irritated by certain aspects of the intracellular environment, particularly the high occupancy exceeding 30% (w/v) (Ellis & Hartl, 1996; Luby-Phelps, 1994) of a wide variety of surfaces and shapes of dissolved and undissolved species. Further, the high occupancy of large molecules causes a phenomenon termed the excluded volume effect; large molecules are excluded by shape and rigidity from occupying spaces interstitial to other large molecules. The excluded volume effect can elevate thermodynamic activities by several orders of magnitude (Hall & Minton, 2003) and significantly increase viscosity. The resulting impact on diffusion and binding coefficients, along with entropic variations (Ellis, 2001a; Ralston, 1990) has a large affect on chemical reaction kinetics and equilibria. See Minton (2005) for statistical-thermodynamic models and Elcock (2010) for a recent review.

1.3.2 Proteins need assistance when nascent, translocating, and stressed

Proteins benefit from folding assistance or stabilization provided by molecular chaperones in at least three different circumstances, these having to do with translation, transport through a membrane, and stress exposure. Folding assistance and stabilization are overlapping but distinct modes of interaction, likewise the three circumstances are distinct situations sharing some commonalities.

Non-native proteins in any of these circumstances may proceed spontaneously to a native conformation per Anfinsen, but may as well irreversibly denature, wasting precious resources, and acting as a nucleation site for aggregation with other proteins in

the cell, eventually obstructing normal cellular traffic. The hydrophobic effect, normally providing the driving force towards attaining and maintaining native conformation also provides the basis for deleterious interactions with other hydrophobic surfaces. That molecular chaperones do participate in each of these circumstances is becoming clearer, but exactly how these roles are played out is not yet clearly defined.

1.4 Heat shock elevates expression of heat shock proteins which produce acquired thermotolerance

Cpn's functionality as a molecular chaperone and ability to come to the aid of thermally stressed client proteins is understood to be the reason for its upregulation as a hsp during heat shock. First observed in Drosophila as a transcriptional chromosome puffing after heating (Ritossa, 1962), heat shock is a biochemical state which is a response to above-optimal or near-lethal temperatures. This phenomenon is correlated with a transcriptional down-regulation of most proteins, upregulation of a handful or two of what are called heat shock proteins, and with acquired thermotolerance, a temporary increased viability at otherwise lethal temperatures. A more generic designation such as stress response might be more appropriate as a descriptor, as a variety of stressors such as toxins (Wolfe, Olsen, Gasuad, Tjeerdema, & Sowby, 1999), saline stress (Bidle, Kirkland, Nannen, & Maupin-Furlow, 2008), and ultraviolet light (Baliga et al., 2004) can indiscriminately induce similar responses, as detection systems sense damage rather than stressors (Kultz, 2005). Nevertheless, terminology involving heat prevails for historical reasons (Macario, Lange, Ahring, & Conway de Macario, 1999). Although *Hydra oligactis* is unable to acquire thermotolerance (Brennecke, Gellner, & Bosch, 1998), and each organism expresses its own unique spectrum of proteins, heat shock is considered a ubiquitous phenomenon, orchestrated by the most conserved genetic systems known (Lindquist & Craig, 1988). For reviews of heat shock, see Richter, Haslbeck, & Buchner (2010), Vabulas et al. (2010), Kultz (2005), Feder & Hofmann

(1999), and Lindquist & Craig (1988). For reviews of archaeal heat shock, see Tachdjian, Shockely, Conners, & Kelly (2008), Macario & Conway De Macario (2001), Macario et al. (1999), Trent (1996) and Conway de Macario & Macario (1994).

Although not all hsps are chaperones, nor are all chaperones stress-inducible, there is considerable overlap between the two sets, and the five (Richter et al., 2010; Trent, 1996) or six (Vabulas et al., 2010; Large, Goldberg, & Lund, 2009) recognized major families of highly conserved chaperones found in a wide range of organisms are named Hsp, with a concatenation of their characteristic (approximate) molecular weight: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and sHSP (small HSPs). See Richter et al. (2010), Kultz (2005) and Feder & Hofmann (1999) for consideration of a larger set of conserved stress response proteins and a minimal stress proteome.

Hsps manage transitions from the typical state of affairs to the heat shock state and back again. This includes sensing, preventatively reducing, minimizing the impact of, and healing damage to, proteins and nucleotides. This requires regulation and control of pathways and expression cascades concerning cell cycle, energy metabolism, stress response and redox state. A little more specifically, hsps target cytoskeletal maintenance, chaperoning, proteolysis, wreckage removal, transport, detoxification, membrane-modulation (Richter et al., 2010; Kultz, 2005).

Heat shock upregulation to high levels is responsible for the first discovery of a cpn in archaea, when electron microscopy revealed oligomers in an accidentally heated culture of *Pyrodictium occultum* (Phipps, Hoffmann, Stetter, & Baumeister, 1991; Klumpp & Baumeister, 1998). Shortly thereafter, heat shock upregulation was also responsible for the discovery of the first *Sulfolobus* cpn (Trent et al., 1991). In this second case, it was not high levels *per se* which attracted attention, but cpn's predominance of hsp expression in *S. shibatae.* This organism depends almost entirely upon cpn for hsp function; thus, an understanding of *Sulfolobus* cpn requires a certain understanding of heat shock.

Cpn in general are abundant proteins, constitutively comprising 1–2% of total cellular protein (Quaite-Randall & Joachimiak, 1999). In archaea, codon usage bias predicts cpn to be among the most highly expressed proteins Karlin, Mrazek, Ma, & Brocchieri, 2005. In *S. shibatae*, cpn is the most abundant protein seen with coomassie-stained 2-D PAGE (Trent et al., 1990), making up 4% of total soluble protein (Trent et al., 1997). Levels increase to 12% at the upper growth temperatures (Trent, 2000). Even a slight heat shock at 88°C for 30 min increases purification yield (Quaite-Randall & Joachimiak, 2000). Although Tachdjian & Kelly (2006) found no transcriptional increase in *S. solfataricus* P2 by whole genome oligonucletide microassay, Kagawa et al. (1995) observe a significant increase in RNA by Northern analysis, and during extreme heat shock (≥ 90°C), cpn appears to be the only protein expressed. The two stage heat shock of Cerchia et al. (2000) increases levels to 30% of the total soluble protein in *S. solfataricus* GΘ; 3.6 mg pure cpn can be isolated per gram of cell mass.

CHAPTER 2 MOTIVATION TO STUDY GUAGLIARDI'S ACTIVITY

The motivations to look closer at Guagliardi et al.'s (1995) (hereafter, study and activity are referred to by Guagliardi's name alone) Surfaces II activity are manifold. The activity itself is incredible, and the study is neglected. Potential biotechnological applications in laboratory and industry are manifest, and further discoveries and details will elaborate current understandings of protein folding, stability, and metabolism, strategies of stress and shock physiology, and thermophily.

2.1 Guagliardi's activity prevents thermal denaturation of enzymes

Guagliardi observed that cpn of *S. solfataricus* strain P2 (Ssocpn) can prevent irreversible thermal inactivation of enzymes, as shown in Figure 1. When heated, malic enzyme (ME) and alcohol dehydrogenase (ADH) lose all enzymatic activity within 15 and 20 minutes, respectively. When incubated with equimolar levels of Ssocpn oligomer, denaturation was entirely prevented for the full observation period reported, 40 and 60 minutes, respectively, or about three times as long as the time to complete inactivation. Molar ratios of 1:10 (cpn:enzyme) gave similar results, but enzymes heated in ratios of 1:20 were ≅ 30% inactivated after 30 minutes.

See APPENDIX A for observations of the mixed results of pilot ADH denaturation studies. Guagliardi's procedural details follow. Enzymes (6 µg in 1 ml) were heated in 10 mM Tris-HCl buffer, pH 8.0. Aliquots withdrawn from the incubation solution were assayed for enzymatic activity at 25 °C by a Cary 1E Varian recording spectrophotometer equipped with a thermostated cell compartment. Absorbance variations were always linear within 2 min; each activity assay was performed in duplicate. ME was assayed in 20 mM Tris-HCl buffer, pH 7.5, 0.05 mM NADP, 1 mM $MgCl_2$, and 1 mM L-malate

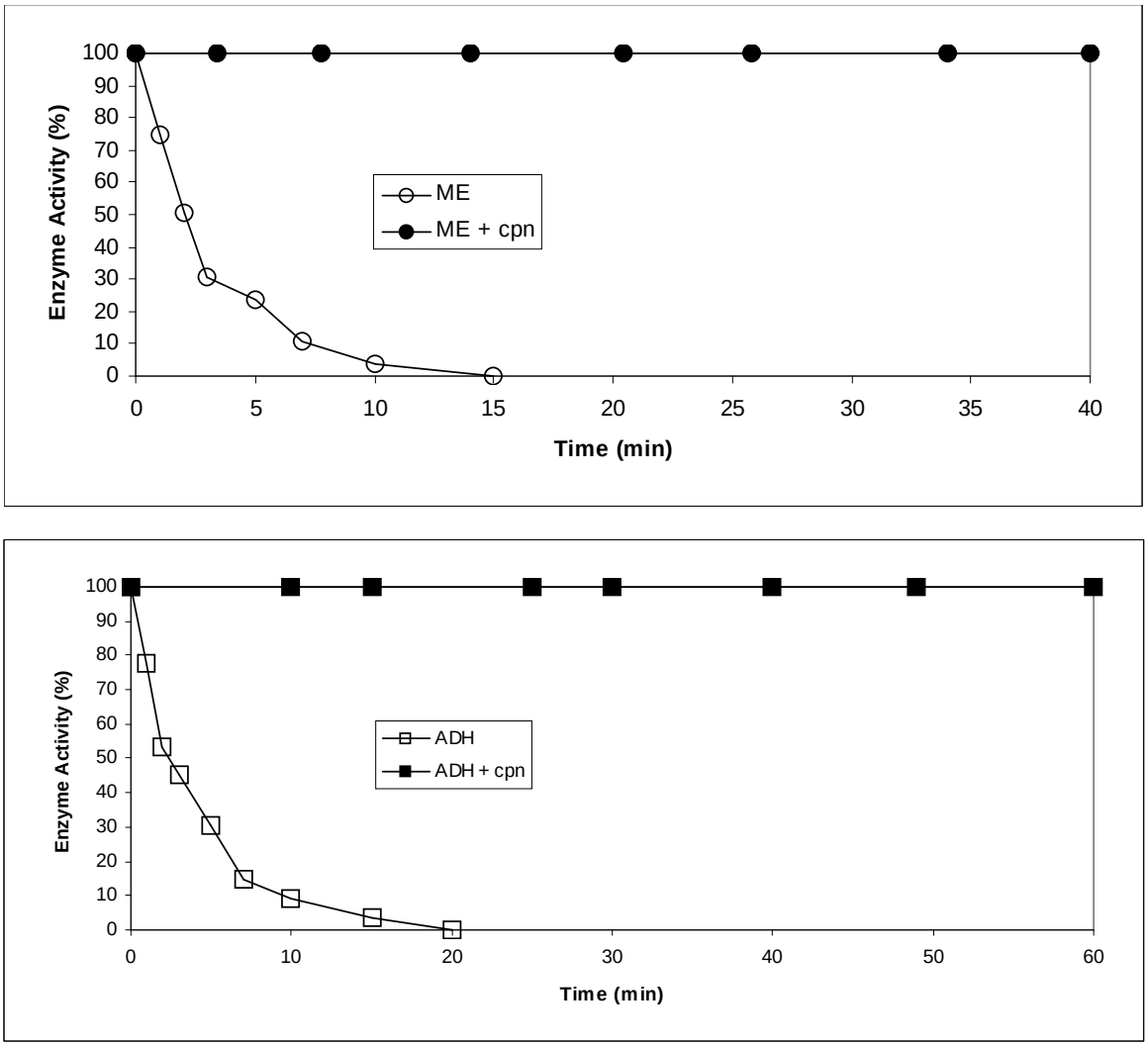

Figure 1. Guagliardi observes Ssocpn prevent irreversible thermal denaturation of chicken liver malic enzyme (ME) and yeast alcohol dehydrogenase (ADH) heated to 50°C in the absence and in the presence of Ssocpn.

(1 ml final volume). ADH was assayed in 50 mM sodium phosphate buffer, pH 8.0, 2 mM NAD, and 1 mM ethanol (1 ml final volume).

The prevention of thermal inactivation is not dependent on ATP. Addition of ATP and Mg^{2+} and K^{1+} cofactors approximately halfway through the observation period had no effect. The activity is not a general stabilization due to protein, as excess bovine serum albumin does not prevent aggregation. The activity is strictly preventative, not reversing in nature. Irreversible aggregation as monitored by light scattering could be prevented,

but not reversed by Ssocpn added after aggregation. The interaction between cpn and enzyme is presumed to be hydrophobic, as 0.1% Triton X-100 eliminated prevention of aggregation. The activity is coincident with a persistent binding. After a 2 h heat treatment, enzyme and Ssocpn elute from a size exclusion column (Superose 6) at 4°C in a single peak. Binding only occurs with denatured enzyme. Native, unheated enzyme and Ssocpn elute as separate peaks. This could imply, incredibly, that enzymatic catalysis occurs while enzyme is bound to cpn.

2.2 Different Cpn Activities with similar features

Although this activity in *S. solfataricus* strain P2 is still rather neglected, this particular line of research has been explored in at least two other organisms. Yan, Fujiwara, Kohda, Takagi, & Imanaka (1997) report that recombinantly expressed β subunit of *Pyrococcus* sp. strain KOD1 similarly stabilizes ADH of *Saccharomyces cerevisiae*. Minuth et al. (1999) report failure to reproduce such activity with cpn from *Methanopyrus kandleri*. These studies are briefly discussed by Gutsche, Essen, & Baumeister (1999).

At one time the activity was novel, even unique, for at least three features. The features include interactions: (a) with an apparently functionally active client, (b) with a client too large to fit inside the central cavity, (c) in an ATP-independent fashion. Other activities with one or more similar features are now reported or argued for cpn of a different strain of *S. solfataricus*, for other Group II cpns, and, perhaps unsurprisingly, even for the prototypical Group I cpn of *E. coli*, GroEL. Client proteins which are too large for the central cavity draw attention to at least three concerns: (a) an unknown binding site may be involved, (b) no 'capping' is possible, and (c) no encapsulation is possible. Capping, whether by Group II built-in iris lid or by separate Group I GroES cofactor cap, corresponds to a single conformational process in a sequence or cascade of sophisticated oligomer-wide conformational movements. These movements are

orchestrated by positive cooperativity and allosteric communication between subunits in each ring such that each ring functions as a unit. Negative cooperativity and allosteric communication between rings causes an alternation of non-equivalent states between rings. It is unclear how the process might proceed without the capping process. Encapsulation of client is one of the more salient features of cpn activity, and is the basis of several important concepts invoked to explain folding catalysis: 'infinite dilution', isolation in an Anfinsen cage, and close confinement. The importance of both capping and encapsulation is perhaps underlined by genetic coding for an alternate cofactor lid in the genome of T4 bacteriophage. The alternate cofactor lid interacts with native host GroEL to produce a larger central cavity to accommodate a major capsid protein which is too large for the native GroEL/ES complex (Hunt, van der Vies, Henry, & Deisenhofer, 1997). However, the chaperoning activity of small, monomeric polypeptides containing the cpn client binding site, attests that neither encapsulation, nor capping is essential in all cases (Hua et al., 2001; Jewett & Shea, 2006; Zahn et al., 1996). Compared to the more well known ATP-dependent, encapsulation-associated activity, these features are not trivially different, and demand a more sophisticated and inclusive conceptual framework.

Condo et al. (1998) report binding of *S. solfataricus* strain MT4 cpn and a large 90 kDa zinc-dependent aminopeptidase. This is too large for the cavity, which will accommodate 50-60 kDa clients (Braig et al., 1994; Ditzel et al., 1998). The peptidase is bound in an active state, and ATP affects neither binding nor peptidase activity. Strain MT4 Ssocpn also binds and participates specifically in the maturation of 16S rRNA (Ruggero et al., 1998). ATP inhibits this binding and maturation process. *S. shibatae* cpn at sufficient concentration forms filaments by orderly stacking upon binding ATP (Trent et al., 1997) and also associates with the membrane (Trent et al., 2003).

The possibility that interactions with proteins too large to be encapsulated occur as domain-wise assisted folding is considered briefly for other Group II cpns by Spiess, Meyer, Reissmann, & Frydman (2004, p. 599, 603), and by Bigotti & Clarke (2008, p.

335) and for the Group I cpn GroEL by Netzer & Hartl (1998, p. 70). Bigotti & Clarke (2008) also note evidence for co-translational interactions by CCT and GroEL. A nascent translating protein may be small enough to enter the central cavity, but a polypeptide tether to a large ribosome presumably prevents tidy encapsulation by either lid or iris. Several lines of research of GroEL are reviewed by Lin & Rye (2006, p. 217, 232), Horwich, Fenton, Chapman, & Farr (2007, p. 121, 123), and Ellis (2003, 2001b). These studies establish that binding of clients larger than 60 kDa does occur, and in some cases is required for productive folding. There is some evidence of repeated, ATP-dependent interaction with the trans ring, whereas the encapsulating activity occurs in the cis ring.

2.3 Evolving project formulation

The constant ultimate focus of this project has been investigation of Guagliardi's Surfaces II activity. The original focus (Seipel & Peeples, 2001) was a comparison of chaperoning activity of cpn from a variety of extreme environments. While the studies of Guagliardi concern cpn from one thermophilic organism, and its interaction with several enzymes during thermal stress, early formulations of this project planned assay of chaperoning activity of cpn from acidic environments in acidic conditions, and assay of halophilic cpn in high salt conditions, and so on. Failure to find such fantastic activities would not obscure useful cross-species comparisons.

Later formulations of the project involved refocusing investigations exclusively onto *S. solfataricus* cpn activity. An attractive prospect is identification of a temperature and a cpn:enzyme ratio which supports stabilization and thermal denaturation prevention lasting the *in vitro* lifetime of the enzyme. An even more attractive prospect is an increase in the enzyme activity's upper temperature limit. Guagliardi did not demonstrate activity at elevated temperatures, but instead demonstrated that thermally stressed cpn-enzyme complexes were catalytically active upon cooling to room temperature for enzyme activity assays. Designs to pursue such an astonishing and non-intuitive activity were

underway before the studies of Yan et al. (1997) came to this author's attention. In those studies, strictly speaking, an increase in temperature range was not directly observed, but cpn did maintain ADH in a catalytically active form at 50°C over a time frame where ADH without cpn became inactive. The crucial difference between this experiment and those of Guagliardi is that ADH was assayed at the same temperature at which it was inactivated. Taken together, these studies are highly suggestive.

2.4 Differences between Surfaces I and II.

The mechanism and surfaces (Surfaces II per Guagliardi) responsible for the ATP-independent activity appear distinct in several ways from mechanism and surfaces (Surfaces I) responsible for the more familiar and well studied ATP-dependent activity for which cpn is generally known. The two surface types exhibit different functionality, are independently saturable, and cater to different clients. The differences may have evolved to meet the needs of different circumstances or different client proteins. The difference in functionality is highlighted by the difference in ATP dependence. Guagliardi notes that the ATP-dependence of Surfaces I is consistent with a constitutive functionality, while the ATP-independence of Surfaces II is consistent with functionality in the dramatically reduced concentration of intracellular ATP during stressful conditions.

2.4.1 Surfaces I and II are individually saturable

Four model enzymes appear in the study. Surfaces I interact with monomeric client enzymes which are smaller in size (chicken egg white lysozyme, 14.4 kDa; yeast α-glucosidase, 68.5 kDa) than the Surfaces II larger tetrameric client enzymes (ME, 260 kDa; ADH, 150 kDa). Each of these enzyme clients are segregated to one or the other Surface type. The surfaces are individually saturable with enzyme, rendering them unavailable to additional enzyme which segregates to the same surface, while the other unsaturated surface type is still available to different enzymes. For example, the larger

oligomeric enzymes ME and ADH each interact with Surfaces II. Surfaces II can be saturated with ME, after which no protection is afforded to ADH, and vice versa. However, Ssocpn with saturated Surfaces II will still protect the smaller, monomeric enzymes lysozyme or α-glucosidase, enzymes that interact with Surfaces I. Similarly, Surfaces I can be saturated, and still protect enzymes that interact with Surfaces II.

2.4.2 Possible segregation criteria includes size and oligomerization

How do clients segregate to the two Surfaces types? It is tempting on the basis of client enzyme size alone to suppose that Surfaces I are inside the Ssocpn cavity, and interact with enzymes that fit inside while Surfaces II are external and interact with enzymes which are too large to fit inside the cavity. Some studies of interaction and binding of proteins too large to be encapsulated, as discussed above, suggest that the binding sites of the trans ring is responsible. Guagliardi notes that activity of enzymes bound to the external surface of Ssocpn is consistent with exposure to the milieu and access to substrates and cofactors. A purely size based rationale satisfactorily explains why larger enzymes do not saturate interior Surfaces I, but requires additional hypothesis about why smaller enzymes do not saturate Surfaces II. Surfaces II may be dispersed, leaving smaller enzymes unable to span enough distance to bind sufficient numbers of sites.

Other cpns show relevant client preferences. Eukaryl cpn shows a clear working relationship with the cytoskeletal proteins actin and tubulin (Lundin et al., 2008), a mild preference for proteins with tryptophan-aspartate repeats (Craig, 2003) and, most relevantly, a preference for oligomeric clients (Spiess et al., 2004). Oligomerization state, rather than size, may be the critical segregation criteria. Surfaces II may interact with intersubunit contacts of oligomeric clients. Interestingly, oligomerization itself appears to be a thermophilic adaptation (Tanaka et al., 2004).

2.4.3 Segregation may be incidental

It must be noted that the segregation of these four model enzymes to the two Surfaces may be incidental, idiosyncratic, or artifactual rather than based on size or quaternary structure. It may be that Ssocpn has a unique relationship with each enzyme, or with different denatured conformations of those enzymes. A heterogeneous population of Ssocpn in different conformational states might exhibit heterogeneous functional consequences. Cpn is capable of a variety of conformations. The ATP-induced conformation of Ssocpn is unable to bind clients of Surfaces I, but the activities of Surfaces 2 are indifferent. Further, *S. shibatae* cpn is comprised of monomers from three different genes (Kagawa et al., 2003) which are differentially expressed with respect to temperature, giving oligomers with varying compositions.

2.4.4 Guagliardi's *in vitro* conditions differ from those *in vivo*

In vitro biochemical work in non-physiological conditions hazards artifactual observations for simple, controlled experiments delivering clear signal. Although physiology may be somewhat beside the point of many biotechnological applications, the development of such is often aided by mindful cognizance of the original, evolved, *in vivo* functions and environment. The study of Guagliardi uses dilute solutions of purified enzymes, while macromolecular crowding due to the high occupancy of cytoplasm is understood to be a major factor in the necessity for evolution of cpn and other molecular chaperones. All incubations and assays occurred in a pH between 7.5 and 8.4, while intracellular pH in this genus is ≅ 6.5 (Moll & Schäfer, 1988). The studies occur below the growth range temperature of the source organism (50-87°C; Zillig et al., 1980) in one instance (α-glucosidase, 40°C), and well below heat shock temperature in all instances. Although constitutive Ssocpn expression implies that some activity occurs at normal physiological growth temperatures, it is relevant that this suite of experiments

concerns interaction of thermally denatured enzymes with cpn at temperatures where Ssocpn would not encounter thermally denatured enzymes. Conversely however, it must be noted that where a simple stabilizing surface is concerned, lower temperatures may lead to greater stability and greater chaperoning activity. Additionally, thermophilic cpn is paired with enzymes from taxonomically distant, mesophilic avian and fungal organisms.

2.5 Guagliardi's study is an attractive starting point

Several features of Guagliardi's findings make it an attractive starting point. The 'completeness' of the protection from thermal denaturation is striking. ADH at 50°C irreversibly lost all activity within 20 minutes, but with Ssocpn remained 100% active for an hour. Similarly striking is the absence of ATP or other energy cofactors in the interaction. The work involves enzyme activity which lends itself to application more directly than the aggregation and folding studies otherwise seen in the literature.

Certainly, there are other pressing biochemical questions which do not appear to lead directly to use or application. The location of Surfaces I and II is of interest; surface bound enzyme may be discerned by traditional size discriminating methods such as light scattering, native PAGE, or size exclusion chromatography. A description of cpn-enzyme interactions in terms of binding coefficients and rate expressions could justify or nullify certain assumptions. This sort of description is likely to involve some understanding of enzyme denaturation.

2.6 Rationale to expect additional, undiscovered Ssocpn function

Some of the rationales to entertain the possibility that Ssocpn harbors valuable functionality yet to be discovered are similar to the rationales for evolution of the already discovered Surfaces II activity. Ssocpn is a hsp from a thermophile. The molecule has evolved to help an organism which is already adapted to extreme heat cope with even

higher temperatures. The widespread distribution of this organism reflects a highly successful life strategy, of which this protein is a significant part. This protein in this organism is constitutively abundant, and especially so during heat shock. Cpn is typically less abundant in other species. Although the abundance could be a compensation strategy for a marginally effective molecule, it could also suggest that this organism is highly dependent upon this protein, for a critically important, or for a multiplicity of, roles or modes of action. The transcriptional portion of heat shock in this organism depends almost entirely upon this single protein (Trent et al., 1991), whereas other organisms typically express one or more handfuls.

Especially noteworthy is the absence of Hsp70 in the transcriptional response of *Sulfolobus*, (Trent, 1996; Gribaldo et al., 1999; She et al., 2001; Macario, Malz, & Conway de Macario, 2004). Similar to cpn, Hsp70 is both a hsp and a molecular chaperone (Zmijewski et al., 2007; Bukau & Horwich, 1998). Hsp70 is one of the most conserved proteins in evolution (Macario & Conway de Macario, 1999), ubiquitous, without exception, in eukarya, endosymbiotic organelles, bacteria, and found absent only in some archaea, most systematically in crenarchaeal hyperthermophiles (Macario et al., 2004). While *S. solfataricus* may do without the functions of Hsp70 and other hsps, it is tempting to suspect that Ssocpn performs some of these responsibilities (Guagliardi et al., 1995; Frydman, 2001). Cpn is an ancient protein with a demonstrated ability to acquire new responsibilities.

The three proteins Hsp70, Hsp40 and Hsp23 (or Hsp24 per organism) usually occur exclusively as a package, although functions of Hsp23 in eukarya are performed by analogs or homologs (Macario et al., 2004; 1999). The bacterial homologs are referred to as DnaK, DnaJ, and GrpE, respectively. This vital triad comprises what is termed the molecular chaperone machine. *Sulfolobus* may do without the functions of the molecular chaperone machine, may rely upon an altogether different strategy such as proteases (Large et al., 2009), or could depend upon a different molecule other than cpn for these

functions, such as prefoldin (Frydman, 2001; Hartl & Hayer-Hartl, 2002), a molecular chaperone known as GimC in the archaea. Less consequential, but pertinent to this discussion is the absence of the molecular chaperones Hsp33, Hsp90, and Hsp100 in nearly all archaeal genomes (Laksanalamai, Whitehead, & Robb, 2004; Large et al., 2009). See Macario & Conway De Macario (2001) for other molecular chaperones absent in archaea.

2.7 Impact, or "Why is thermal stability desirable?"

The primary motivation for this work is biotechnological application in laboratory or industry. The biotechnology industry is very interested in stable enzymes for use in conditions far removed from the original living cytoplasmic conditions. In industry, enzymes are replacing less environmentally-friendly methodologies, and reactions are often heated to increase reaction rates. Increasing thermal stability by simple addition of cpn to a reaction mixture is much easier than other approaches such as directed evolution.

Novel, wider, and simpler applications of enzymes and proteins are to be expected. A novel application due to thermal stability is exemplified by polymerase chain reaction (PCR), a methodology to replicate DNA. PCR has become an essential research tool playing no small part in the overall commercial success of biotechnology. The methodology has generated billions of dollars and a Nobel Prize (Fore, Wiechers, & Cook-Deegan, 2006). The viability of this method rests completely upon the high thermal stability of Taq DNA polymerase which persists through multiple cycles of DNA melting temperature. An example of a simplification can be illustrated with insulin, a medication used to treat diabetes. A typical pharmacy will stock several hundreds of different medications on shelves at room temperature, but insulin, a crucial and popular protein hormone, is stored along with a handful of others in a refrigerator (Grajower et al., 2003; Huus, Havelund, Olsen, van de Weert, & Frokjaer, 2005). An increase in maximum storage temperatures would reduce logistical concerns and costs of transport and storage.

These examples are to be taken as illustrative of the potential of an increased thermal stability, not necessarily as research goals. Similarly, as the enzymes in Guagliardi's studies and in this project's design are not of great commercial or biotechnological interest, the design is to be taken as proof of concept, as a stepping stone to work with more attractive enzymes. Lastly, but certainly not least, descriptions of applications so closely paralleling physiological function will be relevant to physiological understandings of protein folding, stability, and metabolism, strategies of stress and shock physiology, and thermophily.

CHAPTER 3 HEAT SHOCKING CHEMOSTAT TO PRODUCE CHAPERONIN AND STUDY HEAT SHOCK

3.1 Motivations: cautious, native expression of much chaperonin, and a steady-state, homogenous system for heat shock investigation

The heat-shocking chemostat (HSC) is designed to produce cpn and to study heat shock. As a production unit the apparatus synergizes the high productivity of a continuous process with the increased cpn levels produced by heat shock. Cpn is an hsp; relative levels significantly increase during heat shock (see section 1.4 for particulars). Heat shock is used to increase yield of cpn isolation (Cerchia et al., 2000; Quaite-Randall & Joachimiak, 2000; Schoehn, Quaite-Randall, Jimenez, Joachimiak, & Saibil, 2000a; and Knapp et al., 1994). To this author's knowledge, this particular triple-stage configuration of heat shocking chemostat has not been described, but see the recent study by Luders, Fallet, & Franco-Lara (2009) who claim first report of a continuous heat shock study, discussed under 3.2.4 Stress response investigations. The steady states of continuous culture will likely continue to impact heat shock studies, perhaps eventually proving as pivotal in this field as in its growth physiology provenance.

As a production unit, a heat shocking chemostat is an alternative to other common strategies such as large reactors (Worthington, Blum, Perez-Pomares, & Elthon, 2003, 9.5 L; Guagliardi et al., 1994, 25 L; Marco et al., 1994, 120 L; Kagawa et al., 1995, 150 L; Quaite-Randall & Joachimiak, 1999, 160 L) or recombinant expression in *E. coli* (Kagawa et al., 2003; Schoehn et al., 2000b; Yoshida et al., 1998; Koeck, Kagawa, Ellis, Hebert, & Trent, 1998; and Nakamura et al., 1997). Large reactors entail significant downstream scale-up development and challenges, and recombinant expression entails certain hazards compared to native expression. The motivation to bypass recombinant hazards with native expression is caution. Proteins expressed recombinantly in foreign

hosts do not always fold or assemble correctly. The issue is perhaps exacerbated in the current situation; a protein from a thermophilic organism is desired, and the most feasible host is mesophilic (*E. coli*). The disparity in growth temperatures is biologically quite large (75°C - 37°C = 38°C). An investigator whose caution borders on the suspicious might wonder about cpn's prominent relationships with temperature (as a thermophilic heat shock protein) and with protein folding (as a cpn). The fear can be contextualized by imagining a protein folding funnel energy landscape with cpn conformation settled in a non-native local free energy minimum when expressed at the low physiological temperature of *E. coli*. If the low temperature minimum has a trough leading to another non-native local minimum at physiological *Sulfolobus* temperatures, the protein conformation may never find the global minimum associated with native function. Trent et al. (1997) has related that *S. shibatae* cpn stored for >48 h at 4°C lost the ability to form filaments at physiological temperatures in the presence of Mg^{2+} alone and required nucleotides to do so. The relative contributions of elapsed time and reduced temperature are unclear, and the finding does not necessarily speak directly to the concerns under discussion, but does suggest that cpn may have a "temperature memory" with functional consequences. Further complicating matters is the observation that the three different *S. shibatae* cpn subunits have different temperature-dependent expression profiles, resulting in cpn oligomers of varying composition and thermal stability (2003). Varying subunit composition is not prohibitive for recombinant expression, but is a complicating factor for interpretation of data.

3.2 Background

3.2.1 Continuous culture enjoying resurgence

Continuous culture as a method was simultaneously and independently developed by Monod (1950) and Novick & Szilard (1950). Using this method, microbial growth rate can be fixed in a steady-state physicochemical environment, effectively decoupling

growth physiology from the changing conditions of batch culture. However, note that Ferenci (2008) warns that mutations cause continuous cultures to become heterogeneous populations within 100 generations. See Nelson, (2008a, 2008b) for a brief historical outline of development, and see Sinclair & Cantero (1990) or Tempest (1970) for a fundamental mathematical description. The method at one time was an extremely popular tool (Ricica, 1973) in a variety of fields such as biochemistry, physiology, ecology and evolution. Popularity waned when molecular biology, genomics and associated "omics" methods took center stage. Continuous culture is currently resurging in popularity (Bull, 2010; Hoskisson & Hobbs, 2005) to produce controlled, defined, homogenous, and static environments and populations for testing of systems biology and analysis by omics technologies (Daran-Lapujade, Daran, van Maris, de Winde, & Pronk, 2009; Monk, Pearson, Mulholland, Smith, & Poole, 2008; Wecker et al., 2009).

3.2.2 Thermoacidophile accommodations

Design of continuously culturing systems is significantly more complex (Tempest, 1970; Herbert, Phipps, & Tempest, 1965) than shake flask batch cultures. Further, engineering for thermophiles and acidophiles requires additional constraints and accommodations. The reactor of Pysz, Rinker, Shockley, & Kelly (2001) is similar to the device described in this paper in that it is an assemblage built around a round bottom flask (2 L, 1 L working volume) in a heating mantle. Provisions are made for colloidal sulfur feed, pH control by addition of HCl and NaOH, overhead-driven impeller agitation, and fluid retention by condenser-cooled gas exhaust line. The system has been used to culture a variety of organisms: thermophiles, hyperthermophiles, thermoacidophiles, anaerobes, and sulfate reducers, as seen in the publications of that lab group. Godfroy, Postec, & Raven (2006) describe a glass, jacketed, vertical concentric-tube gas-lift reactor designed for continuous culture of hyperthermophilic *Thermococcales*. Gas lift agitation obviates shear damage that *Pyrococcus furiosus*

experiences with stirrer speeds in excess of 1800 rpm. The reactor supports 2 L of culture with pH control, temperature control by circulating bath (water or mineral oil), and fluid retention by condenser cooling of exhaust gas. Raven, Ladwa, Cossar, & Sharp (1992) describe a similar system with 2 and 5 L glass reactors. Tsao, Kaneshiro, Yu, & Clark (1994) describe a 5 L commercially made and subsequently passivated reactor used to culture the extremely thermophilic and methanogenic *Methanococcus jannaschii*. The system features an overhead-driven impeller, fluid retention by enhanced condenser, and a heated medium feed tank. A less complex continuous culture system is described by Gilbert & Stuart (1977). The design of this Pyrex glass construction addresses scale-down issues associated with its small working volume of 55 ml, such as evaporative losses, siphon dead space, and nutrient fluctuations caused by each discrete drop of medium added. This gas lift reactor is designed to be simple and small enough to operate in parallel with others as part of an array.

Dialysis membrane reactor systems retain cells within the reactor to achieve high cell densities. Although *Sulfolobus* species have been cultured in these reactors (Knapp et al., 1994; Holst et al., 1997; Krahe, Antranikian, & Märkl, 1996), they are not especially relevant to the present discussion. The systems are continuous in the sense that medium flows through the reactor for the duration of the fermentation, but fermentations do not persist indefinitely, nor is the physicochemical environment a static steady state.

3.2.3 Multiple stage continuous culture

Multiple continuous culturing reactors are arranged into cascades or series for different purposes; to decouple processes into separate stages for analysis (Govindaswamy & Vane, 2010), to set up physicochemical gradients (Codeco & Grover, 2001), and, like this work, to subject a single bioprocess stream to a sequence of different environments (Gibson, Cummings, & Macfarlane, 1988; Wimpenny, 1985). See Ričica (1970) for discussion about a variety of multi-stage geometries.

3.2.4 Stress response investigations

Nisamedtinov et al. (2008) investigated *S. cerevisiae* stress response with variable flow single-stage continuous culture (A-stat, D-stat, auxo-accelerostat) by monitoring Hsp12p stress protein expression. Hsp12p was induced by thermal and other types of stress, and was induced more strongly by rapid stress onset compared to gradual onset. Heitzer, Mason, & Hamer (1992) review *E. coli* heat shock in single-stage continuous culture to facilitate replacing industrial batch-wise temperature-inducible recombinant protein expression with continuous processes. The review focuses on the htpG heat shock gene as a response indicator, and states that expression is dependent on both rate and intensity of the stress, but is independent of growth rate. The work of Han, Park, & Kelly (1997) is related to the work described here. A single stage continuous culture was used to investigate the heat shock response of *Metallosphaera sedula*, an organism in the same family (Sulfolobaceae) as genus *Sulfolobus*. An abrupt increase of temperature from the maximum growth temperature of 79°C to 80.5°C initiated a dramatic decrease in cell density and threatened cell washout. However, gradual temperature increase led to a stressed phase growth mode at a greater temperature, up to 81°C. This stressed phase growth was stable, albeit at a reduced cell density, and was accompanied by drastically elevated levels of a 66 kDa hsp immunologically related to *S. shibatae* cpn. The study perhaps most relevant to the work described in this document was performed by Luders et al. (2009). Although the organism is different (mesophilic *E. coli*), and the chemostat simpler by one stage, the two stage continuous culture system is analogous; growth chemostat effluent flows into a heat shock chemostat. Analysis by two-dimensional PAGE showed that the spectrum of hsps produced by chemostat differed from the spectrum produced batch-wise and reported elsewhere. The authors state that no prior report describes the use of this reactor configuration for the study of bacterial stress response.

3.3 Materials and methods

3.3.1 Reactor is a triple-stage chemostat

The HSC is a series of three continuously operated stirred tank reactors. The reactors are separately temperature controlled to grow microbes and apply a two stage heat shock. Temperatures and residence times are designed to replicate the heat shock regimen of Cerchia et al. (2000). Liquid is moved by peristaltic pump and sparged by house compressed air. Culture of the growth reactor is continuously sampled to a fraction collector. The assembly physically appears as a collection of discrete glassware and electronics interconnected by tubing and wires, situated partly inside of a laminar flow hood. The HSC is diagramed in Figure 2 and pictured in Figure 3.

3.3.2 Design

3.3.2.1 Residence Time and Temperature Follows Cerchia's Heat Shock

Heat shocking temperatures and times follow the scheme of Cerchia et al. (2000), as seen in Table 1. Liquid residence time in a chemostat defines microbial generation time. A 24 hour growth reactor (GR) residence time was chosen to define an arbitrarily large generation time to avoid culture washout. The cells do not grow at the elevated temperatures in the heat shock modules. GR volume was chosen arbitrarily per equipment availability. Given residence time and fluid volume of any one reactor (in this case, the GR), the single flow rate through all three in-line flasks (neglecting evaporative losses) is determined by the equation:

$$R_t = \frac{V}{F} = 24(h) = 1440(\min) = \frac{1500(ml)}{F(ml/\min)} \Rightarrow F = 1(ml/\min)$$

where R_t is residence time, V is volume, and F is volumetric flow rate. The flow

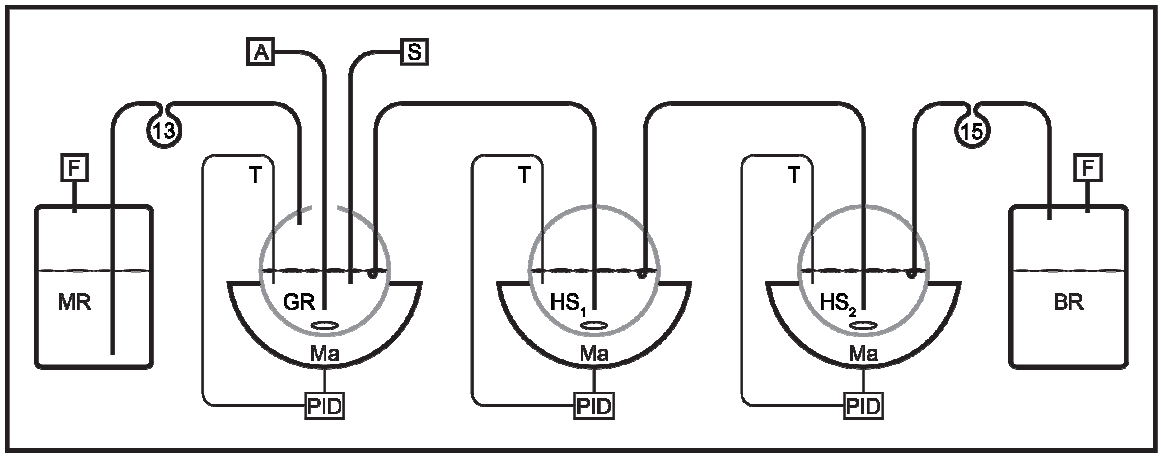

Figure 2. HSC is three reactors in series. Abbreviations follow.

A-Air sparge
BR-Biomass reservoir
F-Filter, 0.2 µm, sterile vent
GR-Growth reactor
HS_1, HS_2-Heat shock reactors #1, #2
Ma-Mantle, heating and stirring
MR-Medium reservoir
PID-Proportional, integral and derivative temperature controller
S-Sampling line
T-Thermocouple
13, 15-Peristaltic pump head
Note unlabelled features: stir eggs, overflow weir u-turn at end of outgoing dip tubes, pressure relief gap between air sparge dip tube and GR, and routing.

rate and desired residence times in the two remaining reactors subsequently defines their volume by the same equation.

The temperature of each reactor is individually monitored by thermocouple, sustained by an electrically heated mantle and stir plate, and controlled by a controller with proportional, integral and derivative (PID) functions.

3.3.2.2 Fluid movement

3.3.2.2.1 Five vessels, only two pumps: non-intuitive design is simpler

Process Fluid follows a linear path from medium reservoir through reactors to biomass reservoir. The house compressed air sparge stream is introduced into the GR and splits into two streams which exit directly from the GR and eventually from the biomass

Figure 3. Picture of the HSC illustrates spatial and organizational challenges. This picture shows long inter-reactor path lengths and accommodations to mix water and medium concentrate by pump, features which were eliminated in later revisions. Note that HS_1 in this picture is an Erlenmeyer flask, while it is diagramed as a round bottom flask in Figure 2.

Table 1. Temperature and residence time in each reactor is different.

Reactor	Growth	Heat Shock 1	Heat Shock 2
Temperature (℃)	75	85	90
Residence Time (h)	24	2	6
Fluid Volume (ml)	1,500	120	360

Note: Underlined values are taken from Cerchia et al. (2000).

reservoir. Gas and liquid flow streams are shown in Figure 4.

Medium is withdrawn and delivered to the GR by the first pump; the second pump suctions culture from the GR, through both air tight heat shock reactors, and delivers fluid to the biomass reservoir. The design calls for only two pumps rather than the more intuitive approach where a pump is situated in-between each of the five vessels, an arrangement which requires 4 pumps. This arrangement is significantly simpler than one with two additional pumps and four additional ports for sparge gas entry and exit. The omissions are appreciated as significant when considering the obviated electrical and tubing interconnections, air tight seals and spatial organization concerns. The situation is further complicated in the smaller vessels which offer less space for addition of the relatively large ground glass joints used in this design.

3.3.2.2.2 Difference of pump rates regulates volume, equals sparge rate, and reduces inter-reactor residence time

Although it would be intuitive to design a chemostat with two pumps moving identical volumetric flow rates into and out of a reactor, this is difficult to realize without a sensor and response feedback loop or some other complication. Even small flow rate differences accumulate to significant errors over the desired time spans. Peristaltic pumps are used here for cleanliness and contamination control, but are not as precise or

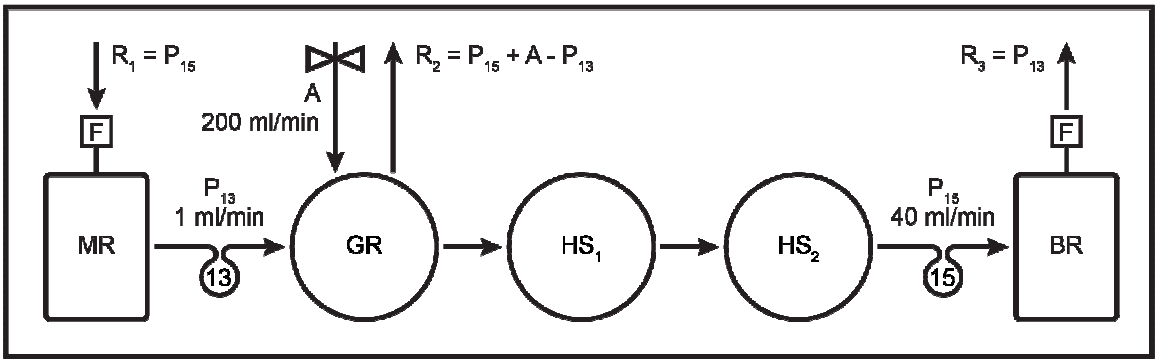

Figure 4. Gas and liquid flow is controlled by air valve and two pumps while three relief vent streams equalize pressure with atmosphere. Abbreviations follow Figure 2, additional abbreviations follow.
R_1, R_2, R_3-Pressure relief venting; R_1 and R_3 cross filters, R_2 exits through gap
13, 15-Pumps named after tubing size
P_{13}, P_{15}-Denotes flow rate defined by pump 13 or 15
Bowtie symbol denotes house air valve.

stable as other pumping technologies. It is easier for fluid height to be regulated by an outlet orifice in a fixed height position connected to a draining pump set for a large flow rate, in which case the net fluid movement through the reactor is metered by the delivery pump.

In the GR, the difference in flow rates between the supply and the larger drainage is the flow rate of reactor atmosphere mixed with the liquid stream suctioned up the GR exit dip tube. This reactor atmosphere is the exclusive gas sparge for both subsequent heat shock reactors. The difference in flow rates is due to the use of two peristaltic tubing sizes with different cross sectional areas having a ratio of 40:1. The pump heads of both lines are driven at identical rotation rates by installation onto a single drive, so the ratio of flow rates reflects the ratio of cross sectional areas. The use of a single drive for both fluid movement tasks is a simpler design over the more intuitive scheme where each stream is moved by a separate drive.

An additional benefit of a design with pumps absent between each reactor vessel is decreased inter-reactor path length and inter-reactor residence time. Significant inter-

reactor path length and residence time entails hazards of cooling and deviation from the ideal intervals of the desired temperature step function. The discrepancy in delivery and exit pump rates pushes each part of culture along by 39 parts of sparge gas at velocities 40 times greater than the net liquid movement through the reactor. The inter-reactor residence times between GR and HS_1, and between HS_1 and HS_2 is calculated to be < 0.5 seconds from a flow rate of 40 ml/min and an inter-reactor volume of 0.3 ml (1/32" ID, < 60 cm)

3.3.2.2.3 Suction preferred over blowing

The draining pump was intentionally placed to maximize path length and complexity of the negative pressure 'suction' leg relative to the positive pressure 'blowing' leg. The pump inlet is connected so as to suction the inter-reactor stream through the reactor rather than moving the stream by blowing. Glassware vessels in general, and some types of seals, are more resistant to vacuum than to positive pressure. Although intended positive pressures can be reduced by design, unintended clogs are an ever-present danger. These clogs can be due to heat treatment-associated precipitation of culture, or stray bits of tubing shed from the wear of the peristaltic pump action. An implicit design choice is to increase the chance that any clogs leaks that do arise will occur in the suction leg rather than the blow leg. While a leak in the suction leg will be harder to detect and will potentially introduce contaminants into the reactor, a clog or leak in the blow leg will eject hot acidic culture, creating a mess or damaging electronics.

3.3.3 Design Realization: materials for hot acid, parts for air-tight mating, design for a modular system

3.3.3.1 Material choices for hot acid

Wetted materials must remain inert and maintain integrity, rigidity or elasticity during continuous and prolonged exposure to hot acid (90°C, pH 3.3). Metals quickly

oxidize in such environments and are carefully excluded from wetted surfaces. Materials must also repeatedly endure temperature and pressure of steam autoclaving, and cooling to room temperature for assembly. Wetted surfaces used in this apparatus include glass and a number of polymers. The transparency of borosilicate glass allows visual inspection, the elasticity of silicon tubing forms air tight seals. Norprene tubing is flexible enough to be easily routed but rigid enough to withstand prolonged peristaltic stroking and to avoid kinking, collapse and bursting. Quick connects (Colder Products miniature SMC series, purchased from Cole Parmer) are made of ABS (acrylonitrile butadiene styrene), acetal, polycarbonate and polypropylene, with Buna-N seals. Bottle reservoirs are topped by polypropylene pour rings and circular port panels. Teflon coats magnets in sir eggs.

3.3.3.2 Mating, assembly, and connection for air tight seals

Pieces are semi-permanently *mated* to make discrete parts. Parts are *assembled* to make reactors and vessels. Reactors and vessels are *connected* to make a functional whole. Mating in many cases is between different materials, accomplished by friction or compression fits of elastic material against rigid parts. Some fits required the lubrication of a small amount of glycerin. Parts are designed to be easily exchanged upon failure, ideally without stopping reactor operation. Figure 5 distinguishes parts. The chemostat is designed to be modular such that the reactors and vessels assembled from parts pass through a small autoclave separately, and are transported through non-sterile atmosphere to the reactor site. Aluminum foil applied prior to autoclaving protects orifices from contamination during transport. Much of the assembly involves ground glass joints and port panels fixed onto bottles. Quick-connect fittings provide for easy, daily exchange of empty medium reservoirs for full reservoirs, and full biomass reservoirs for empty.

3.3.3.3 General physical organization

Reactors are housed in a laminar flow hood as pictured in Figure 3. Supporting

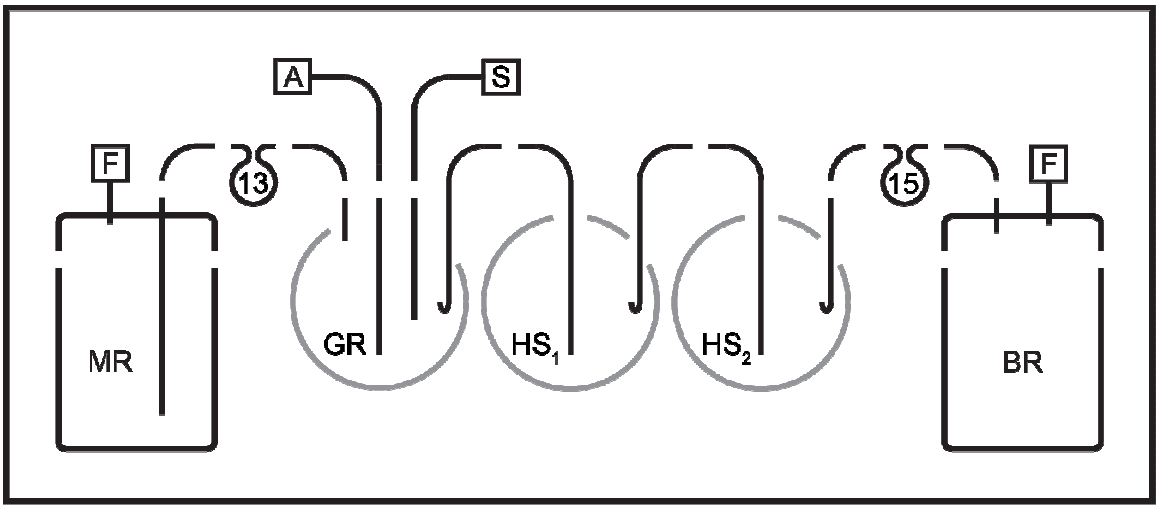

Figure 5. Chemostat is modular for easy disassembly and passage through autoclave. Note direct connection of dip tube adapters without intervening inter-reactor tubing segment. Thermocouple, PID controller and heating omitted for clarity. Abbreviations follow Figure 2.

electronic units rest on an adjacent, waist high, low temperature incubator (Fisher 146E 97-990E), bottle reservoirs rest on an adjacent table. Bricks resting on conical cap legs provide elevation to match reactor adapter heights for short inter-reactor paths. A simple latticework of aluminum rods and clamps secure the three reactors together as a unit, to protect against movement caused by vibrations of the laminar hood fan. Wiring and cords are routed to avoid hot surfaces and electric fields of the stirring and heating mantles and plates. Inducted current errors in thermocouple wires are undesired.

3.3.3.4 Modular system: parts list and descriptions

3.3.3.4.1 Reactors

Reactors are borosilicate glass with multiple necks and female ground glass joints (size 24/40). The GR is a round-bottomed flask (3 L) with four necks. The HS_1 reactor is an Erlenmeyer flask (250 ml) with three necks added by a glass blower. HS_2 is a round bottomed flask (1 L) with three necks. Reactor volumes are approximately twice the desired fluid volumes. In a half-full flask, liquid air interface and gas exchange is

maximized, and the spherical surface portion of the fluid is entirely covered and insulated by the heating mantle. The excess capacity also delays overflow spill when a reactor (the GR) fills due to leaks or clogs.

3.3.3.4.2 Reactor adapters

Each reactor port receives a glass adapter bearing both a male ground glass joint and some sort of interfacing structure such as a hose barb. Many bear integrated dip tubes which reach into the reactor. Each reactor has separate adapters for process stream entry, for exit, and for a thermocouple. The GR medium delivery adapter is the simplest; tubing is attached to a hose barb, there is no dip tube, and medium simply dribbles down the side of the reactor. Traditional concerns about microbial back-growth into medium feed lines are not germane when feeding cold medium to obligate thermophiles. There are at least two dip tubes in each reactor, directing sparging streams onto stir eggs to scatter bubbles, and positioning exit orifices to determine fluid height. The GR sparging dip tube is terminated by a glass frit. Some of the glass dip tubes are extended with polymer tubing, which is easier than glass to lengthen or shorten.

3.3.3.4.3 Exit adapters

A functional dip tube of flexible, small diameter (1/32” ID, 3/32” OD) silicone tubing runs down through a physically supporting, integrated glass dip tube and relatively rigid (Norprene) extension tubing. A small glass tube u-turn (bent in a gas flame) is connected to the silicone, and is held in position straddling the rigid tubing by silicone elasticity. The u-turn serves as an overflow weir, is fixed in place to determine fluid height, and is connected to a small diameter tubing for negligible residence time. The annulus between the tubes forms a blind cavity and will fill with (stagnant) fluid to a level far above desired reactor level unless the outer tube is punctuated by a rather large hole. The flexible silicone tubing is fitted onto a quick-connect fitting. The fitting is held in place butted up against the glass hose barb by a larger piece of tubing. The basal glass

adapter (similar: Ace glass vacuum adapter take-off 5193-08) used for the GR exit has two paths, one for the process stream and one for sampling.

3.3.3.4.4 Reservoirs for medium and biomass

Reservoirs of medium and biomass are 2 L media bottles (Pyrex 1395). Interfacing is made to a single-piece circular panel with three hose barbs. The circular panel is secured against the polypropylene pour ring of the bottle by an open-top cap (Corning 1395-32HTSC), in an arrangement similar to the lid and screw band used in home canning. The polypropylene panels are popped out of carboy caps (Nalgene 2162-0831) and serendipitously make air tight seals with media bottles. Glass pipettes serve as dip tubes, connected to the hose stems by silicone tubing supplied with the carboy cap. Sterile pressure relief venting is provided by an in-line hose-barbed 0.2 µm filter (PTFE, either Gelman Acro50, or Whatman L#J668). Both remaining ports are fitted with filters.

3.3.3.4.5 Stirring and temperature control

Thermocouple probes (ex.: Omega JTSS-116U-12) are sheathed in silicon tubing to protect the metal sheath from the hot acidic culture. Probe and tubing both pass through a glass hose barb or glass thermocouple adapter. Probe, tubing and glass adapter are chosen so a tight seal is made with a minimum of difficulty. Layering of tubing is sometimes necessary. The tubing is plugged with a small segment of fire-polished glass rod. If the plug is too thick, the tubing becomes noticeably stressed, eventually losing elasticity. Probes are immersed to at least ten diameters to minimize stem effect error. Round bottom flasks are heated by a stirring and heating mantle (Glas-Col TEM series). Mantle stirring requires a stir controller unit (Glas-Col 104A EMC4). A matched flask and mantle pair were not available in every desired volume, so an Erlenmeyer flask and a conventional stirring and heating plate (Fisher Isotemp 11-600-16SH) was used for HS_1. Independent heating and stirring control is desired; heating element connections were brought outside the chassis for connection to the temperature controller. Three different

PID Temperature controllers were used (Cole Parmer DigiSense R/S 89000-10, Barnant 689-0000, Omega Micromega CN7734-62).

3.3.3.4.6 Process stream lines and pump segments

Interconnecting process stream lines are extensively segmented by quick connect fittings. Lines between reactors are small diameter silicone tubing. Lines up and downstream of the sequence of reactors are larger diameter (Cole Parmer Masterflex Norprene size 15, 6404-15) except for an intermediately sized pump segment (Masterflex size 13, 6402-13) for medium delivery. Larger diameter tubing must be fitted over a stub of intermediate sized tubing to make a seal with quick connect fittings. Fluid is moved by peristaltic pump (Cole Parmer L/S Variable-Speed Economy Drive PM-07524-50). Two pumps heads for different sized tubing are powered by the single pump drive. Tubing wears when repeatedly squeezed by peristaltic pump, eventually deforming, shedding small slivers into the stream and leaking. Wear is confined to small, foot-long sections of tubing which are replaced as needed. Lines connected to the medium and biomass reservoirs are made long enough to move from their respective resting places to inside the laminar flow hood, for aseptic replacement.

3.3.3.4.7 Sampling and sparge lines

House compressed air is routed by Tygon tubing (Cole Parmer R-3603, 5/16” ID, 7/16” OD) through a sterilizing filter. House air is obtained from the fume hood fixture, as the pressure is much greater than that of the wall fixtures. The sampling line is a small diameter tubing (Masterflex Norprene size 13) interrupted by a short segment of silicone tubing and nylon double hose barb adapters. The silicon tubing is acted upon by peristaltic pump (Control Co 13-876-1) which moves fluid to a fraction collector (Biorad 2110) filled with plastic test tubes (Fisher polystyrene 13×100 mm, 14-956-8E).

3.3.4 Operation

3.3.4.1 Thermoacidiphile aseptic technique depends upon ethanol and slow contaminant growth

The developed technique relies upon the synergism of a laminar flow hood, conscientious cleanliness, disinfectant, and procedures designed to outrun slow growth of contaminants. The laminar flow hood is kept on continually, blanketing both reactor and work area in filter-sterile air. All surfaces brought near the reactor are wiped down with disinfectant, and all work on or near the reactor is done with disinfected gloved hands. 70% ethanol is liberally dispensed from a spray bottle. This disinfectant is mild, but offers several advantages over other options. A 10% bleach solution, combined with heat, tended to render some polymers brittle. Ethanol presents low toxicity towards personnel, and towards hot culture, from which it evaporates. Its solvent properties are used to rinse contaminants away. 100% ethanol dispensed from a squirt bottle is used to rinse medium and biomass bottle reservoirs several times between each use.

Continuous process stream flow carries contaminants away much faster than they grow. Reservoir bottles are changed out daily, and replaced with a freshly rinsed bottle. Bottles are labeled and dedicated to either medium or biomass. The entire chemostat is shielded from atmosphere contaminants by air tight seals and 0.2 µm filters, except for GR sparge gas exit. The sparge gas escapes through the loose ground glass joint of the sparging adapter; escaping gas blows contaminants away from the pressure relief vent gap.

3.3.4.2 Reactor and culture requires daily attention

Reactor operation demands at least daily attention to confirm proper cell density, replenish medium and remove biomass. Proper reactor function must be confirmed with respect to: liquid height, temperature, fluid movement, stirring and sparging. Problems such as contaminant growth, clogs and leaks can be identified before becoming larger

Figure 6. Larger scale HSC uses a 10 L Nalgene carboy as GR.

issues. High quality materials are used but do wear and age faster in such extreme duties. Kinking or pinching of tubing can lead to rupture; loss of tubing elasticity, occurring more quickly when stretched, can lead to leaks. The short lengths of tubing which grasp hose barbs or stems are occasionally cut off and the fittings are replaced onto the new end. Tubing worn by peristaltic pump action must occasionally be replaced. Ground glass joints must be regreased (Dow Corning High Vaccuum Grease) after each autoclaving.

Cell density and growth is monitored by optical density measured at 600 nm (Spectrophotometer Beckman DU-640) in disposable cuvettes. Culture is drawn continuously from the GR at 1 ml/h and delivered to a fraction collector at room temperature with a one hour fraction time. Such small volumes of culture cool quickly to

room temperature, and growth of thermophiles stop. Optical density measurements are made daily, after resuspending cells by a brief vortex.

3.3.5 Past configuration development

The chemostat has developed from a simple single-stage growth reactor, to a double-stage, and then to a triple-stage chemostat in a bid to increase cpn levels to assist the development of an effective protein isolation procedure. The reactor of each of these steps has been configured many ways in response to different aims and shortcomings. Figure 3 and Figure 6 show two contrasting forms. The reactor shown in Figure 6 is a larger scale reactor with filtration harvest. The 10 L carboy (covered in shiny insulation on left) is fitted with a black rubber stopper, cored to accommodate a residential aquarium heater (200 Watts) with temperature control circuitry removed. A round bottom flask heat shock reactor hangs at an angle to minimize inter-reactor path length. The Grant circulating water bath (tall, grey, next to blue cooler) cools culture passing through the horizontal Graham condenser before delivery to a tangential flow filtration unit for concentration (on top of the stack of electrical units; stir control, temperature controls, and pump). Cool, concentrated culture is collected in a flask in an ice bath in the cooler to the right.

3.3.5.1 Reactor vessels

Different vessels have served as reactors with varying success. Media bottles (Pyrex 1395) heated by a soldering iron element thermally coupled by an automotive type hose clamp worked well. The long neck of volumetric flasks offered a rudimentary integrated condenser for hot moist sparging gas to vent. These were abandoned for vessels with easier interfacing options, that is, multiple ground glass joints. The working volume and hence residence time of graduated cylinders is easily determined and adjusted by vertical adjustment of exit dip tube, but such a fluid geometry is problematic to maintain in a well mixed state by conventional stir bar.

3.3.5.2 Medium mixing

For a long time before the adoption of daily recharging with fresh medium, medium concentrate and water was mixed by pump. Two different sizes of tubing and pump heads on a single drive moved fixed ratios of the fluids; concentrate was made to a similar concentration factor of 40. This movement of two process streams at a fixed ratio of volumetric flow rate is also discussed above under 3.3.2.2.2 Difference of pump rates regulates volume, equals sparge rate, and reduces inter-reactor residence time. The medium mixing pump was run as needed to charge the medium reservoir. This was helpful for larger scales and operation in less clean environments outside the laminar flow hood. Daily charging with water is less problematic than charging with medium or concentrate, which must be carefully processed and protected from microbial contamination. This arrangement is seen in Figure 3, a glass 'Y'-shaped manifold tube where water and concentrate meet is connected to the bottom pump.

3.3.5.3 Odor control

Sulfolobus cultures can smell offensive. Odor has been controlled variously by routing effluent gas to a fume hood, bubbling through a 2 M sodium hydroxide base bath, and most recently, released within the laminar flow hood.

3.4 Observations

3.4.1 HSC produces heat shock: acquired thermotolerance and increased cpn

The heat shock induced by the treatments in the heat shock reactors is evidenced by a striking acquired thermotolerance, and by increased levels of cpn, two phenomena which are quantified in a table and a figure following. See B.9 Viability measurement after heat challenge and B.10 Measurement of relative cpn levels in HSC for procedural details. Table 2 shows quantification of acquired thermotolerance. Each of the four data

columns represents different conditions; different inocula origin (GR, HS_1, HS_2) and whether or not the inocula were heat challenged (HC). The numbers indicate the number of tubes (of five) to show growth at inoculation dilution factors from 10^1 to 10^7. The first column (GR) shows the growth of serially diluted culture inoculated from the GR. Growth of all 5 tubes at the 10^7 dilution indicates that at least one viable cell was transferred to each of the tubes and suggests that the GR contains at least 10^7 viable cells per 0.5 ml, the inocula volume. The zeros in the second column (GR+HC) column shows that the heat challenge (90°C, 6 h) is lethal to all the cells in the inocula. Growth of tubes in the third column (HS_1+HC) indicates that the heat treatment of HS_1 generates an acquired thermotolerance which enables cells to survive the otherwise lethal heat challenge. The numerical similarity of the first column, representing GR cells which are not heat shocked, and the third column, representing HS_1 cells which have been heat challenged indicates a saturated measure. To distinguish the viable cell densities, greater dilutions are necessary, or data concerning how long each tube took to grow. Figures in the fourth column (HS_2) confirm viability of cells eluting from the HSC.

Western Blot was used to visualize and quantify the increasing levels of cpn in samples taken from each of the reactors (see Figure 7). Western blot cpn band intensities of samples from the GR, HS_1, and HS_2 are 599, 648 and 877, respectively, per Biorad Quantity One software, indicating increasing levels of cpn.

3.4.2 Much biomass produced

The chemostat in various forms has proved to be an effective method of archaeal biomass production. Culture volumes of two strains of *Sulfolobus* approaching 1 kL have been produced, from which many tens of grams of pellet have been isolated. The apparatus has also produced 30 L of halophilic *Haloferax denitrificans* culture. Upkeep of the continuous process required a surprising amount of effort and time, especially during development of the design and protocol. Some of this time passed as the reactor

Table 2. Measurement of acquired thermotolerance produced by HSC

Condition	GR	GR+HC	HS_1+HC	HS_2
Dilution 10^1	5	0	5	4
Dilution 10^2	5	0	5	5
Dilution 10^3	5	0	5	4
Dilution 10^4	1	0	4	5
Dilution 10^5	4	0	4	5
Dilution 10^6	4	0	4	5
Dilution 10^7	5	0	5	4

Note: Abbreviations in column header row follow Figure 2; HC indicates heat challenge.

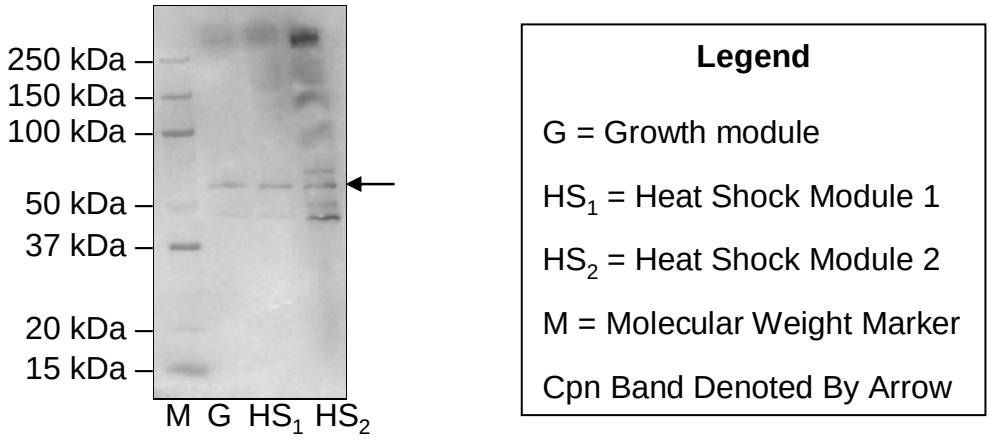

Figure 7. Western blot of cpn in samples taken from each of the reactors in the HSC

was allowed to settle to steady state for a period of time allowing 3 to 6 working volumes to flow per Sinclair & Cantero (1990). With care, the chemostat has been run continuously, uncontaminated, for several months before shut down and cleaning was necessary. Such prolonged continuous operation is likely not possible with all organisms, especially those that form biofilms. Small changes can cause unforeseen issues, even a medium change can invite problems; the richer medium of Kelly formed pieces of beige or brown congealed curd which clogged the small bore of the exit overflow weirs.

Occasionally, brown particles or films proved to be harmless and non-growing, perhaps medium precipitate or a microbe product, cooked to a solid consistency. Given exposure, room temperature culture does support the growth of a smelly off-white pellicle, and white immersed “fuzzies” resting on the bottom of the container. This contamination growth takes a week or two to become visible to the eye. Exposed medium grows the immersed fuzzies after two or three weeks.

The temperature control system typically kept process values of round bottomed flasks within 0.1°C of the set point, with rarer excursions to a 0.3°C discrepancy. The Erlenmeyer flask, with its exposed sides, experienced more frequent and slightly larger excursions. Temperature control during growth of a halophile at 37°C was more difficult and required insulation to achieve a stable process value. Some temperature controllers acquire temperature data to personal computer; this was a tremendous aid for heater sizing and controller parameterizing.

Evaporative losses approached 10% per comparison of medium and biomass volumes. The figure could be decreased by certain measures, for instance, sparging with water-saturated sparge gas, or routing escaping sparge gas through cooling condensers. Because fluid volumes are fixed, and 80% of the moist sparge gas vents from the GR, fluid residence times in both HS reactors are increased. A complete mathematical description of the impact would require knowledge of the amount of water vapor entering the reactor with the sparge gas, and the amount of water leaving the reactor from the vent streams, whether as vapor or as an entrained mist.

Foaming and subsequent fouling were issues during halophile growth, requiring a sparge rate reduction. Foaming was not an issue for *Sulfolobus* medium at physiological temperatures, although medium did foam problematically at room temperature.

3.4.3 Overflow weir eliminates problematic fluctuating fluid level

Figure 8 illustrates a problematic fluctuating reactor level. The fluctuation is caused by a hysteretic fluid column formed by surface tension underneath the exit dip tube. The long interval when fluid flow exiting the GR ceases while the reactor is "Filling", depicted middle, is greater than one quarter the desired residence time of HS_2. Further, although the height change is small, the hysteretic thermal load perturbs fluid temperature by several degrees. Affixing a u-turn fitting to the end of the dip tube to serve as an overflow weir completely obviates this issue.

Although surface level falls below bottom of outgoing dip tube, fluid continues to flow up a column maintained by surface tension ("Draining", on left, liquid levels 1-3). After column breaks (4), flow stops until surface rises ("Filling", on right, liquid levels 5-6) and eventually contacts dip tube (1), beginning cycle again. The exit stream consequently alternates between fluid for 2 minutes (while "Draining") and atmosphere for 40 minutes (while "Filling"). The reactor receives 40 ml medium during the filling, but the resultant calculated fluid flow rate of fluid exit, 20 ml/min, is only half the suctioning pump setting, suggesting that time is necessary to reduce pressure sufficiently to cause flow, if pump slippage or leaking is not to blame. The target fluid height is the widest point of the spherical flask; hence the greatest volume change is accomplished with the least change of height. Liquid agitation and sparging makes height measurement difficult. Purely geometric calculations show that a height change of only 1.5 mm accounts for the 40 ml volume change.

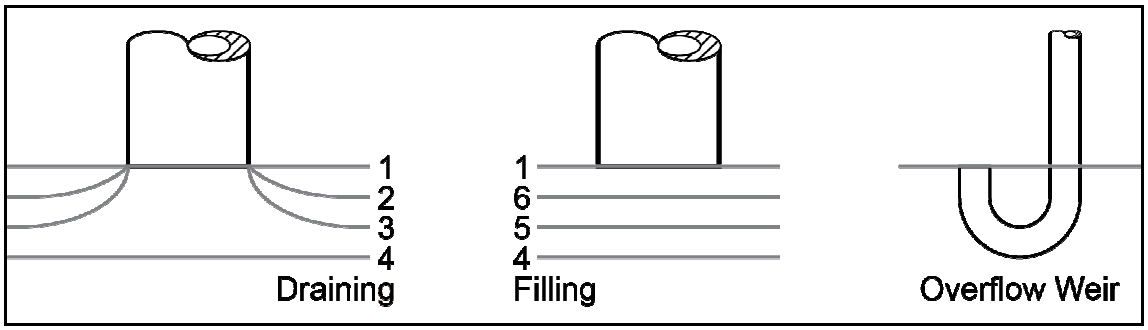

Figure 8. Fluctuating fluid levels are caused by a hysteretic fluid column formed by surface tension underneath the exit dip tube. This phenomenon is depicted left and middle. A u-turn overflow weir, depicted right, completely obviates this issue.

3.5 Discussion

3.5.1 First steady state HSC to be used with archaea may produce excess culture

The HSC produces acquired thermotolerance and increased levels of cpn. To the author's knowledge, this HSC is the first reactor to apply a static, steady state, continuous heat shock to any archaea or extremophile. This description is one of the first of a small group of relatively recent studies of continuous heat shock. The study of continuous heat shock, or the use of a continuous process to study heat shock, is likely to continue and expand.

As a production unit, the reactor produces a small, steady stream of culture, which can be more manageable than an equivalent number of large batches. However, this is not necessarily the case, and the fixed volumetric production rate combined with the daily attention required to maintain the reactor may swamp downstream capacity for processing or protein isolation. As a result of such a bottleneck, culture may be sewered, which represents a waste of time, materials and efforts. The other option is to stop the flow, resulting in a 3-6 day wait after startup to reach settle to steady state. This waiting period also consumes time, materials and effort during which no usable culture is

produced. During the tenure of the current project, the reactor produced culture at a rate that far exceeded downstream demand for the current studies.

A concern about freshness arises from the purely logistic issue of placing a batch-wise process downstream of a continuous process. After a fresh biomass reservoir bottle is put in place, the culture immediately eluting must wait for a full batch volume to accumulate. In typical practice during this project, daily harvest meant that some culture in each batch aged as much as 24 h. In contrast, the entire volume of a batch-wise grown culture may immediately be processed without delay. Not only is aging reduced in batch culture, but culture age can be considered homogenous.

3.6 Conclusions

The heat shocking chemostat is an effective way to produce much biomass, especially thermally stressed cells, with elevated levels of hsps. The reactor is also a tool to study heat shock in a steady state condition, and to study the difference between batch heat shock and continuous heat shock. More generally, the reactor produces a controlled and steady state experimental platform for studies of continuous processes. Materials have been chosen to withstand fairly aggressive conditions of hot acid, operation in other chemically aggressive environments is possible. The reactor is a modular assemblage, easily accommodating design changes or improvements, and different research directions, discussed following.

3.6.1 Possible improvements

The reactor could be refined in numerous ways while keeping its basic organization. In many ways, the current configuration is one moment in a continually evolving assembly cobbled together from available equipment. Insulation of the inter-reactor lines should be prioritized. A large baffle in the laminar flow hood separating the chemostat from the hood work area would prevent temperature fluctuations due to disturbances in the laminar flow. The HS_1 Erlenmeyer could be replaced with a round

bottom flask and mantle, a system which is more temperature stable when surrounding air flow is disturbed. Thinner thermocouples would result in faster response and better temperature control. Sparging with ambient air would yield more certainty about air quality than the house compressed air currently in use. House air may carry contaminants from the compressor and from the building lines. Although the current dip tubes work well, more durable units could be blown with small inner diameter, thick walled glass tubing. Enlarging the lines and increasing the flow rate might prevent the clogging encountered with the richer Kelly medium. Transparent process stream lines could be visually inspected for cleanliness and contamination.

In an extravagant improvement scenario, the reactor would be fitted with greater instrumentation, data collection and control. The pH and dissolved oxygen of each vessel could independently be subject to a control loop feedback mechanism, similar to the PID temperature control each vessel currently enjoys. Data acquisition of each process value and the control loop response could provide experimental dependent-variable data as well as to confirm stability and proper function when reactor is unattended. Conversely, in the case of pH control, buffering the medium (tartrate, phthalate, etc.) may accomplish a related goal. Agitation could be increased or improved with baffles or over-head driven stirrers. At great expense of simplicity, each reactor could be fitted with individual sparging streams and fluid-retaining, cooling condensers on exhaust lines. At some point during the proliferation of probes and process lines, the number of interfacings will become difficult with round bottom flasks and a move to a commercial or custom made reactor will be desired. The issue is commonly addressed by interfacing through a flat plate serving as a reactor lid which seals against a glass cylinder.

3.6.2 Possible further research directions

Future directions may be articulated as a series of questions. What is lowest growth rate possible before lag phase and washout (Novick, 1955)? Does growth rate

affect medium utilization or heat shock? Heat shock is used loosely here to refer to acquired thermotolerance, and the spectrum of expressed proteins, especially cpn. Also see whole-genome oligonucleotide microarray analysis of *S. solfataricus* strain P2 heat shock by Tachdjian & Kelly (2006) giving greater time course details of heat shock transcription than most other heat shock studies. What is the limiting nutrient? Ferenci (2008) and Tempest & Wouters (1981) discuss the starvation responses associated with different limiting nutrients. Does starvation stress, limiting nutrient identity or oxygen limitation have any effect on heat shock? Current reactor design uses two abrupt step function changes to maximize cpn production. What affect does gradual versus abrupt changes have on heat shock? See Nisamedtinov et al. (2008) for studies on yeast. The current research direction is biomass production for cpn isolation, which is discussed next.

CHAPTER 4 PURIFICATION

4.1 Development overview

Great and repeated efforts were taken to develop a purification procedure for cpn. Several strategies were pursued. This chapter is a description of the rationale and trials of protocol design; for materials, methods and procedural details of separations and analysis see APPENDIX B. The general purification strategy aims to exploit the uniquely large 1 MDa size of the cpn oligomer. Promising results were obtained with centrifugal ultrafiltration, while an undesired and unexplained disassembly to monomer, frustrated the size basis of other methods, most relevantly, size exclusion chromatography (SEC).

The purification strategy could target isolation of monomers to be re-assembled later, but it is unclear whether disassembled and reassembled cpn would be functionally equivalent to never-disassembled oligomers. Further, the disassembly might reflect serious issues in protein metabolism or purification.

Results of separation methods are summarized in Table 3. Each entry in the table is discussed following, embedded in a treatment which parallels efforts towards a comprehensive conceptual protocol development. More concretely, each step, method and condition of the entire process, from growth to separations and detection was varied and refined in an attempt to remove what is assumed to be a single and persistent error. A summary of this attempt towards a comprehensive consideration is presented in Table 4. Some of these conditions are confounded with other experimental conditions, these are indicated in parenthesis. Nearly all list entries are explicitly discussed later in this document.

Although it is possible that further development will deliver a protocol with a single centrifugal ultrafiltration step, or a different type of ultrafiltration (for example, pressurized Amicon stirred cell), most efforts included SEC chromatography because of the method's power and throughput, following the example of Cerchia et al. (2000).

Table 3. Summary of results of separation methods

Method	Potential	Comments
Centrifugal Ultrafiltration	Good	Cpn retained, undersired proteins decreased
SEC	Poor	No separation, due to disassembly
Preparative native PAGE	Unknown	NaCl interfered with method
Dialysis	Poor	No separation, due to disassembly
$(NH_4)_2SO_4$ Precipitation	Poor	Gave clean fractions, but very low yield

Table 4. Purification methods and conditions

Organism	• *S. acidocaldarius* *S. solfataricus* strains: • P1, • P2
Medium formulations	• Peeples, • Kelly
Cell growth	• Batch-grown, • Chemostat
Heat shock	• No heat shock (batch-wise) • Mild (90°C, 1 h; chemostat) • Intense (two stage: 85°C, 2 h; 90°C, 6 h; chemostat)
Temperature	Culture collection: • 0-4°C, • 24°C Purification: • 0°C, • 24°C, • 40°C
Cell storage	• Stored at -80°C for one to three days (chemostat grown, heat shocked cells) • Freshly harvested, never frozen (batch grown, not heat shocked)
Buffer formulation (constant ingredients: 50 mM tris-HCl, pH 7.5, nuclease, protease inhibitor, 1 mM DTT, 1 mM EDTA)	Constant ingredients with: • 0 and with • 250 mM NaCl Constant ingredients with: • 5% glycerol, 10 KCl, 5 $MgCl_2$, and with: • 0 and • 150 mM NaCl
Cell lysis	• Sonication, • Triton X-100, • French press
SEC resin	• Superdex 200, • Sephacryl S500-HR
SEC fraction collection tube	• Glass, • Polypropylene

Conceptual development, rationale and trials proceeded with a careful examination of published protocols, which are presented under heading 4.2 Published purifications. Although virtually no description ever satisfies absolutely every desire for detail, these examples are invaluable to deduction of themes and highlighting of specifics related to effective purification procedures.

It is possible that the problematic disassembly concerns a particular combination or permutation of conditions or methods, rather than an error. Some choices are easily judged to be beneficial, such as movement towards fresher cell mass and buffer. Other choices are more difficult to judge. The movement toward colder temperatures, while typically good for protein purifications, might be detrimental when working with a thermophilic hsp. Some choices that may make little difference, such as lysis by French press or sonication, were nonetheless explored in a spirit of trial and error. Measurement of progress and comparison of successive methods was problematic since many conditions led to a similar extremely small amount of oligomer, or small amount of separation barely detectable by a very sensitive method of silver stained PAGE.

The duration of protein purification, combined with equipment capabilities, precludes running procedures in parallel and side-by-side real time comparisons. Consequently, changes were made to experiments run sequentially, rather than the more desirable large-scale full factorial design where multiple conditions are run in parallel. Some attempts were made (4.7 Experiments to determine cause of disassembly) to identify factors promoting disassembly in crude cell lysate, but concerns about relevance favored a return to using the actual purification as an assay.

4.2 Published purifications

Published purifications are summarized in Table 5, Table 6, and Table 7. Also see the detailed account of purification with *S. shibatae* by Quaite-Randall & Joachimiak (2000), and purification reviews by Quaite-Randall & Joachimiak (1999) and

Table 5. Buffer formulations in which cells are lysed in published purifications, followed by five used in this work.

Publication		Org[a]	System[b] (mM)	pH	Mg^{2+} (mM)	KCl (mM)	NaCl (mM)	NH_4Cl (mM)	Nuc[c]	Lysis[d]	ProtInh[e]	Disulfide[f] (mM)	EDTA (mM)	Glyc[g] (%v/v)
Trent	1991	*shib*	H 50	7.5	5					0.1% T				
Guagliardi	1994	*solf*	T 50	8.0			200							5
Knapp	1994	*solf*	T 10	8.8			20							
Marco	1994	*solf*	T 10	7.7	5				D					
Kagawa	1995	*shib*	T 20	7.5	5	10				0.5% T				
Quaite-	1995	*shib*	T 50	7.5						0.25% T		10 M	1	
Joachimiak	1997	*shib*	T 50	7.5						0.25% T		0.5 D	0.5	
Nakamura	1997	7	T 50	8.0							P		1	
Trent	1997	*shib*	H 25	7.5	10	10			D					
Ruggero	1998	*solf*	T 20	7.0	10			40	D			3 D		
Cerchia	2000	*solf*	T 50	8.0	15					0.1% T		1 D	1	
Quaite-	2000	*shib*	T 50	7.5						0.5% T				
Kagawa	2003	*shib*	H 25	7.5	10	10			B	L				
1		*solf*	T 50	7.5								1 D	1	
2		*solf*	T 50	7.5					B		R	1 D	1	
3		*solf*	T 50	7.5			250		B		R	1 D	1	
4		*solf*	T 50	7.5	5	10			B		R	1 D	1	5
5		*solf*	T 50	7.5	5	10	150		B		R	1 D	1	5

Note: Abbreviations: a Organism: *shib*, *shibatae*; *solf*, *solfataricus*; 7, *Sulfolobus*. sp. strain 7. b Buffer System: H, Hepes; T, Tris. c Nulease: D, DNAase; B, Benzonase brand. d Lysis Reagent: T, Triton X-100; L, Lysozyme. e Protease Inhibitor: P, PMSF; R, Roche brand. f Disulfide Bond Breaker: M, β-mercaptoethanol; D, DTT. g Glycerol.

Table 6. Scale, buffer rate, lysis method and temperature of published purifications.

Publication		Organism	Scale	Buffer (ml/g)	Lysis Method	Temperature
Trent	1991	*shibatae*			0.1% Triton	Lyse 4℃; DensGrad 4℃
Guagliardi	1994	*solfataricus*	10 g	1	Homni Mixer, FrezThw	Clarify 4℃; Chrom atography 24℃
Knapp	1994	*solfataricus*	100 g		French Press	
Marco	1994	*solfataricus*	1.5 g	2	Vibrogen Cell Mill	Clarify 4℃; DensGrad 4 ℃
Kagawa	1995	*shibatae*	"large"		0.5% Triton	Lyse 24℃
Quaite-Randall	1995	*shibatae*	160 L		0.25% Triton	
Joachimiak	1997	*shibatae*	50 g	4	0.25% Triton, Sonic	Lyse Ice
Nakamura	1997	sp. strain 7		2*	French Press	Lyse 4℃; Clarify 5℃
Trent	1997	*shibatae*		3*	Sonic	
Ruggero	1998	*solfataricus*		2.5	Grinding Alumina	
Cerchia	2000	*solfataricus*	2.5 g	3*	0.1% Triton	Lyse 0℃
Quaite-Randall	2000	*shibatae*	150 L	5	0.5% Triton, Sonic	Lyse Ice; Clarify 15°C; all else 24℃
Kagawa	2003	*shibatae*		2*	Lysozyme, FrezThw, Sonic	Lyse Ice; Clarify 4℃; D ensGrad 4℃

Note: Scale column lists g of wet cells or L of culture. Buffer column lists rate of buffer addition to cell suspension in ml buffer/g cell pellet. Entries marked with an asterisk (*) described buffer additions in volumes or volume fold, with no specified units on pellet measurement. Abbreviations: Triton, Triton X-100; FrezThw, freeze and thaw cycles; Sonic, sonication; DensGrad, centrifugal density gradient separation.

Table 7. Major purification steps of published purifications.

Publication		Organism	Step 1	Step 2	Step 3	Step 4	Step 5
Trent	1991	*shibatae*	IEX (Q S-rose)	DEN (Gradient)			
Guagliardi	1994	*solfataricus*	SEC (S-rose 6)	AFF (Red A)			
Knapp	1994	*solfataricus*	SOL	IEX (Mono Q)	DEN (Gradient)		
Marco	1994	*solfataricus*	IEX (TMAE650S)	DEN (Gradient)	SEC (S-rose 6)		
Kagawa	1995	*shibatae*	IEX (Q S-rose)	SEC (S-cryl 300)	IEX (Mono Q)		
Guagliardi	1997	*solfataricus*	UF(300 kDa)	AFF (Red A)			
Nakamura	1997	sp. strain 7	IEX (DE52)	IEX (BToyoP)	SOL	SEC (CL-6B)	
Trent	1997	*shibatae*	IEX (DEAE S-rose)	IEX (Mono Q)			
Ruggero	1998	*solfataricus*	DEN (pellet)	DEN (Gradient)	SOL	SEC (S-cryl S200)	IMM
Cerchia	2000	*solfataricus*	SEC (S-rose 6)				
Kagawa	2003	*shibatae*	IEX (DEAE)	DEN (Gradient)	IEX (Mono Q)		

Note: Major separations steps abbreviated as follows: AFF, affinity chromatography; DEN, density based separation; IEC, ion exchange chromatography; Imm, immunoprecipitation; SEC, size exclusion chromatography; SOL, solubility based separation (ammonium sulfate precipitation); UF, ultrafiltration. Chromatography resins abbreviated: BToya, Butyl-Toyopearl; CL-6B is Sepharose based; S-rose, superpose; S-cryl, sephacryl; S-dex, superdex; H-appatite, hydroxyapatite. DEN abbreviations: gradient, sucrose or glycerol gradient; pellet, cpn is pelletted in ultracentrifuge

Joachimiak et al. (1997). The tables reflect a NCBI Pubmed search (http://www.ncbi.nlm.nih.gov/pubmed/) using the terms: “sulfolobus chaperonin”. Purifications of recombinant cpn expression in *E. coli* are not included in the tables; Nakamura et al. (1997), Yoshida et al. (1998), Koeck et al. (1998). Publications not in English (Chu, Wang, He, & Dong, 2008, in Chinese; Kirino-Kagawa, Yaoi, & Trent, 1998, in Japanese) have also been omitted. Redundancy has been reduced as follows: Guagliardi et al., 1997 and Guagliardi et al. (1995) both cite Guagliardi et al. (1994), although the first replaces the first SEC step with ultrafiltration. Condo et al. (1998) and Ruggero et al. (1998) both use the buffer of Londei, Altamura, Cammarano, & Petrucci (1986) to break cells. Yaoi et al. (1998) cite Trent et al. (1997). Schoehn et al. (2000a) cite the purification review of Quaite-Randall & Joachimiak (1999), but may have meant Quaite-Randall & Joachimiak (2000) or Kagawa et al. (1995); no two of these three similar procedures are identical. Valle et al. (2001) cite Ruggero et al. (1998). Concerning the major purification steps on Table 7: Quaite-Randall, Trent, Josephs, & Joachimiak (1995), Joachimiak et al. (1997), and Quaite-Randall & Joachimiak (2000) are identical to Kagawa et al. (1995), except that all three used FastQ ion exchange for the first step instead of Q Sepharose. Ellis et al. (1998) cites Knapp et al. (1994), substituting DEAE Sepharose for the first step.

4.3 Microorganism Growth

4.3.1 Four organisms of Family Sulfobaceae

This work began with *Metallosphaera sedula* cultures, but development of a growth protocol was impeded by an iridescent precipitate which interfered with acridine orange facilitated microscopic cell count. Three different organisms of genera *Sulfolobus* were cultivated next. *Acidocaldarius* was first, followed by two different strains (P1 and P2) of *S. solfataricus*, the species used by Guagliardi et al. (1994) and Guagliardi et al. (1995). The particular strain used by those authors, strain MT-4, was not grown; three

separate shipments from ATCC were either not viable or arrived with broken test tubes. However, the P1 and P2 strains and the MT-4 strains are “probably identical … or similar” (Zillig et al., 1980).

A growth curve is necessary to identify an appropriate harvest window. Figure 9 shows an extremely well-behaved curve giving a high coefficient of determination (R^2) due to an auto-sampling assembly and a spectrophotometer. See B.2.5 Growth curve by peristaltic pump sampling and fraction collector for details. In general, the highest density culture still in logarithmic growth phase is desired to maximize yield of healthy, actively growing cells.

4.3.2 Medium formulations

Two media formulations were used. Peeples formulation was used initially, and for the bulk of the work. A move to the richer Kelly formulation yielding more biomass was made for the most recent work. This formulation was used for batch-wise grown cells, but tended to clog the small tubing of the reactor; only several chemostat pellets were generated with Kelly formulation medium. Initial sterilization by autoclave was later replaced by the more gentle method of 0.2 μm filtration.

Large Nalgene carboys of single strength medium were eventually replaced by 2 L glass bottles of forty-fold concentrate for convenience. Large carboys of medium and bottles of concentrate sat at room temperature while attached to the chemostat. Most recently, due to concerns about freshness, single strength bottles of medium were stored at 4°C and discarded after several days.

4.3.3 Batch and chemostat growth

An initial growth strategy targeted generation of a relatively large amount of several liters of culture per day by chemostat operated close to washout, in a bid to compensate for low percentage yield with great throughput. The largest chemostat employed a 10 L Nalgene carboy as a growth reactor. This eventually proved unwieldy,

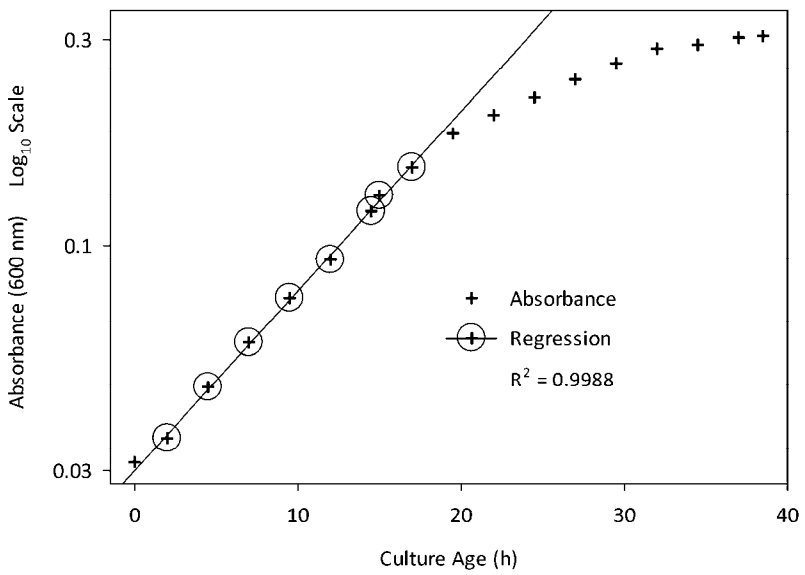

Figure 9. Growth curve of *S. solfataricus* strain P2.

and large culture volumes were exchanged for heat shocking to produce much cpn. Flow rate was reduced to give an approximate 24 h doubling time (1 ml/min, 1.5 L working volume; 0.0417 h^{-1} doubling time; compare to batch data of Figure 9: 7.2 h, 0.139 h^{-1}), and a heat-shocking vessel (1 h, 80°C, 10°C above growth temperature) was added. After some time, a second heat-shocking vessel was added. In that configuration, organism was grown at 70°C, heat shocked for 2 h at 85°C, and then heat shocked for 6 h at 90°C per Cerchia et al. (2000). For details, see Chapter 3 Heat Shocking Chemostat to produce chaperonin and study heat shock. Most recently, due to suspicions that either continuous growth or heat shock was promoting cpn disassembly, cultures only several generations removed from the original gift of Kelly were grown batch-wise, with no heat shock in 1 or 2 L cultures in 2 or 4 L Erlenmeyer flasks in a shaking oil bath, without heat shock. These suspicions were based upon the following published observations. The spectrum of hsps expressed in continuous culture is different from batch cultures, at least for *E. coli* (Luders et al., 2009), indicating that heat shock is not necessarily identical for these two growth modes. Continuously grown cultures are constantly nutrient limited and exhibit a

nutrient-specific starvation stress response (Ferenci, 2008; Tempest & Wouters, 1981). In contrast, batch cultures become limited towards the end of growth, perhaps by nutrient exhaustion, or perhaps by waste accumulation. Ferenci (2008) warns that mutations cause continuous cultures to become heterogeneous populations within 100 generations. In this system, 100 generations at a 24 h generation time is over 3 months, a time period which is attainable, but seldom realized for this work. Note that White et al. (1997) were unable to purify GroEL in particulate form after mutation of a single amino acid, Glu(glutamic acid)386. The residue occurs 14 times in the homooligomeric GroEL 14-mer, and normally forms two salt bridges evidently important for oligomer stability. Purified cpn has been observed to disassemble *in vitro* at high temperatures, by Kagawa et al. (2003) at 94°C, and by Yaoi et al. (1998) at 90°C. Trent et al. (1991) observed the purified complex become labile *in vitro* at 80°C.

4.4 Purification conditions

4.4.1 Scale and concentration

Typical cell weight in this work has been 0.5-1 g wet weight. This is smaller than any of the amounts seen in Table 6, and at least one procedure is one hundred fold greater. But even increasing the yield of this project by a similar factor would not yield a significant, or even a nominal amount of oligomer. Scale is not the issue. However, the related parameter of concentration may be the issue. Lissin (1995) notes differences in extent of Mg-ATP induced re-assembly of chloroplast cpn between concentrations of 15 and 60 μM, and Quaite-Randall et al. (1995) were able to reassemble diluted *S. shibatae* subunits to oligomers simply by concentration to levels over 10 μM. Overlaid on top of the expected dynamic chemical equilibrium of association is the possibility that the allosteric binding cooperativity produces a steep association curve describing a process behaving somewhat like a sharp threshold value. Cooperative association is not specifically discussed in the *Sulfolobus* literature, but is observed in other cpns

(Kusmierczyk & Martin, 2001). Cooperative association might be expected if assembly and disassembly occurs as steps in cpn activity, as suggested by Quaite-Randall et al. (1995).

The volume of buffer (4 ml) added to cell pellet per g of cells in this work is several fold larger than figures in Table 6, with two exceptions. Application to SEC in high resolution or analytical amounts might reduce further concentration enough to favor monomers. This is the view of H. K. Kagawa (personal communication), who was not familiar with the single step SEC purification of Cerchia et al. (2000) which is discussed later in this document.

4.4.2 Temperature: 0 to 40°C

Temperature is a crucially important parameter in many processes. Purification work with this thermophilic organism initially began at room temperature, was later reduced to refrigerator and ice bath temperatures for the bulk of the work in accordance with general protein purification procedures. It is assumed that cooler temperatures favor stability. The purification was also once conducted at 40°C. This hot purification used freshly harvested batch-grown culture which was harvested, washed, clarified and chromatographed all at 40°C, the maximum recommended temperature of Sephacryl SEC resin. Purified cpn has been observed to disassemble at high temperatures as discussed at the end of 4.3.3 Batch and chemostat growth. Even the moderately high temperature of 55°C will cause Ssocpn disassembly in 4 M urea, while cpn is stable in 8 M urea at 24°C (Knapp et al., 1994). This finding might argue against purification at higher temperatures. A temperature incubation experiment showed no disassembly in lysates at 0, 4 or 24°C. The assay detected monomers in filtrates of 100 kDa filters. This negative finding suggests that this experiment may not reproduce what occurs during SEC.

Quaite-Randall & Joachimiak (2000) state that since *S. shibatae* is thermophilic, purifications may be carried out at room temperature and probably at 4°C as well. These

authors sonicated on ice and clarified lysate at 15°C. Protease activity of thermophiles is presumed to be low at room temperature. Table 6 shows several temperature indications during published purifications. Approximately half of the procedures list reduced temperatures during lysis, probably to decrease protease activity, or to counteract heat generated by lysis methods such as sonication. Approximately half of the procedures indicate reduced temperature for centrifugations during clarification or density gradient separation, perhaps due to the relatively long time spans. Four procedures indicate no temperature. It is not clear whether the omissions are due to an assumption that reduced temperatures are standard during protein purification, or an assumption that absence of prescription indicates room temperature.

4.4.3 Buffer formulation

Buffer formulation is crucial in any protein work. Formulation in this work has been carefully reviewed with respect to the published literature. The most recent formulation uses the most common buffer system and includes all stabilizers. Table 5 lists published buffer formulations, followed by the sequence of five buffer formulations used in this work. Buffers listed are those used when cells are broken. Formulation variations are often encountered later in the purifications. The tris buffer system is by far the most popular seen in the table. A molarity of 50 mM is used in over half of the tris buffers. A pH of 7.5 is used in over half of all the procedures. The buffer system used throughout this work is 50 mM tris, pH 7.5. The ions Mg^{2+} and K^{1+} were included in the last two buffer formulations because of their role in phosphorylation, ATP hydrolysis, assembly and disassembly. Magnesium is included in half of the procedures, and in three of the five *S. solfataricus* procedures. Potassium is listed three times, each time with *S. shibatae*. If cpn of *S. shibatae* and *S solfataricus* are sufficiently different, it is possible that potassium leads to disassembly. This would not, however, necessarily explain the disassembly that occurred when neither ion was included in the buffer formulation.

NaCl is commonly used to adjust ionic strength and elute bound proteins from ion exchange chromatography (IEC) resin. Guagliardi et al. (1994) and Knapp et al. (1994) both use NaCl (200, 20 mM, respectively) with *S. solfataricus*. Other salts are also used for these purposes. NH_4Cl is used by Ruggero et al. (1998) in general buffer (40 mM) and is used to resuspend ultracentrifuge pellet (500 mM) by both Condo et al. (1998), and by Ruggero et al. (1998). In this project, after some work with no NaCl, 250 mM was included for some time after the post-IEC buffer formulation of Joachimiak et al. (1997). A return to a no-NaCl condition was made with the addition of Mg^{2+} and K^{1+} to the formulation. Later a modest 150 mM NaCl was re-added to the formulation, to prevent ionic interactions with the size exclusion resin per manufacturer's recommendation (Sephacryl, Amersham Biosciences, 1996).

Benzonase brand (> 90% purity, Merck) nuclease was used at a rate of 1 µl (25 units) per batch (one to several ml) throughout this work to reduce viscosity; the reduction was marked. Less than one third of the purifications published include nucleases. Two of the four instances concern *S. solfataricus*. Only Kagawa et al. (2003) used Benzonase (1 unit per ml); with *S. shibatae* (and *E. coli*). Phipps et al. (1991) note that one benefit of IEC is the removal of nucleic acids "without resorting to the use of DNase and RNase". Benzonase is probably dispensable, but makes fluid handling easier, and is not suspected to contribute to disassembly.

Roche Complete Mini (EDTA free) protease cocktail inhibitor tablets were used throughout the current work although indicated in the table only once. Whether or not due to the activity of the cocktail, protease activity is not suspected to play a role in the problematic cpn disassembly, since the characteristic molecular weights of the cpn monomers (as shown by PAGE) are observed in sharp bands, while proteases would be assumed to cleave a protein into sizes different from the monomer.

Disulfide bond breakers (DTT, 2-mercaptoethanol) are listed four times, twice with *S. solfataricus*. A modest 1 mM amount of DTT was used throughout this work.

Quaite-Randall & Joachimiak (2000) use 5 mM for storage of *S. shibatae* cpn. Quaite-Randall & Joachimiak (1999) use 5 mM DTT for storage of GroEL and the cpn of *T. thermophilus*. *S. solfataricus* strain P2 cpn has a single cysteine in each of the α and β subunits, and none in the γ gamma subunits; this structure is not expected to form disulfide bonds.

EDTA is added to purification buffers to chelate metal ions and inactivate metalloproteases, or to inhibit microbial contamination. It is listed four times in the table, once with *S. solfataricus*. It has been used throughout this work. In formulations with Mg^{2+} or K^{1+}, it is probably saturated with these ions.

Glycerol can stabilize proteins (Lissin, 1995) and promote oligomerization (Darke et al., 1996). Glycerol is listed once, with *S. solfataricus*. An amount of 5% was added in the later parts of this work. Ellis et al. (1998) stored *S. solfataricus* cpn with 20% glycerol. Quaite-Randall & Joachimiak (1999) note the use of 50% glycerol in the storage of both Type I and Type II cpn at -80°C. These same authors (2000) use 50% to store *S. shibatae* cpn. See discussion under 4.5.6.3 Cerchia et al.'s purification for a discussion about the possibility that glycerol was used in that protocol. An increase in concentration might be beneficial, but is not suspected to be the controlling factor in the disassembly.

4.5 Particular methods explored

4.5.1 Cell harvest by centrifuge

Chemostat culture is cooled by elution onto ice or into a cold incubator. Batch cultures were initially cooled while spinning by refrigerated centrifuge rotor. Most recently, batch culture is chilled prior to harvest per Zaparty et al. (2010). Culture cooling is not specified in any of the *Sulfolobus* cpn purifications surveyed for this document. Harvest by centrifugation was found to be more efficient and less invasive than tangential filtration (Minitan, Millipore).

4.5.2 Cell lysis: five methods

Among the five *S. solfataricus* entries on Table 6, six different lysis methods are listed, with no method listed twice. This suggests that a variety of cell lysis methods produce acceptable yields of intact oligomer. Several cell lysis procedures were tried in this work. Freeze and thaw cycles were ineffective as measured by Lowry assay and phase-contrast microscopy. A microfluidizer (Microfluidics 110-Y) was effective, but the holdup demanded an undesirably large scale. Probe sonication was effective and used for the bulk of the work. Sonication is seen on the table four times (each time with *S. shibatae*). French pressing is listed twice (once with *S. solfataricus*) on the table. Suspension was French pressed for the most recent experiments in this work.

The most popular method, Triton X-100, is listed six times, (once with *S. solfataricus*). However, Triton X-100 (0.5% v/v) was found to cause disassembly to monomers in this work, as seen in Figure 10, where monomers (60 kDa) are detected by SDS-PAGE Western Blot analysis of 100 kDa centrifugal filtrates of cell lysate. In contrast, no monomers are detected in filtrates of lysate produced by sonication (120 W, 10 min) or French Press (3 passes at 18,000 psi), suggesting that cpn remained as oligomer (1 MDa) and was completely retained by the filters.

It is not surprising that a detergent (Triton X-100) promotes disassembly of subunits held together partially by the hydrophobic effect, but it is surprising that it does so at a level comparable to those seen on Table 6. Trent et al. (1991) used 0.1% Triton X-100 to lyse cells, then diluted to 0.01% prior to centrifugal clarification and IEC. The dilution step could be due to a reversible cpn disassembly, or could be due to difficulties using IEC with Triton X-100 treated sample, as suggested by the IEC procedure of Shimamura et al. (2003). In that procedure, sample from cells lysed in 3% Triton X-100 was loaded onto DEAE Sephacel rinsed with 0.1% Triton X-100. The column was rinsed with 0% Triton X-100 prior to elution by NaCl.

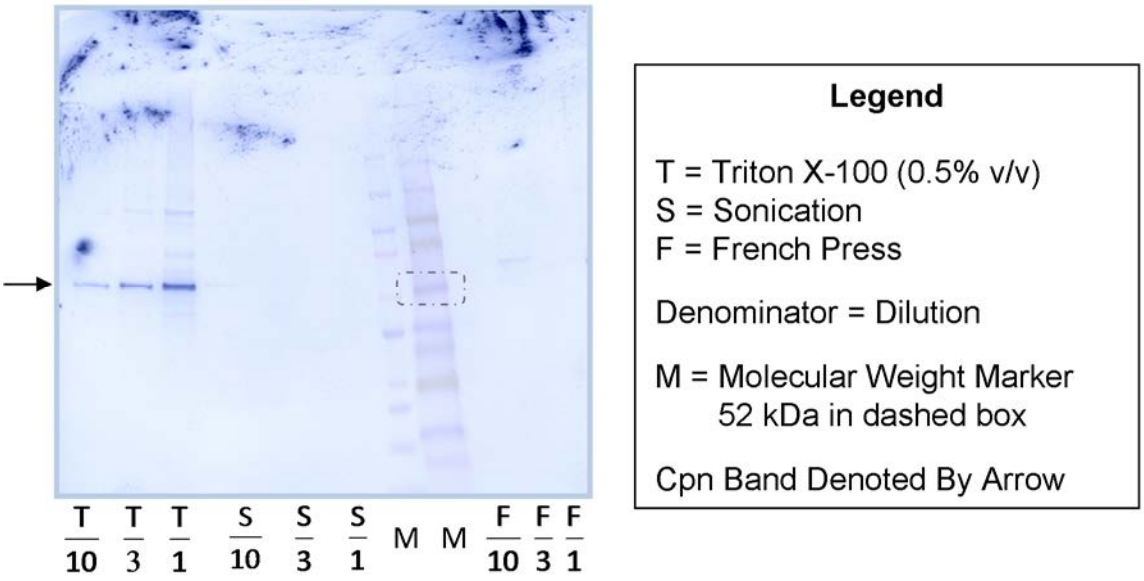

Figure 10. Western blot detection of monomer in filtrates of cell lysate generated by three different lysis methods

4.5.3 Lysate clarification: centrifugation preferred over filtration

Cell Lysate is clarified by centrifugation. Initial purifications involved much filtration and pre-filtration to clarify and to rinse away low molecular weight cell constituents and isolate cpn. Several types of filters (pressurized stirred cell and centrifugal filters, both by Amicon; syringe filters; vacuum filters) were used in sequences with decreasing pore sizes. Later, and for the bulk of the work, filtration was minimized due to concerns about adsorptive losses and surfaces possibly catalyzing disassembly.

4.5.4 Major purification steps in the literature

The published purifications appear straightforward, perhaps typical. Few details are given, giving a sense of a routine procedure. A notable detailed exception is Quaite-Randall & Joachimiak (2000). The major purification steps of published procedures are summarized in Table 7. IEC is the most popular technique, listed seven times, six times

as the first step. Four procedures list IEC twice. SEC is almost as popular and is listed six times. Given the uniquely large size of cpn, it is curious that SEC isn't more popular. erhaps resin sized for such a large molecule isn't often pre-existing in lab inventory, or perhaps density based separations are used instead. Only one procedure uses neither SEC nor a density based separation. Density based separations such as glycerol or sucrose gradients are listed five times, almost as many times as SEC.

Permutation of separation steps could affect cpn disassembly. Protein stability depends in a general way upon solvent character and co-solutes and in a specific manner by particular ligands. Different separation techniques concentrate, dilute, or separate target proteins from stabilizers and de-stabilizers in different ways. Considering this in conjunction with the table, it might be argued that IEC be the first step in cpn purification. Conversely, the prevalence of IEC as a first step may simply be a reflection of the ease with which IEC handles large sample volumes, or due to commonly available resin inventory.

4.5.5 Diafiltration

Perhaps the most promising separations seen are those of a 100 kDa diafiltration experiment seen in Figure 11. Diafiltration effectively rinses undesired protein away from cpn, but requires vortexing every five minutes to prevent visible solute polarization. Vortexing can be detrimental to proteins. This decreases the attractiveness of this method.

The figure shows analysis after lysate was concentrated to half volume and re-diluted ten times. Although cpn is detected by Western blot ("Overview", upper left) eluting to filtrate in the first round of filtration ("F0"), which represents loss of material, none was detected in the tenth round ("F10"). Comparison of lanes "0" and "10" in the coomassie gel of "Retentate" (bottom left) shows a significant reduction in undesired protein. Note that all retentate lanes are diluted ten times relative to filtrate lanes, and that the coomassie stain used for the "Retentate" is much less sensitive than the silver stain

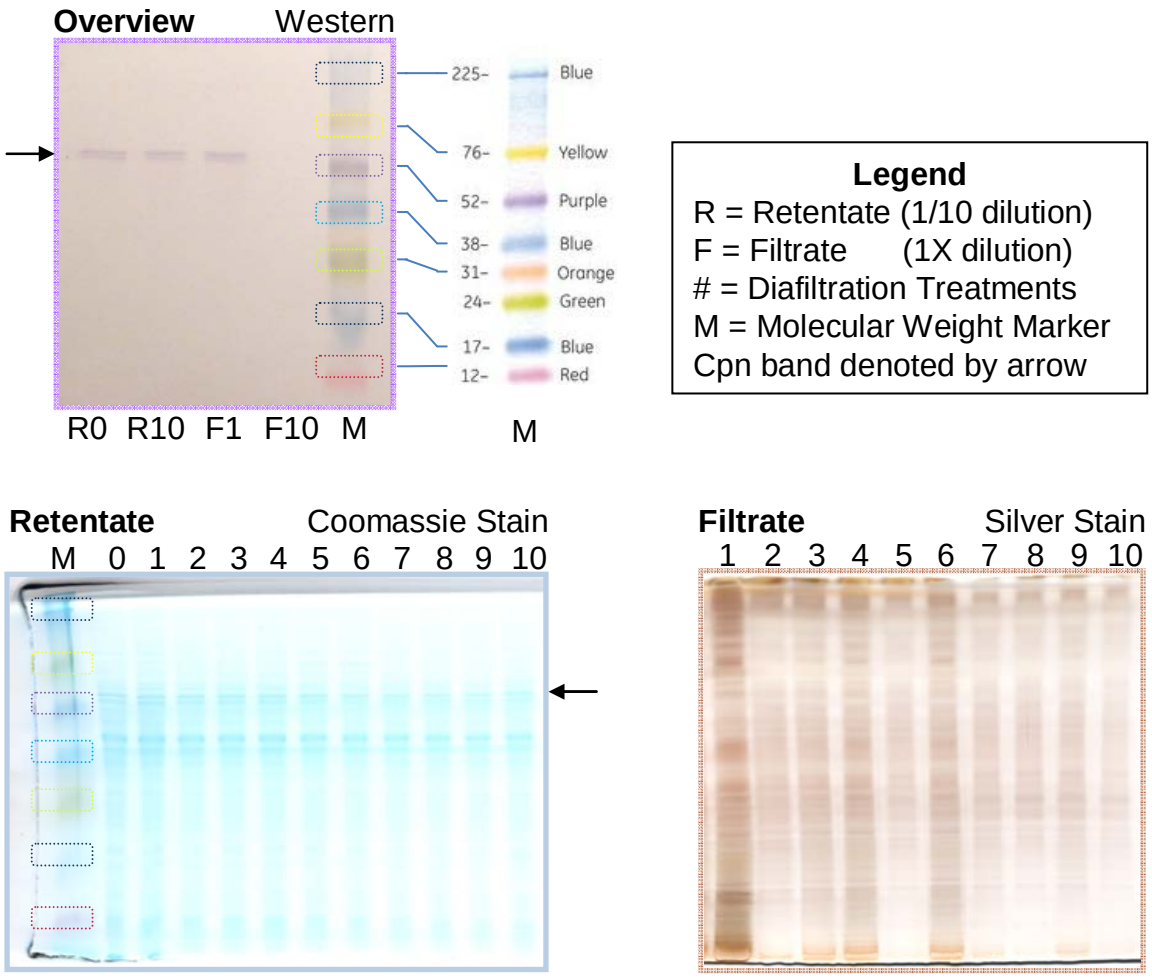

Figure 11. Centrifugal 100 kDa diafiltration of lysate

used for the “Filtrate” (bottom right). The variation in the filtrate gel may be due to centrifugal polarization, or to sampling or dilution error.

Note that Quaite-Randall & Joachimiak (2000) report losses of 10-20% when using some of the first 300 kDa filters on the market, suggesting a distribution of pore sizes in the filters, or particles of cpn < 300 kDa, perhaps due to an assembly and disassembly equilibrium.

4.5.6 Chromatography in this work: IEC and SEC

Most efforts of purification development included SEC. Four different chromatography resins were used in this work. Initial work commenced with DEAE IEC, but was quickly shelved in favor of exploiting the unique and large size of cpn with SEC. The first SEC resin used was Superdex 200 resin, eventually abandoned in favor of the more appropriately sized (GE Healthcare Bio-Sciences AB, 2007) Sephacryl S500HR resin. Buffer flow was 1 ml/min for most of the work, but was decreased to 0.5 ml/min to increase resolution. Clarified lysate is typically diluted below recommended resin concentration limits and applied in small 1% volume loads for high resolution per GE Healthcare Bio-Sciences AB (2007). A larger load, double SEC procedure is described in B.8 Chromatography.

The often-seen repeated result of SEC is illustrated in Figure 12. See calibration curve in B.8 Chromatography. No cpn is detected by Western Blot of SDS-PAGE (bottom panel) where large molecules are expected, towards the beginning of the elution (top panel); instead, much cpn is detected later in the elution, mixed with the bulk of the undesired proteins. A reasonable interpretation is oligomer (1 MDa) disassembly to monomers (60 kDa) before or during SEC. This SEC trace is further discussed in the following two sections: 4.5.6.1 Abs280 Preliminary peak and 4.5.6.2 Early Monomer Elution.

SEC has been a constant for most of this work. Although rigorous cleaning methods were eventually adopted, it is possible that the in-house resin still harbors material from the earliest work, which may be catalyzing disassembly. This would not explain two SEC runs at the Protein Crystallography Center, described in B.8 Chromatography.

Cpn disassembly during purification is mentioned in the literature, Marco et al. (1994), working with *S. solfataricus*, and Waldmann et al. (1995a) working with *T. acidophilum*, both note that 5-10% of cpn eluted from SEC as monomers.

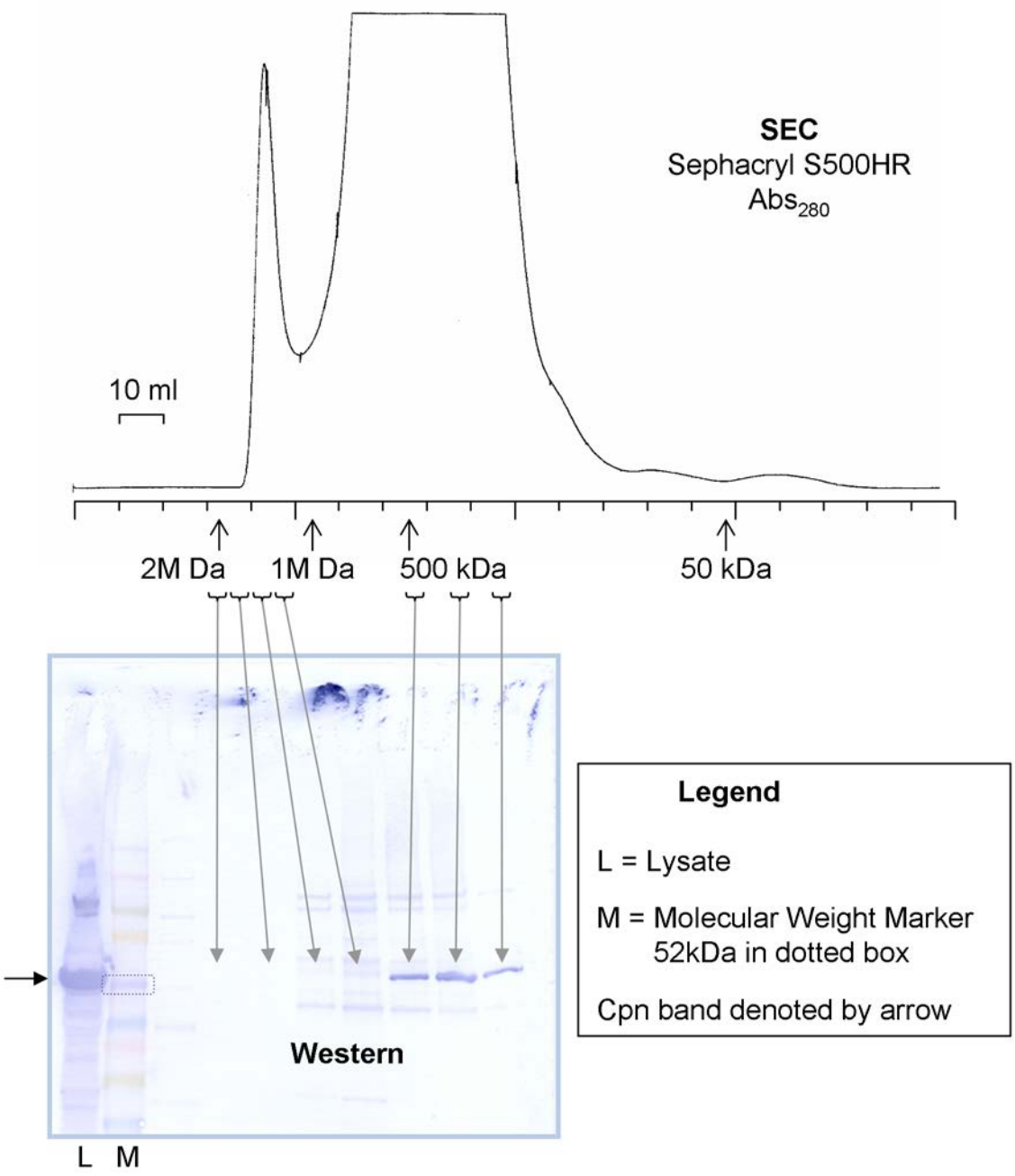

Figure 12. Western blot analysis of SEC fractions.

Kagawa et al. (1995) notes the presence of both oligomer and monomers present in IEC fractions. Yaoi et al. (1998) observed both subunits and oligomers in purified cpn. Quaite-Randall et al. (1995) purified monomers directly from lysate.

4.5.6.1 Abs_{280} Preliminary peak

The first Abs_{280} peak elutes from SEC where oligomer is expected, and also elutes with a residence time of cpn monomers after heating with SDS. However, analysis by silver-stained SDS-PAGE and Western blot shows a mixture of proteins and only the incredibly small amount of cpn accessible to the high sensitivity of silver stain. This small amount of cpn may be considered background noise, as it is detected in many of the fractions.

The first peak is prominent in the Figure 12 Abs_{280} trace generated by chart recorder set to highlight this peak, at the expense of truncating the top of the second peak. The precise composition of the Abs_{280} peak is interesting insofar that it might be cpn which is somehow unavailable to SDS-PAGE analysis, by loss due to surface adsorption, or by a size-changing phenomenon such as aggregation or proteolysis. Similarly, Marco et al. (1994) report an Abs_{280} peak eluting prior to the oligomer during SEC, later found to be empty by Coomassie blue stained SDS-PAGE.

4.5.6.2 Early Monomer Elution

Cpn monomers detected by Western blot elute from SEC earlier than expected per calibration curve. This is interesting insofar that it is possibly related to the absence of oligomer. Note that the early, immunologically detected cpn peak elutes with the bulk of the undesired cellular components of the crude lysate. The calibration curves may not be analogous, or the bulk of the components may be associating, appearing enlarged per elution time. Early cpn elution is consistent with at least four other scenarios.

The first is an increase in effective size by association to another particle. Condo et al. (1998) observed co-purification of cpn with several polypeptides (120, 90, 30, and 20 kDa), Ruggero et al. (1998) observed co-purification with the 16s rRNA (nucleotide and some proteins). The second possibility is in-column disassembly. Oligomer entering the column and subsequently disassembling mid-column would elute as monomers earlier

than monomers entering the column. The third possibility is an oligomer with an unexpected number of subunits. Fourthly, it is possible that what is interpreted as monomer eluting along with low molecular weight constituents, is actually oligomer which has experienced a delayed elution. Delayed elution could be due to a non-specific adsorption between resin and cpn or even between the resin and something bound to cpn. This would be heartening after a fashion, but note that, nonetheless, little separation is indicated. The later peaks expected to be monomers are wider than the earlier ghost peak. Peak spreading is expected with longer elution times, but could point to a non-homogenous population of particles externally bound to cpn, or to an ongoing, in-column, size changing process such as disassembly and assembly.

4.5.6.3 Cerchia et al.'s purification

The most notable published procedure is the single step SEC purification of Cerchia et al. (2000). That work is especially relevant because of its simplicity and because the bulk of the current work was also a single SEC step. Cerchia et al. applied 30 mg crude extract to a SEC column approximately 300 ml in volume to obtain 3.6 mg pure cpn / g cell mass. This yield is over ten times the yield of 0.25 mg cpn / g cell that Cerchia et al. obtained with the method of Knapp et al. (1994). Although the ratio of SEC load column volume approximates that used in this work, (generally 10-20 mg / 100 ml) dilution in SEC may still account for disassembly as Cerchia et al. designed and applied a two stage batch-wise heat shock to increase cpn levels. Although a similar two stage heat shock was eventually implemented in this work in continuous culture (Chapter 3 Heat Shocking Chemostat to produce chaperonin and study heat shock), continuous and batch-wise heat shock may not be identical.

Cerchia et al. cite Hüdepohl, Reiter, & Zillig (1990) for lysis, who add glycerol to the crude extract for storage at -70°C. In the most literal and strict reading, the protocol of

Cerchia et al. does not appear to call for glycerol, but a different understanding may lead to such addition.

4.6 Miscellaneous Methods

Ammonium sulfate precipitation of crude lysate produces pellet fractions which appear to contain only cpn by coomassie stain, but the yield is extremely low, < 1%, as seen in Figure 13. The figure shows coomassie gels of “Supernatant” (upper) and “Pellet” (middle) of 11 different lysate aliquots containing different amounts of ammonium sulfate. Faint, isolated cpn bands are seen magnified in the “Pellet Detail” (lower). The low yield combined with the possibility of damage by this invasive procedure reduces the attractiveness of this method.

PAGE analysis of 100 kDa dialysis retentate of crude lysate over several days showed a cpn band which faded at a rate similar to other bands; cpn did not become enriched, as seen in Figure 14. The figure shows retentate composition at three time points, 0, 2 and 16 hours, with each at two dilutions, 1/10 and 1/30, left and right of the central molecular weight lane.

Preparative native PAGE was thwarted by the low conductivity of buffer with 150 mM NaCl. This method may be viable upon further development, but SEC is generally considered to be a more effective and less laborious method.

4.7 Experiments to determine cause of disassembly

Preliminary experiments to investigate what aspect of the SEC was causing disassembly targeted monomers eluting to 100 kDa centrifugal filtrates. The results were not easily interpreted. The only two conditions yielding detectable subunits after 2 h were the conditions of dilution and incubation with SEC resin. However, dilution in the presence of resin did not yield detectable subunits. Overnight incubation produced subunits in all conditions. The experiment should be repeated for confidence, perhaps redesigned to detect native oligomer directly. The 100 kDa separation or the 10 kDa

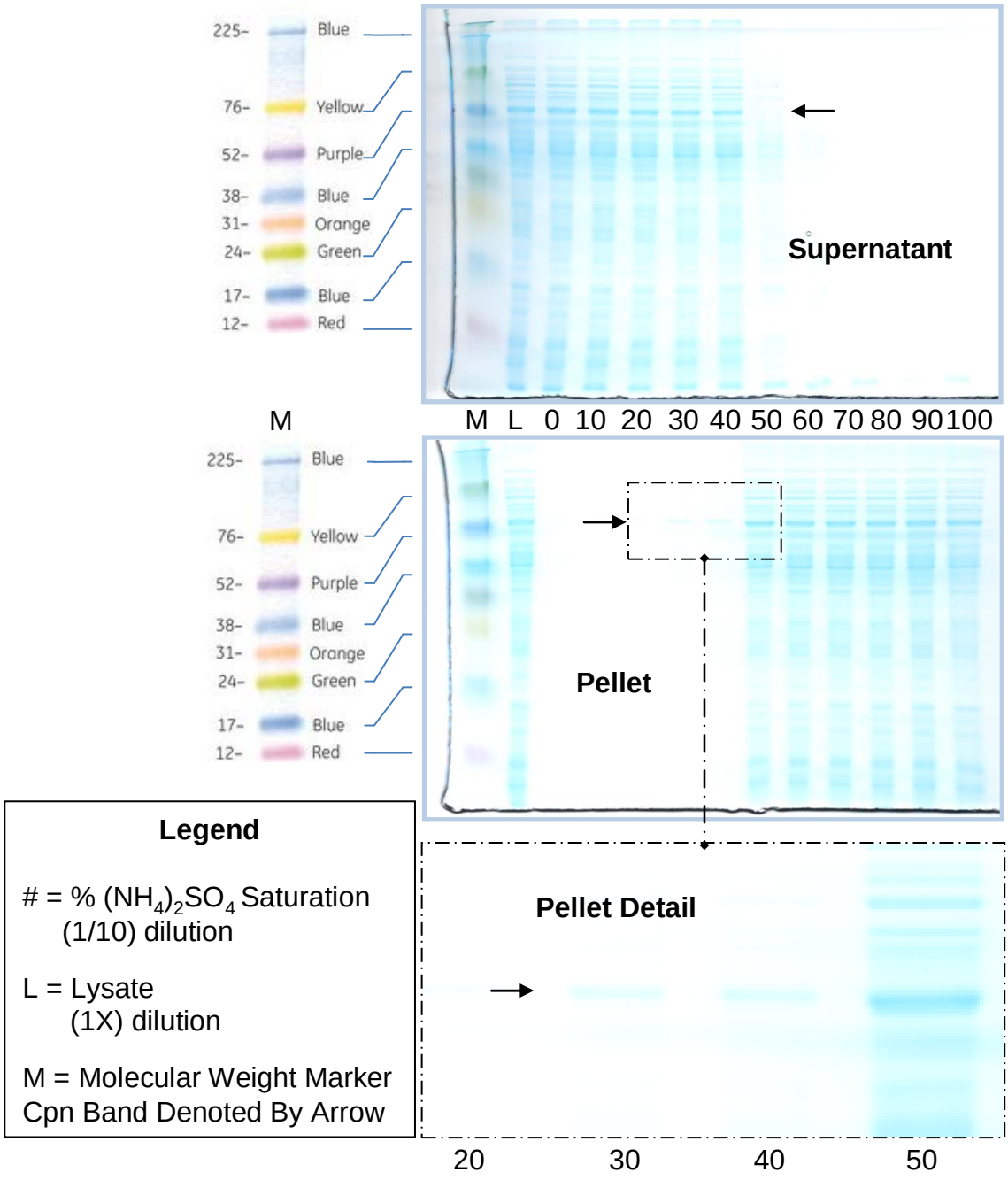

Figure 13. Ammonium sulfate precipitation analyzed by SDS-PAGE and coomassie of 11 solutions with different concentrations of ammonium sulfate.

concentration of the resulting filtrate may be confounding results, by providing adsorptive surfaces, or by separating cpn from stabilizers or destabilizers in a manner different from SEC. This sort of disassembly assay may or may not be directly applicable

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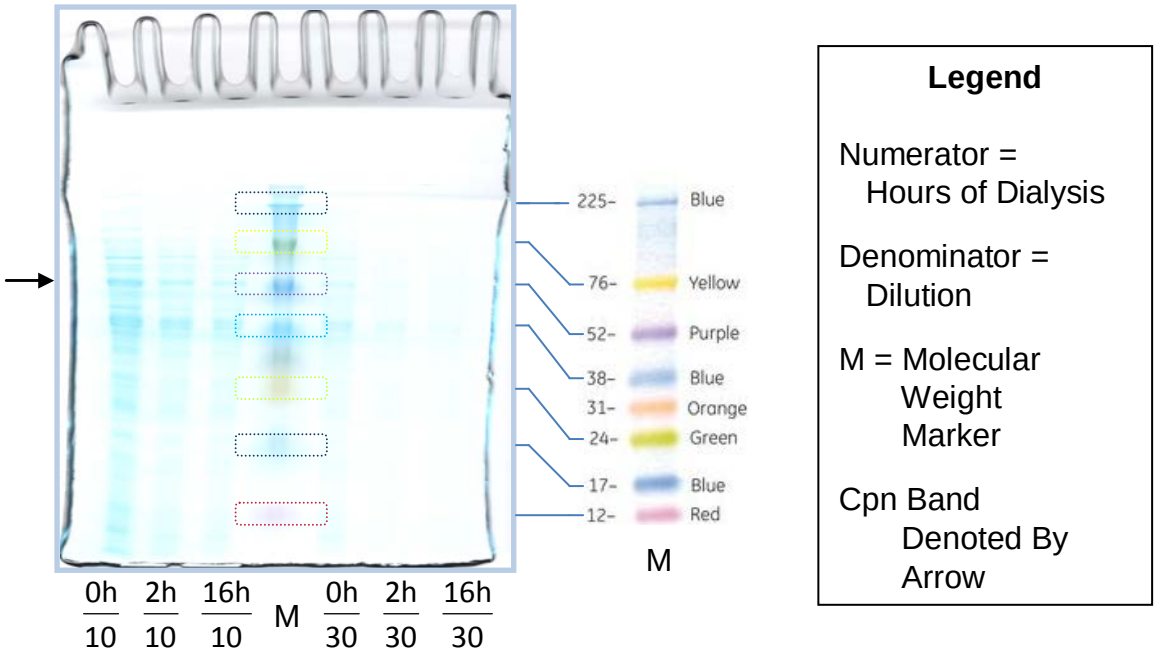

Figure 14. Dialysis of lysate analyzed by SDS-PAGE and coomassie at three different time points and two different dilutions.

to what happens during SEC chromatography. Taking each individual observation at face value suggests that dilution and resin both destabilize oligomers. This suggests that larger or more concentrated loads be applied to the resin, or suggests movement away from Sephacryl S500HR resin.

4.8 Conclusions

Many trials along with many variations of each step in the process have produced fractions with disassembled cpn monomers mixed with like-sized lysate undesirables. A persistent error or a combination of conditions promotes disassembly. The currently suspected cause is low cpn concentration, discussed following.

4.9 Recommendations for further work

Recommendations are prioritized following:

1) More SEC based attempts appear warranted. Cerchia et al.'s purification should be replicated, or perhaps used as a guide with an eye towards increasing cpn concentration (per H. Kagawa's suspicions, 4.4.1 Scale and concentration) throughout the procedure. This could include batch-wise two stage heat shock, reduced rate of buffer addition to cell mass and larger, preparative SEC loads ignoring manufacturer's concentration limits (per L. Gakhar, 4.5.6 Chromatography in this work: IEC and SEC). All SEC fractions should be analyzed by PAGE, in case of triple peak phenomenon as encountered by Marco et al. (1994) (empty ghost peak, oligomer, monomer, see 4.5.6.1 Abs280 Preliminary peak). Fractions believed to contain monomers should be analyzed for oligomer content. Faithful reproduction would entail ultracentrifugation clarification, Superose 6 resin and *S. solfataricus* strain GΘ. Cerchia et al. lysed cells with 0.1% Triton X-100, but this has been observed to cause disassembly in this work. Their buffer formulation included magnesium but no potassium, a combination which has not been tried in this work.

2) SEC has been constant in the work. Other methods could be explored, such as IEC, affinity chromatography (Guagliardi et al., 1994) or glycerol gradient separation (per recommendation of H. Kagawa, personal communication).

3) Work could proceed by trying to identify what factors are contributing to loss of oligomer (disassembly, aggregation, adsorption). This could be done with non-denaturing PAGE and Western blot, or SDS-PAGE analysis of 100 kDa filtrates, although relevance may be questionable (4.7 Experiments to determine cause of disassembly).

4) ATP could be added to the buffer formulation, but it is not clear what effect this would have.

5) Cpn could be purified as monomers, and reassembled without ATP per Quaite-Randall et al. (1995), or with ATP. See Kusmierczyk & Martin (2001) for a review of cpn assembly.

APPENDIX A OBSERVATIONS OF PILOT ADH THERMAL DENATURATION STUDIES

ADH (Sigma A3263) was surprisingly measured to be only ≅ 4% active by weight (data not shown) per manufacturer's quality assurance assay performed during this work. Bryce Plapp (University of Iowa, personal communication) is unsurprised and relates with surety that commercial preparations are not only inactive, but also a mixture of more than one type of ADH.

Inactivation curves are less reproducible than the individual constituent enzyme assay points, irregardless of whether the manufacturer's or Guagliardi's protocol was used. The assay or the enzyme is sensitive to buffer identity and seems to respond differently to each. Citric acid buffer in particular, presents markedly lower and variable data although the enzyme is formulated to contain "<2%" citrate buffer salts. The enzyme is sensitive to solvation history. Enzyme is diluted several times during the work. Changing buffer identity of any of these diluents affects the measured activity. In some instances, a short-lived aberration was seen immediately following dilution, as though the enzyme hydration layer was only slowly replaced with new dilution buffer. Thermal inactivations at 45°C and 55°C are consistent on a gross level with Guagliardi's 50°C denaturation (section 2.1). Inactivations at higher temperatures are inconsistent with those of Guagliardi et al. and another published study (He, Bai, & Zhou, 1997); the enzyme is more stable than expected at the higher temperatures. Similarly, attempts to thermally aggregate and/or precipitate ADH were successful only at temperatures approaching 100°C and over long time periods approaching weeks. Stabilizers packaged with the enzyme may be the cause, and may need to be removed; the enzyme is formulated as 90% protein. Inactivation curves with bovine serum albumin added as a stabilization reference standard showed it to be a surprisingly effective stabilizer.

APPENDIX B METHODS AND MATERIALS

B.1 General lab procedures

Great care is taken to exclude contaminants and to maintain meticulously clean reagents, equipment, and environment. See B.4 Buffer making for additional similar details. Much of the glassware used is labeled and dedicated to task. Clear containers are preferred over opaque for visual inspection. Work is performed in the protective environment of a laminar flow hood (Labconco Purifier Class 2 Safety Cabinet 36209-02) when possible, with washed and alcohol disinfected gloves. This work includes labeling tubes, loading French press cell, and making dilutions. Polypropylene epi tubes (Fisher 05-408-138, Axygen MCT-150-C) and disposable pipette tips (initially Fisherbrand: 200 µl, 02-681-140; 1,000 µl, 02-681-172, 02-707-507; and later, ART brand: 200 µl, 2069; 1,000 µl, 2279) are used.

B.2 Organisms, medium, and growth

B.2.1 Organisms

Sulfolobus acidocaldarius and *Haloferax denitrificans* are strains maintained in the laboratory. *S. solfataricus* strain P1 (35091) was purchased from American Type Culture Collection (ATCC, Manassas, VA). *S. solfataricus* strain P2 was a kind gift of R. Kelly (North Carolina State University).

B.2.2 Medium formulations

Two medium formulations are listed in Table B-1, one of T. Peeples (University of Iowa, personal communication) and R. Kelly (North Carolina State University, per Charlotte Cooper, personal communication). The indicated 1 N acid strength is that used in this work, and is not indicated in the original formulation sources. C. Cooper noted that better growth was obtained with Difco yeast extract over Fisher Scientific brand.

Table B-1. Medium formulations

Chemical	Brand, Grade	Peeples	Kelly
KH_2PO_4	RPI P41225, ACS grade	0.4	3.1
$(NH_4)_2SO_4$	Fisher Sci A702, ACS	0.4	2.5
$CaCl_2 \cdot 2\ H_2O$	Fisher Sci C70, USP/FCC	-	0.25
$MgSO_4 \cdot 7\ H_2O$	EM Science MX0070, ACS	0.4	0.2
Tryptone	Bacto 211705	1	-
Yeast Extract	Difco 212750, rec over Fisher Sci	-	2
1 N H_2SO_4, to pH:	Mallinckrodt 2468 KXAK, CS anal	3.3	4.0

Note: Values are prescriptions of g/L in dry chemical rows, and are target pH values in the sulfuric acid row.

B.2.3 Medium methods

Despite autoclave specification in most medium recipes, medium was filter-sterilized with 0.2 μm bottle top filter due to concerns about contamination with autoclave steam. The autoclave is difficult to meticulously clean to levels matching the cleanliness of other wetted surfaces and the attention and efforts expended overall. Although most of the autoclave interior is shiny stainless steel, the seams, heating element and thermocouple are complex and rough surfaces bearing the inevitable burnt-on spills and oxidation. Even if 18 MΩ water is used (rather than distilled water), unidentified contaminants can be carried inside bottles by water vapor. Recall that single-distilled water is not as clean as double-distilled water, which is not as clean as the 18 MΩ water used throughout the rest of this work. Medium is often made as a concentrate to reduce volume and thereby simplify production. Concentrate is filter sterilized, and later mixed with 18 MΩ de-ionized water in dedicated bottles rinsed with ethanol (3 times), house distilled water (2 times), and de-ionized water (3 times). Peeples formulation medium was made as concentrates as high as 40×, but Kelly formulation

won't easily dissolve even at 10× concentration, so is made as a 5× concentrate. Medium is stored in refrigerator before discarding after approximately one week.

B.2.4 Frozen organism stocks

Frozen stocks of cells are made per procedure of Charlotte Cooper (of the Kelly lab, personal communication). Freshly grown cells pelleted in centrifuge are suspended in medium with 7% Dimethy sulfoxide. Cells are suspended in a volume giving a target Abs_{600} of ≅ 5 (ignoring the limited range of Beer's law). For example, cells from 10 ml of culture of Abs_{600} ≅ 0.5 are re-suspended in 1 ml. Single ml volumes aliquoted to cryo-tubes are flash frozen in slurry of dry ice and ethanol. Suspension becomes milky as it freezes. Tubes are stored at -80°C.

B.2.5 Growth curve by peristaltic pump sampling and fraction collector

Points in the growth curve (Figure 9) are Abs_{600} measurements of fractions collected continuously by peristaltic pump. One L of filter sterilized Kelly lab formulation medium in an autoclaved 2 L Erlenmeyer flask was inoculated (10% v/v) with a seed culture of *S. solfataricus* strain P2. Flask was placed in a 75°C bath (New Brunswick G 76 Gyratory) in oil (Dow Corning 200 fluid, 100 CST) rotating at approximately 90 RPM. Culture was delivered at a rate of 8 ml/h to a rotary fraction collector set to collect 30 min fractions. Fractions were vortexed briefly before dispensing to disposable cuvettes and reading by Beckman Du-640 UV-Vis spectrometer. Assembly details follow: one hole of a 2-hole, #10 stopper fitting the Erlenmeyer held a syringe (1 ml Norm-ject Tuberculin), narrow end up, and the other hole held a thick walled glass dip tube. Dip tube was bent at 90°C outside of flask for strain relief. The syringe was fitted with a filter (0.2 μm, Millex-FG, Pall #4225) for pressure relief venting. The glass dip tube was connected to small Tygon tubing (1/32" ID, Norton Fisher 14-169-1A) via a 3 cm section of Masterflex tubing (4.8 mm ID, L/S-15 06404-

15) fitting over the glass and an adapter (1/8” hose barb to 1/16” hose barb, Cole Parmer 30703-41). A small length of Silastic tubing (0.03” ID, Dow Corning 508-004) inside the adapter served to reduce volume holdup and eliminate stagnant dead space. Tygon tubing ran for several feet to a peristaltic pump (Control Co 13-876-1) which operated on a section of Silastic (0.03”) tubing. Tygon and Silastic were connected by a hose barb adapter (1/6” to 1/16”, Cole Parmer 30703-00). Tubing ran as Tygon from pump for several feet to the fraction collector (Biorad 2110). Tubing was secured with binder clips for strain relief, and creep and kink prevention.

B.2.6 Cell harvest, washing and storage

Prior to harvest, culture is chilled for 10 minutes in a 5 gal bucket ice bath on a rotary shaker set to ≅ 80 RPM. Cells are harvested by centrifugation (Sorvall RC 5Bplus, GS3 rotor) at 13,700 × G RCF for 10 minutes at 4°C in 500 ml bottles (Nalgene polypropylene copolymer). Rotor is stored in refrigerator. Culture of Abs_{600} ≅ 0.3 (de-ionized water blank) yields ≅ 0.3 g/L. For batch sizes exceeding a single centrifuge run, pellets are stacked by decanting spent culture fluid, refilling with fresh culture, and positioning bottles such that new pellets are deposited directly onto old pellets. Pellets are combined into one bottle by metal spatula for 2 rounds of cell rinsing in refrigerated buffer. Cells are washed twice in buffer with Teflon pestle and glass mortar cell homogenizer (Wheaton 55 ml), or, more recently, with a less invasive method of shaking cell pellet in centrifuge bottle until resuspended, followed by centrifugation. Washing buffer is tris buffer without additives (50 mM, pH 7.5), or, more recently, lysis buffer with additives, but without protease inhibitor or nuclease. Washed cell pellets are stored wet in centrifuge tubes at -80°C and used within several days or discarded. Most recently, when cells are to be used immediately and without freezing, transfer holdup losses are reduced by performing final rinsing resuspension in the same 50 ml conical centrifuge

tube where lysing resuspension will take place. In this case, the tube is spun in an Eppendorf benchtop centrifuge 5403.

B.3 Cell suspension and lysis

B.3.1 Suspension

An appropriate portion of protease inhibitor tablet (Roche Complete EDTA-free, Cat. 11 836 170 001, in 1/8 tablet increments, recommended 1 tablet per 10 ml) is dissolved in refrigerated lysing buffer. These tablets are marketed for "the complete inhibition of serine and cysteine proteases during extractions from animal and plant tissues or cells, yeast and bacteria". It is unclear how appropriate these are for archaea. Nuclease (1µl, Novagen Benzonase, >90% purity) is added. Frozen pellets are retrieved by running cold tap water over bottle and scraping pellet off in a single piece by a metal spatula. Buffer is added to cell pellet in 50 ml disposable conical centrifuge tube at a rate of 4 ml/g. Conical tube is kept in an ice and water slurry and suspension is worked with a glass rod and vortexed well until homogenous.

B.3.2 Lysis by sonication

Cells are lysed by probe sonication (Fisher Scientific Sonic Dismembrator 550, Misonix XL2020, with micro-tip) at power setting 5 (0.5 × 600 Watts = 300 Watts) and 33% duty cycle at 1 s resolution for 6 min (2 min sonication time) on ice. Lysate is vortexed halfway through sonication, which increases efficiency. Samples are taken prior, halfway through, and after sonication for later protein analysis.

B.3.3 Lysis by French press and lysate clarification

Cells are lysed by two passes (Spectronic press in mini-cell) at 18,000 psi in a 4°C pressure cell. Pressure cell is loaded and cleaned in laminar flow hood. Cell is cleaned with ethanol and 18 MΩ water, and allowed to dry overnight in a laminar hood before storing, cleaned and covered, in refrigerator. Lysate is clarified by centrifuge for

30 min at 50,000 × G RCF at 4°C (SS34 rotor). Supernate is decanted by pipette to one or more epi tubes.

B.4 Buffer making

Great care is taken to make clean buffer. Gloves (nitrile powder-free, Kimberly-Clark, KC500 55082) are worn, washed with soap (Decon Bac Down antimicrobial hand soap, 7001) and tap water, dried with paper towel (Kimberly Clark multifold, 09-54-43-0-06) and changed often. Gloves and rough surfaces (example: bottle caps with ridges) are patted dry rather than wiping to prevent towel tearing and release of paper particles. The entire work area is wiped down with ethanol (70%, Decon 2701) and paper towel. Buffer is made with 18 MΩ water (Thermo Scientific NANOpure Diamond Life Science, D11931) which is dispensed to dedicated 2 L glass serum bottles (Pyrex 1395). Bottles are wiped down with wet paper towel prior to opening. Buffer is made in a 2 L dedicated beaker (Kimax 14000) with ACS or biotech grade chemicals, see Table B-2. See 4.4.3 Buffer formulation for buffer formulations. Dry chemicals are pre-aliquoted to 50 ml polypropylene conical centrifuge tubes (initially Fisher 05-539-6, later Dow Corning 430290). Dry chemicals are weighed in plastic weighing dishes (polystyrene, Fisher 2-202B) previously wiped with wet Kim Wipe (Kimberly-Clark EX-L), or on weighing paper (Fisher low nitrogen, 09-898-12A). Any Kim Wipe exposed to atmosphere and dust overnight is discarded. Bottles of fluids and dry chemicals are wiped down with wet paper towel prior to opening to remove dust, even if kept in a cabinet with doors. Care is taken when working to observe an ‘air space’ above open bottles, chemicals, and fluids exposed to air, so as to avoid contamination by particles falling off of, for example, lab coat sleeves. Glycerol is stored at 4°C as a 50% aqueous solution. Fluid volumes are measured with a 100 ml graduated cylinder (Kimax 20030) dedicated to buffer making. Buffer is pH-adjusted with 1 N hydrochloric acid. The entire pH probe, from tip to cord, and the portion of the probe holding arm to be suspended above the

Table B-2. Buffer ingredients

Ingredient	Brand, Grade
Tris Base	RPI T60040, Ultrapure Mol Bio >99.9%
NaCl	Fisher BP358-212, Enzyme
Glycerol	RPI G22025, ACS
KCl	Sigma P9541, Mol Bio >99.0%
$MgCl_2$	Sigma M0250, Reagentplus >99.0%
Dithiothreitol	RPI D11000, Mol Bio >99%
EDTA	Fisherchemical E478, ACS
HCl	Fisher A144s-212, ACSplus
Protease Inhibitor	Roche Complete Mini EDTA-Free Tablets
Nuclease	Benzonase
Triton X-100	Sigma Life Sci T8532, For Electrophoresis

fluid, is rinsed with 18 MΩ water and patted dry with Kim wipes. Probe (Accumet 13-620-96) and pH meter (Accumet 50) is calibrated with pH standard solutions (pH 10, Ricca 1601-16; pH 7, 1551-16; pH 4, 1501-16). Plastic film (Saran Premium Wrap) is used to protect buffer from air-born dust during lengthy pH adjustment. Buffer is filtered (0.2 μm, PES, bottle-top vacuum filter, Nalgene 595-4520) into a dedicated 2 L serum bottle. Bottle was initially washed with three rinses fresh aqua regia (1 volume part nitric acid, 3 parts hydrochloric acid) or Nochromix (Godax) and two rinses each of chloroform, acetone, and ethanol. Between batches, bottle is rinsed three times with ethanol and five to seven times with 18 MΩ water. Glassware (beakers, graduated cylinders, bottles) is rinsed and stored wet under plastic film. Buffer is stored at 4°C and discarded after several days.

B.5 Protein concentration assay

Rough protein concentration estimates (mg/ml) are made by Abs_{280} measurements in a Beckman Du640 spectrophotometer in a quartz cuvette with a 1 cm path length using an extinction coefficient of 1. The ratio of Abs_{260}/Abs_{280} was never large enough to warrant use of more involved calculations that accommodate absorbance of nucleic acid.

Samples are sufficiently diluted to mind Beer's law and return readings less than 1. Cuvettes are cleaned with multiple rinses of 95% ethanol and 18 MΩ water. More precise measurements, requiring less sample volume but more time, are made by Lowry assay (Biorad Dc, BSA standard, 500-0007). Samples with particulates (for example, lysate prior to clarification), are spun at 13,000 RPM for 3 min in flat-bottomed cuvettes, by Sorvall Biofuge Pico prior to decanting by pipette and analysis.

B.6 Gel electrophoresis

Proteins are separated by gel electrophoresis per Laemmli (1970). The Mini-Protean Gel 3 Electrophoresis system (Biorad) is used for casting and running gels, and for Western Blot transfers. 18 MΩ water is used exclusively throughout the electrophoresis procedure. Non-denaturing gels are run similarly, omitting SDS, DTT, and heating.

B.6.1 Gels are purchased or made

Purchased gels (Ready gel, Biorad) are 4%-20% gradient, tris-HCl buffered, 15 lane, 15 µl wells. The gradient helps to focus bands and allow visualization of the entire protein size spectrum while separating cpn oligomers and monomers. When the dye front comes off the bottom of the gel, cpn monomers are mid gel and oligomers are several mm from the top. Single percentage gels are made 1.5 mm thick according to standard methods (Biorad), occasionally using multi-gel casting chamber (Biorad). The separation gel is 12% acrylamide (Biorad 2.6% C, 161-0158) in 1.5 M tris, pH 8.8 with 10% SDS, 0.05% Temed, and 0.05% ammonium persulfate (APS). The focusing gel is 4% acrylamide in 500 mM tris, pH 6.8, 10% SDS, 0.05% Temed, and 0.05% APS. APS is made fresh weekly and added as a 10% stock solution.

B.6.2 Sample preparation

Samples are diluted 1:1 into Laemmli sample buffer (Biorad) formulated without 2-mercaptoethanol, which is added at a rate of 5% (v/v) immediately before use. Samples are heated in a boiling water bath for 5 minutes and spun at 13,000 RPM for 1 min in Sorvall Biofuge Pico to retrieve liquid on sides of the tubes after heating, and then vortexed.

B.6.3 Electrophoresis separation runs

Samples are applied with gel-loading pipette tips, preferably in volumes as low as 5 µl to increase focus. Molecular weight ladder standards (Amersham Rainbow) dilutions are aliquoted (10 µl) to decrease freeze-thaw cycles. 5 µl of a 1/10 dilution is visualized with silver stain. Tank buffer contains 25mM tris base, pH 8.3, 192mM glycine, and 0.1% SDS. Tank buffer is made as ten fold concentrate and stored at 4°C. SDS gels are run at 200 V for 65 minutes with tank buffer initially at 4°C, approximately as long as dye front takes to exit gel. Non-denaturing gels are run for 130 minutes to distance oligomer bands from staining edge effects. Protein is stained by Coomassie (Biorad Bio-Safe) or silver stain.

B.6.4 Silver stain

Ammoniacal silver stain is used to visualize protein bands. Procedure is adapted from P. Rose (University of Iowa, personal communication). Some silver stains can detect as little as 0.02 ng protein per band (Merril & Washart, 1998). The sensitivity of this method was confirmed to exceed that sensitivity with BSA (standard from Biorad Dc assay). Gels are stained in polypropylene containers (24 oz, Glad) on a rotary shaker (approximately 60 RPM). Wash volumes are 100-200 ml. Stain wash schedule is: 7% acetic acid, 7 min; 50 % methanol, 20 min, twice; water, 10 min, twice; silver stain, 15 min; water, 5 min, twice; developer; stain stop. Gel is kept in developer until bands are visible, typically 5-10 minutes. Half-strength developer is useful for gels with large loads

or high background. Silver stain is made by addition of silver to a pre-made mixture of bases immediately before use. A volume of 100 ml is used per gel with the following composition: 0.8 g silver nitrate, 1.4 ml concentrated ammonium hydroxide, 2.4 mM NaOH. Developer, 100 ml per gel, is made immediately before use with the following composition: 10 mg citric acid anhydrous, 50 µl formaldehyde. Stain development reaction is quenched with stain stopper: 5% tris base, 2.5% glacial acetic acid. Gels are stored in stain stopper. Staining containers are washed with 10% bleach and rinsed with distilled water. See Rabilloud, Vuillard, Gilly, & Lawrence (1994) for review of silver stain methods.

B.7 Western Blotting

B.7.1 Protein blotting

Protein is transferred wet (Biorad Mini Trans-Blot) according to Towbin, Staehelin, & Gordon (1979) and manufacturer's recommendations. Each Western blotted gel is run as a duplicate of a silver stained gel. Transfer buffer is 25 mM tris, pH 8.3, and 192 mM glycine. Transfer buffer is made as ten-fold concentrate and stored at 4°C. Gels are soaked in transfer buffer for 15 min prior to incorporation in wet sandwich. PVDF membrane (Millipore Immobilon-P 0.45µm) is wetted with methanol and rinsed twice with transfer buffer prior to sandwiching with Whatman Filters (#1, 110 mm, cut to size). Buffer is applied to sit as a layer on top of gel prior to placement of membrane. Transfer is made with 350 mA for 20 min in hot buffer. Transfer buffer is heated to 80-90°C by microwave oven per Kurien & Scofield (2002). 5 µl of full strength molecular weight markers (Amersham Rainbow) is visible on transferred membranes, confirming transfer. Ponceau-S (two different manufacturers; Boston BioProducts ST-180, Fisher Biotech BP103-10) appears to interfere with subsequent detection, and is used sparingly to confirm transfer.

B.7.2 Immunological Detection

Immunological detection of proteins blotted on membranes is based on McCubbin & Frank (1997). Membranes are developed in covered detection containers (RPI brand) on an orbital shaker set at ≅ 60 RPM. Schedule of solutions is: rinse; block, 30 min; rinse; 1° antibody, 30 min; rinse, 4 × 5 min; 2° antibody, 30 min; rinse, 4 × 5 min; developer. Rinses are made with TBSTA buffer (10 mM tris pH 8.0, 150 mM NaCI, 0.05% Tween-20, 8 mM sodium azide), made as ten-fold concentrate and stored at room temperature. Blocker is 10% Donor Equine Serum (HyClone) in TBSTA. Horse serum is aliquoted (5 ml) to conical centrifuge tubes and frozen. A 'cap' of TBSTA is later layered on top and frozen to prevent sublimation and serum protein denaturation. TBSTA (45 ml) is added on the day of use. 1° antibody is polyclonal anti-*S. shibatae* cpn antibodies raised in rabbit (kind gift of J. Trent, NASA Research Center, Moffet Field, CA). The antibodies were grown in response to *S. Shibatae*, but the highly conserved nature of this molecule almost guarantees cross reactivity to a close relative. See Kagawa et al. (2003, 1995) and Trent et al. (1990) for details about these antibodies. The vial received is labeled "#62 8wk", and contained 200 µl – 250 µl of a beige and light pink solution. 2° antibody is affinity-isolated anti-rabbit IgG(H+L) alkaline phosphatase conjugated antibody from goat (Sigma A3687). 1° and 2° antibody solutions are diluted 1:10,000 in TBSTA, carefully retrieved by pipette and reused. In an appropriately sized container, 15-25 ml fully covers a membrane. Membrane is developed in NBT/ BCIP solution (MP Biomedicals, Liquid Substrate Plus) diluted 1:30 in 18 MΩ water immediately prior to use. Depending upon target load, antibody solution exhaustion, and developer concentration, development takes several minutes to 2 hours.

B.8 Chromatography

The chromatography system is manufactured by Amersham Biosciences (currently GE Healthcare Biosciences AB) and includes the following components: LKB

Controller LCC-500 Plus, LKB Pump P-500, Valve MV-7, Monitor UV-M, and Chart Recorder REC-481. The size exclusion resin used most recently is Sephacryl S-500HR (Amersham Pharmacia, now GE Biosciences) packed in lab into a XK16/70 column, which is ≅ 100 ml. Superdex 200 resin was used for the first half of this work. A calibration curve is shown in Figure 15, made per manufacturer's recommendations (Amersham Biosciences, 2002). The standard molecules include proteins, a carbohydrate and an immunoglobulin, evidently presenting differing interactions with the resin and deviations from the expected straight line on the calibration curve. M_r (relative molecular weight) is plotted versus $K_{av} = (V_e - V_o)/(V_t - V_o)$ where V_e is elution volume of the molecule, V_o is void volume, the V_e of molecules eluting in the void volume (in this case, IgM), and V_t is resin volume. The standards used were part of a calibration kit (Sigma-Aldrich MWGF1000: blue dextran, 2 MDa; thyroglobulin, 669 kDa; apo-ferritin, 443 kDa; β-amylase, 200 kDa), and immunoglobulin IgM (970 kDa), purchased separately.

Chromatography buffer is 0.2 μm filtered to protect resin, and degassed for 1 h at 500 mm Hg vacuum with stirring to minimize detrimental air bubbles. All chromatography samples are 0.2 μm filtered or centrifuged for 30 min at 50,000 × G RCF to extend resin life. Buffer is pumped through SEC columns at 0.5 ml/min (0.25 cm/min) to 1 ml/min. Column jacket is cooled by circulation of ice-cooled water through column jacket by peristaltic pump (Masterflex 7520-50, size 15 tubing). Eluent is fractionated by X-Y fraction collector (Foxy Jr., Isco) into disposable borosilicate glass test tubes (13×100 mm, VWR 47729-572) or polypropylene conical centrifuge tubes (15 ml, Fisher 05-539-12). Fractions are transferred to ice slurry or refrigerator as eluted. A standard reference curve was made from a marker kit (Sigma MW-GF-1000) and GroEL, the cpn of *E. coli* (Sigma). Freeze dried or powdered proteins are dissolved and allowed to fully hydrate for an hour before filtration by 0.2 μm syringe filter. Fresh standard solutions were made day of, since some deteriorated overnight in the refrigerator. Signal level is

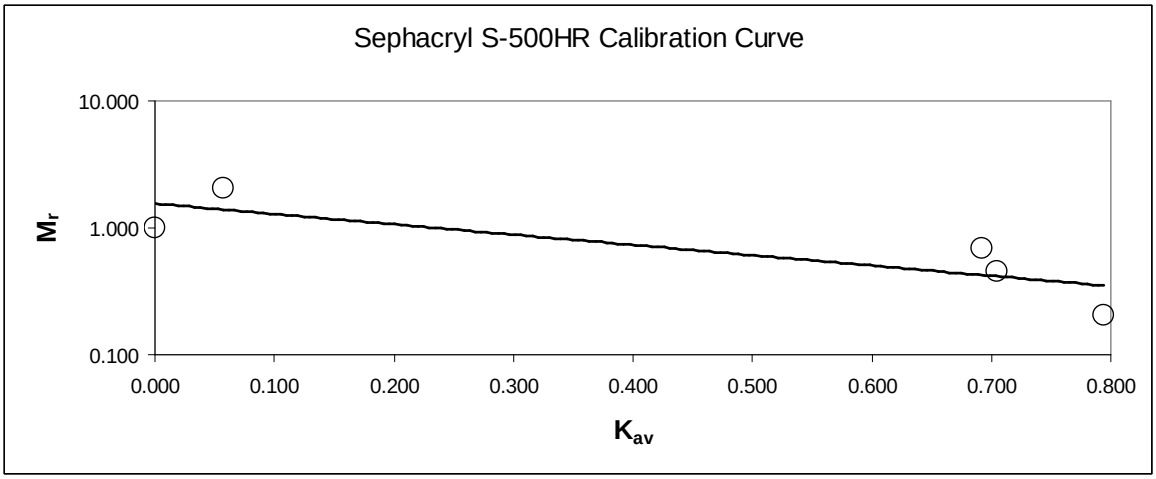

Figure 15. Calibration curve of Sephacryl S-500HR size exclusion resin

determined by Abs_{280} of fluid passing through detector, line pressure, and detector and chart settings. Chart is typically set to 100 × 1 mV and 1 mm/min. Sample is applied with buffer pumps running after signal settling to a flat baseline to avoid start-up pressure and signal fluctuations. Loading loops for sample injection volumes one ml or less are put in-line by electric MV-7 valve. Sample loads greater than one ml in volume are injected by syringe directly into FPLC line toward column. The manufacturer recommends (GE Healthcare Bio-Sciences AB, 2007) that sample volumes be 1-4% of column volume for analytical work, and that concentrations be less than 70 mg/ml at room temperature due to deleterious viscosity effects on separation and resin packing. The concentration recommendation is halved during 4°C runs due to the increase of aqueous buffer viscosity at reduced temperatures.

A higher concentration, double SEC procedure was performed under guidance of L. Gakhar at the Protein Crystallography Center at the University of Iowa. A large load (2 g) of batch grown cells were broken in an equivalent volume of buffer and run, undiluted, on a Superdex 200 column. Fractions containing cpn were pooled, concentrated and rerun on a Superdex 75 column. Note that the concentration of lysate

(≅ 200 mg/ml) in the first SEC run exceeds the manufacturer's recommended concentration limit (35 mg/ml, 4°C) by a factor greater than 5.

Recommended resin cleaning procedures vary per document; the procedure below is an amalgam of concentrations, flow rates and contact times found in the following documents: Amersham Biosciences (1996); GE Healthcare Bio-Sciences AB (2006); GE Healthcare Bio-Sciences AB (2007); and GE Healthcare Bio-Sciences AB (2008). The column is not inverted; fluid flow is not reversed. Rinsing water and cleaning solutions are run at 10 cm/h (20 ml/h for XK16/70 column). Resin is regenerated with one-half column volume of 1 M NaCl, and rinsed with two column volumes of water. Resin is cleaned with one-quarter column volume of 0.5 M NaOH, and rinsed immediately with four column volumes of water. Resin is cleaned with one-half column volumes of 30% isopropanol, and rinsed with two column volumes of water. Resin is rinsed with five column volumes of buffer prior to sample application. See Quaite-Randall & Joachimiak (2000) for alternate procedures.

B.9 Viability measurement after heat challenge

Tolerance to heat challenge was measured by classic five tube most probable number method (Brock, 1979, table A2.3). Great care was taken to exclude contaminants. Dilutions were made immediately in front of the reactor inside the laminar flow hood. Screw cap test tubes prevented evaporation. A metal dry bath served as a heated (75°C) work space. Medium was pre-heated to 75°C. Serial dilutions and inoculations were made by dilution of 0.5 ml into 4.5 ml culture. 1:10 stock serial dilutions served as inocula for 5 tubes at each dilution from 10^1 to 10^7. Dilutions to be "heat challenged" were transferred to a 90°C metal dry bath for 6 hours. Tubes were transferred to a 75°C heated air incubator for growth (Fisher Isotemp 625D). All growth occurred within two weeks, although tubes were inspected for growth periodically over the next month.

Growth observations were made by eye. Turbidity and/or pellets from settled cells were taken as positive growth data.

B.10 Measurement of relative cpn levels in HSC

Eluting culture from each of the three vessels was collected onto ice, pelleted and frozen at -20°C over a period of several days. Pellets were processed in a single day. Pellets (≅ 0.1 g) were lysed by sonication in 1 ml buffer (50 mM tris-HCl, pH7.5, 250 mM NaCl, 1 mM DTT, 1 mM EDTA). Each HSC PAGE lane was loaded with 10 ng protein per Lowry assay (Biorad). Samples were electrophoresed for 30 min at 300 V. Protein blotting was confirmed by Ponceau S stain.

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