DEGRADATION KINETICS OF AN ABNORMAL BETA-GALACTOSIDASE IN *ESCHERICHIA COLI*

Thesis by

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The tree the tempest with a crash of wood
Throws down in front of us is not to bar
Our passage to our journey's end for good,
But just to ask us who we think we are,

Insisting always on our own way so.
She likes to halt us in our runner tracks,
And make us get down in a foot of snow
Debating what to do without an axe.

And yet she knows obstruction is in vain:
We will not be put off the final goal
We have it hidden in us to attain,
Not though we have to seize earth by the pole

And, tired of aimless circling in one place,
Steer straight off after something into space.

Robert Frost, *On a Tree Fallen Across the Road*

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ABSTRACT

Many recombinant proteins expressed in *Escherichia coli* are identified by the cell as abnormal and are degraded rapidly. As a model protein, we employed an unstable fragment of *E. coli* β-galactosidase, the CSH11 mutant, coded by a single copy in the chromosome. This experimental system enabled identification of the process and cellular variables having the greatest impact on the degradation rate of abnormal proteins.

The *in vivo* degradation rates were measured using pulse-chase radioactive labeling techniques, and intracellular concentrations were determined using α-complementation assays. The degradation of the CSH11 fragment was sensitive to the culture temperature and approximately followed Arrhenius behavior, but the apparent degradative rate constant for its 90 kDa degradative intermediate increased by an order of magnitude between 37^o and 40°C. The apparent rate constants for both β-galactosidase fragments inexplicably decreased with increasing induction level. There was no aggregation of the fragments to account for this decrease in rate constants.

Cellular protease levels were determined using an *in vitro* assay of cell extracts. The ATP-independent proteases were not induced when the abnormal β-galactosidase was expressed, and their level decreased by 20% from 37^o to 42°C. Expression of the abnormal protein caused a doubling in the ATP-dependent proteolytic activity. This ATP-dependent activity also increased 2.5-fold from 30^o to 42°C with induction of the abnormal protein.

Stress (heat shock-like) responses were detected by radioactive pulse- labeling of cellular proteins and analysis by one- and two-dimensional PAGE. There was no response to induction of the abnormal β-galactosidase at 30°C, even though the concentration of abnormal protein was approximately the same as

at 37°C where a stress response was observed. Determined with the protease assay, ATP-dependent proteolytic activity was associated with the stress proteins independent of protease La.

Proteolytic susceptibility to α-chymotrypsin correlated well with the thermal stability of the affinity purified β-galactosidase fragments. Thermal instability of the 90 kDa intermediate is the likely cause of the significant increase in its *in vivo* degradation rate between 37° and 40°C.

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CHAPTER 1

Introduction

1.1 Protein Degradation in *Escherichia coli*

All proteins are synthesized to be degraded. This is true in both eukaryotic as well as prokaryotic cells, even though only 25 years ago protein degradation was not thought to occur in growing bacteria. Proteolysis is now widely accepted as an important cellular process responsible for turnover of cellular proteins (some much faster than others), cleavage of the signal peptide from secreted preproteins, and control of intracellular levels of "regulatory" proteins (for review see: Goldberg and St. John, 1976; Gottesman, 1989; Miller, 1987). In addition to these common functions, proteases are critically important in helping the cell adapt to new environments. The most-well known adaptation is the transition of cells from exponential phase growth to stationary phase because of starvation for important nutrients (Brunschede and Bremer, 1971; Reeve et al., 1984; St. John and Goldberg, 1980). This limitation invariably causes an increase in the overall degradation rate, partly due to degradation of abnormal proteins generated by premature termination of translation (Brunschede and Bremer, 1971; Goldberg and St. John, 1976). These protein fragments as well as other types of abnormal proteins can typically be recognized by a disrupted conformation and/or high proteolytic susceptibility (Goldberg and Goff, 1986).

Bacterial cells efficiently target these "abnormal" proteins so that they are usually degraded rapidly. In fact, *Escherichia coli* appears to have a separate degradative pathway for abnormal proteins (Goldberg and St. John, 1976). Interestingly, ATP-energy is generally required for abnormal protein degradation despite the thermodynamic favorability of the reactions. This ATP requirement

is a stringent one as 80 - 90% reduction in ATP levels is necessary to affect the rate of abnormal protein degradation (Olden and Goldberg, 1978). It has been proposed that the ATP-dependent proteases make the first cleavages of abnormal proteins and that the ATP-independent proteases and/or peptidases rapidly degrade the resulting fragments (Goldberg and Goff, 1986). Probably the majority of endoproteases in *Escherichia coli* have been isolated and identified (Fig.1; Cook, 1988; Miller, 1987). However, all but two have been only initially characterized (Goldberg et al., 1981; Miller, 1987) so their specificity and role in proteolysis are still unknown. The two that have been studied in more depth are the ATP-dependent proteases Clp and La. Protease La is positively regulated by abnormal protein presence (Goff and Goldberg, 1985), and *lon* (La) mutations are well known to reduce the degradation rate for many abnormal proteins (Chung and Goldberg, 1981; Gottesman, 1990). Alternatively, the activity of the two component Clp protease has not yet been shown to be influenced by abnormal proteins, and *Clp* mutations do not significantly affect abnormal protein degradation (Maurizi et al., 1990).

Bacterial cells also respond to abnormal protein presence by increasing the synthesis of their stress (heat shock) proteins. Many of these critically important proteins, including the ATP-dependent protease La, have been shown to affect abnormal protein proteolysis (Goff and Goldberg, 1985; Straus et al., 1988). It is not surprising that some stress proteins are involved in proteolysis since abnormal protein levels typically increase when the cell is stressed (Goldberg and St. John, 1976). Many of the components of the cell's degradative mechanisms have been identified, and it is now necessary to learn when they function and

how they are regulated.

1.2 Thesis Motivation

Unfortunately for the biotechnology industry, some recombinant proteins expressed in *Escherichia coli* are recognized as abnormal and are highly labile. In some cases, controlling degradation is essential if any of the recombinant protein is to be recovered. Not only does degradation then result in lower product yields, but by generating similar degradative products it can also make purification of the desired protein more difficult. Because protein purification is both very expensive and already difficult, reductions in *in vivo* degradation can be beneficial beyond the obvious increases in product yield.

While labile abnormal proteins can sometimes be stabilized by secretion to the periplasm or by insolubilization to form inclusion bodies, their degradation must otherwise be controlled to obtain worthwhile yields. Inactivation of the responsible proteases is increasingly becoming an option with the advent of newer and more powerful recombinant DNA technology. Even some of the adverse phenotypes of these mutations can be overcome (Gottesman, 1990). However, this approach will not always be available to the designers or operators of a recombinant protein production process.

Abnormal protein degradation must then be controlled by more indirect means. A useful tool for this approach would be a mathematical model relating the parameters of the process: growth conditions, host genotype, and recombinant protein characteristics to the *in vivo* degradation rate of the protein.

Before such models can be written, a better understanding of the cell's degradative mechanisms is needed. Particularly, we need to identify which process parameters can significantly affect abnormal protein degradation, and what is the relationship between proteolysis and these parameters. Only then will informed decisions be made as to how to minimize proteolysis of recombinant proteins. Similarities between prokaryotic and eukaryotic cellular processes suggest some results for *Escherichia coli* will also be applicable for eukaryotic expression systems.

1.3 Thesis Scope

The objective of this study is to provide parametric information on abnormal protein degradation in *Escherichia coli*. A systematic investigation is conducted on the proteolysis of a model protein. This protein is a premature termination fragment resulting from the CSH11 nonsense mutation of *Escherichia coli* β-galactosidase (McKnight and Fried, 1981). It is endoproteolytically cleaved (McKnight and Fried, 1981), probably by an ATP-requiring reaction (Kowit and Goldberg, 1977) that produces a detectable degradative intermediate. Since both fragments are degraded at moderate rates, they are analyzed simultaneously. Experiments are performed in early exponential phase growth while the cells are in balanced growth and the effect of the investigated parameters can best be determined. The β-galactosidase fragment is produced from a single gene copy in the chromosome of an otherwise wild-type strain of *Escherichia coli*. The degradation of the abnormal β-galactosidase is investigated in the

absence of high abnormal protein concentration or high plasmid copy number; both of which cause significant stress to the cell.

Chapter 2 describes our investigations on the effect of culture temperature and induction level on the *in vivo* degradation rate of the abnormal β-galactosidase and its degradative intermediate. Dependence of these rates on both the specific growth rate and the intracellular concentration of abnormal protein is also examined.

The cellular protease activities are lumped by ATP-requirement and assayed, as shown in Chapter 3, to test for correlations with the *in vivo* degradation rates of the β-galactosidase fragments. This chapter also questions the involvement of the stress proteins in abnormal β-galactosidase degradation. Also, the protease assay is used to determine stress protein contribution to the proteolytic activity of *Escherichia coli.*

The results presented in Chapter 2 suggest a strong dependence of the *in vivo* degradation rate on the protein properties of the β-galactosidase fragments. Chapter 4 describes an analysis of the structural properties of the β-galactosidase fragments, and how these properties influence the fragments' protease sensitivity and *in vivo* proteolytic susceptibility.

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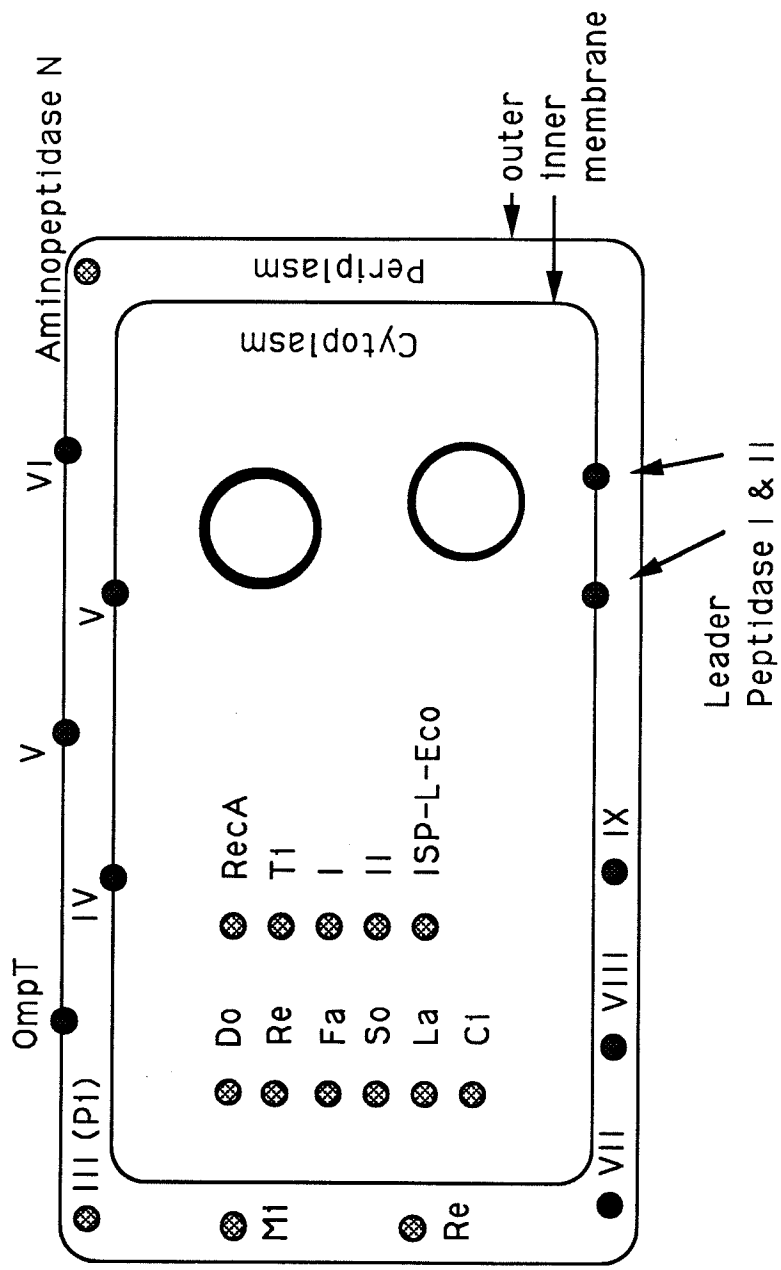
1.5 Figure

Figure 1. Cellular location of the known endoproteases in *Escherichia coli.* The darkly-shaded circles represent metalloproteases, the lightly-shaded circles are the serine proteases, and Aminopeptidase N is a thiolprotease. The protease type for the leader peptidases has not yet been determined. The large circles in the cytoplasm represent plasmids.

Figure 1.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Chapter 2

Temperature and Induction Effects on the Degradation Rate of an Abnormal β-Galactosidase in *Escherichia coli*

Source: Kosinski, M.J. and J.E. Bailey (1991) *J. Biotech.* 18:55-68.

2.1 Summary

Intracellular protein degradation was investigated using an unstable fragment of *E. coli* β-galactosidase, the CSH11 mutant, as a model protein. This abnormal protein was expressed from a single copy gene in the chromosome and is converted to a detectable degradative intermediate. The *in vivo* degradation rates of both β-galactosidase fragments were measured using pulse-chase radioactive labeling techniques, and their intracellular concentrations were determined using α-complementation assays. In the physiological range of 30^o to 37^{o}C, the apparent degradation rate constant for the CSH11 fragment follows Arrhenius behavior; while the intermediate's apparent degradation rate constant is unchanging. However, above 37^{o}C the degradation rates of both fragments increase significantly. Analysis of the labeled intermediate's rate of change above 40^{o}C reveals that the CSH11 fragment is being degraded by a second pathway which does not produce the intermediate. When the induction level of the abnormal β-galactosidase was varied, the degradation rates of both fragments behaved similarly, but they unexpectedly decreased with increasing IPTG concentration. The two parallel degradation pathways for CSH11 apparently operated at only the lower IPTG levels. The measured degradation rates did not correlate directly with the intracellular concentration of abnormal proteins.

2.2 Introduction

Many recombinant proteins expressed in bacteria are identified by the cell as abnormal and are degraded rapidly. This instability may be a consequence of the absence of stabilizing ligands or, more likely, due to a different cellular environment which is incompatible with correct protein folding. Effective large-scale production of recombinant proteins using bacteria requires strategies for stabilizing these proteins. One method is to employ secretion to the periplasm, which is particularly effective for some naturally secreted proteins such as insulin (Talmadge and Gilbert, 1982) and mT antigen (Palme and Eckhart, 1986). Proteins can also be protected from proteases by genetic fusion to a portion of a bacterial protein such as β-galactosidase (Flores et al., 1986; Itakura et al., 1977), or through the formation of inclusion bodies (Cheng et al., 1981; Kitano et al., 1987).

Regardless of the expression approach, it is important to understand the cell's response to abnormal protein presence and the factors influencing that response. Culture conditions such as temperature (Chesshyre and Hipkiss, 1989) and growth medium composition (Carr et al., 1987; Kowit and Goldberg, 1977) and genetic factors including *lon* and *htp*R mutations (Gottesman, 1990) are known to have significant impact on abnormal protein degradation in some cases. By better understanding the degradation process and what controls it, we may better design cells and processes for recombinant protein production.

About half of the research on abnormal protein degradation has been done by introducing amino acid analogs into the proteins of the cell (Goldberg, 1972;

Prouty et al., 1975). While this method produces some general information, it is so disrupting to the cell that it is difficult to make definite conclusions concerning protein stability. We, therefore, chose to study a single abnormal protein. Our model protein is a nonsense mutant of *E. coli* β-galactosidase and is one of three similar mutant proteins that have been previously studied (Goldschmidt, 1970; McKnight and Fried, 1983; Kowit and Goldberg, 1977). It appears all three mutants produce a common detectable degradative intermediate (McKnight and Fried, 1981). The mutant we studied is degraded at a conveniently observable rate (7.4 minute half-life at 37°C) and is produced from one gene copy in the cell's chromosome, thereby avoiding effects of plasmid presence on cell response.

Using α-complementation assays to quantitate the amount of abnormal poly- peptides and radioactive pulse-chase experiments to measure degradation rates, we studied both β-galactosidase fragments to elucidate the degradation process in the cell. We investigated two important parameters: the culture temperature and the level of abnormal protein induction. Temperature has been shown by preliminary (Straus et al., 1988) and nonspecific measurements (Lin and Zabin, 1972) to significantly impact degradation of mutant β-galactosidase. Also, the amount of a single abnormal protein affects the cell's heat shock protein expression (Parsell and Sauer, 1989a). Accordingly, we varied the induction level at one temperature to observe the effect on degradation rates.

2.3 Materials and Methods

Bacterial Strains

The *Escherichia coli* strain CSH11 contains a nonsense mutation in the C-terminal portion of the *lacZ* gene. Strain BGF1 ($F^-\lambda^-$ *lacZ*) was obtained using P1 transduction to transfer the *lacZ* mutation to wild-type strain MG1655.

Growth Conditions

Most studies of cellular protein kinetics use the radioactive pulse-chase technique which requires growth in medium essentially free of the labeled amino acid. The majority of experiments have been done in minimal medium without amino acids, but under these conditions cell physiology and protein composition are markedly different from those in cells grown in the presence of amino acids (Maaloe and Kjeldgaard, 1966). We therefore chose to perform our experiments in M63 minimal medium supplemented with 0.2% glycerol and all the amino acids except methionine and cysteine (Minkley and Ippen-Ihler, 1977). Cultures were started from overnights grown at the same temperature without IPTG (isopropyl β-D-thiogalactopyranoside). A 0.5% inoculum was used for all the experiments, and the indicated amount of IPTG was added at the time of inoculation to ensure a steady state by the time of labeling. This avoids any of the transient effects following induction which have been observed (McKnight and Fried, 1981; Hipkiss, 1979). Cells were grown in 30 mL medium in baffled 250 mL Shake flasks (Bellco Glass Inc.) using a New Brunswick G24 Incubator at 250 rpm. All measurements were done with cells in early exponential phase. These cells can be considered in balanced growth, and this is a good basis for

comparison between batch cultures. Culture growth was monitored using a Klett meter with a green filter. When the culture reached 60 Klett Units, some was transferred to test tubes for radioactive labeling and the remainder put on ice to prepare samples for α-complementation assays. After 10 - 20 minutes on ice, seven mL of culture each was transferred to two 16 $\times$ 125 mm test tubes. The cells were spun down at 1500 g for 20 min, the supernatant discarded, and the pellets stored at $-20°$C. All chemicals were from Sigma Chemical Company.

Labeling

Labeling was done in 25 $\times$ 150 mm test tubes in a shaking water bath (Reichert Scientific) at 100 strokes/min to ensure aerobic growth. Culture volumes of 1.5 mL were labeled with 22.5 μCi of ^{35}S-methionine (Amersham Corp.). For experiments done at the temperatures 30, 33.5, 37, 40.5, and 44°C, the respective pulse times were 12, 9, 6, 4, and 3 minutes. The chase was begun by the addition of unlabeled methionine at a concentration of 20 mM. Eight test tubes were used per experiment. The respective intervals between the chased samples were 2.5, 2, 1, 0.5, and 0.5 minutes for the first six and twice these times for the last two. After the pulse, sample taking was delayed by twice the above chase interval to allow the chase to become effective.

Analyses

Degradation Rate

The degradation rate of the β-galactosidase fragments was determined by the method of McKnight and Fried (1981). Radioactive labeling was done with

a mixture of mutant and wild type cells (about 4:1). The cell types were grown separately before mixing. Both were in exponential growth in identical medium at the same temperature and IPTG concentration. Chases were stopped by addition of 1/10 volumes of ice cold 100% TCA. The TCA-precipitated proteins were subjected to the immunoprecipitation procedure of Bankaitis and Bassford (1984). Immunoprecipitates were run on 6% homogeneous SDS gels (Laemmli, 1970). Polyclonal antibodies against β-galactosidase and Protein A-Sepharose Resin were obtained from Sigma. Since the antibodies against β-galactosidase recognized both the wild-type protein and the two detectable fragments, they were all brought through the immunoprecipitation and gel electrophoresis procedures together (Fig. 2). Because the wild-type β-galactosidase is stable compared to the fragments, it could be used to normalize the above procedures (Lin and Zabin, 1972). The gels were fixed in 5% acetic acid/ 5% isopropanol solution, washed with distilled water, and soaked for two hours in Autofluor (National Diagnostics). They were then dried onto filter paper using a Bio-Rad Slab Gel Dryer. Fluorography was done at -70^{o}C with preflashed Kodac X-OMAT AR film for two to seven days. Densitometry of the developed films was done using a LKB 2202 Ultrascan Laser Densitometer, and the integration was performed with a multiple Gaussian fit program also from LKB.

α-Complementation

The α-donor samples were prepared essentially by the method of Kowit and Goldberg (1977). The cell pellets in the 16×125 mm test tubes were thawed and suspended in 400 μL Buffer II (20 mM Tris-HCl (pH 7.2), 0.1 M NaCl, 1 mM

$MgCl_2$, 1% mercaptoethanol, 0.5 mM EDTA). For standards, known amounts of β-galactosidase (Sigma) were added to test tubes containing 400 μL Buffer II and were treated the same as the samples. The tubes were autoclaved at 121°C for 30 min, and then placed on ice for five minutes. After centrifugation to remove precipitates, the supernatant was recovered. Three 100 μL α-donor samples were each added to 50 μL of α-acceptor, prepared by the method of Lin and Zabin (1972). The mixtures were incubated in a water bath at 28°C for about eight hours. They were then placed on ice until assayed by the method of Miller (1972). The measured activity was corrected for differences in the total amount of supernatant recovered.

2.4 Results

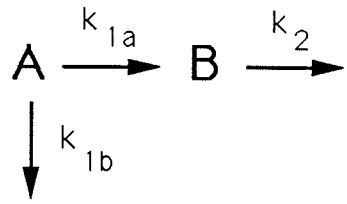
Degradation Rate Determination

Overall degradation of the CSH11 fragment (denoted A) is pseudo-first-order (see Fig. 1a) in agreement with previous work on related proteins (McKnight and Fried, 1981; Kowit and Goldberg, 1977; Goldschmidt, 1970). Thus, the amount of radioactively labeled A during the chase can be described by:

$$A = A_o e^{-k_1 t} \qquad [1]$$

and a linear least squares fit of the data yields the effective rate constant. This effective rate constant is expected to depend on the amounts and activities of intracellular proteases. All degradation assays were conducted on mid-exponential phase cells so both protease levels and activities could achieve steady states.

The most general degradative mechanism for large nonsense fragments of β-galactosidase is



in which A is degraded to undetected peptides and to a 90 kDa degradative intermediate (denoted B). McKnight and Fried (1981) concluded that A is converted to B by a specific endoproteolytic cleavage, but also suggested that apparent discrepancies in their data might be due to a second degradative pathway for A. Both the A and B fragments appear to be degraded by an energy-dependent process *in vivo* (Kowit and Goldberg, 1977).

Fragment B is also degraded by a pseudo-first-order reaction (McKnight and Fried, 1981; Kowit and Goldberg, 1977). Letting B_o denote the initial amount of radioactively labeled B, the time-trajectory of the amount of labeled B is given by:

$$B = \frac{\epsilon\alpha k_1 A_o}{k_2 - k_1}\left[e^{-k_1 t} - e^{-k_2 t}\right] + B_o e^{-k_2 t} \qquad [2]$$

where time zero is defined after the chase becomes effective (B is nonzero then because it is being produced during the pulse). The sum of $k_{1a} + k_{1b}$ equals the k_1 determined from the data on A degradation, and ϵ is defined as the ratio of k_{1a} to k_1. The correction factor α accounts for the difference in specific radioactivity between A and B. We estimate α to be 0.885 using the apparent molecular weights (McKnight and Fried, 1981) and the nucleotide sequence for

β-galactosidase to evaluate the difference in number of methionines between A and B.

In determining k_1 and A_o, the intensity of the A band was normalized by the amount of coprecipitated wild-type β-galactosidase (Fig. 2; see Materials and Methods). Because the wild type and A bands were not well resolved (Fig. 2), the intensity of the B band was normalized by the sum of the A and wild type bands. A least squares fit of $B/(A+1)$ yields ϵ and the effective rate constant for the degradation of B.

Degradation and Temperature

Shown in Table 1 are the determined effective rate constants for five different temperatures from 30° to 44°C. For fragment A, k_1 increases with temperature between 30° and 37°C, and then increases 2.5-fold above 37°C. The effective rate constant for fragment B (k_2) is nearly constant between 30° and 37°C, but then it also increases, by an order of magnitude, above 37°C.

At physiological temperatures, 30° to 37°C, the majority of A is degraded by the pathway which produces the intermediate B. The fit of the $B/(A+1)$ data for the 30° experiment was insensitive to the value of ϵ, and so it may be that only one pathway operates in this temperature range. However, above 37°C a much better qualitative and quantitative fit of the data was obtained with the inclusion of another pathway for the degradation of A (Fig. 1b). The values of ϵ and the fit of the data suggest that this second degradation pathway becomes important at these higher temperatures.

There appear to be two regimes of temperature dependence for the degra-

dation of the β-galactosidase fragments. The boundary of these two regimes is between 37^o and 40.5^{o}C, but the nature of the transition is unclear. It should also be noted that the effective rate constants do not correlate with the specific growth rate (Table 1).

Degradation and Abnormal Protein Expression

To determine the influence of induction level on the degradation rates, pulse-chase experiments were performed with different amounts of IPTG at 37^{o}C (results are given in Table 2). As opposed to the temperature study, both effective rate constants respond similarly, but they unexpectedly decrease with increasing IPTG concentration. With excess IPTG (1 mM), fragment A is degraded only by the pathway which produces intermediate B. Near 0.1 mM IPTG the fit of the data is insensitive to the value of ϵ, but the majority of A is still degraded to B. However, at 0.05 mM IPTG the second pathway bypassing the B intermediate is significant and essential for a good fit of the data.

β-Galactosidase Fragment Concentrations

Because the fragments are not enzymatically active, we used an α-complementation assay to determine the steady-state intracellular concentrations. This assay is a nonspecific measurement because it measures the amount of all fragments containing the initial portion of the β-galactosidase protein (Miller and Hershberger, 1984; Villarejo and Zabin, 1974; Morrison et al., 1971). Kowit and Goldberg (1977) used α-complementation with gel electrophoresis to study the degradation of individual β-galactosidase fragments. For the nonsense mutation X90, which produces a larger β-galactosidase fragment than CSH11, Kowit

and Goldberg observed only the X90 fragment and the 90 kDa intermediate for various growth conditions. We base our assumption that only the CSH11 and 90 kDa fragments are contributing to α-complementation activity upon this observation.

During early exponential phase there is balanced growth, and we can assume quasi-steady-state. A material balance on B:

$$\epsilon k_1 \alpha A_{ss} = (k_2 + \mu) B_{ss} \qquad [3]$$

where A_{ss} and B_{ss} are the steady-state intracellular concentrations, and α and ϵ are as described above. Since A and B are the only fragments contributing α-donors, we have:

$$A_{ss} + B_{ss} = C \qquad [4]$$

where C is the total α-complementation concentration in equivalent β-galactosidase per culture OD (μg (60 Klett Units)$^{-1}$). Thus from [3] and [4]:

$$A_{ss} = C\left(\frac{k_2 + \mu}{k_2 + \mu + \epsilon\alpha k_1}\right) \qquad [5]$$

$$B_{ss} = C\left(\frac{\epsilon\alpha k_1}{k_2 + \mu + \epsilon\alpha k_1}\right) \qquad [6]$$

The measured values of C and the calculated values for A_{ss} and B_{ss} are given in Table 3 as a function of temperature and in Table 4 as a function of induction level.

The total α-complementation activity decreases steadily with increasing temperature. Between 30^o and 37^{o}C there is an increase in the steady-state

level of B and a decrease in A. Above 40°C the behavior of the steady-state levels is markedly different. The α-complementation activity saturates as function of IPTG concentration near 0.1 mM. The steady-state level of B increases with increasing IPTG concentration, while the amount of A gradually decreases except for a jump near 0.1 mM. A discontinuity also occurs at 40.5°C for the steady-state amount of A as a function of temperature. Both of these irregularities occur near the apparent activation point of the second degradative pathway.

Fragment A Synthesis Rate

With the quasi-steady-state assumption, a material balance on fragment A allows the calculation of its synthesis rate:

$$R_{syn} = (k_1 + \mu)A_{ss} \qquad [7]$$

Using the values in the above Tables and Eqn [7], the calculated synthesis rates for different temperatures (Fig. 2A) and induction levels (Fig. 2B) are unexpected. The synthesis rate should monotonically increase to approach a saturated value as a function of IPTG concentration (Dalbow and Bremer, 1975a), and it should be increasing and not constant between 30° and 37°C. Some explanations are proposed in the discussion section.

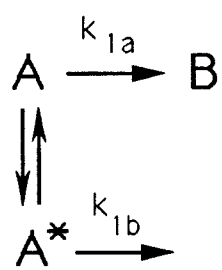
2.5 Discussion

Temperature and Degradation

In the physiological range of temperatures, from 30° to 37°C, the CSH11 β-galactosidase fragment is mainly degraded by a specific endoproteolytic cleavage.

In studying cells grown at different temperatures, Herendeen and Neidhardt concluded that in the nominal temperature range the intracellular concentration of enzymes does not change, but their activity is modulated via the temperature (Herendeen et al., 1979). Making the assumption that the level of protease(s) acting on fragment A remains constant, a plot of Ln k_1 vs T^{-1} (K) does in fact exhibit Arrhenius behavior. A linear least squares fit yields an activation energy of 20,250 cal mol^{-1}. This behavior does not continue at temperatures greater than 40°C.

Comparison of the steady-state levels of A, B, and their total amount above and below 40°C reveals that they do not increase at the higher temperatures (Table 3). Thus, the degradation rate does not seem to be responding to the amount of abnormal protein in the cell. The increase in degradation of A and B above 40°C could be a consequence of an altered protein state. The thermal stability of a protein can be important in determining its susceptibility to proteases *in vivo* (Parsell and Sauer, 1989b; Chesshyre and Hipkiss, 1989). This suggests a possible mechanism for the degradation of A:



where the folded form A is in equilibrium with an unfolded form A*. This unfolded form would presumably be more susceptible to proteases and could be degraded without producing any detectable products. It is even possible that the

same protease(s) degrade both forms. Increasing the temperature would shift the equilibrium towards the unfolded state and effectively decrease the half-life of A.

Because fragments A and B are so similar, it may be thermal instability which causes both of their degradation rates to increase significantly at higher temperatures. Protein stability and susceptibility may also explain the unusually constant degradation rate for B between 30° and 37°C. McKnight and Fried (1981) also observed this insensitivity of the β-galactosidase CSH10 mutant's k_2 to conditions, such as different lengths of induction before and the addition of chloramphenicol during degradation rate measurements, which affected the k_1.

The higher proteolytic activity above 40°C could alternatively be a cellular response such as the activation of additional degradative pathways. A second pathway might be related to the heat shock proteins since their levels are increased at higher temperatures (Ingraham, 1987), perhaps due to the presence of more denatured proteins (Ananthan et al., 1986; Goff and Goldberg, 1985; Parsell and Sauer, 1989a). For about five years it has been known that many abnormal proteins induce a heat shock-like response in *E. coli* (Goff and Goldberg, 1985; Carr et al., 1986; Parsell and Sauer, 1989a). Protease La is one of these heat shock proteins (Goff et al., 1984) and is important in the degradation of abnormal proteins (Gottesman and Zipser, 1978; Gottesman, 1990). More recent work suggests that there is more than one degradation system for abnormal proteins. Carr and Hipkiss showed abnormal proteins could induce proteolytic activity that was independent of the heat shock response (Carr et al., 1987). The regulation of this activity might be related to catabolite repres-

sion via cAMP. Secondly, Straus and Gross have shown that at least four heat shock proteins other than La are involved in the degradation of abnormal proteins. Mutations in their genes tended to stabilize the β-galactosidase nonsense mutant X90 (Straus et al., 1988). The role of these proteins is still unknown, but suggestions include their action as proteases, regulators of proteases, and/or "unfoldases." The preferred hypothesis is that they act as "unfoldases" which use ATP-energy to either stabilize unfolded proteins or make them susceptible to proteases (Straus et al., 1988; Keller and Simon, 1988).

Therefore the cell may have more than one pathway for the degradation of abnormal proteins, and their relative activities might depend on cellular environment. At 30°C mutations in the heat shock proteins do not stabilize β-galactosidase X90 as much as at 37°C (Straus et al., 1988). Also, at low temperatures some abnormal proteins do not induce heat shock proteins (Carr et al., 1986). However, increasing the levels of heat shock proteins at either 30° or 37°C does increase the degradation rate of abnormal proteins (Straus et al., 1988). Protease La plays a larger role at lower temperatures based on stablilization of abnormal β-galactosidase in *lon* mutants (Straus et al., 1988; McKnight and Fried, 1981). Thus the influence of temperature on degradation kinetics of abnormal proteins could be due to a regulation of the proteases involved or a shift from one degradation pathway to another.

Induction and Degradation

The degradation rates of both β-galactosidase fragments do not directly correlate with the steady-state amounts of A, B, or their total as the induction

level is increased (Table 4). However, the decrease in degradation rates might be explained by the fragments forming aggregates at higher intracellular concentrations. Prouty and Goldberg (1972) observed that abnormal proteins could form aggregates, and while this did not stop their degradation, it is possible that this could reduce the abnormal β-galactosidase fragment's susceptibility to proteases. When β-galactosidase X90 is produced in large amounts, from high copy number plasmids, it forms inclusion bodies and is stabilized (Cheng et al., 1981).

Higher abnormal protein concentrations may induce higher levels of heat shock proteins. Some of these proteins may act as "chaperones," binding to unfolded portions of the β-galactosidase fragments to effectively stabilize them. Under some growth conditions, *grp*E and *dna*K mutations increase the degradation rate of β-galactosidase X90, suggesting they act to stabilize the abnormal protein *in vivo* (Straus et al., 1988).

Common to the appearance of a second degradative pathway and higher degradation rates for both fragments is a low steady-state level of the total abnormal protein. The transition between these two regimes lies between 4.5 and 5.5 equivalent β-gal per OD (μg (60 KU)$^{-1}$) (Tables 3 and 4). These low abnormal protein levels may be a result of the high degradation rates, but they could alternatively be causing the cell to regulate its specific protease activity. The cell might normally degrade small amounts of abnormal protein with the help of a faster but more energy consuming pathway, but when the abnormal protein concentration is increased, regulation would shift the task away from this second pathway so that the cell could conserve energy. The cell usually sees high

abnormal protein concentrations when it is under some external stress, and it is reasonable to expect that the proteolytic systems in the cell are tightly regulated. In studying the degradation of the β-galactosidase mutants CSH10 and CSH11, McKnight and Fried (1981) performed pulse-chase experiments during which chloramphenicol was added at the beginning of the chase. Because the induction time was short and the chase times were long, it is reasonable to assume that the level of abnormal protein was lower in the cells to which chloramphenicol was added. The effective rate constants for both mutants were unexpectedly equal and greater than those determined without chloramphenicol addition. These experiments confirm that the specific protease activities are regulated *in vivo* because these greater degradation rates do not normally prevail.

Synthesis Rate

We do not find an obvious explanation for the decrease in fragment A synthesis rate at high IPTG concentrations. The synthesis rate of β-galactosidase is known to be influenced by the cell's specific growth rate (Dalbow and Young, 1975b). Additional experiments at 37°C suggest the growth rates of both the mutant BGF1 and the wild-type MG1655 strains depend on the concentration of IPTG as well as the medium (data not shown). If maximum induction occurs at 0.1 mM IPTG, then at higher IPTG concentrations the synthesis rate of fragment A should respond as the growth rate, and they in fact do exhibit qualitatively similar behavior (Table 2). Alternatively, the cell's proteolytic system may be involved. A relationship between proteolysis and catabolite repression has been implied (Carr et al., 1987), and we have observed that BGF1 always

has a greater specific growth rate than MG1655 when the *lac* operon is induced (data not shown). Since the mutant protein is not active, this growth effect probably involves the proteolytic system.

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2.8 Tables

TABLE 1

DEGRADATION RATES FOR DIFFERENT CULTURE TEMPERATURES

Temp (C)	Specific Growth μ (hr^{-1})	$k_{1_{eff}}$ (A) (min^{-1})	ϵ [a]	$k_{2_{eff}}$ (B) (min^{-1})
30.0	0.40	0.028 ± 0.005	0.74 ± 0.20	0.012 ± 0.011
33.5	0.52	0.045 ± 0.014	1.0	0.011 ± 0.010
37.0[b]	0.71	0.094 ± 0.013	1.0	0.015 ± 0.007
40.5	0.91	0.25 ± 0.04	0.21 ± 0.03	0.15 ± 0.03
44.0	0.80	0.31 ± 0.03	0.35 ± 0.05	0.12 ± 0.02

[a] ϵ is $k_{1a}/(k_{1a} + k_{1b})$ (see text).

[b] Values for 37^o are the average of 2 experiments.

Effective rate constants for Fragments A & B given with 90% confidence limits.

TABLE 2

DEGRADATION RATES FOR DIFFERENT IPTG LEVELS AT 37°C

IPTG (mM)	**Specific Growth** μ (hr^{-1})	$k_{1_{eff}}$ **(A)** (min^{-1})	ϵ [a]	$k_{2_{eff}}$ **(B)** (min^{-1})
0.05	0.73	0.17 ± 0.02	0.40 ± 0.07	0.071 ±0.021
0.1	0.78	0.12 ± 0.01	0.74 ± 0.11	0.094 ± 0.019
0.2	0.72	0.087 ± 0.018	1.0	0.026 ± 0.008
1.0	0.75	0.091 ± 0.015	1.0	0.010 ± 0.010
1.0	0.68	0.096 ± 0.010	1.0	0.019 ± 0.005

[a] ϵ is $k_{1a}/(k_{1a} + k_{1b})$ (see text).

Effective rate constants for Fragments A & B given with 90% confidence limits.

TABLE 3

FRAGMENT A & B LEVELS FOR DIFFERENT CULTURE TEMPERATURES (CONSTANT IPTG CONCENTRATION AND CULTURE O.D.)

Temp (C)	**Total α-Complementation** μg β-Gal	**A** μg β-Gal	**B** μg β-Gal
30.0	6.5	3.8 ± 0.7	2.8 ± 0.7
33.5	6.3	2.1 ± 1.8	4.2 ± 1.8
37.0[a]	5.7	1.4 ± 0.5	4.3 ± 0.5
40.5	4.4	3.4 ± 0.3	0.97 ± 0.27
44.0	2.6	1.5 ± 0.3	1.1 ± 0.3

[a] Values for 37^o are the average of 2 experiments.

Experiments performed with 1 mM IPTG in the culture medium, and samples taken at 60 Klett Units. Total α-complementation known to ± 0.3 μg equivalent β-galactosidase. Levels of Fragments A & B are given with 90% confidence limits.

TABLE 4

FRAGMENT A & B LEVELS FOR DIFFERENT IPTG CONCENTRATIONS AT 37°C

IPTG (mM)	**Total α-Complementation** μg β-Gal	**A** μg β-Gal	**B** μg β-Gal
0.05	3.5	2.3 ± 0.4	1.2 ± 0.3
0.1	6.3	3.7 ± 0.7	2.7 ± 0.6
0.2	6.3	2.2 ± 0.6	4.1 ± 0.6
1.0	5.7	1.3 ± 0.4	4.4 ± 0.4
1.0	5.8	1.4 ± 0.6	4.4 ± 0.6

Samples taken at 60 Klett Units. Total α-complementation known to ± 0.3 μg equivalent β-galactosidase. Levels of Fragments A & B are given with 90% confidence limits.

2.9 Figures

Figure 1. (a) Natural logarithm of normalized A vs. chase time for 30° (□), 37° (•), and 40.5°C (○) experiments. The solid lines are the least squares fits for determining the effective rate constants. (b) Normalized B values vs. chase time for the 44°C experiment. The dashed line represents the best fit possible without the second degradative pathway for A. The solid line is the fit including the additional pathway.

Figure 2. Fluorogram of the 30° experiment shows three immunoprecipitated polypeptides at different times in the chase. It is difficult to see the decrease in fragment A on this time scale, but the increase in fragment B is clear. The molecular weights are those observed by McKnight and Fried (1981).

Figure 3. Calculated fragment A synthesis rates for the experiments investigating temperature (**A**), and abnormal protein induction level (B) (error bars represent 95% confidence limits).

Figure 1.

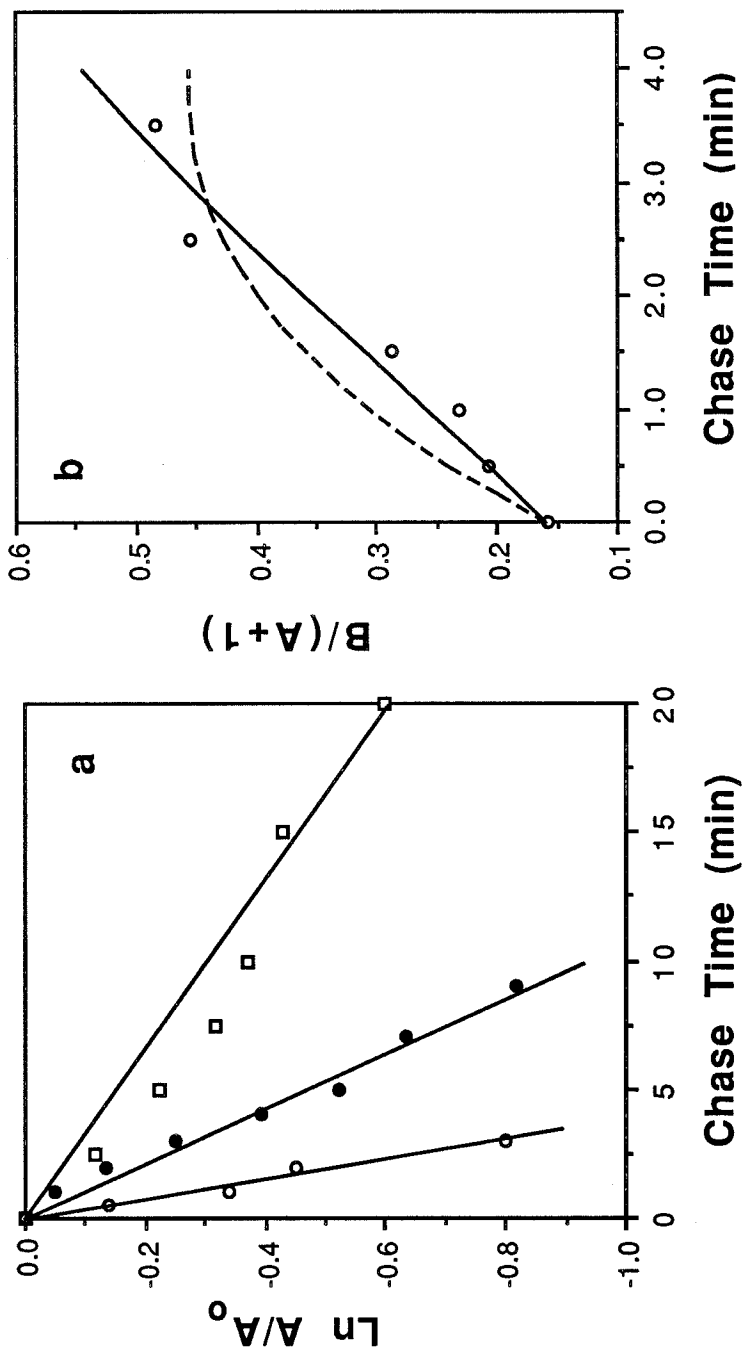

a
Ln A/A$_o$
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
0
5
10
15
20
Chase Time (min)
b
B/(A+1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.0
2.0
3.0
4.0
Chase Time (min)

Figure 2.

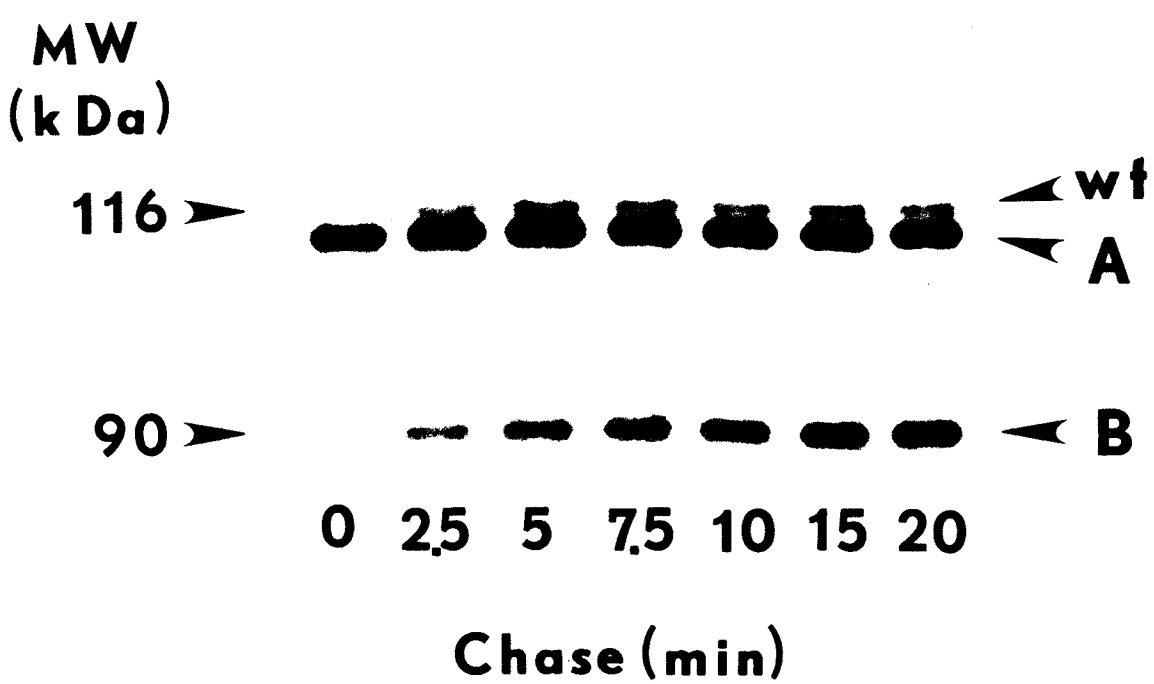
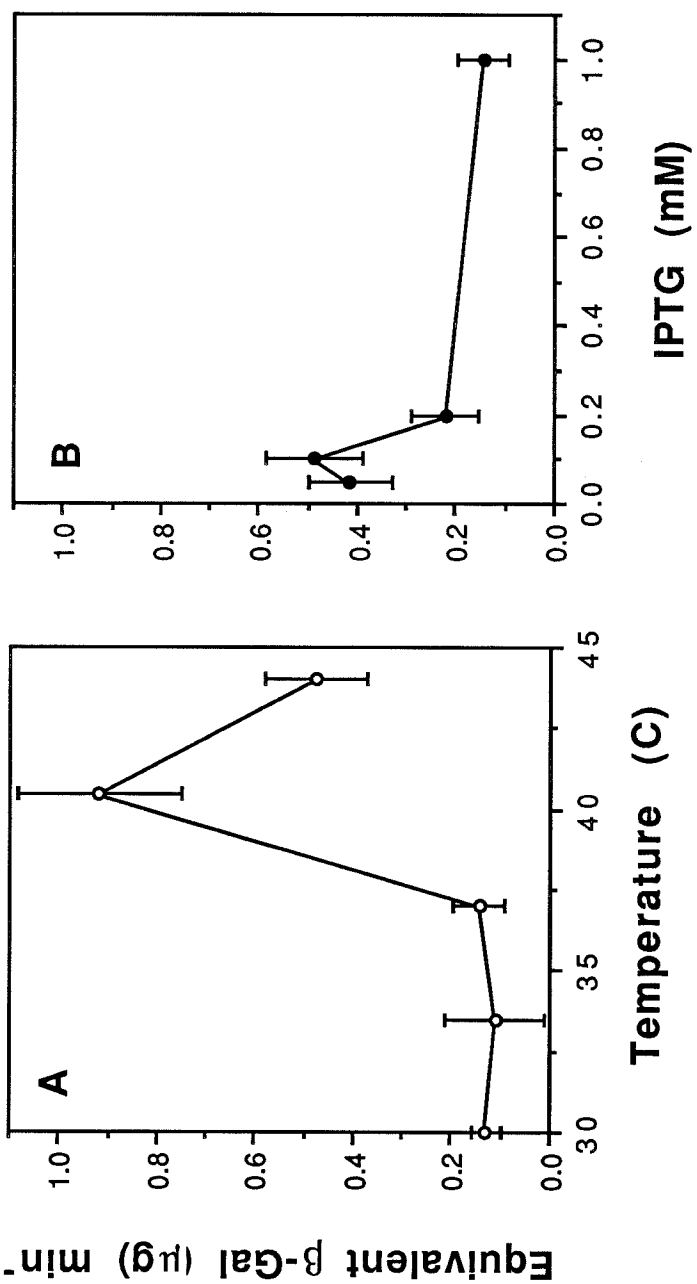


Figure 3.


A
B
Equivalent β-Gal (μg) min⁻¹
Temperature (C)
IPTG (mM)
0.0
0.2
0.4
0.6
0.8
1.0
30
35
40
45

Chapter 3

Proteolytic Response to the Expression of an Abnormal β-Galactosidase in *Escherichia coli*

3.1 Summary

Steady-state expression of low levels (0.5% total cellular protein) of an abnormal β-galactosidase causes a measurable increase in the cell's ATP-dependent proteolytic activity. The ATP-independent activity remains unchanged, even with overexpression of the stress proteins. Growth at temperatures above 40°C results in a significant decrease in the level of ATP-independent proteolytic activity compared to growth at 37°C, and the ATP-dependent activity increases 2.5-fold from 30° to 42°C. A stress response occurs following induction of the abnormal β-galactosidase at 37°C, but induction at 30°C fails to stimulate a detectable response. Increasing the level of stress proteins, even without protease La, results in higher ATP-dependent proteolytic activity in the cell.

3.2 Introduction

Expression of certain recombinant proteins in *Escherichia coli* results in their rapid degradation (Kitano et al., 1987; Tsai et al., 1987). Sometimes the proteolysis is so severe that none of the desired protein is recovered (Miyamoto et al., 1985; Sambucetti et al., 1986). The important cellular process of abnormal protein degradation is found in both prokaryotes and eukaryotes (Ananthan et al., 1986; Goldberg and St. John, 1976). Therefore, it is important to understand how abnormal proteins are recognized, how they influence the protease levels, which proteases are involved in their degradation, and most importantly, how to regulate the levels of these proteases.

The best known apparatus of abnormal protein degradation is that in *Escherichia coli*. The majority of abnormal proteins are initially cleaved endoproteolytically (Kowit and Goldberg, 1977; Miller and Green, 1981) by an ATP-requiring process (Olden and Goldberg, 1978; Voellmy and Goldberg, 1981). Many of the proteases in *Escherichia coli* have been identified and initially characterized (for review, see Goldberg et al., 1981 and Miller, 1987). Among these, protease La plays an important role in the degradation of many abnormal proteins (Maurizi et al., 1985; Simon et al., 1979). Its expression can be induced by abnormal protein presence (Goff and Goldberg, 1985), and its cellular concentration correlates with the rate of abnormal protein degradation (Goff and Goldberg, 1987). Other stress proteins have been implicated in the energy-dependent degradation of abnormal proteins (Keller and Simon, 1988; Straus et al., 1988). It has been proposed that these stress proteins may regulate prote-

olytic activity (Gottesman, 1989), act as ATP-dependent proteases like La (Goff and Goldberg, 1985), or function as "unfoldases" which make abnormal proteins accessible to cellular proteases (Keller and Simon, 1988).

We have further investigated the process of abnormal protein degradation in *Escherichia coli* by studying the CSH11 nonsense fragment of β-galactosidase encoded by a single copy chromosomal gene. This aberrant protein is a good model protein since two similar fragments of β-galactosidase exhibit all the degradative characteristics mentioned above (Kowit and Goldberg, 1977; McKnight and Fried, 1981; Straus et al., 1988). We have previously studied the effects of culture temperature and the level of induction on the *in vivo* degradation rate of the CSH11 β-galactosidase fragment and its detectable degradative intermediate (Kosinski and Bailey, 1991). The observed differences in proteolytic susceptibility might be due in part to changes in the aberrant protein's conformation (Parsell and Sauer, 1989a). These differences might also result from a cellular response to the presence of the abnormal protein. Goff and Goldberg (1985) reported induction of protease La by several types of abnormal proteins which should cause an increase in the cell's ATP-dependent proteolytic activity. Additionally, Carr et al. (1986) observed higher proteolytic activities in cells grown with amino acid analogs. In the experiments described here we measure the cellular proteolytic activity levels to determine if they are influenced by either temperature or the presence of the abnormal β-galactosidase. The induction of stress proteins is examined since these are likely involved in the degradation of β-galactosidase fragments (Straus et al., 1988).

3.3 Materials and Methods

Bacterial strains and plasmids

The strain BGF1 is a derivative of the essentially wild type strain MG1655 ($F^-\lambda^- thi$) (Guyer et al., 1980) differing only by carrying the CSH11 nonsense mutation of *lacZ* (Kosinski and Bailey, 1991). Strain CAG11054 is *ara* Δ(*lac pro*) *thi* $supC^{ts}$/[F' $lacI^Q$ *lacZ* :: *Tn*10], and strain CAG775 is *lacZX* – 90 *lon* :: *Tn*10 (C.A. Gross). Plasmid pDS2 has a *tac* promoter controlling the expression of *htpR* (Grossman et al., 1987).

Cellular fractionation and cell-free extracts

Cells were grown as described previously (Kosinski and Bailey, 1991). Culture growth was monitored using a Klett meter with a green filter. When the culture reached 60 Klett Units, cell growth was stopped by placing the culture on ice for 10 minutes. Cell fractionation was carried out by a modification of the protocol of Randall and Hardy (1986). Cells from 10 ml of culture were collected by centrifugation at 5000$\times g$ for 12 min at 4°C. The pellet was resuspended in 2.5 ml of 100 mM Tris-acetate (pH 8.2) containing 0.5 M sucrose and 5 mM EDTA. To this, 200 μl of a 2 mg/ml lysozyme solution was added followed immediately by 2.5 ml ice cold water; this preparation was then left on ice for five minutes. After addition of 187.5 μl of 0.5 M $MgSO_4$, the spheroplasts were pelleted by centrifugation as above. The spheroplasts were then resuspended in 400 μl of 10 mM Tris-HCl (pH 7.8), 5 mM MgCl and lysed by the addition of 20 μl of 10% triton solution. This technique produced efficient lysis as verified by sonication of the spheroplasts and total protein assays (data not shown). Unlysed

spheroplasts and cell debris were removed by centrifugation at $14,000 \times g$ for five minutes. The supernatant was frozen at -70^{o}C until assayed. All chemicals were from Sigma Chemical Company.

To prepare cell-free extracts, cells were grown in 500 ml of the same medium in five one-liter Erlenmeyer flasks. Culture growth was monitored using a Shimadzu UV-160 Spectrophotometer, and the cells were collected when the culture reached an OD_{590} of 0.4. The protocol of Murakami et al. (1979) was used to produce the cell-free extract. The total protein concentration of the extracts was determined using the Bio-Rad Protein Assay with bovine serum albumin (Sigma Chemical Co.) as a standard.

Protease assays

The protocol used was essentially as described by Twining (1984). The substrate was also FITC-casein (Type-III, Sigma), but the assay buffer was similar to that used in Goldberg's lab [125 mM Tris-HCl (pH 8.0 at 37^{o}C) and 22.5 mM $MgCl_2$ (Goldberg et al. 1981) with 0.2% NaN_3]. The ATP-dependent and -independent protease activities were distinguished by separately incubating the cell extract with and without 3 mM ATP (Murakami et al., 1979). The fluorescence of the supernatant was measured at 25.0 ± 0.1^{o}C. Each assay was done using 8 or 10 tubes which were removed from the 37^{o}C water bath in four hour intervals. Stock solutions of substrate, assay buffer, and buffer for measuring the fluorescence were made and stored at -20^{o}C until needed.

Stress protein induction experiments

Thirty milliliters of culture were grown at either 30^{o} or 37^{o}C until early

exponential phase. A 10 ml aliquot was transferred to a prewarmed flask containing IPTG (0.05 or 0.5 mM final concentration). The heat shock control was done by transferring an aliquot of culture grown at 37°C to a flask in a 42°C shaker. At the indicated times, 2 ml of culture was transferred to a test tube containing 40 μCi of ^{35}S-methionine (Amersham Corp.). The labeling was performed in 25 × 150 mm test tubes in a vigorously shaking water bath to ensure aerobic growth (Kosinski and Bailey, 1990). The cells were incubated with the radioactive label for five minutes for the 37° and 42°C samples, while the 30°C samples were labeled for 10 minutes. The incorporation of the label was stopped by placing the test tubes on ice for 10 minutes. The culture was then transferred to microcentrifuge tubes and spun five minutes at 14,000 × g at 4°C. The cells were washed with ice cold 0.9% NaCl to remove unincorporated label, and then pelleted again. The pellet was stored at -70°C until analyzed.

One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Samples were prepared in 4% (w/v) SDS, 20% (w/v) glycerol, 150 mM DTT (dithiothreitol), 125 mM Tris/HCl (pH 6.8), boiled for 10 minutes and immediately electrophoresed on 11.5% (fluorography) or 9-16% gradient gels (silver staining). Silver staining was done as described by Hochstrasser et al. (1988). Two-dimensional gel electrophoresis was performed using the O'Farrell technique (1975) as modified by Hochstrasser et al. (1988). Samples were prepared in 2% (w/v) SDS, 30 mM DTT and boiled for 10 minutes. Isoelectric focusing was performed to equilibrium (4 h 200 V, 4 h 500 V, and 8 h 2000 V). The ampholines used were 2% pH 3-10 (Serva), 2% pH 3-10 (Bio-Rad) and 1% pH 5-7 (Bio-Rad).

The second dimension was run on a 11.5% SDS-polyacrylamide gel at constant current (40 mA, 160 × 200 × 1.5 mm).

After fixing and washing, the gels were soaked for two hours in glycerol: Autofluor (5:95, National Diagnostics). The gels were then dried onto filter paper using a Bio-Rad Slab Gel Dryer. Fluorography was done at −70°C with Kodac X-OMAT AR film for 2 to 7 days.

3.4 Results

Proteolytic activity levels

The endoproteolytic cleavage of the β-galactosidase fragments in the cytoplasm of *Escherichia coli* is apparently done by serine proteases (McKnight and Fried, 1983) which are also capable of degrading casein (Miller, 1987), and thus these proteases can be detected by the casein assay described in the Methods section. Figure 1 shows the effect of temperature and *lac* operon induction on the cytoplasmic ATP-independent proteolytic activity. The presence of this abnormal protein in low concentrations does not measurably change the ATP-independent activity. However, this proteolytic activity is significantly influenced by the growth temperature. It increases slightly from 30° to 37°C, but then decreases by about 25% from 37° to 40.5°C. At high growth temperatures the lack of abnormal β-galactosidase influence on the ATP-independent activity is especially apparent. Here the ATP-independent proteolytic activity is nearly constant (Fig. 1) while the abnormal protein concentration decreases by more than 50% from 40.5° to 44°C (as determined by α-complementation assays, data not shown).

Studies with the similar β-galactosidase X90 polypeptide imply that the CSH11 fragment is initially cleaved by an energy-dependent process (Kowit and Goldberg, 1977). Thus, we assayed for ATP-dependent proteolytic activity in extracts of whole cells. Note that this ATP-dependent proteolytic activity in *Escherichia coli* is localized to the cytoplasm (Murakami et al., 1979), and it constitutes a minor fraction of the cell's total proteolytic activity (Table 1). The steady-state expression of the abnormal β-galactosidase induced higher ATP-dependent proteolytic activity (Table 1). Expression of the wild type β-galactosidase in MG1655 also stimulated ATP-dependent proteolytic activity but not to the extent that the abnormal β-galactosidase did. The ATP-independent activity level (for the whole cell) was nearly the same for the uninduced and induced BGF1 (Table 1).

The growth temperature has a more pronounced effect on the ATP-dependent proteolytic activity than the ATP-independent activity in cells producing this abnormal β-galactosidase (Fig. 2). The level of ATP-dependent activity increases more than 2.5-fold from 30° to 42°C (Fig. 2). Because of the decrease in the ATP-independent activity, the ATP-dependent activity makes up a greater portion of the cellular proteolytic activity at high growth temperatures. The total ATP-independent protease concentration in the cell varies by 25% over this temperature range (Fig. 2), and these levels qualitatively match the trend for the cytoplasmic ATP-independent protease activity in Figure 1.

Stress response

The induction of the wild type β-galactosidase in an isogenic strain produces

a barely detectable response (Fig. 3, lanes 11-13; see discussion). However, the expression of the abnormal β-galactosidase fragment from a single gene copy in the chromosome at 37°C definitely induces a stress (heat shock-like) response (Fig. 3, lanes 6-10). Two-dimensional gel analysis (Fig. 4) revealed the proteins that are induced by the abnormal β-galactosidase expression are also induced by a temperature shift to 42°C (Neidhardt and VanBogelen, 1981). However, the magnitude of the stress response to the abnormal β-galactosidase is not the same as that for a 37° to 42°C temperature shift (Fig. 3, lanes 14,15). It also is a different response temporally, since stress protein levels normally peak 5 - 10 minutes after a temperature shift (Fig. 3, lanes 14,15; Lemaux et al., 1978; Neidhardt and VanBogelen, 1981) while the response to the abnormal β-galactosidase is more delayed (Fig. 3 lanes 7-10). The steady state levels of the major stress proteins in either mutant or wild-type cells are not noticeably different (Fig. 5). Although approximately the same level of abnormal protein is produced at 30° as at 37°C (Fig. 3), there is essentially no stress response at the lower temperature (Fig. 3, lanes 1-5). In a separate experiment we observed no detectable stress response during 90 minutes following IPTG addition to BGF1 at 30°C (data not shown). Culture temperature is the major determinant of the steady state stress protein level in both the mutant and the wild-type cells (Fig. 5).

To investigate the contribution of the stress proteins to the cellular proteolytic activity, we assayed the extracts of cells overproducing the stress proteins. Induction of the *htpR* gene on plasmid pDS2 causes a considerable overproduction of the stress proteins (Grossman et al., 1987). This increase in stress protein

level did not increase the ATP-independent proteolytic activity level (Table 2), but it did increase the ATP-dependent activity (Table 2). Since the increase in ATP-dependent activity may be due solely to protease La, we repeated the experiment with a *lon* mutant strain which lacks a functional protease La. Table 2 shows that again the ATP-independent activity is not increased while the ATP-dependent proteolytic activity is. Unexpectedly, both specific proteolytic activities are about double for the *lon*$^-$ strain with pDS2. The ATP-dependent activity may also be more sensitive to IPTG addition in this genetic background.

3.5 Discussion

Expression of abnormal proteins which are typically degraded rapidly can increase the cellular proteolytic activity via the stress response (Goff and Goldberg, 1985). Because both protease La and the ClpP component of the Clp protease are stress proteins (Goff and Goldberg, 1985; Kroh and Simon, 1990), we expect an increase in stress protein synthesis to increase the cellular level of proteases. Though the stress response is transient (Lemaux et al., 1978), Goff and Goldberg (1985) have shown that steady-state synthesis of recombinant TPA (human tissue plasminogen activator) and HSA (human serum albumin) caused a doubling of protease La's synthesis rate, which strongly suggests a higher proteolytic activity. However, these heterologous proteins were produced in large quantities from multicopy plasmids, and, while small levels of abnormal protein have been shown to induce stress protein synthesis, it has not yet been shown that their steady-state expression causes higher proteolytic activity. Our results prove that even when the abnormal protein is a native protein

mutant and constitutes only approximately 0.5% of total protein, it can cause a measurable increase in the cell's proteolytic activity.

Only the ATP-dependent portion of cell's proteolytic activity responds to the expression of the abnormal β-galactosidase. We have shown that the ATP-independent protease levels respond to neither the induction or the steady-state expression of the abnormal protein nor to overexpression of the stress proteins. The emphasis on the ATP-dependent proteolytic activity agrees well with Goldberg's model of abnormal protein degradation (Goldberg and Goff, 1986). He hypothesized that the first and most important step is an ATP-dependent proteolytic one, which is then followed by the action of the ATP-independent proteases and the peptidases. Interestingly, there is no concurrent response by the ATP-independent protease levels. Perhaps this level would be increased for higher abnormal protein concentrations. Note also that some abnormal proteins are degraded in an ATP-independent manner (Parsell et al., 1990), proving that there is at least one other proteolytic pathway in the cell for abnormal proteins.

The important ATP-dependent proteolytic activity increases with temperature in contrast to the response of ATP-independent protease levels (Figs. 1 & 2). The ATP-dependent activity is also more responsive to both the presence of abnormal protein and the culture temperature. This activity correlates with growth temperature and the corresponding specific growth rate (0.37, 0.66, 0.88 hr^{-1} for 30°, 37°, and 42°C respectively) rather than with the abnormal protein concentration, suggesting that regulation of this activity is influenced more by growth conditions than abnormal protein presence. However, it is likely that, while the abnormal protein concentration is decreasing with increasing temper-

ature (Fig. 5), the protein is becoming more unfolded. This might increase the amount of unfolded polypeptide and thus increase the "effective" abnormal protein concentration. Regardless of the induction mechanism, the increase in ATP-dependent proteolytic activity with temperature is not proportional to the larger increase in the *in vivo* abnormal β-galactosidase degradation rate observed at higher temperatures (Kosinski and Bailey, 1991). Possible explanations for this discrepancy include regulation of the *in vivo* specific activity of the protease(s) degrading the abnormal β-galactosidase and significant changes in the abnormal protein's conformation.

The ATP-independent protease level does not monotonically increase with culture temperature. The lower ATP-independent proteolytic activity above 40°C is opposite to the increase in both the β-galactosidase fragment *in vivo* degradation rate (Kosinski and Bailey, 1991) and the total protein turnover rate (St. John et al., 1978). The cell decreases its ATP-independent protease level without abnormal protein expression (Fig. 1), so this must be a normal cellular response to higher temperature. If many proteins do not fold properly at growth temperatures above 40°C, a kinetic competition between degradation and correct folding of proteins may become important. Reduced protease concentration could compensate for the increase in protease specific activity and "substrate" concentration at elevated temperatures, providing more time for some proteins to fold properly. The higher level of stress proteins (Fig. 5) may be another cellular response to assist in protein folding (Beckmann et al., 1990; Rothman, 1989).

Although previous studies of low abnormal protein expression have shown

the induction of a stress response, the protein was still expressed from multicopy plasmids (Parsell and Sauer, 1989b). Presence of common multicopy cloning vectors without heterologous inserts have been shown to affect *E. coli* metabolism (Seo and Bailey, 1985) and cellular protein levels (Birnbaum and Bailey, 1991). Reacting to the synthesis of the abnormal β-galactosidase from a single chromosomal gene confirms the sensitivity of the stress response. The fact that the cell responds to the small level of abnormal β-galactosidase as it does to a temperature shift supports the hypothesis that it is the abnormal protein presence in the cell that triggers the heat shock response. However, we have also observed that IPTG addition to wild-type cells can also induce a slight stress response (Fig 3; unpublished results).

Differences in the stress response observed here from a normal heat shock response are probably due to the amount of abnormal β-galactosidase present. The concentration of abnormal protein in the cell has been shown to affect the level of stress response (Parsell and Sauer, 1989b), and there are likely more abnormal proteins in the cell during a normal heat shock than after the induction of this single-copy mutant β-galactosidase gene. Also, the rate of abnormal protein formation after a temperature upshift is likely greater than after the induction of this abnormal β-galactosidase which might explain the slower stress response observed (Fig. 3). The similarity of the steady state level of stress proteins with and without abnormal β-galactosidase synthesis (Fig. 5) suggest this stress response is also transient.

It is possible that there is a lower temperature limit below which abnormal proteins do not induce a stress response. Carr et al. (1986) found that amino

acid analog-containing proteins did produce a stress response at 37°C but did not at 20°C. Alternatively, a mutant λ repressor protein is capable of inducing a stress response at both 30°C and 37°C (Parsell and Sauer, 1989b). However, the β-galactosidase fragment in this study does not induce a detectable stress response at 30°C (Fig. 3) even though the amount of protein is similar to that at 37°C. This abnormal β-galactosidase may be more folded at 30° than at 37°C which could cause a lower "effective" abnormal protein concentration.

It has recently been shown that the ClpP proteolytic portion of the most abundant ATP-dependent protease in *E. coli* is a stress protein (Kroh and Simon, 1990). However, the activity of the protease has been shown to depend on the level of ClpA (the ATPase) component, and it is not under σ^{32} control (Katayama et al., 1990). Kroh and Simon (1990) did not show that the ATP-dependent proteolytic activity in the cell is increased when the stress proteins are overproduced in a *lon*$^-$ strain. Our results show this is indeed the case. While other stress proteins might play ATP-dependent roles in abnormal protein degradation, the connection of the Clp protease to the stress response leaves their role more uncertain. Because of the hypothesis that some of these other stress proteins act as ATP-dependent unfoldases (Keller and Simon, 1988; Straus et al., 1988) and contribute to abnormal protein degradation, we have been careful to define the ATP-stimulated contribution in terms of proteolytic activity rather than amount of protease.

Finally, the protease assays of the *lon*$^-$ strain reveal a 2-fold higher level of specific proteolytic activity compared to the *lon*$^+$ strain. The *lon*$^-$ strain grew poorly and, while many of its phenotypes can be overcome by secondary

mutations (Gottesman, 1990), there appears to be a significant increase in the level of the remaining proteases in possible compensation for the loss of the critically important protease La.

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3.8 Tables

Table 1. *Effect of abnormal β-galactosidase on the total cellular proteolytic activity*

		Specific Proteolytic Activity		
Strain	IPTG (mM)	without ATP	with ATP	ATP-dependent
BGF1	0.0	47.0 ± 3.6	53.0 ± 2.6	6.2 ± 3.9
BGF1	1.0	51.0 ± 3.0	65.0 ± 2.4	15.0 ± 3.5
MG1655	1.0	55.0 ± 2.6	65.0 ± 2.5	9.9 ± 3.0

Cells were grown to midexponential phase with or without 1 mM IPTG. Proteolytic activities are given as equivalent α-chymotrypsin per total protein (ng per mg). Values are the average of two separate experiments and are given with 90% confidence limits.

Table 2. *Effect of stress protein overproduction on the cell's proteolytic activity*

		Specific Proteolytic Activity	
Strain	IPTG	without ATP	with ATP
CAG11054:pDS2	-	37 ± 4	32 ± 4
	+	32 ± 3	46 ± 6
CAG775(*lon*):pDS2	-	73 ± 8	64 ± 7
	+	60 ± 12	106 ± 10

Cells containing the plasmid pDS2, which bears the *htpR* gene under control of a *tac* promoter, were grown at 30°C to midexponential phase. Half of the culture was harvested while *htpR* expression was induced in the other half with 1 mM IPTG for 25 minutes. Proteolytic activities are given as equivalent α-chymotrypsin per total protein (ng per mg) with 90% confidence limits.

3.9 Figures

Figure 1. ATP-independent protease concentration in the cytoplasm of BGF1. Cells were grown with (•) or without (○) 1 mM IPTG present. The level of ATP-independent activity in 10 ml of culture at 60 Klett Units is reported as ng α-chymotrypsin per ml extract. The 90% confidence limits are $\pm$12% for the 33.5° and 44°C values and $\pm$6% for the other temperatures.

Figure 2. ATP-independent (•) and ATP-dependent (□) specific proteolytic activity levels in BGF1 grown in the presence of 1 mM IPTG. Proteolytic activity given as equivalent α-chymotrypsin per total protein (ng per mg).

Figure 3. Stress response to induction of the *lac* operon with IPTG. The position of the stress proteins dnaK, htpG and groEL are marked, and arrows indicate the position of the abnormal (◄) and normal (◄) β-galactosidase. Lanes 1-5 represent proteins from BGF1 grown at 30°C. Lanes 6-10 and 11-13 contain proteins from BGF1 and MG1655, respectively, grown at 37°C. Lanes 14 and 15 represent proteins from BGF1 culture that was subjected to a temperature shift from 37° to 42°C. β-galactosidase expression was induced with 0.05 (lanes 2,3,7,8) or 0.5 (lanes 4,5,9,10,12,13) mM IPTG. Lanes 1, 6, and 11 contain proteins from uninduced cultures. Cells were pulse labeled at 10 min (lanes 2,4,7,9,12), 30 min (lanes 8,10,13), and 55 min (lanes 3,5) after IPTG addition. Cells subjected to a temperature shift were pulse lableled at 10 min (lane 14) and 30 min (lane 15) after the temperature shift. Only part of the

fluorogram is shown. Each sample loaded had 10,000 counts-per-minute (cpm) total. (Electrophoresis performed by U. Rinas.)

Figure 4. Two-dimensional gel electrophoresis of BGF1 before (**A**) and after (**B**) the induction of the abnormal β-galactosidase. Cells were grown at 37°C and pulse-labeled before (**A**) and 30 minutes after (**B**) IPTG addition. The following stress proteins were identified according to known databases (Phillips et al. 1987): (1) Protease La (H94.0, H94.1); (2) HtpM (F84.1); (3) DnaK (B66.0); (4) HtpG (C62.5); (5) GroEL (B56.5); (6) GrpE (B25.3); and (7) GroES (C15.4). The position of the abnormal β-galactosidase is indicated with an arrow. The right side of the gels corresponds to lower pH values. Samples loaded each had 100,000 cpm total. (Electrophoresis performed by U. Rinas.)

Figure 5. One-dimensional gel electrophoresis of total cell protein of BGF1 (lanes 1,3, and 5) and MG1655 (lanes 2,4, and 6). Cells were grown at 30°C (lanes 1 and 2), 37°C (lanes 3 and 4), and at 44°C (lanes 5 and 6). Arrows indicate the position of the abnormal (◄) and normal (◄) β-galactosidase. Molecular weight standards (lane 0) include rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa, double band), and hen egg white lysozyme (14.4 kDa). (Electrophoresis performed by U. Rinas.)

Figure 1.

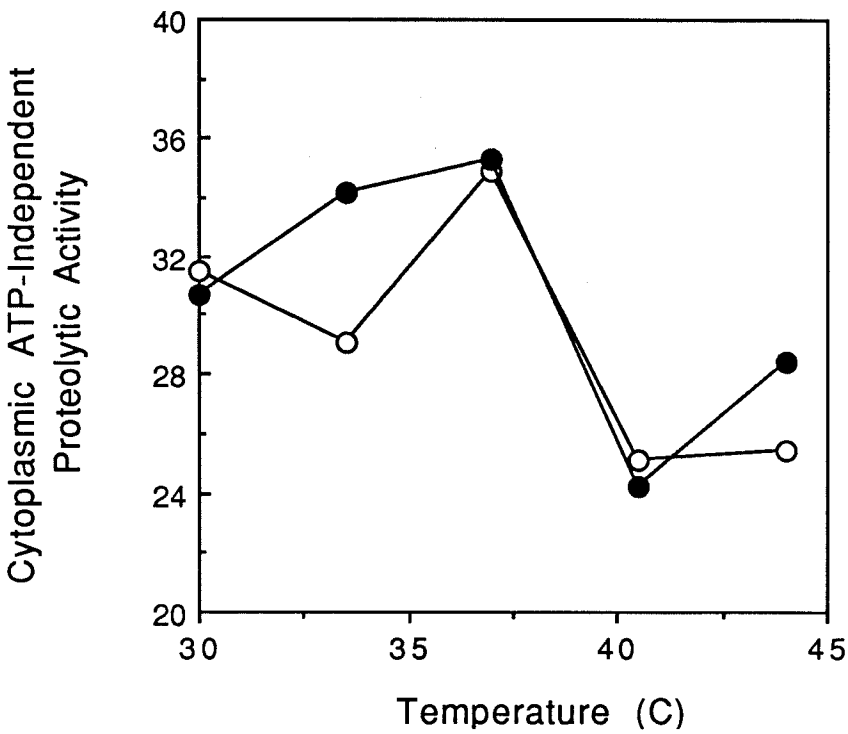

Cytoplasmic ATP-Independent
Proteolytic Activity
40
36
32
28
24
20
30
35
40
45
Temperature (C)

Figure 2.

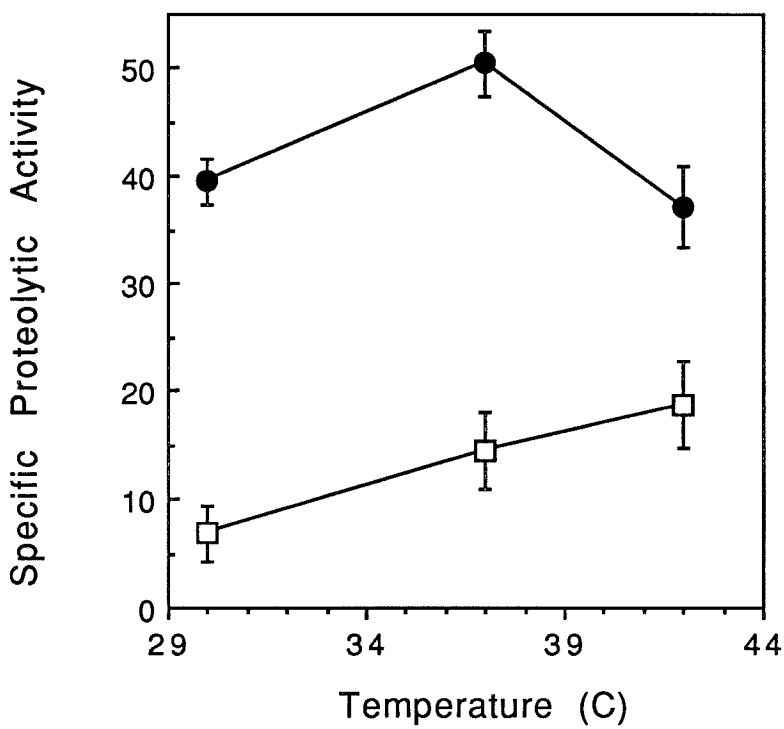

Specific Proteolytic Activity
50
40
30
20
10
0
29
34
39
44
Temperature (C)

Figure 3.

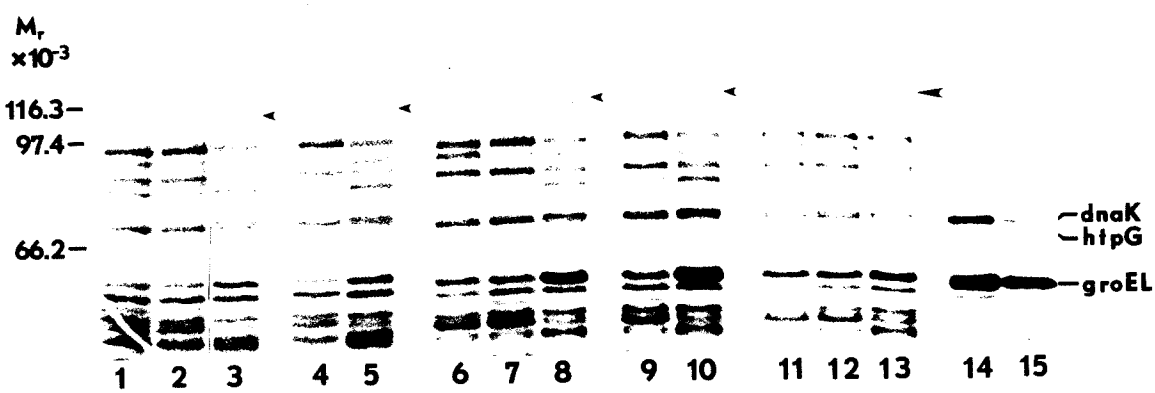
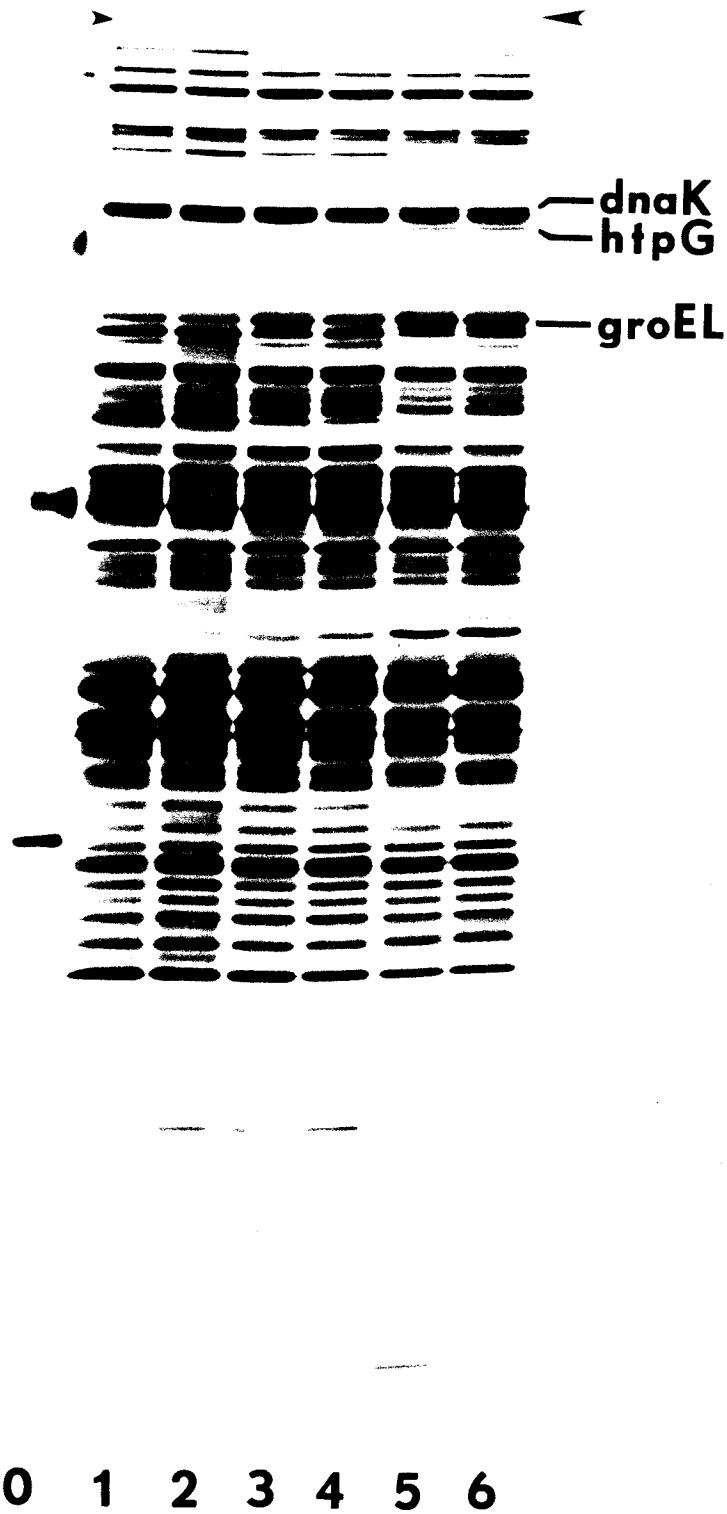


Figure 4.

Figure 5.



Chapter 4

Structural Characteristics of an Abnormal Protein Influencing its Proteolytic Susceptibility

4.1 Summary

Both β-galactosidase fragments, the CSH11 mutant polypeptide and its 90 kDa degradative intermediate, exist predominantly as monomer subunits instead of in the tetrameric form characteristic of the native enzyme. Both fragments also produce small amounts of dimers and tetramers. Though the fragments can bind the enzyme's substrate and can be purified by affinity chromatography, the tetramers formed are not enzymatically active. The tetramer and higher molecular weight aggregate forms of the wild-type enzyme confer greater protection from proteolysis for the protein's α-donor fragment than does the fragments' monomers. The thermal stability of both β-galactosidase fragments correlates with their sensitivity to α-chymotrypsin. The relatively low thermal stability of the 90 kDa degradative intermediate appears to be the cause of the significant increase in its proteolytic susceptibility, both *in vitro* and *in vivo*, at moderately high temperatures.

4.2 Introduction

Perhaps the most important factor determining the proteolytic susceptibility of a recombinant protein is how its conformation is influenced by the cellular environment. The fundamental reason for many protein instabilities is an abnormal conformation due to a nonnative environment (Goldberg and St. John, 1976; Talmadge and Gilbert, 1982) or a mutation (Bowie and Sauer, 1989; Parsell and Sauer, 1989). Even single amino acid substitutions can significantly affect protease sensitivity (Reidhaar-Olson et al., 1990). While the very rapid degradation of some recombinant proteins can be reduced by mutating the genes of critical proteases (Gottesman, 1990), this approach is not always desirable (Tsai et al., 1987) or always available. Therefore, the protein's structural properties can be critically important when designing the expression system for a recombinant protein.

The most investigated proteolytically susceptible proteins are the nonsense fragments of β-galactosidase (Goldschmidt, 1970; Kowit and Goldberg, 1977). Though the wild-type enzyme is an extremely stable tetramer, β-galactosidase fragments are generally very labile (Lin and Zabin, 1972). These protein fragments make good model abnormal proteins since they are degraded endoproteolytically and in an ATP-requiring manner that somehow involves Protease La (Kowit and Goldberg, 1977). Though some of the structural properties of these mutants are known, this study will examine the structural characteristics of two particular β-galactosidase fragments whose *in vivo* degradation characteristics have been determined (Kosinski and Bailey, 1991).

Comparison of the mutant β-galactosidase fragments to the wild-type enzyme will identify important physical attributes influencing proteolytic susceptibility. In particular, the affinity for aggregation will be checked. β-galactosidase is known to have a tendency to form aggregates in addition to its normal tetramer (Appel et al., 1965; Marchesi et al., 1969). Aggregation in the form of inclusion bodies in many cases stabilizes recombinant proteins (Cheng et al., 1981; Kitano et al., 1987), but this phenomenon is true almost exclusively for plasmid-based expression. The β-galactosidase fragments in this study are produced from a single gene copy in the chromosome of an otherwise wild-type strain of *Escherichia coli*. Additionally, the effect of temperature on the β-galactosidase fragments will be investigated by both thermal stability and protease sensitivity analyses. The well-known protease α-chymotrypsin will be used because of its limited specificity and the fact that the proteases in the cell extract are not necessarily active against the β-galactosidase fragments even when gentle lysis techniques are used (McKnight and Fried, 1983).

4.3 Materials and Methods

Gel electrophoresis

All electrophoresis was performed using the Pharmacia PhastSystem. Both SDS-PAGE and Native-PAGE were done according to the protocols suggested by Pharmacia. Samples for SDS-PAGE were prepared by diluting 3-fold with a standard sample buffer (Hames, 1981) and by boiling for five minutes. Samples for Native-PAGE were also prepared by a 3-fold dilution but with a salt-free

solution (15% glycerol (w/v), 1.5 mM $MgCl_2$, 0.04% Bromophenol Blue, and 10 mM mercaptoethanol). Electrophoresis was performed immediately after sample preparation. Electrophoresis standards included SDS-PAGE Molecular Weight Standards (High Range) from Bio-Rad and β-galactosidase (*Escherichia coli*) from Sigma Chemical Company.

Protein detection was accomplished using the procedures suggested by Pharmacia. Coomassie Blue staining of the PhastGels was done by Pharmacia's Fast Coomassie Staining Method (#200). Quantitation was accomplished by scanning the gels with a LKB Laser Densitometer. β-Galactosidase detection following electrophoresis of cell extracts was accomplished by Western Blotting. Tranferring the gel onto nitrocellulose filter was done using Pharmacia's protocol (Development File #220). The electroblotting conditions and detection with peroxidase-labeled antibodies were essentially as described by Towbin et al. (1979).

Chymotrypsin degradation experiments

Degradation of the β-galactosidase fragments by α-chymotrypsin was performed both in cell extracts and with the affinity purified fragments (see below). The wild-type β-galactosidase analyzed was either purchased from Sigma Chemical Company or produced in the wild-type strain MG1655. The β-galactosidase fragments were produced by the strain BGF1 (Kosinski and Bailey, 1991) containing the CSH11 nonsense mutation of *lacZ*. Cell extracts were made from early exponential cells grown in M63 minimal medium supplemented with all the amino acids except methionine and cysteine (Kosinski and Bailey, 1991).

The extracts were produced essentially by the procedure of Randall and Hardy (1986) except for the use of brief sonication to rupture the spheroplasts. Sixty ml of culture produced 400 μl of cell extract in 20 mM Tris:HCl (pH 7.4), 10 mM $MgCl_2$, and 10 mM mercaptoethanol.

To 360 or 500 μl of cell extract was added 2.5 or 10 μl respectively of a 10 mg/ml α-chymotrypsin solution (in 10 mM citrate/HCl (pH 3.0)). The extracts were then incubated with the chymotrypsin at 37°C, and at the indicated times 50 μl was collected and analyzed for α-complementation activity by the previously described assay (Kosinski and Bailey, 1991).

The degradation of the affinity-purified β-galactosidase fragments by α-chymotrypsin was performed by incubating 100 μl of the purified protein solution (see below) with 0.1 μg α-chymotrypsin. Samples of 10 μl were collected every 50 seconds and added to 20 μl cold SDS sample buffer on ice. After all the samples were collected they were boiled simultaneously for five minutes.

Affinity purification

Purification of the β-galactosidase fragments was conducted as by Ullmann (1984) with the following exceptions: cultures were grown in modified LB medium (Dennis et al., 1985) at 30°C, 3 liters of culture (1.5 OD_{590}) were applied to 1 ml of p-aminophenyl-β-D-thiogalactopyranoside resin (Sigma Chemical Company), and, after thorough washing, the resin was incubated with the elution buffer (100 mM sodium borate (pH 10), 10 mM β-mercaptoethanol) for 10 minutes before the bound proteins were eluted. Fractions of 250 μl were collected. The three fractions with the greatest protein concentration (approx-

imately 1.1 mg/ml) were combined and the pH reduced from 10 to 8 by the addition of approximately 20 μl of 6 N HCl. Aliquots of 100 μl were quick frozen in methanol at -70°C and then stored at this temperature.

Thermal denaturation

The thermal denaturation of the β-galactosidase fragments was determined by incubating 15 μl of the affinity-purified protein solution (see above) in a parafilmed glass test tube in a heated water bath. After five minutes at the indicated temperatures the tubes were transferred to a water bath at room temperature and allowed to cool. After the addition of 30 μl of native-PAGE sample buffer the samples were subjected to Native-PAGE on either 12.5 or 20% homogeneous gels.

4.4 Results

Aggregation affinity of β-Galactosidase fragments

Nondissociating or native PAGE is a direct method for detecting protein aggregates in cell extracts. The extracts analyzed were prepared in a "gentle" manner by EDTA/lysozyme treatment followed by either brief sonication or addition of small amounts of triton X-100. Too much sonication or too high a concentration of reducing agents (dithiotreitol, β-mercaptoethanol) detectably increased the amount of aggregation of the β-galactosidase fragments (data not shown). However, triton addition caused no observable change in the way the β-galactosidase fragments ran on the native gels (data not shown). We, therefore,

employed 0.05% (w/v) triton to lyse the spheroplasts in the preparation of the extracts shown in Figure 1.

The positions of the different forms of β-galactosidase on a 12.5% native gel are consistent with those previously observed (Edwards et al., 1990). The identifications of Edwards et al. (1990) have been used to label the different bands in Figure 1. There are tetramers, dimers, and monomers present in the solution of commercial wild-type β-galactosidase (Fig. 1, lane 1). There are also large amounts of high molecular weight aggregates between the tetramer and the sample application point in the stacking gel (Fig. 1, lane 1). The monomer of the wild-type enzyme (lane 1) is indeed slightly larger than the monomer of the CSH11 fragment in lanes 3-6. The solution of commercial β-galactosidase also contains a fragment (lane 1) that matches the 90 kDa fragment in lanes 3-6. The extract from the wild-type cells contains only the tetramer and higher molecular weight aggregates of β-galactosidase (Fig. 1, lane 7). The distinct bands above the tetramer position may correspond to hexamers, octamers, or decamers that have been previously observed (Appel et al., 1965; Marchesi et al., 1969).

Extracts from BGF1 grown in different concentrations of IPTG (Fig. 1, lanes 2-6) predominantly contain only monomers of the CSH11 and 90 kDa fragments. There are no high molecular weight aggregates detectable even with high concentrations of IPTG. However, as can be seen better in lanes 8 and 9, it does appear that both dimers and tetramers of both β-galactosidase fragments are present at relatively very low levels. BGF1 grown on X-Gal plates (5-bromo-4-chloro-3-indoyl-β-D-galactoside) produces very light blue colonies, but can be attributed to a low readthrough frequency of the amber mutation. There was no

detectable β-galactosidase activity in the cell extracts, and there is no indication that the tetramer species in Figure 1 are enzymatically active.

Investigation of protein structure can also be done by proteolytic analysis (Edwards et al., 1988; Zabin, 1982). We employed the protease α-chymotrypsin to determine if there is a difference in proteolytic susceptibility for the β-galactosidase fragments from cells grown with different concentrations of IPTG. The amount of the β-galactosidase fragments was measured using an α-complementation assay, and without chymotrypsin addition there was no detectable loss in α-complementing activity in the cell extract (data not shown). Unexpectedly, the complementing activity increased after short incubation times with the protease (Fig. 2). The activity of the protease is somehow making the generation of the α-donor fragment easier. However, there is no detectable difference in the proteolysis of the β-galactosidase fragments in extracts from 0.05 and 1.0 mM IPTG cultures.

Chymotrypsin digestion of the wild-type and mutant β-galactosidases reveals (Fig. 2) a major difference in their proteolytic susceptibility. The almost constant complementing activity for the wild-type enzyme implies that the tetramer and higher molecular weight aggregates (Fig. 1, lane 7) protect the α-donor fragment from the proteases and peptidases in the cell extract. The faster decrease in complementing activity during the digestion of the β-galactosidase fragments is evidence that they are much less protected (Fig. 2).

Affinity purification

Affinity purification of the wild-type β-galactosidase using a substrate ana-

log, p-aminophenyl-β-D-thiogalactopyranoside, was first done by Steers et al. (1971). Ullmann (1984) modified this procedure and used it to purify β-galactosidase fusion proteins. Most of the β-galactosidase mutants are not enzymatically active, but many can bind the substrate and be reasonably purified by a similar procedure (Villarejo and Zabin, 1973; Villarejo and Zabin, 1974). Using the protocol of Ullmann (1984) we purified the β-galactosidase fragments from extracts of cultures of BGF1 grown either with or without IPTG (Fig. 3A). Both the CSH11 and 90 kDa fragments are purified, but so also is an unknown contaminant protein with a molecular weight of approximately 45 kDa (Fig. 3A). Western blotting of this gel produced no signal for the contaminant band, confirming it is not another fragment of β-galactosidase (data not shown). The purified proteins are eluted at a concentration of approximately 1.1 mg/ml with about 73% contaminant, 16% CSH11 fragment, and 12% 90 kDa fragment as determined by laser densitometer scanning (Fig. 3A). While Coomassie Blue staining shows good purity, silver stained gels revealed many more proteins are present in the column effluent (data not shown). Many of these other contaminant proteins might also be fragments of β-galactosidase.

The affinity bound proteins were eluted using a 100 mM sodium borate (pH 10) buffer (Ullmann, 1984). A 100 mM phosphate buffer (pH 7.8) failed to elute the bound proteins (data not shown). Because the purified β-galactosidase fragments had a great affinity for membranes (only 22% recovery from a Centricon-10 ultrafiltration unit (Amicon)), we did not employ either filtration or dialysis techniques to change the buffer or the pH of the eluted material. We did, however, titrate the sodium borate buffer with concentrated HCl to a final pH

of 8.

The contaminant appears to run as a dimer on a native PAGE (Fig. 3B). At pH 10 it migrates with the CSH11 fragment, but at pH 8 the contaminant and CSH11 are separable: the contaminant at the top and the 90 kDa fragment at the bottom with the CSH11 fragment between these two (Fig. 3B). The contaminant is very hydrophobic and not only binds the Centricon-10 membrane but also causes binding of wild-type β-galactosidase to the membrane (101% recovery of activity versus 64% recovery of activity in the presence of the contaminant protein; data not shown). The following sets of experiments were performed with the affinity purified proteins in 100 mM sodium borate (pH 8) buffer.

Thermal denaturation

When β-galactosidase is exposed to high temperatures, it breaks down to dimers, then to monomers, and then these form high molecular weight aggregates (Edwards et al., 1990). Monitoring the decrease in the amount of tetramer after exposure to increasing temperatures shows when β-galactosidase thermally denatures. Such an experiment with commercial β-galactosidase in 100 mM sodium borate (pH 8) at 1.1 mg/ml gives a melting temperature of approximately 50°C (Fig. 4), matching previous results (Edwards et al., 1990). Exposure of the affinity-purified proteins to increasing temperatures and quantitating the decrease in β-galactosidase fragment monomer concentration gave the results in Figure 4. The monomer of wild-type β-galactosidase may be more thermally stable than that of the CSH11 mutant (Edwards et al., 1990). However, the 90 kDa fragment is less thermally stable than the CSH11 fragment even though

it is less proteolytically susceptible at moderate temperatures (Fig. 4). Under the conditions of this experiment, neither fragment is significantly unfolded at normal growth temperatures.

α-Chymotrypsin degradation of β-galactosidase fragments

Edwards et al. (1988) investigated the degradation of wild-type β-galactosidase by α-chymotrypsin. They found that the monomer was cleaved first in two closely located sites near the middle and that the larger fragment formed was about 68 kDa (Edwards et al., 1988). In Figure 5 can be seen a chymotryptic degradation product of the β-galactosidase fragments that appears to be the same degradative intermediate produced by the wild-type enzyme. This polypeptide was observed following chymotrypsin digestion of the β-galactosidase fragments even at 45°C (data not shown).

A natural logarithmic plot of the rate constants for the chymotrypsin degradation of the β-galactosidase fragments versus 1/T(K) is shown in Figure 6. The degradation of the CSH11 fragment behaves in an Arrhenius fashion over the temperature range of the experiment (Fig. 6A). However, the rate constant for the 90 kDa fragment is nearly constant between 33° and 41°C (Fig. 6B). The much higher degradation rate at 45°C matches the sharp decrease in thermal stability seen in Figure 4.

4.5 Discussion

The β-galactosidase polypeptides produced by the CSH11 nonsense mutation, the CSH11 fragment and the 90 kDa degradative intermediate, exist in

the cell mainly as monomer subunits. While both may form small amounts of dimers and tetramers (Fig. 1), there is no increase in the formation of higher molecular weight aggregates with expression level from a single gene copy in the chromosome. We hypothesized (Kosinski and Bailey, 1991) that an observed decrease in the *in vivo* degradative rate constant might be due to decreased protease sensitivity caused by aggregation of the β-galactosidase fragments. The β-galactosidase mutant X90 synthesized from a plasmid formed inclusion bodies that effectively stabilized this fragment (Cheng et al., 1981), but its expression from the chromosome results in unaggregated monomers (Fowler and Zabin, 1966). Addition of amino acids to the medium, use of higher IPTG concentrations, as well as a different analytical technique (native PAGE versus analytical ultracentrifugation) proves that very high expression levels are required for premature-termination fragments of β-galactosidase to aggregate.

While the C-terminal portion of β-galactosidase is important for oligomerization, detection of both dimers and tetramers for the CSH11 mutant suggests that the C-terminal region is not essential (Fig. 1). Mutants of β-galactosidase lacking a crucial segment in the N-terminal region form dimers but not tetramers (Celada and Zabin, 1979), while deletions of the C-terminal end disrupt even dimerization (Fig. 1). These C-terminal deletions apparently do not eliminate subunit binding but rather shift the equilibrium much farther towards the monomer state.

The importance of this result is apparent when considering genetic modifications (genetic engineering) of proteins. There are critical regions in most proteins such that even single amino acid changes can dramatically increase pro-

tease sensitivity (Ogasahara et al., 1985; Reidhaar-Olson et al., 1990). A possible effect of some mutations is to disrupt subunit binding which can result in increased proteolytic susceptibility. The M15 deletion of 30 amino acids (11-41) from the N-terminal region of β-galactosidase disrupts the normal tetramerzation (Celada and Zabin, 1979), and the resulting mutant is very protease sensitive. However, even though dimerization still occurs, the M15 dimer is much more susceptible to proteolysis than the CSH11 and 90 kDa fragments (Figs. 5; Edwards et al., 1988). Protection of β-galactosidase against proteolysis is due to its oligomerization (Zabin, 1982) and probably also to the nonspecific aggregation. This provides an explanation for the decreased proteolytic susceptibility of the wild-type enzyme compared to the fragments in the cell extracts (Fig. 2).

The increase in complementing activity following chymotrypsin digestion may be explained by understanding how the α-donor fragments are generated. We used autoclaving to release the α-donor from both the wild-type and mutant polypeptides. Incubation with chymotrypsin should open up the polypeptides' structure which could make release of the α-donor by autoclaving easier. In support of this explanation, Lin and Zabin (1972) found that CNBr treatment of β-galactosidase polypeptides caused about an 8-fold increase in complementing activity. The action of the CNBr could be considered similar to that of chymotrypsin. This freeing of the α-donor by proteolytic action can apply for aggregates as well as monomers. Thus the proteolysis experiments (Fig. 2) may actually be showing that some of the β-galactosidase fragments do exist as aggregates and were not detected by native PAGE. Even if there are aggregates present, there appears to be no increase with higher expression levels (Fig. 2).

Both β-galactosidase fragments bind the substrate analog and can be purified by affinity chromatography (Fig. 3A). This is understandable considering the fragments present with the commercial β-galactosidase must have been copurified (Fig. 1). However, the purification procedure used here (Ullmann, 1984) was not reported to copurify any additional *E. coli* proteins. It is possible that a specific mutation is necessary to eliminate the presence of this contaminant, but Ullmann (1984) used several different strains and made no mention of any essential genotype. The expression of the abnormal β-galactosidase might cause higher cellular levels of this contaminant protein which could explain why it was not detected during purifications of wild-type or fusions of β-galactosidase (Ullmann, 1984). While other β-galactosidase fragments have been purified by affinity chromatography, there was no SDS-PAGE analysis to show the presence of any contaminants (Villarejo and Zabin, 1973). It is unlikely though that the contaminant is purified due to any binding to the β-galactosidase fragments since about the same amount is recovered from extracts grown without IPTG (Fig. 3A).

We tried to identify this contaminant both to identify it and try to eliminate it from the purification. A likely candidate was the *lacY* gene product, the *lac* permease. It has the right size, 46.5 kDa, it should bind the substrate analog, and the binding to the Centricon-10 filter would be consistent with a hydrophobic membrane protein (Beckwith, 1987). However, the contaminant protein appears to be a dimer (Fig. 3B), which the *lac* permease is not. We therefore transferred by P1 transduction a Tn10 transposon into the *lacY* gene in BGF1 to form BGF2 ($lacZ^-$ $lacY^-$). The transposon inactivated the *lac*

permease as confirmed by the failure of BGF2 to grow on melibiose at 42°C (Beckwith, 1987). If the contaminant was the *lac* permease, then we should expect to either eliminate the contaminant from the purification by destroying the binding region of the protein or at least change its molecular weight. Neither occurred (data not shown), so it is very unlikely that the contaminant is the *lac* permease.

Affinity purification of the β-galactosidase fragments did allow for more direct investigations of their thermal properties. The degradation characteristics of the β-galactosidase fragments by chymotrypsin *in vitro* do indeed match qualitatively the *in vivo* results previously observed. The Arrhenius behavior of the rate constant for the CSH11 fragment as well as the insensitivity of the 90 kDa fragment's rate constant to increasing temperature are consistent with the *in vivo* trends (Fig. 6; Kosinski and Bailey, 1991). Even the transition between low and high proteolytic susceptibility of the degradative intermediate (Fig. 6) was seen *in vivo* (Kosinski and Bailey, 1991). The idea that thermal unfolding of the 90 kDa fragment is the reason for the sudden increase in protease sensitivity is supported by the occurrence of both transitions at apparently the same temperature (Figs. 4 and 6).

There is, however, a quantitative difference between the temperature at which the 90 kDa fragment makes this transition *in vivo*, 37° - 40.5°C (Kosinski and Bailey, 1991), and *in vitro*, 41° - 45°C (Fig. 6). A plausible explanation for this shift may well be differences in the protein environments. The sodium borate buffer and the higher pH did not affect the thermal stability of the wild-type β-galactosidase (Fig. 4; Edwards et al., 1990), but the β-galactosidase fragments

can be more sensitive to pH. Understandably, both the nature of the protein and the protease employed significantly impact protein sensitivity (Ogasahara et al., 1985). There are many other effectors of β-galactosidase stability and activity whose influence could cause the observed shift in the transition temperature. For instance, the presence of Mg^{+2} can increase the melting temperature by 7°C (Edwards et al., 1990). Thus, differences between the *in vivo* and *in vitro* protein environments (such as concentration of monovalent and divalent ions, pH, and presence of IPTG) may well be the reason for the difference in the transition temperature of the 90 kDa fragment.

While most "normal" proteins are thermally stable at physiological temperatures, certain "abnormal" proteins are not. The β-galactosidase mutant M15 is a very extended molecule at moderate temperatures (Zabin, 1982) and the 90 kDa fragment becomes much more so at sightly elevated temperatures. It is both useful and important to know the physical properties of a protease sensitive recombinant protein before designing the process for its production.

4.6 Acknowledgements

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4.8 Figures

Figure 1. Western blot of homogeneous 7.5% native PAGE of β-galactosidase fragments in cell extracts. Commercial wild-type β-galactosidase as a standard (lane 1) showing positions of: tetramer (T), dimer (D), monomer (M), and the 90 kDa fragment (90) (see text). Extracts from cells grown with 0, 0.025, 0.05, 0.1, and 1.0 mM IPTG are in lanes 2, 3, 4, 5, and 6 respectively. Extract from induced wild-type cells is in lane 7. Lanes 8 and 9 are respective samples as in lanes 3 and 4 except 4 μl instead of 1 μl was applied to the gel. The stacking gel and its boundary with the separating gel are evident at the top.

Figure 2. α-Chymotrypsin degradation of α-complementing activity in cell extracts. Extracts from wild-type MG1655 (△) grown with 1.0 mM IPTG, from BGF1 with either 0.05 (■) or 1.0 (□) mM IPTG, or from BGF1 (▲) with 1.0 mM IPTG in complex medium and lysed by sonication.

Figure 3. PAGE analysis of affinity-purified proteins. (**A**) A homogeneous 7.5% SDS-PAGE of affinity column eluents. Lane a contains molecular weight standards, in decreasing size: myosin, 200,000; β-galactosidase, 116,250; phosphorylase b, 97,400; bovine serum albumin, 66,200; and ovalbumin, 42,700 Da. Lane b contains commercial wild-type β-galactosidase. Lane c and d have column eluents after purification of induced and uninduced cultures, respectively. (**B**) A gradient 8 - 25 % native PAGE of affinity-purified proteins. The commercial wild-type β-galactosidase as a standard (lane g) showing positions of:

aggregated protein (Ag), tetramer (T), dimer (D), monomer (M), and 90 kDa fragment (90). The purified proteins are analyzed at either pH 8 (lane e) or pH 10 (lane f).

Figure 4. Thermal denaturation of β-galactosidase fragments. Percentage of commercial wild-type β-galactosidase tetramer ($\triangle$) or affinity purified CSH11 fragment (o) and 90 kDa fragment ($\bullet$) monomers remaining after exposure to indicated temperatures.

Figure 5. α-Chymotrypsin degradation of affinity-purified proteins at pH 8. Purified protein (100 μl) was incubated at 33°C with 1 μg chymotrypsin. Lane 1 contains the proteins before protease addition. Lanes 2, 3, and 4 are 2.5, 5.0, and 7.5 minute proteolytic digestions. Molecular weights (in kDa) are shown at left and the chymotrypsin degradative product is indicated by the arrow.

Figure 6. Arrhenius plots of rate constants for the α-chymotrypsin degradation of the affinity-purified β-galactosidase fragments. Natural logarithm of the rate constants for the degradation of the CSH11 fragment (**A**) and the 90 kDa fragment (**B**) determined at temperatures 45, 41, 37, and 33 °C from left to right.

Figure 1.

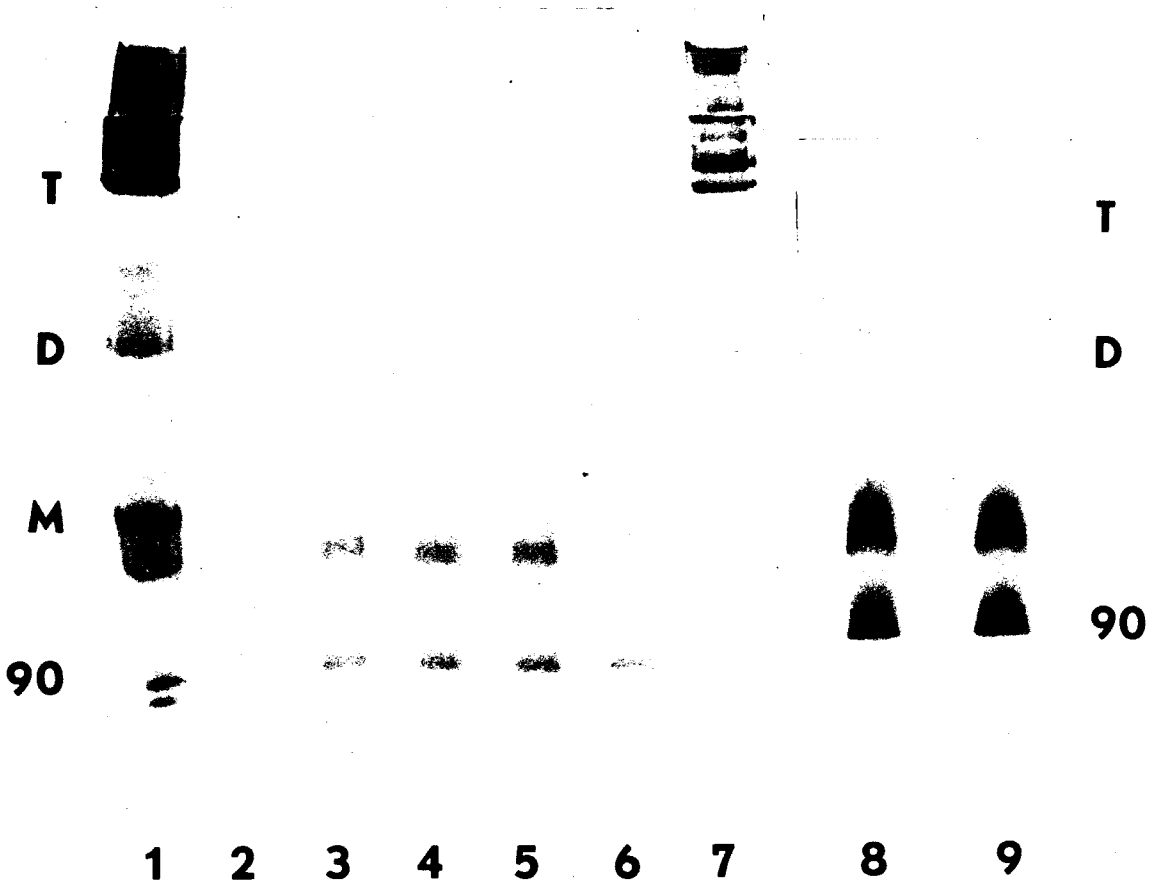


Figure 2.

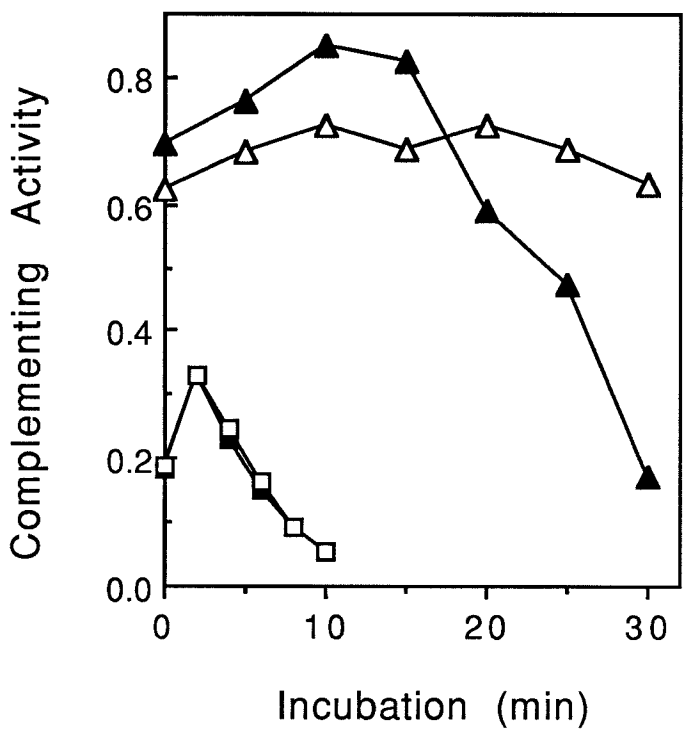

Complementing Activity
0.0
0.2
0.4
0.6
0.8
0
10
20
30
Incubation (min)

Figure 3.

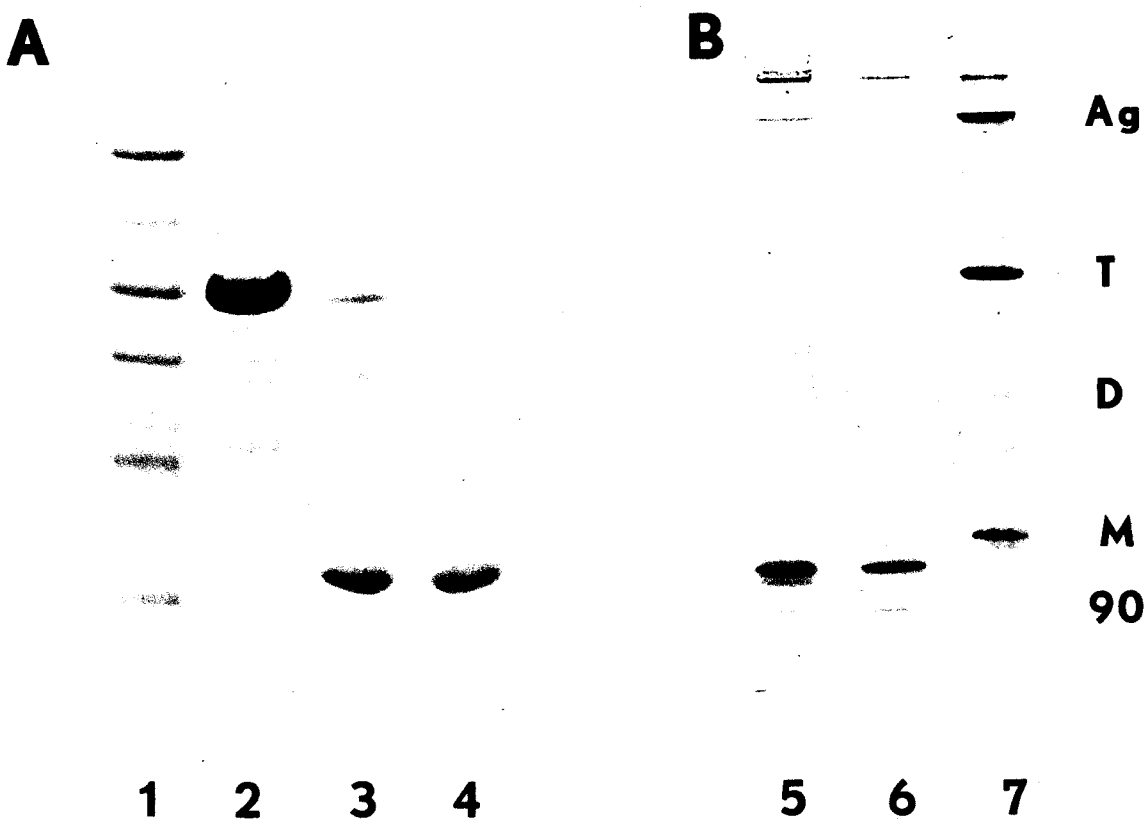
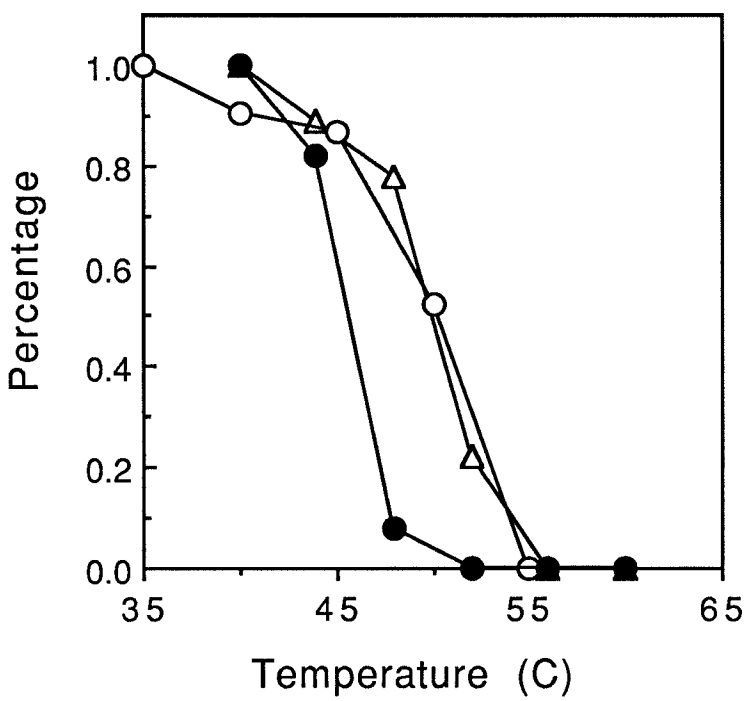


Figure 4.



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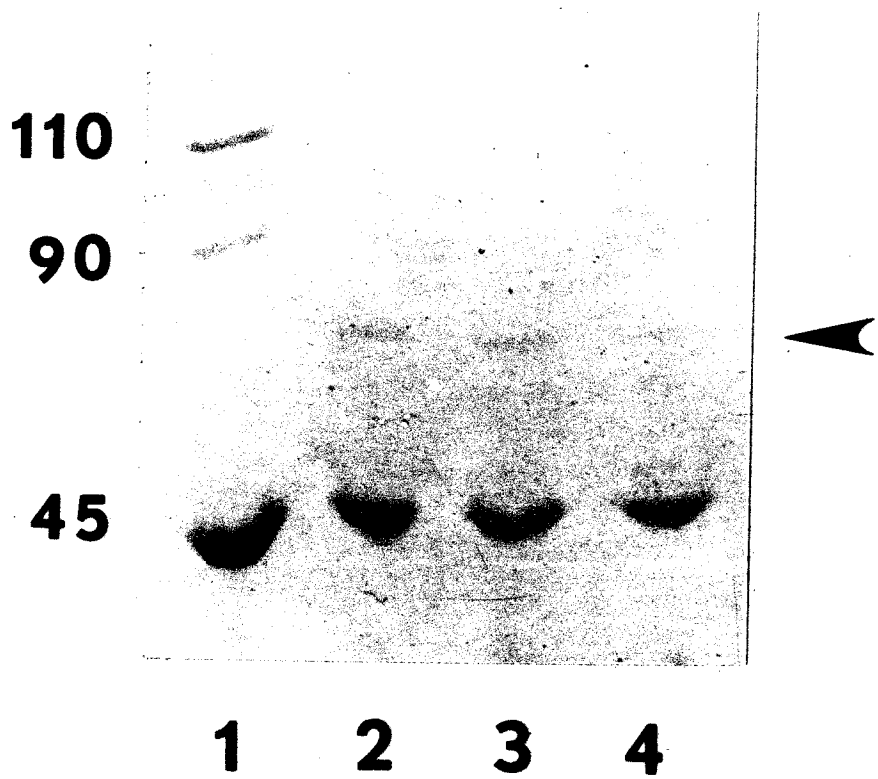
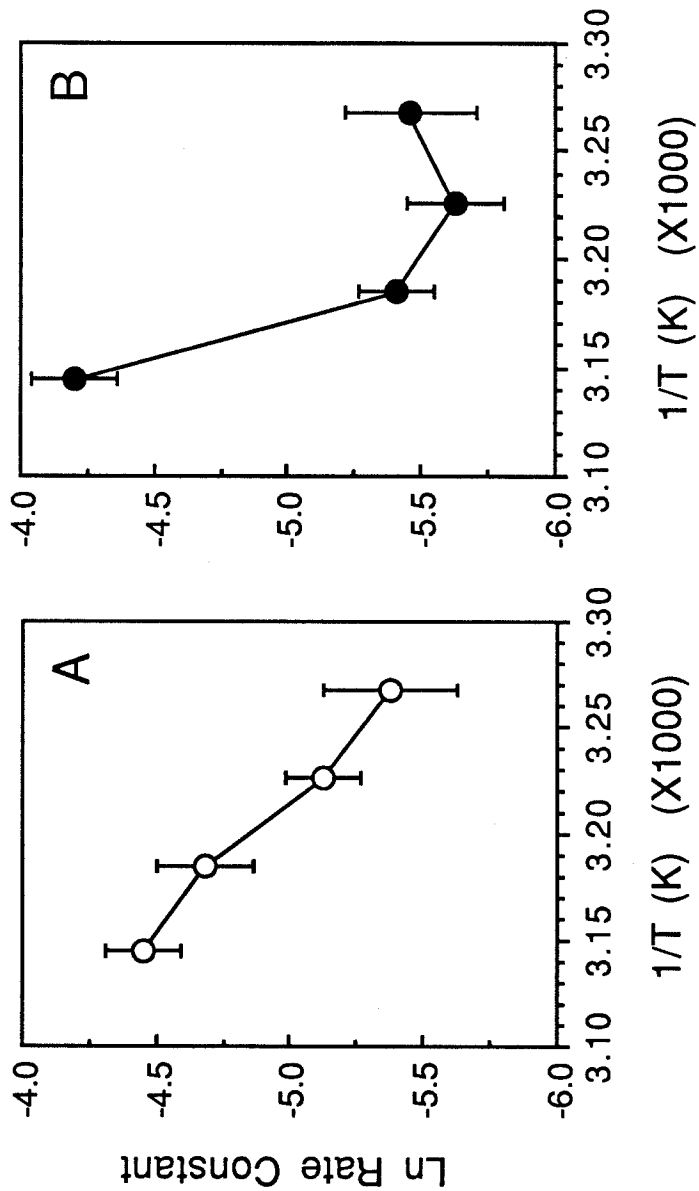


Figure 6.



Chapter 5

Conclusions

5.1 Implications for Recombinant Protein Production

The most well-known degradative pathway for abnormal proteins is that for large fragments of *Escherichia coli* β-galactosidase. Apparently, three different nonsense mutations of the *lacZ* gene (X90, CSH10, and CSH11) produce polypeptides that are degraded to a common intermediate (Kowit and Goldberg, 1977; McKnight and Fried, 1981). Formation of these detectable intermediates at 37°C is caused by an endoproteolytic cleavage of the fragments in both minimal medium (McKnight and Fried, 1981) and complex medium (Kowit and Goldberg, 1977). The degradation rates of the initial fragments are less than the intermediates and depend on the induction regimen. The effective rate constant for the cleavage of the CSH10 polypeptide is 2.5 times less under steady-state induction than immediately following *lacZ* induction (McKnight and Fried, 1981). ATP-energy is required for both proteolytic steps (Kowit and Goldberg, 1977) and protease La is very important for these *in vivo* cleavages (McKnight and Fried, 1983).

Using one of these β-galactosidase fragments (CSH11) as a model abnormal protein, the goal of this work was to determine a relationship between abnormal protein degradation and various process variables. While many of the quantitative results are specific for this abnormal β-galactosidase, there are several qualitative trends influencing *in vivo* proteolysis that may be more general. Of the parameters investigated, the one with the greatest impact on abnormal protein degradation is culture temperature. While higher temperatures (greater than 40°C) are used to induce recombinant protein synthesis (λ pL promoter)

and to obtain greater protein synthesis rates (to a limit), high temperature may also cause greater proteolysis in several different ways.

The abnormal protein degradation rate is more sensitive than the specific growth rate to the culture temperature. From 30° to 40.5°C the growth rate of BGF1, expressing the CSH11 fragment, increases 130%, while the effective degradative rate constant for the β-galactosidase fragment increases 790%. At high temperatures, the degradation rate of labile abnormal proteins will be much greater than their synthesis rate since protein synthesis scales with the growth rate (Cooper, 1988). Therefore, the culture temperature which maximizes recombinant protein concentration may be less than 37°C, as shown by the steady state level of abnormal β-galactosidase in Chapter 2.

Higher temperatures not only increase the specific activity of the cell's proteases, but they also increase the level of ATP-dependent proteolytic activity. While the ATP-dependent activity level increases 2.5-fold from 30° to 42°C in cells expressing the abnormal β-galactosidase, the dependence of this level on temperature may be greater for cells producing higher levels of labile recombinant protein. However, the ATP-independent proteolytic activity is not affected. These results confirm that the ATP-dependent proteolytic activity is most responsible for abnormal protein degradation. Planned protease mutations should concentrate on eliminating the ATP-dependent proteolytic activity in *Escherichia coli*.

Temperature influences the folding of the abnormal protein. Thermal stability of the protein is one of the two most important determinants of its degradation rate. The other is presence of the proteases. Thermal unfolding will not

be significant at moderate temperatures for all recombinant proteins, but the decrease in thermal stability of the 90 kDa intermediate correlated well with the increase in its sensitivity to α-chymotypsin. The qualitative match between these results and the fragment's *in vivo* degradation rate strongly suggest that thermal instability is the cause of the order of magnitude increase in the proteolysis rate at higher temperatures. The thermal stability of a recombinant protein should be determined before a temperature is chosen for its production.

Higher temperatures also result in higher levels of stress proteins. These proteins are known to be involved in abnormal protein degradation and shown to contribute to the ATP-dependent proteolytic activity (independent of protease La), the level of many stress proteins should be kept low to minimize proteolysis. Results in Chapter 3 indicate a significant difference in stress responses to abnormal protein expression between 30° and 37°C. Thus, for reducing the level of stress proteins, the optimal production temperature for labile recombinant proteins is closer to 30° than 37°C and certainly not greater than 40°C.

Finally, high expression levels of abnormal β-galactosidase from a single gene in the chromosome may resemble labile recombinant protein production from multicopy number plasmids. Although expression of the abnormal protein induced higher ATP-dependent proteolytic activity, the effective rate constants for both fragments decreased with increasing induction level. Stabilization due to aggregation was demonstrated not to be the cause of this decrease. However, together with the stabilization caused by inclusion body formation at high expression levels, these results imply that labile proteins should be produced as fast as possible to minimize their degradation.

5.2 Recommendations for Future Work

The work in this thesis has identified important process variables impacting abnormal protein degradation and described the magnitude of their effect. To complete a picture-model of the degradation process and provide a good basis for the creation of a mathematical model, three additional and important process variables need to be examined.

First, medium composition, which determines the protein composition of the cell, greatly affects the types of proteases and their level (Carr et al., 1987; Cook, 1988). Medium type has been shown to not affect the level of proteases in cells expressing abnormal proteins (Carr et al., 1987), but the presence of peptides in the medium may have an important influence on the degradation of some abnormal proteins. Addition of certain peptides to yeast cultures reduced the proteolysis of labile proteins by the N-end rule pathway (Baker and Varshavsky, 1991). This may also be true for bacteria, as others have found excess organic nitrogen, in the form of yeast extract and tryptone, can inhibit recombinant protein degradation (Tsai et al., 1987).

Second, the phase of growth of the cell can also significantly affect the type and level of proteases (Cook, 1988) as well as the overall degradation rate. Cells grown in fedbatch cultures at high density will almost certainly have different responses than those in early exponential phase as studied in this thesis. This dimension would have to be explored to find how the conclusions of this work translate to conditions used more often in industry.

Finally, to make the results of this work truly applicable for industrial pro-

cesses, the gene for the CSH11 β-galactosidase mutant should be inserted into a plasmid. The resulting high level of abnormal β-galactosidase expression would probably result in inclusion body formation. Both low and high copy number plasmids could be employed and a wide range of expression levels investigated. These systems could also have significantly different behaviors compared to the one used in this study. This thesis has made clearer how *Escherichia coli* responds to changes when producing labile abnormal proteins. With this basis the effect of abnormal protein overproduction and plasmid presence can be deduced.

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Appendix A

IPTG Influences the Metabolism of *Escherichia coli*

A.1 Summary

Addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a strain of *Escherichia coli* with one *lac* promoter in its chromosome causes a repression in synthesis for a set of proteins. One of these proteins, designated H35, is a prominent cellular protein present only during exponential growth. Repression of H35 is transient and delayed following an IPTG pulse. Cellular response to an IPTG pulse during exponential growth shares several features with a heat shock response. Significant increases in the specific growth rate of cells in both amino acid-supplemented minimal medium and complex medium were observed for some IPTG concentrations relative to IPTG-free cultures. Other IPTG concentrations caused a reduction in specific growth rate.

A.2 Introduction

Of the known controllable promoters, the most studied and best understood is that of the lactose operon of *Escherichia coli* (Zipser and Beckwith 1970; Beckwith 1987). The *lac* promoter and its derivatives, most notably the *tac* promoter, are therefore widely used in constructing prokaryotic expression vectors for biological experiments and biotechnological purposes. The common method of induction of these promoters is addition of isopropyl β-D-thiogalactopyranoside (IPTG). The IPTG molecule is the strongest known inducer of the *lac* promoter and is not metabolized by the cell (Zubay et al. 1970; Beckwith 1987).

The unability of the cell to catabolize IPTG may be why it is generally assumed that IPTG does not influence the cell in any other way. This assumption is especially important when investigating how expression of an IPTG-induced protein influences cell function. We provide evidence that IPTG does in fact influence *E. coli* metabolism substantially, altering both the synthesis of certain proteins and the specific growth rate.

A.3 Materials and Methods

Strains. The *Escherichia coli* strain BGF1 (Kosinski and Bailey 1991) is isogenic to the wild-type strain MG1655 (Guyer et al. 1980) except for a nonsense mutation in *lacZ*.

Media and growth. Unless otherwise noted, cells were grown in M63 minimal medium supplemented with all the amino acids except methionine and cysteine

(Kosinski and Bailey 1991). Some experiments were performed in a modified LB complex medium (Dennis et al. 1985). Cell growth was monitored using a Klett meter with a green filter. Radioactive labeling of cellular protein prior to gel electrophoresis was performed in early exponential phase at approximately 60 Klett Units. All chemicals were from the Sigma Chemical Company.

Pulse labeling and gel electrophoresis. At the indicated times, 1 ml of culture was transferred to a test tube containing 15 μCi of [^{35}S]methionine (Amersham Corp.). Labeling was performed in sufficiently large test tubes shaking at sufficient speed to ensure aerobic growth. Cells were incubated with radioactive label for five minutes, and incorporation was stopped by placing the test tubes on ice for 10 minutes. The culture was then transferred to microcentrifuge tubes and spun five minutes at $14,000 \times g$ at 4^{o}C. Cells were washed with ice cold 0.9% NaCl to remove unincorporated label, and then pelleted again. The pellet was stored at -70^{o}C until analyzed.

One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 11.5% acrylamide gels. Samples were prepared in 4% (w/v) SDS, 20% (w/v) glycerol, 150 mM DTT (dithiothreitol), 125 mM Tris/HCl (pH 6.8), boiled for 10 minutes and immediately electrophoresed. Two-dimensional gel electrophoresis was performed using the O'Farrell technique (1975) as modified by Hochstrasser et al. (1988). The second dimension was run on a 11.5% SDS-polyacrylamide gel at constant current (40 mA, 160 $\times$ 200 $\times$ 1.5 mm). Samples were prepared in 2% (w/v) SDS, 30 mM DTT, and boiled for 10 minutes. For 2D gel analysis the sample

loaded had 150,000 counts-per-minute (cpm) total, and 15,000 cpm per sample was loaded on 1D gels. After fixing and washing, the gels were soaked in Autofluor (National Diagnostics) for two hours and then dried onto filter paper using a Bio-Rad Slab Gel Dryer. Fluorography was done at -70^{o}C with Kodac X-OMAT AR film for two to seven days.

A.4 Results

Synthesis of a set of proteins is reduced when IPTG is added to *Escherichia coli* MG1655 growing exponentially in amino acid supplemented M63 minimal medium (Figs. 1A and 1B). Addition of IPTG also causes a slight increase in synthesis of some major stress proteins (Figs. 1A and 1B). Similarly, a shift in culture temperature from 37^{o} to 42^{o}C (Figs. 1A and 1C) results in both the well-known increase in stress protein synthesis and repression of the proteins similarly influenced by IPTG addition.

One of the proteins in this set with reduced synthesis is also a prominent cellular protein present during exponential growth (Fig. 1, marked by a $\diamond$). This protein constitutes about 1-2% of the total cellular protein but has not yet been identified nor mapped on 2D gels. Using Neidhardt's nomenclature (Phillips et al. 1987) we shall refer to it as "H35". H35 has a molecular weight of approximately 35-36 kDa and is stable with no degradation apparent during a 30-minute chase during exponential phase growth (data not shown). However, H35 is not present during stationary phase in either amino acid-supplemented minimal medium or LB complex medium (Khosla et al. 1990).

Repression of H35's synthesis following IPTG addition is a delayed response. The expression level of H35 is unchanged 10 minutes after IPTG addition at 37°C (data not shown), but synthesis is completely repressed 30 minutes after IPTG addition (Fig. 1B). This delay is clearly seen in Figure 2 which shows proteins labeled for five minutes with ^{35}S-methionine at the indicated times following IPTG addition at 30°C. Complete repression of H35 synthesis was observed 30 minutes after IPTG induction even though β-galactosidase production is maximized 10 minutes after induction (Fig. 2; Dalbow and Young, 1975). However, the repression is transient; synthesis of H35 is resumed 90 minutes after IPTG addition (Fig. 2). The protein slightly larger than β-galactosidase in Figure 2 also appears to be regulated as H35 is. Both the synthesis rate and cellular level of H35 are similar in steady-state growth with or without IPTG (Fig. 2, lanes 1 and 9, and two-dimensional gel analysis not shown).

In addition to inducing the *lac* promoter and repressing the synthesis of a group of proteins, IPTG also affects the specific growth rate of *E. coli*. Table 1 shows influence of IPTG on the specific growth rates for the strain MG1655, which produces wild-type β-galactosidase, and BGF1 which synthesizes the CSH11 β-galactosidase mutant. Errors represent the uncertainty of the specific growth rate calculated from a single set of growth curve data. Results in each column in Table 1 are from growth measurements done simultaneously using the same batch of medium. While a similar response of growth rate to the level of IPTG was obtained in other shake flask experiments, the IPTG concentrations corresponding to transitions from growth inhibition to growth enhancement varied by 0.05 mM (data not shown). Thus the influence of IPTG

on growth is extremely sensitive to the culture conditions.

Repeating the growth rate experiment using IPTG from a different supplier (Boehringer Mannheim instead of Sigma Chemical Co.) resulted in the same dependence of growth rate on IPTG concentration being observed. To check if the influence was medium-dependent, we repeated the experiment in LB medium; a similar effect was observed (Table 1). The percent decrease in growth rate between 0.05 mM and 0.1 mM IPTG was the same as for amino acid-supplemented minimal medium (Table 1).

A.5 Discussion

Repression of certain proteins' synthesis is known to be part of the stress response (Lemaux et al. 1978; Yamamori et al. 1978). However, a lag in repression of protein synthesis has not been previously reported. Both temperature upshift and IPTG addition cause delay in repression of H35 production (data not shown; Fig. 2). Addition of IPTG appears to elicit a typical stress (heat shock-like) response (Fig. 1). The difference between the reaction to IPTG addition (Fig. 1B) and to heat-shock (Fig. 1C) is a lesser increase in the stress protein synthesis rate despite the same extent of protein synthesis repression. This dissimilarity could be explained if the repression of protein synthesis was a more sensitive part of the stress response than the characteristic increase in stress protein synthesis.

IPTG increases the efficiency of the cell's metabolism in the sense that, the specific growth rate in certain concentrations of IPTG is greater than the growth rate without IPTG (Table 1). The enhancement in growth is not due to the

activity of β-galactosidase since the effect is also observed with BGF1, which produces the nonfunctional enzyme (Table 1). However, the β-galactosidase enzyme could still be playing an important role. Since IPTG binds β-galactosidase (Steers and Cuatrecasas 1974), the amount of active enzyme will influence the intracellular concentration of free IPTG. The mutant strain, producing a β-galactosidase fragment which might have a lower IPTG affinity and is at a lower steady state level compared to the wild type (Brown et al. 1967), should have a higher intracellular concentration of free IPTG. In fact, the mutant BGF1 exhibits an approximately 15% greater growth rate than the wild-type MG1655 in the presence of 1 mM IPTG (data not shown).

A.6 Acknowledgements

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A.8 Table

Table 1. Effect of IPTG on specific growth rate.

IPTG (mM)	Specific Growth Rates (hr^{-1})		
	MG1655 (aa-M63)	BGF1 (aa-M63)	BGF1 (LB)
0.0	0.67 ± 0.02	0.69 ± 0.03	1.18 ± 0.12
0.05	0.73 ± 0.03	0.79 ± 0.06	1.31 ± 0.13
0.1	0.65 ± 0.01	0.64 ± 0.01	1.05 ± 0.07
0.5	—	0.74 ± 0.05	—
1.0	0.74 ± 0.03	—	1.12 ± 0.08

MG1655 (wild-type) and BGF1 (lacZ) cells were grown in either amino acid- supplemented M63 minimal or LB medium at 37°C. The errors represent 95% confidence limits.

A.9 Figures

Figure 1. Repression of protein synthesis following either the addition of IPTG or an increase in culture temperature. Two-dimensional gel electrophoresis of ^{35}S-labeled proteins from MG1655 grown at 37°C without IPTG (**A**), 30 minutes after the addition of 0.5 mM IPTG (**B**), and 30 minutes after a temperature shift from 37° to 42°C (**C**). The marked proteins include those repressed both by IPTG addition and temperature shift (□), the most prominent of these proteins, H35 (◇), and three of the stress proteins (○): dnaK, groEL, and groES in order of decreasing molecular weight. The position of β-galactosidase is indicated by an arrow. (Electrophoresis performed by U. Rinas.)

Figure 2. Transient repression of H35 synthesis in MG1655 at 30°C. One-dimensional gel electrophoresis of ^{35}S-labeled proteins before (lane 1) and 10,20,30,40,50,60, and 90 minutes (lanes 2,3,4,5,6,7, and 8 respectively) after addition of 0.5 mM IPTG. Lane 9 shows labeled proteins from cells grown in steady state presence of 0.5 mM IPTG. Shown are different exposures of separate sections of the same fluorograph. (Electrophoresis performed by U. Rinas.)

Figure 1.

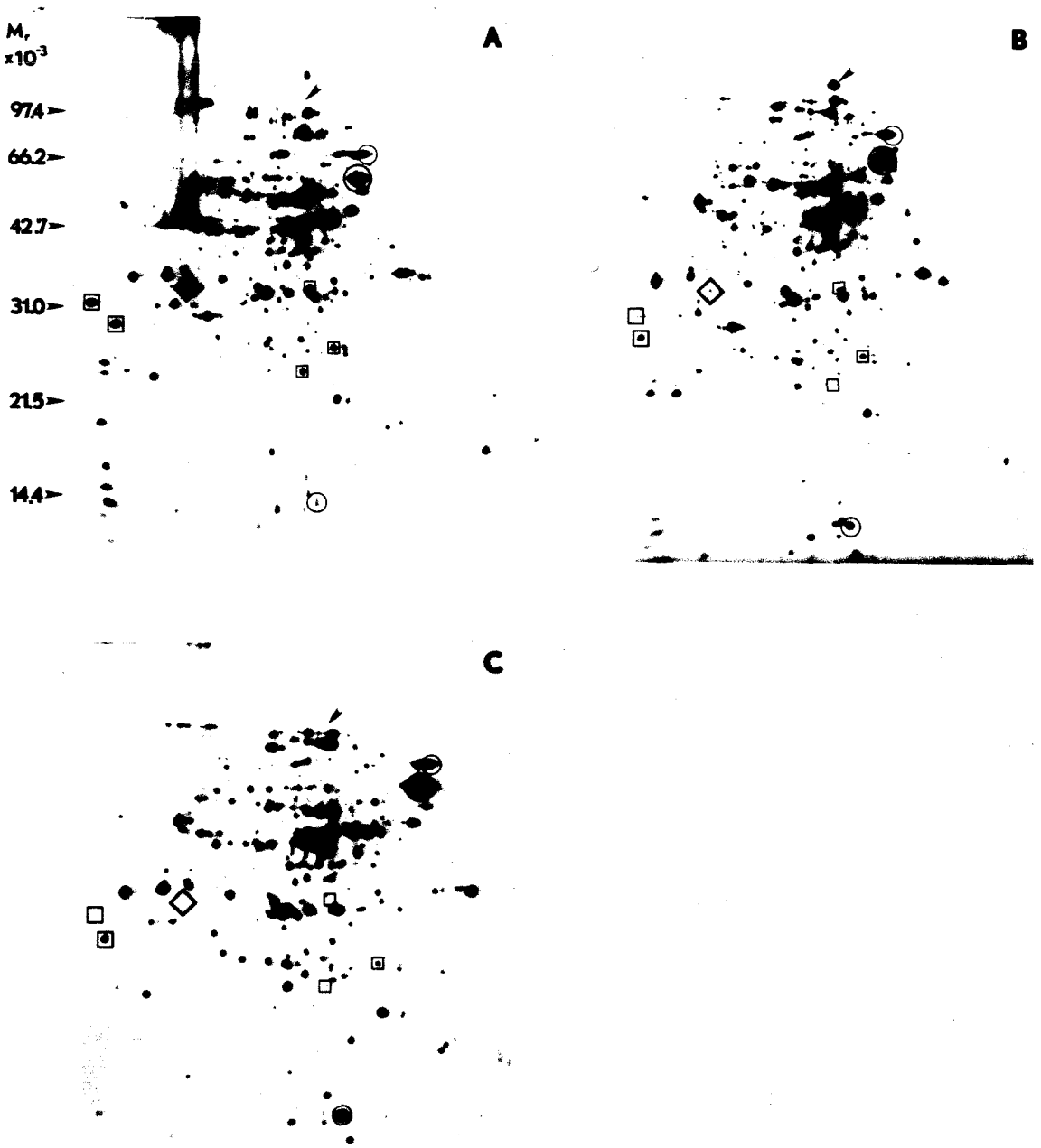
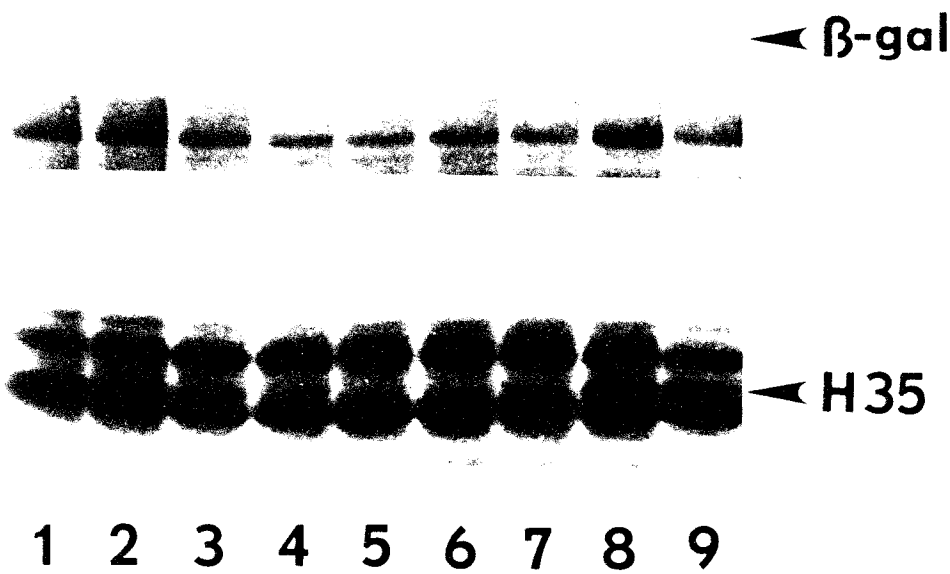


Figure 2.



Appendix B

Effect of Abnormal β-Galactosidase Expression on Specific Growth Rate

B.1 Specific Growth Rates

An important characteristic of a bacterial strain is its specific growth rate. Balanced growth during exponential phase is specified by the specific growth rate which in turn is determined by the nutrients in the medium. In this thesis, we investigated abnormal protein degradation in the *Escherichia coli* strain BGF1, which is isogenic to the wild-type strain MG1655 except for the *lacZ* gene (see Chapter 2). As mentioned in Chapters 2, 3, and in Appendix A, the specific growth rate of these two strains was measurably different when grown in shake flasks side-by-side. This appendix presents a compilation of the growth rate data collected over the course of the experiments for the thesis. The cells were grown as described in Chapter 2, and cell growth was monitored using a Klett meter with a green filter.

Figure 1 presents the specific growth rates for both BGF1 and MG1655 grown in amino acid-supplemented M63 medium. The values shown are the average of at least three separate experiments, and both strains were grown in the same preparation of medium and side-by-side in the shaker for all of the experiments. The difference between the average growth rates ranges from 14% at 30^o to its maximum of 20% at 40.5^{o}C. The two strains had essentially identical growth rates in IPTG-free medium at 37^{o}C (data not shown).

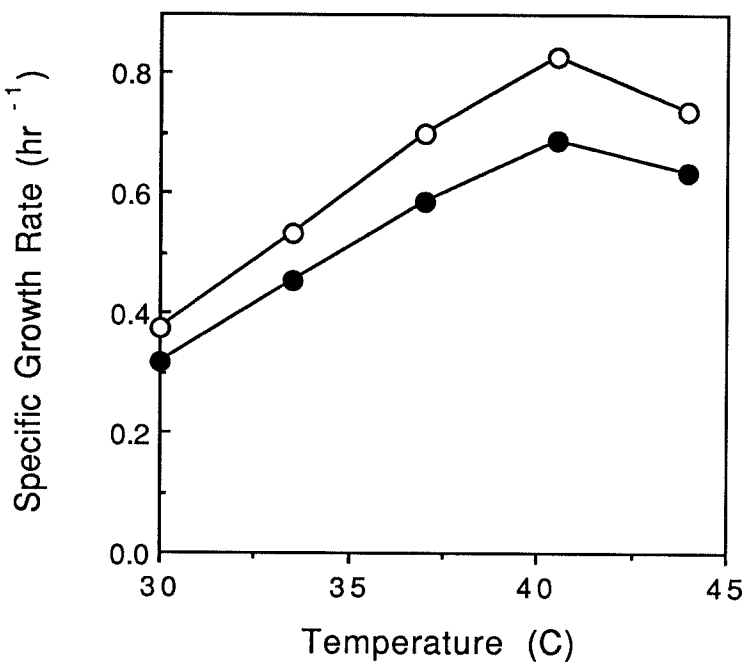
How could the expression of a relatively low level of abnormal β-galactosidase ($< 0.5\%$ of the total cellular protein) result in a 20% increase in the specific growth rate? The greater effect at higher temperature hints of possible stress protein involvement as levels of stress proteins increase with temperature

(Chapter 3). However, the absence of stress response to the induction of the abnormal β-galactosidase at 30°C (Chapter 3) rules out this explanation for low temperatures. The stress proteins contribute to higher proteolytic activity that might affect important regulatory proteins and change the growth rate. Probably the most plausible explanation is based on the hypothesis in Appendix A. The lower concentration of abnormal β-galactosidase, compared to the wild-type, and its lower binding capacity for IPTG should result in a higher free IPTG concentrations in the cell. If IPTG stimulates cell metabolism, then higher IPTG levels would enable the mutant to grow faster.

B.2 Figure

Figure 1. Specific growth rate for BGF1 (○) and MG1655 (●) grown at the indicated temperatures in the presence of 1 mM IPTG. Each point represents the average of at least three separate experiments. The 90% confidence limits are ± 0.06 hr^{-1} or better for the values except BGF1 at 40.5^o and 44°C which were ± 0.11 hr^{-1}.

Figure 1.


Specific Growth Rate (hr -1)
0.0
0.2
0.4
0.6
0.8
30
35
40
45
Temperature (C)

Appendix C

Specific Activity of Cellular Proteases Degrading the α-Donor Fragment

C.1 Protease Specific Activity

In Chapter 4, the amount of complementing activity in cell extracts was shown to increase after addition of the protease α-chymotrypsin. We hypothesized that the action of the protease was "opening" the protein molecule and making the release of the α-donor fragment easier. This breakup of the protein would also make it more susceptible to other proteases, which was especially true with the mutant β-galactosidase fragments (Chapter 4).

The rate of decrease in complementing activity, after the maximum was reached, was independent of the concentration of α-chymotrypsin (Figure 1A). Therefore, the slope of this decrease depends on the activity of the proteases or peptidases in the cell extract (from BGF1). By using the same extract and the same amount of α-chymotrypsin, the slopes from digestions performed at different temperatures (Figure 1B) relate the specific activity of the proteases acting on the α-donor fragment. Figure 1B clearly shows a maximum specific activity at approximately 37°C.

That the protease specific activity should decrease with temperature greater than 37°C is inexplicable. Given the higher turnover rate for cellular proteins at elevated temperatures, the specific activity of the proteases and/or peptidases should be higher at these temperatures. The physical properties of the substrate do not explain this decrease either. Higher temperature will make the substrate more proteolytically susceptible and would increase the degradation of the complementing activity.

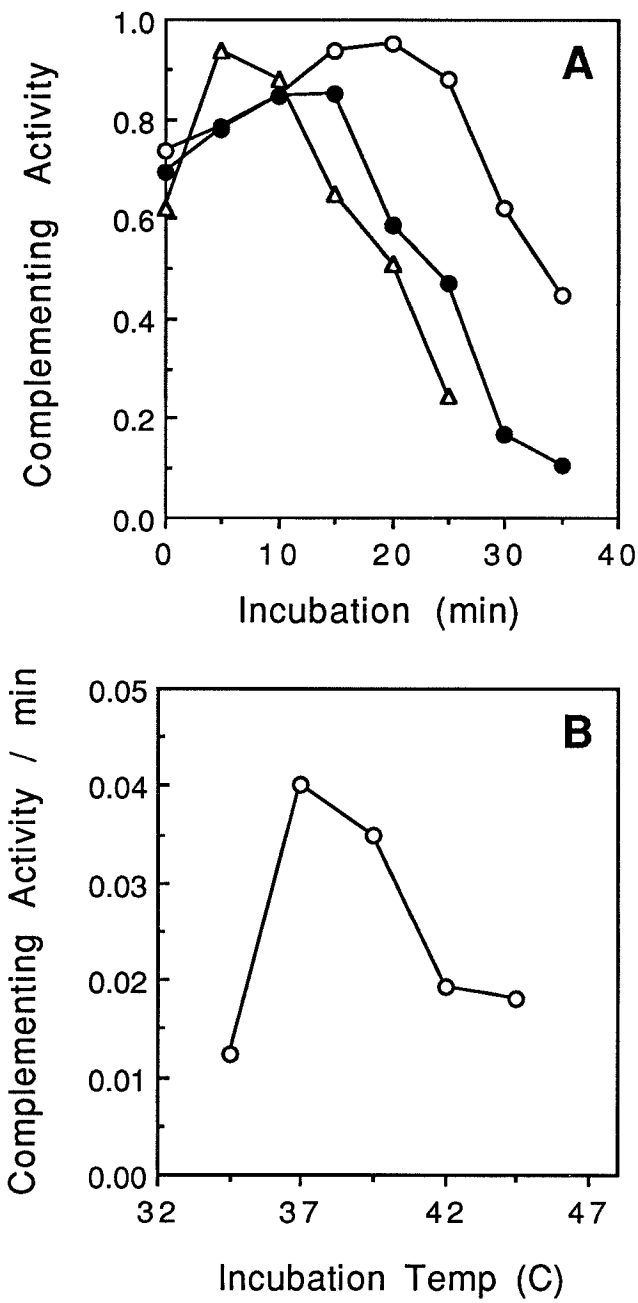
Both the level of ATP-independent proteases (Chapter 3) and the specific

activity of the proteases degrading the α-donor polypeptide are lower at 42° than at 37°C. The contradiction between these decreases and the greater rates of *in vivo* proteolysis at elevated temperatures implies it is protein properties and not protease activity that governs degradation at these temperatures.

C.2 Figure

Figure 1. α-Chymotrypsin initiated degradation of complementing activity in cell-free extracts at 37°C. (**A**) To 500 μl of extract containing the β-galactosidase fragments was added 5 ($\circ$), 10 ($\bullet$), or 15 ($\triangle$) μl of a 10 mg/ml solution of α-chymotrypsin. At the indicated times, samples were withdrawn and assayed (as in Chapter 2) for complementing activity. (**B**) The rate of decrease, following the maximum, in complementing activity is shown for incubation with 5 μl α-chymotrypsin at the indicated temperatures.

Figure 1.


A
Complementing Activity
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Incubation (min)
B
Complementing Activity / min
0.05
0.04
0.03
0.02
0.01
0.00
32
37
42
47
Incubation Temp (C)