Stabilization of a Proteolytically Sensitive Cytoplasmic Recombinant Protein During Transition to Downstream Processing

Aleksei Rozkov, Sven-Olof Enfors

Department of Biochemistry and Biotechnology, Royal Institute of Technology (KTH), S-100 44 Stockholm, Sweden; telephone: +46 8 790 8743; fax: +46 8 723 1890; e-mail: aleksei@biotech.kth.se

Received 6 February 1998; accepted 18 September 1998

Abstract: The influence of aeration and glucose feeding on the stability of recombinant protein A in Escherichia coli during the transition period from a fed-batch cultivation to downstream processing was studied. Neither interruption of the feeding under aerobic conditions nor anaerobic conditions in presence of glucose could stabilize protein A completely and the intracellular ATP pool did not decrease to less than 0.75–1 mM by this treatment. On the other hand, the absence of both oxygen and glucose resulted in a decrease of the ATP pool to less than 0.5 mM and almost complete stabilization of protein A. The decrease of ATP was more severe when sulfite was used instead of nitrogen gas to create anaerobic conditions in presence of glucose. This also resulted in nearly complete stabilization of protein A, which might be explained by an inhibiting effect of sodium sulfite on fermentation. Therefore, protein stabilization and decrease of the ATP pool were correlated in experiments in vivo. The concentrations of ADP and AMP increased during starvation and may also play a role in stabilization of the protein in vivo. ATP may be a limiting factor of proteolysis also during further steps of downstream processing. Its concentration decreases by 80–90% during harvesting and centrifugation of biomass and even further during disruption of cells. However, neither addition nor regeneration of ATP in cell disintegrate was enough to restore degradation of protein A, indicating that an additional factor limits proteolysis in vitro. © 1999 John Wiley & Sons, Inc. Biotechnol Bioeng 62: 730–738, 1999.

Keywords: proteolysis; Escherichia coli; protein A; ATP; sulfite

INTRODUCTION

Proteolysis of recombinant proteins is often a major problem in industrial processes, resulting in decreased yield of the product and contamination by degradation products that may be difficult to separate from the full length protein. Many methods to control proteolysis have been described in the literature (Enfors, 1992; Makrides, 1996), but most of the efforts were applied to controlling proteolysis on the cell level during cultivation, as formation of inclusion bodies (Hellebust et al., 1989b) and secretion to periplasm (Talmadge and Gilbert, 1982), or on the protein level, by altering the primary protein structure, e.g., by removal of sensitive sequences (Hellebust et al., 1989a), or by protective fusion (Murby et al., 1991).

The accumulation of a proteolytically unstable protein in a cell depends on the balance of two opposite processes: its synthesis and degradation. Interruption of cultivation may result in conditions that do not support high synthesis rate of the protein but still are favorable for its degradation. For example, recombinant staphylococcal protein A, which was used as a model protein in the present study, has half-lives ranging from 15 to 30 min, depending on temperature, host strain (Kandror et al., 1994; Yang and Enfors, 1995), and process conditions (Yang and Enfors, 1996). Therefore, inappropriate conditions during the transition to downstream processing, which in large-scale production could last for hours, might result in large loss of yield before the product is recovered.

The recombinant staphylococcal protein A is known to be degraded by an energy-dependent mechanism (Kandror et al., 1994; Yang and Enfors, 1995), as most abnormal proteins (Goldberg, 1992). Thus, depletion of ATP might be a strategy to slow down the rate of proteolysis. However, ATP is also needed for synthesis of the product and the ATP pool in living cells, being well controlled, decreases only by 45–60% even under conditions of starvation for carbon, potassium or phosphate (St. John and Goldberg, 1978). Therefore, control of proteolysis on the ATP level can hardly be applied during the cultivation process, but might be feasible during the transition to downstream processing.

Protein A was shown to be stabilized by absence of glucose and respiration inhibition by addition of sodium azide (Yang and Enfors, 1995). Similar results were obtained by inhibition of glycolysis and oxidative phosphorylation (Kandror et al., 1994). Earlier reports described inhibition of proteolysis of some other proteins by the same method (Bejarano et al., 1993; Hershko and Tomkins, 1971; St. John and Goldberg, 1978). These methods of stabilization of recombinant proteins, in spite of apparent simplicity and...
robustness, are not suitable for application in large-scale fermentation processes because of the costs and the problem of disposing of large quantities of cultivation broth containing toxic substances used to inhibit ATP production. Therefore, a simple, inexpensive and environmentally friendly technique is needed to increase the stability of recombinant proteins by ATP depletion during the transition to downstream processing.

The objectives of this study were to investigate the influence of anaerobic starvation in the absence of glucose upon the stability of a recombinant protein in vivo, to elaborate strategies for minimizing proteolysis in the transition period from cultivation to product recovery and to investigate the role of ATP in protein A stabilization.

MATERIALS AND METHODS

Micro-organism

_Escherichia coli_ W3110 containing the plasmids pRITcI857 and pRIT2 was used unless otherwise indicated. Plasmid pRITcI857 encodes the temperature-sensitive λ repressor cI857 and contains a kanamycin-resistance gene. Plasmid pRIT2 encodes a recombinant protein A and contains an ampicillin-resistance gene. The expression of protein A was controlled by the λPR promoter and the temperature-sensitive repressor cI857.

Medium

In all experiments a minimal mineral medium was used with the composition (per liter): 7 g (NH₄)₂SO₄, 1.6 g KH₂PO₄, 6.6 g Na₂HPO₄·2H₂O, 0.5 g (NH₄)₂·H-citrate. A 2 mL amount of a 1 M solution of MgSO₄, 2 mL of a trace element solution (Holme et al., 1970), 70 mg of ampicillin, 20 mg of kanamycin, and 10 g of glucose were sterile filtered and added to the medium after its sterilization and cooling to the temperature of cultivation. Feeding solution, containing 300 g/L of glucose, was autoclaved for 15 min at 121°C.

Apparatus

A 10-L standard bench-scale bioreactor was used. Initial volume of the culture medium was 5 L. The concentration of dissolved oxygen was measured by a polarographic electrode and was maintained above 30% of saturation unless otherwise indicated. Feeding with a glucose solution was performed by a peristaltic pump under computer control. Carbon dioxide and oxygen in outlet gas were measured by infrared and paramagnetic analyzers, respectively.

Culture Conditions

The microbial culture was maintained in sterile tubes at −80°C. For preparation of inoculum the microbial culture was inoculated into a shake flask with a medium of the same composition as the batch medium in fermenter. The ratio between the volume of culture medium and the total volume of flask was 1/10. An overnight culture was re-inoculated in fresh medium and incubated until the OD₅₈₀ reached 2–3. 400 mL of this culture were inoculated into the fermenter medium. Temperature 30°C was maintained throughout the experiments except for the induction period. A 25% solution of ammonia was used to control pH at 7.0. Exhaustion of glucose in the end of the batch cultivation phase was monitored as a sudden rise of concentration of dissolved oxygen and, then, feeding with glucose was started. The maximum specific growth rate in batch culture was 0.4 h⁻¹. Therefore, the rate of acceleration of the feeding rate was chosen 0.35 h⁻¹ to avoid formation of acetate by overflow metabolism. The exponential feeding phase lasted for 5–6 h until the desired cell density was achieved. Then, the feeding rate was established at a constant value and the production induced by shifting of temperature from 30 to 40°C. The transition period from 30 to 40°C lasted 10 min. The induction temperature (40°C) was kept for 3 h in all experiments.

Analyses

Biomass was monitored by measuring of optical density of the culture at 580 nm after dilution to the optical density range 0.2–0.6. Biomass concentration was also assessed by measurements of dry weight of suspended solids, which were centrifuged at 4500 RPM for 10 min and washed once by distilled water and dried overnight at 105°C. Concentrations of glucose, acetate and formate were measured using enzymatic kits from Boehringer Mannheim GmbH according to the instructions of the manufacturer. Respiring and nonrespiring bacteria were quantified by the staining technique used by Andersson and co-workers (1996) with exception that instead of using of a 2% solution INT (iodonitrotetrazolium) a solution, saturated at 0°C was used. Visible count was performed using Nutrient Agar (Difco, Detroit, MI) plates and 1% solution of sodium chloride for dilution of cell suspension. Protein concentration in the culture medium was measured by the Bradford method (1976). Samples for this analysis were taken from the fermenter, centrifuged, and filtered before freezing at −20°C.

Analysis of Adenine Nucleotides

Adenine nucleotides were extracted from the cells by rapid (in less than 4–5 s) addition of culture broth to ice-cold 10% solution of trichloroacetic acid (TCA). The ratio of broth to TCA was less than or equal to than 1:1 and efficiency of extraction did not depend on the ratio in this range. The exact amount of added sample was determined by the difference in weight before and after sampling. Tubes with extracted samples were kept for 1 h on ice and stored at −25°C prior to the analysis, which was carried out using enzymatic kit from BioOrbit Oy (Turku, Finland) according to the instructions of the manufacturer.
to the instructions of the manufacturer. Extracellular adenosine nucleotides were sampled as follows: culture broth was centrifuged at 4300 g for 10 min, and supernatant was frozen at −25°C. The ADP and AMP were analyzed essentially as described by Lundin (1986) with the exception that double the amount of adenylate kinase but no CTP was used in assay of AMP.

Analysis of Protein A and Its Degradation Products

Cells were harvested by centrifugation at 4°C and 6000 g for 10 min and resuspended in buffer (50 mM Tris, 10 mM MgCl₂, pH adjusted to 7.4 by acetic acid) to a concentration 50 g dry weight per liter and stored at −80°C prior to analysis. Thawed suspension of cells was disintegrated twice in a French press at 16,000 psi (110 MPa), diluted by TST (50 mM Tris buffer, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20) buffer in a ratio 1:1, clarified twice by centrifugation at 48,000 g for 20 min (each run) and subjected to IgG affinity chromatography. A 10 mL amount of sample (containing crude extract from 250 mg of dry weight of cells) were loaded on a chromatographic column, containing 4 mL of IgG-sepharose gel 4B (Pharmacia, Sweden). The column was washed by TST and 5 mM ammonium acetate. Buffer containing 0.2 M of acetic acid (pH 4.7, adjusted by ammonium acetate) was used to elute heat shock proteins, which are co-purified (Gustavsson et al., 1997; Hellebust et al., 1990) with IgG-binding proteins. Protein A was eluted with 0.2 M acetic acid (pH 3.3, adjusted by ammonium acetate). The protein A concentration was determined from the absorbance data at 280 nm using an extinction coefficient 0.33 mL mg⁻¹ cm⁻¹. The eluted fraction was collected and freeze-dried. SDS–PAGE was performed with 3.5% stacking gel and 11% separation gel according to the method of Laemmli (1970). Proteins were stained with Coo massie Brilliant Blue R-250.

Analysis of protein A by Western blotting was performed essentially as described by Yang and Enfors (1995) with the exception that 0.5% of gelatin was used for blocking. Membranes were scanned and evaluated by computer software (ImageMaster, Pharmacia Biotech). Protein A in this case was quantified by integration of its density peak and expressed as percentage of the initial concentration.

Study of Stability of Protein A in Vivo

The aim of this part of study was to study the influence of culture conditions on in vivo proteolysis of protein A after cultivation (product formation phase) has finished. For this purpose various situations were created with respect to energy metabolism (Table I). In all cases culture broth was kept in the bioreactor at the temperature of induction (40°C).

The first case considered was stop of glucose feeding and continued aeration. Since glucose was the limiting nutrient in the fed-batch culture it was rapidly depleted when the feeding was interrupted. Continued aeration enabled the cells to oxidize acetate, which was formed during the induction phase, although at a slow rate and with smaller energy gain compared to glucose metabolism.

The second case was stop of aeration and continued glucose supply. Under these conditions glucose can only be fermented, yielding considerably less energy per unit of glucose in comparison with respiratory metabolism. In order to eliminate dissolved oxygen in the culture and prevent aeration from headspace either nitrogen was sparged through the medium or sodium sulfite was added (280 or 560 mg/L).

In the third case conditions of complete energy starvation were created by elimination of both glucose and oxygen. The acetate which was produced after the induction by temperature cannot be metabolized by E. coli under anaerobic conditions. The residual oxygen was eliminated by either nitrogen sparging or sulfite addition as described above. To take into account the possible de novo protein synthesis, chloramphenicol was added in some experiments to a concentration of 100 mg/L. The experimental conditions are summarized in Table I.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glucose feed</th>
<th>Aeration</th>
<th>Dissolved oxygen elimination</th>
<th>Protein synthesis inhibition by chloramphenicol</th>
<th>Expected energy metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Off</td>
<td>Off</td>
<td>N₂</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Off</td>
<td>Off</td>
<td>Na₂SO₄, 280 mg/L</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>Off</td>
<td>Off</td>
<td>Na₂SO₄, 560 mg/L</td>
<td>–</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>Off</td>
<td>On</td>
<td>None</td>
<td>–</td>
<td>By acetate oxidation</td>
</tr>
<tr>
<td>5</td>
<td>Off</td>
<td>Off</td>
<td>Na₂SO₄, 560 mg/L</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Off</td>
<td>On</td>
<td>None</td>
<td>+</td>
<td>By acetate oxidation</td>
</tr>
<tr>
<td>7</td>
<td>On</td>
<td>Off</td>
<td>Na₂SO₄, 560 mg/L</td>
<td>+</td>
<td>By fermentation</td>
</tr>
<tr>
<td>8</td>
<td>On</td>
<td>On</td>
<td>None</td>
<td>+</td>
<td>By respiration</td>
</tr>
<tr>
<td>9</td>
<td>On</td>
<td>Off</td>
<td>N₂</td>
<td>+</td>
<td>By fermentation</td>
</tr>
</tbody>
</table>
ATP Stability During Downstream Processing

*E. coli* W3110 with and without the plasmid encoding protein A was used in this part of the study in order to compare the influence of the presence of recombinant protein A on the stability of the ATP level during downstream processing.

The nonrecombinant strain was pregrown in shake flasks at 37°C until reaching the late exponential phase. The culture was centrifuged at 6000g for 20 min. Cells were resuspended in Tris/MgCl₂ buffer (pH 7.4) to 50 g of cells per liter. Part of this suspension, referred to as “washed cells”, served as a control. The remaining part of the washed cells was subjected to disintegration in a French press at 20,000 psi (138 MPa). The percentage of viable cells in the homogenate, as measured by viable count, was about 0.1% of the total. Culture, washed cells and disintegrate were incubated at room temperature (22–23°C) and 0.1 mL were withdrawn for ATP analysis at regular intervals and added to 2 mL of 10% trichloroacetic acid solution. All samples were kept at −80°C until analysis. Similar experiments were carried out with the plasmid pRIT2 harboring strain, which was grown and induced for production as described in the previous section. The subsequent procedures were the same as in the case with nonrecombinant strain.

### Attempt to Reconstitute Proteolysis of Protein A in Cell Disintegrate

Cell disintegrate was divided into five parts and dispensed into test tubes. Test tube A was a control that did not receive any additions; ATP was added into test tubes B, C, and D at time intervals and to concentrations shown in Table II; in the test tube E ATP was initially added up to 1.58 mM and then regenerated by pyruvate kinase (Boehringer Mannheim, Germany) (330 mg/L) and phosphoenolpyruvate (Sigma) (3.33 mM added every 5 min). Analysis of protein

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.58</td>
<td>3.16</td>
<td>3.16</td>
<td>1.58</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.47</td>
<td>0.94</td>
<td>1.58</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0.47</td>
<td>0.94</td>
<td>1.58</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>0.47</td>
<td>0.94</td>
<td>1.58</td>
<td>0</td>
</tr>
<tr>
<td>Etc. every 10 min</td>
<td>0</td>
<td>0.47</td>
<td>0.94</td>
<td>1.58</td>
<td>0</td>
</tr>
</tbody>
</table>

Measured ATP, ADP, and AMP (mM)

- ATP at 0 min: 0.10, 0.47, 0.87, 1.03, 0.10
- ATP after 60 min: 0.06, 1.38, 2.78, 4.02, 1.10
- ADP after 60 min: 0.03, 0.90, 2.75, ND, 0.10
- AMP after 60 min: 0.05, 0.72, 2.34, ND, 0.17

*ATP was regenerated by pyruvate kinase and phosphoenolpyruvate. ND, not determined.

### RESULTS AND DISCUSSION

### Stability of Protein A in Vivo in the Transition Period from Cultivation to Product Recovery

Accumulation of a proteolytically susceptible protein is a function of both synthesis and degradation rates. Degradation rate constants above 1 h⁻¹ have been reported (Kandror et al., 1994; Yang and Enfors, 1995). Thus, inappropriate conditions in the transition period from cultivation to product recovery, when product formation is ceased can result in large loss of protein due to proteolysis. Since most of the proteolysis of cytoplasmic recombinant proteins is ATP-dependent (Goldberg, 1992), control of the ATP level in cells by manipulating the culture conditions might be a feasible method to reduce proteolysis. Glucose feeding and aeration are two important parameters, influencing the energy status of the cell, and can be easily controlled under process conditions.

#### Interrupted Glucose Feeding and Continued Aeration

In the experiments where only the glucose feeding was interrupted while aeration maintained a high dissolved oxygen concentration, the cells were exposed to glucose starvation, but instead the acetate (from 1.5 to 3.6 g/L in different experiments) that was formed during the temperature induction (Strandberg and Enfors, 1991) could serve as an energy source. Acetate concentration in one of the experiments decreased from 1.5 to 0.9 g/L during 90 min incubation. These conditions caused a gradual reduction of the ATP pool from 3 mM to about 1 mM where it stabilized after about 30 min (Fig. 1). The product concentration declined slowly, apparently suggesting a low rate of proteolysis. However, addition of chloramphenicol showed that the product degradation rate was considerable: 50% of the protein A disappeared within 20 min, indicating that the apparent stability of the product was due to compensation of degraded product by de novo synthesis (Fig. 1). This is surprising since no glucose was available and growth on acetate alone requires induction of gluconeogenesis. A possible explanation would be that due to the very strong APR promoter a certain rate of protein synthesis could be supported by energy from acetate metabolism with amino acids provided by turnover of other proteins. The other possibility that chloramphenicol stimulated proteolysis was excluded in earlier experiments with the same protein (Yang and Enfors, 1995).

#### No Aeration and Continued Glucose Feeding

To investigate the product stability when oxygen alone was eliminated glucose feeding was continued while aeration
was stopped and oxygen was depleted by nitrogen sparging. The de novo protein synthesis was inhibited by addition of 100 mg/L of chloramphenicol. The ATP pool gradually declined from 3 to 0.75 mM during 90 min (Fig. 2). This ATP drop may be explained by the shift from aerobic to anaerobic conditions, in which the lower ATP level was ensured by fermentation of glucose. Analysis of the product concentration in the cell during this period showed rapid decrease of about 40% during the first 10 min of anaerobic conditions (Fig. 2). The period of rapid initial degradation of protein A coincides with a sharp decrease of the ATP pool in cells from 3 to 1.8 mM during this time (Fig. 2). Protein degradation almost ceased when ATP decreased to less than 1.8 mM. The experiment described above was carried out with glucose-limited culture. Excess of glucose did not significantly influence the response of ATP on the shift to anaerobic conditions (data not shown).

Similar experiments were carried out with addition of sodium sulfite instead of nitrogen for elimination of dissolved oxygen. Sodium sulfite caused a more sharp and severe decrease of the ATP concentration (Fig. 4), if compared to the experiment with nitrogen sparging (Fig. 2), which may be a reason for the higher stability of protein A in the case of sulfite (Figs. 2 and 3).

Since sulfite addition seemed to have an additional effect on proteolysis compared to removal of oxygen alone, its effect was compared with that of the respiration inhibited by azide. Cells were grown in shake flasks and induced for 3 h. Sodium sulfite, 560 mg/L, was added in one of the flasks to eliminate dissolved oxygen and 1300 mg/L of sodium azide to the other to inhibit respiration. Both cultures were supplemented with 10 g/L of glucose, which should provide energy by fermentation sufficient for proteolysis. Both flasks were incubated at 40°C for 90 min. Analysis made by Western Blotting (Fig. 4) showed that protein A in the culture with sodium sulfite was more stable than in the experiment with azide. However, protein A in both sulfite- and azide-treated cells was more stable than in the culture where oxygen was eliminated by nitrogen sparging (Fig. 2). The protein loss during 90-min incubation was 11%, 37%, and 63%, respectively. Corresponding ATP levels were 0.14–0.24, 0.41–0.52, and 0.5–1.5 mM. Thus, lower ATP levels correlated with slow proteolysis. The relatively high ATP level in nitrogen-flushed culture can be explained by energy generation by fermentation. However, the azide- and sulfite-treated cells did not ferment during the incubation, since no acetate was formed (Fig. 4). Thus, sulfite and azide effects seem to be inhibition of energy generation by fermentation, which might explain the lower ATP levels and the higher protein stability.

**Complete Energy Starvation**

When the culture was starved for both oxygen and glucose, the protein A level was completely stabilized (Fig. 5). In this case, the stable level should not be caused by compen-
sating synthesis of product, since acetate cannot furnish the cells with energy under anaerobic conditions. Thus, the stabilization should be caused by inhibited proteolysis. This was verified from the stability of the protein when incubated with chloramphenicol, which inhibits de novo protein synthesis (Fig. 6). The absence of glucose and oxygen resulted in a rapid (less than 2 min) decrease of the ATP pool from 2–3 mM to less than 0.5 mM (data not shown). The stabilization of the protein and decrease of the ATP pool could not be attributed to death of the cells. The viable count assay did not reveal any effect of anaerobiosis, caused either by nitrogen or sulfite, on the number of viable cells (data not shown). However, the number of bacteria able to respire, enumerated with INT staining, decreased by about 20% but this decrease cannot account for the extensive decrease of ATP concentration. Furthermore, the concentration of extracellular proteins increased insignificantly (by 20%) or remained constant at the level about 100 mg/L, which corresponds to 0.8% cell lysis. The decrease of the ATP pool and stabilization of protein A were reproducible in 3 replicate experiments.

Our data show that elimination of glucose or oxygen alone did not stabilize protein A completely. In both cases the rate of proteolysis during the first 10–20 min was very high, leading to a loss of about half of the protein yield. On the contrary, the complete starvation caused by absence of both oxygen and glucose stabilized protein A almost completely. The ATP pool in these energy-starved cells decreased to about 0.5 mM within less than 2 min, which could be the reason for the stability of protein A that is reported to be degraded by an ATP-dependent mechanism (Kandror et al., 1994). Moreover, even the decrease of the ATP pool during glucose starvation under aerobic conditions (to about 1.4 mM) or during oxygen starvation in the presence of glucose (to about 1.8 mM) seemed to decrease the rate of proteolysis considerably. However, the $K_m$ value for ATPase activity of the protease Clp (Ti), which is responsible for most of the degradation of protein A (Kandror et al., 1994), is 0.21 mM (Chung et al., 1996; Hwang et al., 1988). Therefore, if ATP was the only factor regulating degradation of protein A, the rate of proteolysis would be considerable at the ATP concentration 0.5 mM which was obtained in the absence of both glucose and oxygen. Therefore something else than simply the low concentration of ATP could determine the low rate of proteolysis. ADP was reported to inhibit the proteolytic action of the proteases Lon (La) (Goldberg and Waxman, 1985; Larimore et al., 1982; Menon and Goldberg, 1987) and Clp (Chung et al., 1996). Moreover, ADP inhibits the assembly of the ClpA/ClpP complex (Seol et al., 1995). During severe energy starvation the energy charge declines (Chapman et al., 1971) and the ADP pool increases, and therefore it could play a significant role in the rate of proteolysis in vivo. According to our data, the ADP concentration 0.12–0.29 mM did not interfere with rapid proteolysis in

Figure 4. Influence of sodium sulfite and azide on proteolysis of protein A. Analysis by Western blotting of cells incubated with chloramphenicol showed that protein A was more stable in sulfite-containing culture (○) than in the culture with azide (□). The acetate concentration in sulfite-containing (○) and azide-containing (×) cultures.

Figure 5. Normalized concentration of recombinant protein A (purified by affinity chromatography) after stop of glucose feeding and aeration. Oxygen was removed by nitrogen sparging (○) or addition of sodium sulfite (□, 280 mg/L; ○, 560 mg/L). Other experimental conditions as experiments 1, 2, and 3, respectively, in Table I.

Figure 6. Concentrations of recombinant protein A (○) and adenine nucleotides (○, ATP; ×, ADP; +, AMP) after stop of both feeding and aeration. Sodium sulfite (560 mg/L) and chloramphenicol (100 mg/L) were added to eliminate oxygen and to stop de novo protein synthesis. The energy charge (□) declined from 0.7 to 0.2 within 2 min and then remained constant. Conditions are according to experiment 5 in Table I.
control experiment (Fig. 7) but raised ADP levels may be responsible for stabilization of protein A during complete energy starvation. Concentrations of both ADP and AMP increased to 0.5–1.1 and 1.4 mM, respectively (Figs. 3 and 6), resulting in decrease of calculated energy charge from 0.8–0.9 to 0.2. The effects of ATP decrease and ADP and AMP increase under the conditions of absence of glucose and oxygen could not be separated and, possibly, both contributed to the stabilization of protein A.

Fate of ATP in the Course of Primary Downstream Processing

It has been shown in our previous work that proteolysis of protein A from pRIT2 in cell disintegrate is much less extensive than in intact cells (Yang and Enfors, 1995). The availability of ATP was assumed to be the reason for this. The first step in downstream processing is centrifugation of the biomass, which results in a large loss of ATP (by up to 88%) (Chapman et al., 1971; Cole et al., 1967; Lundin and Thore, 1975). This decrease corresponds to the ATP decrease during energy starvation when protein A was almost stable (Fig. 6), and thus, centrifugation might be sufficient for stabilization of proteolytically sensitive recombinant proteins in the cell pellet. Our data showed that after centrifugation and suspension of biomass in buffer the ATP pool in the cells dropped several times from a normal physiological value of 1.7–2.8 to 0.7–0.4 mM and 0.17–0.15 mM in nonrecombinant and recombinant cells, respectively (Fig. 8), apparently due to starvation.

The lower ATP levels in recombinant cells raises the question whether this is due to the on-going proteolysis consuming ATP. The energy requirement for degradation of 1 peptide bond by the Clp protease is 6–8 ATP molecules (Maurizi, 1992). Since Clp protease degrades protein A (31 kDa) into 1.5 kDa polypeptides (Goldberg, 1992), the estimated ATP requirement for degradation of 1 mole of SpA is about 145 mol of ATP. The average yield of protein A in our experiments was about 10 mg/g of biomass dry weight. Therefore, if all SpA was degraded it would require intracellular ATP concentration of about 20 mM. In our experiment the ATP level in starving SpA-containing cells decreased from about 3 mM to about 0.2 mM, which is the $K_m$ value of Clp for ATP. This result shows that energy-dependent proteolysis could explain the more severe decrease of ATP in starving recombinant cells.

The ATP concentration in a suspension of washed cells that served as a control, did not change considerably for at least 80 min of incubation. Disruption of cells in a French press resulted in further decrease of the ATP concentration due to the dilution by disintegration buffer. During 60 min incubation of disintegrate at the room temperature (22–23°C) the amount of ATP decreased from 0.11–0.15 to 0.02–0.03 mM in three replicate experiments (data of one of the experiments are given in Fig. 9), which is less...
than $K_m$ for ATP of the proteases Lon (0.02–0.03 mM) and Clp (0.21 mM). There was no significant difference in ATP concentration in disintegrate of recombinant and non-recombinant cells (Fig. 9), which suggests that presence of protein A did not contribute to additional ATP degradation in disintegrate. Since the ATP quantity remained approximately the same after disintegration but the concentration declined due to the dilution, extra ATP was added to the disintegrate of recombinant cells up to the normal in vivo concentration, 1.2 mM, to measure the rate of ATP degradation in disintegrate. Analysis showed that in less than 5 min the ATP concentration dropped 10-fold to 0.11 mM and continued to decrease slowly to 0.08 mM during further incubation (data not shown). Thus, disintegrate has a strong ATPase activity.

Results of the experiments above show that ATP may be the limiting factor for proteolysis during downstream processing during which the ATP concentration decreases as a result of three processes: centrifugation, dilution during cell disruption and hydrolysis by ATPases.

**Attempt to Reconstitute Degradation of Protein A in Vitro**

A plausible explanation for the fact that proteolysis stops after disruption of the cells is a lack of ATP. Thus we investigated the possibility to reconstitute proteolysis in cell disintegrate by repeated supply of extra ATP approximately to the levels supporting proteolysis in vivo (Table II). Addition of ATP alone resulted in build-up of concentrations of ADP and AMP to levels possibly inhibiting proteolysis (Table II). Therefore, another variant of the experiment was made, in which ATP was regenerated from ADP by added pyruvate kinase and AMP by adenylate kinase already present in disrupted cells. ATP regeneration enabled to maintain relatively high levels of ATP (from 0.81 to 1.45 mM) and to keep ADP and AMP low (Table II). However, analysis of the protein revealed no degradation in any of the experiments (Fig. 10), showing that the degradation of protein A in vitro requires an additional factor which is active only in intact cells. This result suggests that ATP may be not the only limiting factor for proteolysis in vitro, which is in agreement with the results of Goldberg and co-workers (Kandror et al., 1995, 1997).

**CONCLUSIONS**

Availability of oxygen and glucose were found to be important parameters, determining the degradation rate of the proteolytically sensitive protein A. Elimination of either oxygen or glucose alone was not enough to achieve complete stabilization of protein A, but a combination of these actions did result in stabilization. Addition of sulfite in combination with cessation of the aeration, stabilized the protein in vivo even in presence of glucose evidently due to elimination of dissolved oxygen and inhibition of fermentation. Furthermore, the absence of both glucose and oxygen caused a decrease of the ATP pool to less than 0.5 mM and an increase in concentrations of AMP and ADP, which could be a reason for the reduced proteolysis. The attempts to reconstitute proteolysis in cell disintegrate by addition or regeneration of ATP were unsuccessful, showing that some factor other than ATP limits the protein A proteolysis in vitro.

**REFERENCES**


Hershko A, Tomkins GM. 1971. Studies on the degradation of tyrosine aminotransferase in hepatoma cells in culture. Influence of the com-

**Figure 10.** Western blot showing the concentration of protein A in three disintegrates afer 0, 30, 60, and 90 min of incubation at room temperature. Lanes 1–4, control without any additions; lanes 5–8, attempts to reconstitute proteolysis in cell disintegrate by addition of ATP; and lanes 9–12, regeneration of ATP. Scanning of the membrane did not detect proteolysis in any of the disintegrates.