Elevated Levels of Asparagine Synthetase Activity in Physiologically and Genetically Derepressed Chinese Hamster Ovary Cells Are Due to Increased Rates of Enzyme Synthesis*

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The activity of asparagine synthetase in Chinese hamster ovary (CHO) cells is increased in response to asparagine deprivation or decreased aminoacylation of several tRNAs (Andrulis, I. L., Hatfield, G. W., and Arfin, S. M. (1979) J. Biol. Chem. 254, 10629-10633). CHO cells resistant to β-aspartylhydroxamate have up to 5-fold higher levels of asparagine synthetase than the parental line (Gantt, J. S., Chiang, C. S., Hatfield, G. W., and Arfin, S. M. (1980) J. Biol. Chem. 255, 4808-4813). We have investigated the basis for these differences in enzyme activity by combined radiochemical and immunological techniques.

The asparagine synthetase of beef pancreas was purified to apparent homogeneity. Antibodies raised against the purified protein cross-react with the asparagine synthetase of CHO cells. Immunotitration studies show that the amount of enzyme protein in physiologically or genetically derepressed CHO strains is proportional to the level of enzyme activity. Measurement of the relative rates of asparagine synthetase synthesis by pulse-labeling experiments demonstrate that the difference in the number of asparagine synthetase molecules is closely correlated with the rate of enzyme synthesis. In contrast, the half-life of asparagine synthetase in wild type cells and in physiologically or genetically derepressed cells is very similar. It appears that the increased levels of asparagine synthetase can be attributed solely to an increased rate of enzyme synthesis.

Cultured Chinese hamster ovary cells modulate the specific activity of asparagine synthetase in response to the degree of aminoacylation of a number of species of tRNAs (1). CHO cell mutants affected in asparagine synthetase activity were previously isolated by selection for resistance to β-aspartylhydroxamate (2). The activity of asparagine synthetase in some of these β-aspartylhydroxamate-resistant lines is 5-fold higher than that in the parental line and is no longer subject to regulation by either the asparagine content of the medium or the extent of aminoacylation of tRNA's (3). Initial studies, which compared some physical and catalytic properties of the enzyme from mutant and parental lines, failed to show any significant differences in these properties. This suggests that the differences between these lines are the result of differences in the number of enzyme molecules rather than differences in catalytic activity per molecule.

In addition to mutants containing constitutively elevated levels of asparagine synthetase activity, Chinese hamster cell lines containing low or undetectable levels of activity have been isolated as asparagine auxotrophs (3, 4). Thus, the asparagine synthetase system appears to be an attractive one for genetic and biochemical studies of the regulation of enzyme levels in animal cells.

In this study, we describe the purification of asparagine synthetase from beef pancreas and the preparation of rabbit antibody to asparagine synthetase. Using this antibody, we show that the differences in enzyme activity levels resulting from both physiological manipulation and genetic alteration are due to differences in the content of asparagine synthetase molecules. Elevated levels of asparagine synthetase are achieved through alterations of the rate of enzyme synthesis with no change in the rate of enzyme degradation.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyapatite (Hypatite C) was purchased from Clarkson Chemical Co., and agarose-linked Cibacron blue 3GA (Mextex gel blue A) was purchased from Amicon Corp. PMSF, ATP, and dithiothreitol were from Sigma. Freund's complete and incomplete adjuvants were from Grand Island Biological Co. Formaldehyde-fixed Staphylococcus aureus was prepared by the method of Kessler (5). L-[1-14C]Aspartic acid (222 mCi/mmol) and L-[15N]methionine (500 to 1400 Ci/mmol) were purchased from Amersham Corporation, Arlington Heights, IL. The sources and purity of all other chemicals were identical to those described earlier (1, 2, 6).

Cell Culture—The properties of the parental CHO cell line, tsH1, which contains a temperature-sensitive leucyl-tRNA synthetase, and lines AH-2 and AH-5, which contain increased asparagine synthetase activity, have been described (1, 2). Cells were grown in monolayer or suspension culture in α-ME medium (7) as previously described (1, 2, 6).

All cell lines were tested periodically and found to be free of mycoplasma contamination.

Enzyme Assay—Asparagine synthetase activity was measured by following the conversion of [14C]aspartic acid to [14C]asparagine, as previously described (2). Labeled aspartic acid was purified in order to establish low assay backgrounds. [14C]Aspartic acid was adsorbed to a Dowex 1-acetate column and washed thoroughly with water. The adsorbed aspartic acid was eluted with 2 N HC1 and neutralized with Tris base. One unit of activity catalyzes the formation of 1 pmol of asparagine/min. Protein was assayed by the method of Lowry et al. (8), or by the dye-binding method described by Bradford (9), using crystalline bovine serum albumin as a standard.

Enzyme Purification—All steps were carried out at 0-4 °C unless otherwise noted. Beef pancreata, obtained fresh from a local slaughterhouse, were trimmed of fat, minced, and homogenized in 2 volumes of 10 mM Tris, 1 mM dithiothreitol, 5 mM EDTA, 20% glycerol, 0.5 mM PMSF, pH 7.5 (Buffer A), using a Brinkmann Polytron.
homogenate was centrifuged at 15,000 × g for 30 min and then at 105,000 × g for 60 min, and the supernatant solution was saved (Fraction I). Cetyltrimethylammonium bromide (1%) was added drop-wise while stirring to a final concentration of 0.1%. After 10 min, the precipitate was removed by centrifugation. The supernatant (Fraction II) was mixed with concentrated solutions of sodium ATP, MgCl₂, and urea* to achieve final concentrations of 105 M, 30%, and 7.5%, respectively. This solution was rapidly raised to 55 °C, held at that temperature with gentle stirring for 8 min, and then rapidly chilled in an ice bath. Heat-denatured protein was removed by centrifugation. (NH₄)₂SO₄, saturated at 4 °C and adjusted to pH 7.5 with NH₄OH, was added to the supernatant solution while stirring to a final saturation of 60%. After stirring for an additional 15 min, the precipitate was collected by centrifugation and dissolved in 1/4 of the original homogenate volume of buffer A (Fraction III). Hydroxyapatite, previously equilibrated with buffer A, was added to Fraction III in the ratio of 10 mg of hydroxyapatite/mg of protein. After stirring for 15 min, the hydroxyapatite and adsorbed protein were collected by centrifugation for 10 min at 10,000 rpm in a Beckman JA-10 rotor. The pellet was suspended in 1/4 of the original homogenate volume of buffer A containing 12 mM ATP and stirred for 15 min. The suspension was centrifuged for 10 min at 15,000 × g and the supernatant was saved. **Determination of Asparagine Synthetase** Antibody during this time were negative. Twelve weeks after this final injection, a γ-globulin fraction was prepared from the serum as described by Palacios et al. (11). The antibodies were visualized as described by Hanford and Arfin (11). Preparation of Anti-Asparagine Synthetase—Homogeneous beef pancreas asparagine synthetase (200 μg/ml in buffer A) was mixed with an equal volume of complete Freund’s adjuvant. Three New Zealand white rabbits were each injected with about 100 μg of protein divided into four equal portions, two given intramuscularly in the hind legs and two subcutaneously along the backbone. Each rabbit received three injections. The injection of the protein in incomplete adjuvant at 3-week intervals. Tests for circulating antibody during this time were negative. Twelve weeks after the immunization schedule was begun, each rabbit was similarly injected with 1 mg of protein in complete adjuvant. Maximal antibody titers were obtained 10 days after this injection. The rabbits were bled from the ear vein every 2 weeks after this final injection. A γ-globulin fraction was prepared from the serum as described by Palacios et al. (12), adjusted to a protein concentration of 20 mg/ml and stored at −20 °C. A control γ-globulin fraction was prepared in an identical manner from preimmune serum.

Characterization of Antibody Directed Against Asparagine Synthetase—Cell extracts were prepared by 2 cycles of freeze-thawing in Buffer A, followed by centrifugation at 30,000 × g for 15 min. Protein concentration was adjusted to 10 mg/ml. Fifty μl of cell extracts were incubated with various dilutions of antiserum for 30 min at 4 °C. A sufficient amount of 25% (w/v) formaldehyde-fixed *S. aureus* suspended in Buffer A was then added to each tube and incubated for 50 min at 4 °C. The suspension was then centrifuged for 1 min in an Eppendorf 5412 centrifuge and the activity remaining in the supernatant was determined by the usual assay procedure.

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<tr>
<th>Table I</th>
<th>Representative purification scheme for bovine asparagine synthetase</th>
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<tr>
<td>Fraction</td>
<td>Total protein</td>
</tr>
<tr>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>I. 100,000 × g supernatant</td>
<td>11,414</td>
</tr>
<tr>
<td>II. 0.1% cetyltrimethylammonium bromide</td>
<td>8,384</td>
</tr>
<tr>
<td>III. Heat denaturation: 35 °C, 8 min</td>
<td>2,120</td>
</tr>
<tr>
<td>IV. Hydroxyapatite</td>
<td>316</td>
</tr>
<tr>
<td>V. Dextran blue 3GA agarose</td>
<td>5.7</td>
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<tr>
<td>VI. Glycerol gradient</td>
<td>0.6</td>
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Synthesis of Asparagine Synthetase in CHO Cells

Fig. 1. SDS-acrylamide gel electrophoresis of bovine asparagine synthetase. 30 μg of Fraction VI protein were loaded onto a 6-cm, 10% gel and electrophoresed as described under “Experimental Procedures.” The gel was stained with Coomassie brilliant blue R-250.

cell extracts and removing the resulting complex with fixed S. aureus. These titration experiments (Fig. 2) show that all CHO cell asparagine synthetase activity is removed by the antibody. The specificity of the antiserum preparation is demonstrated by SDS-acrylamide gel electrophoresis analysis of the immunoprecipitate from [35S]methionine-labeled cells (Fig. 3). A single, sharp peak of radioactivity is observed. This peak can be eliminated by preincubation of the antiserum with an excess of purified bovine asparagine synthetase. No peaks of radioactivity are observed when an equivalent amount of preimmune serum is used. When the mobility of this band is compared with proteins of known molecular weights, a subunit molecular weight of 56,500 is determined.

Immunotitration of Asparagine Synthetase from Physiologically and Genetically Derepressed Cells—We have previously demonstrated that the specific activity of asparagine synthetase is increased in CHO cells grown under conditions leading to decreased aminoacylation of several tRNAs (1). We have also shown that cell lines AH-2 and AH-5, which are resistant to the toxic effects of β-aspartylhydroxamate, have constitutively elevated asparagine synthetase activity (2). Extracts from tsH1 cells grown under different conditions leading to elevated enzyme activity (Fig. 2A) and extracts from tsH1, AH-2, and AH-5 cells (Fig. 2B) were titrated with rabbit anti-asparagine synthetase antiserum to determine whether this

Fig. 2. Immunotitration of asparagine synthetase. 50 μl of cell extracts containing 500 μg of protein were mixed with the indicated volume of antiserum and incubated at 4 °C for 30 min. Following precipitation with fixed S. aureus, asparagine synthetase activity remaining in the supernatant was assayed. In A, extracts were prepared from tsH1 cells growing in complete α-ME medium (●), α-ME medium containing 40 μM leucine (○), or α-ME medium lacking asparagine (◊). In B, extracts were prepared from tsH1 (●), AH-2 (○), and AH-5 (◊) cells grown in complete α-ME medium.

Fig. 3. SDS-acrylamide gel electrophoresis of immunoadsorbed 35S-labeled CHO cell asparagine synthetase. tsH1 cells were grown for 48 h in the presence of [35S]methionine. Asparagine synthetase was immunoprecipitated and electrophoresed in a 7.5% gel. 35S activity was determined following digestion of the gel slices with H2O2.
increase in enzyme specific activity results from an increase in catalytic efficiency or an increase in enzyme protein.

Fig. 2A shows that the amount of antiserum required for complete neutralization of asparagine synthetase activity in extracts from cells grown in complete medium, medium lacking asparagine, or medium containing 1/2 of the normal leucine concentration is proportional to the specific activity of the enzyme. Similarly, Fig. 2B shows that for the tsH1, AH-2, and AH-5 cell lines, the amount of antiserum required to neutralize the enzyme activity is also directly proportional to the specific activity of asparagine synthetase in these cell lines. Incubation of these extracts with control serum had no effect on enzyme activity. These results demonstrate that the increased enzymatic activity results from an increase in the amount of an immunologically identical enzyme protein and is not due to an alteration in catalytic efficiency.

Turnover of Asparagine Synthetase—The increased asparagine synthetase content in the physiologically and genetically derepressed cells could result from changes in the rate of enzyme synthesis or degradation, or a change in both. To properly assess the relative contribution of rates of synthesis and degradation in determining the steady state concentration of asparagine synthetase, it is necessary to avoid conditions which may alter these rates. Since asparagine synthetase levels are known to be affected by the degree of aminoacylation of a number of tRNAs (1), labeling conditions which changed the degree of acylation of any tRNA were avoided. The concentration of methionine used during the labeling period allowed cells to grow normally for at least 48 h. In addition, asparagine synthetase specific activity remained unchanged throughout this period. It is, therefore, unlikely that the labeling conditions change the steady state rate of synthesis or degradation.

Turnover studies were performed on tsH1 and AH2 cells growing in complete medium and tsH1 cells growing in medium lacking asparagine or medium containing a reduced amount of leucine. To measure the relative rate of enzyme synthesis, short term incorporation of [35S]methionine into asparagine synthetase and total cytosolic proteins was determined as described under "Experimental Procedures." [35S]Methionine is incorporated into total cytosolic proteins and asparagine synthetase in a linear fashion for at least 8 h under these conditions. Two h after addition of label, cell extracts were prepared and incubated with rabbit antiserum. Following binding to fixed S. aureus and washing, the 35S-labeled enzyme was subjected to SDS-acrylamide gel electrophoresis. The gels were sliced, and the 35S activity was quantitated as described under "Experimental Procedures." The relative rate of 35S incorporation into asparagine synthetase is expressed as the ratio of the counts per min in the enzyme to the counts per min in total cytoplasmic proteins. Table II shows that the differences in asparagine synthetase specific activities are completely accounted for by increases in the relative rates of enzyme synthesis.

The rate of degradation of asparagine synthetase was investigated by pulse-decay analysis. Cells were labeled using the same medium as described for the experiments used to determine the rates of enzyme synthesis. Fig. 4 shows the decrease of radioactivity in asparagine synthetase due to degradation after transfer to a medium containing an excess of unlabeled methionine. Loss of radioactivity in asparagine synthetase followed first order exponential kinetics. The calculated half-life of asparagine synthetase in tsH1 and AH-2 cells growing in complete medium is 43 and 46 h, respectively. Very similar half-lives are found for asparagine synthetase in tsH1 cells growing in medium lacking asparagine (49 h) or in medium containing limiting concentrations of leucine (52 h).

**DISCUSSION**

The asparagine synthetase activity of CHO cells is elevated in a wide range of conditions where asparagine synthetase content is elevated. When wild type cells are grown in a medium lacking asparagine or when mutants containing altered aminoacyl-tRNA synthetases are grown under conditions that lead to decreased aminoacylation of their cognate tRNAs (1). This physiological derepression results in a 2- to 3-fold rise in the activity of asparagine synthetase. Mutants containing constitutively derepressed levels of asparagine synthetase activity have been isolated on the basis of their resistance to the toxic effects of the asparagine analog 2- aspartylhydroxamate (2). These genetically derepressed cells have about 5-fold more asparagine

**Figure 4. Degradation of asparagine synthetase.** The half-life of asparagine synthetase was determined in tsH1 (○) and AH-2 (△) cells grown in complete a-ME media and in tsH1 cells grown in a-ME medium lacking asparagine (□) or containing a limiting amount of leucine (○). Cells were labeled with [35S]methionine for 2 h, divided into four equal fractions, and grown in the appropriate media. Cells were harvested at various times during the following 75 h, and the amount of 35S activity in asparagine synthetase was determined as described under "Experimental Procedures."

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<th>Table II</th>
<th>Relative rates of [35S]methionine incorporation into total protein and asparagine synthetase</th>
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<tr>
<td>Cell type</td>
<td>Medium</td>
</tr>
<tr>
<td>tsH1</td>
<td>Complete</td>
</tr>
<tr>
<td>tsH1</td>
<td>Lacking asparagine</td>
</tr>
<tr>
<td>tsH1</td>
<td>Limiting leucine</td>
</tr>
<tr>
<td>AH-2</td>
<td>Complete</td>
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*Note:* The asparagine synthetase activity of CHO cells is elevated when wild type cells are grown in a medium lacking asparagine or when mutants containing altered aminoacyl-tRNA synthetases are grown under conditions that lead to decreased aminoacylation of their cognate tRNAs (1). This physiological derepression results in a 2- to 3-fold rise in the activity of asparagine synthetase. Mutants containing constitutively derepressed levels of asparagine synthetase activity have been isolated on the basis of their resistance to the toxic effects of the asparagine analog 2- aspartylhydroxamate (2). These genetically derepressed cells have about 5-fold more asparagine
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synthetase activity than wild type cells. No further derepression can be achieved in these drug-resistant lines by limiting the extent to which tRNA\(^{AUG}\) is aminoacylated. In hybrids formed between wild type cells and \(\beta\)-aspartylhydroxamate-resistant mutants, elevated asparagine synthetase activity behaves as a dominant marker (15).

We have investigated the basis for the physiological and genetic derepression of asparagine synthetase in CHO cells. Using antiserum specific for asparagine synthetase, we performed immunotitration experiments (Fig. 2) to quantitate the amount of asparagine synthetase antigen in wild type cells and in physiologically or genetically derepressed cells. The results demonstrate that the increased activity of asparagine synthetase arises from a corresponding increase in immunologically reactive protein.

Direct measurement of the relative rates of asparagine synthetase synthesis in physiologically derepressed tsH1 cells and in the genetically derepressed cell line AH-2 shows that synthesis occurs at an increased rate (Table II). Very similar half-lives were observed for asparagine synthetase under all conditions studied (Fig. 4). The increases in the rate of enzyme synthesis are sufficient to fully account for the elevated steady state asparagine synthetase content of these cells.

tRNAs have been implicated as having regulatory roles in a variety of cellular processes. In prokaryotes, these include the regulation of amino acid biosynthesis (16, 17), amino acid transport (18), and macromolecular synthesis (19). The mechanisms by which tRNA participates in these processes have been studied in detail. There is now abundant evidence (20) that for several amino acid operons, regulation by tRNA occurs via attenuation of transcription. In this process, the rate of translation of a leader polypeptide on the nascent polycistronic mRNA determines whether further transcription will occur. The involvement of tRNA in macromolecular synthesis in prokaryotes, the stringent response, has been shown to be mediated through the compounds ppGpp and pppGpp (21). In both attenuation and the stringent response, the availability of mutations at critical steps has facilitated the elucidation of the regulatory mechanisms.

In the higher eukaryotes, tRNAs appear to play a role in regulating amino acid transport (22), protein degradation (23), and the levels of asparagine synthetase activity (1). The mechanisms through which tRNA exert their regulatory influences in these cases are unknown. The evidence presented here shows that the increased levels of asparagine synthetase found in CHO cells grown under conditions leading to decreased aminoacylation of tRNA are the result of an increased rate of enzyme synthesis. Whether this increased rate of synthesis reflects an alteration in the number of mRNA transcripts or an increased efficiency of transcript utilization is not yet known. A mechanism involving the coupling of transcription and translation, as in prokaryotic attenuation, seems unlikely in a eukaryotic cell, since transcription occurs within the nucleus, while translation occurs on cytoplasmic ribosomes.

The increased rate of asparagine synthetase synthesis in strain AH-2 suggests that this mutant is altered in a regulatory element involved in controlling the rate of enzyme synthesis. The finding that this mutation behaves in a dominant manner in cell hybrids (15) suggests that a diffusible element may be involved. It is clearly too early to postulate any models regarding the mechanism by which asparagine synthetase activity is regulated. However, the ability to select for both structural gene mutants (4) and mutants with defective regulatory mechanisms suggests that the further study of this system will provide insights into the mechanisms of the regulation of gene expression in mammalian cells.

REFERENCES