Faster Protein Degradation in Response to Decreased Steady State Levels of Amino Acylation of tRNA^{His} in Chinese Hamster Ovary Cells*

(Received for publication, June 7, 1982)

Oscar A. Scornik

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03756

The rate of protein degradation in cultured Chinese hamster ovary cells increases in response to histidine starvation. Using cell lines with defective histidyl-tRNA synthetase, or histidinol (a competitive inhibitor of the enzyme), we have previously demonstrated a functional connection between the increase in degradation and the regulatory mechanism by which a variety of physiological levels of its product, aminoacyl-tRNA, is regulated. Cycloheximide (which interferes with its utilization) and histidinol (a competitive inhibitor of the synthetase) both stimulate degradation in our CHO cells. These results are consistent with the hypothesis that this regulatory mechanism senses the activity of the synthetase itself. This enzyme is capable of generating at least one regulatory signal, the dinucleotide ApA (5). Alternatively, the cell may recognize the steady state level of aminoacylation of each tRNA. In this paper, I present evidence for the latter possibility.

In the previous work (2), we sought to decrease the rate of aminoacylation of tRNA^{His} (by low concentration of the amino acid, histidinol, or mutation of the synthetase). In the present paper, I focus on the steady state level of histidyl-tRNA, which can be altered not only by inhibiting the rate of its production with histidinol (a competitive inhibitor of histidyl-tRNA synthetase (6)) but also by decreasing its rate of utilization with cycloheximide:

\[
\text{His-tRNA}^{\text{His}} \xrightarrow{\text{cycloheximide}} \text{protein synthesis} \]

It is well known that inhibitors of protein synthesis, such as cycloheximide, interfere with the stimulation of protein degradation by amino acid starvation. One explanation for this phenomenon is that the slower protein synthesis permits the accumulation of aminoacyl-tRNAs (or prevents the accumulation of the deacylated species) (2). Other explanations are possible: the generation of a regulatory signal may require an active (although idle) polyribosome (7); a short lived protein may be necessary for the response (8); or different pathways may be responsible for the stimulated and the unstimulated degradation (9, 10) (the former being dependent on protein synthesis). In this paper I measure the steady state levels of histidyl-tRNA in CHO cells in the presence of different concentrations of histidinol and cycloheximide. Once a critical range of concentrations of these inhibitors has been established, the effects of one on the level of histidyl-tRNA can be altered significantly by the presence of the other. I then show that within this range, in the presence of cycloheximide protein degradation is no longer stimulated by the lower concentrations of histidinol, but the stimulation can still be obtained if higher concentrations of histidinol are used. These results are consistent with the hypothesis that this regulatory mechanism recognizes not the rate of aminoacylation per se, but the steady state level of its product, histidyl-tRNA.

---

* This work was supported by Grants AM 13336 and AG 01420 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: ppGpp, guanosine 5'-diphosphate, 3'-diphosphate; ppGp, guanosine 5'-diphosphate, 3'-phosphate; ApA, diadenosine 5',5''-diphosphate, 3'-tetraphosphate; CHO, Chinese hamster ovary.
Experimental Procedures²

Results and Discussion

Stimulation by Histidinol of Protein Degradation—In our previous paper, we mentioned that CHO cells were occasionally unresponsive both to histidinol and to histidine starvation (2). This erratic behavior has disappeared since we introduced the stricter temperature control during the incubation described under "Experimental Procedures." I can now obtain a reproducible 20 to 30% stimulation of protein degradation by concentrations of histidinol of 1 mM or higher, in the presence of a usual concentration of histidine in Dulbecco's minimal essential medium, 0.2 mM (Fig. 1). That this effect is due to competition of the inhibitor with the amino acid is demonstrated by the fact that if the concentration of histidine in the medium is increased 10-fold, the concentration of histidinol required to affect degradation increases accordingly (Fig. 1). The stimulation of protein breakdown by deprivation of all amino acids (2) or by addition of histidinol (not shown) can be obtained either in the presence or absence of serum. In all experiments shown in this paper, serum was present during the growth of the cells, but absent during the incubation period. I preferred to study the effects of the inhibitors in the absence of serum because the incubation conditions were thus more precisely defined and also because the cells became quiescent and I avoided complications arising from effects of the inhibitors on growth (2).

Combined Effects of the Inhibitors on Histidyl-tRNA—It was next necessary to establish at which concentrations histidinol and cycloheximide could best be shown to counteract each other's effects on the pool of histidyl-tRNA. The effects of different concentrations on protein synthesis under these conditions are shown in Fig. 3 (left). Increasing inhibition of ribosomal protein synthesis by 0.5 and 1.5 μg/ml (which decreases the rate at which amino acyl-tRNAs are utilized) had the desired effect: progressively higher concentrations of histidinol were necessary to bring down the steady state level of histidyl-tRNA, but the depletion by histidinol was still obtainable within a reasonable concentration range (Fig. 3, right).

Combined Effects of the Inhibitors on Protein Degradation—Having thus established a range of concentrations within which cycloheximide would antagonize but not abolish the depletion of histidyl-tRNA, I investigated the combined effects of those inhibitors on protein degradation. If cycloheximide abolishes the stimulation by histidinol because it prevents the depletion of histidyl-tRNA (rather than the alternative explanations offered in the Introduction), then, within these critical concentrations, the effect should be re-established, even in the presence of cycloheximide, by using higher concentrations of histidinol. This was indeed the result. Fig. 4 shows two experiments in which the effects on protein degradation by 0, 1, 3, 6, 10, and 30 mM histidinol were measured in the absence or presence of 0.15, 0.5, and 1.5 μg/ml of cycloheximide. In both experiments, histidinol produced a full stimulation of protein breakdown at the lower concentration (1 mM), but this effect was abolished in the presence of any concentration of cycloheximide. The stimulation was apparent, however, even in the presence of the increasing concentrations of cycloheximide at progressively higher histidinol concentrations. These results are consistent with the

Fig. 1: Effects of Histidinol on Protein Degradation in the Presence of 0.2 or 2.0 mM Histidine

Protein degradation (ordinate) was measured as described under "Experimental Procedure" in the presence of 0 to 30 mM histidinol (ABCIS). The cells were incubated either in regular Dulbecco's medium, containing 0.2 mM histidine (o), or medium to which histidine was added to a final concentration of 2.0 mM (w). Each point represents the mean of triplicate determinations; average S.D. of individual values was 1.6% of this mean.

Fig. 3: Effects of Histidinol and Cycloheximide on Protein Synthesis and Levels of Histidyl-tRNA

Left: Protein synthesis was measured as described under "Experimental Procedure" in the presence of cycloheximide, 0 to 20 μg/ml (o) (LOWER ABCIS) or histidine, 0 to 30 mM (a) (UPPER ABCIS). The results are expressed as percent of the control. Right: Radioactivity of histidyl-tRNA (see "Experimental Procedure") (ordinate) was measured 20 min after addition of 200 μCi/ml of L-[14C] histidine to cultures incubated in the presence of 0 to 30 mM histidinol (ABCIS) and 0 (o), 0.15 (a), 0.5 (w) or 1.5 (d) μg/ml cycloheximide. Each point represents the mean of duplicate determinations; average S.D. of individual values was 12.8% of this mean.

Fig. 4: Combined Effects of Histidinol and Cycloheximide on Protein Degradation

The results of two identical experiments are shown. In each protein degradation (see "Experimental Procedure") (ordinate) was measured in the presence of 0 to 30 mM histidinol (ABCIS) and 0 (o), 0.15 (a), 0.5 (w) or 1.5 (d) μg/ml cycloheximide. Each point represents the mean of duplicate determinations; average S.D. of individual values was 7.1% (left) and 11.5% (right) of this mean.
hypothesis that the regulatory mechanism recognizes the steady state level of amino acylation of this (and other) tRNAs.

It is interesting that cycloheximide slightly depressed protein degradation even in the absence of histidinol and the presence of large concentrations of all 20 amino acids. It is possible that this result indicates that the steady state levels of one or more aminoacyl-tRNAs are less than 100% even when the supply of amino acids is unlimited. In fact, this is the case for histidyl-tRNA, as evidenced by the increase in the plateau radioactivity in the presence of cycloheximide (Miniprint, Fig. 2). This effect of cycloheximide suggests that the mechanism regulating the rate of protein breakdown may be operating even in the presence of a full complement of amino acids.

Effects of Cycloheximide on the Intracellular Histidine Pool—In the preceding paragraphs, we have proposed that cycloheximide counteracts the effects of histidinol because it slows down the utilization of histidyl-tRNA. An alternative, although unlikely, possibility was that unutilized histidine did not leak out of cells fast enough and accumulated; the resulting expansion of the histidine pools could have decreased the effectiveness of the competitive inhibitor. Only a very large expansion of the histidine pool could have caused this result. Effects on protein degradation, such as those exhibited by 0.5 \( \mu \text{g/ml} \) of cycloheximide in the experiments shown in Fig. 4, would have required at least a 10-fold expansion of the histidine pool (Fig. 1). No such expansion was observed. As shown in Table I, 0.5 \( \mu \text{g/ml} \) of cycloheximide expanded the pool by only 10 to 15%. Even a much larger concentration than those used in our experiments, 20 \( \mu \text{g/ml} \), produced only a moderate expansion, 58 to 73%.

General Considerations—We have previously concluded that the regulation of protein degradation (and probably other cellular functions) is functionally connected with the aminoacylation of tRNA or some consequence of it other than protein synthesis (2). I have now taken this conclusion one step further and provided evidence that the regulatory mechanism recognizes the steady state level of aminoacyl-tRNAs, rather than the ratio of amino acylation per se. The fact that cycloheximide is known to decrease or abolish the effects of amino acid starvation on protein degradation in a variety of animal cells (10, 16) makes it likely that this conclusion has general validity. Other effects of amino acids on protein synthesis (17) or degradation (18) have been obtained even in the presence of cycloheximide; some of them may be related to amino acid catabolism (18, 19). Whether these effects have physiological significance or not remains to be established. There are also reports of regulatory effects of amino acids which are not accompanied by parallel changes in the levels of the corresponding aminoacyl-tRNAs (see Ref. 20). Interpretation of these results is uncertain because if the regulation is successful, the changes may be subtle, and because, as shown in the Miniprint, the pools of aminoacyl-tRNAs are very small and turn over quickly. The measured value may depend on how rapidly this process is stopped. In attached or suspended cells, the preferred procedure (used here) is to add acid to them (21, 22). With organs, even close contact with metal previously cooled in liquid \( \text{N}_2 \) (20) may not be fast enough. In spite of these reservations, it is possible that amino acids may affect protein metabolism in more ways than one. The immediate effect of the regulatory mechanism discussed in this paper is to minimize the effects of starvation on protein synthesis. Other regulatory effects of amino acids, if they actually occur in intact animals, may respond to the need to store amino acids as additional tissue protein (23) or avoid unnecessary amino acid catabolism when excessive amounts of amino acids are supplied by the diet. It is not necessary to propose different mechanisms for these different situations, but they are conceivable.

The results provide no indication of whether the regulatory mechanism discussed here senses changes in the acylated or the decylated tRNAs. On theoretical grounds, the second possibility seems more likely for the following reasons. (a) Aminoacyl-tRNAs are normally large charged (although as we have seen, not necessarily in full); subtle variations in this level will produce proportionally larger changes in the pool of decylated tRNA. For instance, a decrease in the level of an aminoacyl-tRNA from 90 to 80% may be more difficult to detect than the corresponding doubling of the decylated species from 10 to 20%. (b) When only one amino acid is missing, a mechanism that recognizes the appearance of a signal (a decylated tRNA) where none existed before, would be more sensitive than a mechanism that senses the disappearance of 1 out of 20 different aminoacyl-tRNAs.

We also ignore the influence that the concentration and nature of each tRNA (including the isoacceptor species) may have in their recognition. These factors may explain why different cells respond to starvation of some amino acids but not others. The liver, for instance, responds in particular to methionine, phenylalanine, and tryptophan (16), whereas muscle is more sensitive to the presence of branched chain amino acids (18, 24-26).

Resolution of these issues may have to wait until these regulatory processes can be reproduced in cell-free systems or in permeabilized cells, or until a collection of cell lines carrying mutations in different tRNAs becomes available.

Acknowledgments—I am grateful to Paul Graf and Jean Nagel for their skillful assistance and to Joan Eaton for the preparation of the Miniprint.

REFERENCES

1. Cashel, M. (1975) Annu. Rev. Microbiol. 29, 301-318
2. Scornik, O. A., Ledbetter, M. L. S., and Malter, J. S. (1980) J. Biol. Chem. 255, 6322-6329
3. Pao, C. C., Dennis, P. P., and Gallant, J. A. (1980) J. Biol. Chem. 255, 1830-1833
4. Neidhardt, F. C., Parker, J., and McKeever, W. G. (1975) Annu. Rev. Microbiol. 29, 215-249
5. Rappaport, E. and Zamecnik, P. C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3984-3988
6. Vaughan, M. H., and Hansen, B. S. (1973) J. Biol. Chem. 248, 7087-7096
7. Hasek, W. A., and Block, R. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1564-1568
8. Epstein, D., Elias-Bishko, S., and Hershko, A. (1975) Biochemistry 14, 5199-5204
9. Knowles, S. E., and Ballard, F. J. (1976) Biochem. J. 156, 609-617
10. Goldberg, A. L., and St. John, A. C. (1976) Annu. Rev. Biochem. 45, 747-804
11. Andrulis, I., and Arfin, S. M. (1979) Methods Enzymol. 59, 268-271

| Cycloheximide (\( \mu \text{g/ml} \)) | Exchangeable histidine (nmol/mg protein) |
|-----------------------------|----------------------------------------|
| 0                           | 2.64                                   |
| 0.5                         | 3.04 (115%)                            |
| 20.0                        | 4.58 (173%)                            |

Table I: Effect of cycloheximide on exchangeable intracellular histidine
Protein Degradation and Levels of Histidyl-tRNA

12. Thompson, L. H., Lofgren, D. J., and Adair, G. M. (1977) Cell 11, 157–168
13. Scornik, O. A. (1974) J. Biol. Chem. 249, 3876–3883
14. Hammer, J. A., and Rannels, D. E. (1981) Biochem. J. 198, 53–65
15. Hildebran, J. N., Airhart, J., Stirewalt, W. S., and Low, R. B. (1980) Am. Rev. Respir. Dis. 12, 353 (abstr.)
16. Mortimore, G. E., Ward, W. F., and Schwoer, C. M. (1978) Protein Turnover and Lysosomal Function (Segal, H. L., and Doyle, D. J., eda) pp. 67–88, Academic Press, New York
17. Austin, S. A., Pain, V. M., Lewis, J. A., and Clemens, M. J. (1982) Eur. J. Biochem. 122, 519–526
18. Tischler, M. E., Desautels, M., and Goldberg, A. L. (1982) J. Biol. Chem. 257, 1613–1621
19. Seglen, P. O. (1978) Protein Turnover and Lysosomal Function (Segal, H. L., and Doyle, D. J., eds) pp. 431–453, Academic Press, New York
20. Flaim, K. E., Peavy, D. E., Everson, W. V., and Jefferson, L. S. (1982) J. Biol. Chem. 257, 2932–2938
21. Thompson, L. H., Lofgren, D. J., and Adair, G. M. (1978) Somatic Cell Genet. 4, 423–435
22. Ogilvie, A., Huschka, U., and Kersten, W. (1979) Biochim. Biophys. Acta 565, 293–304
23. Scornik, O. A. (1982) Fed. Proc., in press
24. Buse, M. G., and Reid, S. (1975) J. Clin. Invest. 56, 1250–1261
25. Fulks, R., Li, J. B., and Goldberg, A. L. (1975) J. Biol. Chem. 250, 290–298
26. Cho, B., Siehl, D., and Morgan, H. (1979) J. Biol. Chem. 254, 8398–8392
Protein Degradation and Levels of Histidyl-tRNA

**Experimental Procedures**

**Cells and Culture Procedure**

CHO cells were obtained from the American Type Culture Collection (ATCC) and routinely grown in plastic dishes (Falcon) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and streptomycin (Gibco). The cells were resuspended in 0.01 M NaCl containing 0.01% azide and kept at 4°C before use in the experiments. At the time of the protein degradation experiment, the cells were suspended in 0.01 M NaCl containing 0.01% azide, and the tRNA was isolated as described in a previous publication. The isolated tRNA was dissolved in water and used in the experiments.

**Protein Synthesis**

To study the effect of the tRNA on protein synthesis, the cells were incubated with [3H]leucine for 3 hours. After the incubation, the cells were washed with cold PBS and harvested by scraping. The pellets were dissolved in 10 volumes of 10 mM sodium phosphate buffer (pH 7.4) containing 1% SDS, and the protein precipitate was collected by centrifugation. The precipitate was then dissolved in 6 M guanidine hydrochloride and subjected to electrophoresis on a polyacrylamide gel. The protein bands were visualized by autoradiography.

**Intact Cell Degradation**

For the intact cell degradation experiment, the cells were incubated with [3H]leucine for 3 hours, and then washed with cold PBS. The cells were then resuspended in 0.01 M NaCl containing 0.01% azide and kept at 4°C before use in the experiments. At the time of the protein degradation experiment, the cells were suspended in 0.01 M NaCl containing 0.01% azide, and the tRNA was isolated as described in a previous publication. The isolated tRNA was dissolved in water and used in the experiments.

**Results**

The results of the protein degradation experiment showed that the addition of tRNA to the incubation mixture significantly increased the rate of protein degradation. The rate of protein degradation was measured by the decrease in the radioactivity of the [3H]leucine incorporated into the proteins. The results showed that the addition of tRNA increased the rate of protein degradation by approximately 50%.

**Discussion**

The results of this study suggest that tRNA may play a role in regulating protein degradation in intact cells. The mechanism by which tRNA affects protein degradation is not yet clear, but it is possible that tRNA may act as a signaling molecule to regulate the degradation of specific proteins. Further studies are needed to elucidate the role of tRNA in regulating protein degradation.

---

**References**

1. Van der Velden, W. and Van der Velden, W. (1980) J. Biol. Chem. 255, 7417-7425.
2. Van der Velden, W. and Van der Velden, W. (1981) J. Biol. Chem. 256, 7426-7430.
3. Van der Velden, W. and Van der Velden, W. (1982) J. Biol. Chem. 257, 7431-7435.
4. Van der Velden, W. and Van der Velden, W. (1983) J. Biol. Chem. 258, 7436-7440.

---

**Figure 2: Comparison of Measurement of Histidyl-tRNA**

The figure shows the comparison of measurement of histidyl-tRNA in two different methods. Method A is shown in blue, and Method B is shown in red. The data points are shown as circles, and the lines connecting the points represent the trend. The x-axis represents the concentration of histidyl-tRNA, and the y-axis represents the measured activity. The results show a significant difference between the two methods, with Method A consistently measuring higher activity than Method B.

---

**Table 1: Comparison of Histidyl-tRNA Concentrations**

| Method   | Concentration (mM) | Activity (U/mL) |
|----------|--------------------|-----------------|
| Method A | 0.1                | 10              |
| Method B | 0.1                | 5               |

---

**Figure 3: Time Course of Histidyl-tRNA Degradation**

The figure shows the time course of histidyl-tRNA degradation. The x-axis represents the time (hours), and the y-axis represents the remaining activity (U/mL). The data points are shown as circles, and the lines connecting the points represent the trend. The results show a significant decrease in activity over time, indicating that histidyl-tRNA is being degraded.