JOURNAL OF BACTERIOLOGY, Dec. 1973, p. 1253-1257

Vol. 116, No. 3
Printed in U.S.A.

Stringent Control of Intracellular Proteolysis in *Escherichia coli*

MARTIN J. PINE

Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14203

Received for publication 13 September 1973

Regulation of intracellular proteolysis has been compared during amino acid deficiencies in seven double auxotrophs of *Escherichia coli* 9723f with a common phenylalanine requirement. Individual deficienceis were either more effective than, less effective, or equal to phenylalanine deficiency in stimulating intracellular proteolysis. For each amino acid, the same relationship prevailed in inhibiting uracil incorporation into nucleic acids, a reaction series regulated by the *rel* gene for stringent control. The three amino acids least abundant in the cellular protein were the least effective regulators. These findings are interpreted as supportive evidence for stringent control of intracellular proteolysis by the *rel* gene.

Amino acids are general regulators of a number of unrelated metabolic reactions. Amino acid deficiencies stimulate intracellular proteolysis, and the stimulation is largely abolished by chloramphenicol (8, 21). Analogously, amino acid deficiencies inhibit ribonucleic acid (RNA) synthesis and several other biosynthetic reactions, and these inhibitions are also reversed by chloramphenicol (5). The latter regulation is referred to as stringent control and is attributed to the *rel* gene. Generally, the criterion of association of the stringent responses has been their concomitant loss upon *rel* mutation. In one *rel*$^-$ mutant that was starved of a combination of nutrients. Sussman and Gilvarg (24) found intracellular proteolysis to be below normal, but not when glucose was additionally omitted. They raised the possibility that these could be indirect effects of the *rel*$^-$ mutation. Proteolysis in different *rel*$^-$ mutants does not respond consistently to single amino acid deficiencies (8, unpublished experiments). The present study reexamines the question of stringent control of intracellular proteolysis by comparing the normal differences among amino acids in regulating it and also in regulating incorporation into nucleic acids. The rationale for the comparisons is consistent with the most current explanation of the mode of expression of the *rel* gene, and the procedure may be applicable to other investigations of stringent control in organisms for which there are no *rel*$^-$ mutations.

MATERIALS AND METHODS

A thymine-requiring mutant of the phenylalanine-requiring *Escherichia coli* 9723f was obtained by plating with 5 μg of trimethoprim and 50 μg each of thymine, thymidine and adenosine per ml. The mutant and the original strain were mutagenized with *N*-methyl *N'*-nitroso-*N*-nitro-guanidine (1), and auxotrophs with additional amino acid requirements were enriched by penicillin counterselection (9). Cells were cultured and incubated at 37 C in minimal glucose medium as described (20, 21). Cultures at a cell density of 30 units in a Klett-Summerson colorimeter (660-nm filter; 2 × 10^8 cells/ml) were given 3.3 μM L-leucine-*1*-^{14}C, 50 mCi/mmol, which was quickly consumed (20). After 1 h they were filter washed and suspended in an eightfold dilution of amino acid-deficient medium and supplemented with individual amino acids and 100 μg of leucine carrier per ml to trap any free leucine-*1*-^{14}C liberated from cell protein. In the initial experiments, 5 μM uracil-2-^{14}C, 1.1 mCi/mmol, was added after 6 to 10 min to an unlabeled, otherwise identically treated, culture. In subsequent experiments, 5 μM uracil-*6*-^{3}H, 40 mCi/mmol, was added to the same culture. To estimate intracellular proteolysis, 1-ml samples of culture were inactivated with citric acid, steamed, and treated with ninhydrin (21). The $^{14}CO_2$ that was quantitatively evolved overnight from free leucine was trapped in 0.1 ml of NSC solubilizer (Amersham-Searle) and counted with toluene-based scintillant (21). A small correction was made for background decarboxylation of protein (20). A 0.1-ml portion of the ^{14}C-labeled culture was dried and counted directly to determine total leucine incorporation into protein. Intracellular proteolysis is calculated as the percentage of this incorporation that becomes susceptible to decarboxylation. To estimate uracil-2-^{14}C incorporation into total nucleic acid, culture samples were inactivated in cold 10% trichloroacetic acid and filtered and washed with the same fiber disks. To estimate deoxyribonucleic acid (DNA) incorporation, the samples were first in-

cubated overnight in 1 N NaOH before acidification. To estimate uracil-6-^3H incorporation, incorporated activity was recovered on membrane filters, and ^3H was recovered by combustion in a Packard model 305 Tricarb sample oxidizer.

To determine the amino acid composition of *E. coli* 9723f, cells harvested from mid-logarithmic growth were heated 20 min at 90 C in 10% trichloroacetic acid and washed in the same solution, followed by acetone. After overnight in vacuo hydrolysis in 6 N HCl at 100 C, amino acids were determined on a Technicon analyzer with a 57-cm column and eluted with a 5.5-h gradient (11). Cystine and methionine were separately determined after oxidizing the hydrolysate with performic acid (14).

Other experimental details have been previously described (21).

RESULTS AND DISCUSSION

Rates of protein breakdown and incorporation of uracil into nucleic acid are shown in Fig. 1–3 for seven double auxotrophs of *E. coli*, each requiring phenylalanine and one additional amino acid. The incorporation of uracil into RNA, which is the sole product incorporated in the two *thy*$^-$ mutants starved of thymine (Fig. 1), is limited by both the conversion of uracil to

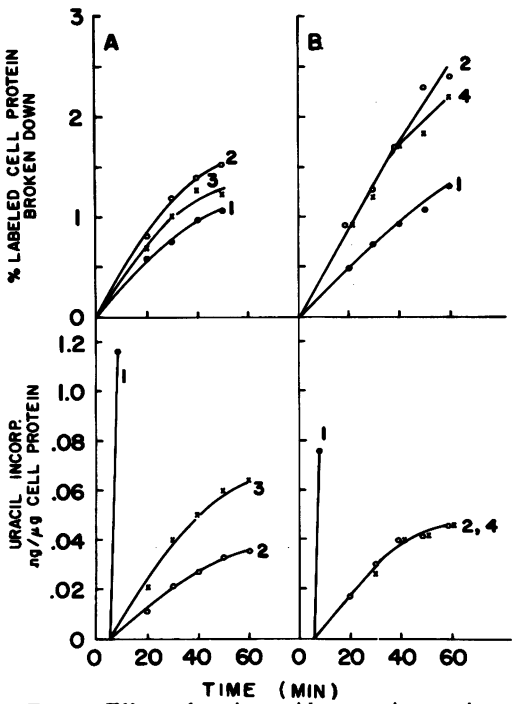

FIG. 1. *Effect of amino acid starvation on intracellular proteolysis and uracil-2-^{14}C incorporation into RNA of his*$^-$*(A) and arg*$^-$*(B) mutants of E. coli 9723 phe*$^-$*thy*$^-$*. 1, Complete growth; 2, phenylalanine absent; 3, histidine absent; 4, arginine absent. Thymine sources were omitted.*

nucleic acid intermediates and the net synthesis of RNA, all of which are under stringent control (5, 6, 25). Intracellular proteolysis is slightly inhibited by thymine starvation alone when growth eventually stops (21). It is unlikely, however, that this comparatively slow effect would be of significance during rapid growth arrestment from the amino acid deficiencies (Fig. 1). In a *thy*$^+$ strain, uracil incorporations into DNA and into total nucleic acid are both affected by individual amino acid deficiencies in an entirely parallel manner (Fig. 2A) and the same is true of RNA incorporation (total minus DNA incorporation). Thus, incorporations into RNA, DNA, or total nucleic acid appear to be equivalent parameters of stringent control. The changes in DNA incorporation, which occur very early upon amino acid starvation (Fig. 2A), must primarily reflect control of the conversion of uracil to nucleotide intermediates because the subsequent assembly of DNA is curtailed more slowly, when rounds of replication are completed (12). Stringent control cannot however be seriously limiting the uptake and exchange of leucine carrier during the proteolytic assay because much greater proteolytic rates can be detected when the labeling schedule is altered. Thus, when proteins are labeled minutes or seconds rather than 1 h before assay, protein half-lives in the order of minutes have been found in *E. coli* 9773f and all other strains that have been examined, either during growth or after prolonged amino acid starvation or other deleterious treatments (3, 17, 19–21, 26). Individual amino acid deficiencies have distinctive regulatory effects (Fig. 1–3). The degree of stimulation of intracellular proteolysis by each of the other amino acid deficiencies correlates with the degree of inhibition of uracil incorporation. If phenylalanine deficiency is taken as the norm, histidine, tyrosine, and tryptophan deficiencies regulate both events less efficiently (Fig. 1, 2). Arginine deficiency is about as effective (Fig. 1) whereas deficiencies of proline, threonine, or serine plus glycine are more effective (Fig. 3). It can be reasonably concluded that both events are controlled by the same regulatory system and that intracellular proteolysis is an additional metabolic process under the regimen of the *rel* locus.

The effectiveness of individual amino acids as regulators can be ranked approximately with their abundance in the cellular protein (Table 1). Tryptophan, histidine, and tyrosine, which are poorer regulators than phenylalanine, are less abundant. Proline, threonine (plus isoleucine), and serine plus glycine are better regulators than phenylalanine and are correspond-

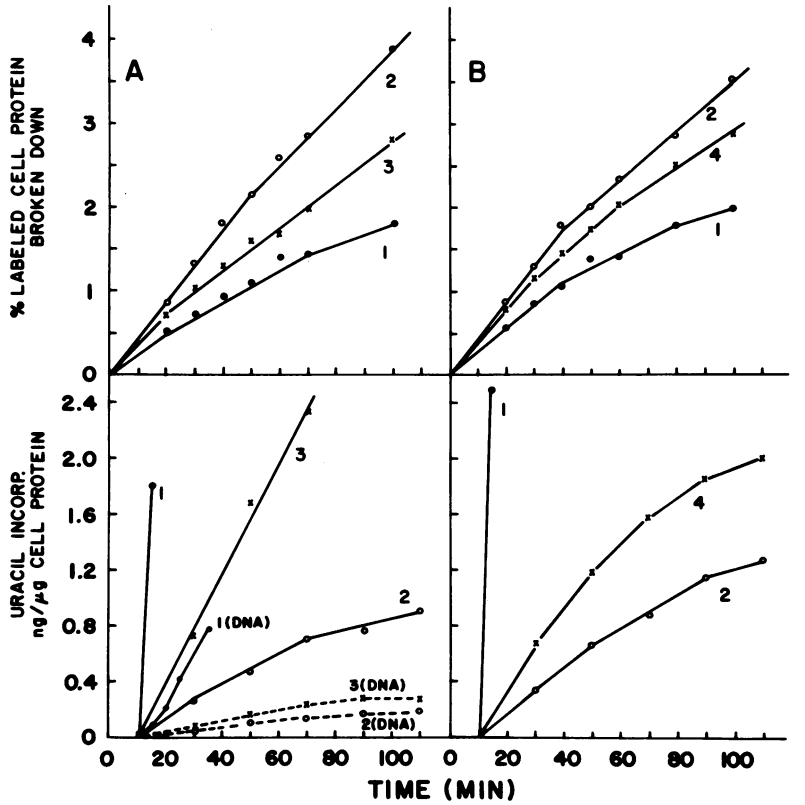

FIG. 2. *Effect of amino acid starvation on intracellular proteolysis and uracil-2-^{14}C incorporation into nucleic acids of tyr$^-$ (A) and try$^-$(B) mutants of E. coli 9723 phe$^-$. 1, Complete growth medium; 2, phenylalanine absent; 3, tyrosine absent; 4, tryptophan absent. Incorporation into DNA is separately indicated in the phe$^-$ tyr$^-$ mutant.*

ingly more abundant. Arginine, somewhat less consistently, is about equal to phenylalanine as a regulator and is more abundant. A similar comparison has been made with a larger number of amino acids as regulators of intracellular proteolysis (21). Ron (22) has shown a consistent correlation between the abundance of six amino acids in cell proteins and the extent to which their deficiency lowers the normal polysomal distribution of the ribosomes. Ribosomes were considered to dissociate prematurely from the complex according to the frequency with which missing amino acids arrest peptide chain growth. Loss of input to the amino acid-accepting site of the ribosome would then lead to a proportionate accumulation of dissociated ribosomes. Both of the dissociated 30*S* and 50*S* components are part of a complex that synthesizes guanosine tetraphosphate (ppGpp) under conditions which are consistent with its possible function as a general regulator of stringent control (2, 7, 13, 18, 26). The connection between cellular abundance of the amino acids and their regulatory capacities would then be apparent. In intact cells, a parallel can be made between the level of ppGpp and the rate of intracellular proteolysis. Both are inhibited by inhibitors of peptide translocation (8, 16, 21) and stimulated by inhibiting initiation of protein biosynthesis (8, 16) or RNA synthesis (16, 21) or by limiting the energy supply in normal and *rel*$^-$ cells (15, 21, 25). Deprivation of different amino acids in *E. coli* B and K-12 produce ppGpp in similar, but not identical, amounts (4). Deficiency of histidine, the rarest amino acid examined, produces the least ppGpp (4).

Sussman and Gilvarg (24) studied the regulation of proteolysis in *rel*$^-$ cells by following the effects of a multiple nutritional deficiency. It is not clear which of the nutritional components was actually limiting and whether this limitation held in all conditions. Also, the proteolytic rate of growing cells, the base line in all present comparisons, was not measured. In other studies, single amino acid deprivations sometimes

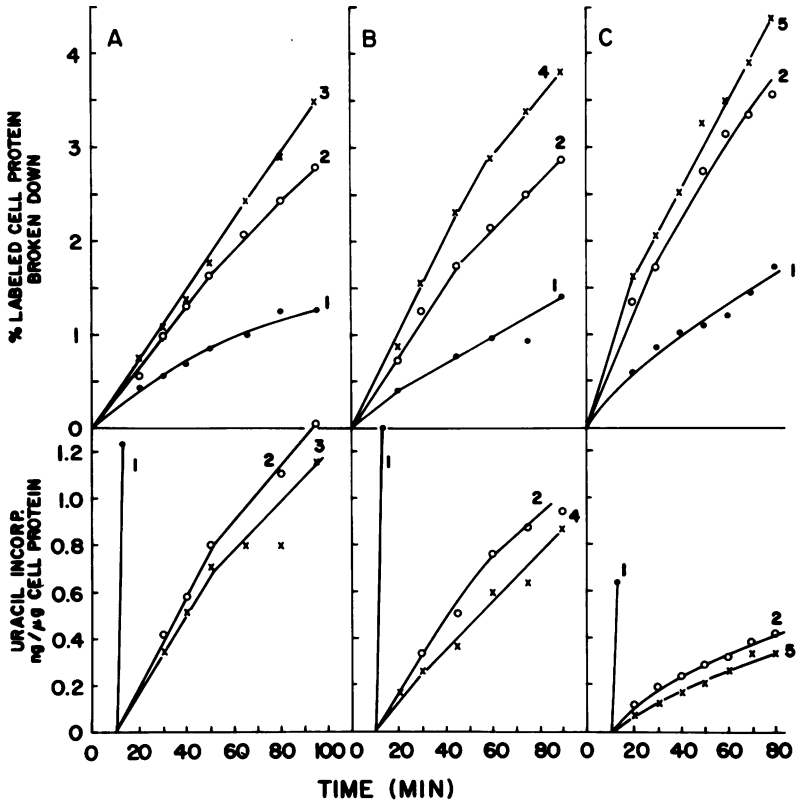

FIG. 3. *Effect of amino acid starvation on intracellular proteolysis and uracil-6-^{3}H incorporation into nucleic acids of pro$^-$ (A), thr$^-$(B), and ser$^-$-gly$^-$ (C) mutants of E. coli 9723 phe$^-$. 1, All amino acids present; 2, phenylalanine absent; 3, proline absent; 4, threonine absent; 5, serine and glycine absent. ^{3}H and ^{14}C activities were measured simultaneously.*

TABLE 1. *Amino acid composition of cell proteins of E. coli 9723f*

Amino acid	Moles (%)
Glu + Gln	11.38
Asp + Asn	11.02
Ala	9.70
Gly	8.12
Leu	7.53
Val	7.00
Lys	6.33
Thr	5.91
Arg	5.47
Ser	5.25
Ile	4.95
Pro	4.49
Phe	3.59
Tyr	2.91
Met	2.67
His	1.91
Try	0.93[a]
1/2 Cys	0.84

[a] Taken from the composition of soluble protein of *E. coli* B (24).

do and sometimes do not affect intracellular proteolysis in *rel*$^-$ mutants (8, unpublished data). Perhaps in some *rel*$^-$ mutants, the loss of translational fidelity (10) and the inability to regulate many biosynthetic reactions give rise to other effects not directly pertinent to stringent control. The present experiments may be less drastic; they provide more supplementary information in following the gradations in normal response to amino acid deficiencies, and they provide a clearer alternative to deciding how deficient the proteolytic response of a relaxed mutant should be. The relationship between the regulatory effectiveness of an amino acid and its abundance in cellular protein is only approximate. The regulatory responses measured may not be simple or linear. There is no assurance, moreover, that all amino acids disappear from transfer ribonucleic acid (tRNA) or are replenished by protein breakdown and conserved identically when their de novo appearance is stopped. The accumulation of abnormal levels of intermediates during indi-

vidual amino acid deficiencies might cause unanticipated changes in metabolites that can further modify intracellular proteolysis. It is unlikely, however, that these would be underlying reasons for the large number of amino acid differences noted here and previously (21, 22). Moreover, the regulatory differences among the amino acids are consistent in virtually all of many mutations of effective and ineffective amino acids that have been examined (17, 21).

ACKNOWLEDGMENT

I am greatly indebted to Allan Grossberg, Department of Biochemistry, Roswell Park Memorial Institute, Buffalo, N.Y., for the amino acid analyses.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli*. Biochem. Biophys. Res. Commun. **18**:788-795.
2. Blumenthal, T., T. A. Landers, and K. Weber. 1972. Bacteriophage Qβ replicase contains the protein biosynthesis elongation factors EFTu and EFTs. Proc. Nat. Acad. Sci. U.S.A. **69**:1313-1317.
3. Brunschede, H., and H. Bremer. 1971. Synthesis and breakdown of proteins in *Escherichia coli* during amino acid starvation. J. Mol. Biol. **57**:35-57.
4. Cashel, M. 1969. The control of ribonucleic acid synthesis in *Escherichia coli*. IV. Relevance of unusual phosphorylated compounds from amino acid-starved stringent cells. J. Biol. Chem. **244**:3133-3141.
5. Edlin, G., and P. Broda. 1968. Physiology and genetics of the "ribonucleic acid control" locus in *Escherichia coli*. Bacteriol. Rev. **32**:206-226.
6. Edlin, G., and J. Neuhard. 1967. Regulation of nucleoside triphosphate pools in *Escherichia coli*. J. Mol. Biol. **24**:225-230.
7. Gallant, J., J. Irr, and M. Cashel. 1971. The mechanism of amino acid control of guanylate and adenylate biosynthesis. J. Biol. Chem. **246**:5812-5816.
8. Goldberg, A. L. 1971. A role of amino acyl-tRNA in the regulation of protein breakdown in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **68**:362-366.
9. Gorini, L., and H. Kaufman. 1960. Selecting bacterial mutants by the penicillin method. Science **131**:604-605.
10. Hall, B., and J. Gallant. 1972. Defective translation in RC^- cells. Nature N. Biol. **237**:131-135.
11. Hamilton, P. B. 1963. Ion exchange chromatography of amino acids. Anal. Chem. **35**:2055-2064.
12. Hanawalt, P. C., O. Maaløe, D. J. Cummings, and M. Schaechter. 1961. The normal DNA replication cycle. J. Mol. Biol. **3**:156-165.
13. Haseltine, W. A., R. Block, W. Gilbert, and K. Weber. 1972. MS I and MS II made on ribosome in idling step of protein synthesis. Nature N. Biol. **238**:381-384.
14. Hirs, C. H. W. 1967. Performic acid oxidation. p. 197. *In* C. H. W. Hirs (ed.), Methods in enzymology, vol. 11, Academic Press Inc., New York.
15. Lazzarini, R. A., and M. Cashel. 1971. On the regulation of guanosine tetraphosphate levels in stringent and relaxed strains of *Escherichia coli*. J. Biol. Chem. **246**:4381-4385.
16. Lund, E., and N. O. Kjeldgaard. 1972. Metabolism of guanosine tetraphosphate in *Escherichia coli*. Eur. J. Biochem. **28**:316-326.
17. Nath, K., and A. L. Koch. 1971. Protein degradation in *Escherichia coli*. I. Measurement of rapidly and slowly decaying components. J. Biol. Chem. **246**:6956-6967.
18. Pederson, F. S., E. Lund, and N. O. Kjeldgaard. 1973. Codon specific, tRNA-dependent *in vitro* synthesis of ppGpp and pppGpp. Nature N. Biol. **243**:13-15.
19. Pine, M. J. 1965. Heterogeneity of protein turnover in microorganisms. Biochim. Biophys. Acta **104**:439-456.
20. Pine, M. J. 1970. Steady-state measurement of the turnover of amino acids in the cellular proteins of growing *Escherichia coli*: existence of two kinetically distinct reactions. J. Bacteriol. **103**:207-215.
21. Pine, M. J. 1973. Regulation of intracellular proteolysis in *Escherichia coli*. J. Bacteriol. **115**:107-116.
22. Ron, E. Z. 1971. Polysome turnover during amino acid starvation in *E. coli*. J. Bacteriol. **108**:263-268.
23. Spahr, F. 1962. Amino acid composition of ribosomes from *Escherichia coli*. J. Mol. Biol. **4**:395-406.
24. Sussman, A. J., and C. Gilvarg. 1969. Protein turnover in amino acid-starved strains of *Escherichia coli* K-12 differing in their ribonucleic acid control. J. Biol. Chem. **244**:6304-6306.
25. Travers, A., R. Kamen, and M. Cashel. 1970. The *in vitro* synthesis of ribosomal RNA. Cold Spring Harbor Symp. Quant. Biol. **35**:415-418.
26. Willetts, N. S. 1967. Intracellular protein breakdown in growing cells in *Escherichia coli*. Biochem. J. **103**:462-466.