ROLES OF PROTEIN SYNTHESIS AND tRNA AMINOACYLATION IN THE REGULATION OF INTRACELLULAR PROTEIN BREAKDOWN IN *E. coli*

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SUMMARY

The previously suggested roles of protein synthesis and tRNA aminoacylation in the regulation of intracellular protein breakdown were examined in strains of *E. coli* temperature-sensitive for aminoacyl-tRNA synthetases. Direct measurements of tRNA aminoacylation show no correlation between the degree of tRNA charging and the rate of protein breakdown. Protein breakdown was accelerated by transfer from 30°C to 42°C to about the same degree in temperature-sensitive mutants as in related normal strains. Deprivation of inorganic phosphate at the high temperature stimulated further protein breakdown in normal, but not in temperature-sensitive strains. It is concluded that the regulation of protein breakdown requires concomitant protein synthesis and is not influenced by the level of aminoacylation of tRNA.

INTRODUCTION

The rate of degradation of intracellular proteins is markedly increased when bacteria are deprived of certain essential nutrients, such as a nitrogen or carbon source (1,2) or inorganic phosphate (3). Several investigators have found that the stimulation of protein breakdown elicited by any of these nutritional deprivations is blocked by inhibitors of protein synthesis (4 - 6).

This action of protein synthesis inhibitors could be interpreted in several ways. One possible explanation is that protein synthesis is directly required for the stimulation of protein breakdown. Alternatively, protein breakdown may be controlled by the levels of precursors of protein synthesis (such as aminoacyl-tRNA). In the latter case, the effect of inhibitors of protein synthesis on protein breakdown would be secondary, due to the accumulation of these precursors.

We have previously investigated the mode of regulation of protein breakdown in stringent (*rel*$^+$) and relaxed (*rel*$^-$) strains of *E. coli* (6). Several lines of experimental evidence have indicated that the control of protein breakdown requires concomitant protein synthesis, and that the inability of aminoacid-starved *rel*$^-$ strains to enhance proteolysis is due to defective protein synthesis (6). In contrast,

Goldberg (7) has proposed that protein breakdown in *E. coli* is controlled by the levels of aminoacyl-tRNA. This suggestion was based mainly on the finding that in mutants temperature-sensitive for valyl-tRNA synthetase, protein breakdown was greatly increased upon transfer to the high temperature. It was also concluded from these results that protein breakdown can be regulated in the absence of protein synthesis. However, the effect of high temperature on protein breakdown in normal related strains was not shown in this report (7).

In view of these contradicting conclusions, we have reinvestigated the mode of regulation of protein breakdown in strains containing temperature-sensitive aminoacyl-tRNA synthetases. Our experiments lead to the conclusion that protein breakdown is not influenced by the level of aminoacyl-tRNA, and provide further evidence supporting the notion that protein synthesis is directly required for the regulation of protein breakdown.

MATERIALS AND METHODS

Strain D2 (*thy*$^-$, *rel*$^+$) of *E. coli* K12 and its derivative 10B6 which contains a thermolabile valyl-tRNA synthetase (*thy*$^-$, *valS*, *rel*$^+$) (8) were kindly provided by Dr. S. Kaplan. Strain 5F2-*rel*$^+$ (*argA*$^-$, *gluS*, *rel*$^+$) which is temperature-sensitive for glutamyl-tRNA synthetase and its relaxed derivative 5F2-*rel*$^-$ (*argA*$^-$, *gluS*, *rel*$^-$) were obtained from Dr. A.G. Atherly. This pair of strains is also derived from D2 strain of *E. coli* (9). *E. coli* K12 CP78 (*arg*$^-$, *his*$^-$, *leu*$^-$, *thi*$^-$, *rel*$^+$) was kindly provided by Dr. J. Gallant. Bacteria were grown in one of the following media: "Medium A" - a Tris-glucose minimal medium (3), supplemented, when required, with 25 µg/ml thymidine, 5 µg/ml thiamine and 50 µg/ml of each required amino acid; "Medium B" - the same minimal medium enriched with 0.05% Casamino acids (Difco); "Medium C" - the minimal medium enriched by replacing one-third of its volume with brain-heart infusion broth (Difco). The temperature-sensitive strains were grown at 30°C, and other strains at 37°C. The growth of all temperature-sensitive strains was completely inhibited at 42°C, and the incorporation of [^{3}H]-leucine into proteins at 42°C was less than 1.5% of the incorporation at 30°C.

Protein breakdown was estimated by the release of trichloroacetic acid-soluble radioactivity from previously labeled cellular proteins, as described earlier (3,6). Briefly, the bacteria were grown for at least three generations in the presence of L-[4,5-^{3}]-leucine and were then harvested, washed and resuspended in media containing an excess of unlabeled L-leucine. The suspensions were incubated under conditions as indicated in the Figures and at timed intervals, aliquots were withdrawn, treated with trichloroacetic acid and repeatedly centrifuged. The released trichloroacetic acid-soluble radioactivities are expressed as the percentage of the radioactivity initially contained in cellular proteins (6).

The level of charged valyl-tRNA was determined by a modification of the method of Lewis and Ames (10). This method involves the use of warm trichloroacetic acid for stopping the incubation, phenol extraction of RNA and measurement of amino acid acceptor activity of periodate- and mock-treated samples of tRNA "stripped" of amino acids. The following modifications were included: Samples of 50 ml bacterial culture with a density of 70-100 units in a Klett-Summerson colorimeter (540 mm filter) were used. The ethanol precipitate was dissolved in 0.5 ml of 0.1M sodium acetate buffer (pH 4.6). The mock and periodate treatments of tRNA were carried out on samples of 150 µl. The concentration of periodate was 20 mM, and the reaction was terminated with a 40-fold excess of ethylene glycol. Assay of amino acid acceptor activity of $tRNA_{val}$ was carried out according to the procedure of Muench and Berg (11), with some modifications. The reaction mixture contained in a final volume of 50 µl the following ingredients: sodium cacodylate buffer (pH 6.9), ATP, $MgCl_2$, KCl, reduced glutathione and bovine serum albumin at concentrations as in the original method, 0.1 nmole of [2,3-^{3}H]-valine (20 Ci/mmole), 0.6 - 2.0 $A_{260_{nm}}$ units of RNA and 14 µg of aminoacyl-tRNA synthetase preparation. The enzyme was prepared from *E. coli* B/r (kindly provided by Dr. R. Ben-Ishai), according to the method of Muench and Berg (11), until the DEAE-cellulose column purification step. It was ascertained that the enzyme preparation was devoid of tRNA and of any ribonuclease activity (11). The results were calculated by dividing the acid-insoluble radioactivity by the adsorbance at 260 nm, and the ratio of acceptor capacity of periodate to mock-treated sample yielded the percentage of charged valyl-tRNA. Accuracy of duplicate samples was within the range of 10% maximal deviation.

RESULTS AND DISCUSSION

Mutants containing temperature-sensitive aminoacyl-tRNA synthetases can be utilized to determine the roles of tRNA aminoacylation or protein synthesis in the regulation of intracellular protein degradation, since in such mutants both tRNA charging and protein synthesis are severely inhibited at the restrictive temperature. If protein breakdown is stimulated by a decrease in aminoacyl-tRNA levels, it is expected that the rate of this process will be greatly enhanced at the restrictive temperature. On the other hand, if concomitant protein synthesis is required for enhancement of protein breakdown, the stimulation will be prevented at the non-permissive temperature. However, when using temperature-sensitive mutants, it is essential to distinguish between the effects of high temperature *per se* and the influence of the temperature-sensitive specific mutation. We had to differentiate, therefore, between the increase in the rate of "basal" protein breakdown due to elevated temperature, and "deprivation-enhanced" protein degradation which might

be elicited by decreased aminoacyl-tRNA levels. ("Basal" protein breakdown is defined as that occuring in normally growing cells or in the presence of chloramphenicol, while "deprivation-enhanced" protein degradation is the regulatory increment over the basal level in nutritionally deprived bacteria). In the experiment shown in Fig. 1, the rates of protein breakdown were measured in a pair of strains which both contain a temperature-sensitive glutamyl-tRNA synthetase, but which differ in their *rel* locus (5F2 rel^+ - 5F2 rel^-). It has been shown previously that amino acid deprivation accelerates protein breakdown only in "stringent" (rel^+) but not in "relaxed" (rel^-) strains of *E. coli* (5,6). In fact, at the permissive temperature (30°C), the deprivation of nitrogen source stimulated protein breakdown in the stringent, but not in the relaxed strain. On the other hand, at the restrictive temperature (42°C), protein breakdown was accelerated to a similar rate in both rel^+ and rel^- strains (Fig. 1).

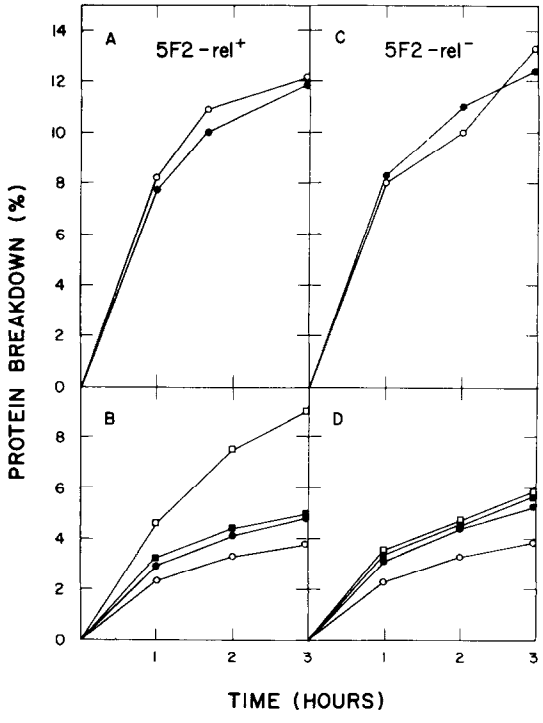

Fig. 1: Protein breakdown in stringent and relaxed strains of *E. coli* containing a temperature-sensitive glutamyl-tRNA synthetase. Bacteria grown at 30°C in "Medium B" were labeled with [^{3}H]-leucine (1 µCi/ml). Protein breakdown was measured as described in "Materials and Methods". A and B, strain 5F2 rel^+; C and D, strain 5F2 rel^-; A and C, incubated at 42°C; B and D, incubated at 30°C; ○—○, complete growth medium "B"; □—□, nitrogen source omitted (omission of ammonium salts and amino acids except L-leucine); ●—●, ■—■, same as above with the addition of chloramphenicol (100 µg/ml).

These results do not appear to be compatible with the hypothesis that protein breakdown is controlled by the levels of aminoacyl-tRNA (7). *Rel*$^-$ cells do not respond to experimental conditions which decrease tRNA aminoacylation. Therefore, the acceleration of protein breakdown at the high temperature in this strain is merely due to the influence of increased temperature on the rate of "basal" protein breakdown. Since there is no further stimulation of protein breakdown in *rel*$^+$ strains at the restrictive temperature, it can be concluded that under these conditions protein breakdown is not influenced by the level of aminoacyl-tRNA.

The data in Fig. 1 also show that chloramphenicol has no influence on the rate of protein breakdown in the temperature-sensitive strain at the nonpermissive temperature. Goldberg (7) has interpreted similar data as indicating that the inhibitor cannot affect protein breakdown under conditions which do not permit the accumulation of aminoacyl-tRNA. We have tested this suggestion in the experiment shown in Fig. 2. In this experiment, bacteria temperature-sensitive for valyl-tRNA synthetase (strain 10B6) were first incubated at 30°C in the presence of chloramphenicol in order to accumulate charged tRNA. The cells were then shifted to 42°C, and rates of protein degradation and the degree of

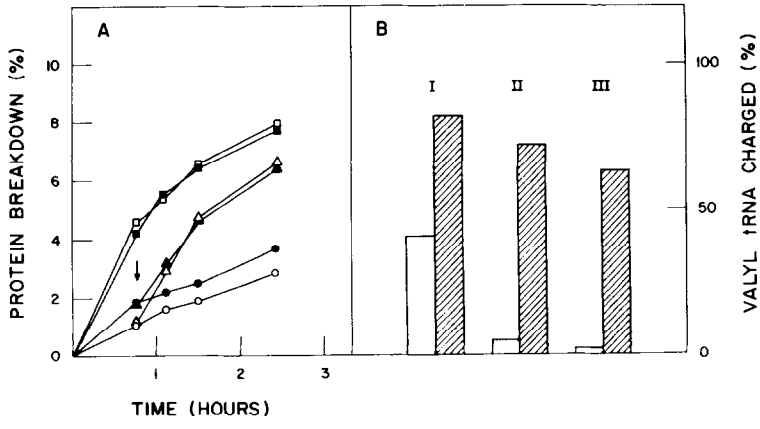

Fig. 2: Comparison of protein degradation and tRNA aminoacylation in a strain containing a thermolabile valyl-tRNA synthetase. A. Protein degradation. Strain 10B6 was grown at 30°C in "Medium C" and labeled with [^{3}H]-leucine (5 µCi/ml). The cells were washed and resuspended in the same medium and protein breakdown was measured as described in "Materials and Methods". ○—○, 30°C; □—□, 42°C; △—△, incubated at 30°C for 45 minutes and then transferred to 42°C; filled symbols, same as above, but chloramphenicol (100 µg/ml) added at the start of the incubation. B. Aminoacylation of valyl-tRNA. Experimental conditions were identical to those described in A, except that radioactive leucine was not added. tRNA charging was determined as detailed in "Materials and Methods". Empty bars - control without chloramphenicol; striped bars - in the presence of chloramphenicol (100 µg/ml). I - incubated at 30°C for 45 minutes; II - as I, and then transferred to 42°C for additional 45 minutes; III - as I and then transferred to 42°C for 90 minutes.

aminoacylation of valyl-tRNA were measured in parallel. As may be seen (Fig. 2B), charged valyl-tRNA which accumulated at 30°C in the presence of chloramphenicol, remained aminoacylated to a large extent during the subsequent incubation at 42°C. In the control culture, which was not preincubated with chloramphenicol, the charging of valyl-tRNA dropped sharply upon transfer to the nonpermissive temperature. If the influence of chloramphenicol were *via* the accumulation of aminoacyl-tRNA, it would be expected that it should slow down protein degradation under these conditions. However, the rates of protein breakdown following the temperature shift-up were similar both in the presence and absence of charged tRNA (Fig. 2A). In similar experiments with strain 5F2 *rel*$^+$, essentially identical results were obtained with regard to protein breakdown (not shown); tRNA charging was not determined in this strain.

The above experiments can be fully explained by the assumption that the regulation of protein breakdown requires concomitant protein synthesis. According to this suggestion, protein degradation cannot be regulated at the nonpermissive temperature in strains containing temperature-sensitive aminoacyl-tRNA synthetases, since protein synthesis is severely inhibited. Thus, the acceleration of protein breakdown at 42°C reflects only the influence of high temperature on the rate of "basal" proteolysis (which does not require protein synthesis), and not

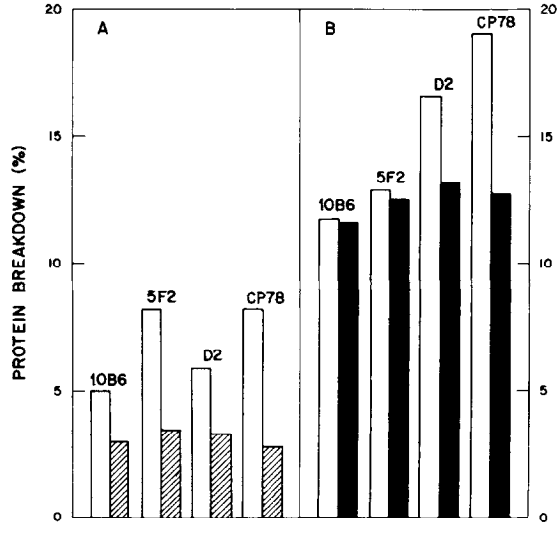

Fig. 3: Protein breakdown in temperature-sensitive and normal strains of *E. coli*: effects of temperature elevation and phosphate deprivation. Bacteria were grown in "Medium A", in the presence of [^{3}H]-leucine (1 µCi and 25 µg per ml medium). Protein breakdown was determined as described in "Materials and Methods", after 3 hours of incubation. A - 30°C; B - 42°C; striped bars - complete "Medium A"; empty bars - inorganic phosphate omitted; filled bars - inorganic phosphate omitted and chloramphenicol (100 µg/ml) added.

"deprivation-enhanced" regulation of protein breakdown (which requires concomitant protein synthesis). Accordingly, chloramphenicol has no influence on protein breakdown at the restrictive temperature since protein synthesis is already inhibited.

This interpretation was further supported by the comparison of the rates of protein degradation of temperature-sensitive mutants with that of normal related strains which do not contain temperature-sensitive enzymes (Fig. 3). The control strains used were D2, a K12 strain from which both 5F2 and 10B6 had been derived and strain CP78, which is also a K12 strain. Protein degradation was measured at the various temperatures in "basal" conditions (in the presence of chloramphenicol) and under the influence of phosphate deprivation. At 30°C, protein breakdown was stimulated by the deprivation of inorganic phosphate in all strains (Fig. 3A). At 42°C, on the other hand, protein degradation was accelerated by phosphate deprivation only in normal, but not in the temperature-sensitive strains. In the presence of chloramphenicol and at the high temperature, the rate of protein breakdown was essentially similar in temperature-sensitive and in normal strains (Fig. 3B). These findings indicate again that it is the "basal" rate of protein degradation which is accelerated by temperature elevation. Most importantly, the lack of stimulation of protein degradation by the deprivation of phosphate in temperature-sensitive strains suggests that the regulatory response is blocked at the restrictive temperature, presumably due to the inhibition of protein synthesis.

The results of this investigation, in conjunction with previous experiments (6), strongly indicate therefore, that the regulatory enhancement of protein breakdown requires concomitant protein synthesis. It remains to be seen whether this requirement represents the formation of a rapidly turning-over protein which may regulate intracellular protein breakdown (6,12).

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