JOURNAL OF BACTERIOLOGY, May 1984, p. 746–748
0021-9193/84/050746-03$02.00/0

Vol. 158, No. 2

Degradation of Aspartate Transcarbamylase in *Bacillus subtilis* Is Deficient in *rel* Mutants But Is Not Mediated by Guanosine Polyphosphates

RICHARD W. BOND AND ROBERT L. SWITZER*

Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

Received 17 November 1983/Accepted 16 February 1984

Degradation of aspartate transcarbamylase in growing and starved *Bacillus subtilis* was deficient in *relA* and *relC* mutants, but these effects were not correlated with differences in the intracellular level of guanosine polyphosphates.

Aspartate transcarbamylase (ATCase [aspartate carbamoyltransferase, E.C. 2.1.3.2]) is stable in exponentially growing *Bacillus subtilis* when the medium contains certain amino acids but is simultaneously synthesized and degraded when NH_4^+ is the sole nitrogen source (1). The enzyme is also degraded when the cells are starved for glucose or for an amino acid (6). The stability of the enzyme in growing cells is a complex function of the particular amino acids present in the medium (1). Ribosomes are capable of sensing starvation for individual amino acids or for a carbon source and respond to such limitations via the stringent response (2, 3). Evidence has been presented that the stringent response, specifically the intracellular level of the nucleotide 3′-pyrophosphorylguanosine-5′-pyrophosphate (ppGpp), regulates the rate of bulk protein degradation in starving *Escherichia coli* (12, 15). Ruppen and Switzer (8, 9) have also presented evidence for involvement of the stringent response in regulating intracellular degradation of glutamine phosphoribosylpyrophosphate amidotransferase in *B. subtilis*. Accordingly, we have examined the rate of ATCase degradation under several nutritional conditions in Rel^+ and Rel^- strains of *B. subtilis*. We have also determined the intracellular levels of ppGpp and 3′-pyrophosphorylguanosine-5′-triphosphate (pppGpp) under the same conditions. Degradation of ATCase was deficient in *relA* and *relC* mutants, but these effects were not correlated with changes in the cellular levels of ppGpp or pppGpp.

B. subtilis BR16 (*rel*$^+$ *trpC2* Lys^-) and BR17 (*relA trpC2* Lys^-) from Swanton and Edlin (13) and strain IS169 (*relC thr-5 trpC2*) from Smith et al. (10, 11) were grown as previously described (1). The stability of ATCase during exponential growth with 15 mM $(NH_4)_2SO_4$ as the sole nitrogen source was determined after uridine addition by the method of Bond et al. (1). In glucose starvation experiments, the medium initially contained 20 amino acids (1) at 100 μg each per ml and glucose at 0.07% (wt/vol). For amino acid starvation, the bacteria were grown to the late exponential phase on a medium similar to that used in glucose starvation experiments but containing 0.15% (wt/vol) glucose. The cells were then collected by centrifugation at 37°C (8) and suspended in warmed (37°C) medium that lacked the required amino acids (tryptophan and lysine for BR16 and BR17; tryptophan and threonine for IS169). ATCase activity was assayed (1) in samples of cells harvested before and during starvation for glucose or for amino acids.

The stability of ATCase in the wild-type and *rel* mutants under various conditions is shown in Tables 1 and 2. The enzyme was degraded as previously observed (1, 6) in the *rel*$^+$ strain. In the *relA* strain, ATCase degradation was detectable but significantly slower than in *rel*$^+$ strains during growth on NH_4^+ and during amino acid starvation. When *relA* cells were starved for glucose, the degradation of ATCase was consistently faster than observed in the isogenic *rel*$^+$ strain during glucose starvation. We have confirmed by specific immunoprecipitation methods (1, 6) that cross-reactive protein disappeared as rapidly as did enzyme activity in the glucose-starved *relA* strain. ATCase degradation was extremely slow or undetectable in the *relC* strain under all conditions examined. These results establish that the protein factors which mediate the stringent response, particularly the one encoded by *relC*, play an important role in regulating degradation of ATCase in vivo.

The possibility must be considered that the apparent differences in rates of degradation of ATCase actually resulted entirely or in part from differences in the rate of synthesis of the enzyme in the strains studied. This possibility can be excluded for the studies presented in Table 1, because the method of analysis involved measuring the loss of enzyme activity after the addition of sufficient uridine to repress ATCase synthesis fully (1). Similarly, the amount of pseudomonic acid added in the experiments presented in Table 2 completely blocked protein synthesis. In the case of glucose-starved cells, Maurizi and Switzer (7) have shown that ATCase synthesis is abruptly and completely shut off before degradation of the enzyme in Rel^+ cells. We confirmed that the same was true for strains BR16, BR17, and IS169 during glucose starvation under the conditions of Table 2 by use of pulse-labeling with [^{3}H]leucine and immunoprecipitation (7). For all three strains, the rate of synthesis was reduced to 6 to 8% of the rate in growing cells within the first 30 min of glucose starvation, and synthesis was barely detectable thereafter. When the same strains were starved for amino acids under the conditions of Table 2, ATCase synthesis was also shown to be abruptly shut off by use of the same methods. For strains BR16 and BR17, synthesis during the first 30 min of amino acid starvation was only 2 to 4% of the rate in exponentially growing cells. The decline in synthesis was slightly slower in amino acid-starved strain IS169, but synthesis had declined to 18% of the rate in exponentially growing cells within 30 min of starvation and to 5% within 60

* Corresponding author.

TABLE 1. Rates of ATCase degradation and levels of ppGpp and pppGpp in exponentially growing *B. subtilis rel*$^+$ and *rel* mutant cells[a]

Strain	Rate and level with nitrogen source:					
	20 amino acids			NH_4Cl		
	Half-life of ATCase (h)	ppGpp	pppGpp	Half-life of ATCase (h)	ppGpp	pppGpp
BR16 (*rel*$^+$)	≥10	14	11	2.4	40	40
BR17 (*relA*)	≥10	≤3	≤3	7	11	7
IS169 (*relC*)	—	≤3	≤3	9	≤3	6

[a] Nucleotide levels are the steady-state values (as measured in picomoles per absorbance unit at 660 nm) of the culture at the time of sampling. —, Not determined.

min of starvation. Taken together, these results indicate that ATCase synthesis did not contribute significantly to the differences in rates of disappearance of the enzyme in Rel$^+$ and Rel$^-$ cells.

The *relA*-encoded protein catalyzes the production of ppGpp and pppGpp when a normal *relC*-encoded protein (ribosomal protein L11) is present (10, 11). Do these polyphosphates mediate the effects of the *relA* and *relC* genes on ATCase degradation? We attempted to answer this question by determining the intracellular levels of these nucleotides in each of the strains under each of the culture conditions listed in Tables 1 and 2. The nucleotides were measured by labeling the cultures with $^{32}P_i$, extraction with 3 M formic acid, and two-dimensional thin-layer chromatography (9). Greater sensitivity was obtained by increasing the amount of formic acid extract analyzed to six times that used by Ruppen and Switzer (9). During exponential growth, the levels of ppGpp and pppGpp in *B. subtilis* were quite low; they were lower in cells grown with amino acids than in cells grown with NH_4Cl as the nitrogen source (Table 1). The level of these polyphosphates was extremely low in the *rel* mutants. Thus, there was a general correlation between very low ppGpp and pppGpp levels and retarded degradation of ATCase. Analysis of specific cases demonstrated that the two parameters were not tightly linked, however. There was a three- to fourfold difference in the levels of ppGpp and pppGpp in exponentially growing cells when they were provided with 20 amino acids as opposed to being provided with NH_4^+ (Table 1). In contrast, the level of ppGpp plus pppGpp in amino acid-starved BR16 (*rel*$^+$) was more than twice as high as in cells growing exponentially on NH_4^+, whereas the rate of ATCase degradation was essentially the same. Likewise, there was a greater-than-fivefold difference in the levels of ppGpp and pppGpp when *rel*$^+$ and *relA* strains were compared during amino acid starvation, whereas the difference in rates of ATCase degradation was 2.5-fold (Table 2). These data suggest that there is no linear or simple relationship between absolute levels of ppGpp and pppGpp and the rate of ATCase degradation. A dramatic demonstration that high levels of ppGpp and pppGpp are not sufficient to induce rapid degradation of ATCase was provided when amino acid starvation was induced with the isoleucyl-tRNA synthetase inhibitor, pseudomonic acid (5). When BR16 cells were treated with pseudomonic acid, very high levels of ppGpp and pppGpp accumulated (Table 2); yet the half-life of ATCase was 5 h, slower than that observed during amino acid starvation (10-fold-lower levels of guanosine polyphosphates).

The levels of ppGpp and pppGpp were examined in glucose-starved *rel* mutant cells (Table 2). ATCase degradation was quite rapid in glucose-starved *relA* cells but was undetectable in glucose-starved *relC* cells. Guanosine polyphosphates were undetectable during growth or during glucose starvation in both *rel* mutant strains. Thus, in glucose-starved *rel* mutants there was no correlation between ppGpp and pppGpp levels (always very low) and the rate of ATCase degradation (very fast or very slow).

Although ATCase degradation was altered in *rel* mutants, the present results exclude a mechanism in which degradation is initiated or continuously activated directly by ppGpp or pppGpp. How might degradation of this enzyme be mediated by the *relC*-encoded protein (and be affected by a *relA* mutation) and yet operate independently of ppGpp or pppGpp accumulation? We suggest that the sensing elements of the stringent response, i.e., ribosomes bearing mRNA and uncharged tRNA, act via a conformational change in the ribosomal protein L11 (the *relC* protein). A conformational change in L11 could activate the stringent factor (*relA*-encoded protein) to synthesize ppGpp and pppGpp and also activate unidentified elements of a proteolytic system. Thus, degradation and guanosine polyphosphate synthesis are activated coincidentally in many situations. It is not yet clear how glucose starvation stimulates ppGpp and pppGpp accumulation or protein degradation. The difference in ATCase stability in the *relA* strain when starvation was for amino acids or for glucose suggests a difference in mechanism. The

TABLE 2. Rates of ATCase degradation and maximal levels of ppGpp and pppGpp in amino acid- and glucose-starved *B. subtilis rel*$^+$ and *rel* mutant cells[a]

Strain	Rate and level under the following conditions:								
	Amino acid starvation			Pseudomonic acid addition[b]			Glucose starvation		
	Half-life of ATCase (h)	Maximal ppGpp[c]	Maximal pppGpp[c]	Half-life of ATCase (h)	Maximal ppGpp	Maximal pppGpp	Half-life of ATCase (h)	Maximal ppGpp	Maximal pppGpp
BR16 (*rel*$^+$)	2	50	170	5	550	1400	1.4	20	15
BR17 (*relA*)	5	≤3	34	≥10	5	40	0.9	≤3	≤3
IS169 (*relC*)	9.5	5	13	≥10	10	10	≥10	≤3	≤3

[a] Nucleotide levels are the maximal values observed during starvation (as measured in picomoles per absorbance unit at 660 nm) of the culture at the time of sampling.

[b] Sodium pseudomonate was added at a final concentration of 1 μg/ml in the late exponential phase of growth. Isoleucine was omitted from the amino acid mixture in the medium in these cases.

[c] The values in this column were taken from reference 8.

results of the similar experiment with the *relC* strain demonstrates a role for *relC* whether ATCase degradation is initiated by amino acid starvation or glucose starvation. This observation provides circumstantial evidence against involvement of ribosome-independent ppGpp synthesis (4, 14) in degradation of ATCase. Finally, it should be noted that our findings do not accord well with the observation from the laboratory of A. L. Goldberg that the rate of bulk protein degradation in starving *E. coli* correlated well with intracellular ppGpp levels (15). This discrepancy may result from differences in mechanisms of regulation of degradation between bulk protein turnover in *E. coli* and degradation of a specific protein in *B. subtilis*. The elucidation of the mechanism by which the stringent response regulates protein degradation will require its reconstruction in vitro.

We acknowledge Simon Rosenzweig for providing purified ATCase, Stanley Bower for helpful criticism of the manuscript, and Beecham Pharmaceuticals, Betchworth, England, for a gift of pseudomonic acid.

This work was supported by Public Health Service grant AI11121 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Bond, R. W., A. S. Field, and R. L. Switzer.** 1983. Nutritional regulation of degradation of aspartate transcarbamylase and of bulk protein in exponentially growing *Bacillus subtilis* cells. J. Bacteriol. **153**:253–258.
2. **Cashel, M.** 1975. Regulation of bacterial ppGpp and pppGpp. Annu. Rev. Microbiol. **29**:301–318.
3. **Cashel, M., and J. Gallant.** 1974. Cellular regulation of guanosine tetraphosphate and guanosine pentaphosphate, p. 733–745. *In* M. Nomura, A. Tissieres, and P. Lengyl (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Fehr, S., and D. Richter.** 1981. Stringent response of *Bacillus stearothermophilus*: evidence for the existence of two distinct guanosine 3′,5′-polyphosphate synthetases. J. Bacteriol. **145**:68–73.
5. **Hughes, J., and G. Mellows.** 1978. Inhibition of isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. Biochem. J. **176**:305–318.
6. **Maurizi, M. R., J. S. Brabson, and R. L. Switzer.** 1978. Immunochemical studies of the inactivation of aspartate transcarbamylase by stationary phase *Bacillus subtilis* cells: evidence for selective, energy-dependent degradation. J. Biol. Chem. **253**:5585–5593.
7. **Maurizi, M. R., and R. L. Switzer.** 1978. Aspartate transcarbamylase synthesis ceases prior to inactivation of the enzyme in *Bacillus subtilis*. J. Bacteriol. **135**:943–951.
8. **Ruppen, M. E., and R. L. Switzer.** 1983. Degradation of *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase in vivo. J. Biol. Chem. **258**:2843–2851.
9. **Ruppen, M. E., and R. L. Switzer.** 1983. Involvement of the stringent response in degradation of glutamine phosphoribosylpyrophosphate amidotransferase in *Bacillus subtilis*. J. Bacteriol. **155**:56–63.
10. **Smith, I., P. Paress, K. Cabane, and E. Dubnau.** 1980. Genetics and physiology of the *rel* system of *Bacillus subtilis*. Mol. Gen. Genet. **178**:271–279.
11. **Smith, I., P. Paress, and S. Pestka.** 1978. Thiostrepton-resistant mutants exhibit relaxed synthesis of RNA. Proc. Natl. Acad. Sci. U.S.A. **75**:5993–5997.
12. **St. John, A. C., K. Conklin, E. Rosenthal, and A. L. Goldberg.** 1978. Further evidence for the involvement of charged tRNA and guanosine tetraphosphate in the control of protein degradation in *Escherichia coli*. J. Biol. Chem. **253**:3945–3951.
13. **Swanton, M., and G. Edlin.** 1972. Isolation and characterization of an RNA relaxed mutant of *B. subtilis*. Biochem. Biophys. Res. Commun. **46**:583–588.
14. **Sy, J.** 1979. Biosynthesis of guanosine tetraphosphate in *Bacillus brevis*, p. 95–106. *In* G. Koch and D. Richter (ed.), Regulation of macromolecular synthesis by low molecular weight mediators. Academic Press, Inc., New York.
15. **Voellmy, R., and A. L. Goldberg.** 1980. Guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and the regulation of protein breakdown in *Escherichia coli*. J. Biol. Chem. **255**:1008–1014.