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Oxygen-Dependent Inactivation of Glutamine Phosphoribosylpyrophosphate Amidotransferase in Stationary-Phase Cultures of *Bacillus subtilis*

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Glutamine phosphoribosylpyrophosphate amidotransferase (ATase) activity is rapidly inactivated in stationary-phase cells of *Bacillus subtilis*. The inactivation of ATase requires both the cessation of rapid cell growth and the presence of oxygen. ATase is inactivated in two protease-deficient mutant strains at a rate similar to that seen in the wild type, and is stable in anaerobic cell-free extracts of the parent strain. These results suggest that the inactivation of ATase is not the result of general proteolysis. The inactivation of ATase in stationary-phase cultures can be inhibited by oxygen starvation. This oxygen requirement does not reflect a dependence on the generation of metabolic energy, but appears to be a direct requirement for molecular oxygen. ATase activity is stable in exponentially growing cells in which further ATase synthesis is repressed by the addition of adenosine, and is inactivated only after the cessation of exponential growth. Addition of chloramphenicol or rifampin to exponential- and stationary-phase cells does not inhibit ATase inactivation, suggesting that protein or ribonucleic acid synthesis is not required for inactivation. ATase is inactivated at the end of exponential growth in cells that have exhausted a required amino acid.

Endospore formation in *Bacillus* cells requires a complex series of biochemical events, which occur in response to carbon or nitrogen starvation. One of these alterations is the loss of the capacity for de novo nucleotide synthesis. Nucleic acids that are synthesized during sporulation are apparently assembled from nucleotides that are formed from turnover of vegetative cell nucleic acids (5). Setlow and Kornberg have shown that free spores are incapable of incorporating aspartate into pyrimidine nucleotides or glycine into purine nucleotides (14). These findings indicate that enzymes of de novo nucleotide synthesis are lost during sporulation. Deutscher and Kornberg (4) reported in 1968 that aspartate transcarbamylase is inactivated in stationary-phase *Bacillus subtilis* cells. Waindle and Switzer (18) extended these observations and concluded that the loss of aspartate transcarbamylase was a specific inactivation that required, directly or indirectly, the generation of metabolic energy.

In this communication we wish to report that the first enzyme of de novo purine synthesis, glutamine phosphoribosylpyrophosphate amidotransferase (ATase, EC 2.4.2.14), is also rapidly inactivated in stationary-phase cells of *B. subtilis*. In this case the inactivation has a specific requirement for oxygen. Studies concerning the nature of this inactivation are presented. (This work was presented in part at the Biochemistry/Biophysics 1974 Meeting, 2-7 June 1974, Minneapolis, Minn.)

MATERIALS AND METHODS

Chemicals. Tetrasodium phosphoribosylpyrophosphate (PRPP), glutamic dehydrogenase (GDH), 3-acetylpyridine adenine dinucleotide, 2-heptyl-4-hydroxyquinoline-N-oxide, lysozyme, and bovine serum albumin were purchased from Sigma Chemical Co. PRPP was assayed before use as described below. GDH was provided as a solution in 50% glycerol containing sodium phosphate buffer, pH 7.3. One unit of GDH was that which would reduce 1.0 μmol of α-ketoglutarate to L-glutamate per min at pH 7.3 at 25 C. Sodium monofluoroacetate was purchased from Calbiochem. Diisopropylfluorophosphate (DFP) was purchased from Aldrich Chemical Co. Rifampin was a gift from Hans Heymann, Ciba Pharmaceutical Co. All other chemicals were reagent grade and commercially available.

Buffer A (4) contained 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride pH 7.9, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM ethylenediaminetetraacetic acid (EDTA).

Bacterial strains. All studies in this paper were conducted with *B. subtilis* strain 168, which requires tryptophan for growth, or derivatives of this strain.

Protease-negative mutant strains L-4 and S-87 were isolated by Hageman and Carlton (7) and were given to us by J. H. Hageman. Strain HS1A11 (aconitase-negative) was isolated and characterized by Carls and Hanson (3) and was provided by R. S. Hanson.

Media and culture methods. Three media were used in this study. The first was supplemented nutrient broth (SNB) containing 0.1% glucose used by Deutscher and Kornberg (4). The second was sporulation minimal medium (SMM) containing 0.1% glucose and 50 μg of tryptophan per ml described by Prestidge, Gage and Spizizen (12). The third was buffered minimal medium (BMM) containing 0.1% glucose used by Anagnostopoulos and Spizizen (1).

Strains 168, L-4, and S-87 were maintained on nutrient agar slants. For growth experiments, 5 ml of SNB containing 0.1% glucose was inoculated from a slant and allowed to grow overnight in a shaking incubator at 37 C. A 5-ml starter culture was transferred to 50 ml of defined growth medium and incubated 2 to 3 h. A 50-ml culture was then transferred to 1 liter of fresh defined medium in a 2-liter Bellco flask with closure and grown with shaking at 37 C. Growth of the culture was measured using a Klett-Summerson colorimeter with a no. 66 filter.

Strain HS1A11 was reisolated on purification plates prior to use as described by Carls and Hanson (3). Mutant colonies were picked from purification plates and streaked on blood agar base plates (3). After 12 h at 37 C, a colony on the blood agar base plate was used to inoculate a 5-ml starter culture. Subsequent inoculations were as described above. At the end of experiments using strain HS1A11, cultures were checked for revertants or contaminants on purification plates.

Preparation of cell extracts. In most experiments, 40- or 60-ml samples were withdrawn from a 1-liter culture at determined times during growth. The samples were immediately centrifuged for 15 min at 20,000 × *g* at 4 C. The pellets were washed with cold buffer A without 2-mercaptoethanol and centrifuged as above. Pellets were then frozen in dry ice-acetone and stored at −20 C for several days with only small loss of enzymatic activity. The pelleted cells were resuspended in 4 or 5 ml of cold buffer A and 40 μg of lysozyme per ml. The mixtures were incubated for 15 min at 37 C and placed on ice. The lysozyme-treated cells were further disrupted by sonic oscillation as described by Deutscher and Kornberg (4) in a Branson probe-type sonicator (model W185). Extracts were centrifuged at 17,000 × *g* for 30 min, and the supernatant fluid was used for enzyme and protein assays.

Enzyme assays: assay I. ATase was assayed by a modification of the method of Shiio and Ishii (15). The standard reaction mixture contained 0.53 mM PRPP, 6 mM L-glutamine, 3.3 mM $MgCl_2$, 50 mM Tris-hydrochloride (pH 7.5), and cell extract (50 to 150 μliters) in a final volume of 0.75 ml. After incubation at 37 C for 20 min the reaction was stopped by heating the mixtures at 100 C for 2 min. The amount of glutamate formed in 0.33 ml of the centrifuged supernatant was measured by adding 0.6 mM 3-acetylpyridine adenine dinucleotide, 50 mM KCl, 100 mM Tris-hydrochloride (pH 7.9), and 15 units of GDH in a final volume of 1.0 ml, incubating at 30 C for 60 min, and observing the increase in optical density at 363 nm. In the control assay, PRPP was omitted from the reaction mixture. ATase activity was the difference between complete and control assays. Assay I yielded a linear relationship between reaction time and glutamate formation. The relationship between volume of extract and glutamate formation was nearly linear, but was always less than expected when small samples were assayed (see Results). The ATase activity of cell extracts was estimated by either (i) measuring the slopes of plots of nanomoles of glutamate formed versus amount of extract assayed or (ii) by using large samples of extract (usually 150 μliters) where the dependence of specific activity on amount of enzyme was small. Assay I was used in all experiments except where noted.

Assay II. In some experiments assay I was modified by using 1.0 mM PRPP and 20 mM glutamine. The glutamate formed was measured by assaying a sample (100 to 300 μliters) of the reaction mixture with the GDH assay above. Use of assay II reduced, but did not eliminate, the nonlinearity between amount of extract and glutamate formation. Use of assay II facilitated enzyme activity determinations of concentrated cell extracts and yielded higher apparent enzyme activities. In both assays one unit of ATase activity was defined as 1 nmol of PRPP-dependent glutamate formed per min.

PRPP concentrations were measured by either the orotate removal assay of Kornberg et al. (8) or the adenine phosphoribosyltransferase assay of Bagnara et al. (2).

Protein was determined by the Lowry method (9) using crystalline bovine serum albumin as the standard.

RESULTS

Inactivation of ATase. Figure 1 illustrates the pattern of ATase activity during growth of *B. subtilis* strain 168 on BMM containing 0.1% glucose. The total enzymatic activity increased rapidly during exponential growth; inactivation began simultaneously with cessation of the exponential phase and appeared to be complete about 4 h after the end of exponential growth. The low enzymatic activity that was observed from 8 to 12 h may be due to new enzyme synthesis associated with slow growth on organic acids (mostly acetate, pyruvate and lactate), which accumulate during growth on glucose. A similar pattern of ATase activity was obtained when *B. subtilis* strain 168 was grown on an unbuffered minimal medium (SMM) containing 0.1% glucose, which supported sporulation. Inactivation of ATase appeared to be complete before the appearance of refractile forespores.

Effect of dilution on ATase activity. ATase activity, as measured by PRPP-dependent glu-

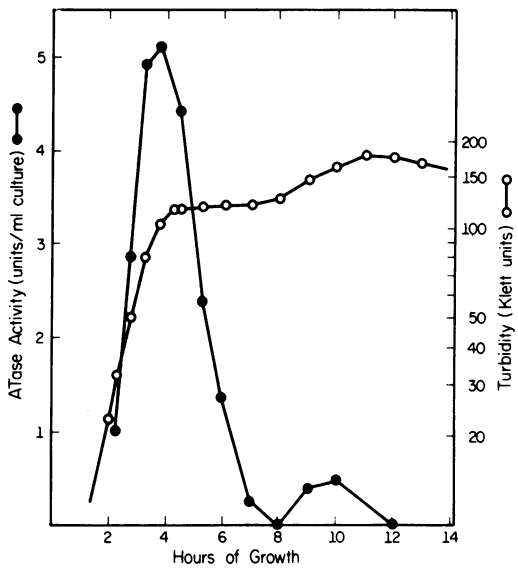

FIG. 1. *Inactivation of ATase activity during growth of B. subtilis 168 on BMM + 0.1% glucose at 37 C.*

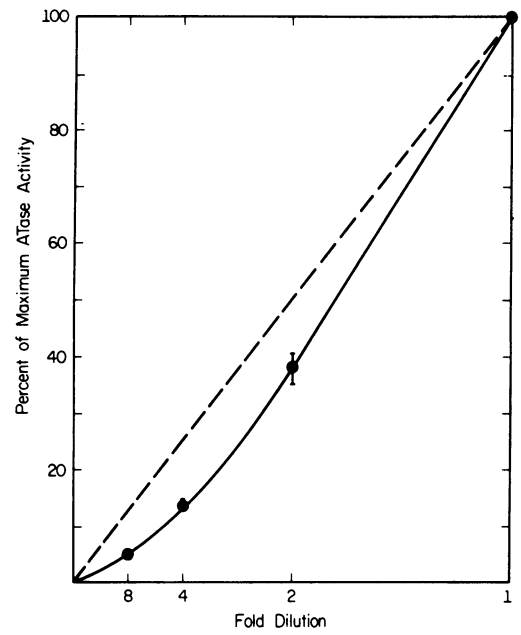

FIG. 2. *Effect of dilution on ATase activity. Dashed line represents ATase activity expected if no loss of activity occurred upon dilution. Open circles are the ATase activities measured after dilution. Error bars show the range of activities obtained from five experiments.*

tamate formation, was not strictly linear with the amount of enzyme assayed. The activity of lower concentrations of enzyme in the assay was consistently lower per milligram of extract than higher concentrations. Addition of 1 mg of bovine serum albumin per ml, preliminary incubation with glutamine or PRPP, or addition of 2-mercaptoethanol did not correct the nonlinearity of the assay. Because it was necessary to have a good estimate of the errors resulting from the nonlinearity of the assay and to establish that the inactivation of ATase was not grossly exaggerated as a consequence of it, the effects of dilution of cell extracts from exponential- and stationary-phase cultures were determined. A cell extract with ATase activity comparable to the amount observed at the peak of activity in Fig. 1 (122 units/ml of extract with assay II) was diluted to several concentrations with buffer A, and 150-μliter samples were assayed with assay II (Fig. 2). Within the range examined, each twofold dilution yielded about 25% less activity than would be expected from dilution without loss of activity. Extracts from exponential- and stationary-phase cultures behaved identically. When whole cells were diluted before extraction, the same loss of activity was observed. ATase activity is not irreversibly inactivated by dilution because the activity of a diluted cell extract can be restored by concentration by pressure dialysis.

The effects of dilution on the ATase assay could be corrected for by assuming that the highest activity in an inactivation experiment represents 100% of the maximum ATase activity and assuming that all lower activities deviate from the actual concentrations of ATase to the extent indicated by Fig. 2. The effects of making such a correction are shown in Fig. 3. While the correction was very substantial at low ATase levels, it is clear that the overall rate of loss of ATase activity from the culture was not greatly overestimated. Hence, subsequent inactivation experiments show ATase activities without correction for dilution effects.

Evidence indicating ATase is not inactivated by general proteolysis. The involvement of proteases in general protein turnover (10) and in the inactivation (4) or proteolytic modification (13) of specific enzymes during sporulation of *Bacillus* species has been demonstrated. It was necessary, therefore, to establish whether proteolysis was involved in the inactivation of ATase.

One approach to the question of protease involvement in the ATase inactivation was to determine whether the enzyme was inactivated in mutant strains lacking proteases. Two well characterized mutants of *B. subtilis* deficient in intracellular proteases were obtained from J. H.

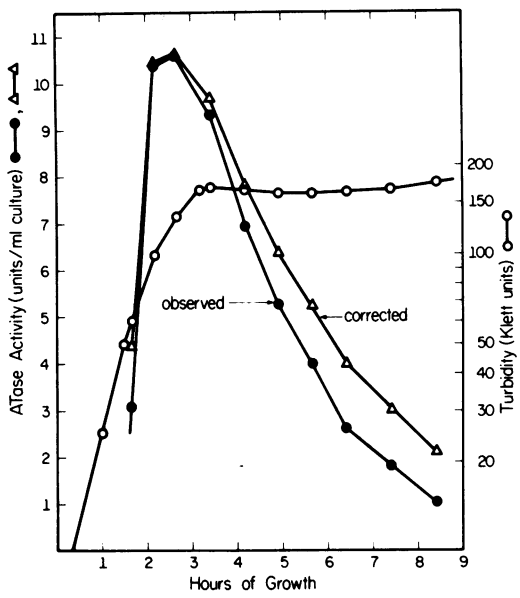

FIG. 3. *Inactivation of ATase activity corrected for loss of activity caused by dilution. Closed circles indicate measured ATase activity and open triangles represent ATase activity corrected for dilution. Culture was grown as in Fig. 1, but ATase activity was measured with assay II.*

Hageman (7). Strain L-4 lacks an EDTA-inhibitable, "neutral" protease and sporulates normally. Strain S-87 is deficient in a DFP-inhibitable protease and is oligosporogenic. Inactivation of ATase in both mutant strains was shown to be the same as in the parent strain, 168, which tended to exclude the involvement of these proteases in the inactivation.

A second approach was to incubate extracts of cells harvested during exponential and early stationary phases of growth at 37 C. Cells were grown on BMM and 0.1% glucose and 40-ml samples were harvested and extracted in 5 ml of buffer A without 2-mercaptoethanol. Extracts of cells harvested during exponential or stationary phase of growth lost 80 to 90% of their ATase activity in 3 h. Similar extracts prepared in buffer A (including 10 mM 2-mercaptoethanol) lost 35 to 50% of initial enzymatic activity after 3 h. When cells were anaerobically extracted (with or without 2-mercaptoethanol) under argon and incubated anaerobically in sealed tubes, no ATase activity was lost after 3 h. These results indicate that the inactivation of ATase is not the result of general proteolysis, but do not exclude the possible involvement of a specialized protease. Such a protease would have to possess an unusual requirement for oxygen and be present at all times during growth, as demonstrated below.

When cells were grown on SMM, some DFP-inhibitable loss of ATase activity in stationary-phase cell extracts was observed, but since BMM supported ATase inactivation as well as SMM, it was unlikely that this activity was required for inactivation.

Oxygen requirement of ATase inactivation. The metabolic requirements of the ATase inactivation were examined by attempting to interrupt the inactivation process after it had begun in stationary-phase cells.

Organic acids accumulated during growth on glucose are utilized via the tricarboxylic acid cycle with concomitant extensive oxygen uptake during the stationary phase (17). Substitution of argon for air that was bubbled through a stationary-phase culture interrupted the metabolism of organic acids and immediately blocked further inactivation of ATase (Fig. 4). The amount of increase in ATase activity seen during anaerobiosis varied in other experiments. This increase may represent reactivation or slow synthesis of ATase. When aeration of the culture was resumed, the inactivation of ATase

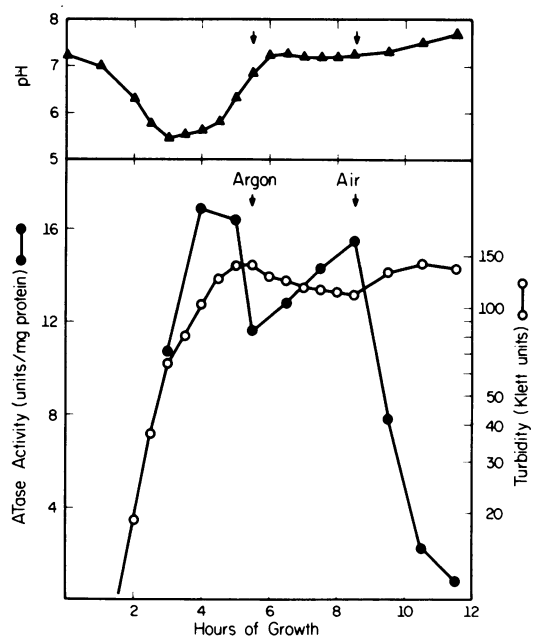

FIG. 4. *Oxygen starvation during inactivation of ATase. B. subtilis 168 was grown on SMM + 0.1% glucose at 37 C. The first arrow indicates when aeration was stopped and argon bubbled through the culture. At the second arrow normal aeration was resumed. ATase activity is expressed as specific activity to compensate for cell lysis.*

proceeded rapidly and the pH of the culture fluid began to rise, indicating the resumption of organic acid utilization (Fig. 4).

There are two explanations for the cessation of ATase inactivation during oxygen deprivation of stationary-phase cells. The inactivation process may require tricarboxylic acid cycle activity and energy-yielding metabolism, or the inactivation of ATase may require molecular oxygen directly. To decide between these possibilities, fluoroacetate, an inhibitor of the tricarboxylic acid cycle, was added to a stationary-phase culture. The addition of 50 mM fluoroacetate did not inhibit ATase inactivation. This concentration of fluoroacetate has been shown to significantly inhibit the adenosine 5'-triphosphate (ATP)-dependent inactivation of aspartate transcarbamylase (18). This result suggests that molecular oxygen rather than some aspect of the tricarboxylic acid cycle is required for the inactivation of ATase. The addition of 50 mM sodium azide or 10^{-5} M 2-heptyl-4-hydroxyquinoline-N-oxide, both inhibitors of the electron transport system (6), to stationary-phase cells also did not inhibit ATase inactivation. It was demonstrated that these inhibitors of electron transport did inhibit the inactivation of aspartate transcarbamylase. These results support the conclusion that energy-yielding metabolism is not required for ATase inactivation.

The above conclusion was tested using a mutant strain of *B. subtilis* 168 that is deficient in an enzyme of the tricarboxylic acid cycle. Strain HS1A11, isolated by Carls and Hanson (3), contains no aconitase. Yousten and Hanson (19) have shown that the ATP content in cells of strain HS1A11 decreases to about 5% of normal ATP levels after the end of exponential growth. When a stationary-phase culture of strain HS1A11 is supplemented with 8 mM gluconate, the ATP level rapidly increases to that found in the wild type. The rate of inactivation of ATase in cells of strain HS1A11 grown on SMM was the same as that measured in the parent strain, 168. The addition of gluconate to stationary-phase cells of strain HS1A11 did not increase the rate of ATase inactivation. In contrast, the rate of ATP-dependent inactivation of aspartate transcarbamylase in strain HS1A11 is less than 20% of the rate in the parent strain, 168, and is markedly stimulated by the addition of gluconate (18). These results support the conclusion that ATP generation is not essential for ATase inactivation. The possibility that the inactivation is ATP dependent and can proceed normally at very low ATP levels cannot be ruled out, however.

Stability of ATase in exponentially growing cells. It is possible that the inactivation of ATase observed during stationary phase also occurs in exponentially growing cells. The inactivation process may be masked by rapid synthesis of ATase during exponential growth and is observed only in stationary-phase cells when rapid synthesis has ended. This possibility was tested by repressing synthesis of ATase in exponentially growing cells by adding 50 μg of adenosine per ml to the culture. This concentration of adenosine prevents further ATase synthesis (11). ATase synthesis stopped after the addition of adenosine and the ATase activity in the repressed cells was constant for approximately 1 h during exponential growth (Fig. 5). Inactivation of ATase began when the cells entered stationary phase.

Effects of inhibitors of macromolecular synthesis and amino acid starvation on inactivation of ATase. The addition of either 100 μg of chloramphenicol or 10 μg of rifampin per ml to exponentially growing cells completely inhibited growth. Rapid inactivation of ATase began immediately after addition of either antibiotic. The initial rate of inactivation after addition of antibiotic was about the same as shown in Fig. 1, but slowed substantially about 2 h after the addition. The addition of the same concentration of either antibiotic to stationary-phase cells

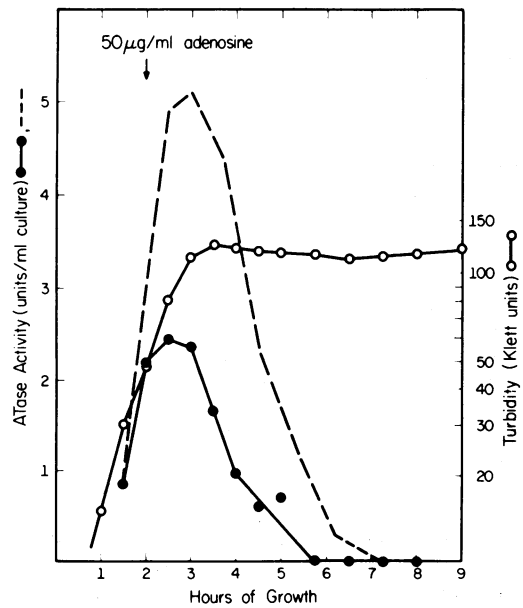

FIG. 5. *Stability of ATase during exponential growth of B. subtilis 168 on BMM + 0.1% glucose at 37 C. Arrow indicates the time of addition of adenosine. Dashed line illustrates the normal enzyme pattern from Fig. 1.*

exerted only a slight decrease in the rate of ATase inactivation. These experiments indicate that protein or ribonucleic acid (RNA) synthesis are not required for the inactivation process to occur. Thus, if a protein or RNA species is involved in the inactivation, it must be present (but inactive) in exponentially growing cells.

In another experiment a culture of *B. subtilis* was grown on BMM + 0.1% glucose and allowed to exhaust a limiting supplement of tryptophan (1 μg/ml). At the end of exponential growth, ATase was inactivated at a rate identical to that of experiments in which glucose was the limiting nutrient. This result, together with the effects of adding antibiotics to exponentially growing cultures, suggests that a variety of conditions causing cessation of rapid growth bring about ATase inactivation.

DISCUSSION

The results of this study have shown that the first enzyme of de novo purine nucleotide biosynthesis is rapidly inactivated when *B. subtilis* cells enter stationary phase. It is not yet known whether other enzymes of this pathway are inactivated at the same time, except for inosine 5'-monophosphate dehydrogenase, which has been reported to be inactivated in stationary-phase cells (4). The first enzyme committed to de novo pyrimidine nucleotide biosynthesis, aspartate transcarbamylase, has also been shown to be inactivated under similar conditions (4, 18). It is reasonable to suggest that these inactivation processes represent a regulatory response designed to conserve metabolic precursors and energy in starving cells as they begin endospore formation. It might be expected that the inactivation of aspartate transcarbamylase and of ATase would be regulated in the same manner and proceed by similar mechanisms.

The inactivation of ATase begins when the culture ceases exponential growth, either because of carbon or amino acid starvation or because a metabolic inhibitor is added to the culture. In this respect ATase inactivation resembles inactivation of aspartate transcarbamylase. It is likely that the signal for inactivation is carried by a change in the concentration of some metabolite or metabolites that reflect the capacity of the cell for growth. The nature of such a metabolite is not known in either case, although results of experiments presented in the accompanying paper (16) suggest that substrates and end product inhibitors of ATase may be involved.

The mechanism of ATase inactivation is clearly different from that governing inactivation of aspartate transcarbamylase (18). Deprivation of stationary cells for oxygen blocks the inactivation in both cases, but the role of oxygen is quite different. In the case of aspartate transcarbamylase oxygen is required to support energy-yielding metabolism (18). Interruption of such metabolism with specific inhibitors or by mutation prevents the inactivation. In the present case, ATase inactivation is not blocked or inhibited by the same inhibitors or in mutants unable to generate ATP during the stationary phase. The conclusion that ATase inactivation is directly dependent on oxygen is strengthened by the demonstration of an oxygen-dependent inactivation in cell-free extracts (16).

The oxygen requirement for ATase inactivation could be an expression of several different mechanisms. Oxygen might serve as a substrate for an oxygen-dependent inactivating enzyme, which is active only after the cessation of exponential growth. Alternatively, oxygen may react directly with a group on the ATase protein to form an inactive, oxidized product, or to form a product that is extremely susceptible to inactivation by proteolysis or some other process. Finally, it is possible that oxygen is required only indirectly, for example, to generate some "signalling" metabolite that actually regulates ATase inactivation. Clearly, these possibilities can be best resolved by reconstructing the inactivation process in a cell-free system. The results of such experiments are reported in the accompanying communication (16).

ACKNOWLEDGMENTS

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