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Inactivation of Isocitrate Lyase During Myxospore Development in *Myxococcus xanthus*

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The inactivation of isocitrate lyase which occurs during late stages of myxospore formation in *Myxococcus xanthus* was studied. Several findings are reported. (i) Protein synthesis is required over a specific time interval in order for isocitrate lyase inactivation to occur at a later time. (ii) Metabolic energy is required at all times during myxospore development if the inactivation is to occur. (iii) It was possible to inhibit protein turnover to a considerable extent without affecting the net loss in isocitrate lyase activity.

Although much has been discovered and is presently being learned about the mechanisms and control of macromolecular biosynthesis, very little is known about the processes of degradation and inactivation of these molecules. Several bacterial systems offer a situation in which the disappearance of certain enzyme activities can be studied as part of a characteristic sequence of biochemical changes during development. Bernlohr and co-workers (10, 11, 13, 21) studied the inactivation of aspartokinase (EC 2.7.2.4) and threonine dehydratase (EC 4.2.1.16) during endospore formation in *Bacillus licheniformis*. They concluded that the inactivation is related to the physiology of the cell, but they did not elucidate its mechanism or control. Deutscher and Kornberg (4) investigated the loss of aspartate transcarbamylase (ATCase) (EC 2.1.3.2) and inosine monophosphate dehydrogenase (IMPDH) (EC 1.2.1.14) activities during sporulation of *Bacillus subtilis*. By using protease negative mutants, they were able to determine that intracellular proteolytic activity is necessary for the loss of IMPDH activity but not for the inactivation of ATCase. Waidle and Switzer (24) have recently extended the work on inactivation of ATCase during sporulation and suggested that the generation of adenosine 5'-triphosphate (ATP) is necessary for this inactivation.

We have previously reported the rapid appearance of the glyoxylate cycle enzymes during the early stages of myxospore formation in *Myxococcus xanthus* (2, 15). Isocitrate lyase (EC 4.1.3.1), the first enzyme in the sequence, loses activity very rapidly during maturation of the myxospore after having attained a peak upon completion of the morphological change. In contrast, malate synthetase (EC 4.1.3.2) activity is relatively stable, a fact which suggests that the mechanism, if not the control, of isocitrate lyase inactivation is somewhat specific. We have recently reported the presence of large amounts of intracellular proteolytic activity at all stages of myxospore formation (submitted for publication). The proteolytic activity was characterized as being mainly due to neutral metal proteases. There were also small amounts of serine protease and unidentifiable activity. All protease activity except the latter, which represented 10% of the total, could be removed with appropriate inhibitors.

The present report presents experiments which investigated the contribution of protein synthesis, protein turnover, and energy metabolism to the process of inactivation of isocitrate lyase during myxospore maturation. The results indicate that protein synthesis is apparently requisite over a specific time interval in order for inactivation to occur at a later time and that ATP is required at all times during the process.

MATERIALS AND METHODS

Organism. *M. xanthus*, strain FB (5), and a mutant (designated TD) with an altered time course of myxospore formation were used.

Cultivation. The organism was grown at 30 C in a medium containing 2% Casitone, 8×10^{-3} M $MgSO_4$, and 10^{-2} M potassium phosphate buffer, pH 7.5 (5).

Myxospore induction. A concentrated suspension of vegetative cells was induced to form myxospores in the presence of vigorously bubbled air in a medium containing 1% Casitone, 8×10^{-3} M $MgSO_4$, and 0.5 M glycerol as previously described (6).

Isocitrate lyase assay. Cell-free extracts were prepared, and isocitrate lyase activity was measured as previously described (15). One unit of activity is

equal to that amount of enzyme which produces 1 nmol of product per min.

Protein determination. Protein in the cell-free extracts was determined by use of the Folin phenol reagent with bovine serum albumin as a standard (14). Total protein per milliliter of culture fluid was determined by the method of Spudich and Kornberg (20).

Radioactive amino acid uptake and incorporation. Radioactively labeled L-[U-^{14}C]valine (New England Nuclear Corp.) was added at a final concentration of 1 μCi/ml (0.46 μg/ml) to dilute, shaking cultures at various stages of myxospore formation. In the absence of any metabolic inhibitors, these cells completed the conversion to myxospores. All cultures were preincubated for 5 min in the presence of the appropriate inhibitor before the addition of L-[^{14}C]valine. For the determination of L-valine uptake, 250-μliter portions of culture fluid were taken at intervals and immediately filtered on membrane filters (Millipore Corp.; pore size, 0.45 μm). These were washed three times with 5 ml of induction medium (at room temperature) and dried in an oven at 55 C. The dried filters were placed into Liquifluor-toluene counting solution (New England Nuclear Corp.) and counted in a Beckman Liquid Scintillation Counter model LS-233. For determination of L-valine incorporation into protein, the 250-μliter portions of culture fluid were precipitated in 5 ml of 5% trichloroacetic acid at 0 C for 30 min, collected on membrane filters, washed with three 5-ml volumes of cold 5% trichloroacetic acid, washed with one 5-ml volume of anhydrous ethyl ether, and dried thoroughly at 55 C. Counts contained on the dried filters were determined as stated above.

Measurement of protein turnover. Vegetative cells were grown for four generations in media without phosphate buffer on a shaking water bath in the presence of L-[^{14}C]valine (0.25 μCi/ml; 0.115 μg/ml). Glycerol was added to a final concentration of 0.5 M during mid-exponential growth. The cells were allowed to convert to myxospores for 2 h. At 2 h the culture was centrifuged, and the cells were resuspended in fresh induction medium containing L-[^{12}C]valine at a concentration of 0.1 mg/ml. Portions (250 μliters) were transferred at intervals into 5 ml of 5% trichloroacetic acid, incubated at 90 C for 30 min, chilled at 0 C for 30 min, filtered on membrane filters (as above), washed with three 5-ml volumes of cold 5% trichloroacetic acid, washed with one 5-ml volume of anhydrous ethyl ether, dried at 55 C, and counted as described above.

Anaerobiosis. The air supply was discontinued at appropriate times of myxospore induction, and compressed nitrogen was vigorously bubbled through the cultures for the duration of such experiments.

Antibiotics and inhibitors. The following compounds were used in all cases at the final concentrations indicated: chloramphenicol (Sigma Chemical Co.), 250 μg/ml; puromycin (Sigma Chemical Co.), 100 μg/ml; rifampin (Sigma Chemical Co.), 20 μg/ml; *o*-phenanthroline (Fisher Scientific Co.), 1 mM; phenylmethanesulfonylfluoride (Sigma Chemical Co.), 1 mM; sodium azide (Matheson, Cole and Bell), 10 mM; and 2,4-dinitrophenol (Matheson, Cole and Bell), 0.2 mM. These compounds were added directly as a dry powder or as an aqueous solution to inducing cultures, except for phenylmethanesulfonylfluoride, which was dissolved in 5% aqueous isopropanol.

RESULTS

Pattern of isocitrate lyase activity in cells with a mutation altering the time course of myxospore formation. The pattern of isocitrate lyase activity previously described (15) for the strain FB was also true of a temporally deranged mutant (TD) of *M. xanthus* which formed myxospores only after a lag of approximately 7 h (Fig. 1). Appearance of isocitrate lyase activity was also displaced by the same number of hours. Furthermore, the specific activity attained a maximum at the time the cells had converted to spheres and declined as the myxospores matured.

Relationship of proteolytic activity and isocitrate lyase activity loss in extract. Previous work has shown that extracts of *M. xanthus* stored at 0 C lose approximately 10% activity of isocitrate lyase per h (15). Other work has shown that there is considerable proteolytic activity in cell-free extracts of *M. xanthus* from all stages of myxospore formation, most of which is inhibitable by *o*-phenanthroline (Orlowski and White, submitted for publication). To test whether this proteolytic activity is responsible for the isocitrate lyase activity loss in extract, we disrupted the cells in buffer containing 1 mM *o*-phenanthroline. The isocitrate lyase activity was stabilized for several hours at 0 C in the presence of *o*-phenanthroline (Fig. 2A). However, when the same extracts were stored at 30 C, activity was rapidly lost both with and without *o*-phenanthroline (Fig. 2B). Because previous data had indicated that most of the proteolytic activity in these extracts

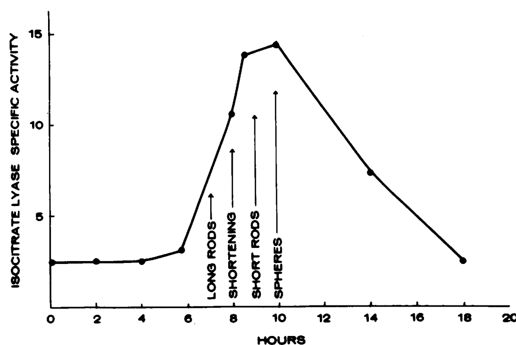

FIG. 1. *Pattern of isocitrate lyase activity during myxospore formation of a temporally deranged mutant (TD) of Myxococcus xanthus.*

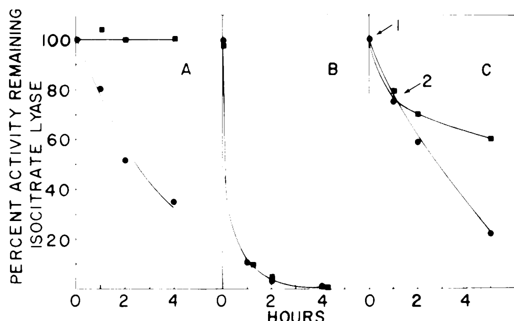

FIG. 2. *Activity loss of isocitrate lyase in extract. (A) Activity loss at 0 C. Symbols: (■), extract containing o-phenanthroline (1 mM); (●), control, no additions. (B) Activity loss at 30 C. Symbols: (■), extract containing o-phenanthroline (1 mM); (●), control, no additions. (C) Effect of high-speed centrifugation on activity loss at 0 C. Symbols: (■), extract centrifuged at 90,000 × g for 60 min (arrow at 1 denotes activity prior to centrifugation, arrow at 2 denotes activity immediately after centrifugation); (●), control, no centrifugation.*

is membrane bound (Orlowski and White, submitted for publication), we subjected the extracts to high-speed centrifugation (90,000 × *g* for 60 min) to remove these proteases. Isocitrate lyase activity was stabilized considerably in such preparations (Fig. 2C). These data suggest that proteases are at least in part responsible for the loss of isocitrate lyase activity in cell-free extracts. All attempts to stabilize the enzyme with Mg^{2+}, cysteine, glutathione, or isocitrate failed.

Effect of protease inhibition on isocitrate lyase activity loss in whole cells. *o*-Phenanthroline and phenylmethanesulfonylfluoride are inhibitors of metal proteases and serine proteases, respectively. The former compound was reported to inhibit at least 80%, the latter up to 10%, of the proteolytic activity in extracts of *M. xanthus* (Orlowski and White, submitted for publication). Both inhibitors were separately added to cultures of developing myxospores (1 mM final concentration) to determine their effect on isocitrate lyase activity loss. *o*-Phenanthroline, when added at 50 min after the start of glycerol-induced morphogenesis, prevented further accumulation of isocitrate lyase activity and also the subsequent loss of activity (Fig. 3A). When added at 2 h after the start of induction, *o*-phenanthroline did not prevent the loss of isocitrate lyase activity despite the fact that protein turnover was appreciably inhibited as described below. The addition of *o*-phenanthroline at any time reversibly inhibited the further morphological development of the cells.

We have previously reported the substantial (25%) net loss of total cellular protein during late stages of myxospore maturation (15). It is possible that this protein loss is due to the functioning of the intracellular proteases we have detected. Because *o*-phenanthroline inhibits the proteases in extract (Orlowski and White, submitted for publication), it was of interest to determine whether *o*-phenanthroline would inhibit protein degradation in whole cells. When *o*-phenanthroline was added to an induced culture at 2 h, the loss in protein was largely prevented (Fig. 4). *o*-Phenanthroline also prevented a considerable amount of the turnover of protein prelabeled with L-[^{14}C]valine (Fig. 5). Phenylmethanesulfonylfluoride had no apparent effect on any aspect of cellular development when added at any time during an induction. Complete morphological conversion took place. Neither the accumulation of isocit-

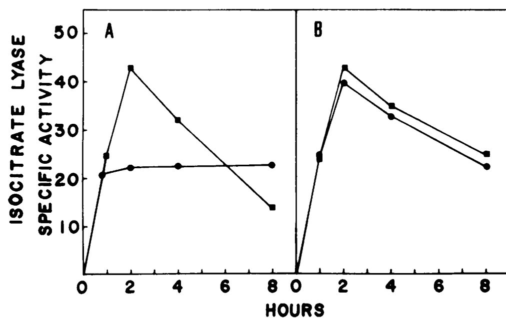

FIG. 3. *Effect of protease inhibitors on the loss of isocitrate lyase activity during myxospore formation. (A) o-Phenanthroline (1 mM). Symbols: (●), addition at 50 min; (■), addition at 2 h. (B) Phenylmethanesulfonylfluoride (1 mM). Symbols: (●), addition at 50 min; (■), addition at 2 h.*

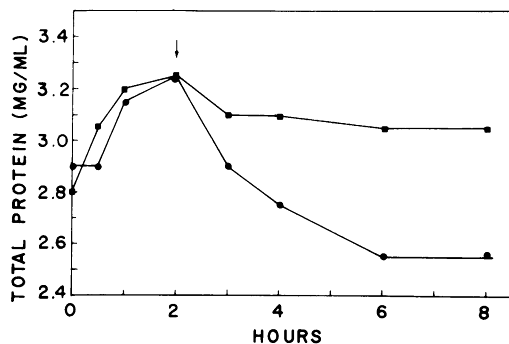

FIG. 4. *Time course determination of total cellular protein during myxospore formation. Symbols: (●), control culture; (■), culture to which o-phenanthroline (1 mM final concentration) was added at 2 h (arrow).*

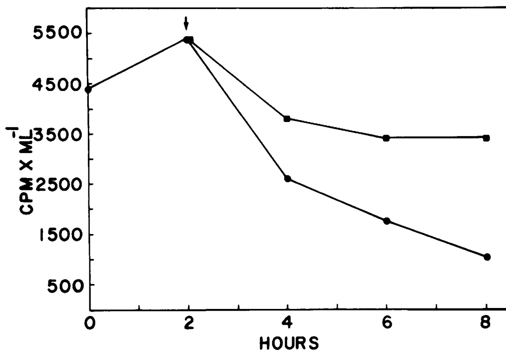

FIG. 5. *Time course of protein turnover (loss of trichloroacetic acid-insoluble L-[^{14}C]valine label) during myxospore formation. The cells were grown for four generations and induced with glycerol for 2 h in the presence of L-[^{14}C]valine label. At 2 h (arrow) the label was removed and a chase of L-[^{12}C]valine was added (see Materials and Methods). Symbols: (●), control culture; (■), culture to which o-phenanthroline (1 mM final concentration) was added at 2 h (arrow).*

rate lyase activity nor its disappearance was affected. Phenylmethanesulfonylfluoride was not inhibitory to vegetative growth of *M. xanthus*. We have no evidence that phenylmethanesulfonylfluoride can enter the cells, although it reportedly permeates other bacterial cells and inhibits intracellular serine proteases (8, 17).

Effect of inhibitors of protein and ribonucleic acid (RNA) synthesis on disappearance of isocitrate lyase activity. We have previously reported that when chloramphenicol was added to an induced culture at the stage of short rods (50 min), it prevented the further accumulation and later disappearance of isocitrate lyase activity (15). When chloramphenicol was added after the cells had already converted to spheres (2 h), no stabilizing influence on isocitrate lyase activity was noticed (15). When this type of experiment was repeated with puromycin, identical results were obtained (Fig. 7A). *o*-Phenanthroline, which also inhibited protein synthesis (Fig. 6), had the same kind of effect on the pattern of isocitrate lyase activity (Fig. 3A). Rifampin, when added to a culture 50 min after the start of induction, did not immediately stop the accumulation of isocitrate lyase activity that continued for several additional minutes (Fig. 7B). This accumulated activity subsequently declined at a rate equivalent to that in a control culture. Rifampin had no effect on the loss of isocitrate lyase activity when added after the cells had already converted to spheres (Fig. 7B).

Effect of chloramphenicol, o-phenanthroline, and rifampin on L-[^{14}C]valine incorporation. Chloramphenicol, *o*-phenanthroline, and rifampin immediately and quantitatively inhibited the incorporation of L-[^{14}C]valine into trichloroacetic acid-insoluble material at 50 min, 2 h, and 5 h after the start of myxospore induction (Fig. 5). Because this effect was unanticipated for *o*-phenanthroline, we wanted to distinguish between a true inhibition of protein synthesis and uptake of L-valine. The data we collected at 30-s intervals over the first 5 min of incubation with L-[^{14}C]valine did not show any difference in uptake kinetics between a control culture and a culture containing *o*-phenanthroline (data not shown). We thus concluded that the observed effect of *o*-phenanthroline on L-[^{14}C]valine incorporation is wholly

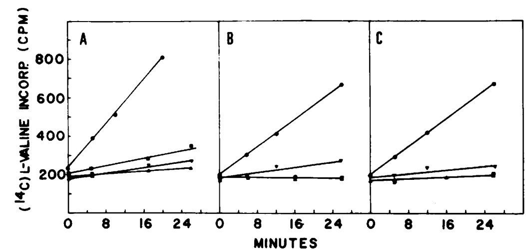

FIG. 6. *Kinetics of L-[^{14}C]valine incorporation (trichloroacetic acid-insoluble counts) at various stages of myxospore formation. L-[^{14}C]valine label was added to cultures (1 µCi/ml) after 50 min (A), 2 h (B), or 5 h (C) of glycerol-induced myxospore formation. Inhibitors were preincubated with cells for 5 min prior to addition of label. Ordinate values represent total counts precipitated from 250 µliters of culture fluid. Symbols: (●), control culture; (▲), culture with chloramphenicol (250 µg/ml); (▼), rifampin (20 µg/ml); (■),.o-phenanthroline (1 mM).*

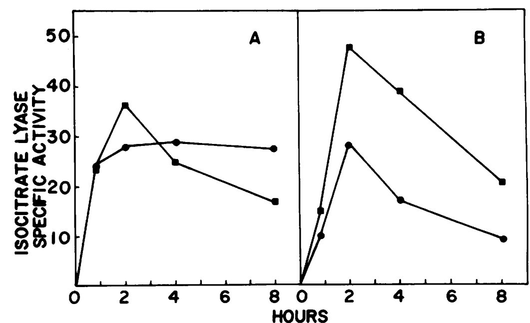

FIG. 7. *Inhibitors of biosynthesis and loss of isocitrate lyase activity during myxospore formation. (A) Puromycin (100 µg/ml). Symbols: (●), addition at 50 min; (■), 2 h. (B) Rifampin (20 µg/ml). Symbols: (●), addition at 50 min; (■), 2 h.*

at the level of protein synthesis.

Effect of anaerobiosis on disappearance of isocitrate lyase activity. Previous investigations have shown that cultures of sporulating *Bacillus* deprived of oxygen by bubbling nitrogen or argon through the cultures failed to display the usual activity loss of aspartokinase (10) or aspartate transcarbamylase (24). Inducing cultures of *M. xanthus* made anaerobic by bubbling with nitrogen do not show the characteristic decline in isocitrate lyase activity at later stages (Fig. 8A). This response could indicate a requirement for either the continuous presence of molecular oxygen or a source of energy (ATP) dependent on aerobic metabolism in order for inactivation of isocitrate lyase to occur. The following experiments were designed to distinguish between these possibilities.

Effect of inhibitors of energy metabolism on disappearance of isocitrate lyase activity. Sodium azide, an inhibitor of terminal oxidation, and 2,4-dinitrophenol, an uncoupling agent of oxidative phosphorylation, were tested for their effect on the pattern of isocitrate lyase activity during myxospore development. Sodium azide completely stabilized isocitrate lyase activity whenever added to a developing culture (Fig. 8B). This could reflect a need for continuous ATP synthesis or electron transport (possibly to eliminate accumulated reducing power from the cell) in order for inactivation of isocitrate lyase to proceed. Addition of 0.2 mM 2,4-dinitrophenol to the cultures (higher concentrations caused the formation of spheroplasts and cell lysis) at any time completely stabilized the isocitrate lyase activity (Fig. 8C). This suggests the dependence of isocitrate lyase inactivation upon continuous ATP synthesis rather than electron transport per se. Attempts to reestablish the inactivation of isocitrate lyase by addition of 3 mM ATP (7) to cultures containing sodium azide were unsuccessful presumably because the nucleotide did not enter the cells.

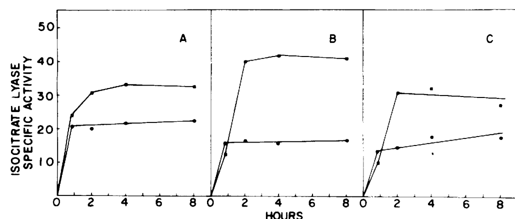

FIG. 8. *Inhibitors of energy metabolism and loss of isocitrate lyase activity during myxospore formation. (A) Anaerobiosis. Symbols: (●), N_2 sparge beginning at 50 min; (■) 2 h. (B) Sodium azide (10 mM). Symbols: (●), addition at 50 min; (■) 2 h. (C) 2,4-Dinitrophenol (0.2 mM). Symbols: (●), addition at 50 min; (■), 2 h.*

DISCUSSION

The ability to stabilize isocitrate lyase activity in extracts at 0 C with the metal protease inhibitor *o*-phenanthroline was useful in that it allowed us to further substantiate the statement made earlier that the loss of activity observed during myxospore maturation occurs in vivo and not during preparation of the extracts (15). The extremely rapid activity loss in extract at 30 C compared with the rate in whole cells during myxospore maturation (ca. 10%/h) suggests that the process is influenced by various undefined parameters differing in both cases. Failure of *o*-phenanthroline to prevent in vitro loss of activity at 30 C may be due to an inactivation process other than proteolytic digestion operative at 30 C. However, because the proteolytic activity at 0 C is only 2% of the value at 30 C (unpublished data), it may be that *o*-phenanthroline is considerably more effective in preventing proteolytic cleavages of isocitrate lyase at 0 C than at 30 C. The stabilization of isocitrate lyase upon removal of particulate proteolytic activity implicates the observed proteases as the cause of isocitrate lyase activity loss in extracts. Whether these membrane-bound proteases have access to and can act upon isocitrate lyase in the whole cells is a moot point, especially because isocitrate lyase occurs only in soluble form (unpublished data).

When *o*-phenanthroline was added to an induced culture at a time before the usual net loss of protein occurred, the loss was largely prevented, a reversible arrestment of morphogenesis was observed, and both protein synthesis and protein turnover were inhibited. These facts suggest that the agent can enter the cell and inhibit the proteolytic enzymes responsible for the characteristic turnover and disappearance of protein. However, *o*-phenanthroline did not prevent the loss in isocitrate lyase activity when added to spherical cells (2 h). Neither did phenylmethanesulfonylfluoride have any apparent effect. One possible conclusion of these experiments is that the loss of isocitrate lyase activity in the whole cell involves some mechanism other than, or in addition to, degradation by the proteolytic enzymes we have detected and inhibited.

The data from experiments involving inhibitors of protein synthesis (including *o*-phenanthroline) suggest that protein synthesis (perhaps the synthesis of a specific protein) must

occur between the stage of short rods (50 min) and spheres (2 h) in order for the subsequent inactivation of isocitrate lyase to occur. It was previously suggested that the different time-dependent effects of chloramphenicol on the disappearance of isocitrate lyase activity might be due to the manner in which protein synthesis was stopped (15). The present experiments utilizing three different inhibitors of protein synthesis (two with known, differing mechanisms of action) do not support the latter idea but rather suggest that it is the complete stoppage of protein synthesis at 50 min, irrespective of the way in which it is inhibited, that is critical to stabilizing isocitrate lyase activity. The data for the rifampin experiments are interesting and on first inspection seem to suggest the existence of a stable or large pool of messenger RNA specific for isocitrate lyase. However, such experiments must be interpreted with much care for the question of stable messenger RNAs in bacterial systems has been frought with controversy. It is clear, however, that if isocitrate lyase activity accumulates beyond 50 min in rifampin-treated cells, its later inactivation still occurs. If isocitrate lyase synthesis escapes the general inhibition of protein synthesis by rifampin, so must the synthesis of protein responsible for later isocitrate lyase inactivation. This hypothetical protein need not be a specific proteolytic enzyme or "inactivation protein" but could conceivably be a developmentally specific labile isoenzyme form of isocitrate lyase. Further testing of this hypothesis awaits purification and characterization of the isocitrate lyase present at various stages of myxospore development. An alternative hypothesis, based upon the assumption that isocitrate lyase is synthesized and inactivated at 50 min but merely inactivated at 2 h, is that the inhibition of protein synthesis at 50 min inhibits the synthesis of isocitrate lyase to the extent that the rate of synthesis equals the rate of degradation. This would lead to an apparent stability of the enzyme when protein synthesis is inhibited at 50 min but not at 2 h. Only after the rates of synthesis and inactivation of the enzyme are measured directly can these hypotheses be evaluated.

The stabilization of isocitrate lyase activity in anaerobic cultures and cultures containing sodium azide or 2,4-dinitrophenol demonstrates the need for continuous supply of metabolic energy (ATP) in order for the inactivation of isocitrate lyase to occur. The requirement of a continuous supply of energy for enzyme inactivation has been described in other systems (16, 24) and may have a basis in several possible mechanisms, some of which are discussed below.

(i) A constant energy supply may be required for the synthesis of a specific protease or some other "inactivation molecule." This is ruled out because the activity of isocitrate lyase decreases in spite of the presence of inhibitors of protein or RNA synthesis after 2 h of myxospore induction. The energy-linked inhibitors effectively stabilize the enzyme activity at this time.

(ii) Energy may be required to excrete the enzyme from the cell. Such a mechanism of enzyme excretion is known to be involved in the control of intracellular uridine 5'-diphosphate-galactose polysaccharide transferase activity during fruiting body formation in the cellular slime mold *Dictyostelium discoidium* (22, 23) and of phosphodiesterase activity during myxospore germination of *M. xanthus* (S. Z. Sudo, D. Weisberg, and M. Dworkin, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, G28, p. 30). We could never detect isocitrate lyase activity in the culture medium; however, this possible mechanism cannot be ruled out until the enzyme protein is purified and assayed immunochemically in the culture fluid.

(iii) The adenylate energy charge of the cell may directly or indirectly modify the susceptibility of the enzyme to the inactivating mechanism or the functioning of the inactivating system toward the enzyme. Adenylate nucleotide species or other metabolites in flux with energy charge may act as effectors or modifiers of the pertinent molecule. Effects of energy charge on enzyme behavior have been reported in bacterial systems (1, 3). An inherently "labile" enzyme, as postulated earlier, may respond to the energy charge by change of conformation or subunit aggregation (19). The energy charge generally remains at levels found in vegetative cells throughout myxospore formation in *M. xanthus* (C. W. Hanson and M. Dworkin, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, G26, p. 30). The addition of respiratory inhibitors and uncoupling agents undoubtedly has the effect to lower the energy charge in the organism although this was not tested.

(iv) Inactivation of isocitrate lyase may be due to direct covalent modification such as an ATP-dependent adenylation or phosphorylation (12). Adenylation has been reported in the mechanism of inactivation of glutamine synthetase in *E. coli* (18). Purification of the enzyme protein will help test this possibility.

(v) Energy metabolism has been reported to

be necessary for protein turnover in *E. coli*, but the basis for this requirement has not been established (9, 16). As stated previously, the data suggest that even when intracellular proteases are substantially inhibited by *o*-phenanthroline, the isocitrate lyase is not stabilized. However, a definitive statement regarding the relationship between the loss of activity of isocitrate lyase and possible degradation by proteolytic enzymes cannot be made with available data.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

More recent experiments have shown that the net loss of protein indicated in Fig. 4 does not always occur; however, further experiments of the type described in Fig. 5 confirm that *o*-phenanthroline significantly inhibits loss of protein prelabeled with [^{14}C]valine.

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