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Regulation of the Dicarboxylic Acid Part of the Citric Acid Cycle in *Bacillus subtilis*

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The regulation of α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase, and malic enzyme has been studied in *Bacillus subtilis*. The levels of these enzymes increase rapidly during late exponential phase in a complex medium and are maximal 1 to 2 h after the onset of sporulation. Regulation of enzyme synthesis has been studied in the wild type and different citric acid cycle mutants by adding various metabolites to the growth medium. α-Ketoglutarate dehydrogenase is induced by glutamate or α-ketoglutarate; succinate dehydrogenase is repressed by malate; and fumarase and malic enzyme are induced by fumarate and malate, respectively. The addition of glucose leads to repression of the citric acid cycle enzymes whereas the level of malic enzyme is unaffected. Studies on the control of enzyme activities in vitro have shown that α-ketoglutarate dehydrogenase and succinate dehydrogenase are inhibited by oxalacetate. Enzyme activities are also influenced by the energy level, expressed as the energy charge of the adenylate pool. Isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malic enzyme are inhibited at high energy charge values, whereas malate dehydrogenase is inhibited at low energy charge. A survey of the regulation of the citric acid cycle in *B. subtilis*, based on the present work and previously reported results, is presented and discussed.

The control of the citric acid cycle in *Bacillus subtilis* has been studied by several authors. Most investigations have concerned the first three enzymes of the cycle, citrate synthase (EC 4.1.3.7, citrate oxaloacetate-lyase [coenzyme A acetylating]), aconitase (EC 4.2.1.3, citrate [isocitrate] hydro-lyase), and isocitrate dehydrogenase (EC 1.1.1.42, *threo*-D$_S$-isocitrate: nicotinamide adenine dinucleotide phosphate oxidoreductase [decarboxylating]). These enzymes are responsible for the synthesis of α-ketoglutarate, and they have been shown to be subject to feedback repression by α-ketoglutarate or glutamate (12, 14, 22, 30). In addition, the synthesis of aconitase is induced by citrate (30). In vitro studies on citrate synthase and isocitrate dehydrogenase have shown that these two enzymes are inhibited by adenosine triphosphate (ATP) (12, 13).

In contrast, very little is known about the control of the dicarboxylic acid part of the citric acid cycle, responsible for the oxidation of α-ketoglutarate to oxalacetate. Mutants of *B. subtilis* defective in α-ketoglutarate dehydrogenase, succinate dehydrogenase (EC 1.3.99.1, succinate:[acceptor] oxidoreductase), fumarase (EC 4.2.1.2, L-malate hydro-lyase), or malate dehydrogenase (EC 1.1.1.37, L-malate:nicotinamide adenine dinucleotide oxidoreductase) have been isolated and characterized genetically (33) and enzymatically (8, 33), but their regulatory properties have not been studied.

The finding that *B. subtilis* mutants lacking malate dehydrogenase are able to convert malate to glutamate, led Carls and Hanson (8) to postulate the existence of malic enzyme (EC 1.1.1.40, L-malate:nicotinamide adenine dinucleotide phosphate oxidoreductase [decarboxylating]) in this organism. Malic enzyme catlyzes the oxidative decarboxylation of malate to pyruvate, an essential anaplerotic reaction during growth on C_4 compounds. This activity was recently demonstrated in extracts of *B. subtilis* (10).

In this work, I describe the regulation of the dicarboxylic acid portion of the citric acid cycle as well as the regulation of malic enzyme in *B. subtilis*.

MATERIALS AND METHODS

Bacteria. All bacterial strains used in this study were derived from *B. subtilis* 168. The isolation and characterization of the citric acid cycle mutants have been reported earlier (31, 33). Mutants lacking malic

enzyme (Mal^-) were obtained by mutagenesis of the wild-type strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine essentially by the method of Adelberg et al. (2) followed by penicillin enrichment in minimal malate medium. Deoxyribonucleic acid from Mal^- mutants was used to transform strain BR26 (*trpC2 leu-2*) to leucine independence as previously described (31). Leu^+ Mal^- transformants were purified and used for subsequent studies.

Media. Nutrient sporulation (NSMP) medium contained 8 g of nutrient broth (Difco) per liter plus 0.001 mM $FeCl_3$, 0.7 mM $CaCl_2$, 0.05 mM $MnCl_2$, 1.0 mM $MgCl_2$, and 0.1 M potassium phosphate buffer, pH 6.5 (15). Solutions of citric acid cycle intermediates and amino acids were adjusted to pH 6.5, filter sterilized, and added separately to the medium.

Purification agar plates were made from the pH indicator medium containing bromocresol purple described by Carls and Hanson (8). Wild-type colonies are blue on purification agar plates, whereas acid-accumulating mutants form yellow colonies.

Penicillin enrichment medium contained minimal salts (34), plus 0.5% L-malate, 0.05% Casamino Acids (Difco), and 10 IU of penicillin G per ml.

Growth conditions. Bacteria were grown in 50 ml of medium in 250-ml Erlenmeyer flasks or in 500 ml of medium in 3-liter Fernbach flasks. Cultures were inoculated with cells from overnight purification agar plates. Incubation was at 37 C on a rotary shaker at 200 rotations per min. Growth was followed by measuring the absorbance at 600 nm (A_{600}). The initial A_{600} after inoculation was 0.03 to 0.05. The purity of mutant cultures was routinely checked by spreading samples on purification agar plates at the end of each experiment.

By convention, t_0 is defined as the time when growth starts to decline from the exponential rate and when sporulation is initiated; t_1, t_2, etc., indicate the number of hours elapsed since t_0.

Preparation of extracts for enzyme assays. Crude extracts were prepared as described earlier (31), except that the buffer contained 50 μg of chloramphenicol per ml. Fumarase and malic enzyme were assayed within 2 h after the preparation of extracts because of instability of these enzymes.

Protein was determined according to Lowry et al. (27) with crystalline bovine serum albumin as standard.

Enzyme assays. The enzyme reactions were followed in a Hitachi 101 spectrophotometer. The assays were carried out at room temperature. Specific activities were calculated as milliunits per milligram of protein, i.e., nanomoles of substrate converted per minute per milligram of protein.

α-Ketoglutarate dehydrogenase was assayed by the method of Bachmann et al. (5), except that antimycin A was excluded from the reaction mixture. The complete reaction mixture contained 50 mM potassium phosphate buffer, pH 6.5, 1 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetate, 2 mM reduced glutathione, 1 mM nicotinamide adenine dinucleotide, 0.1 mM coenzyme A, 0.2 mM thiamine pyrophosphate, 1 mM α-ketoglutarate, and 50 μg of extract protein per ml. The molar extinction coefficient for reduced nicotinamide adenine dinucleotide, or reduced nicotinamide adenine dinucleotide phosphate was taken to be 6.22×10^3 cm^{-1} M^{-1} (25). Succinate dehydrogenase was assayed as described by Ohné et al. (31). Fumarase was assayed by the method of Hanson and Cox (22). The molar extinction coefficient for fumarate was taken to be 2.4×10^3 cm^{-1} M^{-1} (6). Malate dehydrogenase was usually measured by following the oxalacetate-dependent oxidation of reduced nicotinamide adenine dinucleotide by the method of Ochoa (29). In the energy charge experiments, malate dehydrogenase was also measured by following the malate-dependent reduction of nicotinamide adenine dinucleotide according to Yoshida (37). Malic enzyme was assayed in a mixture containing 50 mM potassium phosphate buffer, pH 7.2, 2.5 mM $MgCl_2$, 0.25 mM nicotinamide adenine dinucleotide phosphate, and 50 mM L-malate. Aspartase (EC 4.3.1.1, L-aspartate ammonia-lyase) was assayed by the method of Freese et al. (19). Reduced nicotinamide adenine dinucleotide oxidase (EC 1.6.99.3, reduced nicotinamide adenine dinucleotide:[acceptor] oxidoreductase) was determined as described by Tochikubo (35).

RESULTS

Enzyme levels during vegetative growth and sporulation. It has been shown earlier (30) that the level of aconitase increases markedly during the transition from vegetative growth to sporulation in cultures grown in NSMP medium. The levels of α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase, and malic enzyme were also found to increase at the end of the exponential growth phase (Fig. 1). The levels of α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase were maximal at t_1 to t_2 and thereafter declined, whereas the levels of fumarase and malic enzyme remained high to t_6 when the experiment was terminated.

Freese et al. (16) have suggested that the apparent loss of α-ketoglutarate dehydrogenase activity in sporulating cells depends on the synthesis of a soluble reduced nicotinamide adenine dinucleotide oxidase which interferes with the α-ketoglutarate dehydrogenase assay. The following observations argue against such an explanation. (i) In agreement with Tochikubo (35), the specific activity of soluble reduced nicotinamide adenine dinucleotide oxidase was found to be very low (less than 5 nmol of reduced nicotinamide adenine dinucleotide oxidized per min per mg of protein) and remained unchanged during sporulation. (ii) The addition of quinacrine hydrochloride (an inhibitor of soluble reduced nicotinamide adenine dinucleotide oxidase) to the assay mixture did not increase the activity of α-ketoglutarate dehydrogenase in extracts of sporulating cells. (iii) The α-ketoglutarate dehydrogenase activity in

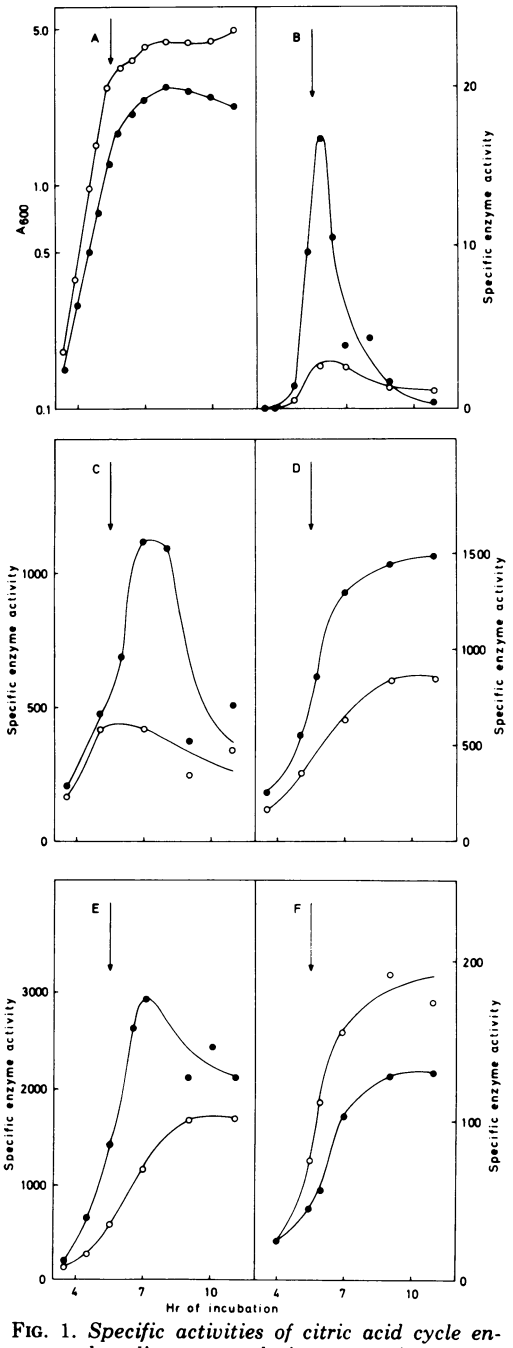

FIG. 1. *Specific activities of citric acid cycle enzymes and malic enzyme during vegetative growth and sporulation of B. subtilis wild type. Panels: A, growth; B, α-ketoglutarate dehydrogenase; C, succinate dehydrogenase; D, fumarase; E, malate dehydrogenase; F, malic enzyme. Symbols: ●, unsupplemented NSMP medium; ○, NSMP plus 0.5% glucose. Arrow indicates t_0 in both cultures.*

extracts of cells harvested at t_1 (maximal activity) was unaffected by the addition of extracts of cells harvested at t_6 (little or no activity). It therefore seems improbable that a substance interfering with or inhibiting the α-ketoglutarate dehydrogenase reaction is present in extracts of late stationary-phase cells. Alternative explanations of the disappearance of α-ketoglutarate dehydrogenase activity during sporulation will be considered in the Discussion.

The addition of 0.5% glucose to NSMP medium reduced the levels of the citric acid cycle enzymes to about 20 to 50% of the levels observed in the control culture (Fig. 1). In agreement with previous findings (10), malic enzyme was not repressed by glucose.

Effect of different metabolites on enzyme levels in vivo. The control of the citric acid cycle enzymes in vivo was studied by growing the wild-type strain and different mutants in NSMP medium with succinate, fumarate, L-malate, alanine, glutamate, or aspartate added to a final concentration of 25 mM. Alanine and glutamate serve as precursors of pyruvate and α-ketoglutarate, respectively (these keto acids are not taken up by our wild-type strain). The catabolism of aspartate will yield fumarate (via aspartase) as well as oxalacetate and glutamate (by transamination with α-ketoglutarate). The results are reported below for each enzyme separately.

(i) α-Ketoglutarate dehydrogenase. The level of α-ketoglutarate dehydrogenase was more or less increased by all the metabolites tested. Glutamate was found to be the most efficient inducer, followed by aspartate (Fig. 2). The other metabolites caused a smaller and somewhat variable increase in the level of α-ketoglutarate dehydrogenase. Glutamate is the predominant intracellular free amino acid under a variety of growth conditions (28), and the other metabolites can be expected to further increase the glutamate pool by transamination (in the case of aspartate) or by being metabolized via the citric acid cycle (8, 20). The results are thus consistent with the hypothesis that α-ketoglutarate dehydrogenase is induced by glutamate (or possibily by α-ketoglutarate). The control of α-ketoglutarate dehydrogenase has not been studied in any citric acid cycle mutants because of the highly variable levels of this enzyme in most mutants.

(ii) Succinate dehydrogenase. In the wild-type strain, the level of succinate dehydrogenase was repressed by fumarate and by malate (Fig. 3 C), but not by any of the other metabo-

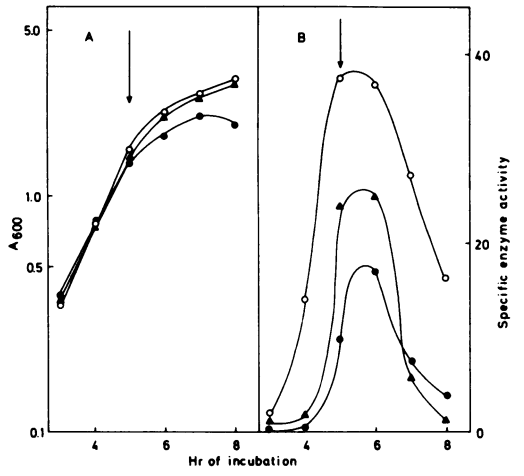

FIG. 2. *Effect of glutamate and aspartate on growth (A) and the level of α-ketoglutarate dehydrogenase (B) in B. subtilis wild type. Symbols: ●, unsupplemented NSMP medium; ○, NSMP plus 25 mM glutamate; ▲, NSMP plus 25 mM aspartate. Arrow indicates t_0 in all three cultures.*

lites tested. When the same experiment was done with a mutant lacking fumarase activity, only malate repressed succinate dehydrogenase (Fig. 3 D). This shows that malate and not fumarate is the true corepressor for succinate dehydrogenase. This conclusion was confirmed by the finding that malic enzyme-defective mutants, which accumulate malate, showed severely reduced levels of succinate dehydrogenase (Table 1). Furthermore, mutants unable to synthesize normal quantities of malate, e.g., mutants lacking α-ketoglutarate dehydrogenase or fumarase, had derepressed levels of succinate dehydrogenase (Table 1).

(iii) Fumarase. The addition of fumarate was found to increase the level of fumarase (Fig. 4). The increase was quite variable, ranging from 10 to 50% in different experiments. The level of fumarase was also slightly elevated by malate, while the other metabolites were without effect. Succinate dehydrogenase mutants had lower levels of fumarase than the wild type (Table 1), and the addition of fumarate increased the level of fumarase about twofold in these mutants (data not shown). This indicates that fumarase is induced by fumarate.

(iv) Malate dehydrogenase. The level of

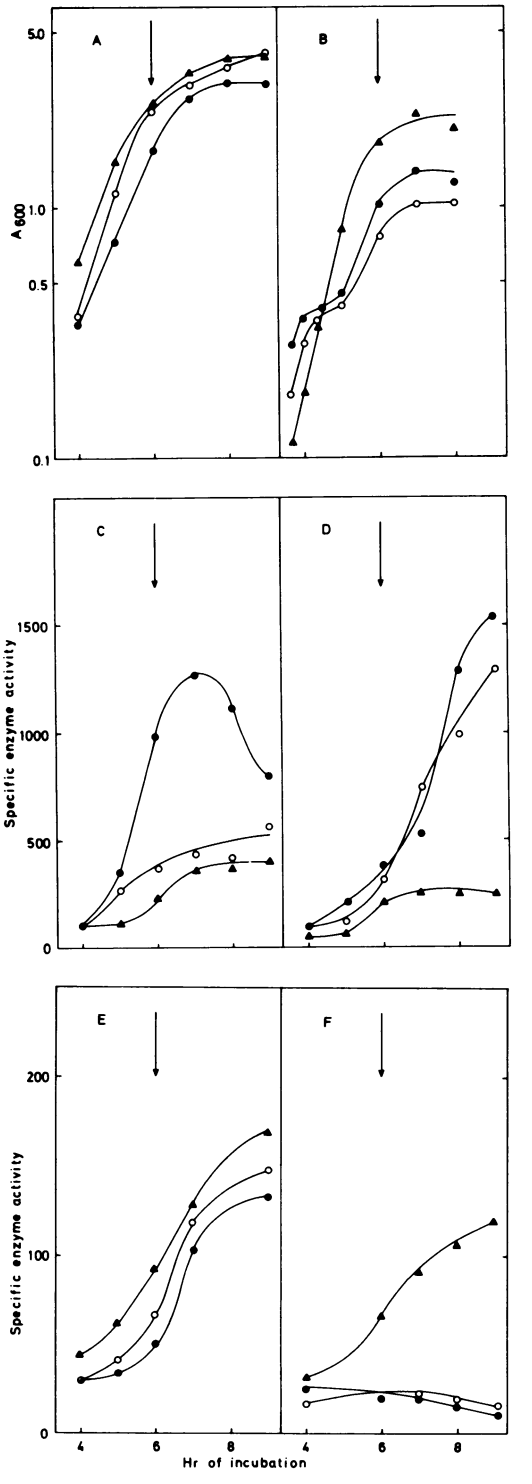

FIG. 3. *Effect of fumarate and malate on growth (A, B) and the levels of succinate dehydrogenase (C, D) and malic enzyme (E, F) in B. subtilis wild type (A, C, E) and CitG4 (Fum⁻) (B, D, F). Symbols: ●, unsupplemented NSMP medium; ○, NSMP plus 25 mM fumarate; ▲, NSMP plus 25 mM L-malate. Arrow indicates t_0 in the unsupplemented cultures.*

TABLE 1. *Specific activities of succinate dehydrogenase, fumarase, and malate dehydrogenase in B. subtilis wild type and different mutants*

Strain[a]	Enzymatic defect[b]	Specific activity[b]		
		SDH	FUM	MAL
Wild type	None	1,170	1,500	130
Cit K5	KDH	2,235	830	23
Cit F78	SDH	<1	920	32
Cit G4	FUM	1,925	<1	15
M471	MAL	180	ND[c]	<1

[a] Bacteria were grown in NSMP medium and were harvested at t_2.

[b] KDH, α-Ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; FUM, fumarase; and MAL, malic enzyme.

[c] ND, Not determined.

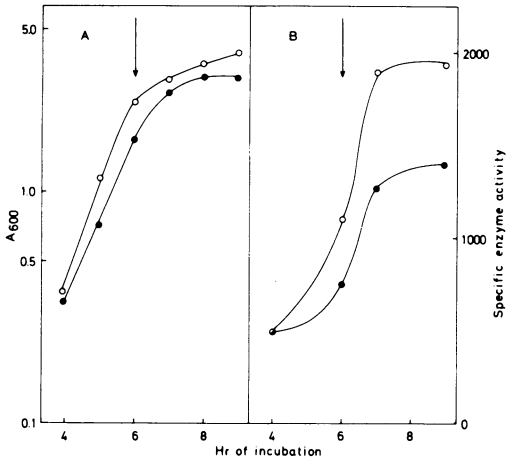

FIG. 4. *Effect of fumarate on growth (A) and the level of fumarase (B) in B. subtilis wild type. Symbols: ●, unsupplemented NSMP medium; ○, NSMP plus 25 mM fumarate. Arrow indicates t_0 in the unsupplemented culture.*

malate dehydrogenase was not changed by the addition of the six metabolites tested either in the wild type or in any citric acid cycle mutants.

(v) Malic enzyme. Both fumarate and malate caused a slight, but reproducible, increase in the level of malic enzyme in the wild type (Fig. 3 E). Again, the true effector could be identified in an experiment with the fumarase negative mutant. In this mutant, only malate increased the level of malic enzyme (Fig. 3 F). It is concluded that malic enzyme is induced by malate. This is consistent with the results reported by Diesterhaft and Freese (10). As expected, mutants impaired in the synthesis of malate because of a defect in α-ketoglutarate dehydrogenase, succinate dehydrogenase, or fumarase showed abnormally low levels of malic enzyme (Table 1).

(vi) Aspartase. The control of aspartase was considered to be of interest because this enzyme catalyzes the interconversion of aspartate and fumarate. It was found that the specific activity of aspartase remained constant throughout the growth cycle. The presence of aspartate caused a twofold increase in the level of aspartase (Table 2). It thus seems likely that aspartase functions primarily in the catabolism of aspartate and that the reverse reaction (the amination of fumarate to aspartate) is of little importance under physiological conditions. This conclusion is supported by the finding (20) that when fumarase negative mutants are supplied with ^{14}C-labeled fumarate, labeled succinate and fumarate is accumulated but labeled aspartate cannot be detected.

Effect of different metabolites on enzyme activities in vitro. A number of compounds were tested for effects on enzyme activities in vitro by the following procedure. In each enzyme assay, the extract was first incubated for 3 min with the compound to be tested and a mixture of all assay components except substrate. Enzyme activities were then measured after the addition of substrate. The results are summarized in Table 3.

The activity of malic enzyme was not significantly affected by any of the metabolites tested, but inhibition of citric acid cycle enzymes was observed in many cases. In general, inhibition did not exceed 25% even when the inhibiting metabolite was added to a final concentration of 1 mM. An exception is the very strong inhibition of α-ketoglutarate dehydrogenase and succinate dehydrogenase by oxalacetate. Half-maximal inhibition of succinate dehydrogenase was observed at 0.03 mM oxalacetate when the substrate concentration was 10 mM (Fig. 5). Preliminary data indicate that oxalacetate in-

TABLE 2. *Specific activity of aspartase in B. subtilis wild type grown in NSMP medium plus different supplements*

Supplement (25 mM)	Sp act[a]
None	44
Succinate	35
Fumarate	52
L-malate	41
Aspartate	80
Alanine	38

[a] Each value represents the average of 7 samples taken at 1-h intervals during exponential growth and stationary phase.

TABLE 3. *Effect in vitro of different metabolites on the specific activities of citric acid cycle enzymes and malic enzyme in B. subtilis wild type*

Compound	Concn (mM)	Inhibition (%) of enzyme activity[a]				
		KDH	SDH	FUM	MDH	MAL
Pyruvate	0.1	—[b]	3	0	12	0
	1.0	—	6	9	20	8
Citrate	0.1	0	0	—	0	—
	1.0	12	8	—	14	—
Isocitrate	0.1	3	0	—	0	—
	1.0	24	0	—	14	—
α-Ketoglutarate	0.1	—	1	0	4	0
	1.0	—	5	4	25	6
Succinate	0.1	0	—	6	0	0
	1.0	10	—	19	5	8
Fumarate	0.1	0	0	—	0	0
	1.0	16	18	—	8	6
Malate	0.1	16	4	—	7	—
	1.0	19	12	—	23	—
Oxalacetate	0.1	94	78	2	—	0
	1.0	100	95	3	—	5
Glutamate	0.1	4	0	13	0	0
	1.0	14	8	21	7	6
Aspartate	0.1	2	0	9	0	0
	1.0	11	0	6	12	6

[a] The extract was preincubated at 25 C for 3 min with the test compound and all assay components except substrate. The reaction was initiated by the addition of substrate. MDH, Malate dehydrogenase; other abbreviations as in Table 1.

[b] (—), Not determined.

hibits succinate dehydrogenase competitively with respect to substrate. The other cases of inhibition of citric acid cycle enzymes by metabolites have not been studied further.

Effect of adenine nucleotides on enzyme activities in vitro. The cell's energy balance has been shown to control the activity of several enzymes involved in energy metabolism. According to Atkinson (4), the energy charge of the adenylate pool, defined as half of the average number of anhydride-bound phosphate groups per adenine moiety, is an important regulatory parameter.

Figure 6 shows the effect of energy charge on certain citric acid cycle enzymes and malic enzyme in *B. subtilis*. Enzyme activities were measured in a mixture of ATP and adenosine monophosphate (AMP). The total nucleotide concentration was kept constant at 5 mM but the proportions of ATP and AMP were varied. Under these conditions, the energy charge is equal to the mole fraction of ATP, i.e., ATP/(ATP + AMP). The results were the same when the final nucleotide concentration was 2 mM instead of 5 mM.

The inhibition at high energy charge values observed with isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malic enzyme is typical of enzymes with catabolic functions (4). In contrast, malate dehydrogenase was inhibited at low energy charge values, a property generally associated with biosynthetic enzymes (4). The result was the same whether malate dehydrogenase was measured by following the malate-dependent reduction of nicotinamide adenine dinucleotide or the oxalacetate-dependent oxidation of reduced nicotinamide adenine dinucleotide. A rationale for these observations will be suggested in the Discussion.

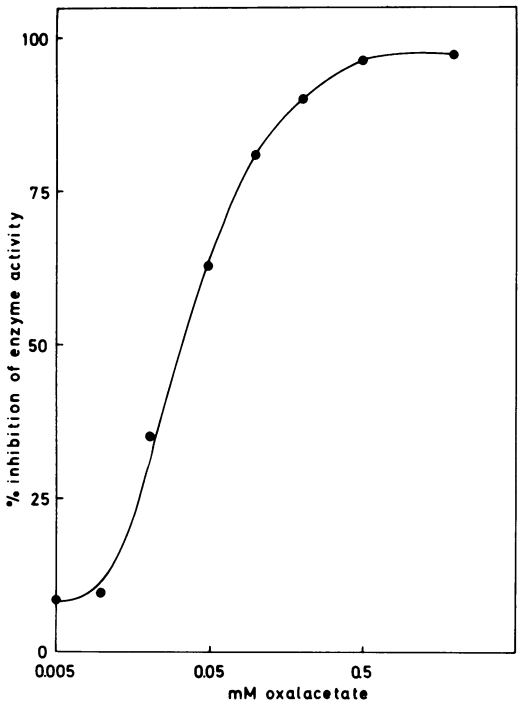

FIG. 5. *Inhibition of succinate dehydrogenase by oxalacetate in extracts of B. subtilis wild type. The extract was preincubated for 3 min with oxalacetate and all assay components except substrate. The reaction was initiated by the addition of 10 mM succinate. The protein concentration in the assay was 10 μg/ml.*

An energy charge response curve such as, for example, that obtained with succinate dehydrogenase could, in principle, be due either to stimulation of enzyme activity by AMP or to inhibition of enzyme activity by ATP. AMP and ATP were therefore also tested singly at a final concentration of 1 or 2 mM (Table 4). The effects of energy charge variations on enzyme activities reported above could in all cases be attributed to varying degrees of inhibition. ATP was found to be the most effective inhibitor of α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malic enzyme, whereas malate dehydrogenase was most severely inhibited by AMP.

The activities of aconitase and fumarase were not altered by the presence of adenine nucleotides.

Activation of α-ketoglutarate dehydrogenase in vitro. In the in vitro studies we observed that α-ketoglutarate dehydrogenase seemed to be activated by some component in the assay mixture. Thus, when the reaction was initiated by the addition of extract, maximal activity was not observed until after a lag of about 4 min. If, however, the extract was first incubated for 4 min in a mixture of all assay components except α-ketoglutarate, the activity was maximal almost immediately after the addition of substrate. The activation of α-ketoglutarate dehydrogenase during preincubation depended on the presence of magnesium ions and reduced glutathione (Fig. 7). Both compounds probably influence the structure of the multi-enzyme complex. Reduced glutathione may affect the conformation of the polypeptide chains by reducing disulfide bridges while magnesium ions may be required for the association of enzyme subunits.

DISCUSSION

This work is an investigation of the control of the dicarboxylic acid part of the citric acid cycle, leading from α-ketoglutarate to oxalacetate, in *B. subtilis*. As mentioned before, the control of α-ketoglutarate synthesis has already been studied in some detail in this organism. Thus, the overall regulation of the citric acid cycle in *B. subtilis* is now quite well understood. The control mechanisms identified up to now will be summarized and discussed below.

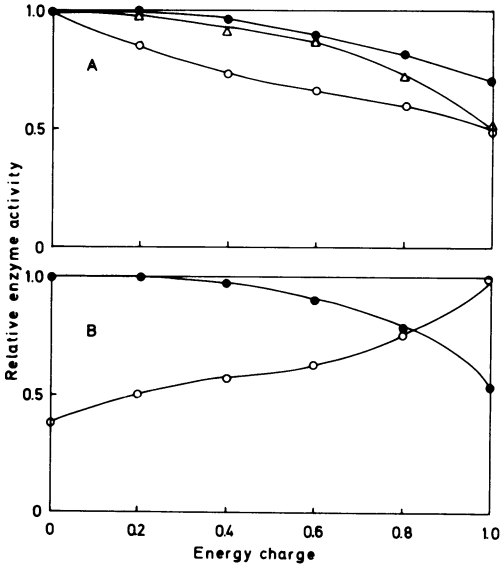

FIG. 6. *Effect of energy charge on citric acid cycle enzymes and malic enzyme in extracts of B. subtilis wild type. Enzymes were assayed in a mixture of AMP and ATP (total nucleotide concentration was 5 mM). The energy charge is equal to the mole fraction of ATP. Enzymes assayed were: (Panel A) isocitrate dehydrogenase (●), α-ketoglutarate dehydrogenase (○), succinate dehydrogenase (△); and (Panel B) malic enzyme (●), malate dehydrogenase (○).*

TABLE 4. *Effect in vitro of AMP and ATP on the specific activities of citric acid cycle enzymes and malic enzyme in B. subtilis wild type*

Compound	Concn (mM)	Inhibition (%) of enzyme activity[a]			
		KDH	SDH	MDH	MAL
AMP	1.0	8	0	38	11
	2.0	25	10	61	17
ATP	1.0	37	12	11	18
	2.0	60	21	33	33

[a] Enzyme abbreviations as in Tables 1 and 3.

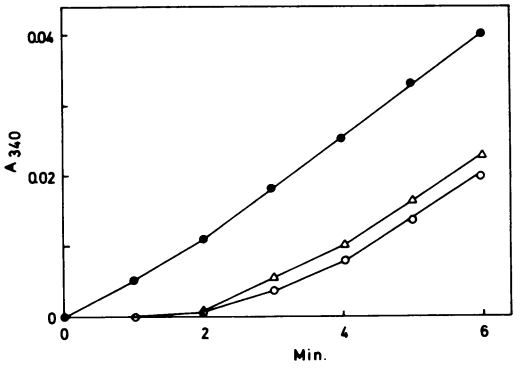

FIG. 7. *Effect of assay components on the activity of α-ketoglutarate dehydrogenase in extracts of B. subtilis wild type. The activity was measured after 3 min preincubation of a mixture containing all assay components except the following: ethylenediaminetetraacetate, CoA, thiamine pyrophosphate, or nicotinamide adenine dinucleotide (●); $MgCl_2$ or reduced glutathione (Δ); extract (O). The reaction was initiated by the addition of the missing assay component. The composition of the complete reaction mixture is given in the Materials and Methods section.*

Control of enzyme activity. The in vitro studies have revealed some interesting feedback inhibition mechanisms. First, oxalacetate is a powerful inhibitor of α-ketoglutarate dehydrogenase and succinate dehydrogenase. Inhibition of succinate dehydrogenase by oxalacetate is also well-known in mammalian systems (1).

Secondly, several citric acid cycle enzymes are inhibited by ATP. Since other adenine nucleotides may also be inhibitory, regulation should rather be expressed in terms of response to changes in the energy charge of the adenylate pool (4). It has been shown here that isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malic enzyme are inhibited at high energy charge values, as expected for enzymes involved in ATP production (4). The effect of energy charge on the activity of citrate synthase has not been investigated, but when the adenine nucleotides were tested singly, ATP was found to be a potent inhibitor of citrate synthase, while AMP was without significant effect (12).

In contrast, malate dehydrogenase is strongly inhibited by AMP and shows an energy charge response usually observed only for biosynthetic enzymes (4). The reason for this unexpected behavior is not clear. The finding that malate dehydrogenase and malic enzyme are differentially controlled by the adenine nucleotides suggests that the energy charge may influence the metabolism of malate. Thus, a drop in the energy charge will result in an increased proportion of malate being metabolized via malic enzyme to pyruvate. This, in turn, will lead to an increased synthesis of acetyl CoA by the action of pyruvate dehydrogenase. Such a control mechanism is clearly useful when the activity of the cycle is limited by the supply of acetyl CoA, as seems to be the case during sporulation in NSMP medium (26).

Studies on a variety of bacterial genera have shown that the energy charge is stabilized near 0.85 in exponentially growing cells but falls rapidly during the stationary phase (9). The extremely low energy charge value of 0.08 has been reported for *Bacillus* spores (9). It would be interesting to study the changes in energy charge during sporulation in relation to changes in the overall activity of the citric acid cycle.

Control of enzyme synthesis. The levels of the citric acid cycle enzymes increase markedly at the onset of sporulation. It is not known what mechanism brings about this increase, but an intact citric acid cycle is obviously required for sporulation (8, 18). The levels of certain enzymes, notably aconitase and α-ketoglutarate dehydrogenase, decrease abruptly after having reached a maximum early during sporulation, whereas the levels of other enzymes, such as isocitrate dehydrogenase (unpublished observations) and fumarase, remain essentially unchanged after t_2 to t_3. The reason for this difference is not known. It is possible that some enzymes are specifically repressed, inactivated, or destroyed (e.g., by proteolytic attack) during sporulation. Alternatively, all the citric acid cycle enzymes may be synthesized at a reduced rate, or not at all, at the later stages of sporulation. In that case, it must be assumed that the rate of turnover varies considerably between different enzymes. It is worth noting that the synthesis of ATP (26) and the rate of oxygen consumption (11) also show maxima early during sporulation and then decrease. These find-

ings suggest that the level of some citric acid cycle enzyme(s) may be the rate-limiting factor for respiration and oxidative phosphorylation during sporogenesis. Similar results have been obtained by Hanson et al. (23, 24) for *B. cereus*.

The addition of glucose to the growth medium leads to the repression of all the citric acid cycle enzymes. It is not known whether this phenomenon is related to the classical catabolite repression of degradative enzymes such as β-galactosidase in *Escherichia coli* (32) or if a novel type of control mechanism is involved. Attempts to increase the levels of the citric acid cycle enzymes during exponential growth in NSMP medium by the addition of cyclic adenosine 3′,5′-monophosphate have not been successful (unpublished observations).

The synthesis of the citric acid cycle enzymes is also regulated by specific induction and repression mechanisms, acting on individual enzymes. These mechanisms are outlined in the model shown in Fig. 8. It has been assumed that a metabolite which affects the specific activity of a certain enzyme when added in vivo, but not when added in vitro, acts primarily at the level of enzyme synthesis.

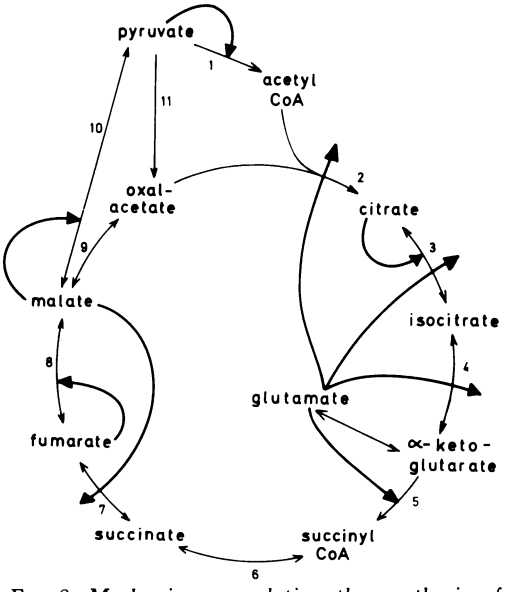

FIG. 8. *Mechanisms regulating the synthesis of citric acid cycle enzymes and some metabolically related enzymes in B. subtilis. The reactions shown are catalyzed by the following enzymes: pyruvate dehydrogenase (1), citrate synthase (2), aconitase (3), isocitrate dehydrogenase (4), α-ketoglutarate dehydrogenase (5), succinyl CoA synthetase (6), succinate dehydrogenase (7), fumarase (8), malate dehydrogenase (9), malic enzyme (10), and pyruvate carboxylase (11). (⟶) indicates induction, (⊣) indicates repression.*

The demonstration of induction or repression of enzyme synthesis by exogenously supplied citric acid cycle intermediates is complicated by the fact that these intermediates are apparently already present intracellularly at high concentrations, causing endogenous induction or repression. Another problem is the rapid metabolism of added intermediates. In some cases, these problems have been at least partially overcome by using the appropriate mutant strains.

Pyruvate dehydrogenase (17), α-ketoglutarate dehydrogenase, fumarase, and malic enzyme are all believed to be regulated by substrate induction (Fig. 8). Citrate synthase (12), isocitrate dehydrogenase (14), and succinate dehydrogenase are subject to feedback repression. Aconitase is unusual in that it responds to both these control mechanisms (30). The occurrence of inducible as well as repressible enzymes in the same pathway is not unique to the citric acid cycle in *B. subtilis*. Several amino acid biosynthetic pathways in other bacterial species are regulated in a similar manner (7). It has not yet been possible to determine whether α-ketoglutarate or glutamate is the true effector for the synthesis of citrate synthase, aconitase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. These regulatory functions have tentatively been assigned to glutamate (Fig. 8).

The level of malate dehydrogenase is not influenced by the addition of any of the citric acid cycle intermediates or their precursors. Yoshida (36) has suggested that this enzyme is synthesized constitutively in *B. subtilis*. It is also possible, however, that malate dehydrogenase is maximally induced by endogenously formed malate under the cultural conditions used.

The synthesis of citrate synthase and aconitase may be partially coordinate (12, 30), but the rest of the citric acid cycle enzymes are apparently regulated independently of each other. This is consistent with the finding (33) that the genes specifying the various enzymes are scattered over the chromosome.

Amarasingham and Davis (3) have demonstrated that α-ketoglutarate dehydrogenase is repressed in anaerobically grown *E. coli*. Under these conditions, succinyl CoA is synthesized reductively from oxalacetate rather than by oxidation of α-ketoglutarate. The citric acid cycle is thereby modified to a branched pathway, consisting of the oxidative branch (oxalacetate → α-ketoglutarate) and the reductive branch (oxalacetate → succinyl CoA) (3). We have observed (33) that *B. subtilis* mutants lacking α-ketoglutarate dehydrogenase are able

to grow on minimal glucose plates without added succinyl CoA (or succinate). Since the glyoxylate shunt is not operative in *B. subtilis* (17; unpublished observations), succinyl CoA must be synthesized by reduction of oxalacetate in these mutants. We do not know to what extent the reductive branch contributes to succinyl CoA synthesis in the wild-type strain. The regulatory mechanisms identified so far (Fig. 8) all seem to promote the oxidative functions of the cycle, but it cannot be excluded that the reductive branch plays an important role under other growth conditions than those used here.

In conclusion, the proper functioning of the citric acid cycle in *B. subtilis* depends on a number of regulatory mechanisms responding to the energy balance and the nutritional status of the cell. Substrate induction mechanisms permit the efficient utilization of certain citric acid cycle intermediates as carbon sources. Feedback repression and feedback inhibition mechanisms control the production of intermediates needed in biosynthetic pathways. The energy charge control system adjusts the activity of the cycle to the energy demand of the cell. Finally, when the ATP requirement can be met with glycolysis, the synthesis of the citric acid cycle enzymes is repressed so that only the biosynthetic functions are fulfilled.

Information on the regulation of the citric acid cycle in other bacteria is scarce. It is known, however, that in *E. coli*, aconitase, and isocitrate dehydrogenase are repressed by glutamate (24); α-ketoglutarate dehydrogenase is probably induced by α-ketoglutarate (3); citrate synthase and isocitrate dehydrogenase are inhibited by ATP (13); and all the citric acid cycle enzymes are repressed by glucose (21). Thus, at least some features of the control of the citric acid cycle are similar in as widely different species as the facultative gram-negative *E. coli* and the aerobic, gram-positive, spore-forming *B. subtilis*.

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