Activities of Anaplerotic Enzymes and Acetyl Coenzyme A Carboxylase in Biotin-deficient *Bacillus megaterium*

By R. M. AL-SSUM AND P. J. WHITE

Department of Microbiology, The University, Western Bank, Sheffield S10 2TN

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INTRODUCTION

Morphological deformity of *Bacillus megaterium* NCIB7581 grown at 37 °C in a minimal chemically defined medium could be prevented by addition of either biotin, aspartate or acetate (Al-ssum & White, 1977). The only carbon source in the minimal medium was glycerol or glucose, and the metabolism of either of these two substrates requires an adequate conversion of C₃ compounds [e.g. pyruvate or phosphoenolpyruvate (PEP)] to C₄ compounds anaplerotically, to replace citric acid cycle intermediates that are withdrawn for biosynthesis. Such conversion occurs in bacteria mainly by the action of a CO₂-fixing enzyme, either the biotin-dependent pyruvate carboxylase, or the biotin-independent PEP carboxylase; generally, organisms have one or other (not both) of these enzymes (Kornberg, 1966). Anaplerotic synthesis of C₄ compounds could also occur independently of CO₂-fixing enzymes by the operation of the glyoxylate cycle. Another biotin-requiring enzyme is acetyl coenzyme A carboxylase, which catalyses the ATP-dependent carboxylation of acetyl-CoA to yield malonyl-CoA (see review by Vagelos, 1964). In biotin-deficient organisms, the activity of this enzyme might be lowered, so that synthesis of fatty acids (via malonyl-CoA) might be impaired.

The nutritional evidence suggests that in *B. megaterium* NCIB7581 an active pyruvate carboxylase is necessary unless a source of C₄ compounds (e.g. aspartate) is available or unless the glyoxylate cycle is functioning. In this paper, activities of three anaplerotic enzymes and of acetyl-CoA carboxylase and malic enzyme are compared in organisms grown with and without biotin. A preliminary report of some of these results has been made (Al-ssum & White, 1974).

METHODS

Organism and growth conditions. *Bacillus megaterium* NCIB7581 was maintained on nutrient agar slopes at 37 °C, subcultured monthly and stored at 2 °C. Batches of 500 ml medium A1 (White, 1972), with or without biotin (10 µg l⁻¹) or other supplements, in 2 l flasks were inoculated with 1.0 ml of a suspension of organisms from nutrient agar (about 0.04 mg dry wt bacteria ml⁻¹) and incubated with shaking (200 gyrations min⁻¹) at 37 °C or 30 °C for 12 to 15 h. Early in the exponential growth phase [EEL colorimeter reading with neutral density filter (no. 1.0) about 1.0; about 0.5 mg dry wt organisms ml⁻¹], the bacteria were harvested by centrifuging at 5000 g and washed in 0.05 M-Tris/HCl buffer, pH 7.4. At later stages of growth *B. megaterium* NCIB7581 was much less susceptible to the action of lysozyme (see below).

Preparation of extracts. (i) Organisms were suspended in 0.1 M-Tris/HCl buffer, pH 7.8, and sonicated in an MSE 125 W apparatus for a total of 3 min, with cooling at 2 °C for 1 min between each 1 min burst of sonication. Supernatant liquid was kept after centrifuging at 17000 g at 2 °C.

(ii) Organisms in the same buffer were put through the pressure cell of Milner, Lawrence & French (1950) at 2 °C and 1.4×10^8 Pa. Supernatant liquid was kept after centrifuging at 12000 g at 2 °C.

(iii) Bacterial suspension (about 5 mg dry wt ml⁻¹) containing 120 µg lysozyme ml⁻¹, in the desired enzymic assay system buffer (0.1 M) at the pH value used for the subsequent enzyme assay, was incubated at 30 °C for 30 min. To obtain a homogeneous extract, the viscous lysate was sonicated for 30 s (as above) at 2 °C and centrifuged at 17000 g.

Assays. Pyruvate carboxylase (EC 6.4.1.1) was assayed by a modification of the method of Sundaram, Cazzulo & Kornberg (1969) in which 0.5 µmol acetyl-CoA was used and citrate synthase (1 unit) replaced malate dehydrogenase and NADH. The reaction was started by addition of extract (50 µl; about 0.25 mg protein), and the system was incubated at 30 °C for 10 to 15 min. The reaction was stopped by adding 1.5 ml ethanol. Samples were removed to scintillation vials, acidified, dried and counted in a fluid containing: Triton X-100, 333 ml; xylene, 667 ml; 2,5-diphenyloxazole, 5 g; 1,4-di-2(5-phenyloxazoly)benzene, 0.5 g.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was assayed similarly except that in the assay system the pH value was 8.6, PEP was substituted for pyruvate, and ATP and KCl were omitted.

Acetyl-CoA carboxylase (EC 6.4.1.2) was assayed by the method of Alberts & Vagelos (1968).

Isocitrate lyase (EC 4.1.3.1) was assayed by the method of Dixon & Kornberg (1959).

Malic enzyme (EC 1.1.1.38 with NAD⁺; EC 1.1.1.40 with NADP⁺) was assayed manometrically at pH 7.4 (Scrutton, 1971). After equilibration at 30 °C the reaction was started by tipping the enzyme (about 5 mg protein) from the side arm. After 15 min, 1 ml 0.5 M-H₂SO₄ was tipped from the second side arm and the total CO₂ evolved was measured.

Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Chemicals. Acetyl-CoA was prepared from coenzyme A and acetic anhydride by a method similar to that used for the synthesis of succinyl-CoA (Simon & Shemin, 1953). Citrate synthase and malate dehydrogenase were from Boehringer; lysozyme (egg white) from BDH; and NaH¹⁴CO₃ from The Radiochemical Centre, Amersham.

RESULTS AND DISCUSSION

Pyruvate carboxylase was measured in extracts of *B. megaterium* by fixing ¹⁴CO₂ from bicarbonate to pyruvate – this produced oxaloacetate which, with acetyl-CoA and citrate synthase, was converted to citrate before counting the radioactivity. A gentle preparation of extracts (i.e. by lysozyme digestion of the wall) was essential to obtain an active enzyme; extracts prepared by sonication or the pressure cell did not incorporate ¹⁴CO₂, which suggests that the enzyme was damaged by the last two methods of extraction. Spectrophotometric assays were unsuccessful owing to the presence of NADH oxidase and malic enzyme, which interfered with the method of Utter & Keech (1963), and deacylating activity, which interfered with the method of Martin & Denton (1970).

High activity of pyruvate carboxylase was found only in organisms grown at 30 °C with or without biotin, or at 37 °C with biotin (Table 1). Enzyme activity was decreased by omission of sodium pyruvate (13 % activity), ATP (30 % activity) or acetyl-CoA (3 % activity) from the assay system. The highest measured activities of pyruvate carboxylase were rather lower than might be expected from the observed rate of growth [about 30 nmol min⁻¹ (mg protein)⁻¹] though some enzyme may well be lost during the most gentle extraction.

Table 1. *Enzymic activities in extracts of B. megaterium* NCIB7581
after growth at 37 °C in medium A1

Cultures were grown and extracts were prepared and assayed for enzymes as described in Methods. The three carboxylases were assayed in protoplast lysates; activities are expressed as nmol $^{14}\text{CO}_2$ fixed min^{-1} (mg protein) $^{-1}$. Malic enzyme was assayed in press extracts; activities are expressed as nmol CO_2 evolved min^{-1} (mg protein) $^{-1}$. Isocitrate lyase was assayed in sonic extracts; activities are expressed as nmol glyoxylate formed min^{-1} (mg protein) $^{-1}$.

Enzyme	Addition to medium			
	No addition	Biotin (10 $\mu\text{g l}^{-1}$)	L-Aspartate (100 mg l^{-1})	*Sodium acetate. 3H $_2$ O (0.5 g l^{-1})
Pyruvate carboxylase	8.7 (22)†	25 (24)†	7.8	5.7
PEP carboxylase	1.1	1.6	1.4	1.8
Acetyl-CoA carboxylase	1.7	7.8	2.0	3.9
Isocitrate lyase	3.2	0.9	0.7	16
Malic enzyme, NAD $^{+}$ cofactor	11	13	15	16
Malic enzyme, NADP $^{+}$ cofactor	14	23	26	22

* Only 0.25 % (w/v) glycerol in medium.

† Activities in organisms grown at 30 °C.

If biotin was absent from the growth medium at 37 °C enzymic activity decreased to one-third, which might be a cause of the slower growth and deformity of the organisms. Neither acetate nor aspartate, which allowed normal growth at 37 °C without biotin, increased the activity of pyruvate carboxylase (Table 1), and so the effects of these two compounds are not due to biotin present as an impurity. Indeed, aspartate inhibited pyruvate carboxylase when added to the assay system; 0.6 mM-aspartate caused 50 % inhibition. Cazzulo, Sundaram & Kornberg (1970) found that aspartate inhibited formation of active pyruvate carboxylase from biotin and apoenzyme.

Phosphoenolpyruvate carboxylase. Only low activity of this enzyme was found in extracts (Table 1), even though the same extracts contained pyruvate carboxylase. Since this latter labile enzyme was detected, it seems unlikely that PEP carboxylase had been present in the organisms but lost during extraction. Furthermore, when the assay system was tested with an extract of *Pseudomonas* AM1 grown on methanol (given by Mr A. A. Hancock of this Department), a PEP carboxylase activity of 45 nmol min^{-1} (mg protein) $^{-1}$ was found.

Acetyl-CoA carboxylase. Organisms grown at 37 °C without biotin had less acetyl-CoA carboxylase activity than those grown with biotin (Table 1). The presence of acetate or aspartate in the medium without biotin at 37 °C caused a slight increase in enzymic activity, possibly by sparing the need for biotin as a cofactor of pyruvate carboxylase. Aspartate in the assay system was inhibitory; 0.5 mM-aspartate caused 50 % inhibition.

The activity of acetyl-CoA carboxylase in organisms grown at 37 °C with aspartate but without biotin was considerably lower than might be expected from the observed rate of growth [8 nmol min^{-1} (mg protein) $^{-1}$]. Possibly much of the enzyme is destroyed during extraction, but the inhibition by aspartate suggests that this enzyme need not function if aspartate is available, when malonate might perhaps be formed by a different route. In bush bean roots, oxaloacetate (from aspartate) is decarboxylated by an oxidative system to yield malonate (Shannon, de Villes & Lew, 1963). The bacterial enzymes, aspartate 1-decarboxylase (see review by Meister, 1965), β -alanine aminotransferase (Hayaishi & Nishizuka, 1962) and malonic semialdehyde dehydrogenase (Nakamura & Bernheim, 1961) acting in sequence also might provide a route from aspartate to malonate.

Isocitrate lyase (glyoxylate cycle). The effectiveness of acetate in allowing normal growth

without biotin at 37 °C was probably a result of inducing the glyoxylate cycle (Table 1). Isocitrate lyase was found at relatively high activity only after growth with acetate (plus glycerol). The products of the enzyme reaction were identified as glyoxylate phenylhydrazones (two isomers) by their absorption at 325 nm and by the chromatographic properties of the 2,4-dinitrophenylhydrazones.

Malic enzyme. This enzyme was assayed because it might be present in biotin-deficient organisms and able to convert pyruvate to malate rather than catalysing the reverse reaction. Activity of malic enzyme was highest under growth conditions where the supply of C₄ compounds was presumably adequate (Table 1). This finding supports the view that the role of malic enzyme is to provide a supply of NADPH and to regulate the intracellular concentration of C₄ compounds (cf. Krulwich, Sharon & Perrin, 1976).

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REFERENCES

- ALBERTS, A. W. & VAGELOS, P. R. (1968). Acetyl CoA carboxylase. 1. Requirement for two protein fractions. *Proceedings of the National Academy of Sciences of the United States of America* **59**, 561–568.
- AL-SSUM, R. M. & WHITE, P. J. (1974). Biotin deficiency in *Bacillus megaterium*. *Proceedings of the Society for General Microbiology* **1**, 69–70.
- AL-SSUM, R. M. & WHITE, P. J. (1977). Effects of biotin-deficiency on growth, morphology and sporulation in *Bacillus megaterium* NCIB7581. *Journal of General Microbiology* **99**, 343–351.
- CAZZULO, J. J., SUNDARAM, T. K. & KORNBERG, H. L. (1970). Mechanism of pyruvate carboxylase formation from the apo-enzyme and biotin in a thermophilic bacillus. *Nature, London* **227**, 1103–1105.
- DIXON, G. H. & KORNBERG, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochemical Journal* **72**, 3P.
- HAYAISHI, O. & NISHIZUKA, Y. (1962). β -Alanine- α -alanine transaminase (*Pseudomonas*). *Methods in Enzymology* **5**, 698–702.
- KORNBERG, H. L. (1966). Anaplerotic sequences and their role in metabolism. In *Essays in Biochemistry*, vol. 2, pp. 1–31. Edited by P. N. Campbell and G. D. Greville. London and New York: Academic Press.
- KRULWICH, T. A., SHARON, B. I. & PERRIN, L. S. (1976). Natural paucity of anaplerotic enzymes: basis for dependence of *Arthrobacter pyridinolis* on L-malate for growth. *Journal of Bacteriology* **127**, 179–183.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MARTIN, B. R. & DENTON, R. M. (1970). The intracellular localization of enzymes in white-adipose-tissue fat-cells and permeability properties of fat cell mitochondria. Transfer of acetyl units and reducing power between mitochondria and cytoplasm. *Biochemical Journal* **117**, 861–877.
- MEISTER, A. (1965). *Biochemistry of the Amino Acids*, 2nd edn, p. 335. New York and London: Academic Press.
- MILNER, H. W., LAWRENCE, N. S. & FRENCH, C. S. (1950). Colloidal dispersion of chloroplast material. *Science* **111**, 633–634.
- NAKAMURA, K. & BERNHEIM, F. (1961). Studies on malonic semialdehyde dehydrogenase from *Pseudomonas aeruginosa*. *Biochimica et biophysica acta* **50**, 147–152.
- SCRUTTON, M. C. (1971). Assays of enzymes of CO₂ metabolism. *Methods in Microbiology* **6A**, 479–541.
- SHANNON, L. M., DE VELLIS, J. & LEW, J. Y. (1963). Malonic acid biosynthesis in bush bean roots. II. Purification and properties of enzyme catalyzing oxidative decarboxylation of oxaloacetate. *Plant Physiology, Lancaster* **38**, 691–697.
- SIMON, E. J. & SHEMIN, D. (1953). The preparation of S-succinyl coenzyme A. *Journal of the American Chemical Society* **75**, 2520.
- SUNDARAM, T. K., CAZZULO, J. J. & KORNBERG, H. L. (1969). Anaplerotic CO₂ fixation in mesophilic and thermophilic bacilli. *Biochimica et biophysica acta* **192**, 355–357.
- UTTER, M. F. & KEECH, D. B. (1963). Pyruvate carboxylase. 1. Nature of the reaction. *Journal of Biological Chemistry* **238**, 2603–2608.
- VAGELOS, P. R. (1964). Lipid metabolism. *Annual Review of Biochemistry* **33**, 139–172.
- WHITE, P. J. (1972). The nutrition of *Bacillus megaterium* and *Bacillus cereus*. *Journal of General Microbiology* **71**, 505–514.