Stability of Enzymes in Starving *Arthrobacter crystallopoietes*

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SUMMARY

Cell-free extracts prepared from spherical and rod-shaped cells of *Arthrobacter crystallopoietes* were assayed for enzymes during various periods of starvation. The level of NADH oxidase dropped to 20 and 30 %, respectively, in spherical and rod-shaped cells during the first 1 to 2 days of starvation and then remained constant for 9 days. Catalase activity decreased continuously and reached a low level in 9 days. Enzymes involved in glucose metabolism and the tricarboxylic acid cycle were stable for the duration of the experiment (about 1 week). Succinic dehydrogenase, fumarase and aconitase were stable during 21 days of starvation, which is the longest time enzymes have been shown to be stable in any bacterium under conditions of total starvation.

INTRODUCTION

The genus *Arthrobacter* has been reported to be the most widespread and abundant micro-organism in soil (Gounot, 1967; Mulder, 1963; Mulder & Antheunisse, 1963), and highly resistant to starvation (Boylan & Ensign, 1970a; Zevenhuizen, 1966) and desiccation (Boylan, 1973). When starved, *Arthrobacter crystallopoietes* maintains 100 % viability for 30 days. In general, there is a lack of physiological studies on starving bacteria and few reports on the stability of enzymes during starvation. Since an organism able to maintain essential enzymes during starvation will be better adapted for surviving adverse conditions than its counterpart unable to do so, studies were undertaken to determine the stability of various enzymes in a starvation-resistant organism, *A. crystallopoietes*. A preliminary report of these findings has appeared (Meganathan & Ensign, 1972).

METHODS

Growth conditions. *Arthrobacter crystallopoietes* (ATCC15481) was grown in the minimal salts medium of Boylen & Ensign (1970a). For growing cells in the form of spheres the medium was supplemented with 0.5 % glucose, and for rods glucose was replaced by 0.5 % ammonium succinate. Glucose and succinate were sterilized separately. About 50 to 100 ml of glucose-grown cells were inoculated into 0.5 to 1 l of glucose or succinate media and grown to about 200 Klett units. This cell density corresponds to a dry weight of 0.5 mg ml⁻¹ and approximately 10⁹ cells ml⁻¹ (Petroff-Hauser counts) for spheres, and 1.0 mg ml⁻¹ and 2.5 × 10⁸ cells ml⁻¹ for rods.

Starvation conditions. Bacteria (spheres (cocci) or rods) were harvested by centrifuging at 4000 g for 5 to 10 min at 2 to 4 °C. The bacteria were washed twice with an approximately equal volume of 0.03 M-potassium phosphate buffer pH 7.0, by centrifugation and resuspension. The bacteria were suspended in buffer at room temperature and starved by incubating

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at 30 °C with shaking. No change in viability was observed for the duration of the experiments.

Preparation of cell-free extracts. Samples were withdrawn at various times during starvation, centrifuged and kept frozen. The bacteria were suspended in 0.03 M-phosphate buffer pH 7.0 and extracts used for the assay of NADH oxidase and catalase were prepared either by sonication or by grinding with aluminium oxide. For tricarboxylic acid (TCA) cycle enzymes, extracts were prepared by grinding with aluminium oxide. Bacteria for preparing the extracts used in assaying enzymes of glucose catabolism were washed in 0.02 M-tris-HCl buffer, pH 7.4, suspended in the same buffer, and passed through a cold French pressure cell twice at 41 to 48 MPa. During the preparation of extracts, care was taken to maintain the temperature below 5 °C. The extracts were centrifuged at 3000 g for 5 min and the supernatant was further centrifuged at 17000 g for 15 min. The clear supernatant was used in all enzyme assays.

Preparation of toluenized cells. After various times of starvation, 8 ml of bacteria were centrifuged and suspended in 0.8 ml of 0.03 M-potassium phosphate buffer pH 7.0. After adding 2 drops of toluene, the tubes were shaken at 30 °C for 30 min, 0.5 ml of the toluenized bacteria was mixed with 0.5 ml of 0.03 M-potassium phosphate buffer pH 7.0, and 0.1 ml of this suspension was used in enzyme assays.

Protein determination. The procedure of Sutherland *et al.* (1949) was used, with bovine serum albumin as the standard.

Units of enzyme activity. The activities of NADH oxidase and catalase were calculated as $\Delta E \text{ min}^{-1} (\text{mg protein})^{-1}$. The activity of the zero time sample was taken as 100 and the activities are expressed as the percentage of the initial activity. All the other activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Enzyme assays. The assays were done spectrophotometrically at room temperature using a Cary 15 recording spectrophotometer. NADH oxidase was assayed in a volume of 1.0 ml, and all the other assays in a volume of 3.0 ml.

NADH oxidase. NADH oxidation was measured at 340 nm according to the method of Mackler & Green (1956).

Catalase was measured spectrophotometrically as decrease in extinction at 240 nm (Bergmeyer, 1965).

Aconitase [Citrate (isocitrate) hydro-lyase, EC. 4.2.1.3]. The conversion of isocitrate to *cis*-aconitate was measured as increase in extinction at 240 nm (Racker, 1950). The molar extinction coefficient was taken as $3.3 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Mahler, Wittenberger & Brand, 1958).

Isocitrate dehydrogenase (threo-D₂-isocitrate:NADP⁺ oxidoreductase, EC. 1.1.1.42). The isocitrate-dependent reduction of NADP was measured at 340 nm (Ochoa, 1955). NADP was omitted from the blank. Since the reaction slows down quickly, all components except the enzyme were mixed first, and after mixing in the enzyme, recording was started immediately. Enzyme activity was calculated from the initial rate. The molar extinction coefficient of NADPH was taken as $6.22 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$.

Succinate dehydrogenase [Succinate:(acceptor) oxidoreductase, EC. 1.3.99.1]. The procedure of Owen & Freer (1970), based on the method of Ells (1959), was used. Substrate was omitted from the blank. The position of the cuvettes was reversed (i.e. the experimental compartment contained the blank and the blank compartment contained the experimental cuvette) and the increase in extinction was recorded at 600 nm. The molar extinction coefficient for the reduction of dichlorophenolindophenol (DCIP) was taken as $1.61 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Rutberg & Hoch, 1970).

Fumarase (L-malate hydro-lyase, EC. 4.2.1.2). The reduction of malate to fumarate was followed at 240 nm (Hanson & Cox, 1967). Substrate was omitted from the blank. The molar extinction coefficient for fumarate was taken to be $2.4 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Bock & Alberty, 1953).

Malate dehydrogenase (L-malate: oxygen oxidoreductase, EC. 1.1.3.3). The flavin-linked malate dehydrogenase was assayed according to Phizackerley (1969) except that KCN was added to the reaction mixture (Francis *et al.*, 1963). The position of the cuvettes was reversed and the reduction of DCIP was measured at 600 nm.

Aldolase (fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate lyase, EC. 4.1.2.13). The experimental cuvette contained 66.7 mM-tris-HCl buffer, pH 8, 100 mM-KCl, 1 mM-dithiothreitol, 0.156 mM-NADH, 2.5 mM-fructose 1,6-diphosphate, triose phosphate isomerase and α -glycerophosphate dehydrogenase in excess units, and extract. To correct for the NADH oxidase present in the extract, NADH was added to both the blank and the experimental cuvette. The blank lacked substrate. The positions of the cuvettes were reversed and the increase in extinction was recorded at 340 nm.

Phosphohexose isomerase (D-glucose 6-phosphate ketol-isomerase, EC. 5.3.1.9). The method of Gale & Beck (1967) was used. Substrate was omitted from the blank.

Fructose diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC. 3.1.3.11). The procedure resembled that for phosphohexose isomerase; fructose 6-phosphate was replaced by fructose 1,6-diphosphate.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP⁺ oxidoreductase, EC. 1.1.1.49). The procedure was based on that for phosphohexose isomerase; glucose 6-phosphate dehydrogenase was omitted and fructose 6-phosphate was replaced by glucose 6-phosphate.

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP⁺ oxidoreductase, EC. 1.1.1.43). The procedure was the same as for glucose 6-phosphate dehydrogenase except that glucose 6-phosphate was replaced by gluconate 6-phosphate.

RESULTS

Enzyme stability

Stability of NADH oxidase. The levels of NADH oxidase in both spherical and rod-shaped cells during various times of starvation are shown in Fig. 1.

Stability of catalase. Catalase activity in spherical cells decreased progressively up to nine days and reached a very low level (Fig. 2). In rod-shaped cells, activity decreased but the level was always higher than in spherical cells.

Stability of glucose metabolic enzymes. Two enzymes of the hexose monophosphate pathway (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), two enzymes of glycolysis (phosphohexose isomerase and aldolase), and one enzyme involved in gluconeogenesis (fructose diphosphatase) were assayed during starvation for up to a week (Table 1). The activities increased for the first two to three days and then remained constant for the rest of the experiment.

Stability of TCA cycle enzymes. Aconitase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase were stable during starvation for about a week in extracts of spherical and rod-shaped cells (Table 2). Rod-shaped cells had more enzymic activity than spherical cells. There was an initial increase in the specific activities of all the enzymes in both spheres and rods. The effect of prolonged starvation on three TCA cycle enzymes, succinate dehydrogenase, fumarase and aconitase, was studied during 21 days of

Fig. 1

Fig. 2

Fig. 1. NADH oxidase activity in spherical and rod-shaped cells of *A. crystallopoietes* during various times of starvation. Activity in spheres (●) and rods (○).

Fig. 2. Catalase activity in spherical and rod-shaped cells of *A. crystallopoietes* during various times of starvation. Activity in spheres (●) and rods (○).

Table 1. *Effect of starvation on glucose metabolic enzymes in cocci*

All activities are given as nmol of product formed/min per mg protein, and aldolase as nmol of substrate used/min per mg protein. G-6-P DH, glucose 6-phosphate dehydrogenase; 6-P-G DH, 6-phosphogluconate dehydrogenase; PHI phosphohexose isomerase; FDP, fructose diphosphatase.

Starvation time (h)	G-6-P DH	6-P-G DH	PHI	FDP	Aldolase
0	8.4	53	222	4.4	195
19	10.0	67	285	5.8	167
47	9.7	49	327	4.2	209
125	9.3	50	253	6.0	213
173	7.1	38	266	5.0	221

Table 2. *Effect of starvation on TCA cycle enzymes in cocci and rods*

All activities are given as nmol of product formed/min per mg protein. IDH, isocitrate dehydrogenase; SDH, succinate dehydrogenase; MDH, malate dehydrogenase.

Starvation time (h)	Aconitase		IDH		SDH		Fumarase		MDH	
	Cocci	Rod	Cocci	Rod	Cocci	Rod	Cocci	Rod	Cocci	Rod
0	150	442	219	390	26	51	137	729	80	213
17	242	550	264	516	32	70	264	811	88	224
41	—	560	—	488	—	80	—	753	—	254
45	223	—	284	—	43	—	240	—	108	—
89	219	621	310	572	44	87	285	948	104	227
161	141	427	292	562	37	86	172	701	108	216

starvation (Fig. 3). After an initial increase, which varied between enzymes, the activities of succinate dehydrogenase and fumarase remained constant. There was a slight decrease in the activity of aconitase, possibly due to instability of the enzyme during preparative procedures.

Enzyme activity in toluenized cells

To determine whether the initial increase in specific activity (activity/min per mg protein) was due to new enzyme synthesis or to the selective degradation of proteins (non-enzymic and certain enzymic proteins) resulting in an apparent increase in specific activity

Fig. 3. Succinic dehydrogenase, fumarase and aconitase activities during prolonged starvation of rod-shaped cells of *A. crystallopoietes*. Activity of succinic dehydrogenase (●), fumarase (▲) and aconitase (○).

(due to a decrease in total protein but not the enzymic proteins reported here) succinate dehydrogenase and fumarase were assayed in toluene-treated bacteria. There was no increase in activity up to five days, although the same enzymes have shown an increase when assayed in extracts.

DISCUSSION

The Embden–Meyerhof–Parnas (EMP) and pentose phosphate pathways and TCA cycle operate in *A. crystallopoietes* (Krulwich & Ensign, 1969). After an initial rapid decrease in endogenous respiration to a very low level, the rate remained constant for 24 days (Boyle & Ensign, 1970*b*). The stability of selected enzymes representing the above pathways was examined during cell starvation.

Although the starving bacteria possessed high stable levels of TCA cycle enzymes, the ability of whole cells to oxidize succinate is rapidly lost (Boyle & Ensign, 1970*b*). To reconcile this discrepancy, succinate dehydrogenase activity was measured in succinate-grown whole cells after various periods of starvation. Succinate-dependent reduction of DCIP was rapidly lost during starvation. As the same cells treated with toluene showed high levels of succinate dehydrogenase, the loss of succinate-oxidizing ability in whole cells during starvation is due to the bacteria becoming impermeable.

There is a consistent increase in the specific activities of the enzymes during the first two days of starvation, which can be brought about either by new enzyme synthesis or by the selective degradation of proteins other than the enzymes studied here. Synthesis of new enzymes in the absence of carbon, nitrogen or energy source seemed improbable. Protein degradation occurs during starvation (Boyle & Ensign, 1970*b*), and during this study when cell extracts were assayed for protein content this showed a tendency to decrease irrespective of the method used for preparing the extracts. During the first two days of starvation, when most enzymes increased in specific activity, three enzymes, NADH oxidase, catalase and 2-hydroxypyridine mono-oxygenase (Boyle & Ensign, 1970*b*) decreased in specific activities. Since specific activities are expressed on the basis of the protein content of extracts, if the quantity of enzyme remains constant and the total protein decreases then an apparent increase in specific activity is to be expected. If the enzymes are assayed in whole cells (made permeable by toluene) and if the activities are expressed on the basis of constant volume, the initial increase in activity would not be seen unless there is new synthesis. This is because the number of molecules of enzymes per unit volume of sample should be the same, since

the number of bacteria per volume is the same. Succinate dehydrogenase and fumarase were assayed in whole cells made permeable by toluene and the activities were calculated as units/ml culture. Neither enzyme increased in activity during five days of starvation.

Though the TCA cycle enzymes are stable during starvation in both rods and cocci, the levels of enzymes were always higher in growing and starving rods than in cocci. In *Pseudomonas aeruginosa*, an organism which, like *A. crystallopoietes*, uses TCA intermediates in preference to glucose, the activities of TCA enzymes are about the same in both glucose-grown and succinate-grown cells (Tiwari & Campbell, 1969). The lower level of TCA enzymes in cocci might be due to the very slow growth of *A. crystallopoietes* on glucose with the consequent decrease in demand for TCA intermediates for biosynthetic purposes.

NADH oxidase activity dropped to 20 and 30 % in cocci and rod-shaped cells respectively and then remained constant; endogenous respiration also decreases (Boylen & Ensign, 1970a).

Boylen & Ensign (1970b) reported that 2-hydroxypyridine mono-oxygenase, an inducible enzyme of *A. crystallopoietes*, was completely lost in about two days of starvation. Postgate & Hunter (1962) studied glycerol dehydrogenase in whole cells of *Aerobacter aerogenes* during starvation and reported a 50 % loss of activity in 7½ h, with a comparable loss in viability. Strange (1966) studied β -galactosidase in starving *Escherichia coli* and found a loss of 29 %, while there was only a 5 % decrease in viability in 30 h. Willets (1967) reported a 35 % loss in β -galactosidase activity when *E. coli* was starved of ammonia and leucine for 4 h, while D-serine deaminase and alkaline phosphatase were stable.

In contrast to the above reports, Bentley & Dawes (1974) reported that starvation of *Peptococcus prevotii*, an anaerobic bacterium, for 33 h resulted in complete loss of viability. When the specific activities of enzymes were measured, they found that serine dehydratase and threonine dehydratase showed 32 and 36 % decreases in activity while phosphotrans-acetylase and acetate kinase activities increased by 16 and 28 % respectively during this period.

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