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RIBULOSE 1,5-BISPHOSPHATE DEPENDENT CO_2 FIXATION IN THE HALOPHILIC ARCHAEBACTERIUM, HALOBACTERIUM MEDITERRANEI

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The cell extract of Halobacterium mediterranei catalyses incorporation of $^{14}CO_2$ into 3-phosphoglycerate in the presence of ribulose bisphosphate suggesting the existence of ribulose bisphosphate carboxylase activity in this halophilic archaebacterium.

Light dependent CO_2 assimilation in the surface water samples from Dead Sea was observed by Oren (1) and was attributed to the activity of purple membrane-containing halobacteria. Propionate-stimulated CO_2 fixation was also demonstrated in Halobacterium halobium cells grown anaerobically (2). Their assumption that α-ketobutyrate was formed from propionate and CO_2 was questioned by Kerscher and Oesterhelt (3) since the reaction products were not identified and no electron donor was added.

Halobacteria which are described as chemoheterotrophs (4) have never been shown capable of autotrophic growth. Carbohydrate metabolism in halophilic archaebacteria is a subject of study in our laboratory (manuscript communicated) and at least in one strain ribulose bisphosphate (RuBP) dependent CO_2 fixation was observed after growth in heterotrophic conditions. While we were examining the details of CO_2 fixation, an analytical study by Javor (5) on CO_2 fixation in halobacteria was published. α-ketobutyrate was not detected and the product of NH_4. propionate stimulated CO_2 fixation was identified as glycine and that from pyruvate as pyruvate. However, no RuBP carboxylase activity could be detected in H. halobium. This note presents preliminary data on the RuBP dependent CO_2 fixation which could be attributed to the activity of RuBP carboxylase in the cell extracts of H. mediterranei.

Materials & Methods

RuBP and ribulose 5-phosphate (Ru5P) were purchased from Sigma Chemical Co., U.S.A. $NaH^{14}CO_3$ (48.1 mCi/mmol) was purchased from Isotope Division,

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Bhabha Atomic Research Centre, India and was diluted suitably with cold $NaHCO_3$. The following archaebacterial halophiles: Halobacterium mediterranei, H. vallismortis, H. halobium and Halobacterium strain R-113 were grown as shake cultures at 37°C in a complex medium containing 20-25% concentrations of NaCl and 0.5% glucose. Glucose was not added to H. halobium growth medium. The details of conditions for growth, harvest and preparation of cell extracts by sonication as well as the source of culture were described in our earlier paper (6, 7). The method of Bowien et al (8) was used for the assay of RuBP carboxylase (EC 4.1.1.39). Protein was estimated by the method of Lowry et al (9).

Results & Discussion

$^{14}CO_2$ fixation reactions

Preliminary tests with cell extracts of halobacteria grown on complex medium showed signs of CO_2 incorporation at least in case of H. mediterranei. Subsequently H. mediterranei was grown on a synthetic medium with glucose (7), and factors affecting CO_2 incorporation by the extracts were tested (Table 1). Incorporation of $^{14}CO_2$ into an acid soluble component took place in the presence of RuBP. The incorporation detected in the absence of added Mg^{2+}

Table 1. RuBP dependent $^{14}CO_2$ incorporation in cell extracts of halobacteria

Strains	Conditions	$^{14}CO_2$ incorporated CPM*
(a) H. mediterranei	Complete	3371
	- $NaHCO_3$	0
	- RuBP	137
	- Enzyme	56
	- Mg^{2+}	3136
	+ EDTA, - Mg^{2+}	200
H. halobium	Complete	ND
H. vallismortis	"	ND
Halobacterium R-113	"	ND
(b) H. mediterranei	Complete with Ru5P*	3276

For (a) reaction mixture in 250 µl contained: $MgCl_2.6H_2O$, 10 mM; $NaH^{14}CO_3$, 33.3 mM (0.17 µCi/µM) Tris-HCl (pH 7.8), 150 mM and 1 M with respect to NaCl. Cell extract was used after 30 min activation with bicarbonate at 25°C, reaction was initiated by addition of 2 mM RuBP and stopped after 1 hr at 30°C with 100 µl of 50% TCA. Following removal of any labile CO_2, precipitate was removed by centrifugation. Radioactivity present in supernatant was measured in a 100 µl aliquot which was added to 15 ml scintillation fluid consisting of POPOP and PPO and counted on a LKB Rackbeta Liquid Scintillation Counter. Protein values: H. mediterranei, 0.35 mg and others, 2-4 mg.

ND = Not detected after 4 h incubation.

For (b), RuBP was replaced by Ru5P, 1.6mM and ATP, 5 mM. Other conditions as in (a).

was attributed to endogenous Mg^{2+}, the concentration of which is known to be high in halobacteria (10). This was confirmed by the addition of 10 mM EDTA to the reaction mixture which resulted in suppression of $^{14}CO_2$ incorporation. Addition of 15 mM $MgCl_2$ to EDTA treated extract restored the activity. In contrast in the extracts of H. halobium, H. vallismortis and Halobacterium R-113, the incorporation of $^{14}CO_2$ could not be detected in complete assay systems even when the incubation time was prolonged to 4 h (Table 1).

The dependence of $^{14}CO_2$ incorporation on RuBP was further confirmed in the reactions when RuBP was replaced by Ru5P and ATP (Table 1"b") since Ru5P kinase activity was detected in H. mediterranei and other halobacteria (manuscript communicated). Thus the coupled activities of Ru5P kinase and RuBP carboxylase in H. mediterranei extract were considered responsible for the incorporation of $^{14}CO_2$ shown in reaction "b", Table 1.

Rate of $^{14}CO_2$ incorporation and effect of salt

The rate of $^{14}CO_2$ incorporation in H. mediterranei extract and the effect of protein concentration on the rate are shown in Fig. 1a and 1b.

The enzymatic activity was halophilic for KCl and NaCl. No activity could be detected in the absence of salt though the low concentration of 0.3 M salt carried over in the assay mixture was adequate for activity. The activity declined slowly in higher concentrations of salt. However, 60% activity was evident in 3 M KCl or NaCl (Fig. 2) and remained unaltered in 4 M salt (data not shown). The activity was lost upon storage of extract without salt.

Identification of the product of $^{14}CO_2$ incorporation

The TCA supernatant obtained after termination of the enzyme reaction was concentrated after desalting and analysed initially by 2-dimensional

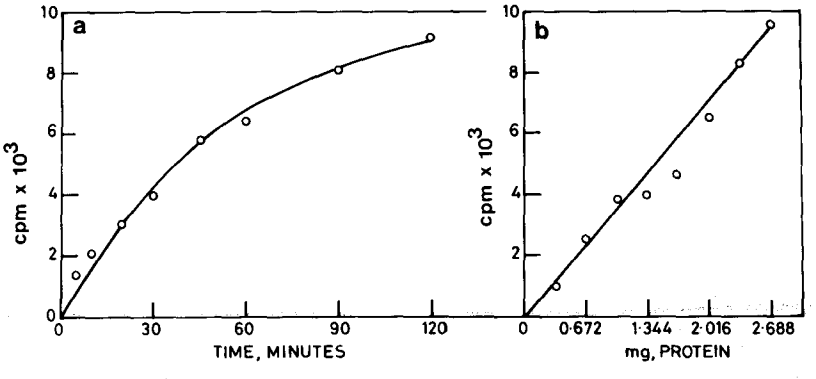
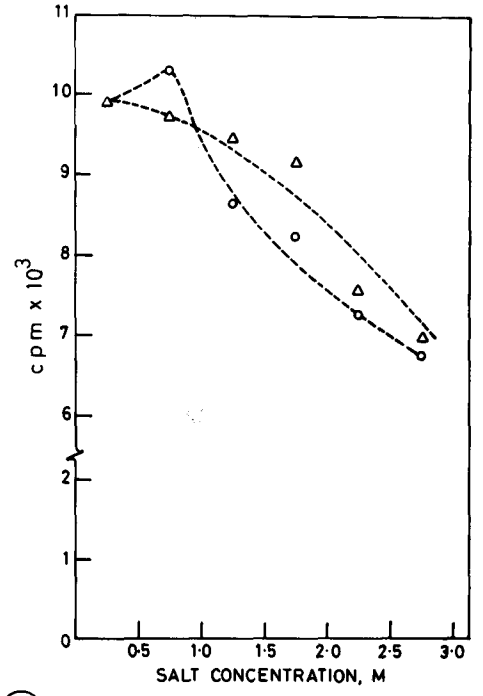
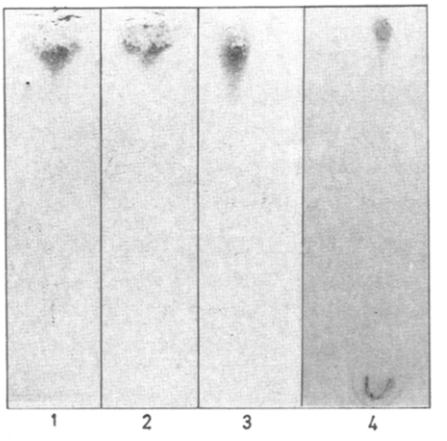


Fig. 1. CO_2 incorporation by extracts of H. mediterranei, conditions as in Table 1. (a) shows the rate of reaction (1.3 mg protein). (b) shows incorporation after 30 min at varying protein concentration.



(2)



(3)

Fig. 2. Effect of salt on RuBP dependent CO_2 incorporation in H. mediterranei. Conditions as in Table 1. Protein, 1.3 mg. Values are corrected for carried over salt. Symbols: O, NaCl, Δ , KCl.

Fig. 3. Identification of product of RuBP dependent $^{14}CO_2$ incorporation in H. mediterranei. Reaction conducted as in Table 1 and TCA supernatant desalted by passage through Sephadex G-10. Radioactive fractions pooled and used. Results of chromatography in descending fashion (Ref. 11) on Whatman paper No. 4, solvent, propionic acid : n-butanol : H_2O = 5:10:7. After drying, paper sprayed with ammonium molybdate reagent (Ref. 12) when phosphate esters turned blue. Lane 1, halobacterial reaction product; Lane 2, 3PGA standard; Lane 3, reaction product of pure spinach RuBP carboxylase, reaction being run for 5 min as in Table 1; Lane 4, autoradiogram of halobacterial reaction product run only in single direction in above solvent, not sprayed, after exposure to Kiran X-ray film for 21 days at 4°C.

chromatography (11) when a major spot corresponding to authentic 3-phosphoglyceric acid (3PGA) was observed. The area of this spot was eluted with water and subsequently rechromatographed in single direction (Fig. 3). The unknown spot on paper showed positive test for phosphate with molybdate spray (12) and its R_f value corresponded to that of authentic 3PGA as well as to the reaction product obtained from the assay of pure RuBP carboxylase from spinach. A second chromatogram run similarly, was autoradiographed when the above halobacterial phosphorylated reaction product i.e. 3PGA was found to be radioactive (Fig. 3). Occasionally minor active spots in the area of sugar phosphates were noticed which are not seen in this Fig.

Though CO_2 fixation due to pyruvate carboxylase activity was present in the extract, it was strictly dependent on the presence of acetyl CoA (data not shown).

The RuBP dependent CO_2 incorporating activity was, however inhibited in the presence of 100 μM 3-keto-2-carboxyarabinitol 1,5-bisphosphate, an inhibitor of the plant enzyme (W. Altekar and R. Rajagopalan, unpublished work). Several metabolites are known to inhibit RuBP carboxylases from plant and microbial sources (13) and the effects of a few were tested on the halobacterial activity. A 30% loss of original activity due to 10 mM fructose disphosphate or sedoheptulose disphosphate was seen, while 10 mM 6-phosphogluconate was not inhibitory.

Thus it is evident from our results that the formation of 3PGA as a result of CO_2 fixation into RuBP, attributable to the activity of ribulose bisphosphate carboxylase indeed took place in the cell extract of H. mediterranei. This activity could not be detected in H. halobium, H. R-113 or H. vallismortis. The halobacterial activity for RuBP carboxylase is low (0.5-1 nMoles/mg/min) as compared to that found for CO_2 fixation in autotrophic eubacteria (e.g. 67 nMoles/mg/min for Hydrogenomonas facilis) (13-15). The low activity could be attributed to the aerobic chemoheterotrophic mode of growth of H. mediterranei and the enzyme may not be required for biosynthetic purposes. Though other carboxylation reactions yielding pyruvate or glycine as the products were demonstrated, Javor (5) could not detect RuBP carboxylase activity in H. halobium, nor could we. The metabolic abilities of H. mediterranei and H. halobium which produces bacteriorhodopsin are very different (7, 16), and while the former can utilize sugars, the latter cannot. Besides RuBP carboxylase, we have detected the activities of rest of the enzymes required for the operation of reductive pentose phosphate pathway to be present in heterotrophically grown H. mediterranei, along with activities of the modified Entner-Doudoroff pathway (manuscript communicated). Rodrigue-Valera's group (17) has shown that H. mediterranei accumulates considerable amount of poly-(β-hydroxybutyrate) while H. halobium does not. The situation is very much reminiscent of the reduced levels of RuBP carboxylase noted by several workers when facultative autotrophic bacteria with metabolic make-up similar to H. mediterranei, are grown organotrophically (14, 18, 19). As a matter of fact RuBP carboxylase from H. facilis and H. entrophus was purified from fructose grown cells (20). Ribulose bisphosphate carboxylase activity has not so far been demonstrated in halobacteria, or indeed in any archaebacteria and it provides the basis for comparing the enzyme from archaebacteria with those from eubacteria and eukaryotes and its evolution. Currently, we are examining the factors affecting its formation in halobacteria.

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References

1. Oren, A. (1983) Limnon Oceanogr. 28: 33-41.
2. Danon, A. and S.R. Caplan (1977) FEBS Letts. 74: 255-258.
3. Kerscher, L. and D. Oesterhelt (1977) FEBS Letts. 83: 197-201.
4. Kushner, D.J. (1985) The Halobacteriaceae. In the Bacteria, Vol. III (Woese, C.R. and R.S. Wolfe, eds.) pp. 171-214.
5. Javor, B.J. (1988) Arch. Microbiol. 149: 433-440.
6. Dhar, N.M. and W. Altekar (1986) FEBS Letts. 199: 151-154.
7. Dhar, N.M. and W. Altekar (1986) FEMS Microbiol. Letts. 35: 177-181.
8. Bowien, B., F. Mayer, G.A. Codd and H.G. Schlegel (1976) Arch. Microbiol. 110: 157-166.
9. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951) J. J. Biol. Chem. 193: 265-275.
10. Kushner, D.J. (1968) Adv. Appl. Microbiol. 10: 73-99.
11. Tyszkiewiez, E. (1962) Anal. Biochem. 3: 164-172.
12. Bandurski, R.S. and B. Axelrod (1951) J. Biol. Chem. 193: 405-410.
13. McFadden, B.A. (1973) Bacteriol. Rev. 37: 1289-1319.
14. McFadden, B.A. and C.L. Tu (1986) J. Bacteriol. 93: 886-893.
15. Anderson, L. and R.C. Fueller (1967) Plant Physiol. 42: 497-502.
16. Torsvik, R. and I. Dundas (1982) Methods in Enzymol. 88: 360-368.
17. Fernandez-Castillo, R., F. Rodriguez-Valera, J. Gonzales-Romos and F. Ruiz-Berraquero (1986) Appl. Environ. Microbiol. 51: 214-216.
18. Dijkhuizen, L., M. Knight and W. Harder (1978) Arch. Microbiol. 116: 77-83.
19. Schlegel, H.G. (1966) Adv. Comp. Physiol. Biochem. 2: 185-236.
20. Kuehn, G.D. and B.A. McFadden (1969) Biochemistry 8: 2394-2402.