Glucose metabolism in the extreme thermoacidophilic archaeobacterium *Sulfolobus solfataricus*

Mario DE ROSA,* Agata GAMBACORTA,* Barbara NICOLAUS,* Paola GIARDINA,†
Elia POERIO† and Vincenzo BUONOCORE††

*Istituto di Chimica di Molecole di Interesse Biologico, C.N.R., Via Toiano 6, Arco Felice, Napoli, and
†Dipartimento di Chimica Organica e Biologica, Università di Napoli, Via Mezzocannone 16, Napoli, Italy

(Received 10 May 1984/Accepted 7 August 1984)

Sulfolobus solfataricus is a thermophilic archaeobacterium able to grow at 87°C and pH 3.5 on glucose as sole carbon source. The organism metabolizes glucose by two main routes. The first route involves an ATP-dependent phosphorylation to give glucose 6-phosphate, which readily isomerizes to fructose 6-phosphate. In the second route, glucose is converted into gluconate by an NAD⁺-dependent dehydrogenation; gluconate is then dehydrated to 2-keto-3-deoxygluconate, which, in turn, is cleaved to pyruvate and glyceraldehyde. Each metabolic step has been tested *in vitro* at 70°C on dialysed homogenates or partially purified fractions; minimal requirements of single enzymes have been evaluated. Identification of the intermediates is based on chromatographic, spectroscopic and/or synthetic evidence and on specific enzymic assays. The oxidative breakdown of glucose to pyruvate occurring in *S. solfataricus* differs from the Entner–Doudoroff pattern in that there is an absence of any phosphorylation step.

The archaeobacteria (Kandler, 1982), comprising extreme halophilic, methanogenic and thermoacidophilic micro-organisms, represent a third line of living organisms in addition to the well-known eubacteria and eukaryotes. The three lines presumably stemmed from the ancient divergence of a putative progenote, and now archaeobacteria appear to be as different from eubacteria as they are from eukaryotes. The ancient divergence of archaeobacteria is reflected in the occurrence of unique features in their biochemical patterns, for example the lipid biosynthesis based on the formation of isoprenoidic ether molecules (Langworthy *et al.*, 1982; De Rosa *et al.*, 1983a,b).

To throw some light on other fundamental biochemical patterns of archaeobacteria, we studied glucose metabolism in the extreme thermoacidophilic archaeobacterium *Sulfolobus solfataricus*, previously named *Caldariella acidophila* (De Rosa *et al.*, 1975; Zillig *et al.*, 1980), which is able to grow on simple media with glucose as sole carbon source. A similar study has been carried out on *Halobacterium saccharovorum* (Tomlinson *et al.*, 1974; Tomlinson & Hochstein, 1972, 1976) an extreme halophile recently recognized as an

archaeobacterium (Langworthy *et al.*, 1982). In this halophile the main route for the pyruvate formation from glucose is through a modified Entner–Doudoroff pathway, in which the phosphorylation step follows the glucose oxidation, as already observed in few other micro-organisms (Szymona & Doudoroff, 1960; Kersters & De Ley, 1968; Andreesen & Gottschalk, 1969; Bender *et al.*, 1971).

Our studies demonstrate that *S. solfataricus* metabolizes glucose through a further modification of the Entner–Doudoroff pathway, in which the phosphorylation step is omitted altogether. To our knowledge, this is the first example of an organism able to convert glucose into pyruvate without involving any phosphorylated intermediate.

Experimental

Materials

Deoxyribonuclease I was obtained from Worthington Corp. (Freehold, NJ, U.S.A.). Baker's-yeast glucose-6-phosphate dehydrogenase, rabbit muscle lactate dehydrogenase, yeast alcohol dehydrogenase and baker's-yeast glyceraldehyde-3-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Affi-

† To whom correspondence should be addressed.

Gel Blue (100–200 mesh) was the product of Bio-Rad Laboratories (Richmond, CA, U.S.A.). [2,6-¹⁴C]Glucose (sp. radioactivity 5.5 mCi/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.). All other reagents were of analytical grade.

Growth of the micro-organism

Sulfolobus solfataricus strain MT-4 was isolated from an acidic hot spring in Agnano, Napoli, Italy (De Rosa *et al.*, 1975). The organism was grown at 87°C either in a 2.5-litre (Chemap, Männedorf, Switzerland) or in a 90-litre (Terzano, Milano, Italy) fermentor with low mechanical agitation and aeration flux of 30 ml/min per litre of broth. The standard culture medium contained (g/l): KH₂PO₄, 3.1; (NH₄)₂SO₄, 2.5; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.25; yeast extract, 2. The defined culture medium had the same salt composition, but the yeast extract was replaced by glucose (3 g/l) as the sole carbon source. The pH of the culture media was adjusted to 3.5 with 0.1 M-H₂SO₄.

Cell growth was quantified turbidimetrically at 540 nm, an absorbance of 0.6 corresponding to 138 mg of freeze-dried cells/l.

The glucose concentration during growth was tested enzymically by the glucose oxidase/peroxidase method (Bergmeyer & Bernt, 1974). Cells were harvested in the early stationary phase of growth, at a concentration of 0.5 g of freeze-dried cells/l, by continuous-flow centrifugation on an Alfa-Laval model LAB 102 B-20 centrifuge. The pellet was washed twice with an iso-osmotic saline solution, pH 6.0, and collected by centrifugation at 9000 g for 30 min. The cells can be stored at –20°C for several months without loss of enzymic activities.

Preparation of homogenate

Unless otherwise stated, the experimentation described was carried out at room temperature (22°C). Wet cells (30 g) were ground with 50 g of glass beads and 50 ml of 20 mM-triethanolamine/HCl buffer, pH 7.0, containing 20 mM-MgCl₂, in the stainless-steel chamber of a Sorvall Omni-Mixer for 5 min at half speed and for 10 min at full speed. After addition of a freshly prepared solution (0.5 ml, 1 mg/ml) of deoxyribonuclease I, the mixture was incubated for 30 min at 30°C with occasional stirring, then centrifuged at 35 000 g for 50 min; the supernatant was designated the crude homogenate. Crude homogenate was extensively dialysed at 4°C against the triethanolamine buffer to remove diffusible material; the dialysis residue was designated the dialysed homogenate. Protein concentration was monitored by the Bio-Rad Protein Assay in accordance with the manufac-

turer's instructions, with bovine serum albumin as standard.

Affi-Gel Blue chromatography

Fractionation of certain enzymic activities was performed by chromatography of crude homogenate (0.5 g of protein) on an Affi-Gel Blue column (1.5 cm × 20 cm) equilibrated with 20 mM-triethanolamine/HCl buffer, pH 7.0, containing 20 mM-MgCl₂. After the column had been washed with the equilibration buffer to remove unadsorbed material, elution was carried out with 0.1 M-triethanolamine/HCl buffer, pH 8.6, containing 20 mM-MgCl₂, and then with a linear gradient formed by mixing 100 ml of the triethanolamine buffer, pH 7.0, containing 0.2 M-NaCl and 100 ml of the same buffer containing 0.75 M-NaCl. The fractions (3 ml), collected at a flow rate of 15 ml/h, were monitored for absorbance at 280 nm and enzymic activities. Glucosephosphate isomerase, gluconate dehydratase and 2-keto-3-deoxygluconate aldolase were detected in the unadsorbed fraction, glucokinase was eluted with the pH 8.6 washing and glucose dehydrogenase with the saline gradient.

Autoradiographic analysis of products of the glucose metabolism

Dialysed homogenate (4.6 mg of protein) was added to NAD⁺ (3 mmol) and 12 μCi of [2,6-¹⁴C]-glucose (5.5 mCi/mmol) and incubated in a sealed vial at 70°C. A control without homogenate was run in a parallel experiment. Samples (50 μl) were withdrawn at intervals; 1 μl of each sample was monitored for radioactivity and the remainder (about 10⁶ d.p.m.) was applied to a thin layer of silica gel. The plate was developed in butan-1-ol/acetic acid/water (3:1:1, by vol.), air-dried and then exposed for 70 h to a Kodak X-Omat XRP-1 film. The radioactive spots were quantified by scraping the adsorbent out of the layer, suspending it in 5 ml of Insta-gel containing Cab-O-Sil (Packard, Downers Grove, IL, U.S.A.) and counting the radioactivity on a liquid-scintillation system (PRIAS Tri-Carb; Packard) equipped with an absolute-radioactivity analyser.

Isolation of products of the glucose metabolism

Glucose (10 mmol) and NAD⁺ (10 mmol) were added to 100 ml of dialysed homogenate (protein concentration 8 mg/ml) and the mixture was incubated at 70°C. Samples (1 ml) were withdrawn at various times and the glucose was assayed by the glucose oxidase/peroxidase method. When no more glucose was detected, the incubation mixture was deproteinized by ultrafiltration on an Amicon XM 10 (Amicon Corp., Danvers, MA, U.S.A.) membrane (nominal *M_r* cut-off 10 000). The ultra-

filtrate was dried under vacuum, dissolved in a few millilitres of water, mixed with 20 g of silica gel, dried again and added to the top of a silica-gel column (5 cm × 34 cm) equilibrated with acetone. After the column had been washed with acetone, each product was eluted stepwise with the following acetone/water (v/v) mixtures: 99:1 (glyceraldehyde), 97:3 (pyruvate), 19:1 (2-keto-3-deoxygluconate), 4:1 (NADH) and 7:3 (gluconate). The products of the glucose metabolism were identified by silica-gel t.l.c. by comparison with authentic standards. After development with butan-1-ol/acetic acid/water (3:3:1, by vol.), the spots were revealed by spraying with ammoniacal AgNO₃ and drying in oven at 100°C (Stahl & Kaltenbach, 1965).

Synthesis and identification of 2-keto-3-deoxygluconic acid

The synthesis of 2-keto-3-deoxygluconic acid was performed from 2-deoxyribose by the procedure described by Weissbach & Hurwitz (1959). The synthetic product was purified on a silica-gel column (5 cm × 30 cm) eluted with a linear gradient of water in acetone from 0 to 10% (v/v). T.l.c. on silica-gel plates of the purified product in propan-1-ol/conc. NH₃/0.2% disodium EDTA (6:3:1, by vol.) gave the *R_f* and the colour reaction reported by Kersters & De Ley (1968) for 3-deoxy-2-keto-gluconic acid.

The 2-keto-3-deoxygluconic acid (50 mg) was acetylated with acetic anhydride (5 ml) and pyridine (0.5 ml) at reflux for 10 min. The reaction mixture was dried under vacuum, redissolved in methanol and resolved by silica-gel t.l.c. in chloroform/acetic acid (19:1, v/v). After exposure to I₂ vapour, the two main products, representing about 28% (*R_f* 0.5) and 15% (*R_f* 0.3) of the reaction mixture, were recovered by methanol elution and characterized by n.m.r. spectroscopy in [2H]-methanol with a Spectrospin (Bruker, Zürich, Switzerland) WH-500 spectrometer.

Assays of enzymic activities

Glucose dehydrogenase activity was measured at 70°C by monitoring NAD⁺ reduction at 340 nm in a reaction mixture (1 ml) that contained: 100 mM-triethanolamine/HCl buffer, pH 9.0, 5 mM-NAD⁺, 40 mM-glucose, 20 mM-MgCl₂ and dialysed homogenate.

Gluconate dehydratase (EC 4.2.1.39) activity was determined at 70°C in a reaction mixture (0.2 ml) containing dialysed homogenate, 4 mM-gluconate and 50 mM-triethanolamine/HCl buffer, pH 7.0; after cooling, the concentration of 2-keto-3-deoxygluconate was measured as described by Weissbach & Hurwitz (1959).

2-Keto-3-deoxygluconate aldolase activity was

determined by incubating at 70°C, in 0.2 ml, 100 mM-2-keto-3-deoxygluconate, dialysed homogenate and 50 mM-triethanolamine/HCl buffer, pH 7.0; the pyruvate and the glyceraldehyde formed were measured at 25°C by using lactate dehydrogenase (Kornberg, 1955) and alcohol dehydrogenase (Racker, 1957) respectively.

Glucokinase (EC 2.7.1.2) activity was assayed by incubating at 70°C, in 0.5 ml, dialysed homogenate, 20 mM-glucose, 1 mM-ATP, 10 mM-MgCl₂ and 50 mM-triethanolamine/HCl buffer, pH 7.0; the amount of glucose 6-phosphate produced was determined at 25°C by using glucose-6-phosphate dehydrogenase (Horecker & Wood, 1957). Glyceraldehyde kinase activity was measured in a similar way by using glyceraldehyde-3-phosphate dehydrogenase (Racker, 1957). The ATP-dependent kinase activities were also monitored at 70°C in a reaction mixture (2 ml) containing 10 mM-glucose, -fructose or -glyceraldehyde, dialysed homogenate, 1 mM-ATP, 10 mM-MgCl₂ and 50 mM-triethanolamine/HCl buffer, pH 7.0; the ADP formed was determined at 25°C by using pyruvate kinase and lactate dehydrogenase as described by Jaworek *et al.* (1974).

Glucosephosphate isomerase (EC 5.3.1.9) activity was assayed by incubating at 70°C, in 1 ml, dialysed homogenate and 50 mM-triethanolamine/HCl buffer, pH 7.0, with either 2 mM-glucose 6-phosphate or -fructose 6-phosphate; after cooling, fructose 6-phosphate was determined by the cysteine/carbazole reaction (Ashwell, 1957) and glucose 6-phosphate by using glucose-6-phosphate dehydrogenase.

Phosphatase activity was determined at 70°C in a reaction mixture (1.2 ml) containing dialysed homogenate, 5 mM-*p*-nitrophenyl phosphate and 50 mM-triethanolamine/HCl buffer, pH 7.0; after addition of 0.1 M-NaOH (2 ml), the *p*-nitrophenate formed was measured at 420 nm.

Results

Growth of the micro-organism

In standard growth conditions (yeast extract plus mineral salts) the doubling time of *Sulfolobus solfataricus* was about 5 h. The amount of nutrient, measured as detectable proteins in the culture broth, was decreased to 27% of the initial value after about 40 h. In a defined medium, when glucose was the sole carbon source, the doubling time increased to about 9 h; in these conditions the glucose was completely utilized in about 70 h.

Specific activities of the enzymes involved in the conversion of glucose into pyruvate were not significantly affected by the culture age (mid-exponential or early-stationary growth phases) and nutrients (yeast extract or glucose). The investiga-

Fig. 1. T.l.c. autoradiography of glucose metabolites produced during incubation at 70°C of *S. solfataricus* homogenate with NAD⁺ and [2,6-¹⁴C]glucose

Dialysed homogenate (4.6 mg of protein) of *S. solfataricus*, NAD⁺ (3 mmol) and [2,6-¹⁴C]glucose (12 µCi, 5.5 mCi/mmol) were incubated in a sealed vial at 70°C. Samples (50 µl; about 10⁶ d.p.m.) were withdrawn at the indicated times and applied on to a analytical silica-gel plate. The chromatogram was developed in butan-1-ol/acetic acid/water (3:1:1, by vol.), air-dried and exposed for 70 h to a Kodak X-Omat XRP-1 film. The standard mixture was obtained by incubating for 60 min at 70°C in 1 ml of 20 mM-triethanolamine/HCl buffer, pH 7.0, 10 mg each of glucose, gluconate, 2-keto-3-deoxygluconate, pyruvate and glyceraldehyde; spots were revealed by spraying with ammoniacal AgNO₃ and drying in an oven at 100°C. Spots 1-6 are: glyceraldehyde (1 and 2), pyruvate (3), glucose (4), 2-keto-3-deoxygluconate (5) and gluconate (6). S and F indicate sample application point and solvent front respectively.

tions reported on glucose metabolism were performed on dialysed homogenates of cells grown on yeast extract and harvested in the early stationary phase.

Products of the NAD⁺-dependent [2,6-¹⁴C]glucose metabolism

A number of radioactive metabolites were produced by incubating at 70°C dialysed homogenate with NAD⁺ and [2,6-¹⁴C]glucose at high specific radioactivity. At various incubation times, samples of the reaction mixture were withdrawn and chromatographed on a silica-gel plate. Fig. 1 shows the autoradiography of a typical thin layer; the spots were identified, by comparison with standards, as glucose, gluconic acid, 2-keto-3-deoxygluconic acid, pyruvic acid and glyceraldehyde. It has to be noted that the glyceraldehyde was not stable in the incubation conditions used; in

Fig. 2. Kinetic analysis of [2,6-¹⁴C]glucose oxidation by *S. solfataricus* homogenate

The radioactive spots, revealed as indicated in Fig. 1, were scraped out of the layer and their radioactivities counted in Insta-gel (5 ml) containing Cab-O-Sil. For the glyceraldehyde evaluation, spots 1 and 2 of Fig. 1 were combined. ●, Glucose; △, gluconate; □, 2-keto-3-deoxygluconate; ■, pyruvate; ○, glyceraldehyde. About 5% of the total radioactivity remained on the application point.

fact, when the pure compound was kept for 60 min at 70°C, a new spot (*R_F* 0.5, corresponding to spot 2 on Fig. 1) appeared in addition to that of glyceraldehyde (*R_F* 0.66, corresponding to spot 1 of Fig. 1). As shown in Fig. 2, after 10 min at 70°C 85% of the glucose was already converted, mostly into gluconate. At incubation times longer than 10 min, the decrease in gluconate almost paralleled the increase in 2-keto-3-deoxygluconate. Significant amounts of pyruvate and glyceraldehyde were observed after 20 min incubation.

Identification of the products of the NAD⁺-dependent glucose metabolism

The products of the NAD⁺-dependent glucose degradation by *S. solfataricus* dialysed homogenate were separated by chromatography on a silica-gel column and identified by t.l.c., instrumental and/or enzymic analyses.

Gluconic acid was identified as the penta-acetyl methyl ester derivative by g.l.c. by using the procedure of Hochstein *et al.* (1976); pyruvic acid and glyceraldehyde were identified enzymically by NADH oxidation in the presence of lactate dehydrogenase and alcohol dehydrogenase respectively, and spectroscopically by ¹H n.m.r. The 2-keto-3-deoxygluconic acid obtained by chemical synthesis and that produced by enzymic degradation of glucose were compared by ¹H-n.m.r. spectroscopy. N.m.r. spectra of the two products were identical but highly complex because of the

presence, in the aqueous solution, of an equilibrium among different forms. After acetylation, both products gave two main derivatives, whose structures and n.m.r. data are reported in Fig. 3. Coupling-constant values of the derivative (b) confirmed that the two chiral centres (C-4 and C-5) had the same configuration as the glucose.

Glucose dehydrogenation

At 70°C, in the presence of glucose and Mg^{2+} , dialysed homogenates of *S. solfataricus* actively reduced NAD^+ at an initial rate of 950 nmol/min per mg of protein; $NADP^+$ also was reduced in the same conditions, but at a much lower rate (200 nmol/min per mg). The addition of 1 mM-ATP did not affect significantly the reaction rate. Mg^{2+} ions could be effectively replaced by Mn^{2+} or Ca^{2+} ; maximum activation was observed at Mg^{2+} , Mn^{2+} or Ca^{2+} concentrations of 20 mM. However, homogenates extensively dialysed against Mg^{2+} -free triethanolamine buffer were still able, although at a lower rate, to reduce NAD^+ in the presence of glucose without any further addition. Optimal conditions of pH and temperature for glucose oxidation were 9.0 and 70°C respectively.

Dialysed homogenates of *S. solfataricus* were also able to promote (i) the NAD^+ -dependent oxidation of 2- and 6-deoxyglucose and D-xylose, and (ii) the $NADP^+$ -dependent oxidation of D-galactose, D-xylose, D-allose, D-ribose and 2- and 6-deoxyglucose. Recent experiments with purified enzyme preparations suggested that only one dehydrogenase is responsible for all the above carbohydrate oxidations (V. Buonocore, M. De Rosa, A. Gambacorta & P. Giardina, unpublished work). The quoted sugars are able to promote singly the growth of the micro-organism as the sole source of carbon atoms.

Dehydration of gluconate

At 70°C dialysed homogenates of *S. solfataricus* converted gluconate into 2-keto-3-deoxygluconate at an initial rate of 74 nmol/min per mg of protein. The gluconate dehydration rate increased in the presence of Mg^{2+} , but was insensitive to the presence of Fe^{2+} and ATP. These results indicated that the dehydration reaction did not occur through the formation of phosphorylated intermediates and that the *S. solfataricus* gluconate dehydratase differed from the enzyme of other prokaryotes, which requires Fe^{2+} ions for full activity (Gottschalk & Bender, 1982).

2-Keto-3-deoxygluconate cleavage

At 70°C dialysed homogenates of *S. solfataricus* cleaved 2-keto-3-deoxygluconate to pyruvate and glyceraldehyde. As observed by enzymic assay, the amounts of pyruvate formed were stoichiometrically equivalent to those of 2-keto-3-deoxygluconate converted, whereas the amounts of glyceraldehyde were constantly higher than the expected value of about 150%. These conflicting results suggested that the compound arising from the thermal degradation of glyceraldehyde (see Fig. 1) was reduced by alcohol dehydrogenase faster than was the parent compound. Support to this hypothesis was obtained by measuring the glyceraldehyde concentrations of standard solutions freshly prepared or kept at 70°C for different times. Because of the thermal instability of the aldehyde, the 2-keto-3-deoxygluconate degradation was then followed by measuring the pyruvate produced. The initial rate of pyruvate formation (110 nmol/min per mg of protein) was not significantly affected by the presence of Mg^{2+} , Mn^{2+} or ATP.

Fig. 3. Chemical shifts and coupling constants of acetyl derivatives of 2-keto-3-deoxygluconic acid. The 2-keto-3-deoxygluconic acid was acetylated and the reaction mixture was resolved by silica-gel t.l.c. in chloroform/acetic acid (19:1, v/v). The two main products, showing R_F values of 0.5 (a) and 0.3 (b), were characterized by n.m.r. spectroscopy. Chemical shifts are given in δ (p.p.m.). The arrows indicate couplings between hydrogen atoms and contain coupling constants (J) in Hz. Ac indicates the acetyl (CH_3CO-) group.

Pyruvate and glyceraldehyde were also formed when homogenates were incubated at 70°C with gluconate or glucose; in the latter case, the presence of NAD⁺ was necessary for the degradation to occur (Table 1). Conversely, low concentrations of pyruvate were detected during incubation of homogenates with glucose 6-phosphate and 6-phosphogluconate (Table 1); in these cases, also, the pyruvate could be derived from the breakdown of the dephosphorylated products regenerated in the incubation mixture by a phosphatase activity.

ATP-dependent glucose metabolism

Dialysed homogenates of *S. solfataricus* were able to promote at 70°C, in the presence of ATP and Mg²⁺, glucose phosphorylation to glucose 6-phosphate at an initial rate of 8 nmol/min per mg of protein. When 2 mM-glucose 6-phosphate was incubated at 70°C with dialysed homogenate of *S. solfataricus*, the carbohydrate concentration decreased at an initial rate of 250 nmol/min per mg of protein; that rate was lowered to about one-third in the presence of 0.1 M-Mn²⁺. The metabolism of glucose 6-phosphate did not require the presence of either Mg²⁺ ions or nicotinamide coenzymes, thus suggesting the absence of a glucose-6-phosphate dehydrogenase activity. Glucose 6-phosphate consumption paralleled the production of a carbazole-sensitive compound that, by silica-gel t.l.c. against standard (Bancher & Washüttl, 1967), appeared to be fructose 6-phosphate. The initial rate of fructose 6-phosphate formation was 250 nmol/min per mg of protein. The hexose isomerization proceeded readily in both directions, and at equilibrium glucose 6-phosphate constituted about 60% of the mixture of the two isomers. Fructose 6-phosphate

was also formed during incubation at 70°C of dialysed homogenate with glucose, ATP and Mg²⁺.

The occurrence in *S. solfataricus* homogenates of the glucosephosphate isomerase reaction at a relatively high rate did not allow a study of the parameters affecting the glucokinase reaction. Therefore the kinase and the isomerase activities were separated and partially purified by chromatography of dialysed homogenates on Affi-Gel Blue columns. Optimal activity of the partially purified glucokinase (114.5 nmol/min per mg of protein) was observed at pH 7.0 in the presence of 1 mM-ATP and 10 mM-MgCl₂; replacement of ATP with dATP, GTP or dGTP decreased this activity by about 50%. Alternatively, the glucokinase activity was followed by measuring the ADP formed. In the presence of glucose, ATP and Mg²⁺, dialysed homogenates and Affi-Gel-Blue-purified fractions produced ADP at initial rates of 20 and 120 nmol/min per mg of protein respectively; the latter value was in agreement with that obtained by measuring glucose 6-phosphate formed by the same fraction.

During incubation at 70°C of dialysed homogenates with glucose 6-phosphate or fructose 6-phosphate, free carbohydrates appeared in the incubation mixture; this could be attributed to the presence of a phosphatase activity. In fact, at pH 7.0 and 70°C, hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenate by dialysed homogenates has been observed (85 nmol/min per mg of protein).

Other ATP-dependent phosphorylations

Other kinase activities were evidenced in dialysed homogenates of *S. solfataricus* by ADP

Table 1. Production of pyruvate by dialysed homogenate of *S. solfataricus* in the presence of various precursors. Dialysed homogenate (200 µg of protein) and 50 mM-triethanolamine/HCl buffer, pH 7.0, were incubated at 70°C with the components indicated. After 15 min, the incubation mixture (0.2 ml) was cooled and the pyruvate was determined at 25°C by using lactate dehydrogenase. For comparative purposes, in this experiment all the putative pyruvate precursors were used at the same concentration.

Addition	Pyruvate formed (nmol/min per mg of protein)
50 mM-Glucose + 5 mM-NAD ⁺	7.1
50 mM-Glucose + 5 mM-NAD ⁺ + 20 mM-MgCl ₂	11.4
50 mM-Glucose + 5 mM-NAD ⁺ + 20 mM-MgCl ₂ + 1 mM-ATP	11.4
50 mM-Glucose 6-phosphate + 5 mM-NAD ⁺	0.4
50 mM-Glucose 6-phosphate + 5 mM-NAD ⁺ + 20 mM-MgCl ₂	0.7
50 mM-Glucose 6-phosphate + 5 mM-NADP ⁺ + 20 mM-MgCl ₂	0.1
50 mM-Gluconic acid	6.0
50 mM-Gluconic acid + 20 mM-MgCl ₂	17.8
50 mM-6-Phosphogluconic acid	0.5
50 mM-6-Phosphogluconic acid + 20 mM-MgCl ₂	1.7
50 mM-2-Keto-3-deoxygluconic acid	86.7
50 mM-2-Keto-3-deoxygluconic acid + 20 mM-MgCl ₂	91.0
50 mM-2-Keto-3-deoxygluconic acid + 20 mM-MgCl ₂ + 1 mM-ATP	88.6

assay. Fructose was phosphorylated at 70°C at an initial rate of 16 nmol/min per mg of protein; the product of the reaction was readily converted into glucose 6-phosphate, as determined by yeast glucose 6-phosphate dehydrogenase. ADP was also formed in the presence of glyceraldehyde at an initial rate of 10 nmol/min per mg of protein; however, no glyceraldehyde 3-phosphate was detected in the incubation mixture by enzymic assay. This result may be due to the poor stability at 70°C of the phosphorylated compound, as observed in control experiments. Conversely, no ADP was detected when dialysed homogenates were incubated at 70°C with ATP, Mg^{2+} and fructose 6-phosphate.

Discussion

Sulfolobus solfataricus is a thermoacidophilic archaeobacterium able to grow on glucose as the sole carbon source. Preliminary investigations with differently labelled [^{14}C]glucoses (De Rosa *et al.*, 1983c) have indicated a quantitative loss of C-1 and metabolic unequivalence of C-1,2,3 and C-4,5,6 fragments of glucose, thus excluding a role of

the glycolysis in *S. solfataricus*. The absence from *S. solfataricus* homogenates of a fructose-6-phosphate kinase, a key enzyme of the glycolytic pathway, gives further support to this hypothesis. Moreover, the lack of a glucose-6-phosphate dehydrogenase activity in the homogenates of the micro-organism suggests the absence of the conventional Warburg-Dickens cycle.

Our studies demonstrate that in *S. solfataricus* the conversion of glucose is accomplished by two main routes, one involving phosphorylation and the other direct oxidation of the carbohydrate. In the first route, an ATP-dependent phosphorylation reaction converts glucose into glucose 6-phosphate (Scheme 1, Ia), which, in turn, is readily isomerized to fructose 6-phosphate (Scheme 1, II); step II is reversible, whereas glucose 6-phosphate can be slowly dephosphorylated by a phosphatase activity (Scheme 1, Ib). Fructose may undergo similar phosphorylation-dephosphorylation reactions (Scheme 1, IIIa and IIIb). The occurrence of steps I-III allows full hexose interconversion and might justify the metabolic equivalence of fructose and glucose in the isoprenoid-lipid metabolism of the micro-organism (De Rosa *et al.*, 1980; Bu'Lock *et al.*, 1983).

Scheme 1. NAD^+ -dependent and ATP-dependent pathways for the metabolism of glucose in *S. solfataricus*

The second route of glucose metabolism involves, as its first step, its NAD⁺-dependent dehydrogenation to gluconate (Scheme 1, IV); this is then dehydrated to 2-keto-3-deoxygluconate (Scheme 1, V), which, in turn, is cleaved to pyruvate and glyceraldehyde (Scheme 1, VI). As the aldolase is saturated by high substrate concentrations, the 2-keto-3-deoxygluconate cleavage may represent the rate-limiting step of conversion of glucose into pyruvate *in vivo*. The unique feature of the oxidative breakdown of glucose to pyruvate is the absence of any phosphorylation reaction; in this aspect this pattern differs from both the conventional Entner–Doudoroff pathway, in which oxidation occurs on glucose 6-phosphate, and the modified one discovered in the extreme halophilic archaebacterium *Halobacterium saccharovorum*, which requires phosphorylation at the level of 2-keto-3-deoxygluconate (Tomlinson *et al.*, 1974; Tomlinson & Hochstein, 1972, 1976). However, the results reported by Tomlinson *et al.* (1974) for *H. saccharovorum* may have to be reconsidered, as, according to the authors, no absolute ATP requirement for pyruvate production was observed. Such a result might imply the occurrence, at least as a parallel route, of an oxidative glucose metabolism identical with that observed in *S. solfataricus*.

The modified Entner–Doudoroff pathway that operates in *S. solfataricus* provides the unique opportunity of obtaining a C₃ fragment (pyruvate) from glucose without ATP involvement. At the moment, we cannot indicate if this pathway is an evolutionary characteristic of the archaebacteria or an individual choice for a few organisms, such as thermoacidophiles, forced to evolve in particularly selective environments. A systematic investigation on a large and representative number of archaebacterial species might contribute to clarify this problem.

We thank Dr. R. Riccio for helpful discussion on interpretation of n.m.r. data, Mr. V. Calandrelli, Mr. E. Esposito, Mrs. I. Romano, Mr. S. Sodano and Mr. A. Trincone for technical assistance, and Mr. R. Turco for drawings. This work was supported by a C.N.R. grant under the Progetto Finalizzato Chimica Fine e Secondaria.

References

Andreesen, J. R. & Gottschalk, G. (1969) *Arch. Microbiol.* **69**, 160–170

- Ashwell, G. (1957) *Methods Enzymol.* **3**, 73–105
 Bancher, E. & Washüttl, J. (1967) *J. Chromatogr.* **29**, D1
 Bender, R., Andreesen, J. R. & Gottschalk, G. (1971) *J. Bacteriol.* **107**, 570–573
 Bergmeyer, H. U. & Bernt, E. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 1205–1215, Academic Press, New York, San Francisco and London
 Bu'Lock, J. D., De Rosa, M. & Gambacorta, A. (1983) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W. & Spurgeon, S. L., eds.), pp. 161–187, John Wiley and Sons, New York
 De Rosa, M., Gambacorta, A. & Bu'Lock, J. D. (1975) *J. Gen. Microbiol.* **86**, 156–164
 De Rosa, M., Gambacorta, A., Nicolaus, B. & Sodano, S. (1980) *Abstr. Soc. Ital. Biochem. Meet.* **26th** 225
 De Rosa, M., Gambacorta, A. & Nicolaus, B. (1983a) *J. Membr. Sci.* **16**, 287–294
 De Rosa, M., Gambacorta, A., Nicolaus, B., Chappe, B. & Albrecht, P. (1983b) *Biochim. Biophys. Acta* **753**, 249–256
 De Rosa, M., Gambacorta, A. & Nicolaus, B. (1983c) *Abstr. Soc. Ital. Biochim. Meet.* **29th** 253–254
 Gottschalk, G. & Bender, R. (1982) *Methods Enzymol.* **90**, 283–287
 Hochstein, L. I., Dalton, B. P. & Pollock, G. (1976) *Can. J. Microbiol.* **22**, 1191–1196
 Horecker, B. L. & Wood, W. A. (1957) *Methods Enzymol.* **3**, 152–154
 Jaworek, D., Gruber, W. & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 2127–2131, Academic Press, New York, San Francisco and London
 Kandler, O. (ed.) (1982) *Archaeobacteria*, Gustav Fisher Verlag, Stuttgart and New York
 Kersters, K. & De Ley, J. (1968) *Antonie van Leeuwenhoek* **34**, 393–408
 Kornberg, A. (1955) *Methods Enzymol.* **1**, 441–443
 Langworthy, T. A., Tornabene, T. G. & Holzer, G. (1982) in *Archaeobacteria* (Kandler, O., ed.), pp. 228–244, Gustav Fisher Verlag, Stuttgart and New York
 Racker, E. (1957) *Methods Enzymol.* **3**, 293–296
 Stahl, E. & Kaltenbach, V. (1965) in *Thin Layer Chromatography: A Laboratory Handbook* (Stahl, E., ed.), pp. 461–469, Springer-Verlag, Berlin
 Szymona, M. & Doudoroff, M. (1960) *J. Gen. Microbiol.* **22**, 167–183
 Tomlinson, G. A. & Hochstein, L. I. (1972) *Can. J. Microbiol.* **18**, 1973–1976
 Tomlinson, G. A. & Hochstein, L. I. (1976) *Can. J. Microbiol.* **22**, 587–591
 Tomlinson, G. A., Koch, T. K. & Hochstein, L. I. (1974) *Can. J. Microbiol.* **20**, 1085–1091
 Weissbach, A. & Hurwitz, J. (1959) *J. Biol. Chem.* **234**, 705–709
 Zillig, W., Stetter, K. O., Wunderl, S., Schulz, W., Priers, H. & Scholz, I. (1980) *Arch. Microbiol.* **125**, 259–269