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Mahua Ghosh · Haripalsingh M. Sonawat

Kreb's TCA cycle in *Halobacterium salinarum* investigated by ^{13}C nuclear magnetic resonance spectroscopy

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Abstract Kreb's tricarboxylic (TCA) cycle was studied in *Halobacterium salinarum* cells grown in the presence of glucose or alanine. The cells were incubated with ^{13}C-labeled substrate and the labeling pattern of various carbon positions in glutamate was monitored by ^{13}C-NMR spectroscopy. [2-^{13}C]pyruvate, when used as a substrate, led mainly to signals for C-1 and C-5 glutamate, with some C-3 glutamate. [3-^{13}C]pyruvate as a substrate produced signals, mainly C-2, C-3, and C-4 glutamate, with some C-1 and C-5 glutamate. The multiplicity of the signals and observation of a C-1 signal in this case indicates extensive cycling of the label in the TCA cycle. Isotopomer analysis of glutamate labeling suggested that of the total pyruvate entering the TCA cycle, the flux through pyruvate:ferredoxin oxidoreductase was 90% while that through pyruvate caboxylase was 10%. Only 53% of the total acetyl-CoA was produced from the added labeled pyruvate, the rest being generated endogenously. In the presence of nitrogen, mainly transamination reaction products were formed in the case of both these substrates.

Key words *Halobacterium salinarum* · TCA cycle · ^{13}C-NMR spectroscopy · Archaea · Halophile

Introduction

Halobacteria are chemoorganotrophic Archaea that need high salt concentrations for growth in addition to a complex growth requirement (Larsen 1962). The organisms are aerobic in nature despite their almost "anoxic" natural habitat (Hochstein 1988) of NaCl-saturated solar salterns with extremely low oxygen solubility. Growth is enhanced in the presence of several carbohydrates in media containing nonlimiting concentrations of potassium (Gochnauer and Kushner 1969). Glycerol and acetate also stimulate growth. Acetate is, however, utilized only when several additional carbon sources are included in the medium (Onishi et al. 1965), and the presence of many amino acids seems to be essential. Enzymes related to Kreb's tricarboxylic acid (TCA) and glyoxylate cycles have been demonstrated in *Halobacterium salinarium* (*H. salinarum*) (Aitken and Brown 1969).

The use of ^{13}C-NMR spectroscopy for studying metabolism in cell systems including eukaryotes, yeast, and bacteria is well established. The technique has been used for studying glucose, acetate, and propionate metabolism in perfused liver (Cohen et al. 1981), heart (Sherry et al. 1988; Malloy et al. 1990; Chatham et al. 1995), brain (Badar-Goffer et al. 1990), *E. coli* (Evans et al. 1993), alanine metabolism in renal proximal tubular cells (Jans and Willem 1989), and glucose and pyruvate metabolism in neuronal primary and tumor cells (Brand et al. 1992a,b) and erythrocytes (Schrader et al. 1993; Berthon and Kuchel 1995). Kreb's TCA cycle and related anaplerotic reactions have been investigated, and mathematical algorithms for evaluating the fluxes through various routes of entry of substrates into the cycle have been reported (Matwiyoff et al. 1982; Chance et al. 1983; Sherry et al. 1988; Jans and Leibfritz 1989; Jans and Willem 1989; Malloy et al. 1990).

^{13}C-NMR studies in Halobacteria are clearly lacking. Mevalonic acid biosynthesis in *Halobacterium cutirubrum* (*H. salinarum*) was reported to occur partially from amino acids (Ekiel et al. 1986). We reported earlier on the metabolism of [1-^{13}C]glucose (Sonawat et al. 1990) and [2-^{13}C]pyruvate (Bhaumik and Sonawat 1994) in *H. salinarum* and found that carbon units from pyruvate enter the TCA cycle by way of both pyruvate:ferredoxin oxidoreductase (P:FOR) and pyruvate carboxylase (PC). Here we present the results of our experiments on the utilization of ^{13}C-labeled pyruvate and alanine by *H. salinarum* cells grown

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M. Ghosh (✉) · H.M. Sonawat
Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400005, India
Tel. +91-22-215 2971, Ext. 2271; Fax +91-22-215 2110 / 2181
e-mail:hms@tifr.res.in

under different nutrient conditions. The data have been quantitated for relative fluxes through P:FOR and PC pathways. In addition, the flux through malic enzyme and the extent of cycling of the label in the TCA cycle have also been investigated.

Materials and methods

Chemicals

[2-^{13}C]Pyruvate, [3-^{13}C]pyruvate, and [3-^{13}C]alanine were procured from Isotec (Miamisburg, USA). Bacteriological peptone (L-37) was from Oxoid (Hampshire, UK). All other chemicals were analytical grade and were used as supplied.

Culture and media

The *H. salinarum* (earlier, *H. halobium* or *H. cutirubrum*) strain M1 cells were grown either in a complex medium containing (per liter) 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, and 10 g bacteriological peptone, or in a defined medium essentially as described by Grey and Fitt (1976). When required, this medium was supplemented with 1% glucose or sodium acetate as additional carbon source. In some experiments for alanine metabolism, the amino acid was not added in the defined medium.

The autoclaved media were inoculated with 5% (v/v) active culture of *H. salinarum*, obtained by at least three successive transfers in the fresh medium, and incubated in a rotary shaker at 37°C and 150 rpm. For frequent use the culture was maintained by transfer to fresh media at 4-day intervals. When not required for prolonged periods it was maintained on slants prepared by solidifying these media with 1.8% (w/v) agar.

The cells were harvested by centrifugation at 2000 *g* at room temperature for 30 min and resuspended (20% w/v) in basal salt medium (complex medium without peptone and the carbon source). ^{13}C-Labeled substrate was added and the cell suspension was incubated for 12 h with continuous aeration by either oxygen or nitrogen. At the end of the incubation period, the cells were sedimented by centrifugation at 2000 *g* for 30 min and cell extracts were prepared as described next.

Perchloric acid extract

The cells were suspended in 10% ice-cold perchloric acid (pca). This suspension was then sonicated (Branson 450 sonifier; CT, USA) for 2 min (4 × 30 s). The sonicate was centrifuged (Beckman L8-60M; Palo Alto, USA) at 35000 *g* for 45 min at 4°C. The supernatant was neutralized with 50% KOH and recentrifuged. The clear supernatant was then lyophilized and dissolved in a minimum volume (~0.60 ml) of D_2O and was stored frozen for NMR experiments.

NMR experiments

^{13}C-NMR spectra of the extracts, prepared as described, were recorded at 125.76 MHz on an AMX500 Bruker NMR spectrometer (Rheinstetten, Germany). The acquisition parameters were 220 ppm spectral width, excitation pulse of 30° (4 μs), with a 2-s delay between the pulses. The transients (1024–6144) were stored in 16384 data points, resulting in an acquisition time of 0.3 s and a digital resolution of 1.7 Hz/point. Gated decoupling of protons was accomplished by applying a power of 18 dB only during acquisition to reduce NOE from the connected protons. The free induction delays (FIDs) were subjected to exponential multiplication, leading to an additional line broadening of 5 Hz, before Fourier transformation. Chemical shifts are in ppm with respect to sodium 3-trimethylsilyl propionate, which was used as an external reference. Resonances were assigned based on chemical shifts reported in literature or by recording natural abundance ^{13}C-NMR spectra of authentic compounds. For quantification, the method reported by Jans and Willem (1989) was adopted with modifications. Essentially, a ^{13}C-NMR spectrum of a pca extract was recorded with the foregoing parameters. A fully relaxed spectrum for the same extract was also obtained. Comparison of these two spectra provided a measure of saturation of each of the resonances. The extent of saturation of each carbon of glutamate was determined and used as a correction factor for the experimental spectra. In addition, a fully relaxed ^{13}C-NMR spectrum of 100 mM unlabeled glutamate was also recorded. This spectrum was used for concentration calibration of the experimental spectra.

Fluxes through various pathways and fractional enrichments were calculated as per established procedures. First, integrals of the various resonances and multiplets were determined using the Bruker software. The values so obtained from the experimental spectra were used in the general equations, reported by Malloy et al. (1990), to calculate the relative flux of PC and P:FOR reactions, fractional labeling of acetyl-CoA, and also the fraction of labeled TCA intermediate derived through anaplerotic routes. These parameters were then used to evaluate the flux through glutamate dehydrogenase, malic enzyme, and recycling of the label in TCA according to Jans and Willem (1989) and Brand et al. (1992a,b). A standard nonlinear least-squares fitting routine was used for this purpose.

Results

Metabolism of [2-^{13}C]pyruvate

Figure 1a shows the ^{13}C-NMR spectrum of the extract of cells, grown in complex medium with glucose as an additional carbon source, and incubated with [2-^{13}C]pyruvate in presence of oxygen. Resonances corresponding to glutamate labeled at the C-1 (176.1 ppm) and C-5 (182.6 ppm) carbons are predominant. Other signals from C-2 lactate and C-2 alanine at 69.6 and 51.7 ppm, respec-

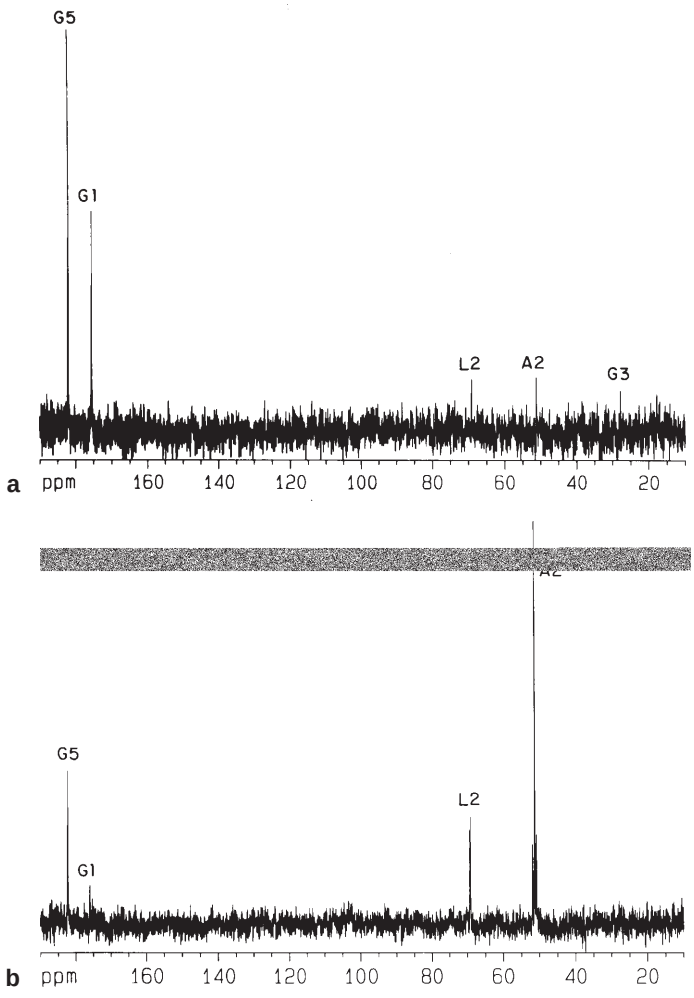


Fig. 1a,b. Proton decoupled ^{13}C-NMR spectrum of perchloric acid extract of *Halobacterium salinarum* incubated with [2-^{13}C]pyruvate in presence of (**a**) oxygen and (**b**) nitrogen. *A2* corresponds to C-2 of alanine; *G1*, *G3*, and *G5* are C-1, C-3, and C-5 of glutamate, respectively; *L2* is C-2 of lactate

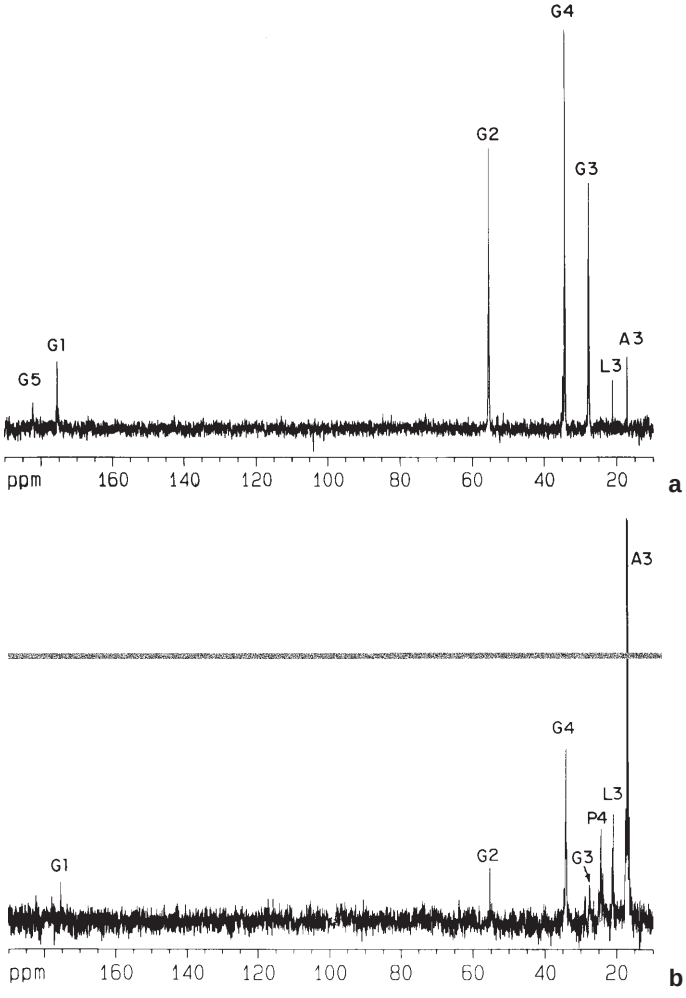


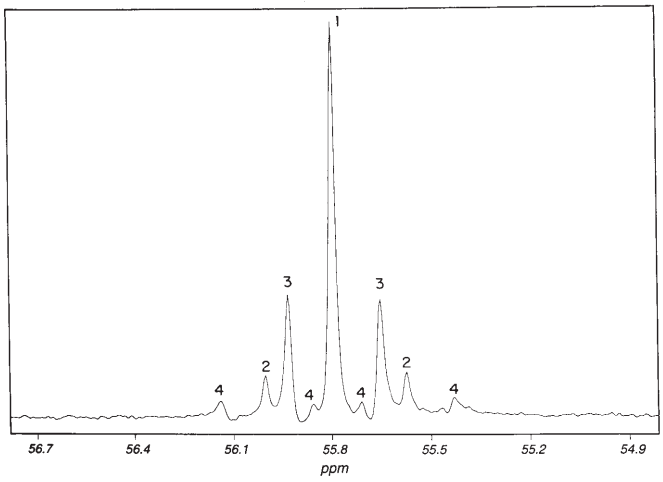
Fig. 2a,b. Proton-decoupled ^{13}C-NMR spectrum of perchloric acid extract of *H. salinarum* incubated with [3-^{13}C]pyruvate in presence of (**a**) oxygen and (**b**) nitrogen. *A3* is assigned to C-3 of alanine; *G1–G5* are C-1 to C-5 of glutamate; *L3* corresponds to C-3 of lactate; *P4* is C-4 of proline

tively, and that of C-3 glutamate, are also observed. Glutamate carbons C-1, C-3, and C-5 are labeled in the ratio 1:0.02:2.17. The intensity of the signal from C-1 glutamate is about half that of C-5 glutamate. The intensity of C-3 glutamate is low. Multiple labeling does not occur, as is evident from the absence of splitting of the carbon peaks. Under nitrogen aeration (Fig. 1b), the levels of C-2 alanine and C-2 lactate are high as compared to those of C-1 and C-5 glutamate. The intensities of glutamate carbons, in this case, are much lower than those in the presence of oxygen. Pyruvate is primarily converted into alanine under anaerobiosis.

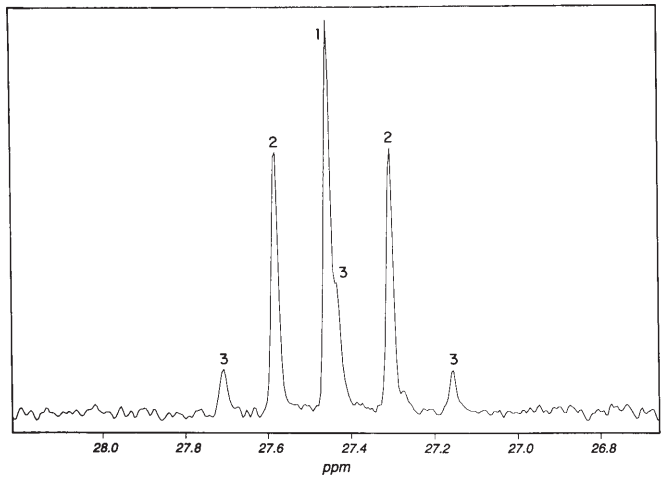
Metabolism of [3-^{13}C]pyruvate

Figure 2a shows the ^{13}C-NMR spectrum of perchloric acid extract of *H. salinarum* cells incubated with [3-^{13}C]pyruvate and aerated with oxygen. The main resonances observed are those of C-2, C-3, and C-4 glutamate, at 55.4, 27.7, and 34.2 ppm, respectively. Signals of C-3 lactate and C-3 alanine, at 21.1 and 17.1 ppm, respectively, and those of C-1 and C-5 glutamate are also seen. The labeling of glutamate C-1 to C-5 is in the ratio 1:2.9:2.6:2.4:0.6. When aerated with nitrogen (Fig. 2b), the peak corresponding to C-3 alanine is predominant. C-2, C-3, and C-4 glutamate and C-3 lactate, although visible, are much lower in intensity as compared to those in Fig. 2a. A new peak corresponding to C-4 proline is observed.

The splitting of the resonances of glutamate carbons as the result of ^{13}C–^{13}C scalar couplings is presented in Fig. 3. Because all the carbon resonances show splitting, it is clear that a significant proportion of labeled carbon positions have ^{13}C-labeling on adjacent carbons in the same molecule. Analysis of the multiplets indicates that of the total acetylCoA that goes into TCA, only 53% is generated from the added labeled pyruvate. Under steady-state conditions, the relative flux through P:FOR is about ninefold that through PC. Of the label entering TCA, the flux from α-ketoglutarate to glutamate (the glutamate dehydrogenase



a



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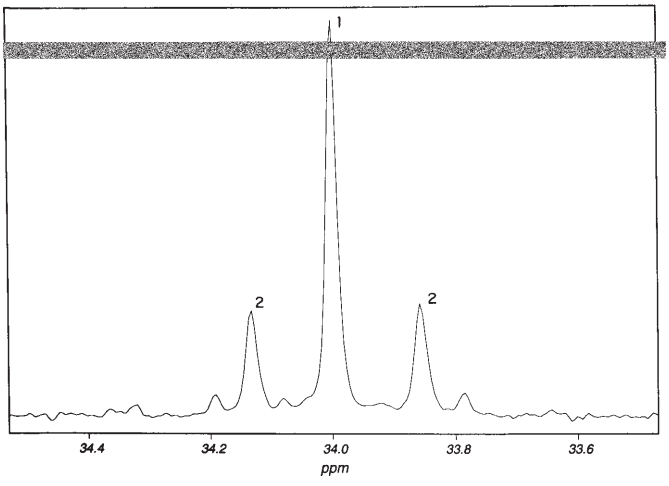

c

Fig. 3a–c. Expanded regions of Fig. 2 showing ^{13}C–^{13}C scalar coupling in (**a**) C-2, (**b**) C-3, and (**c**) C-4 of glutamate. Assignments: (**a**) *1*, singlet of ^{13}C-2; *2*, doublet from coupling with ^{13}C-1; *3*, doublet from coupling with ^{13}C-3; *4*, quartet from coupling with ^{13}C-1 and ^{13}C-3; (**b**) *1*, singlet of ^{13}C-3; *2*, doublet from coupling with ^{13}C-2 or ^{13}C-4; *3*, triplet from ^{13}C-2 and ^{13}C-4; (**c**) *1*, singlet of ^{13}C-4; *2*, doublet from coupling with ^{13}C-3

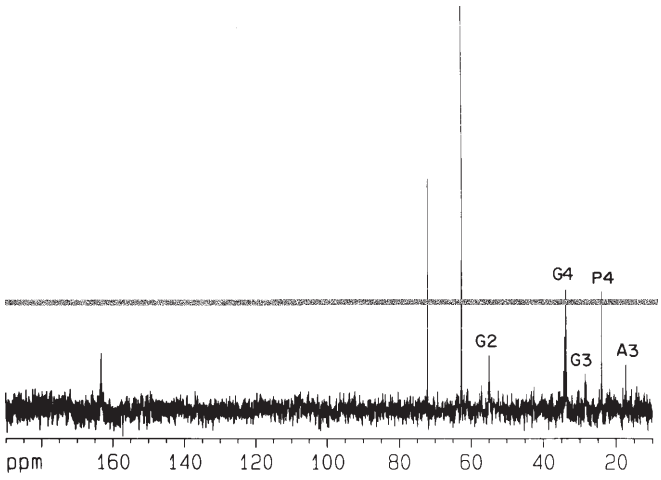

Fig. 4. Proton-decoupled ^{13}C-NMR spectrum of perchloric acid extract of *H. salinarum* incubated with [3-^{13}C]pyruvate in presence of malonate. Assignments as in Figs. 1 and 2

reaction) is 42%. The flux through malic enzyme is about 7%. Again, 41% of the label recycles in TCA through the citrate synthase reaction.

Figure 4 shows the ^{13}C-NMR spectrum of *H. salinarum* cells incubated with [3-^{13}C]pyruvate in the presence of malonic acid. C-2 to C-4 glutamate is low as compared to that seen in Fig. 2a. Also, C-1 and C-5 glutamate are not observed. The spectrum shows additional resonances at 63.3 and 72.8 ppm. Based on reported chemical shifts, these have been assigned to C-6 of α- and β-glucose-6-phosphate and C-2 and C-5 of α- and β-glucose. Resonances corresponding to C-3 alanine and C-4 proline were also observed.

[3-^{13}C]Alanine metabolism

Halobacterium salinarum cells were pregrown in defined medium with or without unlabeled alanine and the cell suspensions were then incubated with [3-^{13}C]alanine. In Fig. 5 are shown the ^{13}C-NMR spectra of perchloric acid extracts of these cell suspensions. When pregrown without unlabeled alanine, C-3 alanine metabolism shows resonances of C-1, C-2, C-3, and C-4 glutamate (Fig. 5a). The labeling of C-1:C-2:C-3:C-4 glutamate is in the ratio 1:1.7:1.9:2.0. C-4 proline and unutilized C-3 alanine are also observed. Similar to the observations for [3-^{13}C]pyruvate, all the glutamate resonances are multiplets. Figure 5b shows the NMR spectra of extracts of cells pregrown with unlabeled alanine. A signal corresponding to C-4 glutamate is observed in addition to unutilized C-3 alanine.

Discussion

Metabolism of ^{13}C-labeled substrates has been investigated in *H. salinarum* by NMR spectroscopy. Kreb's TCA cycle

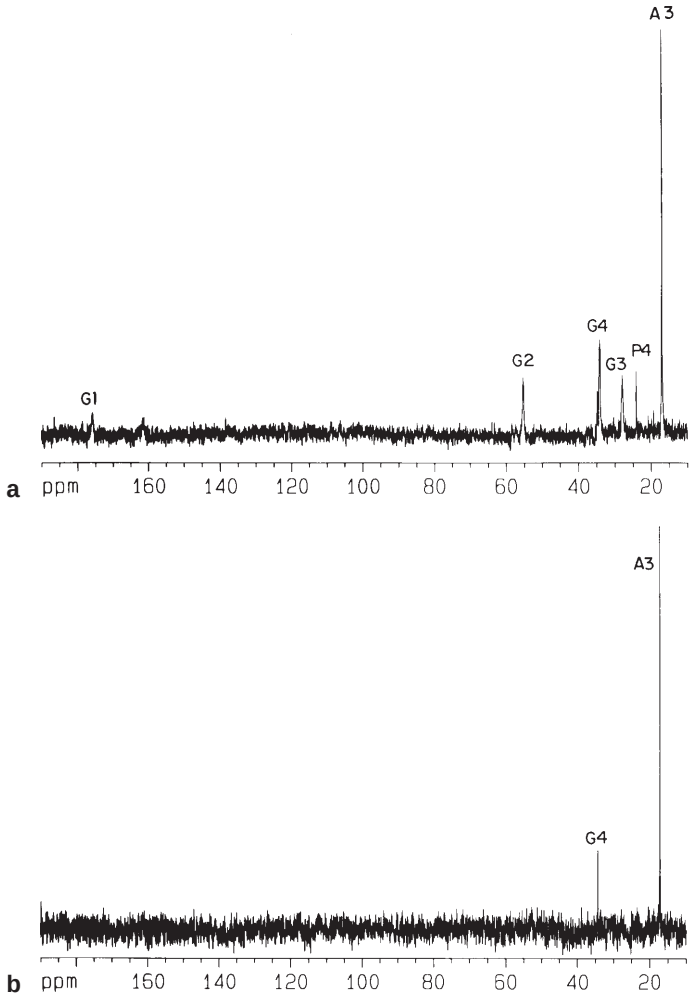

Fig. 5a,b. Proton-decoupled ^{13}C-NMR spectrum of perchloric acid extract of *H. salinarum* cells pregrown in defined medium without (**a**) or with (**b**) unlabeled alanine. After harvesting, both cell suspensions were separately incubated with [3-^{13}C]alanine in presence of oxygen before the preparation of extracts. Assignments as in Figs. 1 and 2

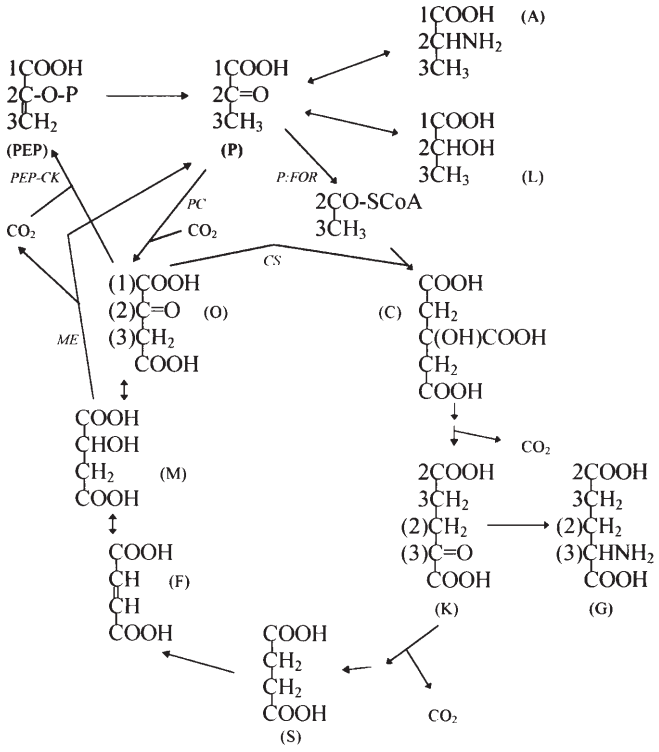

Fig. 6. Metabolic pathways and labeling pattern for pyruvate and alanine utilization by *H. salinarum*. *CS*, citrate synthase; *ME*, malic enzyme; *PC*, pyruvate carboxylase; *PEP-CK*, phosphoenolpyruvate carboxykinase; *P:FOR*, pyruvate:ferredoxin oxidoreductase; (*A*), alanine; (*C*), citrate; (*F*), fumarate; (*G*), glutamate; (*K*), α-ketoglutarate; (*L*), lactate; (*M*), malate; (*O*), oxaloacetate; (*P*), pyruvate; (*PEP*), phosphoenolpyruvate; (*S*), succinate. The carbon numbers are with reference to those of pyruvate (or alanine), and indicate their labeling through P:FOR. *Numbers in parentheses* represent label entering TCA through PC

and related anaplerotic routes have been studied in particular. The NMR results obtained with ^{13}C-labeled pyruvate are fully consistent with the presence of an active TCA cycle in this archaeon. Our observations, thus, corroborate earlier reports (Aitken and Brown 1969; Danson et al. 1985; Danson 1988). Following the label entering the TCA cycle through P:FOR, C-3 pyruvate gives rise to C-2 acetylCoA, which condenses with oxaloacetate to form citrate and further to C-4 α-ketoglutarate (Fig. 6), producing C-4 glutamate as the first spinoff product (Fig. 2a). The fraction of label that continues in the cycle leads to C-3 malate/oxaloacetate. In the second cycle, this would lead to C-2, and C-4 glutamate. The PC reaction also produces C-3 oxaloacetate. This is, again, expected to cause labeling of the C-2 in glutamate. In the ^{13}C-NMR spectrum (Fig. 2a), both C-2 and C-3 glutamate are observed. This is indicative of (i) significant PC activity and (ii) scrambling when the label passes through succinate thiokinase, succinate dehydrogenase, and fumarase. The products and substrates of these enzymes (succinate and fumarate) are symmetric. Because the intensities of C-2 and C-3 glutamate are similar (Fig. 2a), complete scrambling seems to be occurring. This leads to the formation of equimolar C-2 and C-3 malate and oxaloacetate, which, in turn, produce C-3- and C-2-labeled glutamate, respectively, in the second cycle.

The C-3 glutamate signal is split by scalar coupling resulting from labeling at C-2 or C-4 glutamate. The coupling from C-4 glutamate is an indirect measure of the fractional enrichment of C-2 acetylCoA. Any acetylCoA generated endogenously is unlabeled and would not cause any C-4 glutamate labeling; therefore, the splitting would not be observed. A similar splitting of C-4 glutamate signal from scalar coupling with C-3 glutamate in the same molecule is, however, indicative of the extent of label scrambling through the TCA cycle. C-2 α-ketoglutarate, from the third cycle onward, gives rise to C-1 glutamate. The extent of label cycling three times through the TCA cycle is double of that observed as C-1 glutamate because the other half, appearing as C-6 citrate, goes off as $^{13}CO_2$. In subsequent cycles the label is expected to be observed at 1-, 2-, 3-, 4-glutamate.

The malic enzyme seems to play a considerable role in the TCA cycle in *H. salinarum*. Pyruvate labeled at the carbonyl carbon (C-2) is produced by the action of malic enzyme on C-2 malate. C-2 pyruvate gives rise to C-1 acetylCoA by the action of P:FOR, which in turn leads to C-5 glutamate. Glutamate labeling at C-5 is, therefore, an estimate of the flux of the label through malic enzyme and represents 50%, because the other half, generating C-3 pyruvate, is indistinguishable from the exogenous C-3 pyruvate.

Overall, C-2 and C-3 glutamate is an indication of the label traversing TCA twice, while C-1 glutamate signifies cycling of the label in TCA at least three times, and C-4 glutamate is a measure of the initial flux through the citrate synthase reaction. The added pyruvate contributes only about half to the acetylCoA pool of *H. salinarum*. The rest is derived from endogenous and, therefore, unlabeled sources. Essentially similar results have been obtained from experiments with [3-^{13}C]alanine incubation. The only difference is that the metabolism is initiated and perhaps limited by the alanine aminotransferase reaction.

As expected, inclusion of malonate, a competitive inhibitor of succinate dehydrogenase, reduces the cycling of the label in the TCA cycle. This is evident from the absence of C-1 and low levels of C-2 and C-3 glutamate (Fig. 4). However, we would expect a buildup of TCA cycle intermediates before the succinate dehydrogenase reaction. The low levels of C-4 glutamate suggest a decreased flux through the P:FOR route. This decrease could occur if (a) sufficient acetylCoA existed but there was a depleted pool size of oxaloacetate for condensation with acetylCoA in the citrate synthase reaction, or (b) the TCA cycle enzymes existed as a cluster or a metabolon (Srere 1985), as has been suggested by other workers (Evans et al. 1993; Malaisse et al. 1996; Sherry et al. 1994; Sumegi et al. 1993), so that an inhibition of any one enzyme would inhibit the entire cycle. In the presence of malonate, *H. salinarum* shows gluconeogenic activity with pyruvate (Fig. 4), which precludes substantial flux through PC and phosphoenolpyruvate (PEP) carboxykinase.

A comparison of Fig. 2a and Fig. 2b suggests that under aerobiosis pyruvate is utilized mainly in TCA whereas anaerobic conditions lead to an enhancement of its transamination to alanine, the flow through TCA being reduced and limited to formation of C-4 glutamate and C-4 proline, with recycling of the label almost nonexistent. [2-^{13}C]Pyruvate, on the other hand, directly leads to C-1 acetylCoA through P:FOR, followed by C-5-labeled α-ketoglutarate and therefore to C-5 glutamate in the first cycle (Fig. 6). On further passage through the TCA cycle, C-4 malate and oxaloacetate are expected, which give rise to the labeling of C-1 glutamate. Thus at each turn of TCA the incoming C-1 acetylCoA labels C-5 of glutamate and in the subsequent cycle the C-1 position. None of the other carbons is expected to be labeled if P:FOR and citrate synthase are the only route of entry of label into TCA. The extent of scrambling of the label during its passage through succinate thiokinase, succinate dehydrogenase, and fumarase is indicated by the relative intensities of the C-1 and C-5 resonances. A ratio of 0.5 would suggest complete scrambling, as about half the oxaloacetate molecules would be labeled at C-1, which would be lost as $^{13}CO_2$ at the isocitrate dehydrogenase step. Figure 1a yields a C1:C5 ratio of 0.46. Again, C-2 oxaloacetate is derived from C-2 pyruvate through PC, which in TCA leads to C-3 glutamate.

A significant back reaction through malate dehydrogenase and fumarase generates C-3 oxaloacetate also, which in turn labels the C-2 of glutamate. Our results indicate that only C-3 glutamate is produced; C-2 glutamate is not detectable. Thus, the label entering the TCA cycle through PC does not seem to undergo back reactions. Overall, C-5 glutamate indicates P:FOR and citrate synthase flux, C-3 glutamate reflects PC activity, and C-1 glutamate indicates the extent of label scrambling in TCA.

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