Citric acid cycle enzymes of the archaebacteria: citrate synthase and succinate thiokinase

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In eubacteria and eukaryotes, citrate synthase and succinate thiokinase exhibit a species-dependent diversity of structure, activity and regulation. These enzymes have now been examined in the archaebacteria and it is found that each of the three major subgroups, the extreme halophiles, the thermoacidophiles and the methanogens, possesses characteristic types of citrate synthase and succinate thiokinase. The observed patterns of structure and regulation of these two enzymes are compared across the three evolutionary lineages.

Citrate synthase *Succinate thiokinase* *Archaebacteria*

1. INTRODUCTION

Several enzymes of the citric acid cycle possess a diversity of structure, activity and regulation which indicates a strong correlation with the taxonomic status of the source organism [1]. This is particularly marked for citrate synthase [1,2]. The 'large' hexameric [3,4] citrate synthases are allosterically inhibited by NADH and are found exclusively in Gram-negative eubacteria [5]; in the facultative anaerobes of this group the enzyme is also inhibited by 2-oxoglutarate [6]. In contrast, Gram-positive eubacteria and eukaryotes possess 'small', dimeric, citrate synthases which are isosterically inhibited by ATP but are insensitive to NADH and 2-oxoglutarate [1,2]. This pattern is mirrored by succinate thiokinase which also uses an acyl-CoA as substrate. Thus succinate thiokinases of Gram-negative organisms are 'large' enzymes ($\alpha_2\beta_2$) whereas the small ($\alpha\beta$) type is confined to Gram-positive eubacteria and eukaryotes [1,7].

The pattern of these two enzymes is therefore characteristic of the organism's classification. However, they have not been widely studied in the archaebacteria, organisms which, it is proposed, constitute a third primary line of evolutionary descent [8]. It has been reported [9,10] that one of the extreme halophiles possesses a 'small' type citrate synthase but a 'large' succinate thiokinase. In view of this apparent exception to the eubacterial and eukaryotic pattern of diversity, we have extended the investigation to a range of halophilic, thermoacidophilic and methanogenic archaebacteria and so complete the survey of citrate synthase and succinate thiokinase types.

2. EXPERIMENTAL

All chemicals used were of analytical grade. *Sulpholobus acidocaldarius* (DSM639) was grown at 70°C (pH 2.0) as in [11] and *Thermoplasma acidophilum* (DSM1728) at 57°C (pH 1.8) as in [12]. Cell extracts were prepared as in [11]. The halophilic archaebacteria (kindly provided by Dr W.D. Grant, University of Leicester) were the classical halophiles *Halobacterium halobium* (CCM2090), *Halobacterium vallismortis* (ATCC29715) and *Halobacterium volcanii* (NCMB2012) and the alkaliphilic halophiles *Natronobacterium pharaonis* (NCMB2191), *Natronobacterium gregoryi* (NCMB2189) and *Natrononococcus occultus* (NCMB2192). Growth of the halophiles and cell extraction were as in [13]. Cells of *Methanosarcina barkeri* (DSM800) were a kind gift of Miss J. Harris (Food Res. Inst., Norwich)

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and cell extracts were prepared by sonication in 20 mM Tris–HCl buffer (pH 8.0) containing 2 mM EDTA.

Citrate synthase was assayed spectrophotometrically at 412 nm [14] with the inclusion of 2 M NaCl in the assay mixture for the halophilic enzyme. Succinate thiokinase from the halophiles was assayed spectrophotometrically at 412 nm [15] in 0.1 M Na phosphate (pH 7.6), 4 M NaCl, 10 mM $MgCl_2$, 0.15 mM succinyl-CoA, 0.5 mM ADP and 0.1 mM 5,5′-dithiobis(2-nitrobenzoate). Succinate thiokinase from *Sulpholobus, Thermoplasma* and *Methanosarcina* was assayed at 235 nm as in [16]. All assays were performed at 30°C except for extracts of *Sulpholobus* and *Thermoplasma* which were performed at 55°C.

Gel filtration on a column of Sephacryl S-200 was performed at 25°C as in [13]. Filtration of standard proteins and extracts of the thermoacidophiles and *M. barkeri* was performed in 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA and 0.1 M KCl. For halophilic extracts, 4 M NaCl was included in this buffer. Dextran Blue and Dnp-

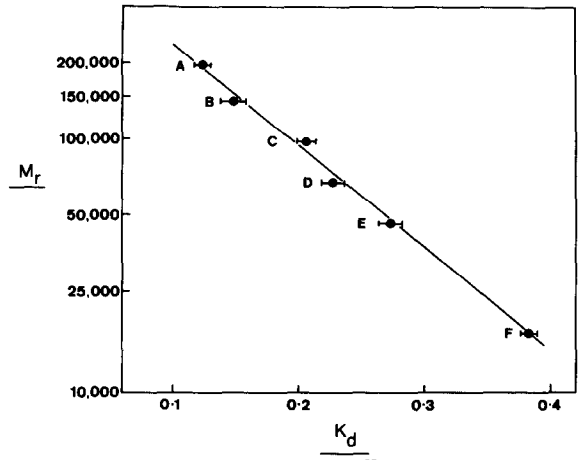

Fig.1. Gel filtration on Sephacryl S-200. K_d, the distribution coefficient, was calculated as described in section 2. Standard proteins: A, pig heart fumarase (194 kDa); B, pig heart lactate dehydrogenase (142 kDa); C, pig heart citrate synthase (98 kDa); D, pig heart malate dehydrogenase (67 kDa); E, yeast 3-phosphoglycerate kinase (47 kDa); F, whale skeletal muscle myoglobin (17 kDa). The bars represent the SE of the K_d values for each protein determined in 5 separate filtration experiments.

Table 1

M_r values of citrate synthase and succinate thiokinase

Organism	Citrate synthase M_r	Succinate thiokinase M_r
Archaebacteria		
H. halobium	111000	–
H. vallismortis	112000	174000
H. volcanii	105000	170000
Nb. pharaonis	106000	174000
Nb. gregoryi	112000	171000
Nc. occultus	105000	184000
S. acidocaldarius	83000	–
T. acidophilum	85000	68000
Gram-negative eubacterium		
Escherichia coli	288000 (hexameric)	136000 (tetrameric)
Gram-positive eubacterium		
Bacillus megaterium	84000 (dimeric)	75000 (dimeric)
Eukaryote		
Pig heart	98000 (dimeric)	75000 (dimeric)

lysine were included in all filtrations and for each protein values of K_d (distribution coefficient) were calculated thus:

$$K_d = (V_e - V_o)/(V_{re} - V_o)$$

where V_e, V_o and V_{re} represent the elution volume of the protein, Dextran Blue and Dnp-lysine, respectively [17].

3. RESULTS

3.1. *Molecular sizes of citrate synthases and succinate thiokinases*

M_r values were determined by zonal gel filtration as described in section 2. The data were plotted as log M_r *vs* K_d, and a good fit to a straight line is observed for the standard proteins (fig.1). Within each group of archaebacteria the elution positions of citrate synthase and of succinate thiokinase were remarkably constant. Thus values of K_d for citrate synthase were 0.184 (± 0.010 SD) from the halophiles and 0.211 (± 0.007) from *Sulpholobus* and *Thermoplasma*. For succinate thiokinase the values were 0.131 (± 0.011) from the halophiles and 0.234 (± 0.010) from *T. acidophilum*. The corresponding individual M_r values are given in table 1 with representative values for the eubacterial and eukaryotic enzymes. Succinate thiokinase from *S. acidocaldarius*, although detectable in cell extracts, appeared to be inactivated during gel filtration. No succinate thiokinase was found in *M. barkeri* and the levels of citrate synthase in this organism were so low (~2 nmol/min per mg protein) that no M_r value could be determined by the present method.

3.2. *Kinetic properties of citrate synthase and succinate thiokinase*

The activity of citrate synthase from all species of archaebacteria depended hyperbolically on the concentrations of acetyl-CoA and oxaloacetate. The data were analysed by the direct linear plot

Table 2

Kinetic parameters of citrate synthase in cell extracts

Organism	Temperature of assay (°C)	Catalytic activity			Inhibition		
		Specific activity (nmol/min per mg)	K_m-acetyl-CoA (μM)	K_m-oxaloacetate (μM)	K_i-ATP (mM)	K_i-NADH (mM)	K_i-2-oxoglutarate (mM)
Archaebacteria							
H. halobium	30	51	211	58	5.6	>10	not inhibited
H. volcanii	30	61	75	38	1.9	>10	>20
Nb. gregoryi	30	14	108	63	4.2	3.2	>20
Nb. pharaonis	30	26	446	210	>30	5.2	not inhibited
S. acidocaldarius	55	280	10	20	0.9	4.6	2.2
T. acidophilum	55	172	6	5	2.2	5.4	5.4
M. barkeri	30	2	7	18	<0.3	<0.1	<0.3
Gram-negative eubacterium							
Escherichia coli	30	150	400 ($S_{0.5}$	55 ($S_{0.5}$)	4.8	0.02	0.06
Gram-positive eubacterium							
Bacillus megaterium	30	100	76	12	1.1	7.5	5.7
Eukaryote							
Pig heart	30	740	7	10	0.3–0.8	4.6	not inhibited

[18] and the determined kinetic constants are given in table 2. The effects on citrate synthase of NADH, ATP and 2-oxoglutarate, potential inhibitors of the enzyme [2], were investigated. Inhibition by NADH and ATP was competitive with acetyl-CoA and where observed, 2-oxoglutarate competed with oxaloacetate. The data were analysed by Dixon plots and the calculated K_i values are given in table 2. Data for eubacterial and eukaryotic citrate synthases are included for reference.

Succinate thiokinase from all halophiles and thermoacidophiles tested was ADP/ATP-specific, no activity being detectable with GDP/GTP. Enzyme activity depended hyperbolically on the concentration of adenine nucleotide. As stated above, succinate thiokinase could not be found in *M. barkeri*, although an ADP-specific enzyme has been reported in *Methanobacterium thermoautotrophicum* [19].

4. DISCUSSION

This work extends the survey of citrate synthases and succinate thiokinases to the archaebacterial kingdom. We find that both classical and alkaliphilic halophiles possess 'small' citrate synthases but 'large' succinate thiokinases, a pattern unique to these Archaebacteria. In contrast to the 'small' citrate synthases from Gram-positive eubacteria and eukaryotes, the halophilic citrate synthases (with the possible exception of that from *H. volcanii*) are weakly sensitive to ATP (K_i > 4 mM). The K_m values for acetyl-CoA are accordingly high, the sensitivity of a citrate synthase to ATP being directly related to the apparent affinity for this substrate [2]. On the other hand, citrate synthase and succinate thiokinase from the thermoacidophiles superficially resemble those from Gram-positive eubacteria and eukaryotes: both enzymes are of the small type and the citrate synthase, particularly from *Sulpholobus*, is sensitive to ATP but not to NADH or 2-oxoglutarate.

In the methanogens, citrate synthase and succinate thiokinase have not been found in the same organism [20]. We have examined the citrate synthase from *M. barkeri* and find it unusual in being sensitive to all three inhibitors, ATP, NADH and 2-oxoglutarate. ADP and AMP also inhibit in the order ATP > ADP > AMP. However, the enzyme was not inhibited by NADPH, suggesting that the effect of NADH is unlike the less specific, isosteric nucleotide inhibition found in 'small' citrate synthases [2]. These data must be viewed with caution as citrate synthase is present in very low amounts in *M. barkeri*. Also, assays of NADH inhibition had to be carried out in extracts where levels of malate dehydrogenase were 30-times greater, although it was shown that 'inhibition' of citrate synthase was not due to a depletion of oxaloacetate by the dehydrogenase. Unfortunately, we could not detect the methanogenic citrate synthase after gel filtration; thus its molecular size is unknown and we have not confirmed the inhibitions on semipurified enzyme. Finally, it should be noted that archaebacterial succinate thiokinases are all ADP/ATP-specific, a situation found elsewhere only in Gram-positive eubacteria [1].

From our observations we conclude that new patterns of structure and regulation are to be found in archaebacterial citrate synthases and succinate thiokinases. It would therefore appear important to begin a detailed study of these enzymes so that the species-dependent diversity can now be compared across all three evolutionary lineages.

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