ON THE REGULATORY PROPERTIES OF A HALOPHILIC CITRATE SYNTHASE

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1. Introduction

Citrate synthase (citrate oxaloacetate-lyase(CoA-acetylating), EC 4.1.3.7) has been shown to have different regulatory properties and molecular weights depending on the taxonomic position of the organism of origin [1–3]. Gram negative organisms have citrate synthases inhibited by low concentrations of NADH and reactivated by AMP [1], with molecular weights in the order of 250,000 [3]. The enzymes from Gram positive organisms and eukaryotes, on the other hand, are insensitive to NADH and their molecular weights are about 80,000 [1, 3].

The Halobacteria are Gram negative bacteria, included among the Pseudomonadaceae which require for growth NaCl concentrations approaching saturation, and have also very high intramolecular salt concentrations [4]. Since the *E. coli* citrate synthase was desensitized towards NADH by KCl concentrations (0.2 M) [5] much lower than those reported as the intracellular concentrations for extreme halophiles (4 M) [4], it was considered of interest to study the regulatory properties of the enzyme from *Halobacterium cutirubrum*.

The results presented in this communication show that the halophilic citrate synthetase behaves unlike the enzymes from other Gram negative bacteria, and indeed resembles the enzymes from Gram positive organisms and eukaryotes in both molecular size and regulatory properties.

2. Material and methods

Deoxyribonuclease I (DNAase), sodium pyruvate and 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, Mo.; oxalacetic acid (OAA), CoA, NADH, citrate synthase, malate dehydrogenase and lactate dehydrogenase from pig heart were purchased from Boehringer, Mannheim; Sephadex G-200 and Blue Dextran 2000 from Pharmacia, Uppsala. Acetyl-CoA was synthesized from CoA and acetic anhydride by the method of Stadtman [6] and assayed with DTNB [7]. The citrate synthase from a marine *Pseudomonas*, used for comparative purposes, was partially purified (about 50-fold, with a specific activity of 4.2 μmoles CoA/min/mg of protein) by the same method used for the purification of NADP-linked malic enzyme [8].

The halobacteria were disrupted by sonic treatment in 0.05 M Tris-Cl buffer, pH 7.6, containing 5 M NaCl and 1 mM EDTA, and treated with DNAase, as previously described [9]. The cell-free extract was then dialysed for 3 hr at 18° against 30 vol of 0.05 M Tris-Cl buffer, pH 7.6, containing 0.2 M NaCl, 1 mM OAA and 1 mM EDTA. Under these conditions most of the proteins in the crude extract, including malate dehydrogenase, were denatured because of the low salt concentration, whereas citrate synthase was protected by its substrate OAA. The denatured proteins could then be precipitated by ammonium sulphate. The 88–100% saturation fraction contained about 50% of the citrate synthase activity in the crude extract, with a purification which varied between 17 and 25-fold in different preparations. A further purification of 2 to 2.5-fold was obtained by gel filtration through Sephadex G-200 equilibrated with the Tris-Cl–0.2 M NaCl–OAA–EDTA solution. The preparations obtained after dialysis overnight of the active fractions

of the eluate against the Tris-Cl–5 M NaCl–EDTA solution had specific activities of about 2.5 μmoles CoA/min/mg of protein (about 50-fold purification with respect to the crude extract) with an overall yield of about 25%, and were free of malate dehydrogenase, the red carotenoid pigment with absorbance at 412 nm, and unidentified substances present in the crude extracts which were able to react with DTNB.

Citrate synthase was assayed spectrophotometrically at 412 nm, as described in the legends to the figure and tables.

3. Results and discussion

The citrate synthase from *H. cutirubrum* behaved as a typical halophilic enzyme, requiring high concentrations of KCl (about 2.6 M) or lower concentrations of $MgCl_2$ (about 50 mM) for maximal activity at saturating concentrations of both substrates, and being rapidly denatured in the presence of low concentrations of salt, except when protected by OAA or, to a much lesser extent, by acetyl-CoA.

Table 1
Inhibition of halophilic citrate synthase by NADH and ATP in the presence of $MgCl_2$ or KCl as activating salts.

		Inhibition (%)		
		45 mM $MgCl_2$	0.175 M KCl	2.3 M KCl
NADH (mM)	0	0	–	0
	5	21	–	9
	10	36	–	18
	20	63	–	33
	30	69	–	47
ATP (mM)	0	0	0	0
	2.5	7	18	10
	5	15	27	16
	10	27	35	26
	20	50	44	39
	30	55	49	44

The reaction mixtures contained (in μmoles) in a final volume of 1 ml: Tris-Cl buffer, pH 7.45, 40; OAA, 0.1; acetyl-CoA, 0.05; DTNB, 0.1; 21 μg of enzyme; $MgCl_2$ or KCl, NADH or ATP, at the concentrations stated on the table. The activities in the absence of inhibitors were 5.3 ($MgCl_2$), 5.5 (0.175 M KCl) and 5.7 (2.3 M KCl) nmoles CoA/min.

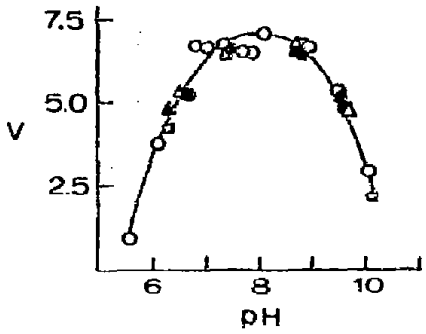

Fig. 1. Effect of pH on the reaction velocity. The reaction mixtures contained (in μmoles) in a final volume of 1 ml: Tris-Cl buffer, at the pH values stated on the abcissa, 40; KCl, 2,800; OAA, 0.2; acetyl-CoA, 0.12; DTNB, 0.1; and 8 μg of enzyme. (○), No additions; (△), 1 mM NADH; (□), 0.5 mM AMP; (●), 5 mM ATP; (▲), 10 mM α-ketoglutarate. The reaction was started by the addition of the enzyme after equilibration of the otherwise complete reaction mixture in the chamber of a Beckman DB-G recording spectrophotometer at 30° for 1 min, and followed as the increase of absorbance at 412 nm due to the reaction of CoA with DTNB. The reaction velocity (v) is expressed as nmoles of CoA/min. The pH values were determined directly in the reaction mixtures with a pH meter fitted with a glass electrode.

NADH at 1 mM did not affect the enzyme activity in the presence of 2.8 M KCl at pH 7.5. Since the effect of NADH and also ATP on the *E. coli* enzyme was strongly dependent on the pH of the reaction mixture [5, 10], the action of the possible effectors NADH, AMP, ATP and α-ketoglutarate was tested over a wide pH range (fig. 1). No appreciable inhibitions were observed. 1 mM NADH was equally ineffective when tested (in the presence of 2.8 M KCl) on the crude enzyme preparation, under conditions such as to detect the inhibition in spite of the presence of malate dehydrogenase [1], or in a preparation partially free from the latter enzyme after gel filtration of the crude extract on Sephadex G-200, in 5 M NaCl. It was also ineffective when the KCl concentration in the reaction mixture was reduced to 0.175 M. These experiments made unlikely the possibilities that desensitization during the purification procedure, or reversion of the inhibition by high salt concentrations, were the explanations of the negative results.

When high concentrations of NADH or ATP were tested in the presence of low concentrations of acetyl-CoA (0.1 × K_m) and OAA (1 × K_m) (table 1) some inhibition was evident. The inhibitions were greater

Table 2
Molecular weight of the halophilic citrate synthase, as determined by gel filtration through Sephadex G-200.

Protein	Amount (mg)	Elution (ml)	Estimated molecular weight	Reference
Malate dehydrogenase	0.050	34.5	55-- 70,000	[11]
Citrate synthase (halophile)	2.800	33.2	70-- 75,000	
Citrate synthase (pig heart)	0.004	32.0	80,000	[3]
Lactate dehydrogenase	0.100	28.0	130 -140,000	[11]
Citrate synthase (*Pseudomonas*)	0.360	24.0	250–300,000	

The sample volume was 1.2 ml. The buffer used for elution of the column (13 × 280 mm) was 0.05 Tris-Cl, pH 7.6, containing 0.4 M KCl, 1 mM OAA and 1 mM EDTA. Fractions (1 ml) were collected at a rate of 10 ml/hr. The void volume of the column, as determined with Blue Dextran 2000, was 19 ml. The halophilic and pig heart citrate synthases could be assayed separately and accurately in the same samples, since the former is nearly inactive in the absence of salt, and the latter is completely inhibited by 3.32 M KCl. The reaction mixtures were as described in the legend to table 1, except for the addition of salt (3,320 μmoles of KCl, only for the halophilic enzyme), and 0.1 ml of eluate. Malate dehydrogenase and lactate dehydrogenase were assayed, and the results plotted and the molecular weights calculated, as described by Andrews [11].

when the salt used as activator was $MgCl_2$ (at 45 mM). The very high inhibitor concentration required, and the fact that the inhibitions were smaller at KCl concentrations approaching that inside the living halophilic cell, make these results of doubtful physiological significance; they show, however, that the enzyme was able to interact to a certain extent with NADH and ATP. The effects of the inhibitors at high concentrations are probably due to the structural similarity between their adenosine moieties and that of the substrate acetyl-CoA. Lineweaver–Burk plots (not presented) showed that both 10 mM NADH (in the presence of 45 mM $MgCl_2$) and 10 mM ATP (in the presence of either 0.175 M or 2.3 M KCl) were competitive with respect to acetyl-CoA. The inhibition by 10 mM NADH was not reverted by 10 mM AMP.

Table 2 shows that the molecular sizes of the halophilic citrate synthase and the enzyme from pig heart are similar. The halophilic enzyme was eluted from Sephadex G-200 between pig heart citrate synthase and malate dehydrogenase, suggesting that its molecular weight is about 70–75,000. This result was not due to dissociation of the enzyme during the purification procedure, since when a crude extract was percolated through a Sephadex G-200 column in 5 M NaCl, the halophilic citrate synthase was eluted in approximately the same volume as in the experiment of table 2. The citrate synthase from a marine *Pseudomonas*, which was inhibited by NADH, reactivated by AMP and desensitized towards NADH by 0.2 M KCl (T.E. Massarini and J.J. Cazzulo, unpublished results) was shown to have a molecular weight of 250--300,000 (table 2). This result is in good agreement with published data on the enzyme from *Pseudomonas fluorescens* [3]. The approximate molecular weights from table 2 suggest the possibility that the *Pseudomonas* enzyme is a tetramer, and the halophilic enzyme a monomer, the basic subunit being of the same order of magnitude in both cases.

The present day Halobacteria are probably derived from Pseudomonad ancesters. It is tempting to speculate that during their adaptation to halophilic life, the inhibition of citrate synthase by NADH became physiologically useless, because of the desensitization caused by the higher intracellular salt concentrations. It would not be surprising, then, that a character no longer important for survival was lost in the course of evolution, together with the subunit structure which allows the regulation in common Gram negative organisms. This would have led to the paradoxical result, from a taxonomic point of view, that the halophilic citrate synthase resembles the same enzyme in Gram positive bacteria and eukaryotes, and differs considerably from all the citrate synthases from Gram negative organisms reported so far.

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