TWO FORMS OF CITRATE SYNTHASE IN A MARINE *PSEUDOMONAD*

Esther MASSARINI and Juan José CAZZULO

Departamento de Bioquímica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario. Suipacha 531, Rosario, República Argentina

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1. Introduction

Citrate synthase (EC 4.1.3.7) is affected in vitro by several effectors, which might be involved in the enzyme regulation in vivo [1]. Weitzman [2–4] has proposed that there is a close relationship between the taxonomical position of a microorganism and the molecular weight and regulatory properties of its citrate synthase. The enzymes from eucaryotes and Gram positive bacteria are 'small' (mol. wt near 100 000) [3] and show little or no sensitivity to NADH [2,3], but are inhibited by ATP [5,6]. The citrate synthases from Gram negative bacteria are 'big' (mol. wt near 250–300 000) [3] and inhibited by NADH [2,3], and, in some instances, by α-oxoglutarate [4]; in some cases AMP is a deinhibitor with respect to AMP [2]. Although exceptions to this regulatory pattern are known [1,7], a general correlation between taxonomical position and regulatory properties seems to exist.

A marine *Pseudomonad* contained a 'big', NADH-inhibited, citrate synthase, which was strongly activated by AMP and KCl in the absence of NADH [8]. We show here that the microorganism contains in addition a 'small', NADH- and AMP-insensitive, ATP-inhibited, citrate synthase. The evidence suggests that the 'small' enzyme might derive from the 'big' enzyme by dissociation.

2. Materials and methods

The microorganism, the conditions for the culture, harvesting and washing of the cells, the suspension of the cells in 50 mM Tris–HCl buffer (pH 7.6) containing 1 mM EDTA 0.2 M KCl and 5 mM $MgCl_2$, the preparation of cell-free extract by sonic disintegration, the DNAase treatment and the ammonium sulphate fractionation were as previously described [9]. The 50–70% saturation fraction was dialysed overnight against 100 vol of 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 70 mM KCl and chromatographed on a column of DEAE-cellulose (1.8 × 35 cm) with a linear gradient of KCl (70–500 mM) in the same buffer. 5 ml-fractions were collected at a rate of 37.5 ml/hr. The K^+ concentration in the eluate was determined by flame photometry. DEAE-cellulose re-chromatography was performed under similar experimental conditions, or with a KCl gradient from 0 to 500 mM in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA; in the latter case the enzyme was previously dialysed overnight against 100 vol of phosphate-EDTA buffer without KCl. The enzyme activity was assayed spectrophotometrically by following the increase in absorbance at 412 nm due to the reaction of the CoA liberated in the reaction with 5,5' dithiobis(2-nitrobenzoic acid) (DTNB), in a UNICAM SP 1800 B recording spectrophotometer, at 30°C. The composition of the reaction mixtures is stated in the legends to the table and figures. In some fractions there was a low oxalacetate-independent deacylase activity, which was assayed in similar reaction mixtures without oxalacetate, and substracted. The rechromatographed citrate synthase of fig.1B (specific activity 44.9 μmol/min/mg of protein, determined in the presence of 10 μM AMP) was purified 160-fold with respect to the crude extract.

The chemicals used were the same as previously described [8].

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3. Results and discussion

When a cell-free extract from the marine *Pseudomonad*, or a 50–70% ammonium sulphate fraction thereof (fig.1 A) was subjected to chromatography on DEAE-cellulose, two main peaks of citrate synthase activity were found. An AMP-insensitive and an AMP-activated enzyme, which are henceforth referred to as

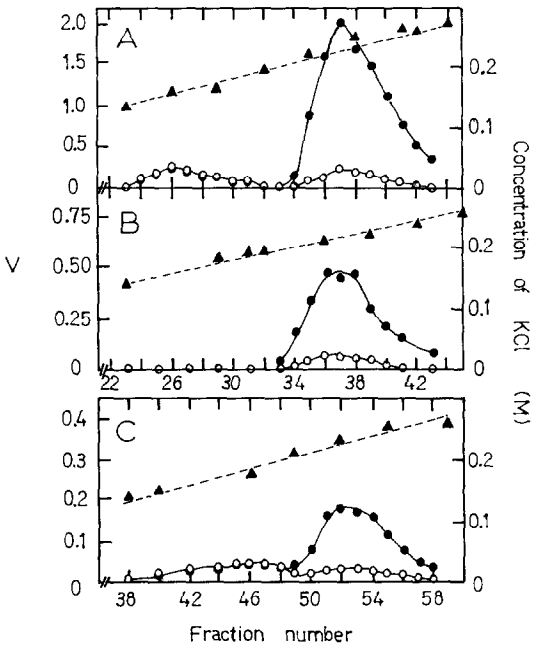

Fig.1. Chromatography of the citrate synthases from a marine *Pseudomonad* on DEAE-cellulose. (A) Chromatography of 2.8 ml of a dialysed 50–70% ammonium sulphate fraction, as described under Materials and methods. Fractions 37–43 were pooled (32.5 ml, total vol). (B) 16 ml of the pool were dialysed overnight against the phosphate-EDTA–70 mM KCl buffer and re-chromatographed with a 70–500 mM KCl gradient. (C) The other half of the pool was dialysed overnight against phosphate-EDTA buffer and re-chromatographed with a 0–500 mM KCl gradient. The reaction mixtures contained (in μmoles) in a final volume of 1 ml: Tris–HCl buffer (pH 7.6), 45; EDTA, 0.9; acetyl-CoA, 0.05; oxalacetate, 0.25; DTNB, 0.1; KCl, 25; and enzyme, which was added last to start the reaction. The reaction velocity (v) is expressed as μmoles of CoA liberated/min/ml of enzyme preparation. The activity was assayed in the absence (○) or in the presence (●) of 10 μM AMP. (▲), KCl concentration in the gradient. Re-chromatography of CS I under the conditions described for (B) (not shown) did not change the elution behaviour, nor the regulatory properties of this enzyme form.

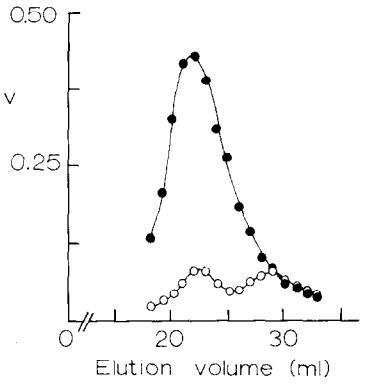

Fig.2. Gel filtration of the citrate synthases from a marine *Pseudomonad* on Sephadex G-200. A mixture of CS I and CS II obtained by DEAE-cellulose chromatography (0.75 ml each), with the addition of 0.05 mg of malate dehydrogenase and 0.1 mg of lactate dehydrogenase, was applied to the top of a Sephadex G-200 column (1.3 × 26 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 0.4 M KCl. Fractions (1 ml) were collected at a rate of 6 ml/hr, at 4°C. The void volume of the column (V_0) determined with Blue Dextran 2000 was 17 ml. The elution volumes (V_e) of CS II, lactate dehydrogenase, CS I and malate dehydrogenase were 21.7 ml; 27 ml; 29.7 ml and 31.6 ml, respectively. The approximate molecular weights were determined according to Andrews [11]. The enzyme activity was assayed and expressed as described in the legend to fig.1, in the absence (○) or in the presence (●) of 10 μM AMP.

citrate synthase I (CS I) and citrate II (CS II), respectively, were clearly separated. CS I and CS II could also be separated by gel filtration on Sephadex G-200 (fig.2); their behaviour was consistent with molecular weights of about 80–100 000 and 250–300 000, respectively. Both enzyme forms were present in cultures obtained from different single colonies, containing exclusively Gram negative bacteria, thus ruling out the possibility that CS I might belong to a contaminant, CS I and CS II were present in cell-free extracts obtained either by sonic disintegration or by grinding with glass powder in a mortar, thus suggesting that they were not an artifact of the breakage procedure.

When CS II was dialysed for 16 hr at 1°C against 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, the AMP activation was almost completely lost. This loss was prevented by the presence of KCl (70–200 mM) during dialysis, and seemed to be dependent on the presence of phosphate.

No 'desensitization' was observed upon dialysis against 50 mM Tris–HCl buffer (pH 7.0) containing 1 mM EDTA, with or without KCl, or similar Tris buffer solutions containing 10 μM AMP. The 'desensitization' of CS II seemed to be accompanied by dissociation. Fig.1B shows that CS II, dialysed and re-chromatographed in the presence of phosphate- EDTA buffer containing at least 70 mM KCl, was indeed free of CS I. Yet when the same preparation was 'desensitized' by dialysis against the phosphate-EDTA buffer without KCl, and re-chromatographed in a KCl gradient from 0 to 500 mM, most of the enzyme was recovered as AMP-insensitive citrate synthase (fig.1C). The 'desensitized' enzyme was eluted in the position of CS I and also in an intermediate position, corresponding to a small 'shoulder' which consistently appeared between CS I and CS II in the first chromatography (fig.1A). Although we have not been able yet to characterize this intermediate form (CS X), which was very unstable, a preliminary study of its regulatory properties, summarized in table 1, shows them to be very similar to those of CS I, but quite different from those of CS II. CS I and CS X were slightly inhibited by AMP, were inhibited by ADP and ATP, and were insensitive to NADH, KCl and inorganic phosphate (P_i). The inhibition of CS I by AMP and ADP was competitive towards acetyl-CoA and non-competitive towards oxalacetate; that by ATP was of the mixed type towards both substrates. The apparent K_M for acetyl-CoA at a saturating concentration of oxalacetate (380 μM) in the absence of adenine nucleotides to 55, 110 and 200 μM in the presence of 3 mM AMP, ADP or ATP, respectively. The apparent K_M for oxalacetate at a non-saturating concentration of acetyl-CoA (100 μM) was 18 μM.

CS II was strongly activated by AMP and KCl, the effects of which were nearly additive [8]; inhibited by NADH and deinhibited by AMP; apparently insensitive to ATP and ADP, at least in the presence of 0.1 mM AMP; and strongly activated by P_i (table 1). The

Table 1
Action of effectors on the activity of the different forms of citrate synthase from a marine *Pseudomonad*

Experiment	Conditions	Enzyme activity (% of control) CS I	CS X	CS II
1	Control (no additions)	100 (15.3)	100 (1.2)	100 (1.5)
	+ 32 mM KCl	85	98	550
	+ 10 μM AMP	96	95	800
	+ 5 mM AMP	85	87	–
2	Control (0.1 mM AMP)	100 (14.7)	100 (1.1)	100 (12.5)
	+ 5 mM ADP	50	53	99
	+ 5 mM ATP	20	42	98
3	Control (2 μM AMP)	100 (14.9)	100 (1.2)	100 (4.2)
	+ 0.8 mM NADH	98	97	44
4	Control (0.26 M Tris–HCl)	100 (7.0)	100 (0.5)	100 (1.8)
	+ 50 mM P_i	80	106	800

The values in parentheses are the activities of the controls, expressed as nmoles/min. The control in Expt.1 was the enzyme activity assayed in the reaction mixture described in the legend to fig.1, without AMP. The control in Expt.2 contained 0.1 mM AMP, in order to saturate CS II with the activator, thus avoiding the activation by the small amounts of AMP present in the ATP and ADP solutions. The control in Expt.3 contained 2 μM AMP, in order to avoid the activation of CS II by the small amounts of AMP present in the NADH solutions, which would otherwise mask the NADH inhibition. The control in Expt.4 contained 0.26 M Tris–HCl buffer in order to compensate for the Tris used to adjust the phosphoric acid solution to pH 7.6; this was necessary since Tris proved to be an activator of CS II although considerably less effective than KCl.

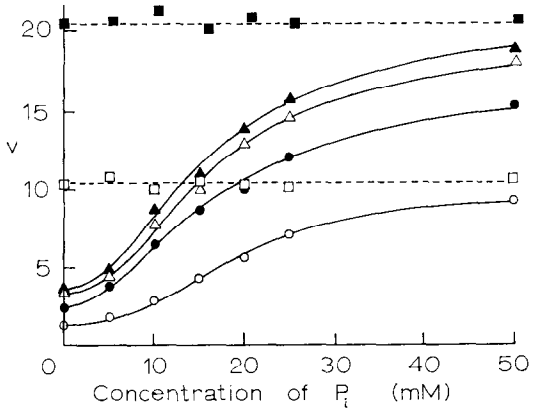

Fig.3. Activation of citrate synthase II by P_i, KCl and AMP. The reaction mixtures were as described in the legend to fig.1, except for the concentration of Tris buffer (pH 7.6), which was 200 mM. 6 μg of enzyme were used per assay. The concentration of P_i was varied as stated on the abcissa. (○), no further additions; (●), 5 mM KCl; (△), 10 mM KCl; (▲), 15 mM KCl; (□), 10 μM AMP; (■), 10 μM AMP + 15 mM KCl. The reaction velocity (v) is expressed as nmoles of CoA liberated/min.

activation by P_i was nearly additive with respect to KCl, but not with respect to AMP (fig.3); the main effect of the activator was an increase in V_{max}, with little change in the apparent K_M values for both acetyl-CoA and oxalacetate, which were considerably higher [8] than the corresponding values for CS I. The inhibition of CS II by NADH concentrations up to 0.5 mM under experimental conditions similar to those of fig.3, except for the acetyl-CoA concentration, which was 0.1 mM, was completely reversed by 0.1 mM AMP, but only partially counteracted by P_i or KCl. The $(I)_{0.5}$ value for NADH was increased from 70 μM in the absence of the activators to 130 and 160 μM in the presence of 50 mM P_i or 0.1 M KCl, respectively. All the curves obtained were sigmoidal.

From a taxonomical point of view, these results imply the paradox of the coexistence in the marine *Pseudomonad* of two enzyme forms, one (CS II) similar to those previously reported for related Gram negative microorganisms, but the other (CS I) showing the molecular weight and regulatory properties of citrate synthases from Gram positive bacteria. However, if the 'small' enzyme (CS I) is an enzyme arising from the dissociation of an oligomer (CS II), CS X being an intermediate dissociation form, not only the apparent paradox disappears, but a direct evolutionary relationship between both types of citrate synthase is suggested. The inhibition by NADH, as well as the activation by AMP, P_i and KCl, seemed to require the interaction between the protomers of the 'big' oligomeric enzyme, being lost when these interactions dissapeared because of dissociation. The inhibition by ATP and ADP, on the other hand, appeared after dissociation of the enzyme, thus suggesting that the corresponding sites were not exposed in the oligomer, but became accessible when the oligomeric structure was disrupted. Since we have no evidence of the allosteric nature of the inhibition by ATP and ADP, which were essentially competitive towards acetyl-CoA, the possibility exists that the nucleotides act at the active site itself; if this were the case, the conformational change induced by dissociation might increase the affinity of the active site towards the inhibitors, thus resulting in a great enhancement of an inhibition apparently not present in the oligomeric state.

If, as our experiments suggest, both forms of citrate synthase coexist inside the living Pseudomonad cell, it is possible that the differences in their regulatory properties reflect different roles in vivo. CS I was inhibited by ATP in a manner which suggests that its response to high energy charge [10] would be the typical one for key enzymes of catabolic pathways. CS II, on the other hand, is probably regulated essentially by the redox potential of the cells through the NADH inhibition, with relatively little effect of the adenylate energy charge, since maximal activation was attained at AMP concentrations lower than 0.1 mM and was not reversed by ATP concentrations 100-folds greater.

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