ACTIVATION OF CITRATE SYNTHASE FROM A MARINE PSEUDOMONAD BY ADENOSINE MONOPHOSPHATE AND POTASSIUM CHLORIDE

Esther MASSARINI and Juan José CAZZULO

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

Received 8 December 1973
Revision version received 2 January 1974

1. Introduction

The regulatory properties of bacterial citrate synthases are related to the taxonomical position of the microorganism [1–3]. The enzyme from Enterobacteriaceae are inhibited by NADH, whereas those from other Gram negative bacteria, like the Pseudomonadaceae, are in addition deinhibited by AMP [1]. The citrate synthases from different bacteria are affected by salts to a different extent; the enzyme from *E. coli* is desensitized against NADH by 0.2 M KCl [4], and that of *Azotobacter vinelandii* is strongly activated by KCl [5]. The latter enzyme, in addition, is activated by 5 mM AMP [5]. A psychrophilic, slightly halophilic, marine Pseudomonad contains an NADH-inhibited, AMP-reactivated citrate synthase [6]. We show in this communication that, in addition to possessing these properties which might be expected from the taxonomical position of the microorganism, the enzyme was strongly activated by KCl (7-fold, at 50 mM) and by very low concentrations of AMP (8-fold, at 10 μM), in both cases in the absence of NADH. The activators affected both the apparent V_{max} and K_M values for the substrates acetyl-CoA and oxalacetate.

2. Materials and methods

The microorganism, the conditions for the culture, harvesting and washing of the cells, the preparation of cell-free extracts by sonication and the DNAase treatment were as previously described [7]. The crude extract was fractionated with ammonium sulphate and chromatographed on a DEAE-cellulose column, with a linear gradient of KCl (0–500 mM), as described for the purification of the malic enzyme from the same microorganism [7]. Citrate synthase activity was eluted between 160 and 220 mM K^+, as determined by flame photometry. The active fractions were pooled and fractionated again with ammonium sulphate. The 66–100% saturation fraction, dissolved in 0.05 M Tris–Cl buffer, pH 7.6, containing 1 mM EDTA, and dialysed against the same buffer for 12 hr at 4°C, was used for the experiments described. The specific activity of the partially purified enzyme was 0.4 (in the absence of effectors) or 14.3 (in the presence of 50 mM KCl and 10 μM AMP) μmoles of CoA liberated/min/mg of protein; this means a 52-fold purification with respect to the crude extract.

The chemicals used were the same as previously described [6], with the exception of CoA, which was purchased from Sigma Chemical Co., St. Louis, Mo., USA. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride [8], using $NaHCO_3$ instead of $KHCO_3$, in order to minimize the K^+ concentration in the reagents. The concentrations of acetyl-CoA and oxalactate were determined with commercial pig heart citrate synthase (obtained from Boehringer, Mannheim), in the presence of an excess of the other substrate and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB).

The small amounts of ADP and ATP free from AMP required to test for a possible activation by the former nucleotides were obtained by paper chromatography [9], followed by elution in 0.05 M Tris–Cl buffer, pH 7.6, containing 1 mM EDTA.

North-Holland Publishing Company – Amsterdam

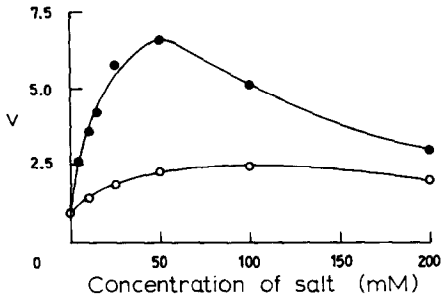

Fig. 1. Effect of KCl and NaCl on the activity of citrate synthase from *Pseudomonas*. The reaction mixtures contained (in μmoles) in a final volume of 1 ml: Tris–Cl buffer, pH 7.6, 45; EDTA, 0.9; acetyl-CoA, 0.044; oxalacetate, 0.14; DTNB, 0.1; enzyme, 13.5 μg; and KCl (•) or NaCl (○) as stated on the abscissa. The reaction was started by the addition of the enzyme to the otherwise complete reaction mixture, and followed as the increase in absorbance at 412 nm in a Beckman DB-G recording spectrophotometer, at 30°C. The reaction velocity (v) is expressed as nmoles of CoA liberated/min.

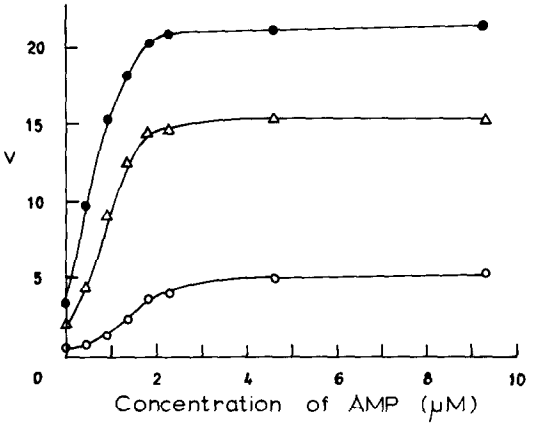

Fig. 2. Effect of AMP on the activity of citrate synthase from *Pseudomonas*. The experimental conditions were as described in the legend to fig. 1, except for the concentration of oxalacetate (0.21 mM), the amount of enzyme (4.5 μg), and the concentration of KCl, which was 0 mM (○), 10 mM (△) or 50 mM (•). The concentration of AMP varied as stated on the abscissa.

3. Results and discussion

In a previous study on the citrate synthase from an extreme halophile [6], we used the enzyme from the marine Pseudomonad for comparative purposes. It became evident that AMP and KCl were able to activate the latter enzyme by themselves, in the absence of the inhibitor NADH.

The effect of KCl, in the presence of a low concentration of acetyl-CoA and a nearly saturating concentration of oxalacetate, is shown in fig. 1. Relatively low concentrations of KCl were able to activate the enzyme, with a maximal activation of about 7-fold at 50 mM. Higher concentrations caused a relative inhibition. NaCl was less effective as an activator, giving a maximal activation of 2.5-fold at 100 mM. The citrate synthase from *A. vinelandii* has been previously reported as activated by KCl and NaCl, with the same order of effectiveness. The enzyme from *Pseudomonas fluorescens* was less affected by the salts [5].

Fig. 2 shows the effect of increasing concentrations of AMP (up to 10 μM), in the absence of KCl or in the presence of the salt (10 or 50 mM). The curves were sigmoidal, and although apparently the sigmoidicity was diminished by KCl, the apparent n values, obtained from Hill plots considering only the portion of activity due to AMP stimulation [10], were 3 irrespectively of the presence or absence of KCl. The $(A)_{0.5}$ values obtained from these plots were 1.7, 0.9 and 0.8 μM AMP, in the absence of added KCl or in the presence of 10 or 50 mM KCl, respectively. The main effects of KCl with respect to the activation by AMP were, therefore, a decrease in the $(A)_{0.5}$ value and an increase in the V_{max}, although the relative extent of the stimulation by AMP slightly decreased as the concentration of KCl was raised (from 8-fold in the absence of added salt, to 7-fold and 6-fold, in the presence of 10 mM and 50 mM KCl, respectively).

ADP and ATP, at concentrations up to 10 μM, were not effective as activators.

In control experiments with pig heart citrate synthase, this enzyme was very little affected by salts and AMP within the same concentration ranges. The enzyme from *A. vinelandii* required for half-maximal stimulation a considerably higher AMP concentration, namely 0.3 mM (5); that from *Ps. fluorescens* was not stimulated by AMP [5].

Table 1 summarizes the results of kinetic experiments on the effect of the activators, alone or together, on the apparent K_M and V_{max} values for both substrates. KCl and AMP, particularly when both of them were together, caused increases in the V_{max} and decreases in the K_M values. Thus the V_{max} obtained from the experiments with oxalacetate as the variable

Table 1
Effect of the activators KCl and AMP on kinetic parameters of citrate synthase from *Pseudomonas*.

Additions	Variable oxalacetate		Variable acetyl-CoA	
	K_M (μM)	V_{max}	K_M (mM)	V_{max}
None	50	0.5	0.40	5.7
50 mM KCl	24	2.6	0.33	20.4
10 μM AMP	7	4.4	0.20	35.1
50 mM KCl + 10 μM AMP	1	18.3	0.03	38.3

The experimental conditions were as described in the legend to fig. 1, except for the concentrations of acetyl-CoA, which was 0.044 mM in the experiments with oxalacetate as the variable substrate, and oxalacetate, which was 0.24 mM in the experiments with acetyl-CoA variable. For the experiments in the absence of AMP, the concentration of acetyl-CoA was varied in the range 0.0044–0.044 mM; under these conditions, Michaelis–Menten kinetics were obtained. At higher acetyl-CoA concentrations, deviations from linearity of the double reciprocal plots were observed, presumably due to very small amounts of AMP present as a contaminant; attempts to eliminate this contamination by chromatography [7] were unsuccessful. This lack of linearity at the higher acetyl-CoA concentrations was not observed when the experiments were performed in the presence of 10 μM AMP.

The apparent V_{max} values are expressed as μmoles of CoA liberated/min/mg of protein. The kinetic constants were obtained from the slopes and intercepts of double reciprocal plots, calculated by the least squares method.

substrate, in the presence of a low concentration of acetyl-CoA, was increased by 36-fold in the simultaneous presence of 50 mM KCl and 10 μM AMP; the V_{max} obtained from acetyl-CoA plots was increased by 6.7-fold under similar conditions. In addition, the apparent K_M values for oxalacetate and acetyl-CoA were decreased by about 12-fold, in the presence of the two activators together. In the case of the *A. vinelandii* enzyme [5], the K_M for acetyl-CoA was decreased to a similar extent in the presence of the activators, but the K_M for oxalacetate was decreased only by 2-fold. The effects on V_{max} were not reported.

The activation of the citrate synthase from the marine Pseudomonad by very low concentrations of AMP suggests that the enzyme would be in an activated state under physiological conditions, and that the regulation in vivo could be accomplished through the inhibition of the AMP-activated citrate synthase by NADH. Preliminary experiments suggest that the activation by AMP and the inhibition by NADH depend on the concentration of the other modifier. In the presence of a high concentration of NADH, the $(A)_{0.5}$ for AMP might rise to a value high enough to allow the enzyme regulation through fluctuations in the concentrations of the adenylates.

Acknowledgements

This investigation was supported by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina). JJC is a member of the Carrera del Investigador Científico of the same institution. EM is a member of the Carrera del Investigador Científico of the Consejo de Investigaciones de la Universidad Nacional de Rosario (Argentina).

References

[1] Weitzman, P.D.J. and Jones, D. (1968) Nature 219, 270.
[2] Weitzman, P.D.J. and Dunmore, P. (1969) Biochim. Biophys. Acta 171, 198.
[3] Weitzman, P.D.J. and Dunmore, P. (1969) FEBS Letters 3, 265.
[4] Weitzman, P.D.J. (1966) Biochem. J. 101, 44 C.
[5] Flechtner, V.R. and Hanson, R.S. (1970) Biochim. Biophys. Acta 222, 253.
[6] Cazzulo, J.J. (1973) FEBS Letters 30, 339.
[7] Cazzulo, J.J. and Massarini, E. (1972) FEBS Letters 22, 76.
[8] Stadtman, E.R. (1957) in: Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 3, p. 931, Academic Press, New York and London.
[9] Paladini, A.C. and Leloir, L.F. (1952) Biochem. J. 51, 426.
[10] Cazzulo, J.J. and Stoppani, A.O.M. (1968) Arch. Biochem. Biophys. 127, 563.