Some Properties of the Citrate Synthase from the Extreme Halophile, *Halobacterium cutirubrum*

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1. Citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] was purified about 400-fold from the extreme halophile, *Halobacterium cutirubrum*, by a method involving $(\text{NH}_4)_2\text{SO}_4$ fractionation, chromatography on DEAE-cellulose and hydroxyapatite and gel filtration on Sephadex G-200. 2. The purified enzyme was best activated by high concentrations of KCl (3 M); the chlorides of other cations and K^+ salts of other anions (Br^- , NO_3^- , SCN^-) were less effective than KCl as activators. The enzyme was best stabilized by high concentrations of NaCl or KCl. Cold-lability was found in the presence of 3 M-KCl, but not in the presence of NaCl at concentrations up to 5 M. The results suggest that both the shielding of negative charges on the enzyme molecule and the stabilization of hydrophobic bonds by high KCl concentrations were required for maximum activity of the enzyme. 3. The double-reciprocal plots for acetyl-CoA or oxaloacetate at several concentrations of the co-substrate intersected at the abscissa in the presence of either KCl or NaCl, at either 1 or 3 M. The K_m for oxaloacetate increased about fivefold with the salt concentration, from 1 to 3 M.

The enzymes isolated from extremely halophilic bacteria require for activity and stability high salt concentrations (Larsen, 1967; Kushner, 1968), similar to those reported to be present inside the living bacterial cells (Christian & Waltho, 1962; Lanyi & Silverman, 1972). The reason for the salt requirement is thought to be related to the shielding of negative charges in the protein molecule by univalent cations (Baxter, 1959), or to the formation of new hydrophobic bonds in the presence of high concentrations of salts that are efficient for salting out (Lanyi & Stevenson, 1970; Lieberman & Lanyi, 1971). Both hypotheses are not mutually exclusive, and they might complement each other (Lieberman & Lanyi, 1972).

Halobacterium cutirubrum contains a citrate synthase [citrate oxaloacetate-lyase (CoA acetylating), EC 4.1.3.7], which differs in both molecular weight and regulatory properties from the similar enzymes isolated from other Gram-negative bacteria of the pseudomonad type (Cazzulo, 1973). The present paper describes the purification of the halophilic citrate synthase, the effects of salts on the activity and stability of the purified enzyme, and the effects of KCl and NaCl on the reaction kinetics. The results suggest that, although charge-shielding effects contribute to the enzyme activation by salts, full activity of the halophilic citrate synthase requires the stabilization of hydrophobic bonds by high concentrations of

KCl. The present results thus agree with the proposal by Lanyi and his co-workers (Lanyi & Stevenson, 1970; Lieberman & Lanyi, 1971, 1972), based on studies performed with other, crude or little purified, halophilic enzymes.

Methods and Materials

Enzyme assay

The enzyme activity was assayed spectrophotometrically, by following the increase in absorbance at 412 nm caused by the condensation of 5,5'-dithiobis-(2-nitrobenzoic acid) with the CoA liberated in the course of the reaction (Sreere, 1969). Unless stated otherwise, the reaction mixture contained (in μmol) in a final volume of 1 ml: Tris-HCl buffer (pH 7.6), 40; oxaloacetate, 1; acetyl-CoA, 0.15; 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1; KCl, 3000; and 0.3–0.6 μg of enzyme, as stated in the legends to Tables and Figures. The reaction was started by the addition of the enzyme to the otherwise complete reaction mixture. The assays were performed at 30°C in a Beckman DB-G recording spectrophotometer. One unit of enzyme corresponds to 1 μmol of CoA liberated/min, under the assay conditions stated above. Specific activities are expressed as units per mg of protein.

The salts solutions used contained 50 mM-Tris-HCl buffer, adjusted to pH 7.6 after the addition of the salt.

Purification of the citrate synthase

Step 1: preparation of cell-free extract. *H. cutirubrum* was grown and the cells were harvested and washed as previously described (Cazzulo & Vidal, 1972). The cells were suspended (0.45 ml/g wet wt.) in 0.05M-Tris-HCl buffer (pH 7.6)-1 mM-EDTA-5 M-NaCl (buffer 1) and disrupted at 5°C by 4 × 15 s treatments in a MSE sonic disintegrator at maximum output. Deoxyribonuclease I (3 µg/ml) and MgCl₂ (1 mM final concn.) were added to the homogenate, the mixture was incubated at 35°C for 30 min and then centrifuged at 37000g for 30 min at 4°C. The supernatant (crude extract) was collected and the precipitate was discarded.

Step 2: (NH₄)₂SO₄ fractionation. The crude extract was dialysed for 3 h at 18°C against 30 vol. of 0.05M-Tris-HCl buffer (pH 7.6)-1 mM-EDTA-0.2M-NaCl-1 mM-oxaloacetate (buffer 2), and fractionated with a saturated solution of (NH₄)₂SO₄ adjusted to pH 7 with NH₃. Four fractions were obtained at 66, 82, 89 and 93 % saturation respectively, and then the supernatant was saturated with solid (NH₄)₂SO₄. The precipitates were redissolved in buffer 1. Most of the protein present in the crude extract was precipitated in the first three fractions, whereas the bulk of the citrate synthase activity was recovered in the last two fractions. When the specific activities of these fractions were similar, as in the preparation described in Table 1, they were pooled.

Step 3: Column chromatography on DEAE-cellulose. The active fraction from step 2 was dialysed for 3 h at 4°C against 20 vol. of buffer 2 and chromatographed at 4°C on a column (17 mm × 100 mm) of DEAE-cellulose previously equilibrated with the same buffer. Stepwise elution was performed by washing the column with buffers of similar composition, containing 0.2M-, 0.28M- and 0.33M-NaCl. Fractions (5 ml) were collected at a flow rate of 30 ml/h. The enzyme was eluted by the 0.33M-NaCl buffer; the active fractions with the higher specific activities were pooled and dialysed overnight against 25 vol. of 10 mM-sodium phosphate buffer (pH 7.0)-0.1 mM-EDTA-4 M-NaCl (buffer 3).

Step 4: column chromatography on hydroxyapatite. The dialysed preparation from step 3 was applied to the top of a hydroxyapatite column (10 mm × 12 mm) equilibrated with buffer 3 at room temperature (20°C). After the enzyme solution was adsorbed, the column was washed with the same buffer, and eluted stepwise with buffers of similar composition, containing 0.1M-, 0.13M- and 0.17M-sodium phosphate, pH 7.0. Fractions (1 ml) were collected at a flow rate of 2.4 ml/h. Usually the bulk of the enzyme activity was eluted by the 0.17M-phosphate buffer; the fractions with the higher specific activities were pooled and used for step 5.

Step 5: gel filtration on Sephadex G-200. The active

fraction from step 4, previously dialysed overnight against buffer 1, was put into a dialysis sac and concentrated with dry Sephadex G-200 to a volume not greater than 1.5 ml, and percolated through a column (13 mm × 260 mm) of Sephadex G-200 equilibrated with the same buffer, at 4°C. Fractions (1 ml) were collected at a rate of 6 ml/h. The elution volume of the citrate synthase was 28 ml; the void volume of the column, determined with Blue Dextran 2000, was 16 ml. The resolution of the step was not improved by the use of a considerably longer column (15 mm × 630 mm), and the specific activity did not increase after a second gel-filtration step on Sephadex G-200, or rechromatography of the active Sephadex fraction on hydroxyapatite.

Protein determinations

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard (steps 1-3), or by the spectrophotometric method of Warburg & Christian (1941) (steps 4 and 5).

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out in a system similar to that described by Dundas (1970), in the presence of 5M-urea and 0.06M-2-mercaptoethanol. The acrylamide concentration in the large-pore and small-pore gels was 2.5 and 7.5 % (w/v) respectively. The relative amounts of protein in the different bands were calculated by densitometry of the gels, stained with Amido Black, by using a Canalco model G densitometer (Canalco, Rockville, Md., U.S.A.).

Chemicals and reagents

CoA, 5,5'-dithiobis-(2-nitrobenzoic acid), deoxyribonuclease I (EC 3.1.4.5) from ox pancreas and DEAE-cellulose were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; oxaloacetic acid, citrate synthase from pig heart and malate dehydrogenase (EC 1.1.1.37) from pig heart were from Boehringer, Mannheim, West Germany; Sephadex G-200 (fine grade) and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden.

Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (Stadtman, 1957), and assayed by the method using hydroxylamine and 5,5'-dithiobis-(2-nitrobenzoic acid) (Cánovas & Kornberg, 1966) or with pig heart citrate synthase, oxaloacetate in excess and 5,5'-dithiobis-(2-nitrobenzoic acid); both methods gave similar results.

Oxaloacetate solutions (about 50 mM) were prepared in 0.5M-Tris-HCl buffer (pH 8.0), and assayed with malate dehydrogenase and NADH in excess (Hohorst & Reim, 1965). Hydroxyapatite was prepared by the method of Tiselius *et al.* (1956).

All the salts used were analytical reagents of the highest purity available.

Table 1. Purification of citrate synthase from *H. cutirubrum*

H. cutirubrum cells (30.3 g wet wt.) were suspended in 13.6 ml of buffer, disrupted by sonic disintegration, and the enzyme was purified as described in the Methods and Materials section. The enzyme activity was assayed as described in the Methods and Materials section, at a non-saturating acetyl-CoA concentration; the specific activity of the purified enzyme (step 5), when determined at saturating concentrations of both substrates in the presence of 3.15M-KCl, was about 70 units/mg (value calculated from the data of the experiment shown in Fig. 5).

Step	Fraction	Total vol. (ml)	Total activity (units)	Recovery (%)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)
1	Crude extract	28	224.4	100	2968	0.07	1
2	(NH ₄) ₂ SO ₄ (89–100% satd.)	6.1	70.9	32	94.55	0.75	11
3	DEAE-cellulose	15	38.0	17	10.65	3.57	51
4	Hydroxyapatite	7	17.9	8	1.71	10.47	149
5	Sephadex G-200	10	12.9	6	0.47	27.45	392

Fig. 1. Effects of salts on the activity of the citrate synthase from *H. cutirubrum*

The reaction mixtures were as described in the Methods and Materials section, except for the concentrations of acetyl-CoA (0.135 mM) and salts, as stated on the abscissa. Some 0.6 μ g of enzyme was used per assay. The reaction velocity (v) is expressed as nmol of CoA liberated/min. (a) Effect of chloride salts of univalent cations: $\circ$, KCl; $\bullet$, NaCl; Δ , NH₄Cl; $\blacktriangle$, LiCl. (b) Effect of chloride salts of bivalent cations: $\bullet$, MgCl₂; $\circ$, CaCl₂. (c) Effect of potassium salts of different anions: $\circ$, KCl; $\bullet$, KBr; Δ , KNO₃; $\blacktriangle$, KSCN.

Results

Purification of the citrate synthase

The procedure described, which is summarized in Table 1, effected a 390-fold purification of the halophilic citrate synthase with a yield of about 6%. When the purified preparation was subjected to polyacrylamide-gel electrophoresis in the presence of 5M-urea and 0.06M-2-mercaptoethanol, a main protein band and several faint bands were observed. The main protein band accounted for 80% of the total protein stained in the gel, as determined by densitometry; it was identified as the citrate synthase by performing the electrophoresis in the absence of urea, then eluting, reactivating and assaying the enzyme. When the eluted enzyme was subjected to electrophoresis in the presence of urea, only one protein band was observed, showing the same mobility as the main band in the electrophoretic pattern of the enzyme preparations obtained from Step 5.

The highly purified citrate synthase lost less than 20% of its activity in 2 months when kept at 4°C in

buffer 1, at protein concentrations of 30–50 μ g/ml. The enzyme stability depended on the protein concentration; diluted preparations, containing about 2.5 μ g of protein/ml, lost half of their activity in less than 3 days, when kept under otherwise identical conditions.

Effects of salts on the activity of the halophilic citrate synthase

Fig. 1 shows the activation of the enzyme by univalent cations (Fig. 1a) or bivalent cations (Fig. 1b), as chlorides. The enzyme activity in the absence of added salt was very low, and all the cations tested gave some activation. Maximum activation was attained in the presence of high KCl concentrations. The curve for KCl showed a plateau from approx. 0.4 to 1.5M, followed by a further increase in enzyme activity at higher salt concentrations; this second activation phase was due to an increase in $V_{\max}$ values (see Table 3). NaCl, on the other hand, was as effective as KCl in the lower concentration range, but did not elicit any further activation over 1M. The

increase in the K_m value for oxaloacetate with the KCl or NaCl concentration (Table 3) did not cause a decrease in the actual reaction velocity, since the enzyme was always saturated with oxaloacetate. NH_4Cl and LiCl were less effective, and caused a relative inhibition at high concentrations. CaCl_2 and MgCl_2 were effective activators at concentrations up to 0.05M, but caused a relative inhibition at higher concentrations.

Fig. 1(c) shows that, when several potassium salts were tested as activators, the nature of the anion proved to be important also. The order of effectiveness was $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{SCN}^-$; further, KBr, KNO_3 and KSCN were able to inhibit the enzyme (in increasing order of effectiveness) when tested in the presence of 1M-KCl (results not shown).

Effect of salts on the stability of the citrate synthase

The halophilic citrate synthase was inactivated in the presence of low salt concentrations; this inactivation was reversible, at least to some extent, by dialysis of the inactivated enzyme against buffer 1. The best recovery values (between 50 and 70% of the original activity) were obtained after dialysis for 2–4h at 20°C; there was also considerable recovery at 4°C.

Table 2 shows the protection of the halophilic citrate synthase against low-salt inactivation, by the different salts tested as activators in the experiment of Fig. 1, at concentrations that elicited nearly optimum activation (except for KCl). KCl was the best protector of the enzyme, closely followed by NaCl;

Table 2. Stabilization of citrate synthase from *H. cutirubrum* by salts

Purified citrate synthase (2.7 μg of protein/ml of incubation mixture) was incubated at 30°C in the presence of the salt concentrations stated in the Table. At different times decay was stopped by pipetting 0.1ml samples into the reaction mixture described in the Methods and Materials section (except for the KCl concentration, which was 2.8M). Under such conditions, neither reactivation nor further inactivation of the enzyme was observed during the assay. The half-life values (in min) were obtained from plots of the logarithm of remaining enzyme activity versus incubation time. The plots were linear in all cases, suggesting that the enzyme decay was a first-order process.

Salt (M)	Half-life (min)
Basal (0.1 M-NaCl)	<0.2
KCl (1)	4.0
NaCl (1)	3.5
NH_4Cl (1)	1.0
LiCl (1)	0.7
KBr (1)	2.0
KNO_3 (1)	2.1
KSCN (1)	<0.2
CaCl_2 (0.1)	<0.2
MgCl_2 (0.1)	<0.2

Fig. 2. Stabilization of the citrate synthase from *H. cutirubrum* by KCl and NaCl

The values of k (apparent first-order rate constant of enzyme inactivation), in min^{-1} , were obtained from semi-logarithmic plots, as described in the legend to Table 2, in the presence of the concentrations of KCl (Δ) or NaCl ($\bullet$) stated in logarithmic scale on the abscissa. The protein concentration in the incubation mixtures was 2.7 $\mu\text{g}/\text{ml}$.

NH_4Cl and LiCl were less effective, and MgCl_2 and CaCl_2 were ineffective. The low effectiveness of the bivalent cations apparently was not due to the lower ionic strength afforded by 0.1M- CaCl_2 or 0.1M- MgCl_2 , as compared with 1M-KCl; in the presence of 0.4M- MgCl_2 (results not shown), which gives a higher ionic strength than 1M-KCl, the half-life of the enzyme was only 0.52min. Table 2 also shows that KBr and KNO_3 were less effective than KCl as enzyme stabilizers, and that KSCN did not protect at all.

Fig. 2 shows the effect of the concentrations of NaCl or KCl on the apparent first-order decay constant (k) at 30°C. Both salts were similarly effective; the double-logarithmic plots shown in Fig. 2 yielded straight lines, with slopes of -2.2 (KCl) or -2.5 (NaCl).

The stabilization of the citrate synthase by KCl or NaCl was markedly influenced by temperature. Fig. 3 shows the Arrhenius plots of enzyme inactivation obtained in the presence of 0.5M-NaCl (Fig. 3a), 3M-NaCl (Fig. 3b), 5M-NaCl (Fig. 3c), or 3M-KCl (Fig. 3d). The plots were biphasic, showing that the activation parameters of the enzyme inactivation process are different at the lower and at the higher

Fig. 3. Arrhenius plots of the stabilization of the citrate synthase from *H. cutirubrum* by NaCl or KCl

(a) 0.5M-NaCl (protein concentration, 2.3 $\mu\text{g}/\text{ml}$ of incubation mixture); (b) 3M-NaCl (2.7 μg of enzyme/ml); (c) 5M-NaCl (2.3 μg of enzyme/ml); (d) 3M-KCl (1.1 μg of enzyme/ml). The enzyme preparations used for experiments (a), (b) and (c) were dialysed against buffer 1 and that for experiment (d) against a similar buffer, containing 3M-KCl instead of 5M-NaCl. k is the apparent first-order rate constant of enzyme inactivation and R the universal gas constant.

Fig. 4. Stabilization of the citrate synthase from *H. cutirubrum* by oxaloacetate

The experimental conditions were similar to those described in the legend to Table 2, except for the presence of 0.5M-NaCl in all the incubation mixtures, and oxaloacetate (μM) as stated on the curves. The protein concentration was 2.3 $\mu\text{g}/\text{ml}$ of incubation mixture.

temperatures tested. In the presence of 3M-NaCl the values of the enthalpy of activation were 123.9 $\text{kJ}\cdot\text{mol}^{-1}$ (29.6 $\text{kcal}\cdot\text{mol}^{-1}$) and 5.9 $\text{kJ}\cdot\text{mol}^{-1}$ (1.4 $\text{kcal}\cdot\text{mol}^{-1}$) at 30° and 0°C respectively. In the presence of 3M-KCl, but not in the presence of any of the NaCl concentrations tested, cold-lability was observed, as indicated by the negative slope of the Arrhenius plot at temperatures below 0°C (Fig. 3d), reflected in a negative enthalpy of activation of -35.2 $\text{kJ}\cdot\text{mol}^{-1}$ (-8.4 $\text{kcal}\cdot\text{mol}^{-1}$) at -5°C.

The halophilic citrate synthase was protected against inactivation in the presence of low salt concentrations, by its substrate oxaloacetate (Fig. 4). The inactivation kinetics were of apparent first order

at all oxaloacetate concentrations tested. The half-life of the enzyme (at 2.3 $\mu\text{g}/\text{ml}$), in the presence of 0.5M-NaCl at 30°C, was increased from 0.55 min in the absence of oxaloacetate to 42.5 min in the presence of 108 μM -oxaloacetate.

Effects of KCl and NaCl on the reaction kinetics of the halophilic citrate synthase

The double-reciprocal plots obtained for the substrate oxaloacetate at several concentrations of the co-substrate acetyl-CoA, and for the substrate acetyl-CoA at several concentrations of the co-substrate oxaloacetate, in the presence of either KCl or NaCl, at 1 or 3M, intersected at the abscissa, showing that the K_m values were independent of the concentration of the co-substrate, within the concentration range tested. Fig. 5 shows the plots obtained in the presence of 1M-NaCl (acetyl-CoA as the variable substrate) and 3.15M-KCl (oxaloacetate as the variable substrate). The secondary plots of intercepts and slopes versus reciprocal concentration of fixed substrate (inserts in Fig. 5) were linear in all cases, and yielded true K_m values that did not differ significantly from the corresponding apparent values obtained from a different experiment presented in Table 3.

Table 3 shows the apparent K_m values for both substrates, determined at several concentrations of KCl or NaCl. The K_m value for oxaloacetate increased with the concentration of KCl or NaCl, from about 18 μM (1M-NaCl or -KCl) to 97.3 μM (3M-NaCl) or 82.8 μM (3M-KCl) (Fig. 5 and Table 3). K_m values in the presence of salt concentrations lower than 1M are not included, since the enzyme instability prevented accurate determinations at low oxaloacetate concentrations. The K_m value for acetyl-CoA, on the other hand, did not show significant variations with

Fig. 5. Double-reciprocal plots for acetyl-CoA in the presence of 1M-NaCl (a) and for oxaloacetate in the presence of 3.15M-KCl (b) obtained at several concentrations of the co-substrate

The nature and concentration of the salt is stated on the plots; the concentrations of the co-substrate (μM) are stated on the lines. Other experimental conditions were as described in the Methods and Materials section. The inserts show the secondary plots of slopes (●) and intercepts (○) as a function of the reciprocal concentrations of the fixed substrate.

Table 3. Variation of kinetic parameters of the citrate synthase from *H. cutirubrum* with the concentration of KCl and NaCl

The concentration of acetyl-CoA, in the experiment where oxaloacetate was the variable substrate, was 0.11 mM; that of oxaloacetate, in the experiment with acetyl-CoA as the variable substrate, was 1.08 mM. Other experimental conditions were as described in the Methods and Materials section. Some $0.4\mu\text{g}$ of enzyme was used per assay, at the salt concentrations stated in the Table. The V_{max} values shown were obtained from the experiment with acetyl-CoA as the variable substrate. The K_m and V_{max} values $\pm$ S.E. ($n = 4$) were calculated by the method of Wilkinson (1961).

Concn. of salt (M)	Apparent K_m for oxaloacetate (μM)		Apparent K_m for acetyl-CoA (mM)		Apparent V_{max} (nmol/min)	
	KCl	NaCl	KCl	NaCl	KCl	NaCl
0.25	—	—	0.29 ± 0.01	0.35 ± 0.04	7.4 ± 0.2	6.4 ± 0.6
0.50	—	—	0.28 ± 0.06	0.31 ± 0.04	7.7 ± 1.1	6.8 ± 0.6
1.00	17.9 ± 0.4	19.2 ± 0.2	0.26 ± 0.04	0.28 ± 0.05	7.7 ± 0.8	7.0 ± 0.9
1.50	26.4 ± 1.0	29.9 ± 2.0	0.33 ± 0.01	0.35 ± 0.07	9.4 ± 0.2	7.7 ± 1.0
2.00	36.8 ± 1.2	54.1 ± 8.9	0.33 ± 0.04	0.31 ± 0.03	10.0 ± 0.8	7.0 ± 0.5
2.50	57.9 ± 1.9	90.4 ± 5.4	0.34 ± 0.07	0.35 ± 0.05	11.8 ± 1.7	7.7 ± 0.8
3.00	82.8 ± 7.1	97.3 ± 5.0	0.36 ± 0.03	0.36 ± 0.04	13.8 ± 0.7	7.9 ± 0.6

the salt concentration, in the presence of either KCl or NaCl (Fig. 5 and Table 3).

Table 3 also shows the variation of the apparent V_{max} values obtained from the plots for acetyl-CoA, at a nearly saturating concentration of oxaloacetate (1.08 mM), as a function of the salt concentration. As in the experiment of Fig. 1(a), where a non-saturating concentration of acetyl-CoA was used, KCl, but not NaCl, above 1.5M elicited a second increase in reaction velocity.

Discussion

The procedure described in this paper allows the preparation of highly purified citrate synthase from the extreme halophile, *H. cutirubrum*, kept in the

native state throughout the purification procedure. Steps 2 and 3 were performed in the presence of one of the substrates of the reaction, oxaloacetate, which is an effective protector of the enzyme against the low-salt inactivation (Fig. 4). Oxaloacetate is known to protect non-halophilic citrate synthases against denaturants such as urea (Srere, 1966). Other halophilic enzymes have been found to be protected by their substrates against low-salt inactivation, and this property has been used for purification (Holmes & Halvorson, 1965; Hubbard & Miller, 1969; Dundas, 1970; Hochstein & Dalton, 1973). The rather low yield obtained in the $(\text{NH}_4)_2\text{SO}_4$ step (32% in Table 1) was acceptable considering the good purification attained, which was never lower than 10-fold, and occasionally was as high as 25-fold.

DEAE-cellulose chromatography seems not to have been applied before to the purification of halophilic enzymes, although other ion exchangers have been used (Aitken & Brown, 1972; Hochstein & Dalton, 1973). Hydroxyapatite chromatography (Norberg & von Hofsten, 1970; Dundas, 1970; Hochstein & Dalton, 1973) and gel filtration (Aitken & Brown, 1972) have been used before for the purification of halophilic enzymes. The elution pattern of the citrate synthase from Sephadex G-200 in buffer 1 was the same as that previously obtained in buffer 2 (Cazzulo, 1973).

The highly purified enzyme preparations were used to study some properties of the citrate synthase, related to its halophilic nature. The best activator of the enzyme was KCl. A biphasic activation curve was observed when either actual reaction velocities, at a non-saturating acetyl-CoA concentration (Fig. 1*a*), or $V_{\max}$ values obtained with acetyl-CoA as the variable substrate (Table 3) were plotted. Similar results were obtained with enzyme preparations of different degrees of purity, from the crude extract (results not shown) to the highly purified enzyme. These results resemble those reported by Lieberman & Lanyi (1971) for the effect of NaCl and other salts on cytochrome oxidase from *H. cutirubrum*. In both cases the activity values corresponding to the first plateau were close to those obtained with bivalent cation salts (cf. Figs. 1*a* and 1*b*). These results suggested the possibility, already advanced for other halophilic enzymes (Lanyi & Stevenson, 1970; Lieberman & Lanyi, 1971, 1972), that the first portion of enzyme activity might be due to charge shielding, whereas the further increase in activity observed at high KCl concentrations might reflect the formation of new hydrophobic bonds by a salting-out effect of the salt. Accordingly, potassium salts of different anions were effective as activators in the order $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{SCN}^-$ (Fig. 1*c*), which is the same order as their effectiveness as salting-out agents (Hatefi & Hanstein, 1969).

The stabilization of the enzyme apparently also required rather high concentrations of a salt effective for salting out. Among the different salts tested (Table 2) KCl and NaCl were the best protectors, with similar effectiveness in the concentration range 0.5–3 M (Fig. 2). The values of the slopes in Fig. 2 suggest the interaction of at least 3 mol of salt/mol of enzyme protein (Aitken *et al.*, 1970). CaCl_2 and MgCl_2 at 0.1 M were effective activators but did not protect the enzyme; such a concentration is supposed to be effective for charge shielding, but is not likely to be effective for salting out (Lieberman & Lanyi, 1971). KBr, KNO_3 and KSCN were less effective than KCl as enzyme protectors (Table 2).

The Arrhenius plots of inactivation of the halophilic citrate synthase at three NaCl concentrations

(0.5, 3 and 5 M), shown in Fig. 3 (*a*, *b* and *c*) were biphasic. Similar results were reported by Lanyi & Stevenson (1970) for the menadione reductase from *H. cutirubrum*, in the presence of 0.05 M MgCl_2 or 2 M NaBr. However, whereas menadione reductase showed cold-lability in the presence of 3 M NaCl, this was not observed with the citrate synthase, even in the presence of 5 M NaCl. On the other hand, the halophilic citrate synthase did show cold-lability in the presence of 3 M KCl (Fig. 3*d*). Thus cold-lability and maximum activation were found in the presence of 3 M KCl, whereas in the presence of 3 M NaCl both cold-lability and the second phase of activation were absent under the experimental conditions used. These results suggest that the halophilic citrate synthase might require the stabilization of hydrophobic bonds by high concentrations of KCl in order to be in the maximally active conformation, although about 60% of the activity can be taken as due to charge shielding by salt concentrations up to 1 M (Table 3). Among the halophilic enzymes shown to exhibit cold-lability under some experimental conditions are menadione reductase (Lanyi & Stevenson, 1970), cytochrome oxidase (Lieberman & Lanyi, 1971), threonine deaminase (Lieberman & Lanyi, 1972) and aspartate transcarbamoylase (Norberg *et al.*, 1973), all of them isolated from *H. cutirubrum*. In all these instances, cold-lability has been thought to support the involvement of hydrophobic bonds in the maintenance of the active enzyme structure. In a study on the effect of bivalent cations on several enzymes partially purified from *H. cutirubrum*, we have recently suggested that the requirement of high salt concentrations for the stabilization of hydrophobic bonds may not be a general property of the enzymes isolated from extreme halophiles (Higa *et al.*, 1974). Some of the enzymes, including citrate synthase, required high KCl or NaCl concentrations for maximum activity and stability. On the other hand, other enzymes isolated from the same organism, such as malate dehydrogenase and glutamate dehydrogenase, were relatively inhibited by high KCl or NaCl concentrations, and were optimally activated by relatively low concentrations of CaCl_2 (0.2 M), thus suggesting that they might be activated chiefly through charge-shielding effects (Higa *et al.*, 1974).

The greater effectiveness of KCl as an activator, as compared with NaCl, is also shown by citrate synthases isolated from non-halophilic bacteria (Flechtner & Hanson, 1970; Massarini & Cazzulo, 1974), and seems to be a general property of many halophilic enzymes (Lanyi & Stevenson, 1969; Aitken & Brown, 1969; Cazzulo & Vidal, 1972; Higa *et al.*, 1974).

The experiments shown in Fig. 5 suggest that the kinetic mechanism of the reaction catalysed by the halophilic citrate synthase is similar to that for citrate synthases from mammalian sources (Shepherd &

Garland, 1969; Matsuoka & Srere, 1973), since in all cases double-reciprocal plots intersecting at the abscissa have been obtained. The reaction mechanism has been thought to be rapid-equilibrium random (Shepherd & Garland, 1969; Matsuoka & Srere, 1973), but Johansson & Pettersson (1974) have presented evidence suggesting that it is ordered, with oxaloacetate binding to the enzyme in the first place. Our results do not allow us to differentiate between the two possibilities mentioned above.

The kinetic pattern was the same in the presence of KCl or NaCl, at either 1 or 3 M, suggesting that the reaction mechanism is identical in the presence of these different salt concentrations. For the NADP-linked isocitrate dehydrogenase from an extreme halophile, on the other hand, Aitken & Brown (1972) have reported a change in the kinetic reaction mechanism with the change in salt concentration. This change, however, occurred at very low salt concentrations, which we have not been able to test with the citrate synthase, owing to the instability of the enzyme. The increase in the apparent K_m value for oxaloacetate with salt concentration (Table 3) resembles that reported for NADP for the halophilic isocitrate dehydrogenase (Aitken *et al.*, 1970; Aitken & Brown, 1972). The K_m values reported here for the halophilic citrate synthase are considerably higher than those for the enzymes from eukaryotes (Shepherd & Garland, 1969), but are of the same order as those reported for other bacterial citrate synthases (Flechtner & Hanson, 1970; Massarini & Cazzulo, 1974).

From a physiological point of view it may be worth noting that the two main intracellular cations, K^+ and Na^+ , were the only two, among the different cations tested, which did not cause a relative inhibition at high concentrations; moreover, optimum activation was attained in the presence of a K^+ concentration close to that reported as present inside living halophile cells (Lanyi & Silverman, 1972). When different anions were tested, as potassium salts, the greatest activation was attained in the presence of Cl^- , the main intracellular inorganic anion (Christian & Waltho, 1962). The enzyme would be stabilized *in vivo* by the high intracellular salt concentration, and by the high local concentration of the enzyme itself.

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