In a separate and identical experiment, without radioactive phosphate, Ca^{2+} determination was also performed.

Results and discussion. The antibiotic X-537A is a carboxilic ionophore which interacts with Ca^{2+} forming a lipophilic complex[20]. It has been demonstrated that such a complex promotes a rapid Ca^{2+} efflux from Ca^{2+} loaded SR vesicles, and a subsequent disruption of the Ca^{2+} gradient created by the pump[14–17].

In Figure 1, it is shown that after the addition of X-537A in the presence of EGTA there still remains about 1.2 nmoles of E~P/mg of protein although all Ca^{2+} has been released. This observation is consistent with results of other investigators, who have demonstrated that phosphorylation of the ATPase of SR may occur in the absence of a Ca^{2+} gradient, and that the E~P formed under these conditions is inhibited by Ca^{2+}, but requires Mg^{2+} [21–22], whereas the presence of acetyl phosphate abolishes the Ca^{2+} inhibition[22]. It has been suggested that this phosphorylation takes place at the external surface of the membrane and that another E~P is also formed at the internal face as soon as Ca^{2+} gradient inside-outside is increased[21].

The addition of EGTA before X-537A (Figure 1) increases the level of E~P, probably due to the increase of the Ca^{2+} gradient and also due to the decrease of Ca^{2+} concentration outside. Once the ionophore is added, the Ca^{2+} gradient is completely destroyed and the E~P decreases to about 1.2 nmoles/mg protein. This level of E~P corresponds to the phosphorylated form which is Ca^{2+} gradient independent. As the presence of EGTA removes external Ca^{2+}, the remaining level of E~P is supposed to be the form which is Mg^{2+} dependent and is inhibited by outside Ca^{2+}. ADP discharges this form which is in accordance with results of other workers[22].

If X-537A is added before EGTA, the E~P is nearly all destroyed (Figure 2). In this case, the E~P which is dependent on the Ca^{2+} gradient is depressed because X-537A abolishes the gradient, and the E~P which is Mg^{2+} dependent is depressed by the extravesicular Ca^{2+}. Acetyl phosphate, which could prevent this inhibition, is in low concentration, since it was hydrolyzed during the previous Ca^{2+} uptake. The further addition of EGTA, on the other hand, decreases the outside free Ca^{2+} concentration and allows the rapid phosphorylation of the external sites to form the Mg^{2+}-dependent E~P which is inhibited by Ca^{2+}.

We conclude that X-537A abolishes the E~P which depends on the Ca^{2+} gradient, but has no effect on the E~P which is Mg^{2+} dependent and inhibited by outside Ca^{2+}.

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Regulatory Properties of the Citrate Synthase from *Rhodospirillum rubrum*

E. MASSARINI, A. I. HIGA and J. J. CAZZULO[1]

Departamento de Bioquímica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario, Suipacha 531, Rosario (República Argentina), 31 October 1975.

Summary. Citrate synthase, purified 600-fold from *Rhodospirillum rubrum*, is activated by KCl and inhibited by ATP and NADH; the effect of the latter inhibitor is completely counteracted by AMP and partially counteracted by KCl.

Rhodospirillum rubrum, a Gram negative, facultatively photosynthetic bacterium, contained citrate synthase (EC 4.1.3.7) both when grown anaerobically in the light or heterotrophically in the dark[2]. The enzyme in cell-free extracts was reported to be inhibited by NADH and deinhibited by AMP[2], as is the case for most citrate synthases from Gram negative bacteria which do not ferment glucose[3], including photosynthetic microorganisms like *Rhodopseudomonas capsulata*[2] and *Rhodopseudomonas spheroides*[4]. However, FLECHTNER and HANSON[5] reported that their partially purified enzyme preparation from *R. rubrum* was not inhibited by NADH, but was inhibited by ATP; they stated that the enzyme regulatory properties were similar to those of the citrate synthase from the Gram positive *Bacillus subtilis*[5,6]. Although a few possible exceptions to the regulatory pattern proposed by WEITZMAN and JONES[3] are known[7,8], we decided to re-examine the regulatory properties of the citrate synthase from *R. rubrum*, particularly with respect to the effect of NADH.

Materials and methods. The blue-green, carotenoid-less, mutant strain BG-1 of *R. rubrum*[9] was grown anaerobically in the light at 30 °C on succinate as carbon source. The cells (41 g wet weight) were disrupted by sonic disintegration, and a cell-free extract obtained by ultracentrifugation of the homogenate[9]. The crude extract was fractionated with ammonium sulphate; the 50–66% saturation fraction, dissolved in 0.05 *M Tris* HCl buffer (pH 7.6) – 1 m*M* EDTA – 0.4 *M* KCl, was subjected to gel filtration on a Sephadex G-200 column (50.5 × 1.8 cm) equilibrated with the same buffer. The elution volume of the citrate synthase was 39 ml; the void volume of the column, determined with Blue Dextran 2000, was 34.5 ml. The fractions with the higher specific activities were pooled and fractionated again with ammonium sulphate. The 50–66% saturation fraction was dissolved in 0.01 *M*

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Table I. Purification of the citrate synthase from *Rhodospirillum rubrum*

Step	Total volume (ml)	Total activity (units)	Recovery (%)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)
Crude extract	128	61.2	100	1152	0.05	1
Ammonium sulphate	1.35	51.6	84	146	0.35	7
Sephadex G-200	5.0	27.2	44	5	5.43	109
Ammonium sulphate	1.0	17.6	29	2	8.80	176
1st hydroxylapatite	6.0	7.0	11	0.32	21.87	437
2nd hydroxylapatite	3.1	2.8	4.6	0.09	31.11	622

Citrate synthase was purified from 41 g (wet wt.) of cells of *R. rubrum*, and assayed, as described in the Methods section. The concentration of acetyl-CoA in the standard reaction mixture was non-saturating; the specific activity of the highly purified enzyme at saturating concentrations of both substrates (Table II) was about 120 μmoles/min/mg protein.

potassium phosphate buffer (pH 7.0)– 0.1 m*M* EDTA, dialyzed against the same buffer solution, and applied to a hydroxylapatite column (0.7 × 0.8 cm), which was eluted stepwise with buffer solutions of similar composition and increasing phosphate concentration. The bulk of the enzyme activity was eluted by the 0.09 *M* potassium phosphate buffer. The active fractions were pooled and re-chromatographed on hydroxylapatite under essentially identical conditions. The enzyme activity was assayed as previously described[10], in the presence of 0.1 *M* KCl. Other experimental conditions are given in the legends to the Tables. The chemicals and reagents were the same as previously described[10,11].

Results and discussion. The 600-fold purified enzyme preparations obtained by the method outlined in Table I were nearly homogeneous when subjected to polyacrylamide gel electrophoresis[12]; the protein band corresponding to citrate synthase was estimated to account for at least 90% of the total protein in the gels.

The enzyme was activated by monovalent cations. Under standard assay conditions[10] KCl was the best activator (6-fold stimulation at 0.1 *M*), whereas NH_4Cl and NaCl, at the same concentration, elicited 3.4-fold and 3-fold activation, respectively. The greater effectiveness of KCl has been reported for other bacterial citrate synthases[4,10,11]. The activation was attained mainly through a decrease in the K_m value for acetyl-CoA, although there was also some increase in the V_{max}; the K_m for oxaloacetate was not changed by the activator (Table II). The double-reciprocal plots obtained for each substrate at several concentrations of the co-substrate, in the absence or in the presence of KCl, intersected at the abcissa, showing that the K_m values were independent of the concentration of the co-substrate. The same kinetic pattern has been reported for citrate synthases from other sources, among them bacterial[11,13].

The highly purified citrate synthase from *R. rubrum* was inhibited by ATP, either in the absence or in the presence of KCl. The inhibition was strictly competitive towards acetyl-CoA and non-competitive towards oxaloacetate (Table III), as previously demonstrated for citrate synthases from other sources[14,15]. The citrate

Table II. Effect of KCl on the kinetic parameters of the reaction catalyzed by the citrate synthase from *Rhodospirillum rubrum*

	Without KCl	105 m*M* KCl
K_m Acetyl-CoA (μ*M*)	780 ± 210	112 ± 4
K_m Oxaloacetate (μ*M*)	13.2 ± 2.6	10.9 ± 0.6
V_{max} (μmoles/min/mg protein)	72.4 ± 13.9	117.2 ± 6.9

The enzyme activity was assayed in the presence of acetyl-CoA concentrations from 50 to 200 μ*M*, and oxaloacetate concentrations from 5 to 50 μ*M*, in the absence or in the presence of 105 m*M* KCl. The true K_m and V_{max} values (± SE, $n = 5$) were calculated from secondary plots of intercepts as a function of the reciprocal concentrations of the fixed substrate[17], by the method of Wilkinson[18].

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Table III. Inhibition of the citrate synthase from *Rhodospirillum rubrum* by ATP

Concentration of ATP (m*M*)	Variable acetyl-CoA		Variable oxaloacetate	
	Apparent K_m (μ*M*)	Apparent V_{max} (μmoles/min/ml enzyme)	Apparent K_m (μ*M*)	Apparent V_{max} (μmoles/min/ml enzyme)
0	109 ± 3	2.25 ± 0.03	10.6 ± 0.7	1.13 ± 0.02
2	202 ± 22	2.46 ± 0.16	9.2 ± 0.8	0.75 ± 0.02
5	368 ± 35	2.73 ± 0.20	10.1 ± 1.2	0.57 ± 0.06

The enzyme activity was assayed in the presence of 105 m*M* KCl. When used as the fixed substrate, the concentration of oxaloacetate was 50 μ*M* and that of acetyl-CoA was 0.1 m*M*. The apparent K_m and V_{max} values (± SE, $n = 4$) were determined according to Wilkinson[18].

Table IV. Effect of KCl and AMP on the inhibition of the citrate synthase from *Rhodospirillum rubrum* by NADH

Concentration of KCl (mM)	$(I)_{0.5}$ (μM)	Apparent n
0	4.7	-2.25 ± 0.07
35	6.1	-2.37 ± 0.24
105	9.6	-1.79 ± 0.19
210	23.0	-1.70 ± 0.04
Concentration of AMP (μM)		
0	6.1	-2.37 ± 0.24
2.5	16.8	-2.03 ± 0.13
5.0	21.5	-2.34 ± 0.20
7.5	31.0	-2.22 ± 0.10
10.0	36.0	-2.14 ± 0.17

The assay conditions were as previously described[11], except for the concentrations of AMP and KCl which were as stated in the Table, and that of NADH, which was varied from 0 to 70 μM. The concentration of KCl in the experiments related to the effect of AMP was kept constant at 35 mM. 0.3 μg of purified enzyme were used per assay. The apparent n values ($\pm$ probable EM; $n = 5$) and $(I)_{0.5}$ values for NADH (NADH concentration for half-maximal inhibition) were calculated as previously described[19].

Table V. Deinhibition of the citrate synthase from *Rhodospirillum rubrum* by AMP

Concentration of NADH (μM)	$(A)_{0.5}$ (μM)	Apparent n
6.4	1.6	1.66 ± 0.05
16.1	4.3	1.84 ± 0.26
32.0	10.4	2.17 ± 0.07
64.0	17.5	2.79 ± 0.11

The experimental conditions were similar to those given in the legend to Table IV, except for the fixed NADH concentrations stated, the concentrations of AMP, which were varied between 0 and 20 μM, and that of KCl, which was kept constant at 105 mM. The apparent n values ($\pm$ probable EM; $n = 5$) and $(A)_{0.5}$ values for AMP (AMP concentration for half-maximal stimulation of enzyme activity) were calculated from Hill plots considering only the portion of the enzyme activity due to the AMP effect, as previously described[19].

synthase from *R. spheroides* has also been reported to be inhibited by ATP, although in the latter case the inhibition was non-competitive towards both substrates[4]. The enzyme from *R. capsulata* was reported not to be inhibited by ATP[2].

The *R. rubrum* citrate synthase, like the enzymes from *R. capsulata*[2] and *R. spheroides*[4], was strongly inhibited by NADH (Table IV); the kinetics of inhibition was sigmoidal. The effect of NADH was counteracted by KCl, which increased the $(I)_{0.5}$ for NADH with some decrease in the apparent n, and by AMP, which also caused an increase in the $(I)_{0.5}$ without significant variation in the apparent n (Table IV). The curves of enzyme de-inhibition by AMP were also sigmoidal; both the $(A)_{0.5}$ for AMP and the apparent n increased with the concentration of NADH (Table V). The inhibition by NADH was non-competitive towards oxaloacetate; the kinetic pattern for acetyl-CoA became sigmoidal in the presence of the inhibitor. The apparent n value for acetyl-CoA increased from 1.08 ± 0.01 in the absence of NADH to 1.73 ± 0.04 in the presence of 6 μM NADH; the corresponding $(S)_{0.5}$ values (concentration of acetyl-CoA required for half-maximal velocity) were 0.6 and 1.1 mM, respectively. The enzyme was not inhibited by α-oxoglutarate at concentrations up to 10 mM, either in the absence or in the presence of KCl. Therefore, the citrate synthase from *R. rubrum* shows the properties to be expected from similar enzymes from Gram negative bacteria which do not ferment glucose[3,16], being distinctly different from those of Gram positive bacteria. The effects of NADH and AMP are similar to those described for the enzymes from *R. capsulata*[2] and *R. spheroides*[4]. There are some differences, however, in the effect of ATP; the activation by KCl has not been reported for the *Rhodopseudomonas* enzymes[2,4].

The inhibitory response of the *R. rubrum* enzyme to NADH and ATP suggests that it will be strongly inhibited under photosynthetic conditions, where both the concentration of NADH and the adenylate energy charge can be expected to be high[2,4]. Although the effects reported have been studied in vitro, it is possible that they are involved in the enzyme regulation in vivo, since they were evident at rather high concentrations of KCl, which might be present inside living bacterial cells.

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Effect of Heat Treatment on the ATPase Activity of Various Sarcoplasmic Reticulum Preparations

C. A. M. CARVALHO and M. S. V. SANTOS[1]

Department of Zoology, University of Coimbra, Coimbra (Portugal), 23 October 1975.

Summary. Ca^{2+}-stimulated ATPase activity of sarcoplasmic reticulum (SR) preparations is activated after a short period of preincubation at temperatures between 40 and 45 °C, but for temperatures higher than 48 °C pronounced denaturation is observed. Heat denaturation is decreased if Mg^{2+} or K^+ are present during heat treatment.

Leaky sarcoplasmic reticulum (SR) vesicles can be prepared by treatment with various agents such as phospholipase A[2], diethylether[3], EDTA[4] and X-537A[5], a Ca^{2+} ionophore. After each of these treatments, the membrane vesicles lose the ability to accumulate Ca^{2+}, although they retain the ATPase activity in some form, and have been utilized by several investigators to study phenomena related to the enzyme[2-7].

In this work we studied the behaviour of SR preparations previously treated with diethylether, EDTA and X-537A with respect to heat denaturation of the ATPase enzyme and its stabilization by cations.

Materials and methods. Sarcoplasmic reticulum membranes were isolated from rabbit skeletal muscle as described previously[8]. Immediatly after isolation, samples of SR were treated with 8% (v/v) diethylether ac-