# ACTIVATION AND INACTIVATION OF CITRATE LYASE LIGASE FROM *RHODOPSEUDOMONAS GELATINOSA*

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## 1. Introduction

*Rhodopseudomonas gelatinosa* is one of the few microorganisms capable of growing on citrate anaerobically in the light [1,2]. The first enzyme involved in citrate breakdown by this bacterium is citrate lyase (EC 4.1.3.6) which carries acetyl groups in thioester linkage at its active sites. Splitting off the acetyl groups results in the inactive form of the enzyme (deacetyl-citrate lyase) [3,4]. It could be shown in our laboratory that citrate lyase of *R. gelatinosa* is subject to covalent modification. Upon depletion of citrate in the medium, the lyase is inactivated by a specific deacetylase. Addition of citrate to the medium causes rapid activation of the lyase by acetylation [5,6]. The enzyme responsible for the latter reaction is citrate lyase ligase:

deacetyl-citrate lyase + acetate + ATP

$\longrightarrow$ citrate lyase + AMP + $PP_i$

This enzyme has been discovered and characterized in *Enterobacter aerogenes* [7] and in *Streptococcus diacetilactis* [8]. But nothing is known about its regulation. The experiments presented in this communication will show that the ligase in *R. gelatinosa* is subject to activation and inactivation reactions.

## 2. Materials and methods

*R. gelatinosa* strain DSM 149 was grown anaerobically in the light as in [5]. After 24 h growth, cells of a 10 liter culture were harvested and resuspended in the same volume of fresh medium without citrate. Following the incubation of this suspension for 1 h with magnetic stirring under anaerobic conditions in the light, citrate was added (final conc. 5 mM or 10 mM). Samples (500 ml) were withdrawn at the times indicated in the figures by means of $N_2$ pressure and centrifuged for 20 min at 20 000 × *g* at 4°C.

For determination of citrate lyase activity (acetylated form of the enzyme) cells were resuspended in 5 ml 0.1 M triethanolamine–hydrochloride buffer, pH 7.2 containing 60 mM $MgCl_2$ [5] and passed through a French press at a pressure of 80 kp/cm$^2$. Cell debris was removed by centrifugation at 20 000 × *g* for 20 min at 4°C. For determination of ligase activity and total citrate lyase (acetylated plus deacetylated form) the cells were resuspended in 5 ml 0.1 M potassium phosphate buffer, pH 7.2 containing 3 mM $MgCl_2$. Cell extracts were prepared as above and dithioerythritol was added to give final conc. 1 mM. In order to stabilize citrate lyase ligase in cell extracts 5 mM ATP and 0.1 M Na-acetate were added immediately after preparation of the extract.

The protein content of cell extracts was determined as in [9] and citrate as in [10]. Citrate lyase activity was measured as in [4]. The incubation mixture for the determination of citrate lyase ligase activity contained in final vol. 0.5 ml: 0.1 M potassium phosphate buffer, pH 7.2; 3 mM $MgCl_2$; 1 mM dithioerythritol; 5 mM L(+)-glutamate; 50 mM sodium acetate; 2 mM ATP; 5 U deacetylcitrate lyase; and approx. 0.5 U ligase. During incubation at 30°C, samples, 20 µl each, were withdrawn and immediately added to a citrate lyase assay mixture.

1 U of citrate lyase ligase is defined as that amount of enzyme which forms 1 U of citrate lyase from deacetyl-citrate lyase in 1 min at 30°C. Citrate lyase (164 U/mg) was purified from *R. gelatinosa* [4]. The enzyme was obtained in the deacetylated form. Malate dehydrogenase (1200 U/mg) was purchased from Boehringer, Mannheim.

## 3. Results

Cells of *R. gelatinosa* which had been grown on 10 mM citrate for 24 h contained only minor amounts of active citrate lyase ligase and citrate lyase. When these cells were harvested, resuspended in fresh medium containing 5 mM citrate, and incubated in the light, ligase activity increased very rapidly (fig.1a). Simultaneously, inactive citrate lyase which had been present in the cells and newly synthesized citrate lyase was acetylated (fig.1b). Upon depletion of citrate after 2.5 h incubation, the ligase was rapidly inactivated and was totally inactive after 6 h. Exhaustion of citrate initiated also the inactivation of citrate lyase by deacetylation as in [5] and after 6.5 h incubation approx. 50% were present as inactive enzyme. At this time citrate was again introduced into the culture to give final conc. 5 mM. It is apparent from fig.1 that activation and inactivation of the two enzyme systems proceeded as following the first addition of citrate.

In order to find out whether protein synthesis was required for ligase activation and inactivation the experiments were repeated in the presence of antibiotics. As shown in fig.2 de novo synthesis of citrate lyase was blocked completely by $10^{-5}$ M puromycin or $5 \times 10^{-6}$ M chloramphenicol. However, the activation and inactivation of the ligase was not affected. The inactivation occurred even when citrate, which was degraded very slowly under these conditions, was still present in the medium.

Figure 3 shows the effect of darkness on the activation and inactivation reactions. When cells of *R. gelatinosa* containing active ligase were incubated further in the dark the ligase activity remained stable (fig.3a). Switching on the light caused immediately a rapid inactivation of the ligase. Figure 3b shows that light was required for ligase activation. It also is apparent that the dark period between the 5 h and 7 h incubation decreased the rate of ligase inactivation

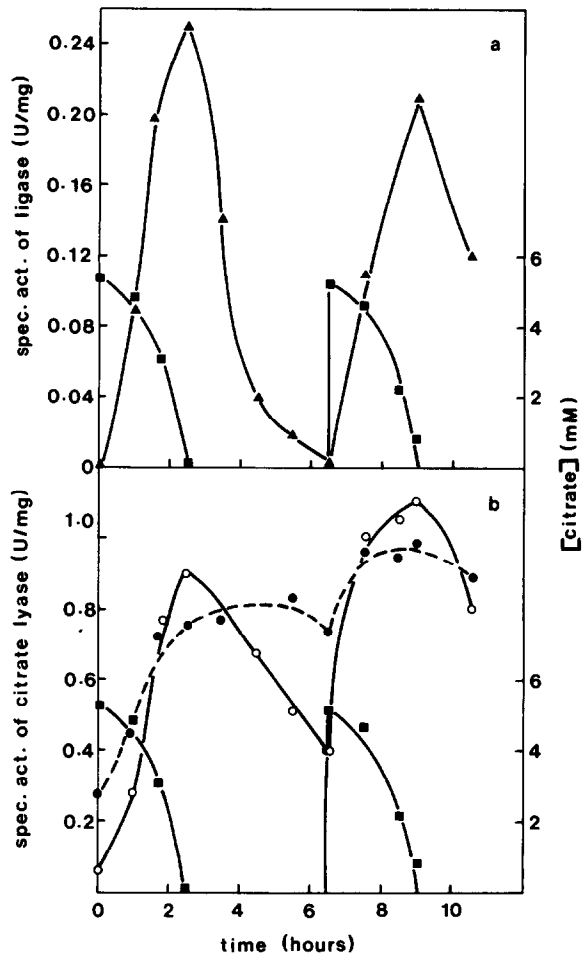


Fig.1. Activation and inactivation of citrate lyase ligase (a) and citrate lyase (b) during anaerobic incubation of *R. gelatinosa* in the light. At zero time and after 6.5 h sodium citrate was added to give final conc. 5 mM. (■) Concentration of citrate; (▲) ligase activity; (○) citrate lyase activity; (●) citrate lyase plus deacetyl-citrate lyase activity.

indicating that light was required not only for the initiation of the inactivation reaction but also for its performance.

## 4. Discussion

When *Rhodopseudomonas gelatinosa* is growing on citrate anaerobically in the light the breakdown of this substrate is initiated by its cleavage into acetate

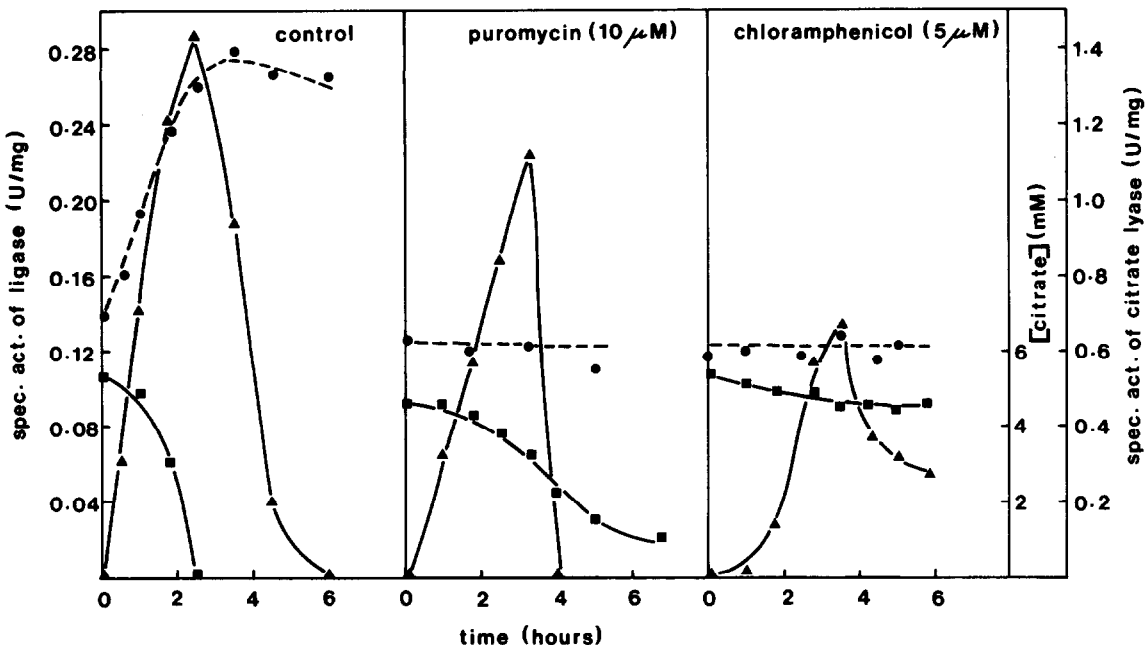


Fig.2. Effect of antibiotics on ligase activation and inactivation. 15 min before the addition of citrate, the antibiotics were added up to the concentrations indicated. (■) Concentration of citrate; (▲) ligase activity; (●) citrate lyase plus deacetyl-citrate lyase activity.

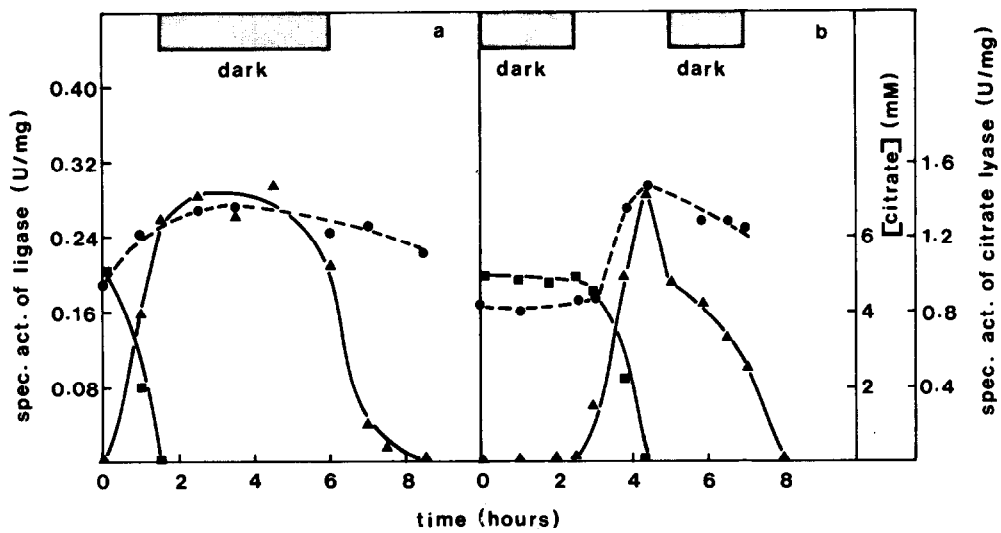


Fig.3. Effect of darkness on ligase activation and inactivation. (■) Concentration of citrate; (▲) ligase activity; (●) citrate lyase plus deacetyl-citrate lyase activity.

and oxaloacetate [2]. It has been shown that three enzymes are involved in this cleavage reaction and its regulation: citrate lyase, citrate lyase deacetylase and citrate lyase ligase [5,6]. The deacetylase is induced along with citrate lyase; it is strongly inhibited by L(+)glutamate and its activity is apparently relieved following the decrease of the intracellular glutamate concentration [6]. Citrate lyase ligase activity must also be regulated. Under the conditions of citrate lyase inactivation its activity would be unnecessary and could give rise to futile cycle reactions.

The experiments reported here show that the ligase, like citrate lyase, is subject to activation and inactivation. It, however, is not yet known what the mechanism of this regulatory process is. Attempts to restore ligase activity in inactivated enzyme fractions by acetylation failed.

Nevertheless, the conditions required for activation or inactivation have been recognized. The activation reaction proceeds only in the light. How light effects this process is unknown but it should be mentioned that a number of regulatory processes have been described as energy dependent [11,12]. The meaning of this energy dependency in terms of the reactions involved is obscure. In addition to light, the presence of citrate and deacetyl-citrate lyase and anaerobic conditions are apparently required for ligase activation. Protein synthesis is not involved as this process proceeds also in the presence of puromycin or chloramphenicol.

The inactivation of the ligase is observed after all the deacetyl-citrate lyase has been converted into the acetylated form. The decrease of ligase activity is delayed in the dark but proceeds in the presence or absence of citrate.

It can be concluded that not only the citrate lyase of *R. gelatinosa* is subject to regulation by activation and inactivation but also its activating enzyme, citrate lyase ligase.

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