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# PURIFICATION AND PROPERTIES OF D-GALACTONATE DEHYDRATASE FROM *MYCOBACTERIUM BUTYRICUM*

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**D-Galactonate dehydratase (D-galactonate hydro-lyase, EC 4.2.1.6) catalyzes the first reaction in the D-galactonate catabolic pathway of non-pathogenic Mycobacteria. As a part of studies concerning the metabolism of D-galactose and related compounds as well as its regulation in saprophytic strains of Mycobacteria, D-galactonate dehydratase has been purified and enzymologically characterized. The enzyme has been purified 325-fold from the crude extracts of galactose-grown *Mycobacterium butyricum* and its molecular weight of about 270 000 has been determined by Sephadex G-200 filtration. Isolation and analysis procedures are described. The dehydratase reaction is optimal within a pH range of 7.8–8.0. The enzyme is strictly specific for D-galactonate; none of the other sugar acids tested serves as a substrate or inhibits the dehydration of D-galactonate. The $K_m$ value for D-galactonate is 1 mM. The enzyme requires $Mg^{2+}$ or $Mn^{2+}$ for activity. The dehydratase is very sensitive to SH-blockers; the most potent inhibitor is $ZnSO_4$, which considerably inhibits the enzyme at a concentration of 2.5–5.0 $\mu$M. Zinc-inhibited enzyme can be reactivated by chelating agents. The dehydratase is heat-resistant but dithiothreitol renders it more sensitive on heating.**

## Introduction

The enzyme D-galactonate dehydratase (D-galactonate hydro-lyase, EC 4.2.1.6) transforms D-galactonate into 2-keto-3-deoxy-galactonate, and it was first described by De Ley and Doudoroff [1] as one of five enzymes engaged in the catabolism of D-galactose in *Pseudomonas saccharophila*. The enzyme was later also found in *Gluconobacter liquefaciens* [2] and *Escherichia coli* [3]. Some of the enzymes of the Doudoroff pathway, i.e., D-galactose dehydrogenase (EC 1.1.1.48), 2-keto-3-deoxygalactonate kinase (EC 2.7.1.58) and 6-phospho-2-keto-3-deoxy-galactonate aldolase (EC 4.1.2.21) have been purified and studied [4–6]. However, there are no data available in the literature concerning the purification and properties of D-galactonate dehydratase. This is in contrast to D-gluconate dehydratase, which has been extensively studied [7,8].

In a search for metabolic reactions of D-galactose and its derivatives in non-pathogenic Mycobacteria, we have identified D-galactonate dehydratase, the enzyme initiating the catabolism of the exogenous D-galactonate in this group of organisms [9,10]. The aim of the present work has been to isolate and to characterize for the first time the D-galactonate dehydratase from *M. butyricum*, an obligate aerobe.

## Materials and Methods

### *Organism and growth conditions*

*Mycobacterium butyricum* was used throughout and the strain was obtained from the Department of Plant Physiology, University of Lublin. The cells were grown statically for 4 days at 37°C in Roux flasks on the surface of a galactose-glutamate-citrate-salts liquid medium [11], but with $ZnSO_4$ omitted. The medium was adjusted to pH 6.8 with KOH prior to steriliza-

tion. After growth, the cells were collected, washed with water and stored at −15°C.

*Enzyme assay*

Galactonate dehydratase was assayed with the ketodeoxygalactonate-formation method [8]. The reaction mixture (1 ml) contained 100 mM Tris-HCl buffer (pH 8.0), 0.5 mM $MgCl_2$, 5 mM potassium galactonate and enzyme. After incubation at 37°C for 20 min, the reaction was stopped with 0.2 ml 20% trichloroacetic acid and the resultant precipitate was discarded by centrifugation. Ketodeoxygalactonate was determined in 0.2-ml aliquots with the 2-thiobarbituric acid method [12]. The absorbance coefficient of 85.55 $mM^{-1} \cdot cm^{-1}$ [7] was taken for calculations. Ketodeoxygalactonate was also measured with the semicarbazide method [13], taking the coefficient of 10.02 $mM^{-1} \cdot cm^{-1}$. Specific activities reported are expressed as μmol ketodeoxygalactonate per min per mg protein.

Protein was estimated by the method of Lowry et al. [14] with bovine serum albumin as the standard. A spectrophotometric method [15] was also used for the protein measurement in the enzyme fractions.

*Polyacrylamide gel electrophoresis*

The method of Davis [16] was used but with the spacer gel omitted. 7% polyacrylamide gels were prepared (0.6 × 8.0 cm) and concentrated enzyme preparation in 10% sucrose solution (45 μg protein in 0.1 ml) was applied to the gel. The electrophoresis was carried out in Tris-glycine buffer (pH 8.3) for 4–7 h at 3 mA per gel and 4°C. Bromophenol blue served as a marker. One gel was stained with 1% amido black in 7% acetic acid. The second gel was cut into slices 2 mm wide, crushed in 0.2 ml of the reaction mixture and incubated for 30 min at 37°C; the reaction was then arrested with trichloroacetic acid solution. Ketodeoxygalactonate was measured as described above.

*Molecular weight determination*

The molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column (1.5 × 85 cm) equilibrated with 50 mM phosphate buffer (pH 7.2). The column was standardized with myoglobin (17 000 daltons), aldolase (149 000), γ-globulin (160 000) and catalase (240 000).

*Purification of* D-*galactonate dehydratase*

Unless otherwise stated, all the operations were performed at 0–4°C and all potassium phosphate buffers (pH 6.8) contained 1 mM EDTA.

*Step 1. Crude extract.* Frozen *M. butyricum* cells (70 g) were thawed in 280 ml 10 mM phosphate buffer and sonicated in 25-ml portions for two periods of 5 min in an MSE apparatus at 100 W and 24 kHz, then cooled with ice. The homogenates were pooled and centrifuged at 16 000 × *g* for 45 min. The resulted crude extract contained about 14 mg protein per ml.

*Step 2. pH 4.5 and streptomycin treatment.* The crude extract was adjusted to pH 4.5 with 1 M acetic acid and kept in an ice-bath for 15 min. A heavy precipitate was centrifuged and suspended in 100 mM phosphate buffer in a volume equal to one-half of the original extract. To the preparation, a 20% solution of streptomycin sulfate was added dropwise with continuous stirring to reach 1% final concentration.

*Step 3. $(NH_4)_2SO_4$ fractionation.* The supernatant solution (158 ml) was brought to 20% saturation with solid $(NH_4)_2SO_4$, stored for 30 min, and centrifuged. The resulting supernatant was then brought to 40% with $(NH_4)_2SO_4$ and centrifuged and the precipitate was suspended in 100 mM phosphate buffer in a volume equal to one-half of that of the preceding step. A third $(NH_4)_2SO_4$ treatment was performed and the proteins precipitating at 35% saturation were collected and dissolved in a minimal volume of the 100 mM phosphate buffer.

*Step 4. Sephadex G-200 column chromatography.* The enzyme solution (6.5 ml) was applied to a Sephadex G-200 column (2.5 × 60 cm) equilibrated with 100 mM phoshate buffer. Fractions of 5.5 ml were collected at a flow rate of 20 ml/h (Fig. 1A).

*Step 5. Heat treatment.* The pooled active fractions (52 ml) were mixed with 26 ml 1 M Tris-HCl buffer (pH 6.8) and adjusted to room temperature. The 2-ml portions of the solution were then kept for 1 min at 70°C, then cooled and centrifuged (Tris-HCl buffer appeared to facilitate the coagulation of proteins). The pooled supernatant was brought to 35% saturation with $(NH_4)_2SO_4$ and centrifuged and the precipitate was suspended in about 20 ml 10 mM phosphate buffer.

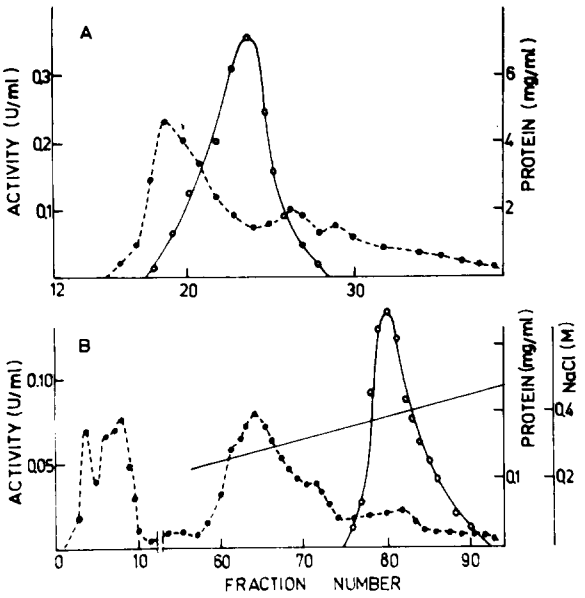


Fig. 1. Purification of galactonate dehydratase. A. Sephadex G-200 filtration of the $(NH_4)_2SO_4$ fraction. The fraction (4.3 ml, 184 mg protein) was applied to the column (2.5 × 60 cm). B. DEAE-cellulose chromatography of the heated (70°C) enzyme preparation. The preparation (30 ml, 48 mg protein) was introduced onto the column (1 × 14 cm). In both graphs: ○, activity; ●, protein concentration.

*Step 6. DEAE-cellulose column chromatography.* The non-dialyzed material from the preceding step was loaded on a DEAE-cellulose column (Whatman DE-52; 1 × 14 cm) equilibrated with 10 mM phosphate buffer. The column was washed with the same buffer and then the enzyme was eluted with a 250-ml linear gradient of NaCl from 0 to 0.5 M in the buffer (Fig. 1B). The dehydratase fractions were pooled, diluted with the same volume of 10 mM phosphate buffer, adsorbed onto a small DEAE-cellulose column (1 × 1 cm) and concentrated by elution with 0.5 M NaCl. Further concentration was achieved by dialysis against solid poly(ethylene glycol) 20 000.

*Step 7. Starch block electrophoresis.* Commercial starch was washed by decantation with water, ethanol and 100 mM phosphate buffer (pH 7.5), then packed into a plexiglass tray to form a block (40 × 8 × 1 cm). The concentrated enzyme preparation (about 1 ml) was applied to the block at the cathode side and the electrophoresis performed for 15 h at about 200 V and 20 mA. After that, the block was cut into 0.5-cm wide segments and eluted with the buffer. The pooled active fractions were concentrated with poly(ethylene glycol) and stored at 0°C.

## Results

The procedure just described resulted in a 325-fold purification with an overall recovery of about 8%. A summary of the purification procedure is presented in Table I. Analysis of the preparation thus obtained upon polyacrylamide gel electrophoresis showed two closely attached protein bands ($R_F$ = 0.3). The amount of enzyme activity corresponded to the area covered by the protein bands.

*Properties of* D-*galactonate dehydratase*

*Reaction product.* Amorphous calcium salt and crystalline potassium salt of ketodeoxygalactonate were prepared from the mycobacterial galactonate dehydratase incubation mixture according to a slightly modified procedure of De Ley and Doudoroff [1]. The $R_F$ values in the three solvent systems [1] were identical with those of authentic ketodeoxygalactonate. The potassium salt was decomposed with charring at 160°C (reported decomposition was at 159–163°C [1]). When analyzed with the method for 2-keto-3-deoxy sugar acids [12], which involves periodate oxidation and 2-thiobarbituric acid treatment, it gave a chromogen with an absorbance maximum a 456–550 nm. The compound formed derivatives typical for α-keto acids with *o*-phenylenediamine (maximum at 330 nm [17]) and semicarbazide (maximum at 250 nm [13]). Incubation of the compound with the purified mycobacterial ketodeoxygalactonate kinase, 6-phospho-2-keto-3-deoxygalactonate aldolase and ATP resulted in formation of pyruvate and glyceraldehyde 3-phosphate, as tested with its crystalline dehydrogenases.

*Molecular weight.* The molecular weight of the enzyme was estimated by chromatography on a Sephadex G-200 column with proteins of known molecular weight. The dehydratase was eluted immediately after the catalase. From the observed $V_e/V_0$ of dehydratase, the molecular weight of the enzyme was estimated to be about 270 000.

*Effect of pH, enzyme concentration and incubation time.* The reaction rate was proportional to the enzyme concentration and was linear with respect to time up to 90 min incubation. The optimum pH was

TABLE I

PURIFICATION OF GALACTONATE DEHYDRATASE

| Purification step | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|---|
| 1 Crude extract | 290 | 4060 | 57 | 0.014 | 100 |
| 2 pH 4.5 and streptomycin-treated | 158 | 2970 | 48 | 0.016 | 84 |
| 3 $(NH_4)_2SO_4$ fraction | 6.5 | 598 | 41 | 0.069 | 72 |
| 4 Sephadex G-200 eluate | 52 | 312 | 34 | 0.108 | 60 |
| 5 Heated at 70°C | 20 | 144 | 33 | 0.230 | 58 |
| 6 DEAE-cellulose eluate | 100 | 12 | 23 | 1.420 | 40 |
| 7 Starch block eluate | 12 | 0.96 | 4.4 | 4.560 | 8 |

found to be in the range 7.8–8.0 in 100 mM Tris-HCl buffer and 50 mM phosphate buffer.

*Cation requirement.* The enzyme activity was absolutely dependent on divalent metal ions, i.e., $Mg^{2+}$ and $Mn^{2+}$. Highest activities were obtained at approx. 0.3 mM $MgCl_2$ and 0.02 mM $MnCl_2$. However, the activity with $MgCl_2$ was almost double that with $Mn^{2+}$. Other divalent cations, such as $ZnSO_4$, $CaCl_2$, $CoSO_4$, $FeSO_4$ and $FeCl_2$ did not activate, and some of them even inhibited, the dehydratase. Increasing the ionic strength did not accelerate the enzyme reaction.

*Substrate specificity.* The enzyme displayed a high degree of specificity towards D-galactonate, and did not act on a variety of other sugar acids including ribonate, xylonate, lyxonate, arabonate, gluconate and galacturonate. These compounds (used as the D-stereo-isomers) were added at concentrations of 10 mM. None of these added at 20 mM affected the dehydration of D-galactonate. The Michaelis-Menten constant for D-galactonate was calculated to be 1.0 mM (Fig. 2).

*Effect of inhibitors.* The galactonate dehydratase was especially sensitive to most-SH-group blockers, but not to *N*-ethylmaleimide (Table II). $Zn^{2+}$ was distinguished by its potent inhibition at micromolar concentrations.

*Storage stability.* The enzyme was quite stable during the initial stages of purification. The cell-free extract, for instance, could be stored in the refrigerator for several days without appreciable loss of activity. However, after purification by DEAE-cellulose chromatography, the enzyme became more sensitive and its activity was abolished when kept in an ice-bath for 1 week. Concentrated phosphate buffer partially protected the dehydratase during the purification procedures.

*Heat resistance and heat activation.* The enzyme preparations at the intial steps of purification (step 3 and 4) exhibited remarkable stability at elevated temperatures. Moreover, it was surprising that the activity increased after brief (1–2 min) heating, by 50–400%, depending on the temperature and the type of preparation used, provided that EDTA was absent from the preparation. Dithiothreitol caused the enzyme to become more sensitive to heating (Fig. 3).

*Reactivation of zinc-inhibited enzyme by chelators.* Chelating agents such as EDTA or *o*-phenanthroline, protected the enzyme from the inhibition

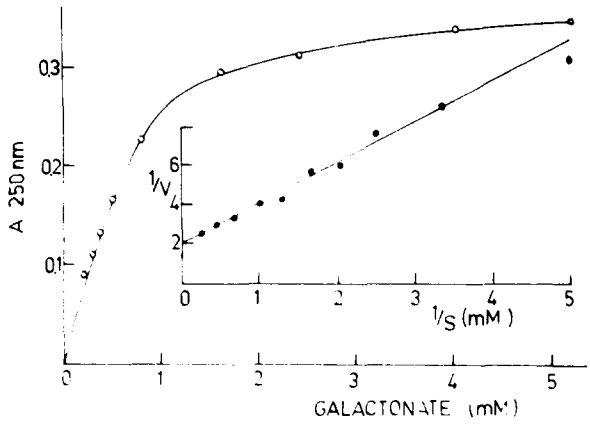


Fig. 2. Galactonate dehydratase activity as a function of substrate concentration.

TABLE II

INHIBITORY EFFECT OF VARIOUS COMPOUNDS ON GALACTONATE DEHYDRATASE ACTIVITY

Prior to testing the compound, the solution was adjusted to approx. pH 7 with 1 M KOH, if necessary. The compounds were added directly to the incubation mixture.

| Compound added | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | | 100 |
| PCMB | 0.01 | 84 |
| | 0.1 | 30 |
| | 1.0 | 0 |
| *N*-Ethylmaleimide | 2.0 | 100 |
| Iodoacetate | 0.25 | 86 |
| | 2.0 | 15 |
| Mercuric chloride | 0.005 | 96 |
| | 0.1 | 15 |
| Sodium fluoride | 5.0 | 66 |
| Copper sulfate | 0.1 | 38 |
| | 1.0 | 0 |
| Cadmium sulfate | 0.01 | 0 |
| Zinc sulfate | 0.0025 | 68 |
| | 0.005 | 0 |

caused by $Zn^{2+}$; they also were able to reactivate it after this inhibition (Table III). It is interesting that the chelators themselves elevated the enzyme activity

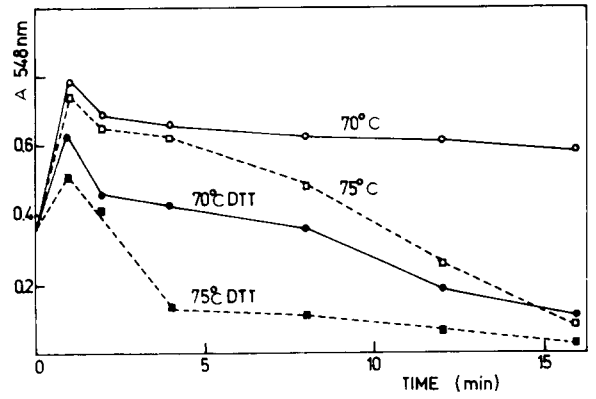


Fig. 3. Effect of dithiothreitol on heat resistance of galactonate dehydratase. Samples of Sephadex G-200 eluate (0.25 ml, 0.32 mg protein in 100 mM Tris-HCl buffer, pH 6.8) were kept at elevated temperatures for indicated time, then collected and the activity was determined. 1 mM dithiothreitol (DTT) was used.

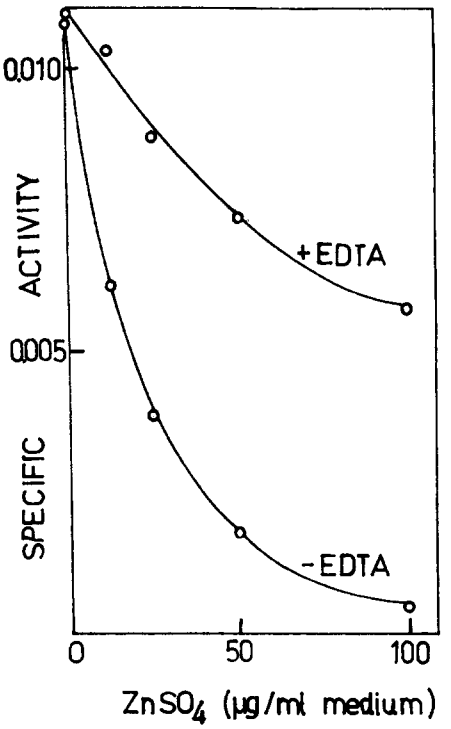


Fig. 4. Partial reactivation of in vivo $Zn^{2+}$-inhibited galactonate dehydratase by EDTA in cell-free extracts of *M. butyricum*. Set of cultures (100 ml) growing at various $ZnSO_4$ concentrations were harvested, washed and sonicated. Enzyme activity in cell-free extracts was determined in the presence of 0.5 mM EDTA and in the absence of the chelator.

even above the level of the heat-activated preparations. It is noteworthy that dehydratase preparations, which had been previously inhibited in vivo by $ZnSO_4$, could also be partially reactivated by EDTA in cell-free extracts (Fig. 4).

## Discussion

Since its discovery in 1957 [1], galactonate dehydratase has been also found in *G. liquefaciens* [2] and *E. coli* [3]. In *P. saccharophila* [1] and *G. liquefaciens* [2], galactonate dehydratase represents a part of the galactose catabolism system. Both of these organisms are devoid of galactokinase and therefore are not able to catabolize galactose via the well-known Leloir pathway [18]. In *E. coli* [3] and non-pathogenic strains of Mycobacteria [19], the Leloir pathway operates. At the same time, both of the

TABLE III

EFFECT OF $ZnSO_4$ AND CHELATING COMPOUNDS ON GALACTONATE DEHYDRATASE ACTIVITY

Activity is expressed as $A_{548nm}$/20 min in 0.2 ml incubation mixture. In the case of the heated enzyme, Sephadex G-200 eluate (1 ml, 6.4 mg protein) was heated for 1 min at 70°C. To form the control, 0.05 ml enzyme preparation was diluted with water to 0.35 ml and incubated at room temperature for 15 min. The reaction mixture was then added and the activity was determined.

| Assay | Concentration (μM) | Addition | Incubation time (min) | Addition | 2nd incubation (min) | Activity | |
|---|---|---|---|---|---|---|---|
| | | | | | | Normal | Heated |
| Control | – | – | 15 | – | – | 0.480 | 0.940 |
| $ZnSO_4$ | 7 | – | 15 | – | – | 0.015 | 0.050 |
| $ZnSO_4$ | 14 | – | 15 | – | – | 0.015 | 0.020 |
| $ZnSO_4$ | 14 | EDTA [a] | 15 | – | – | 1.200 | 1.045 |
| $ZnSO_4$ | 14 | *o*-Phe [b] | 15 | – | – | 1.250 | 1.025 |
| $ZnSO_4$ | 14 | – | 15 | EDTA [a] | 5 | 1.200 | 1.045 |
| $ZnSO_4$ | 14 | – | 15 | *o*-Phe [b] | 5 | 1.150 | 1.030 |
| EDTA | 1 400 | – | – | – | – | 1.200 | 1.040 |
| *o*-Phe | 1 400 | – | – | – | – | 1.200 | 1.040 |

[a] EDTA was used at 1.4 mM here.
[b] *o*-Phe, *o*-phenanthroline, was used at 1.4 mM here.

latter organisms possess a mechanism for the utilization of exogenous galactonate. The mechanism involves the modified Doudoroff pathway, with its enzymes, i.e., galactonate dehydratase, ketodeoxygalactonate kinase and phosphoketodeoxygalactonate aldolase. This event resembles the relationship between the Entner-Doudoroff pathway of glucose [20] and the catabolic route of gluconate [21]. Other sugar acids, i.e., mannonate [22], fuconate [23], arabonate [24] and glucosaminate [25] are also utilized by pathways similar to those for gluconate and galactonate.

This work is the first dealing with purification and characterization of galactone dehydratase. For this reason it is possible to compare it only with other hydrolyases.

The dehydratase from *M. butyricum* has absolute requirements for bivalent metal ions. In this respect, the enzyme is similar to gluconate dehydratase from *Clostridium* [7] and *Alcaligenes* [8], fuconate dehydratase from *Pseudomonas* [23] or 6-phosphogluconate dehydratase from the same genus [26]. Its rather high molecular weight (approx. 270 000) can be compared to that found for gluconate dehydratase from *Alcaligenes* [8]. Sulfhydryl compounds and ferrous ions are known to be obligatory for the activation or reactivation of various hydrolyases [7, 23,27]. The mycobacterial enzyme, as well as the gluconate dehydratase from *Achromobacter* (cf. Ref. 7), is not activated by thiols. In contrast, thiol compounds render the enzyme sensitive to heating.

It is interesting to observe the activation of the enzyme by heat. Collating the results of the experiments in which the behavior of the enzyme under various conditions have been studied, it may be tentatively assumed that the enzyme molecules (being very sensitive to SH-blockers) undergo 'saturation' with heavy metals, which might be present in the ammonium sulfate as a contamination. The exposure to heat releases the enzyme molecules or causes 'translocation' of hypothelical ligands either to groups not essential for the enzyme activity, or to other concomitant acceptors. This supposition is supported by the following observations: (1) EDTA (and also *o*-phenonthroline) activates the enzyme to the level obtained by the heat treatment; (2) no further increase of the activity by heat in the presence of EDTA is observed, and (3) activation of the cell-free extract either by heat ot by EDTA is negligible. High sensitivity of the enzyme to the SH-inhibitors and its complete reactivation by the chelating compounds indicate that the thiol groups could be easily accessible. Further studies are needed to elucidate the question.

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