COOPERATIVE BINDING OF MANGANESE TO CITRATE LYASE FROM *KLEBSIELLA AEROGENES*

H. SIVARAMAN and C. SIVARAMAN

Biochemistry Division, National Chemical Laboratory, Poona 411 008, India

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1. Introduction

Bacterial citrate lyase (EC 4.1.3.6) catalyses the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metal ions such as Mg^{2+} and Mn^{2+} [1].

The enzyme has been obtained pure from several sources [2–5] and shown to be a complex of three non-identical subunits of ~55 000 (α), 30 000 (β) and 10 000 (γ) daltons. The subunit composition and function has been studied extensively in the enzyme from *K. aerogenes* [6–8]. The γ subunit acts as acyl carrier protein (ACP) and carries an essential acetyl moiety [7]. The α subunit functions as an acyl transferase involved in citryl-ACP formation with release of acetate; and the β subunit catalyses the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP [9]. Acetyl-CoA also serves as substrate for the transferase and lyase activities [10,11]. The transferase reaction proceeds in the presence of EDTA, while the lyase reaction requires the presence of Mg^{2+} [9,10].

Relatively little is known about the nature of the interaction between the enzyme and divalent metal cofactors. The formation of a binary Mn^{2+}–protein complex has been suggested from pulsed NMR studies on partially purified citrate lyase from *Streptococcus diacetilactis* [12], although the impure nature of the preparation precludes unambiguous conclusions.

This paper reports studies on the Mn^{2+}-binding properties of pure citrate lyase from *K. aerogenes* by equilibrium dialysis assays using $^{54}MnCl_2$.

2. Materials and methods

Carrier-free $^{54}MnCl_2$ was obtained from the Bhabha Atomic Research Centre, India. Tris was primary standard grade from Sigma Chemical Co., USA. All other reagents were of analytical grade.

Citrate lyase was purified from *K. aerogenes* NCTC 418 as in [13] and preparations were homogeneous in the ultracentrifuge and in polyacrylamide gel electrophoresis. The enzyme assayed by coupling with malate dehydrogenase [14] had spec. act. 70 μmol .min^{-1} at 30°C.

Protein was determined by the method in [15] standardised with pure enzyme.

Sedimentation profiles were recorded on a Spinco Model E ultracentrifuge equipped with phase plate.

2.1. *Metal determinations*

Manganese in stock solutions of $MnCl_2$ (200 mM) was determined chemically [16]. ^{54}Mn was determined on an ECIL Model SC 603 Gamma counter. Magnesium was determined after wet combustion of lyophilized protein samples with conc. HCl/HNO_3, 3:1 (v/v) on a Perkin Elmer Model 303 atomic absorption spectrophotometer.

2.2. *Preparation of Mg^{2+}-free citrate lyase*

Enzyme solutions (4–8 mg/ml) were dialysed 24 h at 0–4°C against 50 mM potassium phosphate–2 mM EDTA (pH 7.5), with 3 changes, 250 vol. each. Enzyme solutions treated in this manner contained no detectable Mg^{2+}.

2.3. *Equilibrium dialysis*

Tris/HCl buffer, 50 mM (pH 7.4), was used in the equilibrium dialysis experiments. Tris under these conditions has been shown to have no detectable interaction with Mn^{2+} in EPR and NMR studies [17]. The absence of Mg^{2+} and Mn^{2+} as impurities in the buffer was established by metal determinations.

All dialysis operations were carried out at 0–4°C. Mg^{2+}-free enzyme solution, prepared as above, was dialysed 16 h against 500 vol. 50 mM Tris–2 mM EDTA (pH 7.5) then equilibrated by dialysis for 36 h against 50 mM Tris (pH 7.5) containing non-radioactive $MnCl_2$ at the desired concentration, with 3 changes, 250 vol. each.

Of the final buffer against which the enzyme solution had been equilibrated 20 ml was treated with 0.01 ml carrier-free $^{54}MnCl_2$ solution. The concentration of the buffer was adequate to neutralise the HCl present in the $^{54}MnCl_2$ with a resulting pH change of $\leqslant$0.1 unit. Addition of the small volume of $^{54}MnCl_2$ was assumed not to alter the total Mn^{2+} concentration in the buffer, being carrier-free. The trace quantities of non-radioactive ^{54}Cr, the decay product of ^{54}Mn, was assumed not to compete with Mn^{2+}-binding.

Dialysis cells were of the type described in [18] with 1 ml capacity compartments. Dialysis membranes were cut from Visking dialysis tubing pretreated by repeated boiling, successively, in 100 mM $NaHCO_3$–10 mM EDTA; 100 mM acetic acid; and several changes of water double-distilled in glass. The membranes were rinsed in the equilibration buffer before use. Cell compartments were filled with enzyme solution on one side and an equal volume of equilibration buffer with added $^{54}MnCl_2$ on the other. The cell-assembly was gently rocked for 24 h at 0–4°C until equilibrium was reached, as determined from preliminary trials. Samples were withdrawn separately from the compartments for both ^{54}Mn and protein determinations. The free-Mn^{2+} concentration was taken to be that in the buffer against which the enzyme had been dialysed extensively. The concentration of bound Mn^{2+} was calculated from the difference in radioactivity counts between the enzyme solution and the corresponding equilibration buffer. The stoicheiometry of binding was calculated for mol. wt 575 000 of the enzyme [13].

Table 1
Sedimentation behaviour of citrate lyase from *K. aerogenes*

Enzyme	Buffer solution (pH 7.4)	$s°_{20,w}$ (S)
Mg^{2+}-free	2 mM EDTA–50 mM Tris–HCl	17.6
Native	2 mM $MgSO_4$–50 mM Tris–HCl	17.8

Speed 59 780 rev./min; temp., 2.2–4.5°C

3. Results

3.1. *Sedimentation behaviour of metal-free and Mn^{2+}-citrate lyase*

Table 1 describes the sedimentation behaviour of Mg^{2+}-free enzyme as well as that of the enzyme in presence of 2 mM $MgSO_4$.

The Mg^{2+}-free enzyme and the enzyme in presence of the metal have almost similar $s°_{20,w}$ values of 17.6 S and 17.8 S, respectively. The sedimentation behaviour of the Mn^{2+}-enzyme obtained by equilibrating the Mg^{2+}-free enzyme with 50 mM Tris–2 mM $MnCl_2$ (pH 7.4), was similar to that of corresponding concentrations of the enzyme in presence of $MgSO_4$. The absence of Mg^{2+} as well as the replacement of Mg^{2+} with Mn^{2+} evidently causes no change in the quaternary structure of the enzyme under the conditions used.

3.2. *Manganese binding*

The saturation curve of Mn^{2+} binding is presented in fig.1a.

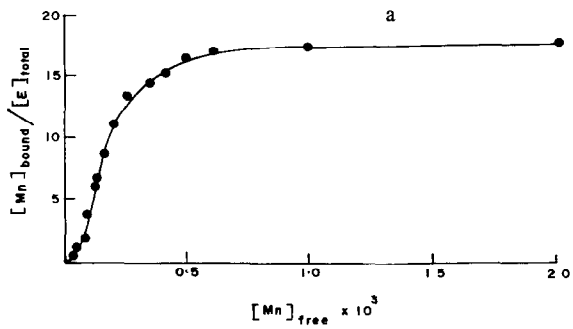

Fig.1a. Binding of Mn^{2+} to citrate lyase from *K. aerogenes*. Equilibrium dialysis performed in 50 mM Tris–HCl buffer (pH 7.4) containing varying concentrations of $MnCl_2$. Protein, 4–8 mg/ml; temp. 0–4°C.

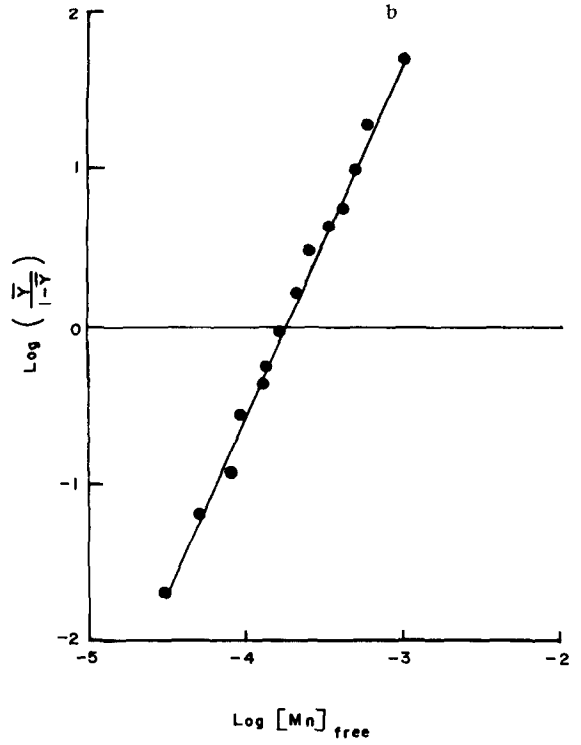

Fig.1b. Hill plot of Mn^{2+}-citrate lyase binding data. $\bar{Y}$ is the fractional saturation of the enzyme with Mn^{2+}, assuming 18 binding sites/mol enzyme under saturating conditions. Data points plotted are for $\bar{Y}$ values in the range 0.02–0.98. The straight line is the least squares fit of experimental data.

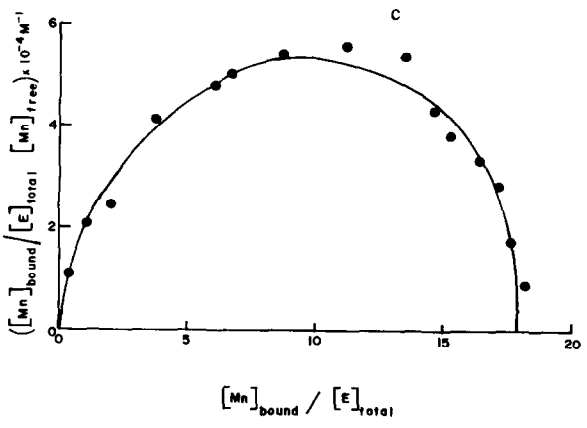

Fig.1c. Scatchard plot of Mn^{2+}-binding to citrate lyase. The solid line is constructed from values calculated from the Hill plot and overlaps approximately curve drawn by eye.

Saturation of binding sites is reached at free-Mn^{2+} concentrations greater than $\sim 1 \times 10^{-3}$ M where 18 g atoms of the metal are bound to 1 mol enzyme. The sigmoidal plot is diagnostic of positive cooperativity. The interacting site behaviour is more apparent in the Hill [19] and Scatchard [20] plots shown in fig.1b and fig.1c, respectively.

The Hill plot (fig.1b) shows linearity over the entire ligand concentration range used. The Hill coefficient, n_H, calculated from the slope of the Hill plot has a value of 2.27 ± 0.05, indicating a significant extent of positive cooperativity in metal binding. The presence of a maximum in the Scatchard plot (fig.1c) is also characteristic of positively cooperative ligand binding [21], particularly since the ultracentrifuge data rule out any metal-dependent dissociation–association effects. The Scatchard plot has an intercept which passes through the origin at $(Mn)_{free} \rightarrow 0$, which would rule out the presence of any independent, non-interacting sites [21,22]. The total number of Mn^{2+}-binding sites/mol enzyme is 18 from the extrapolated value of the intercept of the Scatchard plot with the abscissa axis at $(Mn)_{free} \rightarrow \infty$.

The microscopic dissociation constant $K_{d,n}$ of the Mn^{2+}-citrate lyase complex for the last binding step was calculated from the limiting slope of the Scatchard plot at $(Mn)_{free} \rightarrow \infty$ [23], using the relationship, slope $= -1/K_{d,n}$. A value of 4.5×10^{-5} M was obtained for saturating conditions of divalent metal concentration from the approximately linear region covering the last 3 points of the Scatchard plot with values in the range 17–18 Mn^{2+}-binding sites/mol enzyme.

4. Discussion

These studies show for the first time the cooperative binding of a divalent metal cofactor by citrate lyase. Binding of the divalent metal evidently involves a conformational change in the enzyme complex, probably to a form which is catalytically active in the cleavage reaction.

The allosteric behaviour of the enzyme could be a regulatory mechanism of biological significance. Citrate lyase from *K. aerogenes* undergoes rapid inactivation in vitro during the course of the reaction it catalyses through a process of deacetylation

[14,24]. However such reaction inactivation has been shown to play no role in citrate lyase regulation in vivo [25]. The inactivation in vivo has been shown to be energy dependent [25]. Conformational modulations in citrate lyase complex could be a possible mechanism of regulation, if the requirement of energy is assumed to be for the formation or utilization of metabolite(s) acting either directly as modulator(s) or indirectly through complexing of Mg^{2+}. The earlier speculation [26] that a mechanism for regulation of citrate lyase activity in the cell could be through the ability of ATP to chelate metal ions, could indeed be so through modulation in the conformation of citrate lyase when Mg^{2+} is abstracted from the complex.

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References

[1] Dagley, S. and Dawes, E. A. (1955) Biochim. Biophys. Acta 17, 177–184.
[2] SivaRaman, C. (1961) Biochim. Biophys. Acta 52, 212–213.
[3] Singh, M. and Srere, P. A. (1975) J. Biol. Chem. 250, 5818–5825.
[4] Hiremath, S. T., Paranjpe, S. and SivaRaman, C. (1976) Biochem. Biophys. Res. Commun. 72, 1122–1128.
[5] Giffhorn, F. and Gottschalk, G. (1978) FEBS Lett. 96, 175–178.
[6] Carpenter, D. E., Singh, M., Richards, E. G. and Srere, P. A. (1975) J. Biol. Chem. 250, 3254–3260.
[7] Dimroth, P., Dittmar, W., Walther, G. and Eggerer, H. (1973) Eur. J. Biochem. 37, 305–315.
[8] Singh, M., Srere, P. A., Klapper, D. G. and Capra, J. D. (1976) J. Biol. Chem. 251, 2911–2915.
[9] Dimroth, P. and Eggerer, H. (1975) Proc. Natl. Acad. Sci. USA 72, 3458–3462.
[10] Buckel, W., Ziegert, K. and Eggerer, H. (1973) Eur. J. Biochem. 37, 295–304.
[11] Dimroth, P., Loyal, R. and Eggerer, H. (1977) Eur. J. Biochem. 80, 479–488.
[12] Ward, R. L. and Srere, P. A. (1965) Biochim. Biophys. Acta 99, 270–274.
[13] Mahadik, S. P. and SivaRaman, C. (1968) Biochem. Biophys. Res. Commun. 32, 167–172.
[14] Singh, M. and Srere, P. A. (1971) J. Biol. Chem. 246, 3847–3850.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[16] Cook, J. W. (1941) Industr. Engg. Chem. (Analyt. edn) 13, 48–50.
[17] Mildvan, A. S. and Cohn, M. (1963) Biochemistry 2, 910–919.
[18] Myer, Y. P. and Schellman, J. A. (1962) Biochim. Biophys. Acta 55, 361–373.
[19] Koshland, D. E. (1970) in: The Enzymes (Boyer, P. D. ed) 3rd edn. vol. 1, pp. 341–396, Academic Press, New York.
[20] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[21] Schreier, A. A. and Schimmel, P. R. (1974) J. Mol. Biol. 86, 601–620.
[22] Danchin, A. (1972) Biopolymers 11, 1317–1333.
[23] Bartholmes, P., Kirschner, K. and Gschwind, H. P. (1976) Biochemistry 15, 4712–4717.
[24] Singh, M. and Srere, P. A. (1975) J. Biol. Chem. 250, 5818–5825.
[25] Kulla, H. and Gottschalk, G. (1977) J. Bacteriol. 132, 764–770.
[26] Blair, McD. J., Datta, S. P. and Tate, S. S. (1967) Eur. J. Biochem. 1, 26–28.