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The product inhibition of citrate–oxaloacetate lyase from *A. aerogenes*

Citrate–oxaloacetate lyase (EC 4.1.3.6) has also been known as citrase, citratase, citrate aldolase or citridesmolase. It cleaves citrate to oxaloacetate and acetate and requires divalent metal ions such as Mg^{2+} or Mn^{2+}. It has been obtained in highly purified condition from *Escherichia coli* by BOWEN AND SIVA RAMAN[1], from *Aerobacter aerogenes* by SIVA RAMAN[2] and from *Streptococcus diacetilactis* by HARVEY AND COLLINS[3]. Using partially purified preparations DAGLEY AND DAWES[4] concluded that during the course of the reaction the enzyme became progressively inhibited by accumulation of oxaloacetate in the medium. Such product inhibition is well known and many examples are listed by FRIEDEN AND WALTER[5,6]. The inhibition may be reversible or apparently irreversible; of these the latter case may be of greater quantitative significance[5,6].

The citrate lyase used in the present studies was purified from *A. aerogenes* as described by BOWEN AND ROGERS[7] and used to study the equilibrium constant of the reaction

$$\text{Citrate} \overset{Mg^{2+}}{\rightleftharpoons} \text{Oxaloacetate} + \text{Acetate}$$

HARVEY AND COLLINS[3] have quoted a value for the equilibrium constant for the enzyme from *S. diacetilactis* which differs from that of SMITH *et al.*[8] using the enzyme from *Streptococcus faecalis*. They explain this difference on the basis that the keto form of oxaloacetate is the reaction product and not the enol form, a distinction not made in the earlier work. They also drew attention to inhibition caused by high levels of magnesium ions but made no mention of inhibition caused by the oxaloacetate produced. In the present work on the enzyme from *A. aerogenes* the inhibitory effect of excess Mg^{2+} was noted but the inhibitory effect of oxaloacetate was the major effect. The present communication seeks to show that for this enzyme the inhibition by oxaloacetate is so powerful and irreversible as to make it impossible to calculate an equilibrium constant for the reaction.

The substrate used 20 g/l sodium citrate dihydrate in 0.03 M KH_2PO_4 (pH 7.4), is called the citrate test medium, *i.e.* 2% citrate test medium for the medium described. All solutions contained 1.6 mM $MgSO_4$ since Mg^{2+} was the most efficient

cofactor (*cf.* DAGLEY AND DAWES[4]). Citrate lyase activity was determined from keto acid production at 36° using the method of FRIEDEMANN AND HAUGEN[9] to determine the keto acid.

Preliminary experiments using 2% citrate test medium showed that for a constant amount of enzyme the reaction was linear at first but, after a few minutes, decreased in velocity and then ceased, leaving the bulk of the citrate unattacked. The reaction extent at this point depended only on the amount of enzyme present and hence was not an equilibrium state of the reaction. A linear relationship was evident between the enzyme present and the amount of keto acid produced at the cessation of activity. By using sufficient enzyme the reaction could be made to go to completion. It was found that the amount of citrate undergoing conversion to products was approximately the same for a constant amount of enzyme, whatever the citrate concentration, provided sufficient citrate was present for the reaction to cease well before exhaustion of the citrate. In addition conversion of citrate to products was proportional to the amount of enzyme present irrespective of citrate concentration. The presence of small amounts of acetate (73 mM) did not affect the reaction.

The inhibition of citrate lyase by oxaloacetate appeared to be irreversible since precipitation by ammonium sulphate followed by its solution in fresh buffer (0.03 M KH_2PO_4 (pH 7.4)) did not restore activity to the enzyme. This procedure did not cause loss of activity from enzyme that had not been treated with oxaloacetate, in fact it was the method usually employed to concentrate the enzyme. In further studies, enzyme (2 mg) was incubated at 36° with 2 ml of 2% solutions at pH 7.4 of compounds structurally similar to citrate or oxaloacetate. After several minutes the enzyme was precipitated with ammonium sulphate, taken up in 0.03 M KH_2PO_4 buffered to pH 7.4 and containing 1.6 mM $MgSO_4$ and its actvity assayed. Both oxaloacetate and malate completely inhibited the enzyme but acetate, pyruvate, oxalate, succinate, oxoglutarate, tartarate, oxalosuccinate and isocitrate had no effect on subsequent activity.

TABLE I

DEPENDENCE OF THE REVERSE REACTION ON SUBSTRATE CONCENTRATION

The solutions were of pH 7.4 and the total reaction volume was 0.32 ml. Incubation of enzyme (0.01 mg) with substrates was for 15 min.

Reaction mixture	*Acetate (mM)*	*Oxaloacetate (mM)*	*Citrate formed (percent theoretical)*
A	146.7	44	4.3 (average of three estimations)
B	23.4	4.33	—
C	2.7	0.75	—

In view of the close structural relationship of malate and oxaloacetate the results may prove of significance in connection with the mechanism proposed for citrate lyase by DAGLEY AND DAWES[4]. The purity of the malate had been checked by chromatography and it gave no reaction for keto acids. It was not possible to detect any obvious change in the inhibited enzyme from the normal enzyme as far as its spectrum and sedimentation behaviour were concerned. The inhibition by oxaloacetate was both time and con-

centration dependent. For example, at 36° a 11 mg% concentration of oxaloacetate reduced enzyme activity to half its initial value in 1 min.

The reverse reaction was studied using sodium [2-^{14}C]acetate and oxaloacetate. The formation of radio active citrate after equilibration was detected by using high voltage electrophoresis at pH 2.0 in 0.75 M formic acid[10]. The results are given in Table I. Very little citrate was formed under these conditions and only by using radioautography could it be found when lower concentrations of acetate and oxaloacetate were being used.

The conclusions from these studies are that in the case of citrate lyase from *A. aerogenes* no equilibrium constant can be quoted since (a) for 0.1 M citrate the reaction goes to completion given sufficient enzyme (b) only at high concentrations of oxaloacetate (0.01 M) and acetate (0.1 M) could the reverse reaction be demonstrated (c) the inhibition by oxaloacetate appeared to be irreversible, was time and concentration dependent, and occurred for both forward and reverse reactions. The conditions affecting stability of the enzyme from *A. aerogenes* have been reported in an earlier paper[7]. The purified enzyme was only moderately stable and a fresh batch was used for each experiment. It is possible that the enzymes from *S. faecalis* and *S. diacetilactis* are more stable particularly in regard to oxaloacetate inhibition.

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Department of Biochemistry,
University of Leeds,
Leeds (Great Britain)

T. J. BOWEN
L. J. ROGERS*

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* Present address: Department of Agricultural Biochemistry, University College of Wales, Aberystwyth (Great Britain).