FAILURE OF ETHYLENEDIAMINETETRAACETATE OR MANGANESE TO AFFECT (*R*)-CITRATE SYNTHESIS IN *CLOSTRIDIUM KLUYVERI* AND *CLOSTRIDIUM CYLINDROSPORUM*

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1. Introduction

Extracts of several obligate anaerobes [1–3] possess the unusual property of synthesizing (*R*)-citrate from oxalacetate and acetyl-CoA, i.e. the opposite enantiomer to that synthesized by pig heart citrate synthase, namely (*S*)-citrate. We have shown [4] that the (*R*)-type stereospecificity of the citrate synthase present in extracts of *Clostridium kluyveri* (Barker) was exhibited only under reducing conditions and that oxidizing conditions caused a significant increase in the amount of (*S*)-citrate formed. The stereospecificity of the *C. kluyveri* synthase could also be changed, in a reversible manner, from the (*R*)-type to the (*S*)-type by treatment with p-chloromercuribenzoate. Similar changes have been demonstrated in extracts of *Clostridium acidi-urici* and *Clostridium cylindrosporum* [5].

Gottschalk [6] has partly purified a sulfhydryl enzyme ("(*R*)-citrate synthase") from *C. acidi-urici* which forms (*R*)-citrate under reducing conditions. This enzyme was inhibited by ethylenediaminetetraacetate (EDTA) and stimulated specifically by Mn^{++} among several divalent cations tested. This paper demonstrates that (*R*)-citrate synthesis by extracts of *C. kluyveri* and *C. cylindrosporum* is not sensitive to EDTA or to Mn^{++}. Thus no correlation exists between the capacity to synthesize (*R*)-citrate and sensitivity to these agents.

2. Materials and methods

The three types of dried or frozen cells of *C. kluyveri* employed – Barker, Worthington and Australian (designated A-65) – are described in refs. [2,4]. *C. cylindrosporum* and *C. acidiurici* cells were kindly provided by Dr. J.C.Rabinowitz and *C. thermoaceticum* cells by Dr. H.G.Wood. Cells were suspended in 0.02 M KPO_4 buffer pH 7.5 containing 20 mM mercaptoethanol and subjected to autolysis under hydrogen (dried cells) or sonic disruption (frozen cells). The 26,000 *g* supernatant fraction was prepared and stored (−20°) under hydrogen. Citrate was determined and (*R*)- and (*S*)-citrate formation was measured according to ref. [4].

3. Results

As shown in table 1, citrate synthesis by extracts of three different *C. kluyveri* cells was not inhibited by treatment with EDTA, nor affected by Mn^{++} (or Mg^{++}) added alone or after pretreatment with EDTA. Extracts of Barker cells formed almost exclusively (*R*)-citrate under these reducing conditions while extracts of A-65 cells formed roughly equal amounts of (*R*)- and (*S*)-enantiomers. If a discrete "(*R*)-citrate synthase" of the type described by Gottschalk [6] were present, then one should have observed a large inhibition by EDTA of citrate synthesis by these two

Table 1
Effect of EDTA and divalent cations on citrate synthesis by Clostridial extracts.

Additions	*Clostridium kluyveri* Barker	*Clostridium kluyveri* A-65	*Clostridium kluyveri* Worthington	*Clostridium cylindrosporum*	*Clostridium thermoaceticum*
None	0.018	0.029	0.013	0.208	1.31
Mn^{++}	0.016	–	0.010	0.216	1.27
EDTA	0.019	0.029	0.027	0.210	0.99
EDTA + Mn^{++}	0.017	–	0.029	0.236	1.14
EDTA + Mg^{++}	–	0.031	–	0.163	–
EDTA Inhibition (%)	0	0	0	0	24.1
(*R*)-Citrate (%)	94	57	3	68	0
(*S*)-Citrate (%)	6	43	97	32	100

The reaction mixture contained: potassium phosphate buffer pH 7.5, 100 μmoles; mercaptoethanol, 10 μmoles; CoA, 1 μmole; $MnCl_2$, 1 μmole (where indicated); acetyl phosphate, 20 μmoles; potassium oxalacetate, 20 μmoles; phosphotransacetylase, 5 μg and cell extract (5–30 mg protein). Where indicated enzyme was pretreated with 10 μmoles EDTA (concentration, 9 mM) for 3.5 min at pH 7.5 and 20°, then 11 μmoles $MnCl_2$ (or 30 μmoles $MgCl_2$) were added followed by the other reactants. Final volume 1.7 ml. Final concentrations were: EDTA or metal chelate (6 mM) $MnCl_2$ (0.6 mM) and $MgCl_2$ (12 mM). Incubated 1 hr at 30° (30 min and 45° for *C. thermoaceticum*), under hydrogen. Values are μmoles citrate formed per hour per mg protein. Only part of the oxalacetate and acetyl phosphate disappeared during the incubation. (*R*) and (*S*)-citrate formation was measured in absence of added metal or EDTA.

extracts; an inhibition that would be proportional to the amount of (*R*)-citrate synthesized. Instead, no EDTA inhibition occurred. In the Worthington cell extract, which exhibits the (*S*)-type stereospecificity even under reducing conditions [3,4]. EDTA treatment increased the rate of synthesis, possibly by removing an inhibitory metal; but, clearly, EDTA was not inhibitory.

The citrate synthase of *C. cylindrosporum,* one of three Clostridia known to synthesize (*R*)-citrate [1,5], was also resistant to inhibition by EDTA or stimulation by Mn^{++}. On the other hand citrate synthesis by extracts of *C. thermoaceticum* which forms (*S*)-citrate exclusively [7] was significantly inhibited by EDTA (table 1) and this inhibition was partly overcome by addition of Mn^{++} in excess.

In a control experiment, extracts of frozen *C. acidi-urici* cells were tested. Under the conditions given in table 1, citrate synthesis was inhibited 72% by pretreatment with EDTA and this inhibition was reversed by later addition of Mn^{++} in excess to give 0.6 mM Mn^{++} final concentration. (*R*)-citrate amounted to 70% of the total citrate formed in the absence of EDTA and Mn^{++}.

4. Discussion

Extracts of *C. kluyveri* (Barker and A-65) and of *C. cylindrosporum* which form (*R*)-citrate are not sensitive to EDTA nor stimulated by Mn^{++} as is the "(*R*)-citrate synthase" of *C. acidi-urici.* There appears to be no correlation between the capacity to form (*R*)-citrate and sensitivity to these agents. Even if this lack of EDTA sensitivity merely reflects a metal so tightly bound to the synthase protein as to be unavailable to EDTA, it is clear that the enzyme in *C. kluyveri* and *C. cylindrosporum* differs from that in *C. acidi-urici*. Moreover the present results as well as earlier data [4] are consistent with the occurrence in *C. kluyveri* of a single citrate synthase whose stereospecificity is determined by the degree of reduction (or oxidation) of its exposed sulfhydryl groups. When fully reduced, the enzyme forms (*R*)-citrate, when fully oxidized or alkylated it forms (*S*)-citrate and when partly reduced (or oxidized) a mixture of the two enantiomers results. Indeed since Gottschalk measured stereospecificity only after reduction of the synthase, and crude extracts of *C. acidi-urici* can form significant amounts of (*S*)-citrate [5], a similar

mechanism is not entirely excluded for the *C. acidiurici* citrate synthase.

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