The effect of EDTA on the apparent hysteretic properties of pyruvate carboxylase from *Rhizopus arrhizus*

Stephen A. Osmani† and Michael C. Scrutton

Department of Biochemistry, King's College, Strand, London WC2R 2LS, England

Received 13 September 1984

Pyruvate carboxylase purified from *Rhizopus arrhizus* in the presence of 1 mM EDTA shows markedly non-linear progress curves when assayed in the presence of acetyl-CoA (activator) or L-aspartate (inhibitor). The non-linear progress curves are not abolished by preincubation of the enzyme with substrates, activator or inhibitor. Activation by acetyl-CoA is prevented and can be reversed by the addition of EDTA. Enzyme purified in the absence of EDTA is immediately responsive to activation by acetyl-CoA and inhibition by L-aspartate and shows linear progress curves. Incubation of such an enzyme with EDTA induces properties characteristic of the preparation purified in the presence of this chelating agent. Tight binding of EDTA to the enzyme could not be demonstrated.

Pyruvate carboxylase Rhizopus arrhizus *Acetyl-CoA* *L-Aspartate* *EDTA* *Regulation*

1. INTRODUCTION

Hysteretic enzymes characteristically show non-linear rates of reaction which change slowly over a period of minutes to hours before reaching a steady state [1,2]. Pyruvate carboxylases from various species have been reported to show such a hysteretic response to varying degrees [3–6]. It has been assumed that this response, as for other such enzymes, was an intrinsic property of the pyruvate carboxylase protein which resulted from a slow change in the conformation of the enzyme induced by the presence of its substrates [6].

Pyruvate carboxylase isolated from *Rhizopus arrhizus* has been shown to display an extreme hysteretic response when assayed in the presence of the regulatory modifiers acetyl-CoA or L-aspartate [7]. Here we demonstrate that inclusion of EDTA in buffers employed to purify the enzyme is responsible for the apparent hysteretic responses of pyruvate carboxylase isolated from this organism.

† Present address: Department of Pharmacology, UMDNJ–Rutgers Medical School, Piscataway, NJ 08854, USA

2. METHODS

R. arrhizus (ATCC13310) was grown in a defined medium with glucose as the sole carbon source and mycelium was harvested and stored as in [8]. Pyruvate carboxylase was extracted and purified by ammonium sulfate fractionation and chromatography utilizing an avidin monomer affinity column as in [9] but omitting EDTA from all buffers for some experiments. Enzyme purified in the presence or absence of EDTA gave a single protein band when analysed by polyacrylamide gel electrophoresis in the presence and absence of sodiumdodecylsulphate.

Pyruvate carboxylase was assayed by measurement of the rate of oxaloacetate production in the presence of malate dehydrogenase and NADH as in [10]. The reaction mixture contained, in 0.5 ml, 100 mM K^+ Hepes (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 2 mM NaATP, 20 mM $KHCO_3$, 0.15 mM NADH, 5 μg malate dehydrogenase and acetyl-CoA or L-aspartate at the concentrations indicated. Units are expressed as μmol oxaloacetate formed/min at 25°C. For non-linear progress curves, rates were obtained by drawing tangents to

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

the relationship between the absorbance at 340 nm and time at the times indicated in the figures.

3. RESULTS

Pyruvate carboxylase isolated from beef liver mitochondria [6] does not undergo an hysteretic response if it is pre-incubated in an incomplete assay system containing 100 mM KCl at pH 7.8 and pyruvate is used to initiate the reaction. The use of similar assay conditions in the presence of acetyl-CoA does not eliminate the increase of the reaction rate with time observed for *R. arrhizus* pyruvate carboxylase purified in the presence of EDTA. Similarly, if the assay is initiated by addition of ATP, $KHCO_3$ or Mg^{2+} in the presence of acetyl-CoA, an increase in rate with time is still observed (not shown). Furthermore, as shown in fig.1, addition of a second aliquot of enzyme to a complete assay system gives the same increase in rate with time in the presence of acetyl-CoA and decrease in rate in the presence of L-aspartate as was seen for the initial addition. Hence this response appears to be independent of substrate depletion or product accumulation.

However, the increase in rate with time observed in the presence of acetyl-CoA can be prevented if EDTA is added to the assay system although in such a system the enzyme shows little response to this activator (fig.2). However, if *R. arrhizus* pyruvate carboxylase is allowed to reach a steady-state rate in the presence of acetyl-CoA and EDTA is then added, a slow decrease in rate with time is observed towards the basal value observed on initial addition of the enzyme to the assay system containing acetyl-CoA (fig.2).

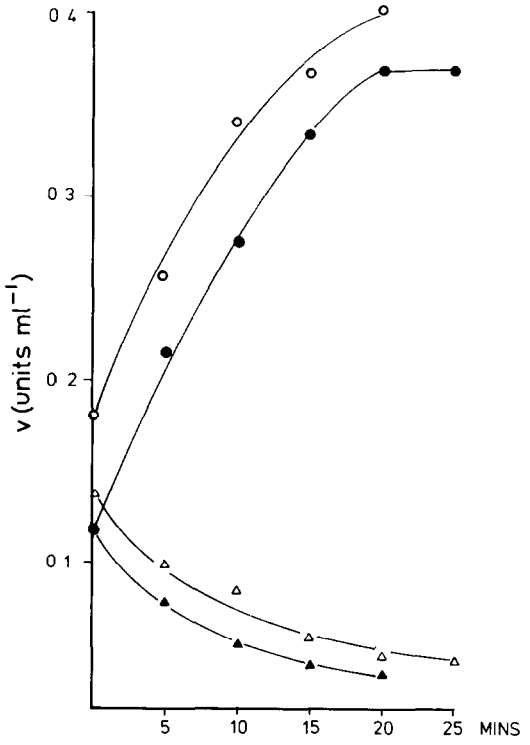

Fig.1. Effect of substrate depletion and product accumulation on the change in the reaction rate with time of pyruvate carboxylase from *R. arrhizus* caused by the presence of acetyl-CoA or L-aspartate. Assay conditions were as described in section 2. The reaction was initiated by addition of 0.4 μg pyruvate carboxylase (spec. act. 8.8 units/mg which had been in the presence of 1 mM EDTA) in the presence of 0.2 μm acetyl-CoA (○,●) or 0.4 mM L-aspartate (△,▲). When the first addition of enzyme (○,△) had reached a linear rate, a second aliquot of enzyme was added (●,▲). The activity expressed by the second aliquot was determined after subtraction of the linear rate expressed by the first.

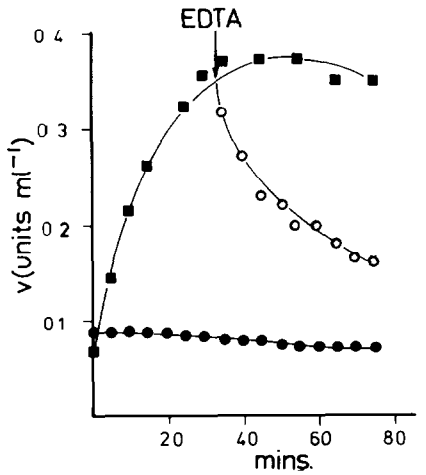

Fig.2. Effect of EDTA on activation of pyruvate carboxylase from *R. arrhizus* by acetyl-CoA. Pyruvate carboxylase was assayed as described in section 2 but with the addition of 0.2 μm acetyl-CoA; 0.1 mM EDTA was added to the assay system either before addition of enzyme (●) or at the time indicated (○) or not at all (■). Pyruvate carboxylase (spec. act. 7.5 units/mg prepared in the presence of 1 mM EDTA) was used to initiate the reaction.

The effect of EDTA on the non-linear relationships of rate with time observed for pyruvate carboxylase isolated from *R. arrhizus* can be more clearly seen if the enzyme has been purified in the absence of EDTA. When such enzyme preparations are used to initiate catalysis in the presence or absence of acetyl-CoA, significant and rapid activation by acetyl-CoA is observed with no significant delay in achievement of the steady-state rate (fig.3A). Furthermore, if EDTA is added to a sample of such an enzyme which is then used to initiate catalysis in the presence and absence of acetyl-CoA, no significant activation is observed initially. However, in the presence of acetyl-CoA, but not significantly in its absence, a marked increase of rate with time is observed (fig.3B). After 30 min the extent of activation by acetyl-CoA at the lower concentration used (5 μM) approximates that ob-

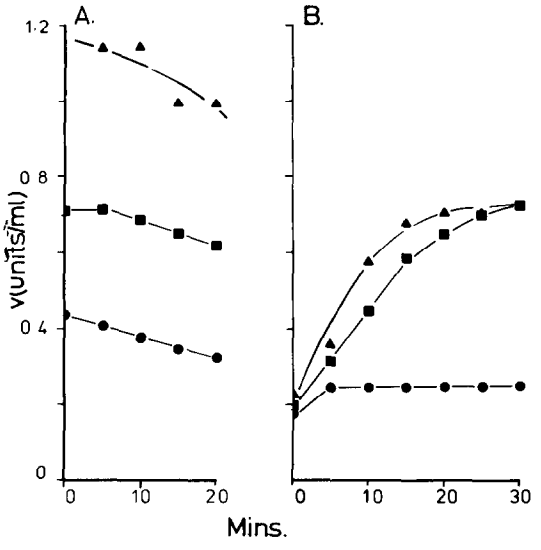

Fig.3. Effect of addition of EDTA on the properties of activation of *R. arrhizus* pyruvate carboxylase by acetyl-CoA. Pyruvate carboxylase was purified in the absence of EDTA as described in section 2. Aliquots of the purified enzyme (spec. act. = 9.1 units/mg) were incubated in the presence (B) or absence (A) of 1 mM EDTA for 2 h at 22°C and then samples taken for assay as described in section 2 in the presence of 0 (●), 5 (■) and 50 (▲) μM acetyl-CoA. The final concentration of EDTA in the assay system for samples incubated in the presence of EDTA was 4 μM. Control studies demonstrated that direct addition of this concentration of EDTA to the assay system had no effect on the properties of activation by acetyl-CoA.

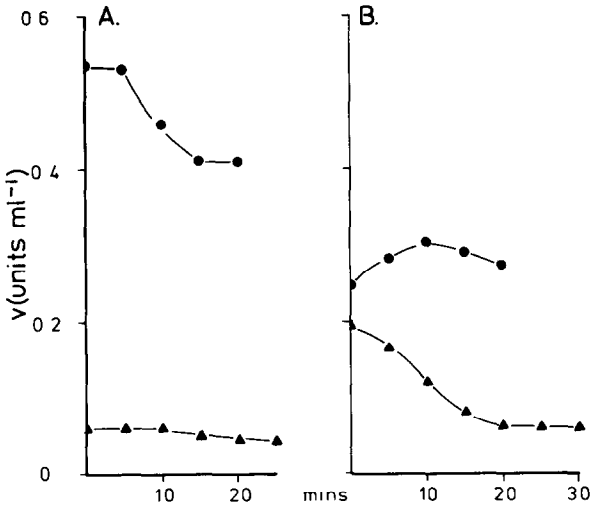

Fig.4. Effect of addition of EDTA on the properties of inhibition of *R. arrhizus* pyruvate carboxylase by L-aspartate. The studies were performed in the presence of 0 (●), or 2 (▲) mM L-aspartate and the data analysed and presented as described for fig.3.

tained immediately for the enzyme which was not pre-treated with EDTA (fig.3).

When similar experiments are performed to determine the effects of EDTA on the decrease in rate with time observed for *R. arrhizus* pyruvate carboxylase in the presence of L-aspartate very similar results were obtained. Thus, the enzyme to which EDTA was not added responds instantly to inhibition by L-aspartate (fig.4A) but after addition of EDTA the initial sensitivity to inhibition by L-aspartate is much decreased. However, the rate observed in the presence of L-aspartate decreases with time to approximate after 20 min that observed initially for the enzyme to which no EDTA was added.

In both figs 3 and 4 it is also apparent that addition of EDTA causes some inhibition of the rate of catalysis observed in the absence of acetyl-CoA or L-aspartate and that small time-dependent increases do occur in this rate for the enzyme treated with EDTA (fig.3B,4B).

4. DISCUSSION

The data presented here demonstrate that the changes in the reaction rate with time primarily observed for *R. arrhizus* pyruvate carboxylase in

response to acetyl-CoA or L-aspartate are generated by the presence of EDTA in the enzyme preparation. Since the isolation of pyruvate carboxylase routinely involves the use of buffers containing 0.5–1 mM EDTA [10] the interpretation of such apparent hysteretic responses of pyruvate carboxylase in the context of metabolic control [6,11] could be erroneous if, as would seem likely, such responses for other pyruvate carboxylases also prove to be an artifact caused by inclusion of EDTA in extraction and purification buffers.

Thus two possible explanations may be considered for them effects of EDTA on the catalytic properties of *R. arrhizus* pyruvate carboxylase shown in figs 2–4. Either this chelating agent may bind to the enzyme and may be displaced by addidion of acetyl-CoA or L-aspartate or alternatively treatment with the chelating agent may remove a bound metal ion which is essential for expression of the regulatory properties and for which rebinding is facilitated in the presence of acetyl-CoA or L-aspartate. Preliminary experiments using [^{14}C]-EDTA indicate that no significant radioactivity is associated with the enzyme-containing fractions when pyruvate carboxylase and [^{14}C]EDTA are separated by gel filtration. Furthermore the effect of exposure of the enzyme to EDTA cannot be reversed by dialysis. Neither of these observations appear consistent with the formation of an enzyme-EDTA complex.

R. arrhizus pyruvate carboxylase has not yet been examined for the possible presence of bound metal ion but studies on other pyruvate carboxylase which are regulated by an acyl derivative of coenzyme A and by one or more dicarboxylic acids have revealed the presence of bound manganese in the enzyme from mammalian and avian liver [12, 13] and of bound zinc in the enzyme from *Saccharomyces cerevisiae* [14]. In contrast, analysis of pyruvate carboxylase from *Pseudomonas citronellolis* which is insensitive to regulation by any metabolite tested thus far, failed to show any evidence for the presence of a bound metal ion ([5], unpublished). When taken together with the data presented here these findings suggest that the bound metal ion in pyruvate carboxylases which are regulated by metabolic effectors may be involved in the correct functioning of the regulatory sites or of catalytic site–regulatory site interaction rather than participating in the catalytic mechanism as suggested previously [15]. Further studies are required to test this latter postulate.

ACKNOWLEDGEMENT

These studies were supported in part by a grant from the Science and Engineering Research Council. S.A.O. holds an SERC studentship.

REFERENCES

[1] Frieden, C. (1968) in: The Regulation of Enzyme Activity and Allosteric Interactions (Kramme, E. and Pihl, A. eds) p. 59, Academic Press, New York.
[2] Frieden, C. (1970) J. Biol. Chem. 245, 5788–5799.
[3] Bais, R. and Keech, D. (1972) J. Biol. Chem. 247, 3255–3261.
[4] McClure, W.R., Lardy, H.A. and Kneifel, H.P (1971) J. Biol. Chem. 246, 3569–3578.
[5] Utter, M.F., Barden, R.E. and Taylor, B.L. (1975) Adv. Enzymol. 42, 1–72.
[6] Yip, B.P. and Rudolph, F.B. (1978) Arch. Biochem. Biophys. 191, 657–665.
[7] Osmani, S.A., Mayer, F.A. and Scrutton, M.C. (1984) Ann. NY Acad. Sci., in press.
[8] Osmani, S.A. and Scrutton, M.C. (1984) Eur. J. Biochem., submitted.
[9] Osmani, S.A., Mayer, F.A., Marston, F.A.O., Selmes, I.P. and Scrutton, M.C. (1984) Eur. J. Biochem. 139, 509–518.
[10] Scrutton, M.C., Olmsted, M.R. and Utter, M.F. (1969) Methods Enzymol. 13, 235–249.
[11] Neet, K.E. and Ainslie, G.R. (1980) Methods Enzymol. 64, 192–226.
[12] Scrutton, M.C., Mildvan, A.S. and Utter, M.F. (1966) J. Biol. Chem. 241, 3480–3487.
[13] Scrutton, M.C., Griminger, P. and Wallace, J.C. (1972) J. Biol. Chem. 247, 3305–3313.
[14] Scrutton, M.C., Young, M.R. and Utter, M.F. (1970) J. Biol. Chem. 245, 6220–6227.
[15] Scrutton, M.C., Reed, G.H. and Mildvan, A.S. (1973) Adv. Expt. Biol. Med. 40, 79–102.