# Pyruvate carboxylase from *Rhizopus arrhizus*

## Analysis of the subunit structure by electron miscroscopy

F. Mayer*, S.A. Osmani and M.C. Scrutton

**Institute für Mikrobiology der Universitat Gottingen, Grisebachstrasse 8, D-4000 Gottingen, FRG* and *Department of Biochemistry, King's College, The Strand, London WC2R 2LS, England*

Received 11 September 1985

Pyruvate carboxylase purified from *Rhizopus arrhizus* exhibits projections when examined in the electron microscope which indicate that this enzyme is a tetrameric molecule in which the subunits are arranged at the corners of a tetrahedron. The tetrameric molecule is stabilised by addition of acetyl-CoA or of pyruvate but is labilised in the presence of 2-oxoadipate. Addition of EDTA causes a decrease in the stability of the tetrameric molecule with a time course similar to that observed for loss of acetyl-CoA-dependent catalytic activity [(1984) FEBS Lett. 127, 157–160]. The data suggest that the hysteretic responses induced by exposure to EDTA are associated with dissociation of the tetrameric molecule to dimers and monomers having a decreased sensitivity to allosteric activation.

*Pyruvate carboxylase* Rhizopus arrhizus *Electron microscope* *EDTA* *Acetyl-CoA* *2-Oxoadipate*

## 1. INTRODUCTION

Previous studies have shown that the cytosolic pyruvate carboxylase purified from *Rhizopus arrhizus* is subject to regulatory activation by acetyl-CoA and long-chain acyl derivatives of CoA and to regulatory inhibition by L-aspartate and by 2-oxoadipate [1]. When purified in the presence of EDTA markedly non-linear progress curves are observed on addition of this enzyme to assay systems containing either acetyl-CoA or L-aspartate. Similar data can be obtained if the enzyme is purified in the absence of EDTA and then preincubated with this chelating agent before addition to the assay system. In order to gain insight into the molecular basis for this apparent hysteretic response we have characterised the effect of EDTA and of the regulatory effectors, both singly and in combination, on the molecular structure of *R. arrhizus* pyruvate carboxylase as viewed in the electron microscope. The results obtained suggest that addition of EDTA promotes dissociation of this pyruvate carboxylase which may be related to the loss of sensitivity to the regulatory effectors.

## 2. MATERIALS AND METHODS

*R. arrhizus* (ATCC 13310) was grown in a defined medium with glucose as sole carbon source and the mycelia were harvested and stored as described [1]. Pyruvate carboxylase was purified from these mycelia as described previously using chromatography on spheroidal hydroxyapatite as the final step. The preparations used here were homogeneous when examined by polyacrylamide gel electrophoresis in the presence or absence of SDS and had specific activities in the range 12–15 $\mu$mol/min per mg protein at 25°C.

Preparation of samples for electron microscopy by negative staining, and imaging of these samples, was performed as described by Osmani et al. [3]. The degree of preservation of the enzyme structure was determined from magnified prints of the electron micrographs obtained from these negatively

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/85/$3.30 

stained samples by counting, in at least 4 independent evaluations per sample, both the preserved tetrameric enzyme particles and the smaller particles which comprise tetramers broken down into dimers or monomers. These numbers were then used to determine a quotient ($Q$) representing, for a given sample, the ratio of preserved tetramers vs small particles. The ratios determined for each of

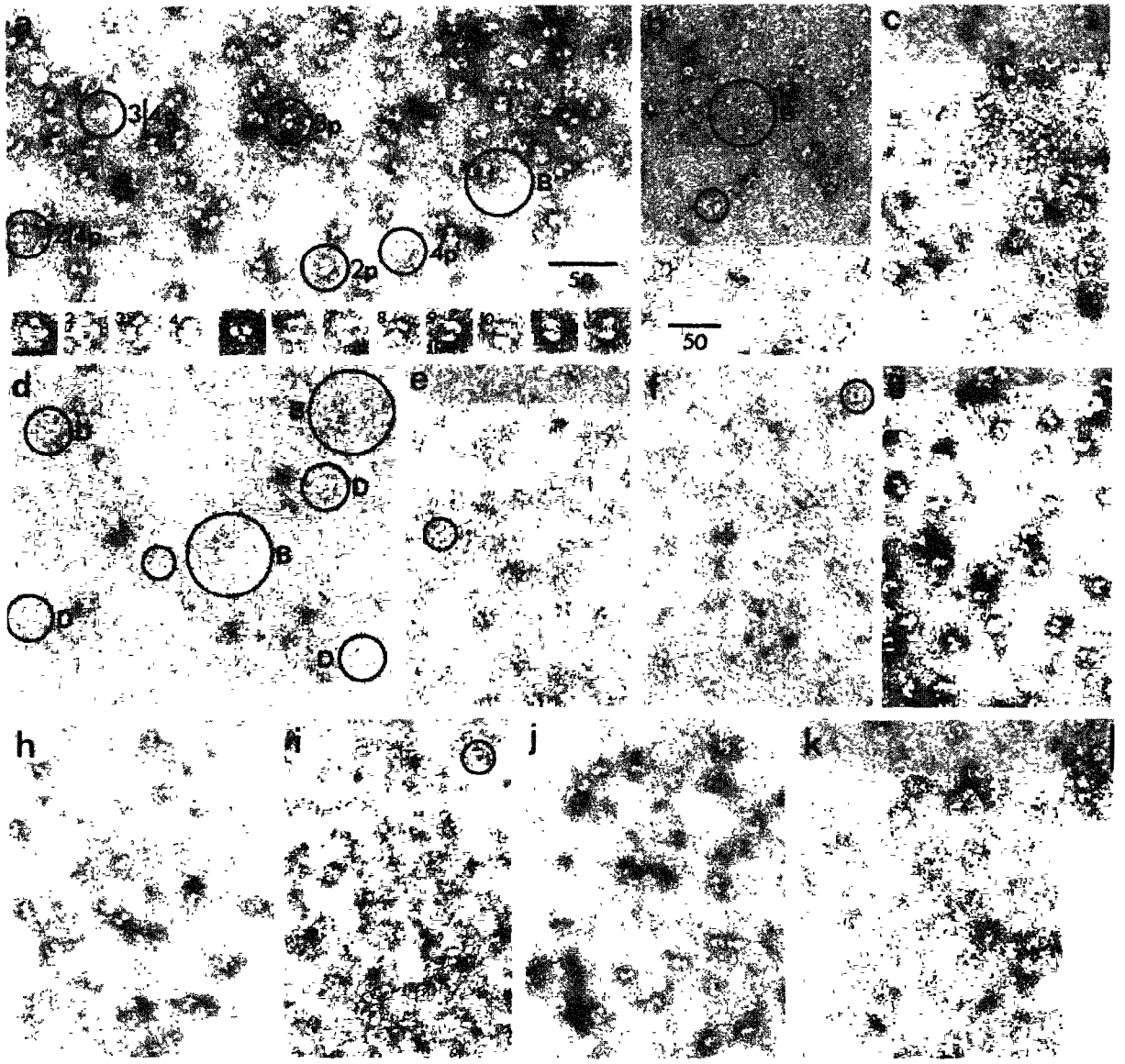


Fig.1. Electron micrographs of *R. arrhizus* pyruvate carboxylase mounted under various conditions. The enzyme samples were negatively stained with uranyl acetate and mounted and the electron micrographs obtained as described in section 2. The bar corresponds to 50 nm in the main micrographs and 20 nm in the gallery. (a) + 50 $\mu$M acetyl-CoA. The gallery shows pyruvate carboxylase molecules at higher magnification in different projections. Rhomboid (4p) 1–4; triangular (3p) 5; two sub-divided projection maxima (2/4p) 6–9; end-on (2p) 10–12. The mean diameters of the particles range from 13.8 nm (2p) to 15.4 nm (4p). (b) No addition, (c) + 20 mM pyruvate, (d) + 1 mM EDTA for 30 min prior to negative staining, (e) + 2 mM L-aspartate, (f) + 10 mM 2-oxoadipate, (g) + 10 mM 2-oxoadipate and 50 $\mu$M acetyl-CoA, (h) + 10 mM 3-oxoadipate, (i) + 10 mM 2-oxoglutarate, (j) + 10 mM oxaloacetate, (k) + 10 mM $MgATP^{2-}$. Inset: polymeric pyruvate carboxylase-avidin complex.

the evaluations for a given sample were averaged. The dimensions of pyruvate carboxylase structures were measured from the magnified prints.

## 3. RESULTS

Fig.1 shows representative samples of *R. arrhizus* pyruvate carboxylase mounted for electron microscopy under various conditions. When the enzyme is mounted in the presence of acetyl-CoA (fig.1a) a high proportion of the particles present show projections consistent with the presence of a tetrameric molecule having its 4 subunits arranged at the corners of a tetrahedron. These projections, most of which are illustrated in the gallery at the bottom of fig.1a, include rhomboid (4p), triangular (3p) and 'edge-on' (2p) presentations of the molecule. In addition projections can be seen in which 3 subunits are clearly visible together with a less clear indication of the presence of the fourth subunit (3/4p) or in which the two main projection maxima are each sub-divided into two sub-maxima (2/4p) indicating the presence of 2 subunits in each maximum which are partially superimposed. Most of the projections observed in the presence of acetyl-CoA are of one of these 5 types although some broken particles (B) are also present. The preservation quotient ($Q$) defined as described in section 2 is approx. 1.5 (fig.2). Effective preservation of the tetrameric molecule is also observed when samples are mounted in the presence of high concentration (10 mM) of pyruvate (fig.1c). Formation of the pyruvate carboxylase-avidin complex at low avidin:pyruvate carboxylase ratios gives polymeric complexes containing chains of 3–4 pyruvate carboxylase molecules (fig.1k, inset) although the preservation quotient estimated by quantitative analysis of the micrographs (1.1) is not as great as that obtained in the presence of acetyl-CoA (fig.2).

When *R. arrhizus* pyruvate carboxylase is mounted in the absence of any additions (fig.1b) or in the presence of L-aspartate (fig.1e) or of $MgATP^{2-}$ (fig.1k) some tetrameric molecules in the various projections indicated above can be seen but the general level of structural preservation is considerably lower and $Q$ approximates 0.2 (fig.2). In addition in the presence of L-aspartate the molecules appear less compact with the mean diameter of the rhomboid projection being 19 nm as compared to 15.4 nm in the presence of acetyl-CoA.

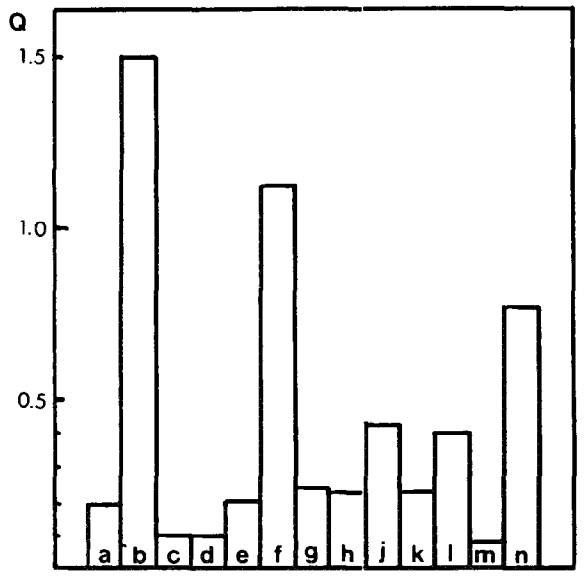


Fig.2. Results of a comparative electron microscopic study on the structural preservation of pyruvate carboxylase isolated in the absence of EDTA and mounted for electron microscopy either without additions or with additions as specified. The preservation quotient ($Q$) was calculated as described in section 2 from electron micrographs similar to those shown in fig.1. In all cases unless otherwise specified the enzyme sample was incubated with the addition for 5 min before mounting. (a) No addition; (b) 50 $\mu$M acetyl-CoA; (c) 1 mM EDTA, incubation for 60 min prior to mounting; (d) 1 mM EDTA, after 60 min 50 $\mu$M acetyl-CoA was added, further incubation for 5 min prior to mounting; (e) 50 $\mu$M acetyl-CoA, after 5 min 1 mM EDTA was added, further incubation for 30 min prior to mounting; (f) 20 mM pyruvate; (g) 2 mM L-aspartate; (h) 10 mM 2-ketoglutarate; (j) 10 mM $MgATP^{2-}$; (k) 10 mM 3-ketoadipate; (l) 10 mM 2-ketoadipate; (m) 10 mM 1-ketoadipate followed (after 5 min) by addition of 50 $\mu$M acetyl-CoA, mounting 5 min later.

An actual decrease in the extent of structural preservation, as compared to that obtained when the enzyme is mounted in buffer, is observed on mounting in the presence of the inhibitory effector 2-oxoadipate (fig.1f). Although a few tetrameric molecules can still be seen the fields are dominated by broken particles and $Q$ is significantly decreased to a value below 0.1 (fig.2). The specificity of this

effect is indicated by the finding that mounting in the presence of either 2-oxoglutarate which is ineffective as an inhibitor of *R. arrhizus* pyruvate carboxylase [1] or 3-oxoadipate has little effect on the extent of preservation (figs 1i,h and 2). Mounting in the presence of 2-oxoadipate + acetyl-CoA yields an extent of structural preservation intermediate between that observed when either of these effectors is added alone (figs 1g and 2).

We have also examined the effect of EDTA on the molecular structure of *R. arrhizus* pyruvate carboxylase as viewed by electron microscopy. Fig.1d indicates that addition of 1 mM EDTA markedly decreases the extent of structural preservation of the tetrameric molecule. Where rhomboid projections can be seen they are usually distorted having diameters up to 23 nm and appear to be in the process of dissociating. The *Q* value observed for a sample mounted in the presence of 1 mM EDTA after incubation for 60 min is 0.1. This value is not increased if acetyl-CoA is added 5 min prior to mounting (fig.2). Furthermore, addition of EDTA abolishes the ability of acetyl-CoA to enhance structural preservation of *R. arrhizus* pyruvate carboxylase. This effect is a function of the time of incubation with EDTA and *Q* falls from a value of 1.5 if the sample is taken for mounting immediately after addition of acetyl-CoA + EDTA to a value of 0.2 if the enzyme is incubated for 30–40 min with these additions prior to mounting (fig.3).

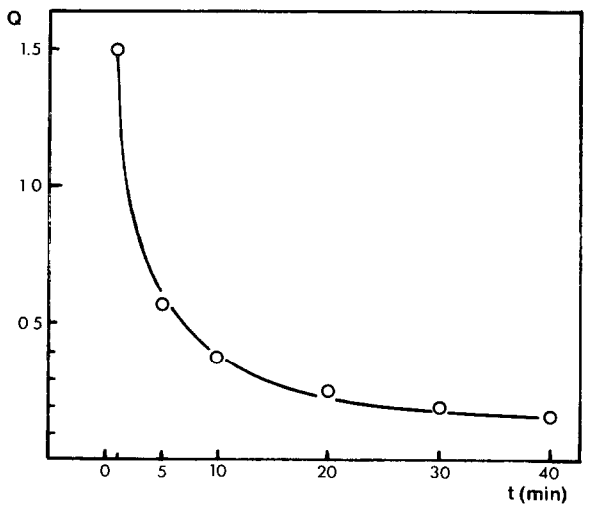


Fig.3. Effect of EDTA on the structural preservation of *R. arrhizus* pyruvate carboxylase. A solution of pyruvate carboxylase (30 μg/ml) was mixed with acetyl-CoA (50 μM final concentration) and EDTA (1 mM final concentration); samples were taken at the times given (1, 5, 10, 20, 30, 40 min) and negatively stained. *Q* is a quotient indicating the degree of structural preservation and was obtained as described in section 2. The data given for each point of the curve are averages from 4 independent evaluations.

## 4. DISCUSSION

The studies shown in figs 1 and 2 indicate that the cytosolic pyruvate carboxylase obtained from *R. arrhizus* is a tetrameric molecule having a structure which is indistinguishable at the level of this analysis from that described previously for the cytosolic enzyme purified from *Aspergillus nidulans* [3] and the mitochondrial enzyme obtained from mammalian liver [4]. The molecular dimensions of these pyruvate carboxylases are also similar, and in all cases addition of acetyl-CoA markedly enhances the preservation of the tetrameric structure (figs 1 and 2) [3,4]. Thus, our findings for *R. arrhizus* pyruvate carboxylase strengthen the conclusion that the regulated pyruvate carboxylases possess the same overall molecular organisation which is independent of both their localisation within the cell and the nature of the effectors which regulate their catalytic activity [5]. These data do, however, provide the first example of destabilisation of the tetrameric structure by an inhibitory effector added alone since such an effect is observed on addition of 2-oxoadipate (figs 1f and 2). This response would be predicted but has previously only been observed as a decrease induced by L-aspartate in the extent to which acetyl-CoA enhances structural preservation of the molecule [3].

The data also suggest that the ability of EDTA to abolish the response of *R. arrhizus* pyruvate carboxylase to acetyl-CoA and L-aspartate [2] is related to a loss of intimate contact between the subunits in the tetrameric molecule often leading to its dissociation. Thus the time course for the decrease in *Q* observed here on addition of EDTA in the presence of acetyl-CoA (fig.3) is very similar to that described previously for the decrease in catalytic activity induced by addition of this chelating agent after full activation by acetyl-CoA

has been achieved [2]. Since EDTA has little effect on the catalytic activity of *R. arrhizus* pyruvate carboxylase expressed in the absence of its effectors [2], we may postulate that expression of the regulatory properties of the enzyme requires the presence of the tetrameric molecule whereas catalytic activity can be expressed by dimers or possibly the isolated subunits. The hysteretic responses described earlier [2] may therefore relate to the assembly or disassembly of the tetrameric molecule.

## ACKNOWLEDGEMENTS

These studies were supported in part by an EMBO short-term fellowship (to F.M.) and an SERC studentship (to S.A.O.).

## REFERENCES

[1] Osmani, S.A. and Scrutton, M.C. (1985) Eur. J. Biochem. 147, 119–128.
[2] Osmani, S.A. and Scrutton, M.C. (1984) FEBS Lett. 177, 157–160.
[3] Osmani, S.A., Mayer, F., Marston, F.A.O., Selmes, I.P. and Scrutton, M.C. (1984) Eur. J. Biochem. 139, 509–518.
[4] Mayer, F., Wallace, J.C. and Keech, D.B. (1980) Eur. J. Biochem. 112, 265–272.
[5] Osmani, S.A., Scrutton, M.C. and Mayer, F. (1985) Ann. NY Acad. Sci. 447, 56–71.