Avidin as a probe of the conformational changes induced in pyruvate carboxylase by acetyl-CoA and pyruvate

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Sheep liver pyruvate carboxylase was mixed with avidin at a molar ratio of 1:1 in the presence of various combinations of the components of the assay systems required for either the acetyl-CoA-dependent or the acetyl-CoA-independent activity and negatively stained samples were examined by electron microscopy. Significant numbers of chain-like polymers of enzyme-avidin complexes were evident only when acetyl-CoA or high levels of pyruvate were present in the media. Similar results were also obtained for chicken liver pyruvate carboxylase despite this enzyme's almost complete lack of acetyl-CoA-independent activity. Thus, although acetyl-CoA and high concentrations of pyruvate may induce pyruvate carboxylase to adopt a 'tight' tetrahedron-like conformation which can interact with avidin to form chains, this structural change alone does not result in an enzymic form that is maximally active. This suggests that the allosteric activation of pyruvate carboxylase by acetyl-CoA is attributable, at least in part to more subtle conformational changes, especially in the case of the chicken enzyme.

Pyruvate carboxylase *Allosteric activation* *Conformational change* *Acetyl-CoA* *Pyruvate* *Avidin*

1. INTRODUCTION

Despite the dramatic effects which acetyl-CoA has on both the activity and the stability of pyruvate carboxylase purified from vertebrate liver, the readily discernable effects which the allosteric effector has directly on the enzyme's structure are mostly subtle (review [1]).

Nevertheless, markedly enhanced preservation of pyruvate carboxylase's tetrameric structure has been observed in electron microscopic studies of this enzyme purified from a thermophilic *Bacillus* [2], vertebrate liver [3], *Aspergillus nidulans* [4], *Rhizopus arrhizus* [5] and *Saccharomyces cerevisiae* [6]. In the presence of acetyl-CoA the pyruvate carboxylases from vertebrate livers were found to exhibit a higher proportion of compact, triangular images enclosing three readily visible intensity maxima. In the absence of acetyl-CoA, besides a higher percentage of particles having no consistent geometry, there was evidence also of a much higher proportion of 'open', predominantly rhomboidal projections [3].

In the course of electron microscopic studies of avidin-enzyme complexes [7] it was noted that only in the presence of acetyl-CoA did the characteristic linear, unbranched polymers form when the avidin:enzyme ratio was between 2:1 and 1:2. It appeared, therefore, as if avidin, with its biotin-binding sites arranged 2 nm apart in pairs on opposite sides of the 4.5 nm diameter molecule [8,9], may represent a sensitive probe of pyruvate carboxylase's conformation in the presence of various substrates and metabolic effectors.

Under suitable conditions (viz., in the presence of high concentrations of enzyme, of NH_4^+, and of the substrates pyruvate and HCO_3^-), sheep liver

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pyruvate carboxylase can exhibit specific activities in the absence of acetyl-CoA up to 25% of that found when this ligand is present at saturating concentrations [10]. Chicken liver pyruvate carboxylase, however, does not exhibit a comparable level of acetyl-CoA-independent activity under these conditions [10]. Therefore, it seemed possible that some component(s) of the acetyl-CoA-independent assay mix may induce sheep liver pyruvate carboxylase to adopt the compact tetrahedral structure found in the presence of acetyl-CoA, whereas this would not be the case for the chicken liver enzyme. This was investigated using electron microscopic observation of avidin-enzyme chain formation as a measure of enzyme conformation. Contrary to expectations, however, it was found that similar changes in conformation were induced in both sheep and chicken liver pyruvate carboxylases by high concentrations of pyruvate (or 2-oxobutyrate) alone.

2. MATERIALS AND METHODS

Avidin and d-biotin were obtained from Sigma, while [^{14}C]biotin and $NaH^{14}CO_3$ were obtained from Amersham (Australia). All other materials were high purity preparations as described earlier [7,11].

2.1. *Preparation of pyruvate carboxylase*

Pyruvate carboxylase was purified from sheep and chicken liver mitochondria according to Goss et al. [11] except that DEAE-Sepharose replaced DEAE-Sephadex. The fractions containing enzyme of the highest specific activities, 25–30 units·mg^{-1} protein (where 1 unit enzyme catalyses the formation of 1 μmol oxaloacetate·min^{-1} at 30°C), were pooled and the enzyme precipitated by the addition of ammonium sulphate (27.7 g/100 ml). This enzyme could be stored at −80°C without loss of activity if dissolved in a buffer composed of 0.1 M *N*-ethylmorpholine acetate, pH 7.0, 0.04 M ammonium sulphate and 1.6 M sucrose. Before use, these salts and sucrose were removed from the enzyme by gel filtration on a TSK G-4000SW column (25 cm × 7.2 mm) equilibrated in 0.1 M Tris-Cl, pH 7.2, containing 0.1 M KCl.

2.2. *Measurement of biotin and biotin-binding sites*

The biotin content of the enzyme and the biotin-binding capacity of the avidin were determined as described by Johannssen et al. [7]. Where ratios of avidin:enzyme are quoted, these refer to [biotin-binding sites]:[biotin].

2.3. *Preparation of enzyme-avidin complexes*

In all experiments the enzymes were diluted to ~16 units·ml^{-1} in the following solutions, each of final volume 96 μl: (a) The complete acetyl-CoA-independent assay mix (viz. 100 mM Tris-Cl, pH 8.4 [sheep] or pH 7.8 [chicken], 2.5 mM ATP, 8 mM $MgCl_2$, 40 mM Na pyruvate, 40 mM $NaHCO_3$, 100 mM NH_4Cl). (b) The acetyl-CoA-dependent assay mix (viz. 100 mM Tris-Cl, pH 8.4 [sheep] or pH 7.8 [chicken], 2.5 mM ATP, 7 mM $MgCl_2$, 10 mM Na pyruvate, 20 mM $NaHCO_3$) with or without 0.25 mM acetyl-CoA. (c) The acetyl-CoA-dependent assay mix containing instead 40 mM Na pyruvate or 40 mM $NaHCO_3$ or 8 mM $MgCl_2$ or 100 mM NH_4Cl or 40 mM 2-oxobutyrate but no acetyl-CoA. (d) 0.1 M Tris-Cl, pH 8.4 [sheep] or pH 7.8 [chicken], containing 40 mM pyruvate. The solutions were incubated at 30°C for 30 min before the addition of 4 μl avidin solution (4 mg·ml^{-1}, so that the ratio of avidin:enzyme was 1:1), and then for a further 2 h at 30°C to ensure as complete avidin binding as possible. At the end of this period, 50 μl of each sample were added to 950 μl of 0.1 M Tris-Cl at the appropriate pH to give a final enzyme concentration of ~40 μg·ml^{-1} (~0.8 unit·ml^{-1}). These solutions were incubated at 30°C for a further hour to allow a large degree of dissociation of unstabilized enzyme to take place. Controls were also prepared which contained no avidin and which were incubated in the acetyl-CoA-dependent assay mix containing 0.25 mM acetyl-CoA, with the final dilution to 40 μg·ml^{-1} being performed in 0.1 M Tris-Cl, containing 0.25 mM acetyl-CoA. Samples were then prepared for and examined by electron microscopy as described [7].

3. RESULTS AND DISCUSSION

Fig.1 shows a control sample of sheep pyruvate carboxylase prepared in the absence of avidin but in the presence of acetyl-CoA. The enzyme

molecules appear to be well preserved and show the 'tight' configuration [3,7]. Fig.2a shows sheep enzyme that had been mixed with avidin in the acetyl-CoA-dependent assay mix including acetyl-CoA. Numerous long chain-like polymers are visible, some of them showing backfolding. Fig.2b shows sheep enzyme that had been mixed with avidin in the acetyl-CoA-independent assay mix. The result is very similar to that in fig.2a. Fig.2c shows a contrast where the sheep enzyme and avidin were prepared in the same way as in fig.2a except that acetyl-CoA was omitted. Only a few typical avidin-enzyme chains are visible. The markedly reduced formation of these chains, the occurrence of distorted enzyme molecules and unspecific aggregates, and the presence of many small particles of different sizes (presumably subunits of broken enzyme molecules and free avidin) (see table 1) are indicative of a low degree of structural preservation of the sheep enzyme under these conditions.

These results demonstrate that some component of the acetyl-CoA-independent assay mix can mimic the structure-preserving effect of acetyl-CoA on sheep liver pyruvate carboxylase and induce this enzyme to adopt the 'tight' tetrahedron-like configuration which interacts well with avidin to form chains of avidin-enzyme polymer. Fig.2d demonstrates that pyruvate is the factor responsible for this effect. This micrograph shows that chain formation occurred when a sheep enzyme sample was mixed with avidin in the presence of pyruvate (40 mM) alone. Similar results (not shown) were obtained with 40 mM 2-oxobutyrate.

Table 1

Summary of electron microscopic observations of the quaternary structure of pyruvate carboxylase-avidin complexes under various conditions

Enzyme[a]/additions[b]	Well-preserved tetramers (%)[c]	Well-preserved tetramers in chains (%)[d]	Tetramers per chain[e]	Number of small particles per unit area[f]
SLPC + acetyl-CoA (≡ fig.1)	93.12 ± 0.84	0	–	43
SLPC + acetyl-CoA-dependent assay mix + avidin (≡ fig.2a)	95.9	88.4	3.53 ± 0.09 (265)	19
SLPC + acetyl-CoA-independent assay mix + avidin (≡ fig.2b)	96.4	87.8	3.03 ± 0.09 (235)	19
SLPC + acetyl-CoA-dependent assay mix minus acetyl-CoA + avidin (≡ fig.2c)	n.d.[g]	25.2	2.42 ± 0.08 (110)	119[h]
SLPC + pyruvate (40 mM) + avidin (≡ fig.2d)	95.8	91.4	3.67 ± 0.13 (278)	19
CLPC + pyruvate (60 mM) + avidin (≡ fig.3b)	97.1	78.5	3.72 ± 0.15 (194)	18

[a] SLPC, sheep liver pyruvate carboxylase; CLPC, chicken liver pyruvate carboxylase

[b] Details of the additions made to the enzyme solutions are given in the legend to the figure indicated in parentheses

[c] The number of well-preserved tetrameric enzyme molecules is given as a percentage of the total number of protein particles observed in 9 areas each 0.25 μm^2 ('unit area')

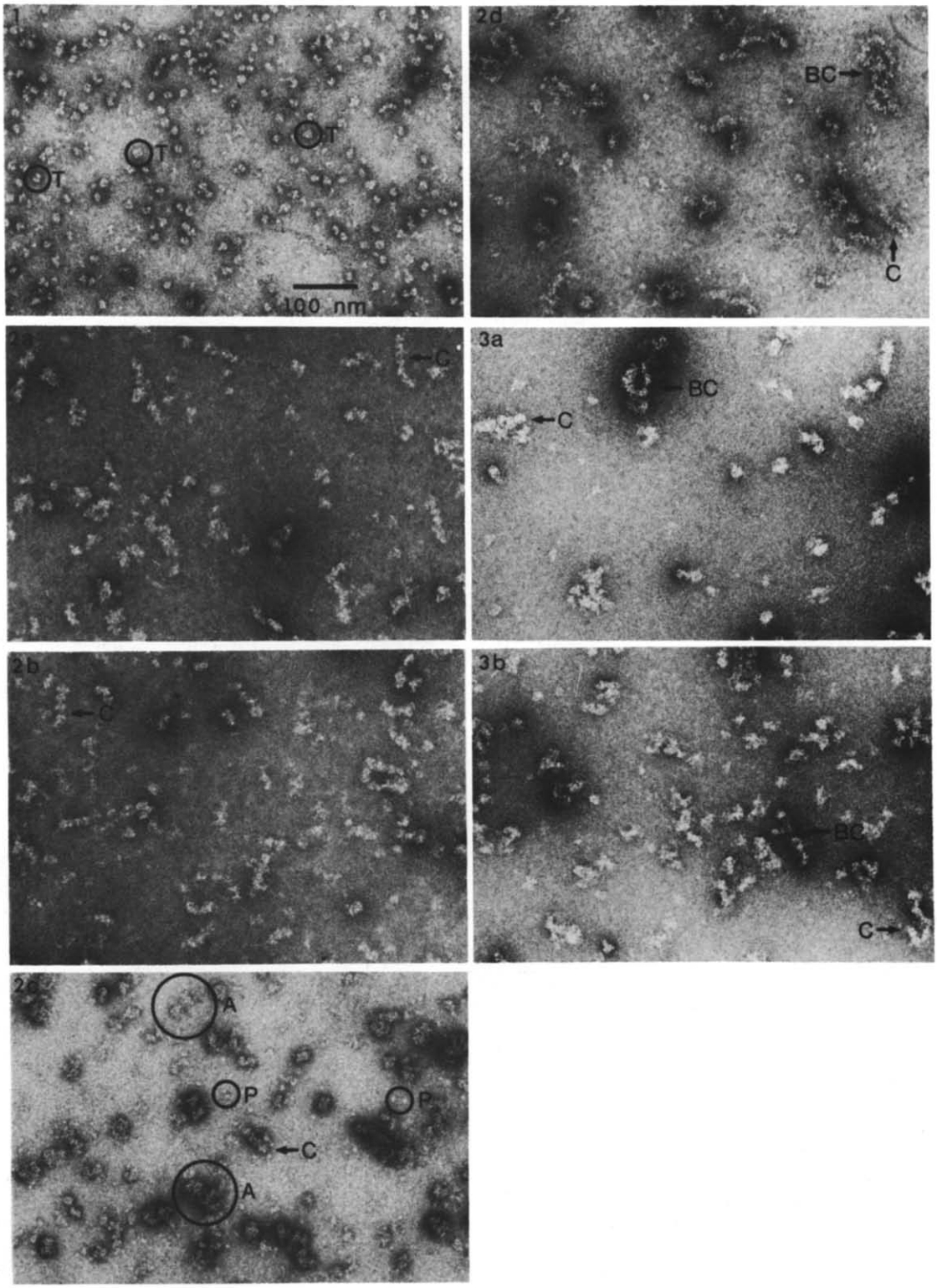
[d] The number of well-preserved tetrameric enzyme molecules present in the chain-like complexes with avidin is given as a percentage as described in [c]

[e] The values given are the means ± SE for the number of chains indicated in parentheses

[f] 'Small particles' are protein molecules (either avidin or broken enzyme molecules) smaller than intact tetrameric pyruvate carboxylase molecules; 'unit area' – as defined in [c]

[g] There were very few intact tetramers evident in the absence of acetyl-CoA, except those stabilized by chain formation with avidin

[h] Accompanied by a number (mean = 14/unit area) of non-specific aggregates of broken enzyme and/or avidin molecules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BC
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Fig.1. Electron micrograph of sheep liver pyruvate carboxylase after negative staining. The enzyme was incubated at 16 units·ml^{-1} in the presence of the components of the acetyl-CoA-dependent assay system and 0.25 mM acetyl-CoA for 2 h at 30°C. The sample was then diluted to a final enzyme concentration of ~40 μg·ml^{-1} (0.8 unit·ml^{-1}) using 0.1 M Tris-Cl, pH 8.4, containing 0.25 mM acetyl-CoA and incubated for 1 h at 30°C before preparation for electron microscopy.

Fig.2. Electron micrographs of preparations of sheep enzyme in the presence of avidin, after negative staining. In all the samples the avidin was present at a ratio of avidin:enzyme of 1:1 ([biotin-binding site]:[biotin]) and final dilution to ~40 μg·ml^{-1} (0.8 unit·ml^{-1}) was performed using 0.1 M Tris-Cl, pH 8.4. These solutions were incubated for 1 h at 30°C before samples were prepared for electron microscopy. The enzyme was incubated at 16 units·ml^{-1} before and during the reaction with avidin in the presence of the following: (a) the components of the acetyl-CoA-dependent assay system together with 0.25 mM acetyl-CoA; (b) the components of the acetyl-CoA-independent assay system; (c) as in (a) except that acetyl-CoA was omitted; (d) 40 mM pyruvate in 0.1 M Tris-Cl, pH 8.4.

Fig.3. Electron micrographs of complexes of chicken liver pyruvate carboxylase and avidin which were present at a concentration ratio of 1:1. The enzyme (16 units·ml^{-1}) and avidin were incubated together in 0.1 M Tris-Cl buffer, pH 7.8, at 30°C in the presence of various concentrations of pyruvate, i.e. (a) 10 mM and (b) 60 mM.

For figs 1–3, the bar is given in fig.1; it represents 100 nm, and on the micrographs, the letters represent: A, non-specific protein aggregates; BC, backfolded chains; C, chains; P, small protein particles (broken enzyme and avidin); T, 'tight' tetrameric pyruvate carboxylase particles.

Samples of the sheep liver enzyme taken prior to the addition of avidin were assayed for catalytic activity under conditions corresponding to figs 2a and b above. In the acetyl-CoA-independent assay system (fig.2b) the enzyme exhibited 10% of the activity which it yielded in the presence of saturating acetyl-CoA (fig.2a). However, when similar assays were conducted on the chicken liver enzyme, even with the pyruvate concentration as high as 80 mM the acetyl-CoA-independent activity was found to be only 0.9% of that in the presence of acetyl-CoA. It was somewhat surprising, therefore, to find, as illustrated in fig.3 that pyruvate alone, especially at 60 mM (see fig.3b), could induce chain formation between avidin and chicken liver pyruvate carboxylase. Such an observation is consistent, however, with the finding of Mildvan et al. [12] that pyruvate enhances the rate of inhibition of chicken liver pyruvate carboxylase by avidin.

From these observations, which are summarised in table 1, it would seem that pyruvate induces a generally similar effect on the quaternary structure of both enzymes. In view of the substantial differences in activity between these enzymes with apparently similar 'tight' tetrahedron-like conformations, it would seem that this type of structural change in pyruvate carboxylase could account for only a small proportion of the stimulating effect that acetyl-CoA has on this enzyme's activity. Thus, more subtle approaches (e.g. use of fluorescent substrate analogues [13]) will be required to probe the structural basis of this allosteric activator's several effects on the catalytic cycle of pyruvate carboxylase [10,14].

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