Volume 231, number 1, 102–106 FEB 05755 April 1988

Pyruvate carboxylase from *Pseudomonas citronellolis*: shape of the enzyme, and localization of its prosthetic biotin group by electron microscopic affinity labeling

Jutta Fuchs, Walther Johannssen, Manfred Rohde and Frank Mayer

Institut für Mikrobiologie der Georg-August-Universität zu Göttingen, Grisebachstr. 8, D-3400 Göttingen, FRG

Received 27 January 1988

Pseudomonas citronellolis is known to contain a pyruvate carboxylase with an $\alpha_4\beta_4$ composition. All the other pyruvate carboxylases investigated so far are made up of four seemingly identical subunits. Nevertheless, this exceptional pyruvate carboxylase exhibits a size and overall shape similar to other pyruvate carboxylases. Electron microscopic affinity labeling with avidin revealed that the prosthetic biotin groups (one per $\alpha\beta$ unit, i.e. four per enzyme particle) are located close to the inter-unit junctions of pairs of $\alpha\beta$ units making up the enzyme. This position of the prosthetic biotin groups is very similar to the location of the biotin in the other carboxylases.

Pyruvate carboxylase; Electron microscopy; Avidin affinity labeling; Prosthetic biotin group; (*Pseudomonas citronellolis*)

1. INTRODUCTION

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-containing enzyme [1,2]. It has been electron microscopically characterized for a variety of organisms [3–17]. The enzyme from *Pseudomonas citronellolis* is exceptional insofar as it is not made up of four identical subunits but contains two different types of subunits [9,12,18–20]. These two different polypeptides are a 65 kDa subunit containing covalently bound biotin, and a 54 kDa subunit. Cohen et al. [9] found an enzyme particle molecular mass between 454 and 530 kDa, indicating an $\alpha_4\beta_4$ composition.

The rhombic appearance for pyruvate carboxylase [10] has recently been demonstrated to be caused by artificial deformation occurring during preparation for electron microscopy by negative staining [11,13–17]. The enzyme isolated from *P. citronellolis* has been shown to exhibit distinct projections with three or four visible protein masses [9]. A general model for the overall structure of pyruvate carboxylase and the location of its prosthetic biotin groups derived from electron microscopy [11] was verified by electron microscopic labeling experiments making use of the high affinity of avidin to biotin [13,17]. The present study demonstrates that this model also holds true, in principle, for the *P. citronellolis* pyruvate carboxylase.

Correspondence address: F. Mayer, Institut für Mikrobiologie der Georg-August-Universität zu Göttingen, Grisebachstr. 8, D-3400 Göttingen, FRG

2. MATERIALS AND METHODS

2.1. *Growth of* P. citronellolis, *and preparation of pyruvate carboxylase*

P. citronellolis (DSM 50332) was grown as described [9]. The cells were harvested close to the end of the logarithmic phase of growth. Purification of the enzyme was performed as described [9]. The final specific activity was 5 U/mg protein. Subunit composition and molecular masses were determined by analytical SDS-polyacrylamide gel electrophoresis according to Laemmli [21]. The enzyme activity was tested according to Taylor et al. [22] and Young et al. [4]. Acetyl coenzyme A (CoA) was prepared as described [23]. Goat anti-biotin antiserum or avidin were applied for enzyme inactivation.

2.2. *Determination of immunological cross reactivities*

Double immuno diffusion [24] and rocket immuno elec-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

trophoresis [25,26] were performed for the analysis of cross reactivities between *P. citronellolis* pyruvate carboxylase and polyclonal antisera raised against pyruvate carboxylase isolated from chicken and *Saccharomyces cerevisiae*. These antisera were kindly supplied by Dr J.C. Wallace, Adelaide, Australia.

2.3. *Preparation of enzyme–avidin complexes*

Aliquots of pyruvate carboxylase and avidin were mixed in molar ratios between 3:1 and 1:3, and incubated for 2 h at 30°C as described [13,17]. Controls were prepared with biotin-treated avidin. The samples were analyzed by negative staining (see below).

2.4. *Electron microscopy*

Electron microscopy of pyruvate carboxylase and of enzyme–avidin complexes was done as described [11] with 4% (w/v) aqueous uranyl acetate, pH 4.8. Electron micrographs were taken with a Philips EM 301 electron microscope at calibrated magnifications.

3. RESULTS AND DISCUSSION

3.1. *Quaternary structure of* P. citronellolis *pyruvate carboxylase*

Analytical SDS gel electrophoresis of purified enzyme yielded bands representing 65 kDa (interpreted to be the α subunit) and 53 kDa (the β subunit) (not shown). These measured subunit molecular masses are in full agreement with published data [9,12].

3.2. *Enzyme inactivation, and immunological cross reactivities*

Application of anti-biotin antiserum in increasing ratios resulted in increasing inactivation of the enzyme; the residual activity at the endpoint of the experiment was 1.4% of the original activity. A remaining activity of less than 1% was found when the enzyme and avidin were incubated together at a molar ratio of 1:1. Both values are in agreement with data published for other pyruvate carboxylases. They indicate that there are no significant differences between the *P. citronellolis* enzyme and pyruvate carboxylases from other sources with respect to the accessibility of the prosthetic biotin group for specific IgG antibodies and avidin [13,17,27]. Goss et al. [12] demonstrated that only antibodies directed against the α subunit of the *P. citronellolis* pyruvate carboxylase could inactivate the enzyme. They also found, by comparison of titration curves for the inactivation of the catalyzed overall enzyme reaction, for the decrease of the ATP/P_i exchange reaction, and for the decrease of the pyruvate–oxaloacetate exchange reaction, that probably both subsites of the active center (i.e. the biotin carboxylation subsite and the transcarboxylation subsite) are located on the α subunit, the polypeptide carrying the prosthetic biotin group. Immunological cross reactivities between *P. citronellolis* pyruvate carboxylase and polyclonal antisera raised against the respective enzyme isolated from chicken and *S. cerevisiae* could not be detected by double immunodiffusion and rocket immunoelectrophoresis (not shown). This finding indicates a very low degree of sequence homology between the exposed epitopes of the enzymes.

3.3. *Shape of the* P. citronellolis *pyruvate carboxylase*

Negatively stained purified pyruvate carboxylase, without (fig.1a), or with (not shown) addition of acetyl CoA, exhibited particle projections of the same types. There were no indications for improvement of the structural preservation of the enzyme by acetyl CoA. It is known [19,20] that the *P. citronellolis* enzyme is not acetyl CoA dependent with respect to its activity.

Electron microscopic enzyme samples contained, besides broken enzyme particles as expected [9], molecular projections with three visible intensity maxima (fig.1a). Rhombic shapes could occasionally be seen (fig.1a). The same type of projection has been observed previously [9]. Cohen et al. [9] concluded that the fourth unit cannot be seen in this position of the enzyme particle because it is located in the center of the projection. In some instances, Cohen et al. [9] could detect four masses. They assumed that the visibility of all four units depends on the position of the enzyme particle relative to the electron beam. Meanwhile, this assumption has been verified for other pyruvate carboxylases [11,14,15] by tilting experiments. Cohen et al. [9] stated that "...the three dimensional structure of this enzyme is quite distinct from all other species of pyruvate carboxylase". Our findings confirm the overall shape of the enzyme as seen by these authors. However, we emphasize that this overall shape is not unique; rather, it is common for pyruvate carboxylase as visualized in a number of cases [11,13–17]. A model developed for the overall enzyme structure of chicken, rat, and sheep pyruvate carboxylase

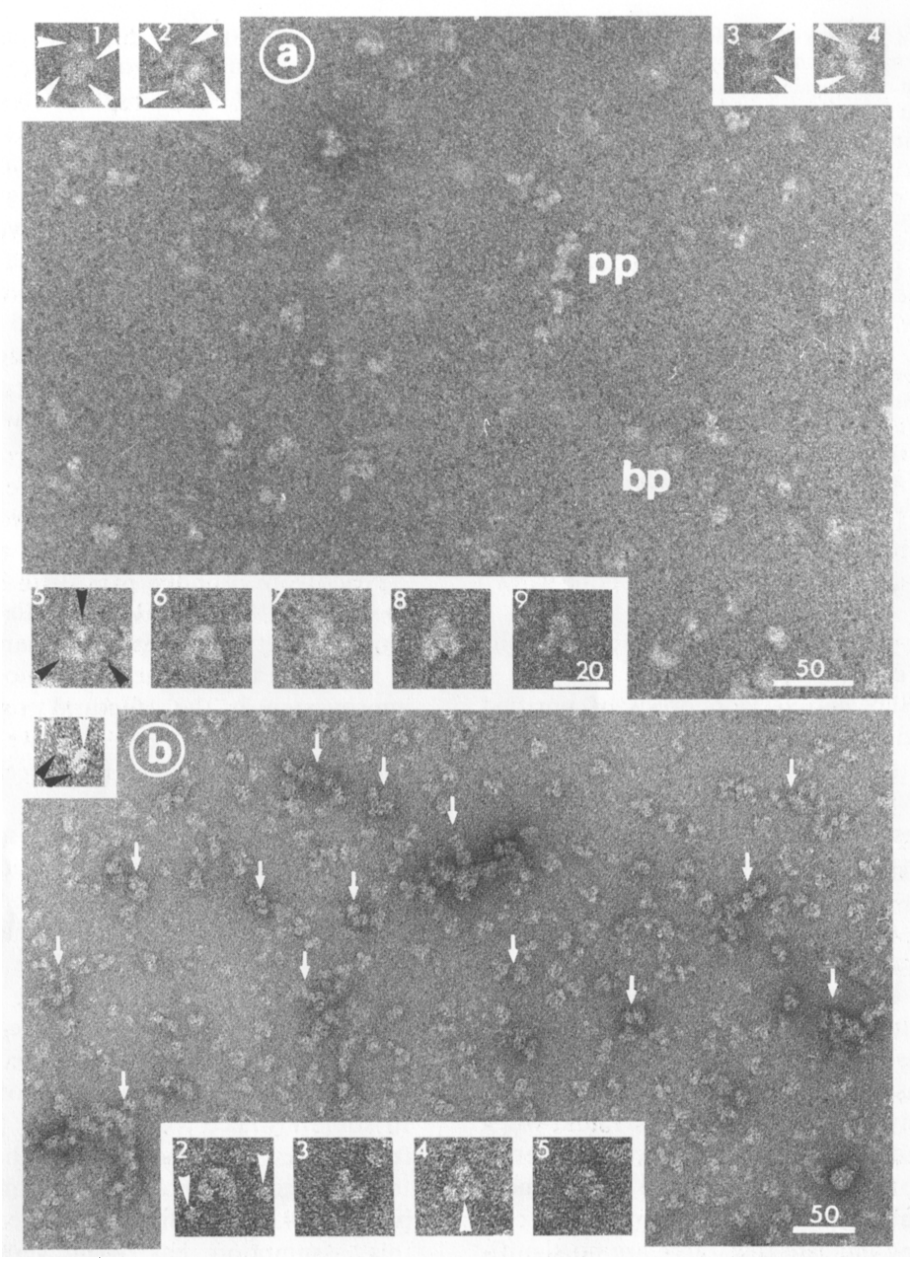

Fig.1. Electron micrographs of negatively stained pyruvate carboxylase isolated from *P. citronellolis*, and of avidin–pyruvate carboxylase complexes. Dimensions are given in nm. All insets have identical magnification. (a) Isolated enzyme particles, unfixed. Besides broken particles (marked bp) of various sizes, preserved particles (marked pp) with a trimer-like appearance are common. Insets: 1 and 2, rhomboid structures with four intensity maxima (arrowheads) interpreted to represent full-size enzyme particles artificially flattened by the mounting procedure for electron microscopy; 3 and 4, particles with two intensity maxima (arrowheads) probably representing halves of full-size enzyme particles; 5–9, full-size enzyme particles, not obviously deformed. Three of the four units making up each of the enzyme particles are visible (arrowheads). The fourth unit in each particle is assumed to be located in the center of the projection; it is not negatively stained (and thus not visible) because it sits above the three other units, in an elevated position. (b) Avidin–enzyme complexes, unfixed. The arrows indicate the presence of a number of 'chains' formed by the interaction of avidin with the prosthetic biotin groups on the enzyme. One avidin is capable of binding to two full-size enzyme particles. Thus, a chain is made up of an alternating sequence of enzyme and avidin molecules. Insets: 1, half of a full-size enzyme particle (the two black arrowheads are pointing to the two units) (compare to panel a, insets 3 and 4), with a centrally bound avidin molecule (white arrowhead); 4, a full-size enzyme particle with one centrally bound avidin molecule (arrowhead); 2, a full-size enzyme particle and two free avidin molecules (arrowheads); 3 and 5, two full-size enzyme particles without visibly bound avidin. The full-size enzyme particles depicted in insets 2–5 are structurally well preserved.

[11], which was shown to be also valid for the enzyme isolated from a thermophilic *Bacillus* [7], from *Aspergillus nidulans* [14], *Rhizopus arrhizus* [15], and *S. cerevisiae* [17] also appears to represent the inherent structural parameters of the *P. citronellolis* enzyme at low resolution. This model specifies that a typical tetrameric pyruvate carboxylase particle is, in fact, made up of two dimers complexed 'back-to-back'. This model applied to the *P. citronellolis* enzyme indicates that a unit formed by one α and one β polypeptide is comparable in size, shape and position within the complex to a typical subunit in other pyruvate carboxylases. The structure of the *P. citronellolis* pyruvate carboxylase could, therefore, be written $(\alpha\beta)_4$ or even $[(\alpha\beta)_2]_2$. Goss et al. [12] provided evidence that the major parts of the α polypeptide chain are exposed whereas the β polypeptide is forming a 'core'.

3.4. *Location of the prosthetic biotin group*

As shown for a number of pyruvate carboxylases [13–15,17], incubation of the enzyme together with avidin resulted in the formation of typical chains. This observation was used for the construction of a model which specifies that the biotin present on each of the four subunits of a typical tetrameric pyruvate carboxylase is located close to the intersubunit junction in the complex. Fig.1b demonstrates that chain formation also took place in samples containing avidin and the *P. citronellolis* enzyme though the overall number of chains, and the number of enzyme particles per chain, were low. Such a low efficiency of chain formation has also been observed for the pyruvate carboxylases isolated from *A. nidulans* [14] and *R. arrhizus* [15]. This typical chain formation mediated by avidin indicates that the location of the biotin relative to the overall enzyme structure is comparable to that found for other pyruvate carboxylases (see above). Our findings taken together with published data on structural and functional parameters of pyruvate carboxylases support the view of Obermayer and Lynen [28] communicated when many of the facts now known had not yet been discovered. These authors stated that "...The various biotin-dependent enzymes catalyze analogous two-step reactions which involve a covalently bound biotin-residue serving as CO_2 carrier. Although similarities among biotin enzymes of the same and different species exist, their primary structures may differ. The various types of carboxylases might represent various stages in the evolution of the enzyme system. In the course of evolution the increasing functional structuring of the cellular interior led to the formation of complexes with multifunctional polypeptide chains, probably as the result of gene fusion".

As described above, the pyruvate carboxylase from *P. citronellolis* is made up of two types of subunits whereas the other known pyruvate carboxylases only exhibit one type of subunit. As also stated above, there are no obvious immunological cross reactions between the *P. citronellolis* enzyme and antisera directed against two 'conventional' pyruvate carboxylases. Nevertheless, the overall three-dimensional organization and the topological location of the prosthetic biotin group of the *P. citronellolis* enzyme are comparable to the other pyruvate carboxylases.

Acknowledgements: We thank J.C. Wallace, Adelaide, Australia, for supplying antisera, and for valuable discussions. Supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

[1] Utter, M.F. and Keech, D.B. (1960) J. Biol. Chem. 235, 17–18.
[2] Utter, M.F. and Keech, D.B. (1963) J. Biol. Chem. 238, 2603–2608.
[3] Valentine, R.C., Wrigley, N.G., Scrutton, M.C., Irias, J.J. and Utter, M.F. (1966) Biochemistry 5, 3111–3116.
[4] Young, M.R., Tolbert, B. and Utter, M.F. (1969) Methods Enzymol. 13, 250–258.
[5] Barden, R.E., Taylor, B.L., Isohashi, F., Frey, W.H., Zander, G., Lee, J.C. and Utter, M.F. (1975) Proc. Natl. Acad. Sci. USA 72, 4308–4312.
[6] Goss, N.H., Dyer, P.Y., Keech, D.B. and Wallace, J.C. (1979) J. Biol. Chem. 254, 1734–1739.
[7] Libor, S., Sundaram, T.K., Warwick, R., Chapman, J.A. and Grundy, S.M.W. (1979) Biochemistry 18, 3647–3653.
[8] Cohen, N.D., Beegen, H., Utter, M.F. and Wrigley, N.G. (1979) J. Biol. Chem. 254, 1740–1747.
[9] Cohen, N.D., Duc, J.A., Beegen, H. and Utter, M.F. (1979) J. Biol. Chem. 254, 9262–9269.
[10] Cohen, N.D., Utter, M.F., Wrigley, N.G. and Barrett, A.N. (1979) Biochemistry 18, 2197–2203.
[11] Mayer, F., Wallace, J.C. and Keech, D.B. (1980) Eur. J. Biochem. 112, 265–272.
[12] Goss, J.A., Cohen, N.D. and Utter, M.F. (1981) J. Biol. Chem. 256, 11819–11825.
[13] Johannssen, W., Attwood, P.V., Wallace, J.C. and Keech, D.B. (1983) Eur. J. Biochem. 133, 201–206.

[14] Osmani, S.A., Mayer, F., Marston, F.A.O., Selmes, I.P. and Scrutton, M.C. (1984) Eur. J. Biochem. 139, 509–518.
[15] Mayer, F., Osmani, S.A. and Scrutton, M.C. (1985) FEBS Lett. 192, 215–219.
[16] Osmani, S.A., Scrutton, M.C. and Mayer, F. (1985) Ann. NY Acad. Sci. 447, 56–71.
[17] Rohde, M., Lim, F. and Wallace, J.C. (1986) Eur. J. Biochem. 156, 15–22.
[18] O'Brien, R.W., Chuang, D.T., Taylor, B.L. and Utter, M.F. (1977) J. Biol. Chem. 252, 1257–1263.
[19] Seubert, W. and Remberger, U. (1961) Biochem. Z. 334, 401–414.
[20] Taylor, B.L., Routman, S. and Utter, M.F. (1975) J. Biol. Chem. 250, 2376–2382.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Taylor, B.L. and Barden, R.E. (1972) J. Biol. Chem. 247, 7383–7390.
[23] Simon, E. and Shemin, D. (1953) J. Am. Chem. Soc. 75, 2520.
[24] Ouchterlony, Ö. (1949) Acta Pathol. Microbiol. Scand. 26, 507–515.
[25] Laurell, C.B. (1966) Anal. Biochem. 15, 45–52.
[26] Weeke, B. (1973) Scand. J. Immunol. 2 (suppl.1), 37–46.
[27] Wood, H.G. and Barden, R.E. (1977) Annu. Rev. Biochem. 46, 385–413.
[28] Obermayer, M. and Lynen, F. (1976) TIBS 1, 169–171.