PURIFICATION OF THE SODIUM TRANSPORT ENZYME OXALOACETATE DECARBOXYLASE BY AFFINITY CHROMATOGRAPHY ON AVIDIN SEPHAROSE

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1. Introduction

Oxaloacetate decarboxylase (EC 4.1.1.3) and methylmalonyl-CoA decarboxylase (EC 4.1.1.41) constitute a group of biotin-dependent enzymes which catalyze decarboxylation reactions. Although these enzymes were detected >10 years ago [1,2] they have attracted little attention until the demonstration that oxaloacetate decarboxylase catalyzes an active transport of Na^+ across the cell membrane [3–5]. Upon decarboxylation of oxaloacetate an electrochemical gradient of Na^+ is established and in this way part of the energy of the highly exergonic decarboxylation reaction is conserved. In accordance with this biological function, oxaloacetate decarboxylase is specifically activated by Na^+ [1] and bound to the cytoplasmic membrane [1,6]. The enzyme has been solubilized from the isolated membranes with non-ionic detergents and purified ~4.5-fold over the crude membrane extract [6]. The purified preparation consisted of one major polypeptide containing the biotin prosthetic group and several minor polypeptides which appeared to be impurities of the enzyme. With phospholipid vesicles and the purified decarboxylase the Na^+ transport has been reconstituted [5]. The catalytic mechanism of the decarboxylase proceeds in a two-step reaction sequence by the Na^+-independent carboxyl transfer from oxaloacetate to the biotin prosthetic group yielding the carboxybiotin enzyme intermediate and by the Na^+-dependent decarboxylation of this intermediate which regenerates the free biotin enzyme [7].

Methylmalonyl-CoA decarboxylase has now been established as another membrane-bound biotin enzyme specifically activated by Na^+ [8]. This enzyme also catalyzes an active transport of Na^+ across the cell membrane. Here, I report on a simple and reliable method for the purification of oxaloacetate decarboxylase using affinity chromatography on a monomeric avidin Sepharose column. Enzyme purified in this way appears to consist of the biotin-containing polypeptide (M_r 65 000) [6] and an M_r 34 000 polypeptide which is comparatively weakly stained after SDS–polyacrylamide gel electrophoresis.

2. Materials and methods

Membranes were prepared from *Klebsiella aerogenes* grown anaerobically on citrate as in [3,4]. Oxaloacetate decarboxylase was solubilized from these membranes with 1.5% Triton X-100 in 2 mM K-phosphate buffer (pH 7.0) and applied to a column of monomeric avidin–Sepharose [9]. The column was washed with ~6 vol. 10 mM K-phosphate buffer (pH 7.0) containing 0.3 M KCl, 0.1% Brij and 0.1 mM diisopropylfluorophosphate. The enzyme was eluted with 10 mM K-phosphate buffer (pH 7.0) containing 0.15 M KCl, 0.05% Brij, 0.1 mM diisopropylfluorophosphate, and 1.5 mM biotin. Oxaloacetate decarboxylase activity was determined spectrophotometrically [6]. Biotin was determined as in [10]. Samples of the enzyme used for biotin analyses were extensively dialysed against 10 mM K-phosphate buffer (pH 7.0). Reconstitution and determination of Na^+ transport were done as in [5]. SDS–Polyacrylamide gel electrophoresis was done as in [11]. The phospholipids of reconstituted enzyme samples were extracted with *n*-hexane prior to the electrophoretic analyses. Protein was determined as in [12].

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3. Results

Because of the very high affinity of avidin for biotin ($K = 10^{-15}$ M) [13] it has not been possible to recover biotin-containing enzymes from avidin affinity columns under non-denaturing conditions. Based on an observation that the monomeric form of avidin has a reduced affinity toward biotin [14], several groups have developed monomeric avidin affinity columns for the purification of biotin-containing proteins [9,15,16].

When a crude membrane extract containing the biotin enzyme oxaloacetate decarboxylase was applied to a monomeric avidin–Sepharose column most of the activity was retained whereas most of the protein appeared in the eluate (fig.1). The decarboxylase was eluted from the column with a biotin-containing buffer. A 15–20-fold purification was achieved and the specific activity raised to 68 U/mg in the best fractions. Analysis of the purified enzyme by SDS gel electrophoresis indicated the presence of one major and one minor polypeptide band but no other visible contaminants (fig.2). The major polypeptide was that observed in [6] with less pure preparations of oxaloacetate decarboxylase. Its M_r was found to be 65 000 by comparison of the electrophoretic mobility with that of marker proteins. The M_r of the minor protein band visible on the gel was found to be 34 000. The staining intensity of this polypeptide was only ~5–10% of the total stain and this was found consistently in several different preparations of affinity chromatographically-purified enzyme. Because of its low staining intensity this polypeptide did not emerge among other contaminating polypeptides in partially purified preparations of the decarboxylase [6]. Several different possibilities had to be considered to account for the occurrence of the minor protein band in affinity chromatographically purified decarboxylase:

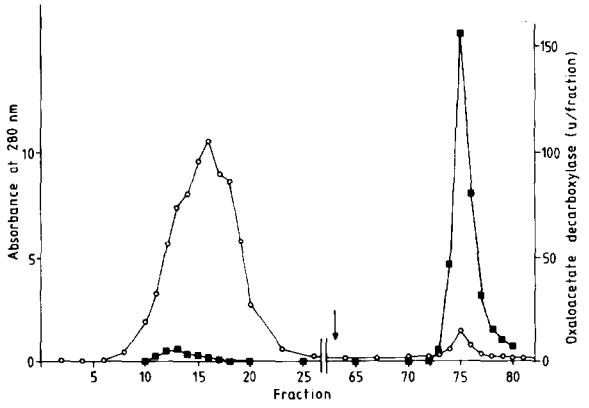

Fig.1. Purification of oxaloacetate decarboxylase on a monomeric avidin–Sepharose column. A crude membrane extract prepared from 40 g (wet wt) *K. aerogenes* cells was applied to the column (2 × 11 cm). At the arrow a biotin containing buffer was applied to elute the enzyme (section 2): (○) absorbance at 280 nm; (■) oxaloacetate decarboxylase activity.

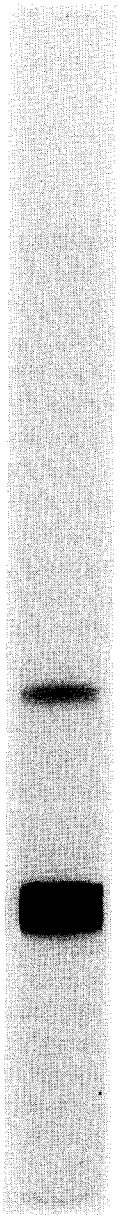

Fig.2. SDS–polyacrylamide gel electrophoresis of affinity chromatographically-purified oxaloacetate decarboxylase (23 μg protein, spec. act. 68 U/mg).

Table 1
Biotin content of oxaloacetate decarboxylase and of the polypeptides of the enzyme

Oxaloacetate decarboxylase sample	Protein (μg)	Biotin content	
		(nmol)	(mol/65 000 g)
Purified enzyme	5.7	0.081	0.92
Purified enzyme	11.4	0.16	0.91
Purified enzyme	17.1	0.29	1.1
Polypeptide M_r 65 000	21.0	0.25	0.78
Polypeptide M_r 34 000	21.0	0	0

The polypeptides of the decarboxylase were separated by SDS gel electrophoresis. The stained protein bands were cut off and hydrolyzed in 0.25 ml 2 M H_2SO_4 for 1 h at 121°C. Samples of the purified enzyme were treated in the same way. The solutions were neutralized with 20% NaOH (~0.18 ml) and analyzed for the biotin content by the competition for [^{14}C]biotin binding to avidin [9]. All analyses were performed in duplicate and average values are given

(i) The minor protein band is a contaminant of the decarboxylase;
(ii) The minor protein band is a functional subunit of the decarboxylase.

In (ii) this subunit could either be present in the purified enzyme samples in a stoichiometry far below 1:1 or could have properties yielding low staining intensities. The most probable contaminants of the enzyme were biotino-proteins, since these would be notoriously copurified on the avidin affinity column. The results of biotin analyses are summarized in table 1. Purified decarboxylase contained ~1 mol biotin/65 000 g protein. This biotin resided exclusively in the large polypeptide and no biotin was found in the small polypeptide or on any other part of the gel. The results were confirmed by SDS gel electrophoretic analyses of oxaloacetate decarboxylase–avidin complexes. Under omission of the usual heating step the avidin–biotin complex will survive the treatment with SDS and any biotin containing protein will be detectable by its displacement toward a high M_r-value. Such a displacement was observed for the polypeptide of M_r 65 000 but position and intensity of the smaller polypeptide remained unchanged. These results confirm the biotin content of the large polypeptide of the decarboxylase [6] and indicate the presence of 1 mol biotin/mol of this protein.

Partial proteolytic hydrolysis of the enzyme could be another source for generating a second polypeptide in purified oxaloacetate decarboxylase preparations. Limited proteolysis of the decarboxylase was therefore conducted to determine whether the minor protein band could be generated by proteolysis of the main protein. However, with 3 different proteases (trypsin, chymotrypsin, thermolysin) no indication for such a hydrolysis was detectable. Whereas the minor polypeptide remained unchanged in presence of either of these proteases, the major polypeptide was readily cleaved into products which were of higher and lower M_r, respectively, than the minor protein. Apparently a region between the catalytic moiety and the biotin carboxyl carrier protein is very susceptible toward proteolytic attack, since trypsin cleaved the major polypeptide into a biotin-free polypeptide of M_r 52 000 and a small M_r biotin carboxyl carrier protein.

Reconstitution experiments were performed to determine whether affinity chromatographically-purified oxaloacetate decarboxylase consists of all proteins required for Na^+ transport. There was no significant difference in the reconstitution of Na^+ transport from these oxaloacetate decarboxylase preparations and those of lower purity which were used in [5]. Both subunits were incorporated in the proteoliposomes, but the relative amount of the smaller polypeptide was considerably increased from ~5–10% to ~20% of the total stain. These results favor the composition of Na^+ pumping oxaloacetate decarboxylase from two different kinds of subunits and disfavor the possibility that the minor protein band visible on the gel is an accidental contaminant of the enzyme.

4. Discussion

Biotin-dependent carboxylases are composed from a biotin carboxyl carrier protein and catalytic subunits for carboxyl transfer and biotin carboxylation reactions [17]. Physically these subunits may be fused into polyfunctional aggregates. The biotin-dependent decarboxylases catalyze avidin-sensitive carboxyl transfer reactions followed by the decarboxylation of the carboxybiotin enzyme intermediates [7,18]. The enzymes therefore require a carboxyl transferase and a biotin carboxyl carrier protein component which may be fused or may occur as separate entities. A separate catalytic subunit for the decarboxylation of the carboxybiotin enzyme appears to be not required, since at least part of the biotin-dependent carboxylases can catalyze abortive decarboxylations of their sub-

strates [7]. To function as a Na^+ transport enzyme, however, oxaloacetate decarboxylase would probably require a protein providing the channel for Na^+ across the membrane. The size of the major polypeptide of the decarboxylase is in the range of other biotin-dependent carboxyltransferases with an attached biotin carboxyl carrier protein and one could therefore suspect that an additional protein would be required for the Na^+ channel.

The above results indicate that oxaloacetate decarboxylase purified by affinity chromatography consists of the major biotin-containing polypeptide and a polypeptide with weak staining intensity on polyacrylamide gels. Because of this weak staining, one must carefully consider this protein as a contaminant. However, the more likely sources for contaminants have been excluded. The minor protein contains no biotin and has therefore no affinity to the avidin column. It is also unlikely to derive from proteolytic cleavage of the main protein. Upon reconstitution of oxaloacetate decarboxylase into phospholipid vesicles both polypeptides were incorporated but the relative amount of the small subunit considerably increased. Part of these small subunits may have been lost during purification, and reconstitution reassembles the enzyme molecules in a stoichiometry which is identical or more closely related to that in the bacterial membrane. Considering the above structure–function relationship between the biotin enzymes, the smaller peptide of the decarboxylase could provide the Na^+ channel through the membrane. This hypothesis should be evaluated eventually using functional assays. An experimental approach to this question seems possible since the purification of the decarboxylase to apparent homogeneity has been established.

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References

[1] Stern, J. R. (1967) Biochemistry 6, 3545–3551.
[2] Galivan, J. H. and Allen, S. H. G. (1968) J. Biol. Chem. 243, 1253–1261.
[3] Dimroth, P. (1980) FEBS Lett. 122, 234–236.
[4] Dimroth, P. (1982) Eur. J. Biochem. 121, 443–449.
[5] Dimroth, P. (1981) J. Biol. Chem. 256, 11974–11976.
[6] Dimroth, P. (1981) Eur. J. Biochem. 115, 353–358.
[7] Dimroth, P. (1982) Eur. J. Biochem. 121, 435–441.
[8] Hilpert, W. and Dimroth, P. (1982) Nature in press.
[9] Liu, F., Zinnecker, M., Hamaoka, T. and Katz, D. H. (1979) Biochemistry 18, 690–697.
[10] Dakshinamurti, K. and Allan, L. (1979) Methods Enzymol. 62, 284–287.
[11] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–267.
[13] Green, N. M. (1963) Biochem. J. 89, 599–609.
[14] Green, N. M. and Toms, E. J. (1973) Biochem. J. 133, 687–700.
[15] Henrikson, K. L., Allen, S. H. G. and Malloy, W. L. (1979) Anal. Biochem. 94, 366–370.
[16] Gravel, R. A., Lam, K. F., Mahuran, D. and Kronis, A. (1980) Arch. Biochem. Biophys. 201, 669–673.
[17] Wood, H. G. and Barden, R. E. (1977) Annu. Rev. Biochem. 46, 385–413.
[18] Galivan, J. H. and Allen, S. H. G. (1968) Arch. Biochem. Biophys. 126, 838–847.