PRO EXPERIMENTIS

A simple method for the preparation of antibodies to the mitochondrial biotin-dependent carboxylases

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Summary. Heterologous antiserum to the 3 biotin-dependent carboxylases was prepared by selective removal of these enzymes from human liver on an avidin-sepharose column. A carboxylase-avidin-sepharose matrix was used as an antigen to produce anti-carboxylase antibodies. The resultant antisera can be used to purifiy the specific carboxylases, to prepare monoclonal antibodies to these enzymes or to study inherited carboxylase deficiencies and biotin-dependent intermediary metabolism.

Interest in biotin-dependent carboxylases has been stimulated by the recognition that certain inherited metabolic disorders are caused by deficient activities of the mitochondrial enzymes propionyl CoA carboxylase (PCC; E.C.6.4.1.3), pyruvate carboxylase (PC; 6.4.1.1) or β-methylcrotonyl CoA carboxylase (βMCC; E.C.6.4.1.4)[3]. A multiple carboxylase deficiency disorder, caused by deficient activities of all 3 carboxylases, has also recently been described[4]. Although it would be desirable to have purified preparations of all the carboxylases to induce antibodies for tissue enzyme studies of affected individuals, only human PCC has been homogeneously purified to date[5]. Thus, to prepare antibodies to all 3 mitochondrial carboxylases, we have developed a technique that exploits the unique property of biotin-containing enzymes to bind specifically and essentially irreversibly to avidin[6]. The carboxylases are selectively removed from human liver extracts on an avidin-sepharose column, which is used, in turn as an antigen to produce antibodies to the enzymes.

Material and methods. A normal infant human liver was prepared within 6 h of the patient's death, suspended in a solution of 1.4 M sucrose and 100 mM Tris-HCl at pH 8.0, and frozen at $-70\,^{\circ}C$ immediately. The liver (200 g) was sonicated and the suspension centrifuged at $5000 \times g$ for 20 min. 100 ml of redistilled acetone (4 °C) were added to 5 ml of supernatant and stirred for 10 min. The suspension was filtered, washed with anhydrous ether and dried. 1 g of acetone powder was dissolved in 10 ml of 100 mM Tris-HCl pH 8.0 and centrifuged at $500 \times g$ for 20 min, resulting in an extract of about 20–30 mg protein/ml. The acetone powder had no detectable acetyl CoA carboxylase (ACC; E.C. 6.4.1.2) activity.

An avidin-sepharose affinity column was prepared by first activating sepharose 4B (Pharmacia) with cyanogen bromide by the method of March et al.[7] and then coupling avidin (Sigma, Lot 40K-9570, 10–15 units/mg) to the activated sepharose according to the procedure of Landman and Landman[8]. After excess activated sepharose was removed with 10 vol. of ethanolamine, the column (0.8 cm × 2 cm) was washed with 100 mM sodium phosphate buffer, pH 7.0, until no protein was detectable in the eluant. The normal liver extract (10 ml) was then passed over the column and the eluants monitored for PCC activity. The column was considered saturated when the PCC activity in the eluants equaled that in the sample applied. The number of avidin sites was colorimetrically determined with the avidin specific dye 2-(4-hydroxyazobenzene)-benzoic acid (Sigma)[8]. Biotin (Sigma) at a concentration that would bind 10 times the calculated number of avidin sites was then passed over the column to saturate any unreacted avidin sites, and the column was washed with 100 vol. of phosphate buffer.

Since avidin-bound biotin-dependent enzymes cannot be removed from the column without denaturation[9], we tested whether the carboxylase in the carboxylase-avidin-sepharose complex retained sufficient activity to permit the entire complex to be used as an immunogen. In fact, 68% of the enzyme activity in the original extract remained on the column, and therefore 1.0 g of the complex was suspended in 1.0 ml of buffer, emulsified with 1.0 ml of Freund's adjuvant, and 0.5 ml of this emulsion injected intradermally in 2 rabbits at each of 2 separate abdominal sites, with 14 days between the 2 injections. Immunoglobulin fractions of the antisera were prepared as described by Crowle[10], and the presence of biotin-containing proteins on Ouchterlony gels was detected using fluorescamine-avidin as described by Swack et al.[9].

The activities of PCC, βMCC, PC and ACC were measured by the amount of ^{14}C-bicarbonate incorporated into the respective products by techniques reported previously[11–14]. Glutamate dehydrogenase activity was assayed by the procedure of Beaufay et al.[15]. All enzyme activities were expressed in nmoles of product formed per min per mg of protein determined by the method of Lowry et al.[16].

Results and discussion. The unique binding of the biotin-containing enzymes to the avidin-sepharose matrix is demonstrated by the following observations: 1. Less than 1% of the protein in the liver extract that was passed through the column adhered to the avidin-sepharose column matrix. 2. The removal of just this small quantity of protein from the extract eluant was nevertheless associated with a complete loss of PCC activity in the eluant; on the other hand, activity of GDH, a non-biotin-dependent enzyme, was fully retained in the eluant. 3. When a known excess of PCC was passed through the column, its concentration in the eluant eventually equaled that in the applied solution, indicating the presence and saturation of specific PCC binding sites. 4. Even after extensive washing, about two thirds of the PCC activity in the original extract could be demonstrated on the column. The ample activity retained by the enzyme within the carboxylase-avidin-sepharose complex reveals that one or more active sites on the enzyme are not involved in binding to the column matrix.

Percentage of carboxylase activity inhibition by anti-human carboxylase immunoglobulin fraction (1:8 dilution) in liver (15 mg/ml) and fibroblast (5 mg/ml) extracts

Enzyme activity	Percentage of enzyme activity inhibited	
	Liver	Fibroblast
Propionyl CoA carboxylase	80	95
β-Methylcrotonyl CoA carboxylase	59	62
Pyruvate carboxylase	73	71
Acetyl CoA carboxylase	0	0
Glutamate dehydrogenase	0	0

Serum obtained prior to primary immunization (control) and 14 days thereafter neither inhibited PCC activity nor caused precipitin lines to form against normal liver extract on Ouchterlony plates. However, sera obtained 8 days after the 2nd immunization (22 days after primary immunization) gave clear indication of anti-human carboxylase antibody formation. Added to normal liver extracts, the serum inhibited carboxylase activities and formed a precipitable complex that was readily removed from solution by centrifugation at 5000 × g for 5 min; non-precipitated complexes retained residual enzyme activity. The anti-carboxylase action was completely eliminated when the serum was passed through a carboxylase-avidin-sepharose column, but not by passage through a avidin-sepharose column. In addition, the antiserum caused a broad precipitin region to form against normal liver extracts on Ouchterlony double-diffusion studies in agarose. The precipitin region retained fluorescamine-tagged avidin, a response indicating the presence of biotin-containing protein. No precipitin arcs or fluorescamine staining regions were detected when pre-immune serum was diffused against normal liver extract or against solutions of avidin.

The specificity of the antiserum was demonstrated by its inhibition only of the mitochondrial biotin-containing enzymes, whereas the activity of the cytosolic enzyme, ACC, that appeared to be removed during the acetone precipitation, was not inhibited. The activity of GDH, another mitochondrial enzyme that does not contain biotin, was also not inhibited by the antibody (table).

These heterologous antibody preparations can then be used to purify individually the 3 human carboxylases on antibody-sepharose affinity columns, to prepare monoclonal antibodies to the individual enzymes, to study biotin-dependent intermediary metabolism or to investigate further the various carboxylase deficiencies.

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A new rapid method for collection and preparation of cell suspensions for electron microscopy

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Summary. We describe a new rapid and simple method for collection and preparation of cell suspensions for electron microscopy; the cells are prefixed with glutaraldehyde in their culture medium, and are then compacted on a filter disc. Post-fixation in osmium, staining and dehydration are performed by transferring the filter disc and the cell pellet from one solution to the next. The pellet is easily separated from the filter disc just before treatment in propylene oxide. This method preserves the fine structure as well as the classical technique. Advantages are that numerous cells have the same orientation in the sections and that many samples can be taken in a very short time.

A number of methods have been described for collecting suspended cells into a compact pellet suitable for ultrastructural studies. Centrifugation requires numerous manipulations and is not well adapted to viscous embedding media. Fibrinogen[2], fresh cock plasma[3], nucleohistones[4] and agar[5] have been proposed. The use of these techniques is delicate and often requires several centrifugations, and the results are not always satisfactory, especially when one has to process numerous samples during a short period of time. During the course of our work on *Tetrahymena pyriformis*, we have developed a new method which seems to be well adapted to the study of cell suspensions.

Material and methods. Tetrahymena pyriformis GL was grown and synchronized according to Zeuthen[6].

1. *Prefixation:* 1 ml of culture was fixed with 1 ml of 1% glutaraldehyde in 0.1 M phosphate buffer (pH: 7.4) for 20 min.

2. *Filtration* (operating system: fig. 1): A filter disc (F in fig. 1, B) cut from a Millipore filter type RA (1.2 µm pore size) was damped with phosphate buffer and adjusted on the top of a glass tube (p in fig. 1 A, external diameter 6 mm, internal diameter 1 mm) and kept in place by vacuum. Prefixed cells were gently drawn up into a Pasteur pipette (Pp in fig. 1, A) with a peristaltic pump (pp in fig. 1,