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Features of Metabolic Pathway Separation

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Gluconeogenesis as a Compartmentalized Activity

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The conversion of pyruvate into glucose in animal tissues involves at least two, and perhaps three, intracellular compartments. The mitochondria furnish the ATP and a major portion of the reducing equivalents which are required for gluconeogenesis, and phosphoenolpyruvate or one of its cytosolic precursors, such as malate, citrate and aspartate. The soluble fraction of the cell carries out the reactions leading from phosphoenolpyruvate to glucose 6-phosphate. The final dephosphorylative step is catalysed by glucose 6-phosphatase, which appears to be entirely membrane-bound, either in the endoplasmic reticulum or perhaps also in the nuclear and plasma membranes (Nordlie, 1976).

The division of the gluconeogenic pathway among intracellular compartments offers the opportunity for regulation of the process. Especially, the requirement for the transport of substances through the inner mitochondrial membrane provides numerous possible control mechanisms. For example, ATP must be transported from the mitochondria by a specific translocator. Also, phosphoenolpyruvate or one of its cytosolic precursors must be transported from the mitochondria by one of the anion translocators. These processes are considered in detail elsewhere in this Colloquium.

The compartmentalization of gluconeogenesis also permits the co-ordination of this metabolic process with others in the same compartments. A case in point is the initial reaction of gluconeogenesis, the carboxylation of pyruvate. This reaction, which occurs entirely within the mitochondria in animal species (Walter, 1976), is inhibited by ADP (Keech & Utter, 1963; Walter, 1976) and activated by acetyl-CoA, thereby providing mechanisms for the co-ordination of the initial step of gluconeogenesis with other mitochondrial activities such as oxidative phosphorylation and the oxidation of fatty acids and pyruvate (Barritt et al., 1976).
As aforementioned, a third intracellular compartment, the endoplasmic reticulum and perhaps other membranes, is involved in gluconeogenesis, since glucose 6-phosphatase is bound to these membranes. It is not clear whether this situation has any significance in the regulation of gluconeogenesis, although it can be postulated that factors affecting the membrane may influence the catalytic competence of the enzyme or its access to glucose 6-phosphate.

As the details of gluconeogenesis and its regulation have been elucidated, it has become clear that there are many species-dependent factors involved. The most striking difference among species with relationship to gluconeogenesis is probably that of the intracellular location of phosphoenolpyruvate carboxykinase. In adult chicken liver the enzyme has been clearly shown to be located entirely within the mitochondrial matrix (Chiao, 1976). Careful intracellular-fractionation studies show that 97% of the enzyme is in the mitochondrial fraction. More importantly, immunological and electrophoretic studies show that adult chicken liver contains a single type of the enzyme.

The situation is essentially reversed in rats, mice and hamsters. The mitochondrial species represents less than 10% of the total enzymic activity in the livers of fed rats, and the proportion of mitochondrial enzymes decreases during starvation or diabetes. Most other species, including guinea pigs, rabbits, cows, pigs, sheep and man, show substantial activities of both types of hepatic phosphoenolpyruvate carboxykinase. The actual activities reported vary according to the methods used and particularly the dietary state of the animal. The data concerning the intracellular concentrations and distribution of this enzyme in various species have been summarized (Söling & Kleineke, 1976).

The functional, structural and genetic relationships of the two varieties of phosphoenolpyruvate carboxykinase are not well understood. Evidence presented previously indicate that there are differences between the two forms, but it was less clear whether there are also similarities. Developmentally, the varieties appear to differ. Foetal rat liver contains only the mitochondrial form, whereas the cytosolic form is synthesized rapidly, starting about the time of birth (Ballard & Hanson, 1967). In a somewhat related finding, it has been shown (B. M. Atkin, N. E. M. Buist, M. B. Weinberg and M. F. Utter, unpublished work) that human fibroblasts cultured from skin or human lymphocytes contain only the mitochondrial form of the enzyme, although human liver and kidney possess both varieties.

Probably the most significant difference between the two forms of phosphoenolpyruvate carboxykinase is their response to endocrine and nutritional stimuli. As shown by Shrago et al. (1963) the amounts of the cytosolic form in rat liver are significantly increased during starvation and diabetes. A large amount of evidence is available to show that the cytosolic form is subject to complex regulation of its rate of synthesis through a variety of stimuli (Tilghman et al., 1976). The situation with the mitochondrial variety is much less clear. Most of the regulatory studies have used rats, and this animal is not satisfactory for the study of possible changes in the mitochondrial form of the enzyme. Earlier studies with guinea pigs appeared to show that the amount of mitochondrial isoenzyme did not change appreciably during starvation or diabetes (Nordlie et al., 1965), but a report by Elliott & Pogson (1977) indicates that both cytoplasmic and mitochondrial activities increased in the guinea-pig liver during starvation. The status of the regulation of the mitochondrial isoenzyme remains to be established, but it is unlikely that the changes in this variety of the enzyme will be found to parallel those of the cytosolic form.

The most convincing evidence that there are structural differences between the cytosolic and mitochondrial forms comes from the work of Ballard & Hanson (1969), who showed that an antibody prepared against the cytosolic variety of the rat liver enzyme did not cross react with the mitochondrial form. Further indication of structural differences was supplied by Dieterhaft et al. (1971), who showed that the two forms from human liver could be separated by ion-exchange chromatography, suggesting that there were charge differences between the two proteins. Apart from this there has been little to show that the two types of the enzyme are different in structure or mechanism. Holten & Nordlie (1965) did find possible slight differences in sensitivity to AMP
inhibition and metal-ion activation between two forms of the guinea-pig enzyme. However, such studies have been rare and inconclusive, because the two types of the enzyme have not been isolated in pure form from the same tissue until now.

As noted, the cytosolic form of phosphoenolpyruvate carboxykinase increases in activity, often dramatically, under conditions of increased gluconeogenesis such as in starvation or diabetes, whereas the mitochondrial form is less affected. On the basis of such observations the inference is often drawn that only the soluble form of this enzyme

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**Fig. 1.** Molecular-weight determination by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the polypeptides of cytosolic (a) and mitochondrial (b) phosphoenolpyruvate carboxykinase from monkey liver.

The molecular weight standards are monomers of: carbonic anhydrase (29000), lactate dehydrogenase (36000), ovalbumin (45000), fumarase (49000), pyruvate kinase (rabbit muscle) (57000), bovine serum albumin (68000) and phosphorylase a (92.500). The phosphoenolpyruvate carboxykinase peak corresponds to a molecular weight of 67000.

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**Fig. 2.** Separation of cytosolic and mitochondrial phosphoenolpyruvate carboxykinase from monkey liver by polyacrylamide-gel electrophoresis.

Gel (M) represents the purified mitochondrial isoenzyme, gel (C) the purified cytosolic isoenzyme and gel (M+C) the mixture of both isoenzymes. The purified proteins were applied to 5% polyacrylamide gels containing 0.05 M-Tris/HCl, pH 8.0. Electrophoresis was run in the same buffer. Protein bands were stained with Coomasie Blue.
Fig. 3. Immunoprecipitation curve of antibody against cytosolic phosphoenolpyruvate carboxykinase from monkey liver as titrated with cytosolic (○) and mitochondrial (△) antigens

Variable amounts of the antibody were mixed with a constant amount of purified cytosolic or mitochondrial phosphoenolpyruvate carboxykinase from monkey liver, incubated at 37°C for 15 min and left overnight at 4°C. After centrifugation at 23500 g for 15 min, the supernatant was assayed for phosphoenolpyruvate-carboxylating activity, which is expressed as μmol of CO₂ fixed/min per ml at 37°C. Rabbit γ-globulin preparation was used as a control with the cytosolic isoenzyme (□).

...participates in glucose synthesis. This view may be true for rat liver, where the mitochondrial activity is very low. However, this situation cannot hold for adult chicken liver, where all of the enzyme is located within the mitochondria (Chiao, 1976). Further, it seems likely that the mitochondrial enzyme participates in gluconeogenesis in guinea pig, rabbit and man, where half or more of the enzyme is found in the mitochondria. There is ample evidence that mitochondria can form phosphoenolpyruvate and that the compound can be transported from the mitochondria, probably via the tricarboxylate transporter (Robinson & Chappell, 1967).

A major hurdle to an understanding of the relationship and physiological roles of the two forms of phosphoenolpyruvate carboxykinase has been the inability to isolate the two isoenzymes from the same source. We have overcome this obstacle by obtaining both varieties of the enzyme in a highly purified state by using rhesus-monkey liver as the starting material. Both types of the enzyme were purified to essential homogeneity. The specific activities of the cytosolic and mitochondrial forms were very similar, 17.6 units/mg of protein for the cytosolic form and 16 units/mg of protein for the mitochondrial form. The two varieties of the enzyme were also very similar or identical in size, as shown by Fig. 1, where sodium dodecyl sulphate/polyacrylamide-gel disc electrophoresis indicated that both forms has a mol.wt. of 67000.
Although the activity and molecular-weight values failed to distinguish between the cytosolic and mitochondrial isoenzymes, they can be separated by polyacrylamide-gel electrophoresis on 5% gels as shown in Fig. 2. These results have both practical and theoretical significance for further studies.

Antibodies directed against the purified cytosolic and mitochondrial isoenzymes have been prepared from rabbits. Immunoglobulin G fractions purified by (NH₄)₂SO₄ fractionation and DEAE-cellulose chromatography show complete identity with the corresponding species of enzyme both by Ouchterlony double-immunodiffusion analysis and by immunoprecipitation studies. Fig. 3 demonstrates the immunoprecipitation of the cytosolic enzyme by immunoglobulin G prepared against this isoenzyme. The cytosolic enzyme can be precipitated completely by the homologous antibody, whereas there is no cross-reaction with the mitochondrial form. The lack of cross-reactivity of the cytosolic antibody with the mitochondrial isoenzyme is similar to the report of Ballard & Hanson (1969) with rat liver. In the present work it was possible to carry out the inverse experiments using the antibody prepared against the mitochondrial isoenzyme. The results (not shown) indicated that the antibody precipitated the homologous antigen and exhibited no cross-reaction with the other isoenzyme, in this case the cytosolic enzyme.

As has been noted previously with the antibody against rat liver cytosolic enzyme (Ballard & Hanson, 1969) and the antibody against the chicken liver mitochondrial enzyme (Chiao, 1976), the antibodies have no effect on the catalytic competence of the enzymes. It is necessary, therefore, to carry out an immunoprecipitation to test the antigen. It is a point of some interest that the antibodies prepared against monkey liver cross-react with the corresponding forms of the enzyme from human liver.

These studies, although preliminary in nature, show that the mitochondrial and cytosolic forms of phosphoenolpyruvate carboxykinase are not structurally identical, since they are readily distinguishable by immunological and electrophoretic means. However, other properties of the two forms of the enzyme suggest that there may be similarities as well as differences. The essentially identical maximal catalytic activities and molecular weights are cases in point. The observation that the antibody-antigen complexes are fully active catalytically suggests that the antigenic determinants may not involve the active sites of the enzyme. The possibility is thus left open that the enzymes may exhibit considerable homology in non-antigenic regions.

It will be of considerable interest to compare the structure of the two isoenzymes by amino acid analysis, peptide 'mapping', investigation of the binding and catalytic sites and eventually by amino acid sequencing. Such information may aid in elucidating the puzzling functional and genetic relationships of the two isoenzymes and on their roles in gluconeogenesis as a compartmentalized activity.

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