IMMUNOLOGICAL SIMILARITY OF THE NADH–CYTOCHROME *c* ELECTRON TRANSPORT SYSTEM IN MICROSOMES, GOLGI COMPLEX AND MITOCHONDRIAL OUTER MEMBRANE OF RAT LIVER CELLS

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Received 15 December 1975

1. Introduction

The structural, functional and biogenetic relationships between different types of biological membranes constitute a subject presently under active investigation. Although different membranes may be functionally related, they have been shown to be biochemically distinguishable entities in various systems [1–4]. Some enzymes, however, have been demonstrated to be localized in more than one type of membrane. Such is the case of the NADH-dependent, rotenone insensitive electron transport system, consisting of NADH–cytochrome b_5 reductase and cytochrome b_5. In liver, this system has been reported to be present not only in microsomes, but also in the outer membrane of mitochondria [5,6] and in Golgi membranes [4].

The localization of the same enzymes in different membranes may be useful as a handle to study their biogenetic relationships. To this purpose, it is of interest to demonstrate that the enzymes present in the different membranes are structurally or at least immunologically similar. So far, the only investigation in this direction concerns the NADH–cytochrome b_5 reductases present in liver microsomes and mitochondrial outer membranes, which have been shown to be immunologically very similar [7]. In this study we have investigated both components of the NADH–cytochrome *c* reductase electron transport system in all kind of rat liver membranes in which they have been found to be present.

2. Materials and methods

2.1. *Materials*

Horse heart cytochrome *c* (grade I), NADH and glucose 6-phosphate were purchased from Boehringer Mannheim GmbH; uridine 5′ diphosphogalactose (UDP-gal) from Sigma Chem Co; [U-^{14}C]UDP-gal (specific activity 300 Ci/mol) from NEN; Sialidase-, βgalactosidase-treated α_1 acid glycoprotein was the kind gift of dr. H. Schachter (University of Toronto). Male Sprague-Dawly rats, weighing 150–200 g, were a gift of Selvi Drug Corporation, Milano. Centrifugations were carried out in Spinco ultracentrifuges.

2.2. *Cell fractionation*

Golgi and microsome fractions were prepared on a discontinuous sucrose gradient as described by Ehrenreich et al. [8], with the following modifications: rats were not treated with ethanol prior to sacrifice, and the discontinuous sucrose gradient consisted of the following 10 ml layers (from bottom to top): load (crude microsomes) in 1.20 M sucrose; 1.10 M sucrose; 0.86 M sucrose and 0.25 M sucrose. The material remaining in the load zone was taken as the final microsome fraction; the material banding at the 1.10–0.86 M sucrose interface corresponds to the heavy Golgi fraction (GF_3) [8], while the material banding at the 0.86–0.25 M sucrose interface corresponds to the intermediate and light Golgi fractions (GF_2 and GF_1) [8]. Mitochondria were prepared by differential centrifugation and washed three times.

2.3. *Purification of enzymes and immunization of rabbits.*

NADH–cytochrome b_5 reductase, solubilized by lysosomal digestion, was prepared as described by Takesue and Omura [9]. Cytochrome b_5 was solubilized by trypsin digestion and purified according to Phillips and Langdon [10], as modified by Omura et al. [11]. The proteins were over 90% pure as assessed by SDS polyacrylamide gel electrophoresis. Before use, they were cross-linked by treatment with 0.01% glutaraldehyde. The excess glutaraldehyde was removed by dialysis. An immunoglobulin fraction was prepared from the serum of immunized rabbits by $(NH_4)_2SO_4$ precipitation. The purity of the antibodies was checked by Ouchterlony double diffusion.

2.4. *Enzyme assays*

The following enzyme assays were used: NADH–cytochrome *c* reductase: according to Sottocasa et al. [5]; glucose 6-phosphatase, essentially as described by Swanson [12]; cytochrome b_5, according to Ernster et al. [13]; galactosyltransferase essentially as described by Schachter et al. [14], using sialidase-, galactosidase-treated α_1 acid glycoprotein as acceptor. Protein was assayed according to Lowry et al. [15].

3. Results and discussion

Table 1 summarizes the results of four cell fractionation experiments. Golgi fractions were identifiable by their high enrichment in galactosyl transferase activity. On the basis of glucose 6-phosphatase specific activities it can be seen that the mitochondrial and light Golgi fractions (GF_{1+2}) are relatively free of microsomal contamination (6.5 and 5.7%, respectively) while the heavy Golgi fraction (GF_3) is considerably impure. This was expected because, in order to avoid possible alterations of liver structure, we did not treat the animals with ethanol before sacrifice, as recommended by Ehrenreich et al. [8].

NADH–cytochrome *c* reductase and cytochrome b_5 specific activities by no means parallel that of glucose 6-phosphatase. On this basis, these activities can be considered to be indigenous to mitochondria and also to Golgi membranes, as has been found by other authors [4,16].

In order to investigate whether these two proteins, common to the four cell fractions, are immunologically similar, we decided to check whether the NADH-cytochrome *c* reductase activity in different membranes could be inhibited by the same antibodies. Since the NADH–cytochrome *c* assay depends on

Table 1
Specific enzyme activities of liver cell fractions[a]

Cell fraction	Glucose 6-phosphatase[b]	NADH–cytochrome *c* reductase[c]	Cytochrome b_5[d]	Galactosyl transferase[e]
Total homogenate	1.564	57.14	–	42.00
Microsomes	4.914	169.71	0.535	73.00
Mitochondria	0.322	60.37	–	–
GF_3	1.623	135.06	0.467	1990.00
GF_{1+2}	0.284	92.64	0.130	1960.00

[a] Values given represent means of four internally consistent experiments.
[b] μmoles glucose 6-phosphate hydrolyzed/20 min/mg protein at 37°C.
[c] nmoles cytochrome *c* reduced/min/mg protein at 25°C.
[d] nmoles/mg protein.
[e] pmoles galactose transferred/60 min/mg protein at 37°C.

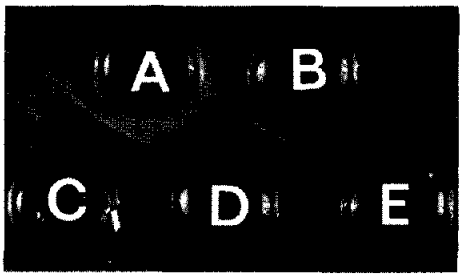

Fig.1. Ouchterlony double diffusion test on anticytochrome b_5 and anti-NADH–cytochrome b_5 reductase antisera with their respective antigens. The wells contained 80 μl of the following samples: (A) anticytochrome b_5 antiserum; (B) antireductase antiserum; (C) and (E) cytochrome b_5 (80 μg/ml); (D) NADH–cytochrome b_5 reductase (63 μg/ml).

the transport of electrons through both the reductase and cytochrome b_5, it might be expected that the enzyme activity would be inhibited by antibodies prepared against either component of the system.

Antibodies against NADH–cytochrome b_5 reductase and against cytochrome b_5 were prepared and the specificity of the two immunoglobulin preparations versus their respective antigens was checked by Ouchterlony double diffusion. As can be seen in fig.1, precipitation lines were single, and formed exclusively between the antisera and their corresponding antigens. When NADH–cytochrome *c* reductase activity was measured in the four cell fractions in the presence of antibodies against either the reductase or cytochrome b_5 the enzyme activity was found to be strongly inhibited (fig.2). At equal concentrations of immunoglobulin the degree of inhibition was approximately the same in all four membrane fractions. In contrast, an immunoglobulin solution prepared from the serum of a rabbit which had not been immunized was ineffective in inhibiting the enzyme activity (fig.2,b). Likewise, neither the antireductase nor the anticytochrome b_5 immunoglobulins affected the activity of another microsomal electron transport enzyme, NADPH–cytochrome *c* reductase (not shown).

It must also be mentioned that NADH–ferricyanide reductase activity, which involves the transport of electrons directly from the coenzyme to ferricyanide, was not inhibited by either of our antibody preparations (results not shown). Presumably in this reaction, the conformational requirements of the reductase are less stringent than in the case of the normal electron transport, involving cytochrome b_5 as well.

The inhibition of NADH–cytochrome *c* reductase activity by the same antibodies in the four membrane

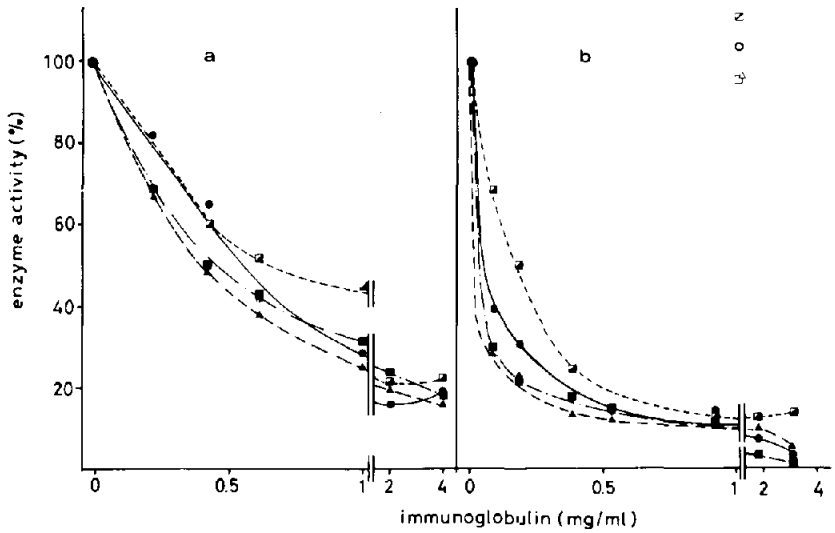

Fig.2. Inhibition of NADH–cytochrome *c* reductase activity in four membrane fractions by antireductase and anticytochrome b_5 antibodies. The enzymes were incubated for 10 min at 25°C in the presence of (a) antireductase and (b) anticytochrome b_5, in the amounts indicated in abscissa. (●—●) Microsomes; (▲---▲) mitochondria; (■---■) GF_3; (▲——▲) GF_{1+2}. Open symbols included in panel b represent values obtained from the same fractions incubated with immunoglobulins obtained from a non-immunized rabbit (~ 1 mg/ml).

fractions we have studied clearly cannot be explained on the basis of cross contamination. We conclude, therefore, that both NADH–cytochrome b_5 reductase and cytochrome b_5 are immunologically similar in microsomes, mitochondrial outer membranes and Golgi membranes. Studies on the biosynthesis of these proteins in the different membranes are now being undertaken in our laboratory.

Acknowledgements

We are grateful to Miss Dominique Granato for helpful suggestions.

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