FIBRIN MEMBRANE ENDOWED WITH BIOLOGICAL FUNCTION

Immobilization of spinach chloroplasts and rat liver microsomes

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Received 19 April 1982

1. Introduction

As an off-shoot of our studies of the mechanism of the thrombin-induced polymerization of fibrinogen [1–4], we thought of using the fibrinogen–fibrin conversion step to immobilize biological materials. Fibrinogen is converted to fibrin monomer by the enzymic action of thrombin and fibrin monomers spontaneously associated with each other to form fibrin polymer. Then, blood coagulation factor XIII catalyses a crosslinking reaction between fibrin monomers to secure fibrin polymer formation [5]. The advantages of fibrin membrane as a supporting material for immobilization are as follows:

(i) The formation of fibrin polymer proceeds under mild conditions;
(ii) This membrane may not be rejected by the immune system when used as a biomedical material.

We have immobilized a variety of biological materials using fibrin membrane, and have successfully immobilized living cells such as *Chlorella* cells and sea urchin eggs [6] as well as enzymes such as asparaginase [7], chloroplast ATPase [8] and ribonuclease [9]. Furthermore, we have been able to immobilize more than one enzyme in a single membrane, for example, the multienzyme system including uricase, catalase, allantoicase and allantoinase [10] and also the multienzyme system of the urea cycle [11,12].

Here, spinach chloroplasts and rat liver microsomes were immobilized into a matrix of fibrin fiber formed from fibrinogen by the concerted action of thrombin and blood coagulation factor XIII. Immobilized chloroplasts retained the ability to synthesize ATP from ADP and P_i by illumination. In a fibrin membrane containing chloroplasts together with hexokinase, glucose 6-phosphate was synthesized from glucose with the help of ATP formed by cyclic photophosphorylation. Microsomes embedded in fibrin membrane retained their functions of the monooxygenase multienzyme system by which aminopyrine was oxidatively demethylated.

2. Materials and methods

Fibrinogen (93% clottable) and thrombin (EC 3.4.21.5) (12.5 units/mg) from human blood were supplied by Green Cross Co. The fibrinogen preparation contained a small amount of blood coagulation factor XIII. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1) and aminopyrine, 4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one, were purchased from Sigma Chemical Co. *N*-Methylphenazinium methylsulfate (PMS) was purchased from Wako Pure Chemical Industries, Ltd. $^{32}P_i$ was obtained from Japan Radioisotope Assoc.

Chloroplasts were obtained from spinach as in [13]. Chloroplasts were suspended in 10 mM Tricine–NaOH (pH 7.8) and broken chloroplast suspension (1.5 mg chl/ml) was used in here. Chlorophyll content was determined as in [14]. The amount of ATP formed by cyclic photophosphorylation was determined using [^{32}P]ATP as in [15]. Hexokinase activity was determined by measuring the absorbance change at 340 nm due to reduction of NADP.

Microsomes were prepared from rat livers as in [16] and suspended in 0.1 M Tris–HCl buffer (pH 7.4) to give 12 mg protein/ml final conc. The activity of aminopyrine *N*-demethylase was determined by measuring the amount of formaldehyde liberated from

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

aminopyrine [17]. Protein concentration was determined by the biuret method [18].

Immobilization of spinach chloroplasts or rat liver microsomes in fibrin membrane was done by modifying the method in [12]. Thrombin (0.5 ml, 40 units) was added to a mixture (20 ml) of 2.5% fibrinogen and chloroplasts (1.5 mg chl) or microsomes (113 mg protein) in the presence of 200 mM glucose, 30 mM $CaCl_2$, 1 mM cysteine and 50 mM citrate buffer (pH 7.0). The fibrin clot containing chloroplasts or microsomes thus formed was pressed to obtain a thin membrane and washed with 10 mM Tricine–NaOH (pH 7.8) or water. The preparation of immobilized chloroplasts was carried out in the dark.

3. Results and discussion

3.1. *ATP formation by immobilized chloroplasts*

Formation of ATP by cyclic photophosphorylation was tested by using 1/3rd of the fibrin membrane prepared as in section 2. The fibrin membrane containing chloroplasts was introduced into 2 ml reaction medium containing 30 mM NaCl, 2 mM $MgCl_2$, 2 mM ADP, 7 mM P_i, $^{32}P_i$ (37 μCi), 0.017 mM PMS and 15 mM Tricine–NaOH (pH 7.8). The sample solution was illuminated by a 300 W tungsten lamp from 15 cm. During illumination, the sample solution was kept in a water bath at 25°C, 30°C and 37°C under gentle stirring. Fig.1a, curves A–C represent the amount of ATP synthesized at 25°C, 30°C and 37°C, respectively. The amount of ATP formed by immobilized chloroplasts at 25°C increases with time and reaching 6 μmol/mg chl upon 30 min-incubation (curve A). Elevation of the incubation-temperature of the reaction mixture leads to a reduction of the amount of ATP formed (curves B,C). A similar result was obtained with chloroplast suspensions without immobilization. To test whether or not ATP formed by immobilized chloroplasts could be consumed in phosphorylating glucose to glucose 6-phosphate, both chloroplasts and hexokinase were immobilized into a single membrane. The fibrin membrane containing chloroplasts (1 mg chl) and hexokinase (1.3 mg) was put into 10 ml reaction medium containing 264 mM D-glucose, 8 mM $MgCl_2$, 8.4 mM P_i, 2.6 mM ADP, 0.021 mM PMS and 50 mM Tris–HCl (pH 7.5). Then the sample was illuminated by a 300 W lamp at 25°C. At a given time, an 0.8 ml aliquot of the sample was withdrawn, and the amount of glucose 6-phosphate determined by measuring the amount of NADPH formed after adding 67 μl NADP (10 mg/ml) and glucose 6-phosphate dehydrogenase (1 unit). Fig.1b shows that the amount of glucose 6-phosphate formed in the sample increased linearly with time after illumination (curve A). In the dark, no appreciable amount of glucose 6-phosphate was formed, as seen in curve B. This indicates that the cyclic photophosphorylation system is well coupled with the hexokinase reaction in the fibrin membrane containing both chloroplasts and hexokinase.

3.2. *Oxidative demethylation of aminopyrine by immobilized microsomes*

Liver microsomes having the electron-transport system containing cytochrome P450 have the ability to hydroxylate or oxidatively dealkylate a number of different substrates. Aminopyrine is one of the most

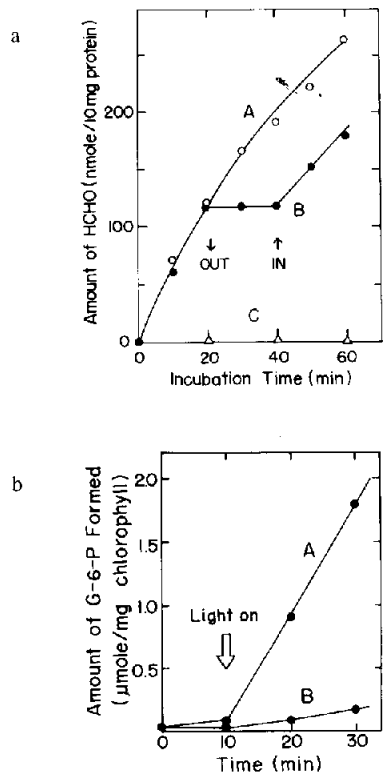

Fig.1. ATP formation by chloroplasts embedded in fibrin membrane (a) and glucose 6-phosphate formation in a fibrin membrane containing both chloroplasts and hexokinase (b): (a) curves (A), (B) and (C) ATP formation at 25°C, 30°C and 37°C, respectively; (b) curves (A) and (B) in the light and in the dark, respectively. For further details see section 3.

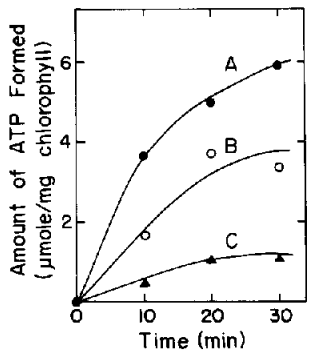

Fig.2. Oxidative *N*-demethylation of aminopyrine with immobilized microsomes: (A) formaldehyde formation from aminopyrine as a function of time; (B) formaldehyde formation when immobilized microsomes were removed from and added to the reaction mixture at the times indicated by arrows; (C) formaldehyde formation in the absence of NADP. For further details see section 3.

popular substrates for investigating the hepatic microsomal monooxygenase multienzyme system [19]. We prepared a fibrin membrane containing microsomes and studied the oxidative *N*-demethylation of aminopyrine. One-ninth of the fibrin membrane (containing 13 mg microsome protein) was introduced into 10 ml of a reaction mixture containing 10 mM $MgCl_2$, 20 mM aminopyrine and 50 mM Tris–HCl (pH 7.4) together with 0.4 mM NADP, 4 mM glucose 6-phosphate and glucose 6-phosphate dehydrogenase (1 unit) (NADPH generating system). The mixture was incubated at 37°C under gentle shaking. At a given time, an aliquot of the reaction mixture was taken out and the amount of formaldehyde determined. The results show (fig.2) that the amount of formaldehyde formed increased linearly with the incubation-time as seen by curve A. When the fibrin membrane was taken out from the reaction mixture (curve B), the reaction did not proceed at all. The reintroduction of the fibrin membrane to the substrate solution resulted in formaldehyde formation at the same rate as that shown by curve A. This indicates that microsomes are completely embedded in the fibrin membrane and free microsomes are not released. In the absence of NADP, no oxidative *N*-demethylation of aminopyrine was observed (curve C). The above function of microsomes embedded in fibrin membrane was retained for >1 month at −70°C. It is concluded that microsomes embedded in fibrin membrane are functioning well and their electron transport system is not impaired. The immobilization of chloroplasts or microsomes in fibrin membrane is, as far as we know, the first attempt of its kind. The former may find application in the utilization of solar energy and the latter for the development of an artificial liver [11,12].

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