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A STUDY OF THE INCORPORATION OF CYTOCHROME OXIDASE INTO PLANAR SYNTHETIC MEMBRANES

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Summary

Cytochrome oxidase molecules were incorporated into black lipid membranes and into a new form of planar synthetic membrane. Studies of these membranes indicated that the incorporation of large membrane bound enzymes into black lipid membranes involves difficulties fundamental to this technique. On the other hand the new method described in this paper is more promising.

Introduction

When Mueller et al. [1] first succeeded in forming black lipid membranes and incorporating a protein in them it appeared that a powerful new technique had been established for investigating membrane bound enzymes. These membranes have in fact proved a valuable tool in the investigation of membrane active antibiotics and in the study of physical properties of lipid bilayers. However, their application to the study of enzymes has proved disappointing. (Montal [2] has reviewed the rather limited progress which has been made in this field). We accordingly thought it worth while to carry out a study of the use of planar synthetic membranes with membrane-bound enzymes incorporated in them, concentrating on the problems of incorporation. We have studied both black lipid membranes and also membranes formed by another technique. We decided to work with cytochrome oxidase both because of the intrinsic importance of this material and also because it has been incorporated into liposomes by several groups [3,4] and thus results from their work are available for comparison. Skulachev and his collaborators [5,6] have also used cytochrome oxidase in a series of experiments in which the enzyme was first incorporated into liposomes and thence incorporated into black lipid membranes. It is not

however clear in this case that true bilayer membranes were employed.

According to the chemiosmotic hypothesis [7,8], cytochrome oxidase molecules incorporated in a membrane and supplied with cytochrome *c* and a suitable substrate on one side should generate a protonmotive force of up to about 180 mV. Measurement of the membrane potential generated under these conditions thus provides a direct measure of the activity of the enzyme. The rate at which the substrate is metabolised does not necessarily measure the activity in the case of an enzyme such as cytochrome oxidase which serves as a transducer converting chemical to electrical energy. A situation can be visualised in which the substrate was metabolised but in which no useful work was done.

Studies using black lipid membranes

Cytochrome oxidase was prepared by the method of Yonetani [9]. After the last fractionation step, the enzyme was stored either in a detergent (1% Tween 80) or as small pellets in separate containers. They were kept at liquid nitrogen temperature and used one at a time. The lipids used were either glycerol monooleate or a mixture of phosphatidylethanolamine, phosphatidylcholine and cardiolipin in a ratio (2 : 3 : 1).

Detergent was first removed from the enzyme by dialysis overnight (12 h) against 50 mM phosphate buffer, pH 7.3. The suspension containing the enzyme was then dehydrated against Ficoll 400 (Pharmacia) for a few hours. 20 mg of lipid was added to 8 mg of the precipitated enzyme and then suspended in 2 ml of *n*-hexane. The whole mixture was briefly sonicated and used for forming the black lipid membranes.

The experimental cell was made from polytetrafluoroethylene and glass and was sealed using Edwards high vacuum silicone grease. The construction and cleaning procedures are given in more detail elsewhere [10]. The bathing solution contained: Tris buffer 50 mM, pH 7.4; Sucrose 200 mM; KCl 10 mM; $CaCl_2$ 5 mM.

Saturated KCl solution in contact with the calomel electrodes was isolated from the cell in two stages to avoid risk of leakage. In the first stage a sintered plug divided the saturated KCl from KCl at 20 mM. A further disposable agar plug divided this solution from the actual cell. The potential was developed across a resistor ranging from 10^9 to 10^{11} Ω and applied to an Analog Devices Varactor Bridge amplifier M311 J and hence via a Keithley electrometer type 602 to a recording millivoltmeter. Provision was made to apply a bias voltage. After fixing the electrodes the membrane was formed by smearing the lipid-enzyme suspension across a 0.5 mm hole in the cell septum using a sterile surgical syringe. The transition to the black state was monitored using reflected light from a fibre optics device and a low powered microscope. Cytochrome *c* was then added to one side of the membrane at a final concentration of 10^{-5} M. After about 10 min ascorbate (to make a final concentration of 2 mM) was added to the same (cytochrome *c* side) and the voltage was monitored.

In this method it ought to be possible to incorporate quite large quantities of enzyme, the limit being set by the quantity which can be incorporated without the membrane becoming unstable. However, another unforeseen effect intervened. Normally when one uses the black lipid membrane technique the first

stage of forming a membrane is to spread a relatively thick layer of lipid in an alkane across the hole in the septum. This septum is made from hydrophobic material and the lipid solution tends to spread across it thus gradually reducing the proto-membrane in thickness. If the region of the hole is monitored by reflected light this region will soon appear coloured as the membrane becomes thin enough for interference phenomena to become important. Bands of the various spectral colours pass across the hole and finally part of this region, usually near the centre, becomes black as the thickness in this region decreases to a value corresponding to the thickness of a lipid bilayer. This black region then rapidly expands until it occupies almost the entire area of the hole. However, when a substantial proportion of cytochrome oxidase was present the situation was quite different. Sometimes the process never proceeded beyond the coloured state. Even when a black area appeared it might fail to expand and sometimes simply co-existed with a substantial region of coloured membrane. If a measurement of the membrane potential was now made while a region of coloured membrane still existed potentials up to 100 mV and of either sign were detected in the absence of cytochrome *c* and substrate. These potentials quickly reappeared after the membrane had been short circuited for a few seconds.

A second artifact could be observed if un-buffered solution was used. In this case the cytochrome *c* ascorbate side of the membrane became negative contrary to what one would expect. This result shows that the membrane is, under those circumstances, slightly permeable to protons.

If a buffer was used and no coloured region was present the ascorbate side became positive with respect to the other side by about 35 mV. Results of this kind were obtained with both types of lipid mixture employed.

A new technique for forming planar synthetic membranes

If our interpretation of our observations using black lipid membranes is correct (see Discussion section) it will always be difficult to use these membranes to study large membrane-bound enzymes. On the other hand the liposome technique has obvious limitations and we thus decided to seek another method for the formation of synthetic planar membranes. One possibility would be to use the method usually associated with Montal and Mueller [11] in which two monolayers are combined together but this way of proceeding presents formidable experimental difficulties even without the added complication of having a large enzyme involved. The method which we finally evolved is related to that of Montal and Mueller [11] but makes use of a supporting structure to carry one monolayer which is then brought into contact with the other.

Initial experiments were made using a hydrophilic Millipore filter as a support but these proved unsuccessful. We thus employed the following technique. A piece of mylar (polyethylene terephthalate) sheet 0.05 mm thick had a hole 0.4 mm diameter punched in it by using a hot needle. A 1% solution of collodion in amyl acetate was used to form a thin film of collodion on water. Part of this film was then picked up on the mylar sheet. Alternatively the sheet was covered on both sides by dipping in 5% solution of collodion and allowing

it to dry after gently breathing on the surface. The collodion film stretched across the hole is porous, having channels in it of the order 20 nm diameter [12]. In order to pick up a lipid monolayer on one side of this structure it was then provided with a temporary backing consisting of a further mylar sheet. In order to form the monolayer we employed a Langmuir trough using the Wilhelmy plate method with a C.1. Electronics balance to measure the surface pressure. This balance was connected to a feedback device so that a predetermined surface pressure could be maintained. Soya bean lipid extract (Sigma) in *n*-hexane was added to the surface of millipore filtered water maintained at pH 6.5 by 5 mM Tris buffer. The hexane was allowed to evaporate and the surface pressure adjusted to 38 $mN \cdot m^{-1}$. The treated mylar sheet was then drawn slowly up through the monolayer via a reduction gear. The velocity of the plate was about 2 mm per min. In this way a lipid monolayer was deposited on the collodion and on the water in the collodion pores. The backing sheet was now removed and a masking sheet of mylar, having a hole in it somewhat larger than 0.4 mm diameter was attached to the front (lipid) side of the main mylar sheet using silicone grease. This precaution was used to reduce the area and to avoid short circuiting the membrane through defective regions.

The next stage in this technique was to place the mylar sheet across a hole in a septum at a slight angle in the specially designed cell shown in Fig. 1. Silicone grease was used to form a watertight insulating seal. A small quantity of the bathing solution described in connection with black lipid membranes was added to the upper compartment while the lower half was partially filled with the same solution.

A second monolayer was now formed from the lipid-enzyme mixture in *n*-hexane prepared as described earlier in this paper and diluted with ten times the quantity of *n*-hexane. 10 μl of this solution was spread on the surface of

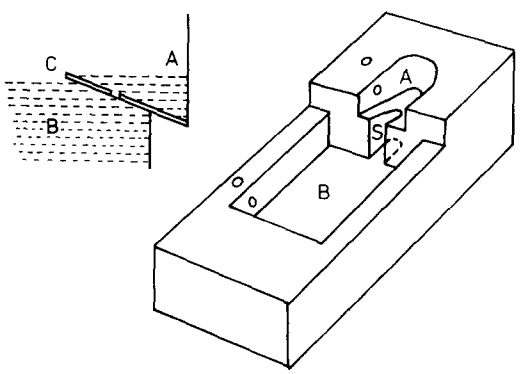

Fig. 1. Schematic diagram of the PTFE cell used in the method of forming bilayer membranes. Compartments A and B have capacities of 1.5 ml and 10 ml, respectively. A mylar sheet with a 0.4 mm hole was covered successively from one side with (1) A collodion film. (2) A lipid monolayer. (3) Another mylar sheet with a larger hole in it. The screened sheet was mounted (monolayer facing down towards the slit S) on the inclined surface which separates the two compartments. A second monolayer on the surface of the liquid contained in compartment B was joined to the first one by raising the level of the bathing solution in this compartment. The final configuration is shown in the small diagram on the left. Here C denotes the mylar sheet.

the electrolyte in the lower half of the cell by means of a micropipette. The area of this surface was 15 cm^2. The degree of dilution and volume required were controlled by preliminary experiments using the Langmuir trough system referred to earlier. The quantity of solution for optimum formation of final bilayer was slightly in excess of the quantity needed to form a monolayer in the lower compartment. Several minutes were now allowed to elapse so that the *n*-hexane could evaporate and the level of the electrolyte in the lower compartment was then gradually raised by adding more electrolyte using a syringe. The lower and upper monolayers were thus brought into contact and usually coalesced to form a bilayer though in some cases the process failed.

Initial experiments were carried out using an electrolyte containing potassium chloride. A small quantity of gramicidin A was added to the lower compartment and a bias of about 50 mV applied across the cell. Characteristic [13,14] current fluctuations were then observed. We believe that this result provides unambiguous evidence that a bilayer membrane is formed in this way, as it is well known that gramicidin will only provide channels bridging bilayers.

In order to test the success of enzyme incorporation membranes containing cytochrome oxidase were formed as described above. Cytochrome *c* to make a concentration of 10^{-5} M was added and after 10 min ascorbate to make a final concentration of 2 mM was also added to the lower portion of the cell. A membrane potential, negative on the upper side, was then developed. On a number of occasions a potential in the region of 55 to 60 mV was developed but this value appeared to represent an upper limit above which it was impossible to proceed. To check that this potential was not an artifact a small quantity of KCN to make a final concentration of $3 \cdot 10^{-4}$ M was introduced on each occasion into the upper part of the cell whereupon the membrane potential rapidly decayed. (We also carried out experiments in which KCN was added

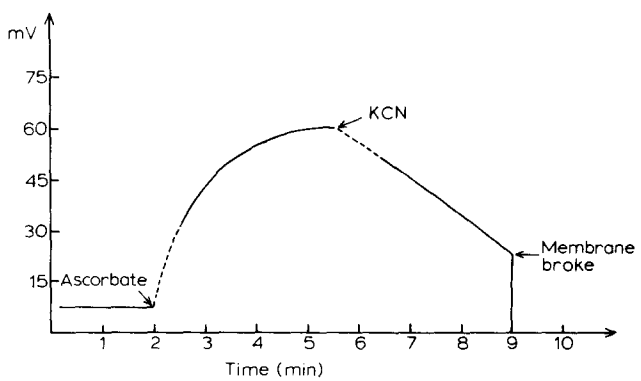

Fig. 2. Membrane potential generated from a bilayer containing cytochrome oxidase.The dotted line corresponds to the periods where solutions were introduced to the cell and the electrometer was clamped. The bathing solution contained 0.2 M sucrose, 10 mM KCl, 5 mM $CaCl_2$ in a 50 mM Tris-HCl buffer (pH 7.4). Cytochrome *c* was added to the lower half of the cell to give a final concentration of 10^{-5} M. The membrane was then formed and after 10 min ascorbate was added to the same side (final concentration 2 mM). KCN solution (final concentration $3 \cdot 10^{-4}$ M) was added to the upper half of the cell (at the point indicated).

before the ascorbate and in these cases no membrane potential was generated.) Typical behaviour is shown in Fig. 2. It may be noted that this fall of potential is much slower than its rise after adding ascorbate. This is presumably due to the time required for KCN to diffuse through the collodion film before reaching the active site of the enzyme.

Discussion

The behaviour using black lipid membranes can tentatively be explained as follows. It is well known that the cytochrome oxidase molecule has a length corresponding to about twice the thickness of a lipid bilayer membrane. Furthermore, there must exist a substantial hydrophobic region around the 'waist' of a membrane-bound enzyme. It thus seems probable that cytochrome oxidase molecules serve to pin adjacent bilayers together and inhibit the thinning process. The origin of the anomalous potentials appearing when coloured regions of membrane are still present must be in some way related to the rearrangement of molecules having a substantial dipole moment which takes place when the thinning process is slowed down by the presence of the enzyme. Normally, when using black lipid membranes for other purposes, the thinning takes place in a few seconds before the electrical measurements are made.

In the case of those membranes in which we succeeded in eliminating coloured regions completely and when a buffer was used we were never able to generate membrane potentials of more than 35 mV. This rather small voltage indicates that the number of enzyme molecules remaining in the black membrane was small. This result is consistent with the pinning hypothesis advanced above. Where a membrane does succeed in becoming completely black most of the enzyme will have been swept into the surrounding torus and thus rendered inactive. These results indicate that the black lipid membrane technique is unlikely ever to be completely successful in the study of large membrane-bound enzymes.

On the other hand, the new technique described in this paper offers obvious advantages. It should be possible to obtain reasonably high enzyme concentrations and to orient all the enzyme molecules in the same direction. However, that it does not lead to an ideal reconstitution in the case which we have studied is evident from the upper limit to the membrane potentials generated. This limit may well result from some chemical change in this large complex molecule brought about by the extraction and reconstitution process rather than from any intrinsic defect in the actual membranes formed. It is also possible that this limit to the membrane potential obtainable arises from voltage dependent H^+ leakage through the membrane.

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