KINETICS OF THE INCORPORATION OF CYTOCHROME b_5, AN INTEGRAL MEMBRANE PROTEIN, INTO UNILAMELLAR DIMYRISTOYLLECITHIN LIPOSOMES

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1. Introduction

The interaction of the so-called integral membrane proteins with lipid bilayer membranes is a subject of considerable interest in membrane biology [1]. Among other interesting aspects of this interaction it is not as yet clearly understood how these proteins, presumably synthesized in the cytoplasm, are incorporated into the various cellular membranes.

An understanding of this process could be of significance in the general understanding of the synthesis of biomembranes. Some attempts have been made in the recent literature to incorporate integral membrane proteins, isolated in a lipid-free state for natural membranes, into preformed lipid bilayer vesicles [2–4]. However, no attempt seems to have been made to investigate the kinetics of this process and the dependence of these kinetics upon the physical state of the lipid bilayer. In this communication we wish to report some results we have obtained on the kinetics of the incorporation of cytochrome b_5, an integral membrane protein isolated from bovine liver microsomal membranes in a lipid-free form, into unilamellar dimyristoyllecithin liposomes. The dependence of the kinetics upon the physical state of the lipid was investigated and a model has been constructed for the insertion of the amphipatic protein into the lipid bilayer. A more detailed kinetic analysis on several different lipid membrane systems will be reported separately.

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2. Materials and methods

The intact form of cytochrome b_5 was extracted from bovine liver microsomal membranes with the help of detergents as in [5]. The purified protein preparation was then concentrated by ultrafiltration using an Amicon 'Diaflo' PM 10 membrane filter and then dialysed against 3 mM Tris–HCl, pH 7.5.

Unilamellar bilayer liposomes of dimyristoyllecithin were prepared in 3 mM Tris–HCl, pH 7.5, according to the procedure in [6] except that centrifugation was done at 150 000 × g for 1 h and the gel filtration step was omitted. The liposome preparation was stored for not more than 4 days at 35°C since single-shelled liposomes are reported to be most stable at a temperature higher than the thermal phase transition temperature of the lipid [7].

In order to study the lipid–protein association kinetics, the lipid preparation at a desired concentration in 3 mM Tris–HCl, pH 7.5, was brought to the desired temperature in a 0.5 cm pathlength fluorescence cuvette placed in the thermostated sample

holder of a Fica 55 absolute spectrofluorimeter. The protein solution was then added in a volume which did not exceed 4% of the lipid suspension volume in the cuvette. The final protein concentration was always 2 μM. After addition of the protein the contents of the cuvette were gently mixed for 10 s, the cuvette was placed back into the sample holder of the fluorimeter and the fluorescence emission of the protein at 330 nm with excitation at 280 nm was followed for several hours. In this work we have used 7.5 nm slitwidths since under these conditions it was possible to get optimal signal to noise ratios while at the same time the contribution of scattered light to the observed fluorescence intensity at 330 nm was negligible. Low salt concentrations were necessary in this study to ensure that the protein was largely in its monomeric form [8].

The lipid phase transition of the pure lipid was observed by monitoring the polarization of fluorescence of the fluorescent hydrophobic membrane probe, diphenylhexatriene (DPH), as in [9]. Temperature scan rates of 30°C/h were employed.

3. Results

Tryptophan fluorescence of cytochrome b_5 increased considerably upon incorporation of the protein into lipid bilayers or micelles [10]. This increase is perhaps due to the transfer of the chromophores from an aqueous to a non-aqueous apolar environment when the hydrophobic tail of cytochrome b_5 is buried into the hydrophobic portion of the lipid structure. Similar increases in protein fluorescence have been reported for the interaction of other proteins and peptides with lipid membranes [11,12]. So far, this useful characteristic seems not to have been exploited for kinetic studies. In fig.1 we show the increase in protein fluorescence quantum yield upon incorporation into the lipid bilayer. This result is similar to [10]. We were able to confirm the validity of the use of this fluorescence increase as a measure of protein incorporation by parallel gel filtration experiments. It is also seen in fig.1 that under the experimental conditions used in this work the light-scattering contribution to the fluorescence intensity at 330 nm is negligible when excitation is done at 280 nm. The light-scattering contribution is also negligible after incorporation of the protein.

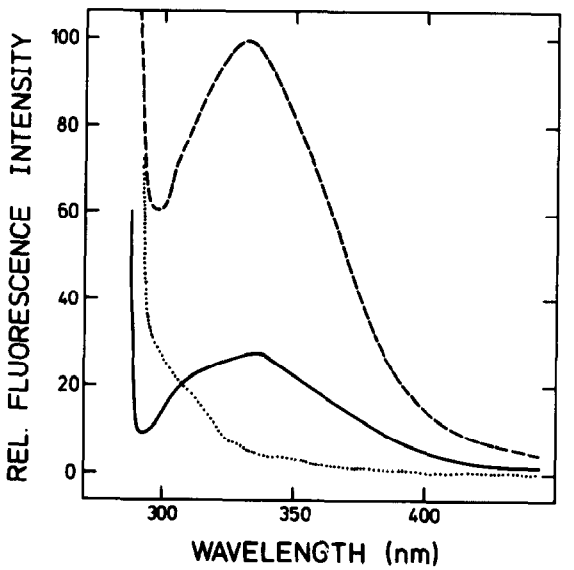

Fig.1. Fluorescence emission of cytochrome b_5 in the lipid-free and lipid-bound states at 35°C. Excitation was done at 280 nm in all cases. Excitation and emission bandwidths were 7.5 nm. All preperations were in 3 mM Tris–HCl, pH 7.5. (——) Fluorescence emission of 2 μM cytochrome b_5 in lipid-free aqueous solution; (– – – –) fluorescence emission of 2 μM cytochrome b_5 in a cytochrome b_5 – dimyristoyllecithin complex at a molar ratio of protein to lipid = 1 : 400; (· · · ·) light-scatter from a 0.8 mM suspension of unilamellar vesicles of dimyristoyllecithin.

In fig.2 we show the increase in protein fluorescence with incubation time for three different incubation temperatures. The 100% value for the relative fluorescence intensity was defined by incubating identical concentrations of the protein and lipid together at 37°C for 24 h, under which conditions all the protein is incorporated into the bilayer liposomes. We have carefully examined curves similar to those shown in fig.2 for different lipid to protein molar ratios. At low lipid to protein ratios biphasic curves with an initial fast non-exponential phase followed by a slow process are observed.

In fig.3A the time required for the incorporation of 50% protein in the incubation mixture into the lipid vesicles is plotted versus incubation temperature. Figure 3B shows the rigidity of the lipid bilayer (expressed as the degree of polarization of DPH in the lipid bilayer) as a function of temperature. As seen, the incorporation process is very slow at temperatures below the lipid phase transition temperature and

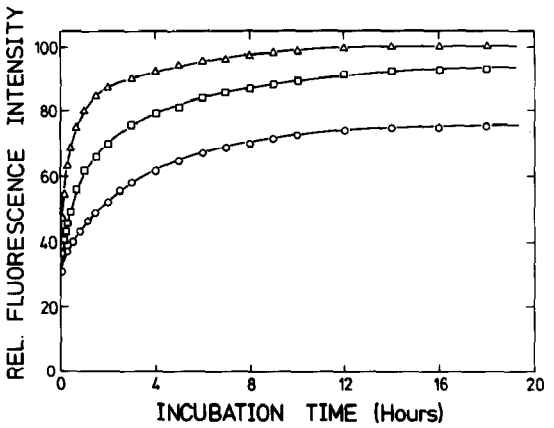

Fig.2. Time course of the increase in fluorescence emission intensity at 330 nm of cytochrome b_5 upon addition to a suspension of unilamellar dimyristoyllecithin vesicles. Excitation was at 280 nm. Excitation and emission slitwidths were 7.5 nm. Protein was 2 μM and lipid was 0.8 mM in all cases. The experiments were done in 3 mM Tris–HCl, pH 7.5 (○) 15°C; (□) 25°C; (△) 35°C. On prolonged incubation the relative fluorescence intensity reached 100% in all cases.

considerably faster above this temperature. There appears to be a distinct correlation between membrane fluidity and ease of incorporation of the protein into the bilayer structure.

4. Discussion

The incorporation of small molecules such as lysolecithin and the phenomenologically-related diffusion of small molecules across phospholipid bilayers has been shown to occur fastest at the lipid phase transition temperature with slower rates above and below this temperature [13,14]. This phenomenon has generally been explained by the existence of 'defects' in the lipid packing of the bilayer due to mismatch at the boundaries of the ordered and disordered lipid phases [14,15].

An alternative explanation may be as follows: If a hydrophobic particle is incorporated into the membrane, on the whole, free energy is gained from the process due to the removal of the particle from water. This process, however, involves distortion of the phospholipid bilayer giving rise to a free energy barrier which determines the rate of incorporation, $t_{1/2}^{-1}$. For

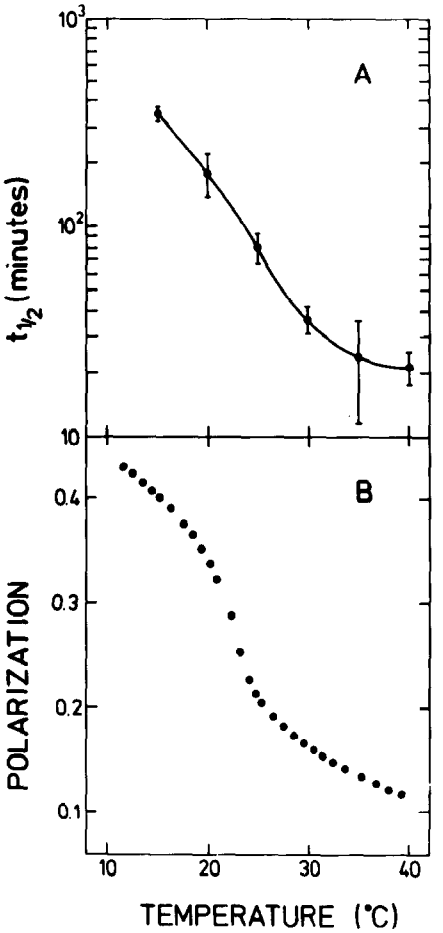

Fig.3. (A) Dependence of the half-time for the incorporation of cytochrome b_5 into unilamellar dimyristoyllecithin liposomes upon temperature. (B) Polarization of the fluorescence of the hydrophobic probe DPH incorporated into the hydrophobic region of unilamellar dimyristoyllecithin liposomes. The polarization is proportional to the fluidity of the lipid hydrocarbon region.

small particles the distortion is assumed to be achieved by changing the lipid order without big changes in the distance between neighbouring lipids. The incorporation in the ordered lipid phase generally involves fluidization of the lipid molecules, and into the fluid phase corresponding ordering. The free energy difference between the ordered and fluid lipid vanishes at the lipid phase transition. At this point the free energy barrier is a minimum and the corresponding incorporation rate is a maximum.

In general, for large particles the same principles apply. However, incorporation of large particles involves a stronger distortion of the lipid phase. Especially, the local compression becomes so great that the lipid molecules have to break up their interaction with neighbours and diffuse away, in the limit create a hole [16]. The temperature dependence of the incorporation rate is then given by the temperature dependence of the lateral diffusion constant which shows an abrupt increase at the phase transition. This mechanism is compatible with our experimental result for the incorporation of cytochrome b_5.

We have built a detailed kinetic model for the incorporation of the protein into the fluid lipid phase. A brief description follows. We assume, on the basis of [17–21], that a protein molecule incorporated into a fluid lipid phase of low order has an extended region of lipid molecules of higher order around itself. The second protein molecule is incorporated into the undisturbed fluid phase because this process is faster than incorporation into the ordered region around the first molecule. In this way protein molecules are built in until there is no more fluid phase. If this process does not lead to the incorporation of all protein molecules available, a second slower process follows which involves the incorporation of the protein molecules in the disturbed lipid regions. Under certain conditions the first process comes to an end when all protein molecules are in the membrane and no more fluid phase is left. This is the case for $L = mP$, where L and P are the number of lipid and protein molecules, respectively, and m is the number of lipid molecules in the disturbed region around one protein molecule. The special case shows characteristic non-exponential kinetics which can be found experimentally by varying the lipid to protein ratio L/P. This yields the number m. Our result at 25°C, slightly above the phase transition, is $m = 400 \pm 50$. Converting this into the number n_{max} of concentric rings of hydrocarbon chains around a protein molecule of diameter 25 Å yields $n_{max} = 5 \pm 1$.

There is evidence that interaction of cytochrome b_5 with small unilamellar liposomes results in asymmetric vesicles with the protein incorporated only in the outer monolayer [22]. In this case, $m = 200 \pm 25$ and $n_{max} = 3 \pm 0.5$. This result reasonably agrees with a theoretical calculation of the disturbing effect of a protein molecule on the lipid ordering [23].

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