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Steady-state nonmonotonic concentration profiles in the unstirred layers of bilayer lipid membranes

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Abstract

Catalytic reactions in the unstirred layers near bilayer lipid membranes can induce nonmonotonic concentration profiles near the membrane surface. In the case of transmembrane diffusion of a substrate immediately followed by its conversion due to the presence of an aqueous soluble enzyme the size of the unstirred layer defined in terms of the concentration gradient at the membrane surface does not correspond to the width of the aqueous layer adjacent to the membrane where the concentration differs from the bulk phase concentration. Deducing of flux values or convection parameters from the concentration gradient at zero distance from the membrane gives misleading results. An empirical equation for the estimation of the size of the concentration boundary layer is proposed. It was derived from pH profiles registered with the help of a microelectrode near a planar bilayer lipid membrane surrounded by a buffer solution containing at one side of the membrane acetaldehyde and sodium acetate and at the other side alcohol dehydrogenase. Since this parameter equals to the thickness of the unstirred layer in the case of exponential concentration profiles it may be applied to estimate both mass transfer restrictions and kinetic of diffusion limited reactions occurring in the immediate membrane vicinity regardless the complexity of the system under investigation.

Keywords: Bilayer lipid membrane; Unstirred layer; pH microelectrode; pH profile; Nigericin; Alcohol dehydrogenase

1. Introduction

Unstirred layers (USLs) near membranes play the role of additional kinetic barriers in transport processes of rapidly permeating substances through natural and artificial membranes [1–3]. The rate and effectiveness of chemical transformations within the USLs are affected by the availability of the reactants. Boundary layers near the membrane are the source of an inaccurate Michaelis constant in membrane transport [4]. The size of the USL seems to have regulatory functions. Variations in epithelial function or luminal stirring can for example readily influence the absorption of small molecules [5].

Therefore, great efforts were made to determine the size of the USL (δ) near various biological objects. δ is defined in terms of the concentration gradient at the membrane surface according to the following equation [6]:

$$\frac{|c_s - c_b|}{\delta} = \frac{\partial c}{\partial x}|_{x=0} \quad (1)$$

where c_s and c_b are the concentrations of the diffusing substance at the membrane surface and in the bulk solution respectively. x is the distance from the membrane. Usually the width of the USLs was estimated by flux measurements [7,8]. Alternatively, microelectrodes placed near the membrane surface were used to determine δ from the time-course of near membrane concentration changes [9]. In the present work microelectrodes were used in order to record concentration values as a function of the distance from the membrane. The microelectrode technique for 'pH-microclimate' measurements was introduced by Lucas et al. [10] and then it was applied to model membranes [11]. Concentration profiles were registered with optical methods too [12]. Independently of the method the concentration always was an exponential function of x [11–14].

Abbreviations: USL, unstirred layer; BLM, bilayer lipid membrane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NAD^+ and NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide; AlDH, alcohol dehydrogenase; PTFE, polytetrafluoroethylene.

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The pH profiles measured in the present work were induced by the simultaneous proceeding of proton producing and proton consuming reactions in the unstirred layers. Acetaldehyde and sodium acid were added individually to one side of the planar bilayer lipid membrane and alcohol dehydrogenase to the other. The system under investigation included:

(a) the acetate permeation followed by proton release [15–17] and

(b) the acetaldehyde permeation followed by proton uptake due to the reaction with NADH in the presence of alcohol dehydrogenase [18]: $CH_3CHO + NADH + H^+ \rightleftharpoons CH_3CH_2OH + NAD^+$.

Since the reaction rates of these transformations are quite different from each other concentration profiles deviating from monotonic ones were expected. Usually local concentrations were estimated from flux parameters making the assumption that the concentration is a monotonic function of the distance to the membrane [19,20]. Using the microelectrode technique there is no need to postulate such a concentration behaviour in order to determine the thickness of the unstirred layer accurately.

2. Materials and methods

Bilayer lipid membranes were formed on a hole in a Teflon partition 1.2 mm in diameter, by a conventional method [21]. The membrane-forming solution contained 20 mg phosphatidylcholine from soy beans (Sigma) and 10 mg cholesterol (Merck) in 1 ml of n-decane (Merck). The experiments were carried out at room temperature (21–23° C). NAD^+, NADH and alcohol dehydrogenase (lyophilized, 470 U/mg protein) were from Fluka. Direct measurements of pH shifts near the bilayer lipid membrane (BLM) were carried out with the help of a pH microelectrode as described earlier [11,14]. Briefly a glass-insulated tip-sensitive bent antimony pH microelectrode was driven perpendicular to the surface of the BLM through the open space in the top part of the cell made of polytetrafluoroethylene (PTFE). Typically the electrode tip was about 10 μm. The smooth approach of the microelectrode to the membrane was carried out using a hydraulic microdrive system. The electric scheme contained a Keithley 617 electrometer connected to a personal computer. Measurements and data analysis were performed using the ASYST software package. Voltages were recorded routinely every second. The microelectrode speed was 4 μm per second. The time of microelectrode response was less than 1 s.

3. Results

Fig. 1 shows pH profiles near a BLM induced by the addition of sodium acetate (curve 1) or alcohol dehydrogenase (curve 5) or both substances together (curves 2–4).

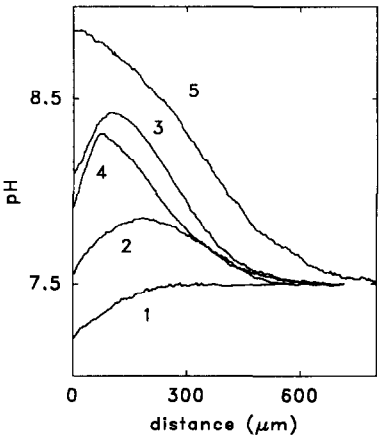

Fig. 1. A series of steady-state pH profiles near a BLM induced by acetate permeation and alcohol dehydrogenase (AlDH) functioning. The solution consisted of 1 mM Tris, 3 mM NADH, 100 mM KCl, pH 7.5. Curves (1), (2) and (3) contained additionally: 20 mM sodium acetate and 4 mM acetaldehyde at the *trans* side; AlDH was added at the *cis* side: (2) 0.04 mg/ml and (3) 0.08 mg/ml; (4) 4 mM acetaldehyde *trans*, 0.18 mg/ml AlDH *cis* and 30 mM sodium acetate *trans*; (5) 4 mM acetaldehyde *trans*, 0.04 mg/ml AlDH *cis*.

The enzyme was given to the compartment where the microelectrode was placed, further called *cis* side. After the transmembrane permeation of acetaldehyde from *trans* to *cis* the substrate was reduced due to the presence of NADH at the *cis* side and a local pH gradient was generated [18]. After sodium acetate was added to the *trans* side it permeated through the membrane in its neutral form and dissociated at the *cis* side releasing hydrogen ions in the aqueous layer adjacent to the BLM [16,17]. As demonstrated in Fig. 1 (curve 2) the pH profile measured for both reactions proceeding simultaneously retained features of every single profile and had a 'bell-like' shape. The distance from the BLM surface to the pH maximum

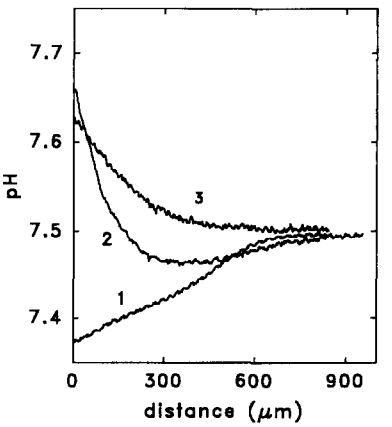

Fig. 2. A series of steady-state pH profiles near a BLM induced by acetate permeation and alcohol dehydrogenase (AlDH) functioning in the case of ethanol oxidation. The buffer solution: 1 mM Tris, 3 mM NAD^+, 100 mM KCl, pH 7.5. The *trans* compartment contained 170 mM ethanol. (1) 0.12 mg/ml AlDH *cis*; (2) 0.12 mg/ml and 20 mM sodium acetate *cis*; (3) 20 mM sodium acetate *cis*.

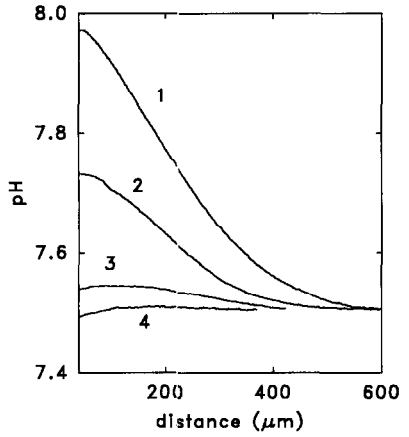

Fig. 3. A group of steady-state pH profiles near a BLM induced by acetate and ammonium permeation. The solution contained 1 mM Tris, 1 mM Mes, 100 mM choline chloride, pH 7.5. 30 mM ammonium nitrate were added to the *trans* side of the membrane. The concentration of sodium acetate at the *trans* side was varied: (1) no acetate, (2) 90 mM, (3) 190 mM, (4) 250 mM.

decreased from 185 μm (curve 2) to 75 μm (curve 4) when the enzyme concentration was increased from 0.04 to 0.18 mg/ml.

Nonmonotonic pH profiles were registered too when acetaldehyde was substituted by ethanol. In this case sodium acetate was added at the opposite side of the membrane (Fig. 2). The difference between an arbitrary chosen pH value and the bulk pH value took on positive and negative values (Fig. 2, curve 2).

Further experiments were carried out in a system where the catalytic proton consuming reaction was replaced by the reaction of proton binding by ammonium which easily permeates through the membrane and shifts the local pH to alkaline values [16]. Fig. 3 shows a series of pH profiles

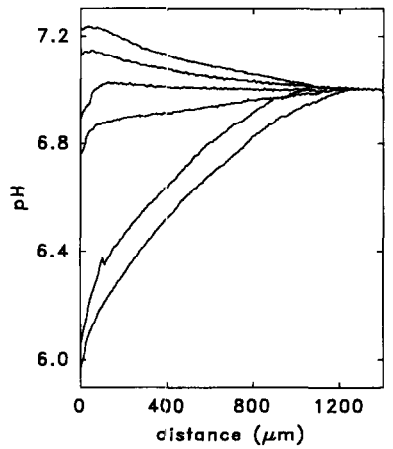

Fig. 4. Set of steady-state pH profiles near a BLM induced by nigericin-mediated K^+/H^+-exchange and acetate permeation. The buffer consisted of 1 mM Tris, 1 mM Mes and 100 mM choline chloride. pH was 7.0. The membrane forming solution contained 140 mM nigericin. The upper curve, 20 mM KCl *trans*; lower curves, 4 mM, 8 mM, 10 mM, 14 mM, 18 mM and 26 mM sodium acetate *trans*, respectively.

near BLMs in the presence of acetate and ammonium at the *trans* side. These pH profiles lack the nonmonotonic character of the ones demonstrated in Fig. 1.

The third kind of profiles studied was originated from the simultaneous proceeding of a hydrogen ion flux across the membrane induced by acetate and an oppositely directed nigericin-mediated proton flux in the presence of a transmembrane KCl gradient [22]. Fig. 4 shows a series of pH profiles in the presence of acetate and nigericin. At high acetate concentrations there were minor shifts from the monotonic course of the pH profiles. A pH maximum appeared at a distance of about 50 μm from the BLM.

All profiles were highly reproducible. The concentration shifts were determined with an error lower than 5% and the distance measurements were made with an absolute error not higher than ±8 μm which arises due to the lack of a definite reference position for the membrane surface (see [14]).

4. Discussion

The proton concentration $c(x)$ appeared to be a very complicated function of the distance x from the membrane when both the catalytic proton consuming reaction and the dissociation of a weak acid take place within the unstirred layer. For convenience a function $F(x)$ is defined as:

$$F(x) = c(x) - c_b \quad (2)$$

In order to understand the origin of the deviation from an exponential profile the velocity of the catalytic reaction was varied (Fig. 1). The finding that higher enzyme concentrations lead to a decrease of the distance where $F(x)$ reaches a maximum enables us to propose the mechanism of the formation of complex pH profiles: the proton release by acetate and the proton uptake by acetaldehyde are separated in space. The former process takes place predominantly near the surface of BLMs due to its high rate whereas the latter process runs at a measurable distance from the membrane. This distance is a function of the concentration of the enzyme. At a low rate of the enzymatic reaction acetaldehyde has to cross a long distance before it can be reduced by NADH.

This assumption was checked by substituting the slow catalytic reaction of proton consumption for a fast one, i.e., using a permeating amine instead of the enzymatic system. Since both reactions have comparable velocities monotonic profiles are expected. As the experimental results confirmed this hypothesis it can be concluded that the complex pH profiles are caused by the low reaction rate of alcohol dehydrogenase.

Besides it is possible to get space separated reactions of proton uptake and release using an ionophore instead of an enzyme or ammonium. Association and dissociation of an ion-ionophore complex are considered to be heterogeneous [23] while proton transfer reactions with acetate homoge-

neous [24]. In this system the observed deviations of the pH profiles from monotonic ones (Fig. 4) are not so pronounced as in the case of the enzymatic reaction (Fig. 1). Obviously, the space resolution of the microelectrode is to low to separate the pH shifts induced by nigericin mediated K^+/H^+-exchange and acetate permeation.

It is generally accepted that the thickness of the unstirred layer is related to the zone where deviations from the bulk phase concentration occur [1,2]. However in the case of nonmonotonic concentration profiles δ derived from Eq. (1) and the zone of concentration shifts δ_s can differ considerably. For example the width of the unstirred layer calculated for curve 2 (Fig. 1) according to Eq. (1) equals 23 μm whereas the zone of pH shifts reaches at least 400 μm. Besides in the case of exponential concentration profiles (Fig. 1, curve 1) the unstirred layer thickness is equal to 94 μm under the same stirring conditions. Thus in order to estimate δ_s under arbitrary conditions another approach should be found. We believe that it is more justified to determine δ_s in terms of the length of the horizontal side of a rectangle, which has the same area as the one included between $F(x)$ and the axes of a rectangular system of coordinates. The vertical side of the rectangle is equal to the largest concentration difference between the interface and the bulk phase, $\max(F(x))$. The mathematical treatment of the problem looks like:

$$\delta_s = \frac{1}{\max(F(x))} \int_{x=0}^{\infty} F(x)\,dx \qquad (3)$$

One possible solution of Eq. (1) is a simple exponential function that can be used to determine δ with the help of a numerical iteration programme [14]:

$$c(x) = |c_s - c_b|e^{-x/\delta} + c_b \qquad (4)$$

As it can be seen easily δ is equal to δ_s in the case of exponential concentration profiles.

After converting the pH profiles of Fig. 1 into concentration profiles δ_s was calculated according to Eq. (3). We got the following values for δ_s: curve 1, 86 μm; 2, 357 μm; 3, 367 μm; 4, 321 μm; 5, 530 μm. As expected the value of δ_s computed for curve 1 is close to 94 μm determinated for δ according to Eq. (4).

Unfortunately, the application of Eq. (3) is also limited. All $F(x)$ must have the same sign. Otherwise one can obtain misleading results when calculating the integral. We observed this kind of pH profiles in the case of both ethanol oxidation and acetate diffusion (Fig. 2). It seems justified to transform Eq. (3) into

$$\delta_s = \frac{2}{\max(F(x))^2} \int_{x=0}^{\infty} (F(x))^2\,dx \qquad (5)$$

Eq. (5) deals with the square of $F(x)$ which makes all values positive in sign. The coefficient 2 was introduced in order to obtain similar values for δ_s (Eq. (5)) and δ (Eq. (4)) in the case of common exponential concentration profiles. Applied to the above mentioned concentration profiles (Fig. 1) Eq. (5) gives the following values for δ_s: 92 μm, 546 μm, 593 μm, 500 μm and 810 μm for the curves 1 to 5, respectively. The values of δ_s calculated with the help of the Eqs. (3) and (5) are equal if the profile is an exponential one (curve 1). Otherwise Eq. (5) gives somewhat higher values (curves 2–4).

The procedure proposed for the estimation of the size of the concentration boundary layer in the case of complex concentration profiles arising due to chemical reactions within the USLs is an empirical one. It allows to overcome the limitations of the classical definition (Eq. (1)) of an unstirred layer but δ_s can not be derived from the transmembrane flux and the reaction rates. The peculiarities of near-membrane concentration profiles due to both mass transfer restrictions and rate limited chemical reactions were predicted by LeBlanc [25]. His theoretical treatment of weak acid diffusion allowed to define a 'reaction layer' assumed to be much smaller than δ. The nanometer scale of the reaction layer was essential for the theory to work but it was impossible to check the presence of this layer experimentally. In the case of a slow catalytic reaction the size of the reaction layer is comparable with the width of the unstirred layer. Apparently for this reason we were able to observe complex concentration profiles near membranes with the help of microelectrodes.

Further efforts should be made to estimate the thickness of δ_s from the flux values and the reaction rates. The present work enables us to believe that the pH microelectrode technique is useful for this purpose at least in the case of chemical reactions the rate of which can be varied by the alterations of the enzyme concentration. The observation that δ_s can differ substantially from the width of the unstirred layer derived according to the conventional definition given in Eq. (1) is important for the study of transport and transformation processes in biological systems where chain reactions are common.

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