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INTERACTIONS OF HAEMOGLOBIN WITH ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS IN MONOMOLECULAR LIPID LAYERS

I. SZUNDI [a], J.G. SZELÉNYI [b], J.H. BREUER [c] and A. BÉRCZI [a]

[a] *Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, P.O.B. 521;* [b] *National Institute of Hematology and Blood Transfusion, Budapest and* [c] *Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest (Hungary)*

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Summary

The role of red cell membrane lipids in the membrane-haemoglobin interaction was studied by measuring the surface potential and surface pressure of monomolecular lipid layers interacting with haemoglobin. Lipids of the outer and inner half of the red cell membrane were compared in respect to their haemoglobin-binding capacity.

It was shown, that haemoglobin molecules interacted readily with the inner layer lipid film in acidic pH regions. This interaction is reduced as pH is increasing but still exists in the physiological pH range. It is in contrast with the findings for the outer layer lipid film, where only a partial interaction could be shown at pH 4, which was reduced to zero reaching the physiological pH range.

It can be concluded from titration experiments that the process of haemoglobin binding as reflected in the measured parameters is irreversible. The result of this model experiments support the hypothesis on phosphatidylserine binding sites for haemoglobin in the inner side of red cell membrane.

Introduction

It has been shown that erythrocyte membrane contains different amounts of haemoglobin (Hb), depending on the conditions of preparation [1—5]. The Hb content is dependent on the type of Hb [2,3] and on the integrity of the membrane as well [6,13]. It has long been a question as to which components of the membrane are involved in this binding. Ghosts are capable of binding a given

Abbreviation: Hb, haemoglobin.

amount of Hb, as was shown by ^{125}I-labelled Hb experiments [7]. The most plausible assumption is that spectrin, a high molecular weight protein on the inner surface of the membrane, may be responsible for Hb binding, but recently contradictory data have been published about the role of this protein [8]. Others, mainly from the number of tightly bound Hb molecules under steady-state condition, deduced that band 3, a carbohydrate-containing protein which spans through the membrane, might have some role in Hb binding [28].

In our previous work it was shown that not the isolated membrane proteins, but the whole membrane, has the most favorable effect on stabilizing Hb [9]. On the other hand, red cell membrane binds Hb at two different sites at least, with binding constants differing from each other by two orders of magnitude. Therefore, it seems that proteins are not the only membrane components involved in this problem [28].

The role of membrane lipids in this interaction has not been investigated to date. Several studies on the interaction of Hb with various lipids of different origins show the rather strong interaction of Hb with phospholipids, but cholesterol lowers this effect in comparison with other soluble proteins [19]. Erythrocyte membrane lipids exhibit a great chemical heterogeneity and an asymmetrical distribution between the inner and outer regions. According to different authors [10—12,16], the outer part is composed mainly of sphyngomyelin and phosphatidylcholine, while the inner part contains predominantly phosphatidylserine and phosphatidylethanolamine. Moreover, each class of phospholipid represents a collection of individual molecules with different properties. There are no general rules, however, which could be used to predict lipid-protein interactions in particular cases, and hence, to draw conclusions on the role of erythrocyte membrane lipids in interaction with Hb, lipids isolated from erythrocyte membrane are needed.

The aim of this work was to study the role of membrane lipids in the membrane-Hb interaction, to decide whether there is any difference between the interactions of Hb with lipids of the outer and inner halves of the lipid bilayer, and to elucidate the mechanism of interaction using Hb and red blood cell lipids as a model system.

Materials and Methods

Preparation of haemoglobin. Hb was prepared from washed normal erythrocytes by haemolysis with 1 vol. distilled water, and subsequent gel filtration on Sephadex G-25.

Preparation of red blood cell lipids. The preparation of lipids from red blood cells was performed by the method of Dodge and Phillips [14]. The individual phospholipid fractions were separated by preparative thin-layer chromatography on silica gel HR 0.5 mm thick, in chloroform/methanol/glacial acetic acid/water (50 : 28 : 8 : 3.5, v/v) to which butylated hydroxytoluene was added to prevent autooxidation during chromatography. The individual phospholipid fractions were scraped off and extracted from the silica gel with

* 40 mM CH_3COOH/40 mM H_3PO_4/40 mM $B(OH)_3$ diluted 50 8 mM and adjusted by 0.2 M NaOH.

chloroform/methanol (1 : 4, v/v). The solutions containing phosphatidylcholine and sphingomyelin fractions were pooled, and evaporated to dryness in vacuo at 37°C. The lipid was transferred with heptane to an ampoule, which was flushed with N_2, sealed and stored at −25°C, and the lipid was used as 'the outer half of the membrane bilayer'. The pooled phosphatodylserine and phosphatidylethanolamine was processed as above, and used as 'the inner layer of the surface membrane'. The total phospholipids were isolated from the original lipid extracts by preparative TLC in petroleum ether/acetone (85 : 15, v/v) containing butylated hydroxytoluene. In this system phospholipids remained at the origin. This area of silical gel was scraped off and extracted as above. The solution was evaporated to dryness, the residue was dissolved in heptane and the solution was stored in sealed ampoules at −25°C. The lipid phosphorus was determined by the method of Chen et al. [15].

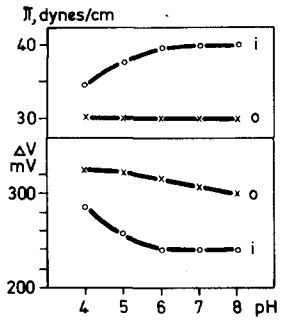
Monolayer measurements. Film-penetration experiments were carried out in a constant-area circular trough equipped with a magnetic stirrer. The trough was made of Teflon and had facilities for exchange of the subphase solutions during measurements. The surface pressure was measured by the Wilhelmy plate method as described earlier [30]. Changes in the surface potential were monitored by an electrometer using an ionizing electrode (1 cm^2 ^{241}Am foil) and a calomel electrode connected to the subphase via a salt bridge. The accuracy of potential measurements was ±5 mV. The subphase water used was tridistilled, the second time from permanganate. Monolayers were spread from heptane solutions by addition of small amounts to the surface, the excess of the film being removed with a capillary tube. Hb was introduced by injections of more concentrated solutions beneath the monolayer or by exchanging the subphase volume by using a peristaltic pump. For pH adjustment, 8 mM Britton-Robinson buffer * was used. Titration of the film was carried out by injections of NaOH and HCl solutions into the subphase buffer or by replacement of the subphase volume using the exchange technique. All experiments were made at room temperature.

Results

The main difference between the inner and outer layer lipid compositions used in this study comes from the presence of negatively charged phosphatidylserine molecules. As shown in Fig. 1, the surface potential and pressure of the inner layer lipid film change in the region pH 4—5 due to the dissociation of carboxyl groups [19]. The outer layer lipid film has practically unchanged characteristics in the pH range studied, i.e. the zwitterionic nature is maintained. The same results were obtained in experiments when the method of the surface potential dependence upon the salt concentration in the subphase [20] was used.

To distinguish between electrostatic and hydrophobic natures of the Hb-lipid interaction, measurements were carried out at different subphase pH values. The initial film pressure in every case was adjusted to 30 dyn/cm, at which the molecular packing in the monolayer is believed to be similar to that in a red cell

* 40 mM CH_3COOH/40 mM H_3PO_4/40 mM $B(OH)_3$ diluted to 8 mM and adjusted by 0.2 M NaOH.



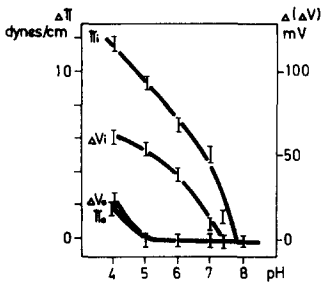


Fig. 1. pH dependences of surface pressure and potential for the inner (i) and outer (o) layer lipid films.

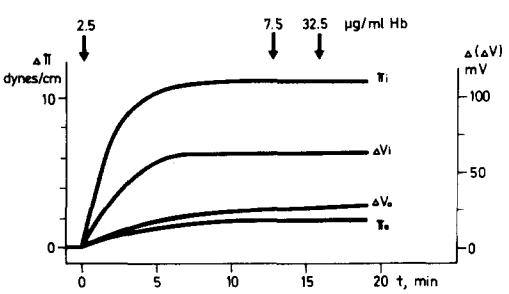
Fig. 2. Dependence of Hb binding upon subphase pH. $\Delta\pi$ and $\Delta(\Delta V)$ represent changes in surface pressure and potential, respectively. The initial surface pressure was 30 dynes/cm.

membrane [21]. Concentrated solutions of Hb were injected into the subphase solution to yield a saturation level in surface pressure and potential changes. The results for both types of monolayers are plotted in Fig. 2. The increase in surface pressure can be taken as a measure of penetration, while the surface potential changes indicate Hb binding in general.

It is seen that Hb molecules interact with the inner layer lipid film in the acidic pH region, and to a much smaller extent at physiological pH. In the case of outer layer lipid film, no effect could be detected except for a small one at pH 4.

Studies on model systems indicate that in interaction with lipids Hb displays features unusual for a soluble protein; its effect is comparable with that of integral membrane proteins [23], and during adsorption no equilibrium seems to exist [23,24]. Additional studies were undertaken to provide a deeper insight into the binding process.

Fig. 3 shows the development of the surface pressure and potential changes during consecutive Hb injections into the water phase at pH 4. The saturation level is reached on the first addition, and a further increase of Hb concentration causes no effect. Saturation was observed even at lower concentration, e.g. 0.6 μg/ml; at very low Hb concentrations the extremely long time intervals



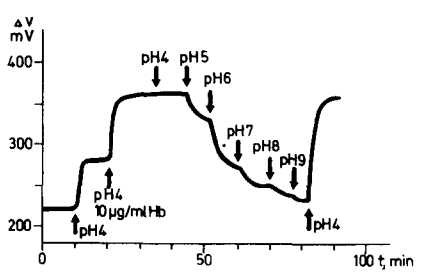


Fig. 3. Adsorption of Hb on the lipid films at pH 4. Arrows indicate addition of Hb to reach final concentration as shown.

Fig. 4. Titration of the inner layer lipid-Hb film. Arrows show pH and concentration values of the solutions used for subphase exchange. The film had 30 dynes/cm initial surface pressure on distilled water.

required for the process to be completed make the measurements rather inaccurate.

From this observation it seems quite probable that the penetration process is a non-equilibrium one; further evidence is given in Fig. 4, where the results of subphase exchange experiments are presented. After formation of the inner layer lipid film on distilled water, the subphase was exchanged for buffer solution (pH 4) and then for 10 μg/ml Hb solution in the same buffer. When the penetration process was completed, Hb was washed out from the subphase with the same buffer solution. Neither surface pressure nor potential changed during this procedure.

On exchange of the subphase buffer for ones with increasing pH values, stepwise changes in surface potential were observed. No similar changes in surface pressure appeared. The surface potential changes were found to be reversible, i.e. no release of bound Hb occurred even at pH 9; only titration of the lipid-protein film took place. Similar results were found in experiments in which titration was carried out by injecting NaOH or HCl solutions into the subphase containing the injected Hb.

Discussion

It is known from studies concerning the binding of Hb to various charged and uncharged monolayers [24,25] and liposomes [23] that interactions between charged groups as well as hydrophobic parts of the protein and lipid molecules take place simultaneously.

Variation of the number of charges on the protein molecules and the monolayer by changing the subphase pH is assumed to alter mainly the electrostatic part of the interaction.

Due to the presence of phosphatidylserine, the inner layer lipid film has a net negative charge, while the outer layer lipid film is zwitterionic in the pH range studied.

The number of positive charges on a Hb molecule increases linearly with decreasing pH below the isoelectric point (pH 6.8) [22], and therefore the surface pressure and potential changes of the inner layer lipid film presented in Fig. 2 suggest that the electrostatic forces are the most important ones in the interaction. Since the surface pH is lower than the bulk one for negatively charged films, the effect is observed even above the isoelectric point of the protein. The measured parameters indicate no interaction in the case of the outer layer lipid film, except at pH 4 where dissociation of Hb to subunits should also be considered.

Similar results concerning the importance of electrostatic interactions in the binding of Hb as shown above have been found for red cell membranes [3]. It has also been concluded [27,28] that only the inner side of the red cell membrane is capable of binding a substantial amount of Hb.

Our results confirm this finding in relation to membrane phospholipids, and show that phosphatidylserine can in fact provide binding sites for Hb, as was assumed [28] on the basis of experiments on red cell membranes.

Although electrostatic forces are of primary importance in the interaction, the binding process seems to be more complicated. There exists a saturation

level of Hb adsorption (at least as reflected in surface pressure and potential changes) at very low Hb concentration (10^{-8} M), a fact which is unusual for a reversible process. Hb also appeared to be unusually effective in inducing ion permeability of liposomes containing phosphatidylserine [23]. From the titration experiments it can be concluded that the process of Hb binding, as reflected in the measured parameters, is an irreversible one.

Long-range electrostatic forces seem to be involved in one of the first steps of the Hb adsorption process, followed by some changes in the conformation of the protein molecule. The extents of such conformational changes are probably dependent on the counter-charge distribution, the hydrophobicity of the adsorbent [26], etc., and determine the reversibility of binding. As far as the functional activity of the adsorbed Hb is concerned, it is shown that in an arrangement similar to that at an air/water interface the redox activity is retained [29]. There seems to be no reason for inactivation in our case either, since Hb adsorbed on a charged lipid film at high surface pressure is considered to have a more intact structure [24] than it has at an air/water interface.

References

1 Hanahan, D.J. and Ekholm, J.E. (1974) Methods Enzymol. 31, 168—172
2 Klipstein, F.A. and Ranney, H.N. (1960) J. Clin. Invest. 39, 1894—1899
3 Fischer, S., Nagel, R., Bookchin, R.M., Roth, E.F. and Teller-Nagel, I. (1975) Biochim. Biophys. Acta 375, 422—433
4 Mitchell, C.D., Mitchell, W.B. and Hanahan, D.J. (1965) Biochim. Biophys. Acta 104, 348—358
5 Hollán, S.R., Szelényi, J.G., Hasitz, M., Szász, I. and Gárdos, G. (1977) Phys. Bohem. 26, 219—224
6 Szelényi, J.G., Breuer, J.H., Györffy, Gy. and Hasitz, M. (1972) Haematologia 6, 327—340
7 Szelényi, J.G. and Hollán, S.R. (1977) IUB Congress Abstr. 284, Hamburg
8 Cassoly, R. (1978) FEBS Lett. 85, 357—360
9 Szelényi, J.G. and Hollán, S.R. (1978) Proc. 8th Congress of the Hungarian Society of Haematology, p. 145
10 Gordesky, S.E. and Marinetti, G.V. (1973) Biochem. Biophys. Res. Commun. 50, 1027—1031
11 Whitley, N.M. and Berg, H.C. (1974) J. Mol. Biol. 87, 541—561
12 Gordesky, S.E., Marinetti, G.V. and Love, R. (1975) J. Membrane Biol. 20, 111—132
13 Palek, J., Liu, S.C. and Snyder, L.M. (1978) Blood 51, 385—395
14 Dodge, J.T. and Phillips, G.B. (1967) J. Lipid Res. 8, 667—675
15 Chen, P.S., Jr., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
16 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83—96
17 Bretscher, M.S. (1972) Nat. New Biol. 236, 11—12
18 Weidekamm, E., Brdiczka, B. and Wildermuth, M. (1978) Mol. Biol. Rep. 4, 25—28
19 Papahadjopoulos, D. (1968) Biochim. Biophys. Acta 163, 240—254
20 McDonald, R.C. and Bangham, A.D. (1972) J. Membrane Biol. 7, 29—53
21 Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97—107
22 Brunori, M., Wyman, J., Antionini, E. and Rossi-Farelli, A. (1965) J. Biol. Chem. 240, 3317—3324
23 Kimelberg, H.K. (1976) Mol. Cell. Biochem. 10, 171—190
24 Fromherz, P. (1971) Biochim. Biophys. Acta 225, 382—387
25 Matalon, R. and Schulman, J.H. (1959) Discuss. Faraday Soc. 6, 27—39
26 Fromherz, P., Petters, I., Müldner, H.G. and Otting, W. (1972) Biochim. Biophys. Acta 274, 644—648
27 Shaklai, N., Yguerabide, I. and Ranney, H.M. (1977) Biochemistry 16, 5585—5592
28 Shaklai, N., Yguerabide, I. and Ranney, H.M. (1977) Biochemistry 16, 5593—5597
29 Scheller, F. (1977) Bioelectrochem. Bioenerg. 4, 490—499
30 Szundi, I. (1978) Chem. Phys. Lipids 22, 153—161