ON THE MOLECULAR SIEVING PROPERTY OF THE HUMAN ERYTHROCYTE MEMBRANE AND THE LOCALIZATION OF SOME GLYCOLYTIC ENZYMES IN THE CELL

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1. Introduction

There are some hints that the glycolytic enzymes could form, in vivo, complexes which allow them to function as a clustered multienzyme system and this would afford them kinetic and regulatory advantages [1,2]. Present evidence suggests that such a multienzyme complex, if it exists, is dissociated when the cell is disrupted and its content is diluted [3]. This problem therefore can be studied only when the cell structure is not far from the living state.

Our approach described here rests on the measurement of the release of several proteins, glycolytic enzymes and others, from human erythrocytes swollen in slightly hypotonic media. In such conditions the cells take up water, their swelling distends the membrane on which pores appear, which make the membrane permeable to otherwise non-penetrating solutes [4]. The osmotic pressure difference which builds up for some time will force some of the erythrocyte content out of the cell whereupon the osmotic difference is reduced and the physiological 'permeability' of the cell is restored. It has been observed that in this stage the pores of the distended membrane confer a molecular sieving property on the membrane [5].

Abbreviations: GAP-DH, D-glyceraldehyde-3-phosphate; NAD oxidoreductase (phosphorylating), EC 1.2.1.12; G6P-DH, D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49; Hb, haemoglobin; LDH, L-lactate; NAD oxidoreductase, EC 1.1.1.27; 6-PGDH, 6-phospho-D- gluconate: NADP oxidoreductase (decarboxylating), EC 1.1.1.44; PGK, ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3

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The cell content which is squirted out during this process must be richer in components localized near the membrane (and not bound to it) than in those localized evenly or further inside. Moreover, as the pore size is not too great, molecular sieving would have an additional effect of retaining molecules of higher molecular weight. There is some reason to believe that the glycolytic enzymes rather than haemoglobin are localized under the membrane of the cell, to be able to interact with the surrounding medium. We present here evidence that 6 proteins including Hb are lost from the swollen cells to a hypotonic solution in amounts which suggest a general behaviour of such an erythrocyte membrane as a molecular sieve for macromolecules. Moreover, the surface near localization of the 3 glycolytic enzymes studied (PGK, GAP-DH and LDH) is reflected in the fact that they are liberated in higher amounts.

2. Materials and methods

Blood freshly drawn from healthy donors of both sexes was obtained from the National Institute of Haematology and Blood Transfusion, Budapest. As an anticoagulant an acid-citrate-dextrose solution pH 7.2 (formula B [6]), was used. Following centrifugation at 900 × *g* for 15 min the plasma and the leukocytes were removed by suction and the sedimented cells were washed 3 times with an equal vol. 143 mM KCl, which contained 10 mM Na-phosphate, pH 7.5.

Total haemolysis: to 1 vol. sedimented cells 9 vol. distilled water were added. Partial haemolysis: to 1 vol.

sedimented cells 4 vol. NaCl solutions, ranging from 154–68 mM were added. The suspensions were gently mixed and allowed to stand for 10 min at 0°C. The non-lysed cells were centrifuged at 900 × *g* for 15 min. All manipulations were carried out at 4°C. To all haemolysates 10 mM NADP and 10 mM dithiothreitol were added to protect G6P-DH from inactivation [7].

Hb and enzyme activities were determined in the haemolysates containing the membrane fragments of the lysed cells in the presence of 0.1% Triton X-100.

GAP-DH [8] was recrystallized 8 times and was free of PGK. NADH and NADP (Reanal, Hungary) were 80% pure as determined enzymatically. Triton X-100 was purchased from Calbiochem and stigmasterol (stigmastadiol-(5,22)-dien-3β-ol) from Merck. PGK and all the other chemicals were Boehringer preparations of reagent grade.

Hb was determined according to [9]. Sedimented cells, 1 ml, contained 260–280 mg Hb. PGK activity was determined by the coupled reaction with GAP-DH [10] and 10 mM hydrazine sulfate was added to trap the glyceraldehyde-3-phosphate formed during the reaction. GAP-DH was assayed in the presence of added excess PGK [11] and LDH in the presence of pyruvate and NADH [10]. G6P-DH was determined in the presence of glucose-6-phosphate and NADP [12] and suitable amounts of haemolysate to obtain about 0.03 A_{340} increase/min in a 1 cm light path cuvette. Under these conditions the 6-PGDH activity of the haemolysates does not interfere with the measurements. 6-PGDH was assayed as in [13]. The specific activities of the enzymes in the total haemolysates were: PGK, 300 ± 25; GAP-DH, 161 ± 16; LDH, 157 ± 10; G6P-DH, 11 ± 1 and 6-PGDH, 11 ± 1 U/g Hb. One unit (1 U) = conversion of 1 μmol substrate/min at 37°C. All measurements were made in a Varian Techtron 635 spectrophotometer equipped with a recorder and a thermostatted cuvette house.

Cholesterol was determined by gas chromatography of Triton-treated, membrane-containing haemolysates with stigmasterol as an internal standard. Chromatography was carried out after trifluoroacetylation of the sterols [14]. The lyophilised samples were suspended in 1 ml distilled water then extracted with 2 ml chloroform:methanol (2:1) containing 5 μg/ml stigmasterol. The membrane-free haemolysates did not contain detectable amounts of cholesterol.

3. Results and discussion

A series of sedimented red cell samples obtained from blood drawn from a single donor was treated with NaCl solutions of decreasing osmolarity and the liberation of 6 proteins and cholesterol into the cell-free supernatants of the partial haemolysates was determined. The data obtained with blood samples drawn from 23 donors are summarized in fig.1, where we plotted the ratio of protein to cholesterol liberation versus the degree of cell disruption. The latter is defined as the fraction of the total cholesterol found in the cell-free supernatants. Liberation of cholesterol is an appropriate measure of cell disruption since Hb

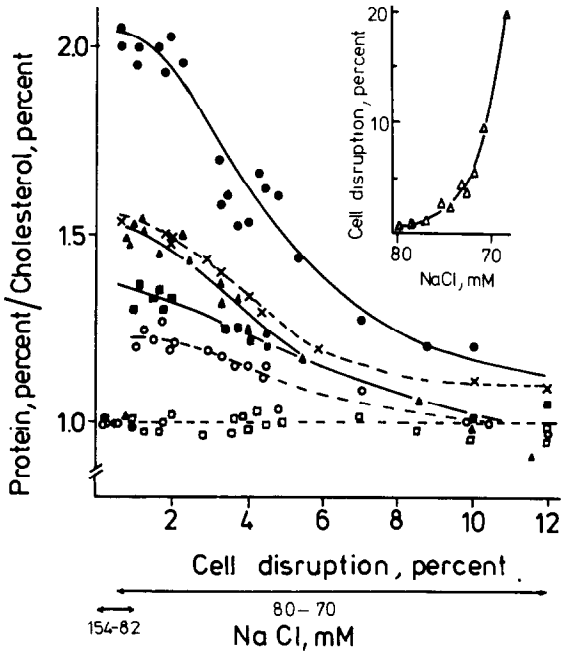

Fig.1. Release of protein and cholesterol into partial haemolysates. Cell disruption is defined as cholesterol determined in the cell free haemolysates. Below the abscissa only the range of NaCl concentrations used are indicated since the percentage of cell disruption varies with the individual blood sample. Data obtained from blood samples drawn from 23 different donors. 100% = values obtained in the total haemolysates. (•) PGK; (×) Hb; (○) 6-PGDH; (▪) LDH; (▲) GAP-DH; (□) G6P-DH. Molecular weights × 10^{-3} for erythrocyte enzymes in the order of the symbols: 48 [16], 68 [17], 104 [18], 140 (rabbit enzyme [19]), 144 [20], 210 [21]. The insert shows cell disruption as a function of NaCl concentration in a typical experiment.

might be lost due to other effects too and cholesterol derives only from the membrane fragments of the disrupted cells. Under the experimental conditions the membrane fragments remain in the cell-free haemolysates.

The decrease in NaCl concentration down to ~82–80 mM (the figure slightly varies with the blood sample) results only in the disruption of a small fraction of the cell population (0.2 – ~1%). This is reflected in the equal release of all cell constituents tested, including cholesterol: their ratio to the latter equals 1. We assume that these lysates derive from cells damaged during manipulations and from the oldest cells which are most sensitive to hypotonicity [15].

However, in the sample treated with slightly more hypotonic solutions (e.g., at/or below 80 mM NaCl) the uniform release pattern disappears: the smaller the molecular weight of a protein the higher the percentage in which it is lost to the medium. Throughout the whole range of hypotonicity studied G6P-DH, the highest molecular weight protein tested, appears in the partial haemolysates in the same percentage as cholesterol, which suggests that this protein is liberated only from disrupted cells. On the other hand, at low hypotonicity all other proteins appear in higher percentage than expected on the basis of cell disruption. This phenomenon occurs in a narrow range of hypotonicity which varies somewhat with the blood sample obtained from different donors. If hypotonicity is further increased cell disruption becomes dominant and the ratio of protein to cholesterol liberation asymptotically approaches unity.

The data suggest that within a certain range of hypotonicity the membrane of the swollen erythrocytes behaves as a molecular sieve for macromolecules.

From the experimental data one can calculate the fraction of any protein released through molecular sieving ($C_i^{\text{non-lytic}}/C_i^{\text{T}}$) according to eq. (1):

$$\frac{C_i^{\text{non-lytic}}}{C_i^{\text{T}}} = \frac{C_i^{\text{measured}}}{C_i^{\text{T}}} - \frac{C_i^{\text{lytic}}}{C_i^{\text{T}}} \qquad (1)$$

where C_i^{T} = the concentration of protein i in the total haemolysate and $C_i^{\text{lytic}}/C_i^{\text{T}}$ is the fraction originating from cell disruption. This latter value is identical with the fraction of the total cholesterol released into the supernatant. If the protein radius r_i is smaller than r the radius of the pores, the molecule can cross a pore only if its center passes within a virtual pore radius $r - r_i$. The probability of crossing for a large molecule is therefore less than the corresponding probability for a small one. If we assume that the proteins are uniformly distributed in the cell then – in a first approximation – the fraction of protein i lost through molecular sieving in a given time interval would be proportional to the cross sectional area of the virtual pore. The relationship holds if a practically unique pore radius is assumed, eq. (2):

$$\frac{C_i^{\text{non-lytic}}}{C_i^{\text{T}}} = k\Pi\,(r - r_i)^2 \qquad (2)$$

where k is a function of the osmotic pressure in the medium. Figure 2 shows the correlation between the protein radii and the square root of the non-lytic liberation of proteins at two different NaCl concentrations.

Practically linear relationships were found that included Hb and 6-GPDH, and the intercepts on the abscissa were around the radius of G6P-DH. This supports the assumption of a practically unique size of the pore radii and suggests that the radius of the transiently-formed pores in the membrane does not exceed

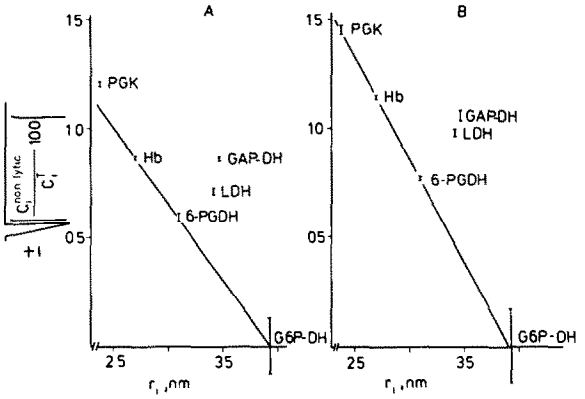

Fig.2. Correlation between non-lytic release of proteins and molecular radius. For the calculation of non-lytic release of proteins cf. text. Molecular radii were calculated from molecular weights and the specific volumes were taken as 0.73 ml/g [22]. (A) NaCl, 75.5–76.5 mM, cell disruption, 1.5% B; NaCl, 72.5–73.5 mM, cell disruption, 4%. Bars represent standard error.

the radius of G6P-DH. However, the points for GAP-DH and LDH fell above the straight lines, and at lower hypotonicity the same held for PGK, too (fig.2A). These proteins are liberated in amounts significantly higher than expected from simple molecular sieving of a homogeneous solution of proteins. This result would be expected if the glycolytic enzymes are localized near the surface of the cell, their concentration being higher at the region where molecular sieving occurs than elsewhere in the cell. Naturally, if cell disruption becomes dominating the contribution of preferential release due to surface-near localization becomes less and less discernible (cf. fig.1).

The results presented argue for the molecular sieving property of the erythrocyte membrane in slightly hypotonic media and the surface-near localization of 3 enzymes of the glycolytic pathway.

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