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Encapsulation of proteins into human erythrocytes: a kinetic investigation

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Moderate osmotic shocks of human erythrocytes by hypotonic dialysis (0.06 mosmol/kg) induce cell swelling and formation of pores, without causing apparent lysis. Using ^{125}I-labeled macromolecules of different molecular weight and net charge, we followed the kinetics and efficiency of their encapsulation into erythrocytes. After a 20–30 min period of cell dialysis, macromolecules of up to 50 kDa begin diffusing into the swollen cells by a process which can be described by a first-order two-compartment kinetics. Adsorption to the external cell surface was insignificant, while adsorption to the inner membrane surface was substantial (15–20%) only for positively charged proteins, at physiological pH. After resealing, pores of a 12–14 kDa cut-off might remain open allowing some release of entrapped material (20–30%), depending on the final cytocrit, while the remaining might be associated with inner membrane or cytosolic components. Although the method of hypotonic dialysis is known to affect minimally the biophysical and immunological properties of red blood cell membranes [1], the interaction of encapsulated material with cell constituents would need to be further assessed when considering red cells as macromolecular carriers.

Introduction

The use of red blood cells (RBCs) as carriers or bioreactors [2–3] demands the selective entrapment of a specific macro or micromolecular component, obtained by a controlled osmotic shock induced by either hypotonic dilution or hypotonic dialysis followed by resealing [4–6]. The hypotonic dialysis, involving a gradual decrease in ionic strength [4,6] seems the procedure of choice because it is the least damaging for the membrane integrity and stability [7,8]. If a moderate osmotic shock is applied and if the volume ratio between dialysis bag and medium is not large (1 : 10–20), swelling and formation of pores (which allow selective entrapment of otherwise impermeant solutes) can be controlled. This technique leads to resealed RBCs which, having a sufficiently long life span (several weeks) and a high encapsulation yield, are potentially suitable for transfusion [7–9]. Although considerable information is available on the morphological and functional properties of loaded resealed RBCs, little is known about the kinetics of entrapment and the size of the membrane pores generated during hypotonic dialysis or remaining after resealing. Clearly quantitative information on these matters may be useful to optimize entrapment yield and to control the release of entrapped proteins. The latter point, in particular, is important if the goal is the dissemination of an entrapped macromolecule in the blood stream.

This paper reports an investigation of the rate of encapsulation and release of different ^{125}I-labelled proteins using human RBCs. The results reported below provide information on: (i) the maximum protein size efficiently entrapped; (ii) the kinetics of encapsulation of proteins of different molecular weight and isoelectric point; (iii) the efficiency of the resealing procedure; (iv) the effect of the hematocrit on the total amount of protein released.

Materials and Methods

Myoglobin (Mb), cytochrome *c* (Cyt *c*), BPTI, ovalbumin (Ov), bovine serum albumine (BSA), insulin, adenosine, inosine and pyruvate were purchased from

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Sigma Chemical Co.; [^{125}I]iodine from Amersham. Labeling of proteins was carried out using the iodogen method [10].

Encapsulation of proteins within human erythrocytes was achieved by the hypotonic dialysis-isotonic resealing method using the moderate hypotonic buffer suggested by Ropars and co-workers [8]. Briefly, washed erythrocytes were mixed with a protein solution (at a final hematocrit of 70% and a protein/RBC ratio = 0.038 mM/1 · 10^6 cells) and added to a dialysis bag (Spectrapor, Spectrum Medical Industries, Los Angeles, CA; molecular size cut-off, 12–14 kDa or 3–3.5 kDa). Swelling was achieved by dialysis against 15 volumes of 10 mM sodium phosphate and 10 mM sodium bicarbonate (pH 7.4), containing 20 mM glucose, and by gentle mixing by rotation at 4° C. Uptake of proteins into RBCs was stopped by incubation of dialyzed cells at 39° C for 10 min, followed by restoration of isotonicity (addition to 10 ml of swollen erythrocytes of 1 ml of 5 mM adenine/100 mM inosine/100 mM sodium pyruvate/100 mM sodium phosphate/100 mM glucose/12% NaCl) and incubation of the suspension at 39° C for 30 min. Thereafter, resealed erythrocytes were washed six times with 0.9% NaCl and once with autologous plasma before each measurement.

Red blood cells in the incubation medium were counted using the Coulter Counter or, in the presence of hemolysis, by the optical method (Burker camera).

The amount of proteins eventually bound to the internal surface of the membrane, was determined by quick hemolysis of loaded erythrocytes followed by centrifugation at high speed. Thus the radioactivity determined on the ghosts, washed three times by centrifugation in low ionic strength medium (0.05% NaCl) in order to minimize removal of protein possibly bound to the membrane, was taken as an indication of the amount of membrane bound labelled protein. It is important to remark that the radioactivity measured on the ghosts was constant after each washing with NaCl. Protein bound to the external surface of the membrane was determined as above, incubating RBCs with labelled proteins for 2 h in a solution (0.7% NaCl) which, although hypotonic, is inefficient for protein entrapment.

The percentage of encapsulation of any given ^{125}I-labelled protein was calculated as number of cpm found in 1 · 10^6 loaded RBCs, with respect to the initial cpm added to the same number of RBC, while the percentage of release was estimated by cpm found in solution with respect to cpm initially present in 1 · 10^6 RBCs. The calculation was referred to 1 · 10^6 RBCells (and not to 1 ml of precipitated cells, as commonly reported), since the shape of loaded cells depends on the procedure used for encapsulation and therefore the total number of cells present in 1 ml may change.

On the assumption of simple diffusion of proteins between the intra- and extracellular compartment during encapsulation and release, data were analyzed by an integrated first-order decay equation:

$$\log \frac{C_{eq} - C_0}{C_{eq} - C_t} = kt \quad (1)$$

where: k is the decay constant, C the concentration of the probe at time zero (C_0) (added or present inside the loaded RBCs), at equilibrium (C_e), and at different times (t, seconds).

Results

The results reported below were all obtained using human erythrocytes, and therefore, in view of well documented species differences [11], the conclusion may not be necessarily valid for RBCs from other species.

The total number of RBCs in the sample (determined by the Burker camera) is decreased by 10–15% after 20–30 min of hypotonic dialysis; thereafter the number remains constant, indicating that most of the erythrocytes swell without hemolyzing. Moreover, swollen cells do not release Hb as shown by the finding that the amount of Hb quantitated in the supernatant corresponds closely to that expected on the basis of the number of lysed cells (10–15% of the total). After resealing, the osmotic fragility of loaded erythrocytes (determined by the stopped-flow method [12]) is quite similar to that of fresh RBCs ($t_{1/2}$ = 8–10 s).

Fig. 1A reports the time course of entrapment of three proteins with different molecular weight, but roughly the same net charge at pH = 7.4. It may be observed that the rate of entry of different proteins and the maximum amount entrapped after 3 h, depend on molecular weight. Entrapment of BSA (66 kDa) is negligible even after 3 h, in agreement with the lack of Hb efflux from swollen erythrocytes (see above); on the other hand Mb is quickly entrapped reaching a plateau after 100 min. We have also found that small molecular weight proteins with high isoelectric point (i.e., BPTI and Cyt c) enter the erythrocytes less readily than Mb, in spite of their smaller size, suggesting an important role of the net charge in the kinetics of entrapment (data not shown).

The time course of entrapment is complex, because of the presence of a 'lag' indicating that relatively little protein is entrapped during the first 20–30 min of dialysis. However, after this 'lag' period, the kinetics of encapsulation is correctly described by a homogeneous first-order diffusion process (Fig. 1B). Erythrocytes predialyzed for 30 min before addition of Mb display, on the other hand, linear entrapment kinetics (as shown by the dotted line in Fig. 1A).

Interpretation of these kinetic data requires that labeled proteins do not bind to the membrane and

TABLE I

Determination of the amount of protein bound to the internal or to external membrane surface of human erythrocytes

Radioactivity measured in the hemolysate and in the ghosts from loaded erythrocytes, according to the method described in the text. The percent values reported in the last two columns refer to the fractional amount of protein bound to the internal surface with respect to the total protein entrapped inside the cell, or to the fractional amount bound to the external surface with respect to the protein added to unswollen erythrocytes.

Protein	p*I*	Total cpm entrapped	cpm in supernatant	cpm in ghosts	% of protein adsorbed	
					internal surface	external surface
BPTI	10.5	25320	18750	5750	22	1.4
Cytochrome *c*	10.6	42320	35375	6230	14	0.8
Insulin	5.4	27250	26875	385	1.7	< 0.2
Myoglobin	7.0	18350	17750	463	1.7	< 0.2

partition exclusively between the extra and intracellular compartments. A summary of relevant results is reported in Table I. It may be seen that negligible amounts of protein are bound to the membrane (< 0.2%), with the exception of BPTI and Cyt *c* which (though less efficiently encapsulated) are bound to a significant extent. This indicates that electrostatic interactions with the membrane are involved, given that at pH 7.4 BPTI and Cyt *c* (p*I* = 10.5 and 10.9, respectively) are positively charged, while insulin and Mb (p*I* = 4.9 and 7.0, respectively) are negatively charged. Moreover, in the case of Cyt *c* experiments carried out at different times shown that after 10-min dialysis the internal surface of membrane is already saturated with bound protein.

It is interesting to know whether the annealing process is sufficient to completely seal the pores induced by hypotonic dialysis. Since loaded erythrocytes prepared by this procedure are stable to hemolysis and because labeled proteins are not adsorbed to the external surface of the membrane (see above), radioactivity measured in the extracellular compartment at different times is a reliable indication of the amount of protein released from loaded intact erythrocytes.

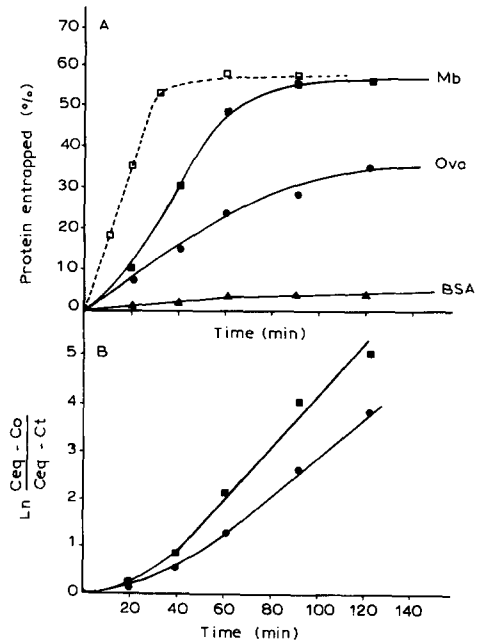

Fig. 1. Time course of encapsulation of myoglobin (■), ovalbumin (●) and bovin serum albumin (▲) into human erythrocytes, as obtained by the dialysis method. Experimental conditions: volume ratio between dialysis bag (hematocrit 70%) and medium (at 0.060 mosmol/kg) 1:15. Initial protein added to $1 \cdot 10^6$ cells 0.038 mM. Dialysis at 4° C and at pH 7.4. Dotted line and (□) indicate data for encapsulation of myoglobin when added to erythrocytes pre-dialyzed in the same buffer for 30 min at 4° C (see text). Panel A reports the time course of entrapment of labeled proteins expressed as percentage of the initial protein added to $1 \cdot 10^6$ cells; data for myoglobin and ovalbumin treated according Eqn. 1 are depicted in panel B.

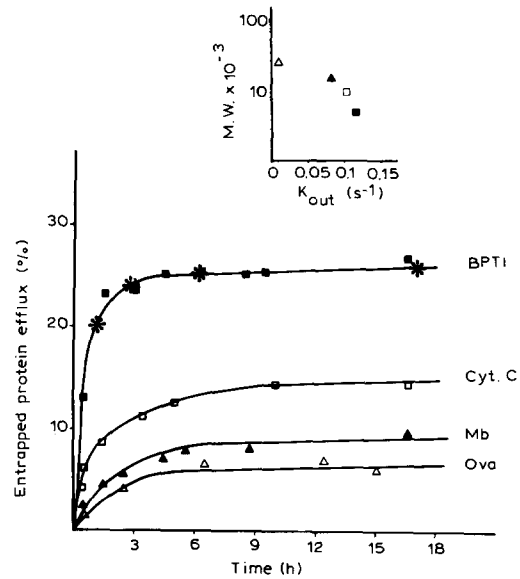

Fig. 2. Time course of release of entrapped proteins loaded into human erythrocytes (expressed as percentage of the initial protein entrapped into $1 \cdot 10^6$ cells). Loaded cells were stored at 30° C in Krebs solution or in autologous plasma. Final hematocrit 15%. BPTI (■), Cyt *c* (□), Mb (▲) and ovalbumin (△). (*) shows the time course of BPTI release from a dialysis bag (cut-off of 12–14 kDa) containing the hemolysate of loaded erythrocytes (■) in the same final volume. The inset shows the dependence of the rate constant of efflux on the molecular weight of the entrapped protein.

Fig. 2 shows the time course of protein released during the first 20 h of incubation of RBCs loaded with different proteins (final hematocrit 15%). For all the proteins, the time course of efflux shows no complexities, and the semilog plot of data is linear, indicating (i) that the kinetics of release is compatible with a first-order diffusion process, and (ii) that the average pore size is constant during the first 20 h of incubation. When incubation is prolonged beyond 20 h, the amount of protein released in the medium increases significantly, presumably due to hemolysis.

The rates and extent of release depend on the molecular weight of the protein. In every case, however, only a fraction (10–30%) of the entrapped protein is released from the resealed RBCs, although the ratio between intra- and the extracellular volume is about 1 : 10.

Fig. 2 (inset) shows the measured rate constant for protein release as a function of the log of the molecular weight of the entrapped protein. It may be observed that the correlation is not linear, and that diffusion is relatively rapid for proteins with molecular weight below 12–15 kDa. These results suggest that the membrane pores of some loaded resealed erythrocytes remain partially open, and that their size is larger than the Stokes radius of BPTI and Cyt *c*, leading to a relatively rapid outward diffusion of these two proteins. Support for this hypothesis is provided by the observation that the kinetics and extent of release of BPTI entrapped in loaded erythrocytes are the same as those measured when the hemolyzate of loaded erythrocytes (obtained by sonication or freezing) is kept in a well stirred dialysis bag, with a cut-off size of 12–14 kDa (see again Fig. 2). This suggests that the distribution of BPTI between medium and hemolyzate depends on interactions with cytosolic components.

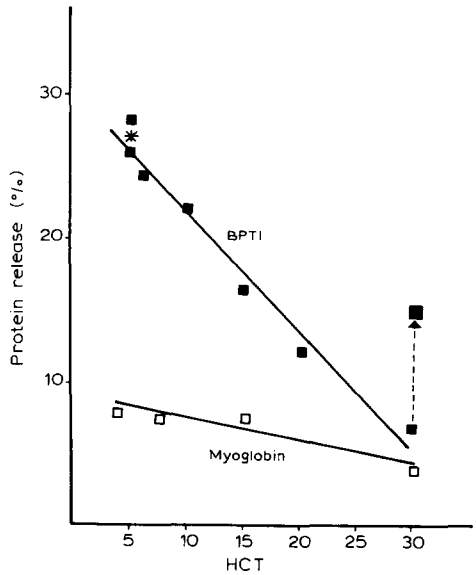

Fig. 3. Percent of protein released as a function of the hematocrit of loaded erythrocytes suspension. Data for BPTI (■) and Mb (□). Loaded erythrocytes were incubated for 8 h at 30 °C either in physiological solution (all the data) or in autologous plasma (*). The arrow indicates the increase of BPTI release when loaded erythrocytes are diluted 1 : 10 with fresh erythrocytes (final hematocrit 30%).

Fig. 3 shows the percentage of protein released in the medium at different haematocrit values. Dilution was achieved adding physiological solution, or autologous plasma, or fresh erythrocytes (see legend to Fig. 3). We have investigated BPTI and Mb, whose stokes radii are, respectively, below and above the presumed pore size of resealed loaded erythrocytes. The fractional amount released increases linearly with increase in the external volume, particularly for BPTI, although the total amount of protein released is always a fraction (5–30%) of the total. Moreover, the increase of protein released is the same whether loaded erythrocytes are diluted with physiological solution or with autologous plasma (see * and full square in Fig. 3). Finally, when loaded erythrocytes are diluted (1 : 10) with fresh erythrocytes, keeping the final hematocrit (30%) constant, the amount of protein released increases (as shown by the arrow), confirming that the effect is related to the increase of the external volume and not to the decrease of the hematocrit.

Discussion

The kinetic data reported in this paper show that by moderate osmotic shock (0.060 mosmol/kg), dialysis for 30 min is sufficient to open membrane pores whose diameter is smaller than the Stokes radius of Hb or BSA, but larger than that of ovalbumin. Thus with Mb 1 h of dialysis is necessary and sufficient to achieve complete equilibration between intracellular and extracellular compartments; after this time, 55% of the externally added Mb is entrapped. Since with an hematocrit of 70% and a conservative available cytosolic space of 70%, the maximal incorporation expected should be 40%, binding of Mb to intracellular components is suggested by the data.

Using 120 min dialysis against either distilled water or 5 mM buffer, and a 1 : 10 ratio of dialysis bag to medium, Sprandel et al. [13] reported that more than 50% of ^{125}I-labeled human serum albumin is entrapped inside human erythrocytes. Under these experimental conditions, however, Hb and other intracellular components are lost during dialysis, with a reduction of the life span of resealed erythrocytes. Similar adverse effects on RBC survival may be expected using moderate ionic strength but large volume of the medium with respect to the dialysis bag. On the other hand, with the direct hypotonic dilution and rapid resealing procedure, quicker (within 60 s) encapsulation of proteins with molecular mass < 90 kDa may be achieved [14], although this is reported [4–6] to be somewhat damaging for the RBCs. Thus, it may be concluded that the use of

the moderate osmotic shock procedure, although yielding a slower entrapment of extracellular proteins and a clear cut size limitation, allows a longer in vivo circulatory half-life [8,9], which may be crucial for possible applications of RBCs as carriers.

A second aspect of our results is that the annealing procedure commonly used [2,3] is insufficient for a complete resealing of the membrane pores: some of these remain partially open, with an estimated cut-off size of approx. 12–14 kDa. This conclusion is based on the fact that in the absence of hemolysis, the kinetics of the efflux are primarily dependent on the molecular weight of the entrapped proteins. Thus smaller proteins (e.g. BPTI or Cyt. *c*, molecular mass < 12–14 kDa) are rapidly lost from resealed RBCs, although the fraction retained at longer times is still quite large; we suggest that BPTI or Cyt *c* remain bound to the internal surface of the membrane, or to cytosolic components of the erythrocyte because of specific binding (the endogenous proteinases for BPTI), or aspecific binding. Consequently, release of entrapped material depends linearly on the increase of the external volume (or final hematocrit), indicating the important role played by binding of entrapped protein to intracellular components of the erythrocyte in the dissemination of entrapped macromolecules. This result is of potential significance for possible therapeutic use of loaded RBCs, because it indicates that results obtained in vitro have to be carefully evaluated to simulate the in vivo condition, where carrier erythrocytes may be diluted 100–1000-fold by reinoculation in the blood stream.

At present, we cannot exclude that the size of pores open after resealing of loaded erythrocytes may depend on the osmotic shock exerted to induce entrapment. In any case, if the radius of the pores could be precisely modulated with reference to that of the macromolecule to be entrapped, erythrocytes may be more effectively used as carriers. However, since rapid (hours) release of entrapped proteins is at best only partial, it may be important and useful to investigate binding of the molecules to cytosolic components of the RBC. In fact this may constitute a strategy to be adopted for carrying proteins or other chemicals to be disseminated in the blood stream. In conclusion, a comprehensive strategy of controlled dissemination of drugs or chemicals from loaded erythrocytes should consider both aspects emerging from this study, i.e. the procedure used, in order to control the size of the membrane pores, and the binding of entrapped chemicals to cytosolic components of the RBC.

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