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Studies on sickled erythrocytes provide evidence that the asymmetric distribution of phosphatidylserine in the red cell membrane is maintained by both ATP-dependent translocation and interaction with membrane skeletal proteins

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In order to study factors which are involved in maintenance of phosphatidylserine (PS) asymmetry within the human red cell membrane, we measured the effect of ATP-depletion and of membrane skeleton/lipid bilayer uncoupling induced by sickling on the distribution of PS within the membrane bilayer of sickle cells. Trace amounts of radiolabeled PS were introduced into the outer membrane leaflet of both fresh and ATP-depleted reversibly sickled cells (RSCs), using a non-specific lipid transfer protein purified from bovine liver. The equilibration of the newly introduced PS over the two halves of the bilayer was monitored by treatment of the cells with phospholipase A_2 which selectively hydrolyzes only those molecules present in the outer membrane leaflet. Within 1 h after insertion into fresh RSCs, only 10% of the labeled PS was accessible to the action of phospholipase A_2. This fraction was markedly increased when the cells were subsequently deoxygenated. Prolonged deoxygenation of RSCs, deprived of their ATP after incorporation of radiolabeled PS, caused enhanced phospholipase A_2-induced hydrolysis of radiolabeled PS. Similarly, phospholipase A_2-induced hydrolysis of endogenous PS in intact RSCs was markedly enhanced when ATP-depleted, but not when fresh cells, were incubated under nitrogen for 3.5 h. Deoxygenated ATP-depleted RSCs markedly enhanced the rate of thrombin formation in the presence of purified coagulation factors Xa, Va, prothrombin and Ca^{2+}. This enhancement appeared to be dependent on the duration of incubation under nitrogen. This phenomenon, indicating the presence of increasing amounts of endogenous PS in the outer membrane leaflet, was not observed when either fresh RSCs or ATP-depleted normal erythrocytes were incubated under nitrogen. Our present observations provide evidence that, in addition to the interaction of PS with the skeletal proteins, an ATP-dependent translocation of PS is required to maintain its absolute asymmetric distribution in the human erythrocyte membrane.

Abbreviations: RSCs, reversibly sickled cells; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Introduction

Phospholipids in the human erythrocyte membrane are asymmetrically distributed over both halves of the lipid bilayer. This asymmetry is absolute for phosphatidylserine (PS) [1–3], with all of the PS molecules residing in the inner leaflet. Two mechanisms have been proposed to explain this finding: (i) a direct interaction of PS with the membrane skeleton [4–7] and (ii) an ATP-dependent unidirectional translocation of PS from the outer towards the inner membrane leaflet [8–12].

Experimental evidence for the first mechanism has been derived from studies on both model systems and human red blood cells. Studies with monomolecular lipid films, constituted at the air/water interface, have demonstrated that purified spectrin specifically interacts with PS [6]. Similar results have been obtained from experiments involving liposomes [6]. Studies demonstrating a similar interaction in the red cell membrane were published by Haest and colleagues [4–6]. Following oxidative cross-linking of spectrin molecules by treatment of intact human erythrocytes with either diamide or tetrathionate, an enhanced accessibility of both amino-phospholipids (phosphatidylethanolamine (PE) and PS) to exogenously added phospholipase A_2 was noted. Since no hemolysis was observed under such conditions, it was concluded that this oxidative treatment had largely abolished the asymmetric distribution of the two amino-phospholipids over both halves of the bilayer. This finding supported the hypothesis that, in the native membrane, PE and PS are retained in the inner membrane leaflet by interactions with spectrin. Very recently, however, it has been shown that the enhanced phospholipase A_2-induced degradation of PE and PS in these oxidized red blood cells might be a consequence of destabilization of their lipid bilayer organization [13], as reflected by an increased 'flip-flop' rate of phosphatidylcholine (PC) molecules [14], rather than a permanently altered phospholipid asymmetry. Enhanced transbilayer mobility of PC has also been shown in hereditary pyropoikilocytosis, where the erythrocytes have a structural defect in the organization of their membrane skeleton [15], and in deoxygenated (sickled) reversibly sicklable cells (RSCs) [16,17]. In the latter case, this abnormality has been assigned to take place only in those areas of the membrane that are in spicular form and where the lipid bilayer has been uncoupled from the membrane skeleton [17]. From the above mentioned studies, it has been concluded that an intact membrane skeleton is essential for stabilizing the lipid bilayer of the red cell membrane. The interaction of amino-phospholipids with the skeletal network is thought to be the primary factor responsible for this stabilization [13,15,17].

The existence of a second mechanism, contributing to maintain phospholipid asymmetry in the red cell membrane, has recently been discovered. Experiments with spin-labeled phospholipid analogs have shown that, once incorporated in the outer monolayer of intact fresh human erythrocytes, sphingomyelin and PC remain mainly in this leaflet, while both amino-phospholipids undergo a rapid translocation to the cytoplasmic half of the membrane [8,10,12]. The latter effect was not observed in ATP-depleted erythrocytes. Studies involving resealed ghosts showed the same phenomenon, viz., a rapid translocation of both spin-labeled PE and PS in favor of the inner monolayer, taking place only when the ghosts have been resealed in the presence of Mg-ATP. Essentially identical results were obtained by Daleke and Huestis [9], using either dilauroyl- or dimyristoyl-species of PE and PS. These observations were confirmed by Tilley and colleagues [11], who used radiolabeled derivatives of naturally occurring sphingomyelin, PC, PE and PS, of which trace amounts were introduced into the outer membrane layer of fresh and ATP-depleted human erythrocytes, using a non-specific lipid transfer protein.

In fresh erythrocytes 95% of the PS and 80% of the PE were translocated to the inner monolayer within 1 and 3 h, respectively. The choline phospholipids, on the other hand, approached an equilibrium strongly favoring the outer leaflet, in which 75% of the PC and more than 90% of the sphingomyelin were found after 21 h at 37 °C.

Once the labeled amino-phospholipids have adopted their inner monolayer positions, subsequent depletion of intracellular ATP does not alter their asymmetric distribution [11]. This latter finding is in full agreement with earlier observa-

tions that the absence of ATP as such, either in intact cells or resealed ghosts, does not affect the transverse distribution of the endogenous phospholipids in the erythrocyte membrane [18–20]. This observation lends support to the above mentioned view that the amino-phospholipids are retained in the cytoplasmic half of the bilayer by (specific) interactions with the membrane skeleton.

Reversibly sicklable cells provide a unique model system to gain further insight into the two potential mechanisms that control phospholipid asymmetry in the red cell membrane, since the effects of both ATP-depletion and uncoupling of the lipid bilayer from the membrane skeleton, can be studied in the same cell. Results of such studies are reported in this paper.

Materials and Methods

Chemicals

Cholesterol was obtained from Merck (Darmstadt), egg phosphatidylcholine, egg phosphatidic acid, bee venom phospholipase A_2, and *Naja naja* phospholipase A_2 were from Sigma (St. Louis, MO). 1,2-Dioleoylphosphatidyl[^{14}C]serine was purchased from Amersham International, U.K. Coagulation factors Xa, Va and prothrombin were isolated and purified as described by Bevers et al. [21]. Chromogenic substrate *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide (S2238) was from Kabi Diagnostica (Stockholm, Sweden).

Non-specific transfer protein. The non-specific transfer protein was partially purified according to Bloj and Zilversmit [22], with adaptations by Crain and Zilversmit [23]. After passage through the carboxymethylcellulose column, the protein was placed on a Sephadex G-50 column and eluted in 10 mM sodium phosphate buffer, containing 10% glycerol and 10 mM mercaptoethanol (pH 6.8). The protein was stored at $-20\,^{\circ}$C in 60% (v/v) glycerol. The glycerol was removed before using the protein by overnight dialysis against 500 vol. of sucrose buffer (280 mM sucrose, 10 mM NaCl, 20 mM glucose, 1 mM EDTA, 10 mM Tris (pH 7.4). The final preparation contained 1 000 IU of activity per mg protein.

Vesicles

Equimolar amounts of cholesterol and phospholipid (egg PC and egg PA in a molar ratio 10:1) were mixed with 2.1 nmol [^{14}C]PS (60 mCi/nmol) per 700 nmol of vesicle PC, dried under nitrogen and dispersed in sucrose buffer at a phospholipid concentration of about 0.15 mM. Vesicles were prepared by ultrasonication under a stream of nitrogen with a Branson sonifier (65 W, 10 min) and centrifuged at $19\,000 \times g$ for 10 min to remove metal particles.

Erythrocytes

After obtaining informed consent, blood from homozygous sickle-cell anemia patients and healthy, normal individuals was collected into acid/citrate/dextrose buffer. The cells were pelleted by centrifugation for 5 min at $2\,500 \times g$ and the buffy coat was removed. The cells were washed three times in sucrose buffer. Cells obtained from sickle-cell patients contained less than 5% irreversible sickle cells. The sickle cells used in this study will therefore be referred to as RSCs.

ATP-depletion of erythrocytes. Erythrocytes were ATP-depleted by incubation for 20–24 h in glucose-free buffer (50 mM Tris-HCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 90 mM NaCl, 0.2 mg/ml streptomycin and 200 IU/ml penicillin (pH 7.4) at $37\,^{\circ}$C. In agreement with previous observations [11], this procedure reduced the ATP content of the cells below the detection limit of the hexokinase/glucose-6-phosphate dehydrogenase assay, i.e., less than 0.01 mg per ml packed cells.

Incubation conditions

Incorporation of [^{14}C]PS. A 33% suspension of erythrocytes was incubated at $20\,^{\circ}$C in a clinical blood rotator, operated at 4 rpm, in the presence of non-specific transfer protein (600 IU/ml packed cells) and vesicles (0.08 mM vesicle lipid) for 30 min. Subsequently, the cells were collected by centrifugation (5 min, $2\,500 \times g$) and washed twice with a glucose containing buffer (10 mM Tris-HCl, 90 mM KCl, 45 mM NaCl, 44 mM sucrose, 10 mM glucose (pH 7.4) in case of fresh cells and with glucose-free buffer in case of ATP-depleted red cells. Cells were resuspended in the same buffer at 5–10% hematocrit and incubated at

37 °C, either under room air or nitrogen. At zero time and after 2.5 h, samples were taken, the cells collected by centrifugation (5 min, 2500 × *g*) and resuspended in a buffer containing 10 mM Tris-HCl, 90 mM KCl, 45 mM NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$, 22 mM sucrose (pH 7.4)). To discriminate between (radiolabeled) PS in the outer and inner halves of the bilayer, the cells were again incubated under room air or nitrogen at 37 °C in the presence of both bee venom phospholipase A_2 (25 IU/250 μl packed cells) and *Naja naja* phospholipase A_2 (25 IU per 250 μl packed cells). After 1 h, the cells were collected by centrifugation, phospholipase action terminated by addition of 50 μmol EDTA in 0.9% NaCl per sample (250 μl packed cells). Finally, the cells were lysed by addition of a cold CO_2-saturated 10 mM EDTA solution. Ghosts were collected by centrifugation at 4500 × *g* for 20 min. Lipids were extracted according to Rose and Oklander [24] and separated on two-dimensional TLC according to Broekhuyse [25]. Phosphorus determination was according to Rouser et al. [26] and radioactivity was determined in a Packard liquid scintillation spectrometer, using emulsifier scintillation solution 299 TM from Packard.

Determination of procoagulant activity. The ability of ATP-depleted sickle and control cells and of fresh sickle cells to enhance the rate of thrombin formation was tested in an assay involving purified coagulation factors Xa and Va, prothrombin and a chromogenic substrate. Full details were described by Bevers et al. [21]. All cells to be tested were incubated under nitrogen at 37 °C as a 1.5% cell suspension in isotonic Hepes buffer (147 mM NaCl, 2.8 mM KCl, 1.7 mM $MgCl_2$, 10 mM Hepes (pH 7.5)). Samples were taken at timed intervals and incubated at 37 °C at concentrations corresponding to 3 μM total phospholipid, in the presence of prothrombin (4 μM), factor Xa (3 nM), factor Va (6 nM), 5.6 mM $CaCl_2$ in isotonic Hepes buffer (pH 7.5). Thrombin formation was measured as described before [21]. All assays were performed under nitrogen.

Data were corrected for the presence of open ghost membranes formed by lysis of some cells (in all cases less than 10% after 7 h of incubation under nitrogen). To that end, percentage of cell lysis was determined in each individual sample from the absorbance at 418 nm of the hemoglobin released in a 2500 × *g* (10 min) supernatant. The corresponding prothrombinase activity was calculated from a calibration curve recorded for cells which had been lysed completely by freezing and thawing of 25 μl of the 1.5% cell suspension, diluted with 1.0 ml of distilled water.

Results

Under the experimental conditions described, the lipid transfer protein introduced an amount of the radiolabeled PS into the cells equivalent to approx. 0.2% of the total erythrocyte membrane phospholipid. 1 hour after its introduction into the outer membrane leaflet of fresh RSCs, only 10% of the radiolabeled PS was accessible to phospholipase A_2 (Fig. 1A, column 1), indicating that 90% of the labeled lipid had been translocated to the cytoplasmic half of the membrane. When, after the introduction of the radiolabeled PS into fresh RSCs, the cells were subsequently incubated for 3.5 h under room air, no marked changes were observed in the accessibility of the labeled compound to phospholipase A_2 (Fig. 1A, column 2). The endogenous PS in fresh intact oxygenated RSCs was minimally accessible to phospholipase A_2 (Fig. 1C, columns 1 and 2), a situation well known to occur in normal erythrocytes [1–3,18]. Incubation of the fresh RSCs under nitrogen caused an enhanced phospholipase A_2-induced hydrolysis of radiolabeled PS (Fig. 1A, column 3) which was even more pronounced for endogenous PS (Fig. 1C, column 3) when compared to the above mentioned situation in oxygenated RSCs. However, the phospholipase A_2 hydrolyzable fractions of either the radiolabeled or the endogenous PS in the fresh deoxygenated RSCs were not significantly enhanced during prolonged incubation of the cells under deoxygenating conditions for up to 3.5 h (Fig. 1A and C, columns 4).

Depleting the RSCs of ATP had no major consequences for the phospholipase A_2-induced hydrolysis of either the previously introduced or the endogenous PS in the cells, provided that cells were incubated under room air (Fig. 1B and D, columns 1 and 2). However, incubation of the ATP-depleted RSCs under nitrogen for up to 3.5 h caused a marked enhancement in the phopsholi-

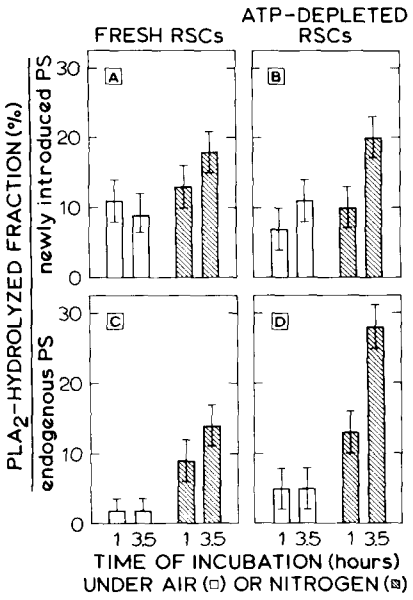

Fig. 1. Phospholipase A_2 (PLA_2) hydrolyzed fractions of newly introduced (A and B) and endogenous (C and D) PS in intact RSCs before (A and C) and after (B and D) ATP-depletion, and subsequent incubation of the cells under oxy- (open bars) and deoxygenating (shaded bars) conditions during the times indicated. Radiolabeled PS was introduced into the outer membrane leaflet of intact cells during a 30 min incubation in the presence of a non-specific lipid transfer protein, as described under Materials and Methods. After careful washing, the cell suspension was split into two fractions. Half of one fraction was incubated under room air, the other half under nitrogen, both at 37 °C. The second fraction was incubated for 20 h at 37 °C in a glucose-free buffer to deplete the cells of their ATP. Half of this fraction was subsequently incubated under room air, the other half under nitrogen, both at 37 °C. The times indicated include a 1 h incubation of the cells with phospholipase A_2 (see Materials and Methods) under either room air or nitrogen. Hemolysis, determined after the phospholipase A_2 treatment of the cells, never exceeded 5%. Hydrolysis of both newly introduced (radiolabeled) PS and the endogenous PS of the cells was determined in the same sample. Data have been derived from duplicate analyses on samples from two independent experiments; bars indicate corresponding SD values.

pase A_2-induced hydrolysis of both the radiolabeled PS (Fig. 1B, column 4) as well as the endogenous PS (Fig. 1D, column 4), when compared to the degradation of these PS fractions observed after 1 h of deoxygenation (Fig. 1B and D, columns 3). These results suggest that a translocation of appreciable fractions of PS from the inner to the outer membrane leaflet in sickled erythrocytes occurs only when cells are both deoxygenated and depleted of their ATP. In agreement with this observation, as well as with previous studies on normal erythrocytes [8–11], over 90% of the radiolabeled PS remained accessible to the action of the phospholipase A_2 when introduced into RSCs previously depleted of ATP (results not shown).

Since RSCs still contain considerable amounts of ATP during the early phase of the 20 h period of metabolic depletion, the lower levels of PS hydrolyzed after 1 h in either oxygenated or deoxygenated ATP-depleted cells (Fig. 1B, columns 1 and 3, respectively) compared to corresponding fractions in fresh RSCs (Fig. 1A, columns 1 and 3), represent the inward translocation of an additional fraction of the newly introduced PS. Similarly, this additional inward translocation may explain why the hydrolysis of newly introduced PS in ATP-depleted RSCs after 3.5 h of deoxygenation (Fig. 1B, column 4) does not markedly differ from that in fresh RSCs incubated under nitrogen for the same time period (Fig. 1A, column 4).

Since phospholipase A_2 has limitations when used to assess the phospholipid distribution in erythrocytes in which the phospholipids are subject to enhanced transbilayer dynamics [13], as

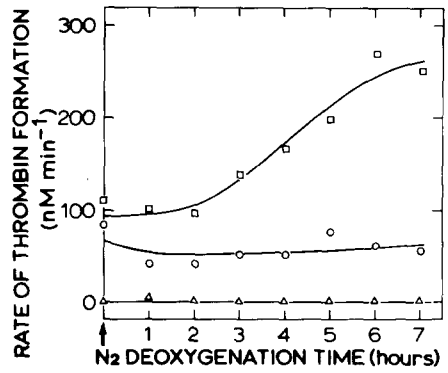

Fig. 2. Effect of deoxygenation on the prothrombinase activity of fresh (○) and ATP-depleted (□) RSCs, as well as of ATP-depleted normal erythrocytes (△). Suspensions of cells were incubated under nitrogen as described under Materials and Methods. At the time points indicated, samples were taken and the prothrombinase activity of the cells determined (see Materials and Methods). Fresh and ATP-depleted RSCs have been derived from the same blood sample. The figure shows the results of a typical experiment.

might occur in the spicules of deoxygenated RSCs [16,17,28], we also used the prothrombinase assay. In this assay, the rate of thrombin formation by the action of a complex of factors Xa and Va on prothrombin is critically dependent on the presence of PS in a membrane surface [17,21]. Normal human erythrocytes, as well as both fresh and ATP-depleted RSCs, were incubated under nitrogen for up to 7 h. Samples were taken at timed intervals and the catalytic activity of these cells with regard to thrombin formation was determined (Fig. 2).

Deoxygenated fresh RSCs caused only little response in the prothrombinase assay, which remained constant during prolonged incubation of the cells under nitrogen for up to 7 h. Prolonged incubation of ATP-depleted cells under nitrogen caused, after a lag period of some 2 h, a marked time-dependent increase in their prothrombinase activity. This indicates the appearance of increasing quantities of PS in the outer membrane leaflet of the deoxygenated ATP-depleted cell. Deoxygenated ATP-depleted normal erythrocytes, on the other hand, appeared to be completely inert when probed in the prothrombinase assay (Fig. 2).

Discussion

Two different mechanisms are believed to be involved in maintaining the asymmetric distribution of phospholipids in the red cell membrane, i.e., (i) the interaction of the membrane skeleton, most specifically spectrin, with the amino-phospholipids, retaining these compounds in the inner monolayer [4–7,16,17,29–31], and (ii) a specific and vectorial ATP-dependent translocation of both amino-phospholipids from the outer to the inner membrane leaflet [8–12]. The RSC provides a unique model to study these two mechanisms, because the interaction between membrane skeleton and lipid bilayer can, at least locally, be broken by transforming the cell into its sickled morphology by deoxygenation [17,28,32,33]. Hence, the contribution of the ATP-dependent translocation process can be studied by comparing the effects of deoxygenation on phospholipid asymmetry in fresh (ATP containing) and ATP-depleted RSCs.

An experimental protocol has been developed recently [11] for the use of the non-specific lipid transfer protein from bovine liver to introduce tracer amounts of radiolabeled phospholipids into the outer membrane leaflet of intact erythrocytes, thereby avoiding the previously described [34] major drawback of this transfer protein in inducing appreciable changes in the native lipid composition of the cell. Thus, this protein was used to introduce trace amounts of ^{14}C-labeled PS into the outer membrane leaflet of intact RSCs. The fate of this newly introduced PS was monitored by subsequent treatment of the cells with phospholipase A_2, converting the PS still present in the outer monolayer into its lyso-derivative.

Our results are consistent with the presence of an ATP-dependent translocation system. This translocation system, which would account for the rapid translocation of PS molecules newly introduced into both normal and sickle red blood cells, has been reported before [8–12,35], though this translocation appeared to proceed at a somewhat lower rate in sickle cells [35].

We originally hypothesized that a permanent rearrangement of amino-phospholipids occurred in deoxygenated sickle cells [27]. In view of the rapid transbilayer mobility of PC which has been reported to occur when the skeletal protein–lipid bilayer interaction is disrupted [16,17], we have revised our previous hypothesis and now believe that the increased accessibility of PS to phospholipase A_2 which occurs in sickled erythrocytes reflects an increase in transversal dynamics, rather than a static redistribution of amino-phospholipids over the bilayer of these cells [16,17,28].

The membrane effects induced by the combination of sickling (dissociation of lipid/skeletal protein interaction) and ATP depletion (inhibition of amino-phospholipid translocation) results in a time-dependent increase in PS in the outer membrane leaflet, as measured by both phospholipase A_2 accessibility (Fig. 1B and D) and the prothrombinase assay (Fig. 2). Due to lateral movements of lipid molecules in each half of the bilayer, PS molecules that have appeared in the outer monolayer of the spicular part of the sickle cell membrane will rapidly diffuse over the entire membrane surface. At the same time, the PS that has disappeared from the inner monolayer of the spicular membrane will be replenished from the

surrounding areas of that leaflet. In the absence of ATP, the PS in the outer monolayer will not be retranslocated to the inner one, and a continuous increase in the amount of PS in the outer monolayer will result. The results of the prothrombinase assay (Fig. 2) are consistent with this model. In principle, this process may proceed until an equilibrium is reached, i.e., a random distribution of PS over both halves of the bilayer. Unfortunately, the prothrombinase assay system does not allow us to quantitate the amount of PS that is actually present in the outer monolayer [17].

The observation that deoxygenated fresh, ATP-containing, RSCs induce only a very slow rate of thrombin formation (Fig. 2), independent of the duration of deoxygenation, supports the above hypothesis. This result seems to be at variance with an earlier observation [17], showing a negligible prothrombinase activity of deoxygenated RSCs. However, it should be noted that, when compared to the former study [17], 6-times higher concentrations of both coagulation factors Xa and Va and 3-times higher numbers of cells have been applied in the assay system used in the present studies. PS molecules that diffuse from the inner to the outer monolayer in the spicular areas of the membrane, will be retranslocated to the inner one by the ATP-dependent system. When this retranslocation takes place in those areas of the membrane where the interaction with the membrane skeleton is preserved, these PS molecules will be retained in the inner monolayer and will have another chance to diffuse to the outer leaflet only when they again reach the spicular areas of the membrane by means of lateral diffusion. Consequently, these mechanisms provide a steady state where only minute amounts of PS are present in the outer membrane leaflet of the sickled fresh RSC (Fig. 2).

Taken together, our findings provide experimental evidence that the localization of PS within the inner monolayer of the red cell membrane is maintained by both an ATP-dependent translocation process and its interaction with the membrane skeleton. In fresh sickled cells, only very small amounts of PS will be exposed in the outer membrane leaflet as the ATP-dependent translocation process will prevent accumulation of PS in that layer. In contrast, when sickling and ATP-depletion occur, a progressive accumulation of PS in the outer membrane leaflet will take place.

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