Eur Biophys J (2010) 39:789–800
DOI 10.1007/s00249-009-0554-6

ORIGINAL PAPER

Rearrangement of erythrocyte band 3 molecules and reversible formation of osmotic holes under hypotonic conditions

Ivana Pajic-Lijakovic · Vesna Ilic · Branko Bugarski · Milenko Plavsic

Received: 21 July 2009 / Revised: 5 October 2009 / Accepted: 9 October 2009 / Published online: 3 November 2009

Abstract The complex phenomenon of rearrangement of band 3 molecules after erythrocyte swelling under hypotonic condition is considered. The rearrangement includes the increase of the mobile fraction and clustering of band 3. The self-associative tendency and the action of the elastic field generated within the lipid membrane after erythrocyte swelling result in equilibration of the number of molecules per cluster and the number of clusters. The local perturbation of the elastic field induces excitation of the cluster in the nearest neighbor and changes its packing state generating changes in the free volume within the cluster. The local perturbation could result in the reversible formation of osmotic hole. We formulated a model to predict changes of the cluster packing states generated by rearrangement of band 3 molecules on two time-scales. The phenomenon is examined on the basis of two experimental sets, i.e. low (5.2 mM Na_3PO_4 solution) and high (46.0 mM Na_3PO_4 solution) hypotonicities at 21°C, from Golan and Veatch (Proc Natl Acad Sci 77(5):2537–2541, 1980). Modeling considerations suggested that lower hypotonic conditions resulted in higher values of: the driving force of agglomeration of band 3 as a measure of self-associative tendency, the specific rate of cluster breaking, the specific rate of increase of the mobile fraction of band 3, and the dispersion of cluster sizes. Lower hypotonic conditions ensure the generation of a higher average value of the free energy within the membrane after erythrocyte swelling, which enables more intensive rearrangement of band 3 molecules.

Keywords Rearrangement of band 3 molecules · Mobile fraction · Packing state of cluster · Formation of osmotic holes · Modeling

I. Pajic-Lijakovic (✉) · B. Bugarski · M. Plavsic
Faculty of Technology and Metallurgy,
Karnegijeva 4, Belgrade, Serbia
e-mail: iva@tmf.bg.ac.rs

V. Ilic
Institute for Medical Research, Belgrade,
Dr. Subotica 4, Belgrade, Serbia

Introduction

Many aspects of the osmotic hemolysis of human erythrocytes has been under investigation for about a hundred years. It is affected by the following processes:

- erythrocyte swelling;
- formation of osmotic holes;
- intracellular hemoglobin diffusion to the membrane holes;
- passage of hemoglobin through the hemolytic holes; and
- diffusion of hemoglobin in the extra cellular medium.

These processes are very sensitive to external conditions, the morphological characteristics of the erythrocyte population, and the experimental measurement procedures (Sheets et al. 1995). This introduces more difficulties into generalization of these complex phenomena and results in the wide range of experimental results reported in the literature. For this reason, deeper insight into the mechanism of formation of the holes would offer the possibility of further optimization of osmotic hemolysis. However, detailed explanation of the formation of osmotic holes in the erythrocyte membrane after swelling in hypotonic media has not yet appeared. Several investigators have studied formation of osmotic holes experimentally over a wide range of experimental conditions (Seeman 1967;

Seeman et al. 1973; Leiber and Steck 1982; Pribush et al. 2002). Two types of osmotic holes can be formed on erythrocytes—reversible osmotic holes and irreversible cracks in the lipid membrane. At low temperatures (21°C) reversible osmotic holes can be formed even under low hypotonic conditions (cation concentration less than 10 mM) as reported by Golan and Veatch (1980). We will consider only reversible formation of holes under hypotonic conditions.

Leiber and Steck (1982) discussed formation of holes after erythrocyte swelling as the consequence of two opposite forces. One is the force which expands the hole (electrostatic repulsion between anionic groups on the membrane) and the other is the force driving hole closure (hydrophobic effects of the lipid–water interface and/or membrane elasticity). Pribush et al. (2002) discussed the reversible formation of holes induced by lipid membrane viscoelasticity after erythrocyte swelling. They discussed the influence of the characteristics of the elastic field generated within the lipid membrane on the formation of holes. The elastic field generated during erythrocyte swelling depends on the hypotonicity of the external medium. Erythrocyte swelling has been correlated with changes of surface strain energy (Li et al. 2005; Pawlowski et al. 2006). However, the effect of trans-membrane protein band 3 rearrangements on lipid membrane strain energy has not been considered. Wong (1999) pointed out that rapid shape transformation of erythrocyte during the process of swelling could be correlated with the rearrangement of band 3. Swelling of erythrocytes in hypotonic medium induces conformational and associative changes in the cytoplasmic domain of band 3 that lead to reversible formation of high-molecular-weight aggregates and the osmotic holes. Sato et al. (1993a, b) also discussed a reversible process of formation of osmotic holes as the result of rearrangement of the population of band 3 molecules within clusters. They reported that a reversible osmotic hole is bordered by a ring of band 3 molecules. They introduced two possible explanations of hole formation during hypotonic hemolysis:

1 a change of membrane fluidity; and
2 a conformational and associative change of band 3 proteins.

Erythrocyte swelling induces conformational changes of the cytoplasmic domain of trans-membrane protein band 3 (Salhany et al. 2000). Such conformational changes result in electrostatic interactions between highly anionic *N*-terminal domains of band 3 molecules and this intensifies their self-associative tendency (Taylor et al. 1999). Conformational changes in the cytoplasmic domain of band 3 also induce long-distance conformational changes in the transmembrane domain (Salhany et al. 2000). This exposes the hydrophobic helices of the transmembrane domain to hydrophilic solution which also intensifies the self-associative tendency of band 3 molecules (Taylor et al. 1999). The self-associative tendency of the mobile fraction of band 3 molecules leads to clustering.

Golan and Veatch (1980) regarded the increase in the mobile fraction of band 3 and intensification of lateral mobility as the consequence of erythrocyte swelling in hypotonic media. However, they did not consider the reversible formation of osmotic holes. Tomishige et al. (1998) considered the mechanisms of lateral diffusion of band 3 molecules. Yeow and Clayton (2007) found the density of clusters per cell surface was $19 \pm 4/\mu m^2$. However, Leiber and Steck (1982) and Sato et al. (1993b) observed only one reversible hole whereas Danon (1961) suggested that a few reversible holes open during the sealing time. What is the possible explanation of the expansion of only a few clusters of band 3 molecules?

The phenomenon of reversible formation of holes as reported by Leiber and Steck (1982) and by Sato et al. (1993a, b) is caused by balancing of two opposite deterministic tendencies. One tendency causes expansion of holes whereas the other causes compression of holes. The equilibration of these tendencies leads all clusters to reach the same equilibrium state. However, only a few clusters expand into the reversible osmotic holes at each moment in time. The specific cluster which expands into the osmotic hole is a random event. In our opinion neither considerations based on deterministic force balance nor those based on the kinetic model as reported by Sato et al. (1993b) are good enough for understanding the complex phenomenon of reversible formation of holes. We need additional consideration of the problem based on statistical mechanics and thermodynamics.

In this paper, we consider the rearrangement of band 3 molecules which results in clustering. Clustering of integral membrane proteins is treated as first-order phase transition (Gil et al. 1998; Destainville 2008). Clustering is caused by the self-associative tendency of band 3 molecules as a result of short-range ionic attractive protein–protein interactions (Taylor et al. 1999). The self-associative tendency on the one hand, and the action of the elastic field generated within the lipid membrane after erythrocyte swelling on the other hand, results in equilibration of the number of molecules per cluster and the number of clusters per erythrocyte surface. The driving tendencies of the elastic field generated within the membrane through long-range membrane-mediated repulsive interactions are discussed by Gil et al. (1998) and Destainville (2008). Evans et al. (2003) and Sens and Turner (2004) modeled the rearrangement of membrane inclusion proteins (e.g. caveolin) induced by thermal fluctuations of the lipid bilayer. However, such modeling considerations have not been used for

explanation of the phenomenon of rearrangement of band 3 molecules under hypoosmotic conditions.

Clusters tend to form a compact structure by close packing of band 3 molecules. This state of the clusters corresponds to the minimum values of both free volume and free energy per cluster. However, local perturbation of the elastic field in the membrane results in excitation of a cluster in that area. Excited clusters change their packing states and free volumes and could rearrange their molecules into ring-like structures. In our opinion, this could be the key phenomenon in the reversible formation of osmotic holes. The phenomenon of rearrangement of the band 3 population will be considered using experimental data from Golan and Veatch (1980) for external medium of various tonicities.

Phenomenological background of the model

The model is formulated to consider the phenomenon of rearrangement of molecules of band 3 induced by erythrocyte swelling. Erythrocyte swelling causes generation of an elastic field within the membrane. Elastic states of the lipid membrane are described by surface free energy, and we can write $E(\tau,t) = E_0 + E'(\tau,t)$ (where τ is the lifetime of local perturbations of free energy—sealing time, t is the long time-scale of osmotic hemolysis duration, $E(\tau, t)$ is the total free energy of the lipid bilayer, E_0 is the surface-averaged value of the free energy after erythrocyte swelling, and $E'(\tau, t)$ is a fluctuating term). We start our consideration with already swollen erythrocyte at $t = 0$. The fluctuating term generated by swelling is expressed as $E'(\tau,t) = \int_0^{\Re(\tau,t)} E'(r,\tau,t) 8r\pi dr$, and the energy of one perturbation is expressed as $E'(r,\tau,t) = d\vec{u}(r,\tau,t) \cdot \vec{\nabla} F(r,\tau,t)$ (where $d\vec{u}(r,\tau,t)$ is the local displacement of the erythrocyte surface after swelling, $\Re(\tau,t)$ is the radius of the swollen erythrocyte, $F(r,\tau,t) = \left[\frac{\kappa}{2}\left(\nabla\left(\vec{\nabla}\cdot\vec{u}(r,\tau,t)\right)\right) + \frac{\sigma^*}{2}\left(\nabla u(r,\tau,t)^2\right)\right] 8r\pi dr$ is the local free energy of the lipid bilayer after swelling, κ is the bending modulus, and σ^* is the surface tension as reported by Evans et al. (2003) and Li et al. (2005). After erythrocyte swelling fluctuations of the lipid membrane are caused by the difference between the interior and exterior pressures and by the elastic characteristics of the lipid membrane. Delano (1995) proposed van't Hoff's Law for the pressure difference. Accordingly, the interior and exterior pressures are expressed as $P_{\text{in}}(\tau,t) = RT\phi_{\text{in}}C_{\text{in}}(\tau,t)$ and $P_{\text{out}}(\tau,t) = RT\phi_{\text{out}}C_{\text{out}}(\tau,t)$ (where R is the universal gas constant, T is the temperature, ϕ_{in} and ϕ_{out} are the average intracellular and external osmotic coefficients, respectively; and $C_{\text{in}}(\tau, t)$ and $C_{\text{out}}(\tau, t)$ are the intracellular and external solute concentrations, respectively). The fluctuations of the erythrocyte surface and volume are described as $dA_C(\tau,t)\sigma^* = \mathrm{d}V_C(\tau,t)(P_{\text{in}}(\tau,t) - P_{\text{out}}(\tau,t))$ (where $A_C(\tau,t) = 4\Re(\tau,t)^2\pi$ is the swollen erythrocyte surface and $V_C(\tau,t) = \frac{4}{3}\Re(\tau,t)^3\pi$ is the swollen erythrocyte volume). When $t \to t_{\text{eq}}$ (where t_{eq} is the time when the interior and exterior pressures are equilibrated) $P_{\text{in}}(\tau, t_{\text{eq}}) \to P_{\text{out}}(\tau, t_{\text{eq}})$.

The action of field E_0 results in an increase in the mobile fraction of band 3 molecules. Associative tendency within mobile band 3 molecules is driven by short-range attractive interactions. This results in clustering. At the same time, the action of field E_0 causes cluster compaction. However, the local action of the perturbation $E'(r, \tau, t)$ on the nearest cluster induces its excitation. The excited cluster changes its packing state, which could result in breakage or formation of a new packing state. The packing state which ensures the maximum free volume within the cluster corresponds to the reversible osmotic hole. On that basis the action of local perturbations is the key phenomenon in the formation of osmotic holes.

For further consideration it is necessary to connect the increase of the band 3 mobile fraction with the dynamics of clustering which results in the formation of osmotic holes. The phenomenon is considered using experimental data from Golan and Veatch (1980) under various hypotonicities of external medium. The total number of band 3 molecules per erythrocyte surface is $n_T \approx 1 \times 10^6$ (Saxton 1990). Such a population represents the sum of two subpopulations of band 3 molecules (Sato et al. 1993a, b). The first subpopulation (20–40%) forms high-affinity complexes with ankyrin (Saxton 1990; Tomishige et al. 1998). The second subpopulation forms low-affinity complexes with other constituents of the cytoskeleton, for example spectrin or band 2.1 (Golan and Veatch 1980). Low-affinity complexes dissociate after introducing the erythrocyte suspension into the hypotonic medium whereas high-affinity complexes survive as a result of the phosphorylation process (Matayoshi et al. 1991). This causes the increase of the mobile fraction of band 3 molecules. The fraction of mobile band 3 molecules, i.e. $f(t) = \frac{n_A(t)}{n_T}$ (where $n_A(t)$ is the number of mobile band 3 molecules) increases as a function of time up to the equilibrium value f_{eq} for the corresponding characteristics of the generated field E_0 within the lipid membrane. Such characteristics depend on the hypotonicity of the external solution and on temperature. Golan and Veatch (1980) determined that f_{eq} is $11 \pm 9\%$ for high ionic strength external medium (46.0 mM Na_3PO_4) at 21°C. For low ionic strength external medium (5.2 mM Na_3PO_4) at 37°C erythrocyte rupture occurred. However, at lower temperatures, for example 21°C this catastrophic event was not observed. The

corresponding equilibrium value of mobile fraction of band 3 molecules was $f_{eq} = 72 \pm 7\%$. The maximum value of the equilibrium mobile fraction of band 3 ($f_{eq} = 83 \pm 6\%$) was obtained at 37°C at the ionic strength of 13.3 mM Na_3PO_4 as external medium. We will consider the rearrangement of band 3 molecules under two hypotonic conditions: 46.0 mM Na_3PO_4 and 5.2 mM Na_3PO_4 at 21°C for which erythrocyte rupture was not observed.

The value of mobile band 3 fraction in the erythrocyte before swelling is small, up to 1%. During the increase of the mobile fraction of band 3, the number of clusters and the number of band 3 molecules per cluster increase up to equilibrium values obtained for $t = t_{eq}$. The equilibrium number of band 3 molecules per cluster results from the action of two opposite tendencies. The first is the associative tendency between band 3 molecules as a result of protein–protein short-range attractive interactions whereas the second is long-range membrane-mediated repulsive interactions (Gil et al. 1998; Destainville 2008). We propose a model which considers rearrangement of band 3 molecules and reversible formation of osmotic holes using a thermodynamic approach.

Model development

We will consider the dependence of the free energy of clusters on molecule packing by introducing the volume function. We assume that we are considering a cluster whose constituents are impenetrable. The volume function depends on the coordinates of grains within a cluster (which, here, are band 3 molecules) and consider their orientations. The volume function is in that way an analog of a Hamiltonian. Averaging over all the possible configurations of the molecules in real space gives us a statistical ensemble describing the random packing of molecules. We have to include some account of this in our formalism in order to reduce the number of possible configurations the cluster may occupy. Also, for a packing that is stable under the applied force generated within the lipid membrane, we must consider the configurations restricting the number of possible volume states that the cluster may occupy to be only those configurations that are stable.

The changes of free energy of a single cluster of band 3 molecules occur on two time scales. The long time-scale t is the time during which the number of molecules per cluster changes. The short time-scale τ (sealing time) represents the life-time of the cluster which consists of $N = const$ molecules. We suppose that the same short time-scale corresponds to the life-time of one perturbation of the elastic field generated within the membrane. During the long time-scale t the cluster represents a grand canonical ensemble whereas during the short time-scale τ the cluster can be treated as a canonical ensemble and described by the partition function.

The partition function for the cluster located at r is formulated for the canonical ensemble as:

$$Z_r(\tau, t) = \mathrm{e}^{-\frac{Y_r(\tau,t)}{\lambda X}} \tag{1}$$

where $X = \frac{\partial V_r(\tau,t)}{\partial S_r(\tau,t)}$ is the compactivity of cluster which represents the analog of temperature, λ is a constant that gives the entropy the dimension of volume, $Y_r(\tau, t) = V_r(\tau, t) + X\frac{\partial Y_r(\tau,t)}{\partial X}$ is the free volume function of a single cluster located at r which represents the analog of free energy, $V_r(\tau,\ t)$ is the total volume of a single cluster located at r, and $S_r(\tau,\ t)$ is the cluster entropy. The partition function, expressed for each set of molecules per cluster $N_r(t)$ for the cluster located at r is:

$$Z_r(\tau, t) = \int_0^1 \int_0^1 \int_0^1 \mathrm{e}^{-\frac{W_T\left(T,\xi_{1r}(\tau,t),\xi_{2r}(\tau,t),\chi_r(\tau,t)\right)}{\lambda X}} d\xi_{1r} d\xi_{2r} d\chi_r \tag{2}$$

where $W_T(T, \xi_{1r}(\tau,t), \xi_{2r}(\tau,t), \chi_r(\tau,t))$ is the total volume function of band 3 molecules within the cluster, which represents the analog of the Hamiltonian. It introduces two properties: orientation and coordination number. On that basis, the total volume function consists of two contributions: the first $W_O(T, \xi_{1r}(\tau,t), \xi_{2r}(\tau,t))$ represents the contribution of molecule orientation and the second $W_C(T, \chi_r(\tau,t))$ represents the contribution of molecule coordination number, i.e. $W_T(T, \xi_{1r}(\tau,t), \xi_{2r}(\tau,t), \chi(\tau,t)) = W_O(T, \xi_{1r}(\tau,t), \xi_{2r}(\tau,t)) + W_C(T, \chi_r(\tau,t))$. To describe molecule orientation, we introduce two degrees of freedom ξ_{1r} and ξ_{2r}. Following similar formalism, we introduce one degree of freedom χ_r to describe the molecule coordination number. All of these contribute to formation of the cluster packing state. We will describe how the two boundary conditions of the packing state depend on the value of the free volume within the cluster. When the free volume within the cluster is minimum, the values of the degrees of freedom are $\chi_r = 0$ (the maximum coordination number) and $\xi_{ir} = 0$, $i = 1, 2$ (the orientation per molecule which corresponds the minimum value of the Stokes radius $\langle r_s \rangle_{\min}$). When the free volume within the cluster is maximum, the values of the degrees of freedom are $\chi_r = 1$ (the minimum coordination number) and $\xi_{ir} = 1,\ i = 1,\ 2$ (the orientation per molecule which corresponds the maximum value of the Stokes radius $\langle r_s \rangle_{\max}$). The total average volume function for the cluster is determined by averaging as:

$$\bar{W}_T(T, \xi_{1r}, \xi_{2r}, \chi_r) = \frac{1}{Z_r} \int_0^1 \int_0^1 \int_0^1 W_T(T, \xi_{1r}, \xi_{2r}, \chi_r)$$
$$e^{-W_T(T,\xi_{1r},\xi_{2r},\chi_r)/\lambda X} d\xi_{1r} d\xi_{2r} d\chi_r \tag{3}$$

The total specific volume function per band 3 molecule is expressed as $\bar{W}(T, \xi_{1r}, \xi_{2r}, \chi_r) = \bar{W}_T(T, \xi_{1r}, \xi_{2r}, \chi_r)/N_r$.

The maximum specific volume function of a single molecule of band 3 in the cluster is realized when the cluster is perturbed and forms the osmotic hole. In that case band 3 molecules form the ring-like structure around the hole. The radius of the reversible osmotic hole could be expressed as $R_H(t) = \frac{N_r(t)\langle r_s\rangle_{\max}}{\pi}$. The total specific volume function per band 3 molecule in further consideration will be presented as:

$$\begin{aligned}\bar{W}(T,\xi_{1r}(\tau,t),\xi_{2r}(\tau,t),\chi_r(\tau,t)) &= \bar{V}_{\min} + \frac{1}{2}\bar{V}_{FV_O}\left(\xi_{1r}(\tau,t)^2 + \xi_{2r}(\tau,t)^2\right) \\ &\quad + \bar{V}_{FV_C}(t)\chi_r(\tau,t)^2 \end{aligned} \tag{4}$$

where $\bar{V}_{\min}$ is the minimum specific volume of a single molecule of band 3 $\bar{V}_{\min} = \frac{4}{3}\langle r_s\rangle^3_{\min}\pi$. The maximum specific free volume per molecule, as a result of its orientation, is $\bar{V}_{FV_O} = \frac{4}{3}\pi\left[\langle r_s\rangle^3_{\max} - \langle r_s\rangle^3_{\min}\right]$. The maximum specific free volume per molecule, as a result of its coordination number, is $\bar{V}_{FV_C}(t) = \frac{R_H^2(t)\pi h_m}{N_r(t)}$ (where h_m is the thickness of the lipid bilayer for the already swollen erythrocyte). When $\chi_r = 0$ and $\xi_{ir} = 0$ at $\tau \to \tau_{\text{eq}}$, the total specific volume function is $\bar{W}_T\left(T,\xi_{1r}(\tau_{\text{eq}},t),\xi_{2r}(\tau_{\text{eq}},t),\chi_r(\tau,t)\right) = \bar{V}_{\min}$. In that state coordination number per molecule within the cluster is maximum. When $\chi_r = 1$ $\xi_{ir} = 1$ at $\tau = 0$, the total specific volume function is $\bar{W}_T(T,\xi_{1r}(\tau=0,t),\xi_{2r}(\tau=0,t),\chi_r(\tau=0,t)) = \bar{V}_{\min} + \bar{V}_{FV_O} + \bar{V}_{FV_C}(t)$. In that state the coordination number per molecule within the cluster is minimum. This is a self-consistent approximation because $\bar{V}_{\min}$ and $\bar{V}_{\max} = V_{\min} + \bar{V}_{FV_O} + \bar{V}_{FV_C}$ are the average volumes of the molecule in the presence of other molecules within the cluster.

The excited cluster after the action of the local perturbed field $E'(r, \tau, t)$ generated within membrane, which satisfies the condition that the free volume per molecule is maximum, in our opinion represents the osmotic hole at time t (time of system evolution—''long time-scale'') during the time sequence, i.e. ''short time-scale'' period $\tau \in [0, \tau_{\text{eq}}]$.

We will interpret the process of compaction of the single cluster as the Ornstein–Uhlenbeck process for all degrees of freedom χ and ξ_i, $i = 1, 2$. This is in accordance with the fact that all band 3 molecules are driven stochastically by random forces with zero correlation time. Therefore, we could describe the phenomenon for the cluster located at r by using the Langevin equation (Edwards and Grinev 1998):

$$\frac{\mathrm{d}\Psi_r(\tau,t)}{\mathrm{d}\tau} = -\frac{1}{\gamma_\Psi(t)}\frac{\partial \bar{W}_\Psi(\xi_{ir}(\tau,t))}{\partial \Psi_r(\tau,t)} + \varphi_{eff\,\Psi}(\tau,t) \tag{5}$$

where $\Psi(\tau, t)$ represent the degrees of freedom: χ and ξ_i, $i = 1, 2$. The stochastic random force $\varphi_{eff\,\Psi}(\tau,t)$ is formulated as white noise with the correlation function $\langle\varphi_\Psi(\tau,t)\varphi_\Psi(\tau',t)\rangle = 2\lambda X\gamma_\Psi(t)\delta_{ij}\delta(\tau-\tau')$, and $\gamma_\Psi(t)$ is the analog of frictional resistance. The Langevin equation can be solved as:

$$\begin{aligned}\Psi_r(\tau,t) &= \Psi_{r0}(E'(r,\tau=0,t))\mathrm{e}^{-\gamma^*_\psi(t)\tau} \\ &\quad + \int_0^\tau e^{-\gamma^*_\psi(\tau-\tau')}\varphi_{eff\Psi}(\tau',t)\mathrm{d}\tau' \end{aligned} \tag{6}$$

where model parameter γ^*_Ψ for $\Psi = \xi_i$, $i = 1, 2$ is expressed as $\gamma^*_\xi = \frac{\bar{V}_{FV_O}}{\gamma_\xi(t)}$. For $\Psi = \chi$ the corresponding parameter γ_Ψ is expressed as $\gamma^*_\chi = \frac{2\bar{V}_{FV_C}(t)}{\gamma_\chi(t)}$. We introduce the assumption that $\gamma^*_\chi \approx \gamma^*_{\xi_i} = 1/\tau_R$. Seeman (1967) reported that $\tau_R \approx 10$ s for the reversible osmotic hole. The initial values of the degrees of freedom $\Psi_{r0}(E'(r,\tau=0,t))$ are determined by the action of the local perturbation field generated within the membrane located at r at short time-scale $\tau = 0$. The initial values for the degrees of freedom are $\Psi_{r0}(E'(r,\tau=0,t)) \le 1$, depending on the value of the local perturbation $E'(r, \tau = 0, t)$. Averaging over the ensemble of molecules per cluster $N_r(t)$ we get:

$$\langle\Psi_r(\tau,t)\rangle = \Psi_{r0}(E'(r,\tau=0,t))e^{-\gamma^*_\Psi(t)\tau} \tag{7}$$

At $\tau \to \tau_{eq}$ the degrees of freedom tend to zero, i.e. $\Psi_r \to 0$, which corresponds to the compact equilibrium structure of the cluster. At the same time, for the long time-scale $t \to t_{eq}$ the degrees of freedom also tend to zero, i.e. $\Psi_r \to 0$. The volume of the single cluster located at r is expressed by using the average values of the degrees of freedom from Eq. 7. In the final form this is expressed as:

$$V_r(\tau,t) = V_{0r}(t) + \Delta V_{FVr}(t)\mathrm{e}^{-2\tau/\tau_R} \tag{8}$$

where $V_{0r}(t) = N_r(t)\bar{V}_{\min}$, and the free volume within cluster is $\Delta V_{FVr}(t) = N_r(t)(\bar{V}_{FV_O} + \bar{V}_{FV_C}(t))$. If the local perturbation $E'(r,\tau=0,t)$ is high enough to induces the initial values of the degrees of freedom equal to $\Psi_{r0}(E'(r,\tau=0,t)) = 1$, this excited cluster becomes the reversible osmotic hole.

The fluctuations of the already swollen erythrocyte surface $dA_C(\tau, t)$ could be correlated with the sum of the surfaces of osmotic holes for time set (τ, t) as: $\mathrm{d}A_C(\tau,t) = \sum_{i=1}^{n_H(t)} A_{Hi}(\tau,t)$ (where the surface of the osmotic hole is $A_H(\tau,t) = R_H(\tau,t)^2\pi$ and $n_H(t)$ is the number of osmotic holes). We introduce the assumption that for $t \to t_{eq}$ the number of osmotic holes $n_H(t_{\text{eq}}) \to 0$. The permeability of the lipid membrane to hemoglobin (Hb) could be expressed by introducing the flow of Hb mass through hemolytic holes as $Q_{\text{Hb}}(\tau,t) = \frac{\Delta m_{\text{Hb}}(\tau,t)}{\Delta t}$. The mass flow of Hb is $Q_{\text{Hb}}(\tau,t) = k(C_{\text{Hb in}}(\tau,t) - C_{\text{Hb out}}(\tau,t))\sum_{i=1}^{n_H(t)} A_{Hi}(\tau,t)$ (where k is the coefficient of mass transfer through the osmotic holes, $C_{\text{Hb in}}(\tau,t)$ is the concentration of Hb in the

erythrocyte, and $C_{\mathrm{Hb\,out}}(\tau,t)$ is the concentration of Hb in the external solution). After erythrocyte swelling and formation of osmotic holes large enough for passage of Hb, continuous mass flow of Hb can be observed. The driving force for Hb release is the concentration difference, which decreases exponentially during the long time-scale t. When $t \to t_{\mathrm{eq}}$ the driving force $\Delta C_{\mathrm{Hb}}(\tau,t_{\mathrm{eq}}) \to 0$. The modeling consideration points to $\Delta C_{\mathrm{Hb}}(\tau,t) = C_{\mathrm{Hb\,in}}(\tau,t) - C_{\mathrm{Hb\,out}}(\tau,t)$ as the driving force for Hb release and the pressure differences which correlate with the total solute concentration difference $\Delta C(\tau,t) = C_{\mathrm{in}}(\tau,t) - C_{\mathrm{out}}(\tau,t)$ as the driving force for the dynamics of formation of reversible osmotic holes. Experimental data for hemolysis (Lieber et al. 1987) indicate that Hb mass flow $Q_{\mathrm{Hb}}(t) = \int_0^{\tau_{\mathrm{eq}}} Q_{\mathrm{Hb}}(\tau,t)\mathrm{d}\tau$ increases up to a maximum value $Q_{\mathrm{Hb}}(t_{\max})$ obtained at $t = t_{\max}$ as the result of the increase in the number of molecules of band 3 per clusters on the erythrocyte surface and the radius of the osmotic holes. At long time-scale $t \in [t_{\max}, t_{\mathrm{eq}}]$ mass flow of Hb decreases as a result of the driving force decrease for Hb release $\Delta C_{\mathrm{Hb}}(t) = \int_0^{\tau_{\mathrm{eq}}} \Delta C_{\mathrm{Hb}}(\tau,t)\mathrm{d}\tau$.

Further model consideration includes the formulation of the long time-scale t evolution of the mobile fraction of the band 3 population which is arranged within clusters which consist of $N(t)$ molecules. The fraction of mobile band 3 molecules is expressed as: $f(t) = \int_{N=1}^{N=N_{\max}} \rho(N,t)\mathrm{d}N$ (where $f(t)$ is the experimentally determined fraction of mobile band 3 molecules). For $N = 1$, molecules of band 3 are monomers, whereas for $N = N_{\max}$, molecules of band 3 are arranged within high-order oligomers. Blackman et al. (1996) suggested a possibility that ~25% of band 3 molecules make aggregates of ~5,000-mers. We introduce the value for $N_{\max} = 1{,}000$ as a first approximation for the fitting procedure. For further model development, we will formulate the long time-scale t-dependent cluster size distribution function $\rho(N, t)$. The mobile band 3 molecules rearrange during the transition from the disordered, randomly mixed phase into the ordered, cluster-like phase. The process of clustering of integral proteins within lipid membrane could be treated as a first-order phase transition, as reported by Gil et al. (1998), Evans et al. (2003), Sens and Turner (2004), and Destainville (2008). Such disorder to order transition through homogeneous nucleation is induced by:

1 short-range attractive protein–protein interactions; and
2 long-range membrane-mediated repulsive interactions between clusters Sens and Turner (2004).

Determination of the nucleation rate during the first-order phase transition is usually reduced to solution of the Fokker–Planck equation for the cluster distribution function in the size space (Bravina and Zabrodin 1997; Fateev 2007). Accordingly, we propose the Fokker–Planck equation for the cluster size distribution function for describing the rearrangement of mobile fraction of band 3 molecules. We introduce the source term due to the increase the mobile fraction as a function of the long time-scale. The corresponding Fokker–Planck equation is expressed as:

$$\frac{\partial \rho(N,t)}{\partial t} = \frac{\partial}{\partial N}(\alpha N \rho(N,t)) + D_N \frac{\partial^2}{\partial N^2}\rho(N,t) + I(N,t) \quad (9)$$

where D_N is the dispersion coefficient such that $D_N \sim E_0$, α is a model parameter which represents the specific rate of membrane-mediated breaking of higher order clusters. Model parameter α depends on the elastic field generated within the lipid membrane after erythrocyte swelling $\alpha \sim E_0$. The reciprocal value of α represents the relaxation time for the corresponding long time-scale, i.e. $t_R = 1/\alpha$. The relaxation time could be identified from the experimental data of Hb release. It represents the time when mass flow of Hb reaches the maximum value $Q_{\mathrm{Hb}}(t_{\max})$. The source term $I(N, t)$ describes the phenomenon of membrane-mediated continual increase of the mobile fraction of band 3 molecules after erythrocyte swelling. It will be expressed as $I(N,t) = \beta\rho(N,t)$ (where β represents the specific rate of formation of the mobile fraction of band 3 such that $\beta \sim E_0$). Further model formulation includes quantitative analyses of changes in the number of band 3 per single cluster based on the Langevin-type equation. This is expressed as:

$$\frac{\mathrm{d}N(t)}{\mathrm{d}t} = -\alpha N(t) + \eta(t) + F_d \quad (10)$$

where the first term on the right hand side of Eq. 10 represents the rate of decrease of unstable high-order clusters. The second and third terms represent $\eta(t)$ the stochastic driving "force" and F_d the deterministic driving "force". The stochastic driving force is induced by protein-lipid short-range attractive interactions. It will be represented as Gaussian white noise which satisfies two conditions, i.e. $\langle\eta(t)\rangle = 0$ and $\langle\eta(t)\eta(t')\rangle = 2D_N\delta(t-t')$. The deterministic driving force quantifies the agglomeration of molecules induced by the self-association tendency. It is driven by protein–protein short-range attractive interactions. After ensemble averaging of Eq. 10, results for the average number of band 3 per cluster can be expressed as:

$$\frac{\partial\langle N(t)\rangle}{\partial t} = -\alpha\langle N(t)\rangle + F_d \quad (11)$$

where $\langle N(t)\rangle$ is the average number of band 3 molecules per cluster which is expressed as:

$$\langle N(t)\rangle = N_0 \mathrm{e}^{-\alpha t} + \frac{F_d}{\alpha}(1 - \mathrm{e}^{-\alpha t}) \quad (12)$$

where N_0 is initial number of band 3 molecules per cluster. We suppose that all band 3 molecules which were initially

mobile were monomers, i.e. $N_0 = 1$. The average number of band 3 molecules per cluster increases up to the equilibrium value $\langle N(t_{eq})\rangle = \langle N_{eq}\rangle$ at $t = t_{eq}$. The equilibrium value could be expressed, from Eq. 12, as $\langle N(t_{eq})\rangle = \frac{F_d}{\alpha}$. The equilibrium number of band 3 molecules per cluster is the result of two opposite actions. On one side, the associative tendency of band 3 molecules causes the formation of clusters which consist of a higher number of molecules whereas the action of the elastic field generated within the lipid membrane through long-range repulsive interactions causes the breaking of higher-order clusters. The variance will be equal to $\sigma^2(t) = \frac{D_N}{\alpha}(1 - e^{-2\alpha t})$. Accordingly, the distribution function $\rho(N, t)$ from Eq. 9 can be expressed analytically as:

$$\rho(N,t) = f(t_{eq})e^{-\beta(t_{eq}-t)}\frac{1}{\sqrt{2\pi\sigma^2(t)}}e^{-\frac{(N-\langle N(t)\rangle)^2}{2\sigma^2(t)}} \quad (13)$$

The number of clusters $v(t)$ increases gradually with time up to the equilibrium value $v(t_{eq})$. This can be expressed as:

$$v(t) = n_T f(t)\sum_{i=1}^{i=N_{max}}\frac{\rho(N_i,t)}{N_i} \quad (14)$$

Initial and boundary conditions for modeling considerations are: at $t = 0$ the fraction of mobile band 3 is approximately $f(0) \approx 0$ and the initial number of band 3 per cluster is $N_0 = 1$. The initial distribution function is $\rho(N, 0) = \delta(N - 1)$. For equilibrium conditions, at $t = t_{eq}$ the fraction of mobile band 3 is $f(t_{eq}) = f_{eq}$. The equilibrium values of the mobile fraction of band 3, f_{eq} for various experimental conditions are introduced from experimental work by Golan and Veatch (1980).

Results and discussion

The modeling considerations should point to some important cause-consequence relationships between the rearrangement of band 3 molecules and the generated elastic field within the lipid membrane after erythrocyte swelling. The elastic influence is described by the surface free energy of the lipid membrane, $E(\tau, t)$, which represents the sum of the surface-averaged value of the free energy after erythrocyte swelling, E_0, and the energy fluctuating term $E'(\tau, t)$. The fluctuating term represents the sum of local energy perturbations $E'(r, \tau, t)$ per swollen erythrocyte surface. The scheme of phenomena considered on two time scales (τ, t) is presented in Fig. 1.

The rearrangement of band 3 molecules induced by the action of the elastic field generated after erythrocyte swelling, E_0, during the long time-scale t is described by the increase of: (a) the mobile fraction $f(t)$, (b) the average number of molecules per cluster $\langle N(t)\rangle$, and (c) the number of clusters per erythrocyte surface $v(t)$. The changes of the cluster packing state occurred on the short time-scale in the range $\tau \in [0, \tau_{eq}]$ during the long time-scale t period obtained in the range $t \in [0, t_{eq}]$. The changes of the cluster packing state are induced by the action of the local energy perturbation of the elastic field, $E'(r, \tau, t)$, generated within lipid membrane. The packing state is expressed in term of the free volume within the cluster. The free volume within the cluster is quantified by the degrees of freedom $\chi_r(\tau, t)$ and $\xi_{ir}(\tau, t)$ (where $i = 1, 2$) as the free volume which corresponds to the molecule orientation and the coordination number, respectively.

The model terms which describe the rearrangement of band 3 molecules are shown in Fig. 1. The increase of the mobile fraction of band 3 molecules is quantified by the specific rate of increase the mobile fraction of band 3 $\beta = \beta(E_0)$. The tendency to self-association of mobile band 3 produces clustering driven by short-range attractive interactions. It is quantified by the deterministic driving force for agglomeration F_d. At the same time, the action of the elastic field causes the breaking of higher-order clusters. It is quantified by the specific rate of cluster breaking $\alpha = \alpha(E_0)$. These two opposite tendencies cause the equilibration of the number of molecules per cluster and the number of clusters per erythrocyte surface. This phenomenon is considered on the basis of experimentally determined fractions of mobile band 3 population $f(t)$ from Golan and Veatch (1980) for various hypotonicities of the external medium. First, we will describe the modeling considerations of short time-scale dynamics and after that the long time-scale dynamics of rearrangement of band 3 molecules.

The local perturbation of the elastic field $E'(r, \tau, t)$ causes the excitation of a cluster located at r as was shown in Fig. 1. This results in changes of the packing state expressed in terms of the degrees of freedom $\chi_r(\tau, t)$ and $\xi_{ir}(\tau, t)$ (where $i = 1, 2$). Such changes could cause breaking of the cluster or reversible osmotic hole formation for $\chi_{r0} = 1$ (average coordination number per molecule is minimum) and $\xi_{ir0} = 1$ (the orientation of molecules per cluster corresponds to the maximum value of Stoke's radius). This is the result of the changes of the free volume within the cluster, which becomes maximum. We introduce the "two-volume model" for consideration of the changes of the free volume within clusters during the process of cluster compaction. On that basis, two boundary conditions of the cluster are defined. The first corresponds to the minimum value whereas the second corresponds to maximum value of the free volume within the cluster. We suppose that if the free volume is maximum, the cluster represents the reversible osmotic hole. Similar modeling is proposed for consideration of packing state changes during the compaction process of powders, as reported by Edwards and Grinev (1998).

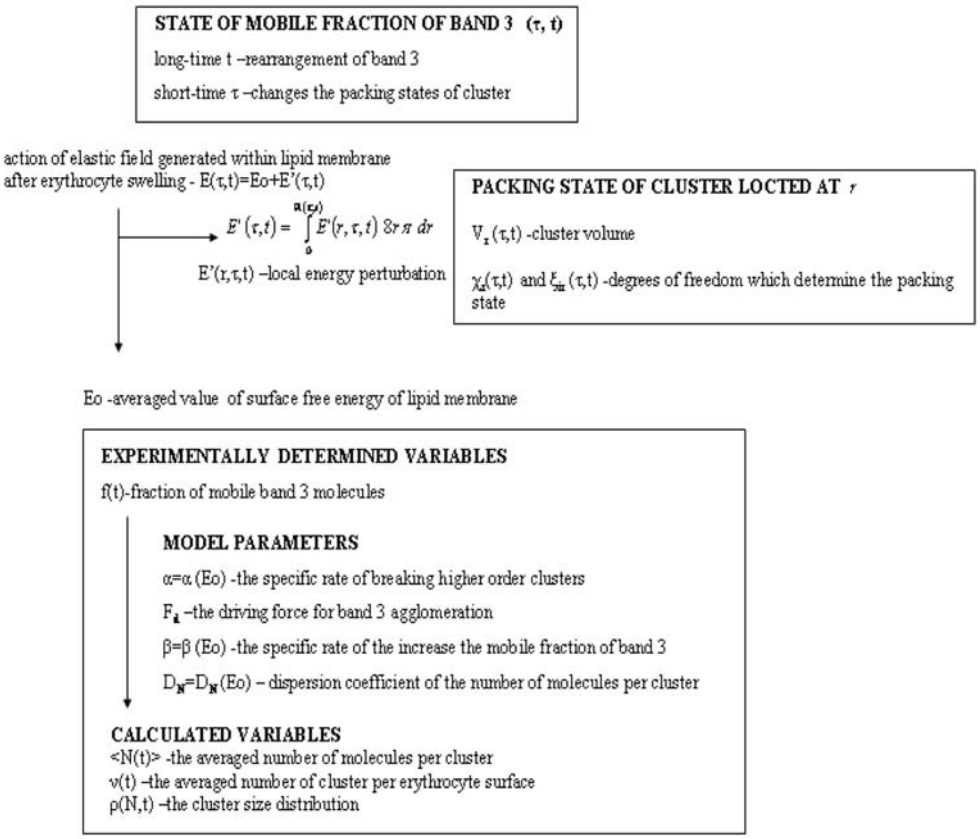

Fig. 1 Schematic representation of the considered phenomena on two time scales

For further modeling considerations it is necessary to introduce the range of the expected values of the equilibrium average number of molecules per cluster $\langle N(t_{\mathrm{eq}})\rangle$ (Eq. 12) for various experimental conditions as the results of the two mentioned tendencies. As Goldstein et al. (1996) reported for skate erythrocytes, the tetramer of band 3 molecules could generate the osmotic hole. Seeman et al. (1973) experimentally determined the diameter of the reversible osmotic holes in the range between 10 and 100 nm for human erythrocytes under hypotonic conditions at pH 7. The reversible osmotic hole is bordered by a ring of band 3 molecules. As Taylor et al. (1999) reported, the value of the Stokes radius of band 3 dimers is approximately 7.6 nm whereas for tetramers it is 11 nm at room temperature and pH 7.2. According to this, four molecules of band 3 could be enough to form the hole structure. Its area corresponds to minimum diameter found for osmotic holes (10 nm). So, we introduce the minimum value of the equilibrium average number of band 3 molecules per cluster as $\langle N(t_{\mathrm{eq}})\rangle_{\min} = 4$. The maximum value of $\langle N(t_{\mathrm{eq}})\rangle$ corresponds to the maximum diameter of the hole, i.e. 100 nm. Similarly as in the previous case, we introduce the maximum value of the average equilibrium number of band 3 molecules per cluster as $\langle N(t_{\mathrm{eq}})\rangle_{\max} =$ 40. We suppose that such clusters could be stable enough to form the reversible osmotic holes without breaking into smaller ones as a consequence of the action of the local energy perturbations $E'(r, \tau, t)$.

The volume changes of such clusters which can form the reversible osmotic holes can be calculated using Eq. 8. The model prediction for volume changes of two clusters, one consisting of four molecules (two dimers) and the other consisting of 40 molecules (20 dimers) as a function of short time-scale τ is shown in Fig. 2. For the calculation, we used the values of $R_{H(N=4)} = 5$ nm and $R_{H(N=40)} =$ 50 nm at $\tau = 0$. The assumed value of the average maximum Stokes radius of the dimer is $\langle r_s\rangle_{\max} \approx 7.6$ nm. The assumed value of the lipid bilayer thickness of the swollen erythrocyte is $h_m \approx$ 3–4 nm (Asami and Yamaguchi 1999). The volume of the cluster decreases exponentially during the time period $\tau \in [0, \tau_{\mathrm{eq}}]$ (Fig. 2).

For consideration of the long time-scale dynamics of rearrangement of band 3, we will use the proposed model for quantitative explanation of the experimental data from Golan and Veatch (1980). We will analyze two experimental sets at 21°C—high hypotonicity (46.0 mM Na_3PO_4 solution) and low hypotonicity (5.2 mM Na_3PO_4 solution). Irreversible, catastrophic effects were not observed for low hypotonic condition at 21°C. As Golan and Veatch (1980) reported for high hypotonicity, the value of the equilibrium

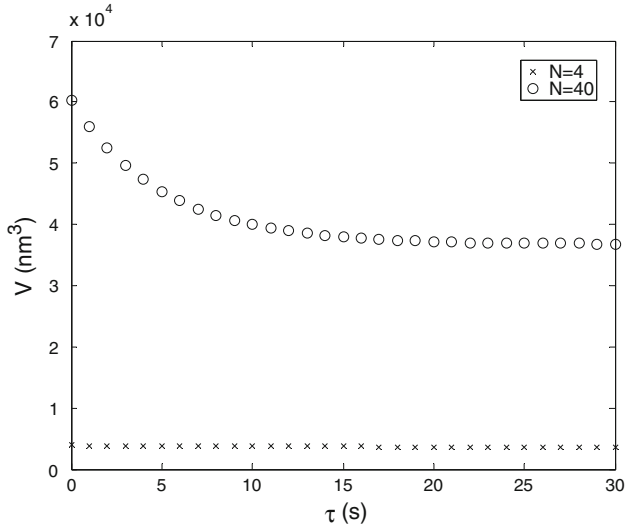

Fig. 2 The model prediction for volume changes for two clusters: one consists of four molecules and the other consists of 40 molecules as function of the short time-scale τ. The considered numbers of molecules of band 3 represent the minimum and maximum values for the stable clusters which can form the osmotic holes

mobile fraction of band 3 molecules is $f(t_{\mathrm{eq}}) = 0.11 \pm 0.09$ obtained after $t_{\mathrm{eq}} \approx 1{,}200$ s. For low hypotonicity, the value of the equilibrium mobile fraction of band 3 molecules is $f(t_{\mathrm{eq}}) = 0.72 \pm 0.07$ obtained after $t_{\mathrm{eq}} \approx 600$ s.

The average equilibrium number of molecules per cluster is expressed as: $\langle N(t_{\mathrm{eq}})\rangle = \frac{F_d}{\alpha}$. The relaxation time is approximately $t_R \approx 0.3 t_{\mathrm{eq}}$. Accordingly, the values of the specific rate of cluster breaking $\alpha = 1/t_R$ under high and low hypotonicity are $\alpha = 2.50 \times 10^{-3}\mathrm{s}^{-1}$ and $\alpha = 5.00 \times 10^{-3}\mathrm{s}^{-1}$, respectively. The choice of the value of $\langle N(t_{\mathrm{eq}})\rangle$ for various experimental conditions is of major importance for further calculation and determination of the model parameters. Additionally needed information can be obtained from Yeow and Clayton (2007). They estimated the density of clusters per cell surface as $19 \pm 4/\mu\mathrm{m}^2$. The total number of clusters is equal to $v = 3{,}100$ (for the radius of the swollen erythrocyte of 3.6 μm, as determined by Pribush et al. 2002). In our case, the equilibrium value of the number of clusters is calculated from $v(t_{\mathrm{eq}}) \approx \frac{n_T f(t_{\mathrm{eq}})}{\langle N(t_{\mathrm{eq}})\rangle}$. We can assume this value is the equilibrium number of clusters under higher hypotonic conditions $v_{\mathrm{high\,tonicity}}(t_{\mathrm{eq}}) \approx 3{,}100$. The corresponding value of the average number of molecules per cluster would be $\langle N(t_{\mathrm{eq}})\rangle_{\mathrm{high\,tonicity}} \approx 36$. If such a cluster forms a ring-like structure because of the action of the perturbation of the elastic field, the diameter of its hole would be 88 nm. This corresponds to the experimentally determined range of diameter of the osmotic hole by Seeman (1967) and Seeman et al. (1973). The deterministic driving force for agglomeration of band 3 molecules is expressed as: $F_{d\,\mathrm{high\,tonicity}} = \alpha_{\mathrm{high\,tonicity}}\langle N(t_{\mathrm{eq}})\rangle_{\mathrm{high\,tonicity}}$. The calculated value of the deterministic driving force for high hypotonicity is $F_{d\,\mathrm{high\,tonicity}} = 0.09\,\mathrm{s}^{-1}$. For further modeling consideration under low hypotonic conditions it is necessary to introduce the value for $\langle N(t_{\mathrm{eq}})\rangle_{\mathrm{low\,tonicity}}$ based on the discussion reported by Leiber and Steck (1982).

Leiber and Steck (1982) experimentally determined that the size of osmotic hole is approximately constant for the hypotonic conditions which affect the reversible formation of osmotic holes. As Golan and Veatch (1980) reported, irreversible effects on erythrocyte membranes are not observed under both hypotonicities considered at 21°C. Accordingly, we assume that the mechanism of formation of holes under these experimental conditions is the consequence of the rearrangement of band 3 molecules without erythrocyte rupture. On that basis, we assume the same value of the radius $R_H(\tau = 0, t_{\mathrm{eq}})$ of the reversible osmotic hole under both hypotonicities. This is indicative of the same value of the average equilibrium number of molecules per cluster at 21°C, i.e. $\langle N(t_{\mathrm{eq}})\rangle_{\mathrm{high\,tonicity}} = \langle N(t_{\mathrm{eq}})\rangle_{\mathrm{low\,tonicity}} = 36$. The average number of molecules per cluster as a function of the long time-scale under both hypotonic conditions is shown in Fig. 3a. The deterministic driving force obtained under low hypotonic condition corresponds to $F_{d\,\mathrm{low\,tonicity}} = \alpha_{\mathrm{low\,tonicity}}\langle N(t_{\mathrm{eq}})\rangle_{\mathrm{low\,tonicity}}$. The calculated value is $F_{d\,\mathrm{low\,tonicity}} = 0.18\,\mathrm{s}^{-1}$. The value of the deterministic driving force for agglomeration of band 3 molecules increases with decreasing tonicity. This is in accordance with the considerations reported by Taylor et al. (1999). They discussed the self-associative tendency of band 3 molecules as the result of short-range, Coulomb attractive protein–protein interactions. Higher concentrations of ions in the external solution could form local concentration gradients of ions near the active groups of band 3 molecules and prevent the self-associative tendency.

The values of β (the specific rate of increase of the mobile fraction of band 3) must be suitable to satisfy the boundary condition: for $t = t_{\mathrm{eq}}$ the equilibrium value of the mobile fraction of band 3 molecules f_{eq} corresponds to the experimental values under high and low hypotonic conditions from Golan and Veatch (1980). The experimental data and model predictions of long time-scale t evolution of the mobile fraction of band 3 molecules are shown in Fig. 3b. As shown in Fig. 3b, model prediction values for the mobile fraction of band 3 molecules correlated rather well with the experimental data, i.e. with relative error of 10% for both experimental conditions. The corresponding, optimum values for β are:

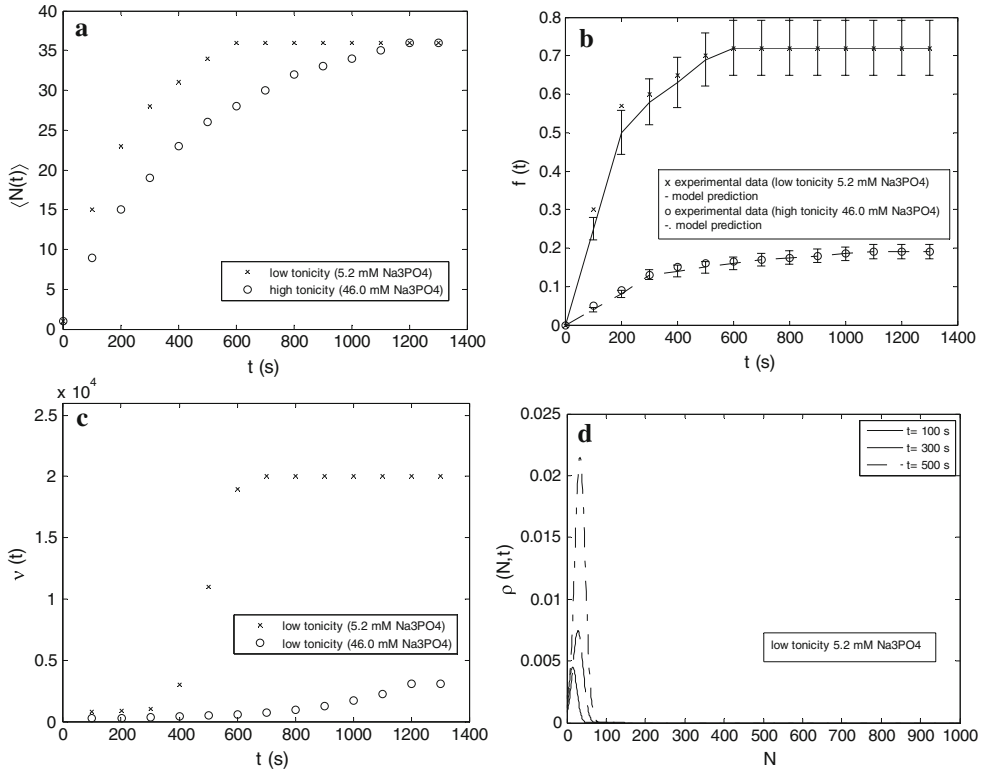

Fig. 3 **a** The model prediction of long time-scale t evolution of the average number of molecules per cluster $\langle N(t)\rangle$ calculated using Eq. 12, under low hypotonic (5.2 mM Na_3PO_4 solution) and high hypotonic (46.0 mM Na_3PO_4 solution) conditions (Golan and Veatch 1980). **b** Comparison of the experimental data and model prediction values for the mobile fraction of band three molecules with the average relative error of 10% under low hypotonic (5.2 mM Na_3PO_4 solution) and high hypotonic (46.0 mM Na_3PO_4 solution) conditions (Golan and Veatch 1980). **c** The model prediction of the number of clusters per erythrocyte as function of long time-scale t calculated using Eq. 14, under low hypotonic (5.2 mM Na_3PO_4 solution) and high hypotonic (46.0 mM Na_3PO_4 solution) conditions (Golan and Veatch 1980). **d** The model prediction of the distribution function $\rho(N, t)$ calculated using Eq. 13, under low hypotonic conditions (5.2 mM Na_3PO_4 solution) (Golan and Veatch 1980)

1 under low hypotonicity $\beta = (3.5 \pm 0.3) \times 10^{-3}\text{s}^{-1}$; and
2 under high hypotonicity $\beta = (3.0 \pm 0.3) \times 10^{-3}\text{s}^{-1}$.

The higher value of β is calculated under lower hypotonic conditions. This condition ensures the higher value of the equilibrium mobile fraction of band 3 molecules.

The values of dispersion coefficient D_N must be suitable to satisfy the boundary condition: for $t = t_{\text{eq}}$ the equilibrium value of number of clusters per erythrocyte surface is $v(t_{\text{eq}})$. As previously calculated, the number of clusters per erythrocyte surface under high hypotonicity is $v(t_{\text{eq}}) \approx 3{,}100$ whereas under low hypotonicity $v(t_{\text{eq}}) \approx 20{,}000$. The model prediction of the number of clusters per erythrocyte surface as a function of long time-scale t calculated using Eq. 14 is shown in Fig. 3c. The corresponding, optimum values for the dispersion coefficient D_N are:

1 under high hypotonicity $D_N = 0.5 \pm 0.2\ \text{s}^{-1}$; and
2 under low hypotonicity $D_N = 0.9 \pm 0.2\ \text{s}^{-1}$.

The higher value of the dispersion coefficient D_N is calculated under lower hypotonic condition. This is indicative of a higher value of the variance of the number of molecules per cluster and intensive rearrangement of the band 3 molecules. The model prediction of the distribution function $\rho(N, t)$ from Eq. 13 under low hypotonic condition is shown in Fig. 3d. The contribution of higher-order clusters can be neglected to a first approximation.

Conclusions

In summary, the results of this study pointed to some important cause–consequence relationships between the rearrangement of band 3 molecules and the generated elastic field within the lipid membrane after erythrocyte swelling. The process can be considered on two time scales. The rearrangement of band 3 molecules obtained on a long time-scale is described by the increase of:

1 the mobile fraction;
2 the average number of molecules per cluster; and
3 the number of clusters per erythrocyte surface.

The changes of the packing state of molecules in the cluster are obtained on the short time-scale. The increase of the mobile fraction of band 3 is induced by the action of the elastic field generated within the lipid membrane after erythrocyte swelling. The tendency to self-association of mobile band 3 causes the micro-phase transition. The micro-phase transition of band 3 molecules results in clustering which could be described as the process of homogeneous nucleation. At the same time, the action of the elastic field causes breaking of higher-order clusters. Such opposite tendencies direct the dynamics of rearrangement of band 3 population into the equilibrium state.

Molecules of band 3 within the cluster tend to form the compact packing which ensures the minimum of free volume. However, the local energy perturbation of the elastic field after erythrocyte swelling induces the excitation of the cluster. This changes the packing states and the value of the free volume within the cluster. This could result in cluster breaking or reversible osmotic hole formation. If the free volume is maximum, the corresponding excited cluster represents the reversible osmotic hole. On that basis the formation of holes could be explained as the consequence of the local energy perturbation of the elastic field generated within the lipid membrane. We developed a simple modeling framework based on some new thermodynamic approaches. This enabled prediction of the complex phenomenon reversible hole formation. The model parameters are:

- the specific rate of cluster breaking $\alpha = \alpha(E_0)$;
- the deterministic driving force of agglomeration of band 3 F_d;
- the specific rate of increase of the mobile fraction of the band 3 population $\beta = \beta(E_0)$; and
- the dispersion coefficient $D_N = D_N(E_0)$.

The phenomenon is considered starting from the experimental data of Golan and Veatch (1980). They determined the mobile fraction of band 3 molecules for two sets of experimental conditions, i.e. low and high hypotonicities at room temperature.

The modeling considerations pointed to the higher values of the parameters:

- the deterministic driving force of agglomeration of band 3;
- the specific rate of cluster breaking;
- the specific rate of increase of the mobile fraction of band 3; and
- the dispersion of the cluster sizes obtained

under lower hypotonic conditions.

Lower hypotonic conditions ensure the generation of a higher elastic field within the membrane after erythrocyte swelling, which enables more intensive rearrangement of band 3 molecules.

If experimental conditions do not provide possibilities for reorganization of band 3 clusters as described above another mechanism can appear leading to crack formation and rupture of the membrane. This usually occurs under low hypotonicity conditions and at higher temperatures. Then it is not possible to realize the two time scale rearrangements of band 3 as described in our model and another mechanism controls the processes. This is not the subject of our model.

Acknowledgments This research was funded by grants (#142075) and Hemiron, Eureka (#4486) from the Ministry of Science and Environmental Protection, Republic of Serbia.

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