Title:
SPIN LABEL STUDIES OF THE HEMOGLOBIN-MEMBRANE INTERACTION DURING SICKLE HEMOGLOBIN POLYMERIZATION

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Short Title:
Hemoglobin-membrane interaction with hemoglobin S

ABSTRACT.
An enhanced hemoglobin-membrane association has been previously documented in Sickle Cell Anemia. However, it is not known how this interaction is modified during the hemoglobin S polymerization process. In this work, we use a model of reconstituted erythrocytes from ghost membranes whose cytoskeleton proteins had been previously labeled with the 4-maleimido Tempo spin label, and that were subsequently resealed with hemoglobin S or A solutions. Using EPR spectroscopy, we studied the time dependence of the spectral W/S parameter, indicative of the conformational state of cytoskeleton proteins (mainly spectrin) under spontaneous deoxygenation, with the aim of detecting the eventual effects due to hemoglobin S polymerization. The differences observed in the temporal behaviour of W/S in erythrocytes reconstituted with both hemoglobins were considered as experimental evidence of an increment in hemoglobin S-membrane interaction, as a result of the polymerization process of hemoglobin S under spontaneous deoxygenation.

Key words: hemoglobin-membrane interaction, hemoglobin S polymerization, reconstituted erythrocytes, 4-maleimido Tempo spin label.

Introduction.

Sickle Cell Anemia (SCA), or homozygous sickle cell disease, is a genetic disorder, which affects about 250,000 children worldwide every year; many of them have multiple strokes and die before they are two years old (1,2), The disease is caused by a mutated hemoglobin (HbS) that unlike the normal HbA, has a high propensity to polymerize when in the deoxygenate state in the venous circulation. The polymerization alters the shape and rigidity of red blood cells (RBC), and triggers a sequence of pathogenic consequences.

Polymerization of HbS constitutes the basic molecular process in SCA (3,4). This process has been widely studied by our group using Magnetic Resonance (5-10). However, the main physiopathological manifestations of this disease are related to the change on the shape and the physical properties of the RBC.

Numerous reports have documented an abnormal enhanced HbS-membrane interaction (11-16). Fung et al. (15), using spin-label technique in ghost membranes, shown that the binding of HbS molecules to the membrane at physiological pH is increased when compared with normal Hb. Eisinger et al. (17), employing measurements of Resonance Energy Transfer efficiencies from fluorescent probes on intact SS oxygenated RBC, showed that HbS concentration proximal to the membrane (h_b) is significantly higher that Hb concentration in the cytoplasm (h_c), and that the ratio between these two concentrations (h_b/h_c) increases with the cellular Hb concentration. This enhanced binding of HbS has been related with a subsequent damage to the RBC membrane (12,13,15), and a significant effect on the HbS polymerization process (14). However, it is not known how this interaction is modified during the HbS polymerization process.

EPR spectroscopy of proteins spin-labeled with maleimide derivatives has been largely used to assess mobility and conformational changes at the sulfhydryl groups of proteins (18-20). The EPR spectra of maleimide spin-labeled proteins are generally composed of two spectral components: the first, a broad anisotropic spectrum, arisen from labels bound in a strongly immobilized environment (S), and the second, a narrower spectrum due to sites in a weakly immobilized environment (W). The W/S ratio is highly sensitive to conformational changes in the spin label environment. For this reason, this ratio has been used to monitor the changes in protein conformation and in the membrane environment. (15,18-20) It has been shown that when RBC membranes are labeled with maleimide derivatives, the spin label is covalently bound to spectrin, the main component of the RBC cytoskeleton.

The Hb-membrane interaction and its eventual modifications as a result of the HbS polymerization process should be a decisive factor in the SCA pathophysiology. In this paper, our objective is to detect these modifications by monitoring the time evolution of the EPR spectrum of model erythrocytes prepared from spin labeled RBC membranes reconstituted either with HbS or HbA. We followed spontaneous deoxygenation at 36ºC, and the changes in the interaction of Hb molecules with the RBC membranes during HbS polymerization process are evaluated through the temporal behavior of the W/S spectral parameter. Spin-labeled erythrocytes reconstituted with phosphate buffer or bovine serum albumin (BSA) were used as control samples where the Hb-membrane association is absent.

Materials and Methods.

Preparation of unsealed erythrocyte ghosts

Fresh human blood from voluntary healthy donors among laboratory personnel was used each day. The blood was collected into heparinized tubes, with approximately 20 ml of blood used for each preparation. The method of Steck and Kant (21) was used with some minor changes for the preparation of unsealed erythrocyte ghosts. Whole blood was centrifuged in a bench centrifuge at 1500 g for 10 min, after which the plasma and buffy coat were aspirated. The packed blood cells were washed 3 times with phosphate buffered saline (PBS) (150 mM NaCl, 5 mM Sodium phosphate, pH=8), and then lysed for 5 min at 4 ºC in 12 volumes of hypotonic phosphate buffer 5 mM, pH=8 (5P8) with constant stirring, followed by centrifuging for 10 min at 22 000 g. The resulting pellet was washed four more times in 5P8 (4ºC, 22 000 g, 10 min) for complete hemoglobin separation. The ghosts were finally resuspended in the same lysis buffer for labeling.

Spin labeling of unsealed erythrocyte ghosts

The unsealed ghosts of 4 mg/mL in protein concentration were labeled with 4-Maleimido-2,2,6,6-tetramethyl-1-piperidinyloxyl (4-Maleimido Tempo, or 4MT). This spin label (from Sigma-Aldrich) was stored in ethanol, which was evaporated with a gentle flow of N_2 prior to the introduction of ghost membranes. A total of 50 µg of 4MT was used per each milligram of membrane protein. After 1 hour of incubation with gentle stirring in the dark, the membrane ghosts were washed 4 times with buffer 5P8 to remove unbound spin-label. The spin-labeled membrane ghosts were stored overnight at 4 ºC in buffer 5P8 (with 3 g/L BSA) until the reconstitution in the next day.

Preparation of hemoglobin solutions.

Hemoglobin solutions were prepared by the method of Clark and Shohet (22) with minor modifications. Red blood cells from healthy volunteer donors (HbA) or from homozygous sickle cell anemia patients (HbS) were washed with PBS (pH=7.4) and then lysed in 10 volumes of dilute buffer (2,5 mM Sodium phosphate, pH=7.4). Hemoglobin was separated from the membranes by two centrifugations at 22000 g, 10 min, 4ºC. Then, the dilute hemoglobin solution was concentrated with Amicon Ultra-4 Centrifugal Filter Devices (30,000 NMWL).

Preparation of reconstituted erythrocytes from Spin-labeled ghost membranes

Erythrocyte reconstitution was carried out using the exchange hemolysis technique described by Clark and Shohet (22). In brief, three volumes of hemoglobin concentrate (HbA o HbS) were added to 1 volume of spin-labeled membrane ghosts. After allowing the membrane-hemoglobin mixture to equilibrate for 5 min, ATP (80 mM) and $CaCl_2$ was added to a final concentration of 1 mM. The solution was mixed, and enough KCl was added to restore molarity to 150 mM. The membrane suspension was incubated at 37 ºC with gentle periodic agitation to promote membrane resealing. The excess of untrapped hemoglobin and other added substances was eliminated by performing several re-suspensions and washings in buffer PBS with glucose (2 g/L) and BSA (3 g/L), pH=7,4.

In order to have a control system with absence of hemoglobin-membrane interaction, spin-labeled erythrocyte membrane ghosts were reconstituted with phosphate

buffer (PBS) or BSA (20 g/dl).

EPR measurements.

Electron paramagnetic resonance (EPR) experiments were performed at 9.7 GHz frequency (X band) in a Bruker ER 200 spectrometer. The samples were placed in fine wall capillaries, subsequently sealed in both ends. Temperature was regulated at (36±1)ºC with a nitrogen flow system Bruker VT1000. The spectra were taken at 2 mW microwave power, modulation amplitude according to the experiment necessities, and 100 kHz modulation. Spectra were obtained at preset times (taking t=0 as the moment when the samples were placed at T= 36ºC), maintaining the samples at constant temperature.

W/S ratio was evaluated as a function of time in samples of resealed spin-labeled erythrocyte membrane ghosts reconstituted with PBS (without proteins), and in samples of resealed spin-labeled erythrocyte membrane ghosts reconstituted with HbA, HbS and BSA respectively.

Microscopic analysis

Immediately after resealing, "fresh drop" images of erythrocytes reconstituted with Hb were obtained. Small aliquots of diluted cells were deposited on optical slides (previously coated with albumin solution in order to avoid the "glass effect"), then covered with a glass coverslip and analyzed at 100 and 400 × magnification (microscope Axiolab, Carl Zeiss, Germany). Images were captured with a digital-video camera (SSC-DC50A, Sony, Japan).

RESULTS

Using the method described above, we have found that it is possible to prepare reconstituted erythrocytes with spin-labeled membranes, refilled with either HbA or HbS, as can be seen in Figure 1.

Figure 2 shows the EPR spectrum of reconstituted erythrocytes labeled with 4MT. There are two spectral components, clearly evidenced in the low field region. This fact implies the coexistence of two spin label populations with different mobilities. The spectral components are quantified through W (peak-to peak amplitude of the low field signature of the weakly immobilized component) and S (baseline-to-peak amplitude at low field of the strongly immobilized component).

An important time decrease of the intensity of the whole EPR spectrum was observed in erythrocytes reconstituted with Hb (A and S). Instead, in labeled membranes resealed with BSA and PBS, EPR intensity remained constant in time. This behavior is shown in Figure 3, where we have plotted the EPR spectral intensity, roughly estimated through S + W (see Figure 2) from spectra acquired in the same experimental conditions.

Figure 4 shows the W/S evolution in samples of erythrocytes reconstituted from membrane samples of different donors and using different resealing mediums, kept at 36ºC and during 600 min of spontaneous deoxygenation. As it can be seen, membranes resealed with PBS or BSA show a linear W/S increase in time, while in HbA resealed membranes W/S remains almost constant, differing from the t=0 value less than ± 0.1.

When ghost membranes were reconstituted with HbS, a marked difference in the time evolution of W/S was observed, as it can be seen in Figure 5. In this case, W/S remains constant at nearly the same value of HbA samples up to a certain time, beyond which a steady decrease in W/S values is observed. The same behavior was obtained when membranes of different healthy donors were used, as seen in Fig. 4. The time beyond which W/S began to decrease was around 250 min. W/S values for erythrocytes resealed with BSA are also included in the figure.

DISCUSSION

We have studied the differences in the time evolution of the EPR spectra of reconstituted erythrocytes prepared from spin labeled membranes of healthy donors, refilled with HbS or HbA solutions. The spin-label was maleimido-Tempo, which binds covalently to the cytoskeleton membrane proteins. Our objective was to detect changes in the membrane behavior related to the spontaneous intracellular polymerization of HbS. We started by labeling ghost membranes in 5 mM phosphate since they represent a well-characterized membrane system (21). The unsealed ghost membranes allow the easy access of the spin label to the cytoplasmic surface, necessary to establish the binding to the cytoskeleton proteins. Subsequent cell resealing was promoted in four different media: phosphate buffered saline solution, and BSA, HbA, and HbS solutions. Resealing with Hb solutions yielded reconstituted cells that closely resemble the intact erythrocytes in their shape, except for a decreased mean cell volume.

The EPR spectrum of reconstituted erythrocytes labeled with 4MT are similar to that of spin-labeled membrane ghosts reported in the literature (23,24), and verified in our laboratories. The effect of many experimental factors, such as pH, ionic strength and temperature on W/S ratio has been previously reported in the literature (25,26). For these reasons, we worked under controlled conditions, using for each experiment fresh membranes, and taking the EPR spectra at the same time delay.

The different reconstituted cells showed a distinctive time evolution in their EPR spectra. As shown in Figure 3, in the case of membranes reconstituted with PBS and BSA, total spectral intensity remained constant during the time span of the experiment, but a steady decrease was observed in membranes reconstituted with HbA and HbS. A similar behavior has been reported by other authors when working with maleimide spin labeled Hb and BSA. It was demonstrated that the presence of SH groups was responsible of that behavior, and it was assumed that spin label reduction took place (27). In our work, it is remarkable the similar EPR time decrease for erythrocytes reconstituted with both hemoglobins A and S. However, the EPR intensity remained constant when BSA solutions are used in cell resealing. Assuming that a SH mediated reduction as that proposed by Perussi JR et al. (27) is active, our results indicate that when BSA is the protein present in the reconstituted cells, its SH groups do not interact with the spin labeled membrane cytoskeleton. On the other hand, when Hb(A or S) is present, they both interact with the membrane in a similar fashion regarding the reduction of the spin label.

We found a distinctive time behavior of W/S depending on the resealing medium, independent of the membrane donor: W/S increased steadily in the whole time span in a linear fashion when resealing was performed with PBS or BSA, and remained constant at

short times when resealing was performed with Hb. One possible explanation for this increment was given in terms of an increase in the opening of the sulfhydryl cavity, favoring the access of the solvent to the spin label surroundings and thus increasing the proportion of weakly immobilized label (20).

It is established that the high-affinity sites for hemoglobin binding to the cytoplasmic surface of the RBC membrane are the inner segments of band 3. [11,28] The hemoglobin-linked band 3 may favor electrostatic interactions between Hb molecules and 4MT bound to neighbor SH groups, contributing to decrease the mobility of the spin label and the membrane segments in which Hb binding takes place. This could compensate the W/S increment provoked for the opening of the sulfhydryl cavity, explaining the constant W/S ratios observed at short times when resealing was performed with Hb.

A striking difference between HbA and HbS was observed at times around 250-300 min: while W/S remains constant for HbA, it begins to decrease in HbS resealed samples. This decrease in the ratio of high mobility to low mobility spin-labeled sites suggests a decrease of spectrin segmental motions. As the time when this effect happens is closely related with the beginning of the HbS polymerization process under spontaneous deoxygenation (5), and doesn't happen in samples with HbA, we propose that the modification in spectrin dynamics is due to the changes in HbS-membrane interaction when HbS aggregates are formed.

In previous works we have reported studies of the HbS polymerization process by using Proton Magnetic Relaxation, both in HbS solutions and in packed erythrocytes of SCA patients, under spontaneous deoxygenation conditions. Proton spin-lattice (T_1) and spin-spin (T_2) relaxation showed a time behavior with sigmoidal characteristics (5,7-10,29,30). The delay time (t_d) (where the irreversible development of the polymerization begins) obtained in these studies was (355±82) min from T_1, and (325±68) min from T_2 (29). Other works inform the presence of a spontaneous deoxygenation process in HbS and HbA samples, without the need of forced deoxygenation. It is shown that 10 % deoxygenation is reached in times around 250 min, similar to those reported for the irreversible beginning of the polymerization (31,32).

We have the hypothesis that HbS linked to the membrane can be involved in the formation of polymeric fractions or constitute active places of polymerization, in agreement with other authors [14]. In consequence, this process may include an increment in the Hb-membrane interaction by the incorporation the new HbS molecules, increasing the binding sites or creating another links on the cytoplasmic surface. This would contribute to decrease the mobility of the spectrin segments to which the spin label is associated, thus decreasing the W/S ratio.

We consider that the changes in the time evolution of the spectra of erythrocytes reconstituted with HbS shown in Figure 5, are due to a strengthening of the Hb-membrane interaction related to the polymerization process, what could constitute a direct link between the abnormalities present in the sickle erythrocyte membranes and the polymerization of HbS.

In the course of the erythrocytes in the circulatory system, repetitive cycles of polymerization-depolymerization, due to the oxygenation-deoxygenation cycle, take place. Therefore, according to our results, simultaneous cycles of Hb-membrane association

increment would also take place, that should have a deleterious effect on the organization and functionality of the cell membrane. These effects can be irreversible if the formation of irreversibly sickled cells (ISCs) takes place. Cells that don't recover their normal shape after oxygenation are poorly deformable, dehydrated, of short life span, prone to hemolysis, and contribute to the pathophysiology of vaso-occlusive episodes (33). Some SCA patients have 5-40% of the circulating erythrocytes as ISCs. It is also known that all sickle cells become ISCs when cycled through many times of deoxygenation and oxygenation. Thus, polymerization of HbS is reversible, but sickling of the cells is irreversible. For that reason, we believe that the increment in the Hb-membrane interaction during HbS polymerization process should have a direct impact in the membrane abnormalities, influencing the main pathological manifestations of the SCA. New studies of the polymerization role in the Hb-membrane association and their transcendence in the membrane abnormalities are necessary. Aprelev et al. (14) showed an increased effect of membrane interaction on HbS polymerization when ghosts from SCA patients were used. In this line, it would be interesting to extend our study to reconstituted erythrocytes from sickle cell membranes.

Acknowledgements

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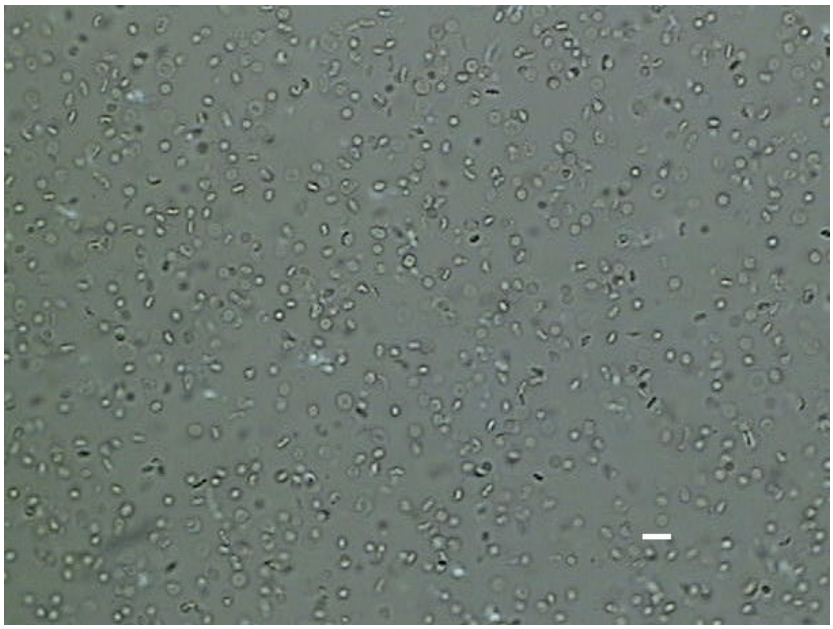

Figure 1: Optical microscopy of erythrocytes reconstituted with HbA. Magnification, 400 ´. Photograph was take by digital video imaging. Bar = 10 micrometers.

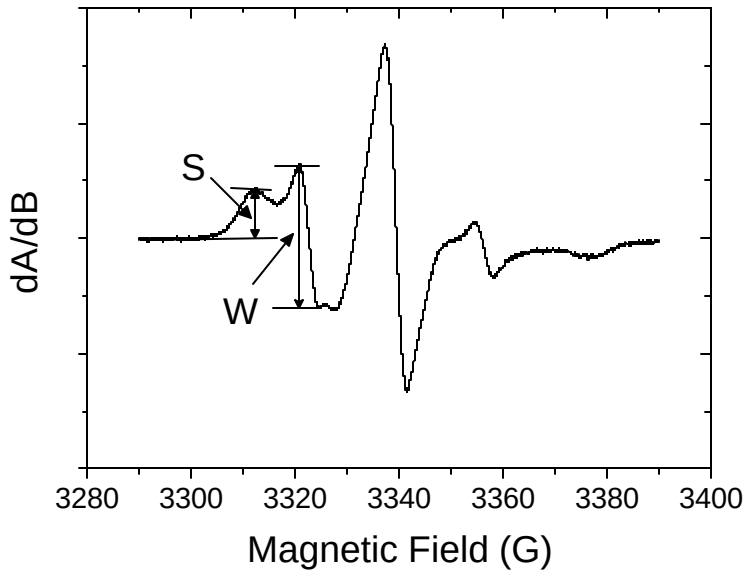

Figure 2: Typical composite EPR spectrum of spin-labeled erythrocyte membranes reconstituted with HbA .W is the height of the low field peak of the weakly immobilized spectral component, and S that correspondent to the strongly immobilized component. Spectrum obtained at 9.7 GHz (X band), 4 G modulation amplitude, 2 mW microwave power, temperature: 36 ± 1ºC.

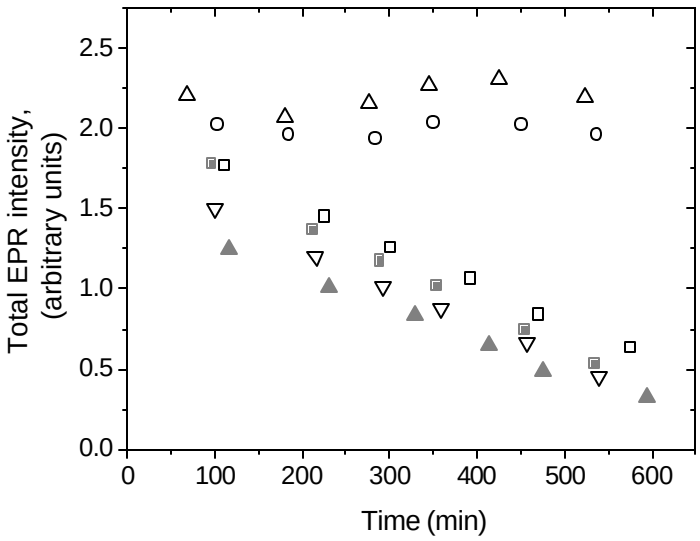

Figure 3: Total EPR signal intensity (estimated through S signal height + W signal height), calculated from the EPR spectra of the different erythrocytes reconstituted as a function of time, adquired in the same experimental conditions. (△) spin-labeled erythrocyte membranes reconstituted with PBS, (○) spin-labeled erythrocyte membranes reconstituted with BSA, (□,■) spin-labeled erythrocyte membranes reconstituted with HbA, (▽,▲) spin-labeled erythrocyte membranes reconstituted with HbS. Open and closed symbols represent membranes from two different healthy donors.

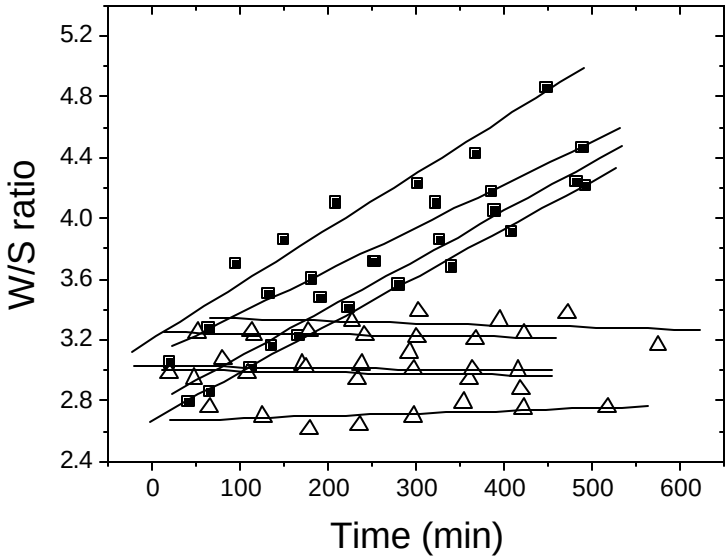

Figure 4: Time behavior of W/S ratio in reconstituted erythrocytes from different donors. (■) Values from spin-labeled erythrocyte membranes reconstituted with PBS or BSA, (?) values of spin-labeled erythrocyte membranes reconstituted with HbA. The lines correspond to linear fits.

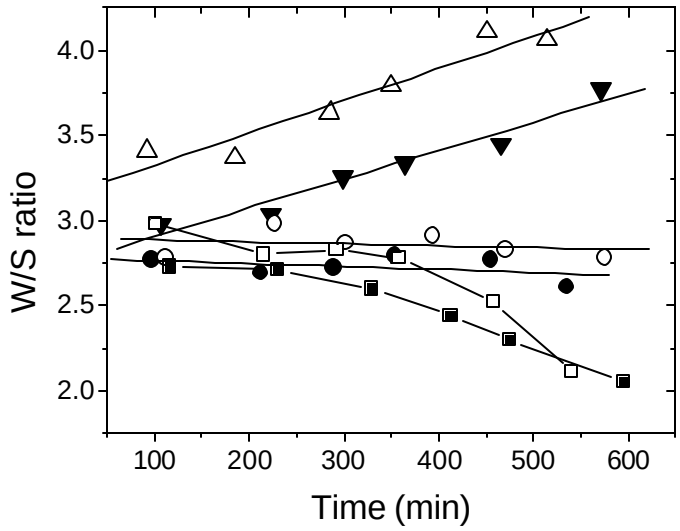

Figure 5: Time behavior of W/S ratio in different reconstituted erythrocytes. (△,▼) spin-labeled erythrocyte membranes reconstituted with BSA, (○,●) spin-labeled erythrocyte membranes reconstituted with HbA, (□,■) spin-labeled erythrocyte membranes reconstituted with HbS. Open and closed symbols represent membranes from two different healthy donors. The lines correspond to linear fits in the cases of BSA and HbA, and are guides to the eye in the case of HbS.