ELSEVIER

Biochimica et Biophysica Acta 1371 (1998) 284–294

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The effect of oxidative stress on structural transitions of human erythrocyte ghost membranes

Vladimir R. Akoev *, Alexander V. Matveev [1], Tatiana V. Belyaeva, Yuri A. Kim

Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russian Federation

Received 27 February 1998; accepted 5 March 1998

Abstract

Differential scanning microcalorimetry was used to study the effect of oxidative stress induced by cumene hydroperoxide (CHP) and Fe^{2+} on structural transitions of membranes of human erythrocyte ghosts. The CHP homolysis was shown to cause: (a) reduction of the intensity of all structural transitions with the disappearance of B_1- and D-transitions; (b) decrease in the enthalpy of oxidized membrane denaturation; (c) negative slope of thermograms; (d) anomalous growth of heat absorption by membranes above 72°C. All these changes occurred until the ratio Fe^{2+}/CHP/membranes < 0.02:0.05:1 was reached, i.e., prior to the moment of maximal level of TBA-RS in membrane ghosts. We interpret changes in the character of heat absorption by oxidized membranes as perturbations in the structural organization and interactions inside the spectrin–actin-protein 4.1 domains, the spectrin-protein 4.2 domain, as well as inside the domain of spectrin–ankyrin–cdB3 and the domain formed by the msdB3. These perturbations are associated mainly with the decrease in the concentration of native protein in the domains because of oxidative aggregation of proteins, as evidenced by SDS electrophoresis of oxidized membranes. Preincubation of membranes with tocopherol did not block the aggregation of proteins in electrophoresis and the decrease in the intensity of structural transitions, whereas it blocked completely the formation of TBA-RS, changes in the thermogram slope and the sharp rise in the heat absorption above 72°C. This proves that these processes are determined by the thermotropic properties of the oxidized lipid bilayer of membranes and also provides evidence that the degradation of PUFA of phospholipids modifies both the structure of protein domains and the physical properties of the lipid bilayer of membranes. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Scanning microcalorimetry; Structural transition; Erythrocyte membrane skeleton; Lipid peroxidation; Cumene hydroperoxide

Abbreviations: CHP, cumene hydroperoxide; *t*-BHP, *tert*-butyl hydroperoxide; TBA-RS, thiobarbituric acid reactive substances; PBS, phosphate-buffered saline; DSC, differential scanning calorimetry; ΔH, enthalpy of denaturation; T_{max}, temperature of transition maximum; ΔC_p^{max}, intensity of transition; $\Delta T_{1/2}$, transition half-width; dry wt., dry weight of membranes; Band 3, band 3 protein; msdB3, membrane-spanning domain of band 3 protein; cdB3, cytoplasmic domain of band 3 protein; HMWA, high-molecular-weight aggregates of proteins; PUFA, polyunsaturated fatty acids

* Corresponding author. E-mail: akoev@venus.iteb.serpukhov.su

[1] Present address: Institute of Theoretical and Experimental Biophysics, Russian Academy of Science, Pushchino, Moscow Region 142292, Russia.

0005-2736/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII S0005-2736(98)00037-6

1. Introduction

Singe-electron reduction of hydroperoxides of fatty acids catalyzed by iron and/or hemoproteins is one of the major sources of free lipoxyl and lipodioxyl radicals in cell membranes [1,2]. Numerous studies, based on the use of organic hydroperoxides such as cumene hydroperoxide (CHP), *t*-butyl hydroperoxide (*t*-BHP) [3–6] and hydroperoxides of fatty acids [5,7–9], made it possible to elucidate many details of the mechanisms of free radical-induced lesions of erythrocyte membranes. Hydroperoxides are known to induce hemolysis [10–17] and to modify antigenic properties of erythrocytes [12,16,18,19] only in the presence of catalysts, such as hemoproteins or free iron ions. The prehemolytic process includes: Hb degradation [8,12–14,20,21] and a stronger binding of degraded Hb to membrane [13–16], disintegration of PUFA of phospholipids [3,5,12,15,22,23] and changes in the asymmetric distribution of lipids in membranes [15], accumulation of fluorescent chromolipids in membrane [6,21,24], changes in the activity of membrane-bound and cytosolic enzymes [20,21], and modification of the ionic permeability of membrane [21,25–27], decrease in the deformability and mechanical stability of erythrocytes [10,16,28]. Homolytic degradation of hydroperoxides was shown to induce the disappearance of electrophoretic bands of spectrin, ankyrin, actin, Band 3, band 4.1 protein, and glycophorin, as well as the aggregation of these proteins into HMWA [6,8,10,12,15,20,24,29,30]. Apparently, the oxidation and aggregation of proteins can be regarded as the final stage of the oxidative degradation of the membrane skeleton of erythrocytes. It is also obvious that the oxidation and aggregation of proteins is accompanied by global conformational restructuring of protein domains of the membrane skeleton. Recently, Carpari et al. [30] showed that *t*-BHP induces redistribution–marginalization of spectrin that, in the authors' view, is associated with changes in the structure of the junction complex of the membrane skeleton. According to the data of Sato et al. [31], derived using circular dichroism, radicals modify the conformation of the cdB3, whereas the inhibition of the Band 3 protein oxidation inhibits the aggregation and oxidative hemolysis. Nonetheless, few data have been reported which present direct evidence of changes in the conformation of membrane domains during peroxidation. Differential scanning microcalorimetry (DSC) is the most adequate method for solving this problem. DSC makes it possible to reveal five structural thermal transitions in ghost membranes called A-, B_1-, B_2-, C- and D-transitions [32]. These transitions are determined by thermal denaturation of membrane domains composed of a set of proteins of the membrane skeleton [33,34]. The A-transition is due to denaturation of the domain formed by α- and β-spectrin–actin [33,35], the B_1-transition is induced by denaturation of the domain formed by ankyrin and proteins of bands 4.1, 4.2, and 4.9 [33]; the B_2-transition is determined by denaturation of the cdB3 [36,37]; the C-transition is associated with denaturation of the msdB3 [37,38]. The nature of the D-transition remains still unclear, though it is known to be related to denaturation of proteins [32]. The present study has shown that the oxidation induced by homolytic degradation of CHP causes changes in the structural organization of all domains of the membrane skeleton of erythrocytes.

2. Materials and methods

2.1. Materials

Reagents: $FeSO_4 \cdot 7\ H_2O$, puriss., from Merck (Darmstadt, Germany); NaCl, Na_2HPO_4 all of the reagent grade, and glycine, Tris-base, dodecyl sulfate–Na salt all of the electrophoresis grade, cumene hydroperoxide were purchased from Sigma Chemical (Milwaukee, MO). 2-Thiobarbituric acid, purum., was from Fluka (Buchs, Switzerland). DL-α-Tocopherol, pharm., 1 IE/mg, was from Serva (Heidelberg, Germany). All solutions were prepared with twice distilled (glass) water. Stock solutions of $FeSO_4$ in water, CHP and tocopherol in 95% ethanol were prepared shortly before the experiment.

2.2. Preparation of membranes

Red cells were isolated from fresh donor blood using glucose citrate solution as anticoagulant. Erythrocyte ghost membranes were obtained via hemolysis in cold 5 mM sodium phosphate buffer, pH 8.0 ($5P_i8$) as described in [39] with extensive washing in the buffer $5P_i8$. In the final stage the ghosts were

suspended in isotonic 310 milliosmolar sodium phosphate buffer, pH 7.4, (310P_i7). The concentration of membranes was measured in mg (dry wt.) after drying the suspension at 105°C for 30 min. The heme content in each extensively washed ghost preparation was determined from the absorbance (at 398 nm) of ghosts dissolved in formic acid according to Ref. [40].

2.3. Electrophoresis

Electrophoresis was carried out in gradient gels (5–15% acrylamide) in a modified buffer system of Laemmly as described in Ref. [39]. To reveal HMWA, membrane samples were solubilized in buffer without SH-reagents (dithiothreitol or mercaptoethanol).

2.4. Microcalorimetry

The temperature dependence of excess specific heat absorption (thermograms) by suspension of erythrocyte membrane was recorded using a differential adiabatic scanning microcalorimeter DASM-4 (Bureau of Biological Instrumentation of the USSR Academy of Sciences, Pushchino). The design, operation principle and basic characteristics of this instrument are described in Ref. [41]. All measurements were done in buffer 310P_i7 at a heating rate of 1 K/min. The noise level did not exceed 30 μJ/K, the reproducibility of the baseline was not worse than 160 μJ/K. Analysis of thermograms was performed with the help of MicroCal Origin Software (Northampton, MA). The relative specific enthalpy of denaturation of membranes (ΔH) was determined by comparing the area under the sample thermogram normalized to membrane concentration with the area of electrical calibration marker of the instrument. The ΔH value of oxidized membranes was determined after subtracting the straight line connecting the point of heat absorption beginning with the point of visible minimum between the C- and D-transitions. The intensity of transition was determined as the value of relative specific heat capacity (ΔC_p^{max}) in the maximum of heat absorption peak at the corresponding maximum temperature (T_{max}). The half-width ($\Delta T_{1/2}$) of the A- and C-transitions was determined as the double width of the left shoulder of the A-transition and the double width of the right shoulder of the C-transition. This mode allows comparison of $\Delta T_{1/2}$ values when it is impossible to determine the true magnitude of $\Delta T_{1/2}$ due to low intensities of A and

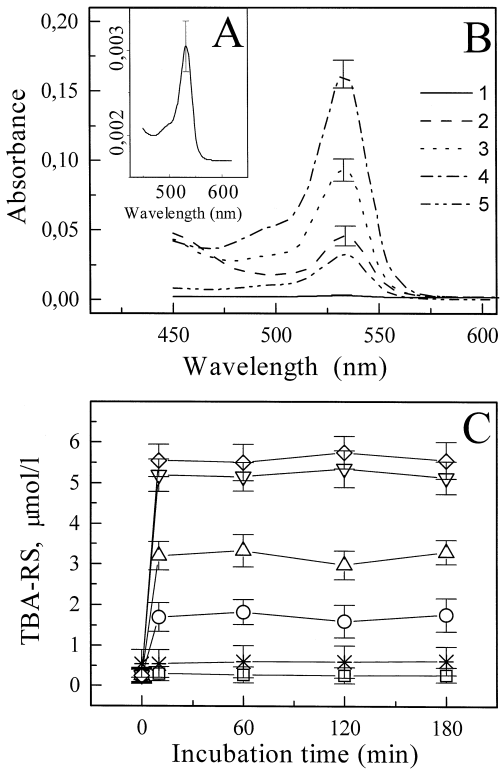

Fig. 1. (A) The representative absorption spectrum of TBA-RS formed in the suspension of erythrocyte ghost membranes without Fe^{2+} and CHP. Vertical bars on the maximum of spectra are S.D. of the spectrum maximum ($n = 4$). (B) Representative absorption spectra of TBA-RS formed in the suspension of erythrocyte ghost membranes. 1, Control membranes without Fe^{2+} and CHP. In the presence of: 2, Fe^{2+} 500 mmol/l. 3, In the presence of CHP 850 mmol/l. 4, In the presence of Fe^{2+} 100 mmol/CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.02:0.05:1). 5, After triple washing of membranes oxidized with Fe^{2+}/CHP/membranes = 0.02:0.05:1. Vertical bars on the maxima of spectra are S.D. of the maxima of spectra ($n = 4$). (C) Time course of TBA-RS formation in the suspension of erythrocyte ghost membranes: □, control membranes without Fe^{2+} and CHP. In the presence of: ○, Fe^{2+} 500 mmol/l; △, CHP 850 mmol/l; ◇, Fe^{2+} 100 mmol/CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.02:0.05:1); ▽, Fe^{2+} 250 mmol/CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1); ∗, TBA-RS formation in the presence of α-tocopherol (850 mmol/l) and Fe^{2+} 250 mmol/l CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1). Conditions of oxidation: suspension of membranes in 310P_i7, 2 ml, 5 mg/ml dry wt., heme iron ≤ 2 μmol in the incubation mixture (≤ 200 nmol per 1 mg dry wt. of membranes or ≤ 100 nmol per 1 mg of membrane protein).

C-transitions and/or a strong overlapping with the B_1- or B_2-transitions. The data obtained were statistically processed using the Student's *t*-test (MicroCal Origin Software).

2.5. Oxidation of membranes

We used for experiments only freshly prepared solutions of CHP and $FeSO_4$. Suspension of membranes (2 ml, 5 mg/ml dry wt., $310P_i7$) was incubated with CHP for 5 min with subsequent addition of Fe^{2+} to the required ratio Fe^{2+}/CHP/membrane = mmol/mmol/g dry wt. of membranes in liter. Then membranes were incubated under aerobic conditions at gentle stirring at 25°C. In the process of incubation membrane aliquots of 100–500 μl were taken for the determination of TBA-RS and electrophoresis. TBA-RS quantity was estimated by the technique of Ohkawa et al. [42]. For calorimetric studies membranes were oxidized for 1 h under the same conditions. After incubation the membranes were subjected to at least triple washing with cold (4°C) buffer $310P_i7$ by centrifugation.

2.6. Oxidation of erythrocytes

Red cells (Ht = 10%) in PBS were mixed with 50 mmol/l CHP and 2.5 mmol/l Fe^{2+} and incubated at 37°C for 1 h at stirring. Already in 20 min hemolysis took place with the formation of methemoglobin. After oxidation, the erythrocytes were washed four times in PBS. Ghosts were obtained from the oxidized erythrocytes using the technique described above. It was difficult to remove hemoglobin by washing from the ghosts obtained from the oxidized erythrocytes. We used for calorimetric studies the pink ghosts membranes after 10-fold washing in $5P_i8$ and triple washing in $310P_i7$.

3. Results

3.1. Membrane oxidation

As one can see from the TBA-RS spectrum shown in Fig. 1A, the control ghost preparations (without Fe^{2+} and CHP) contain small quantities of TBA-RS

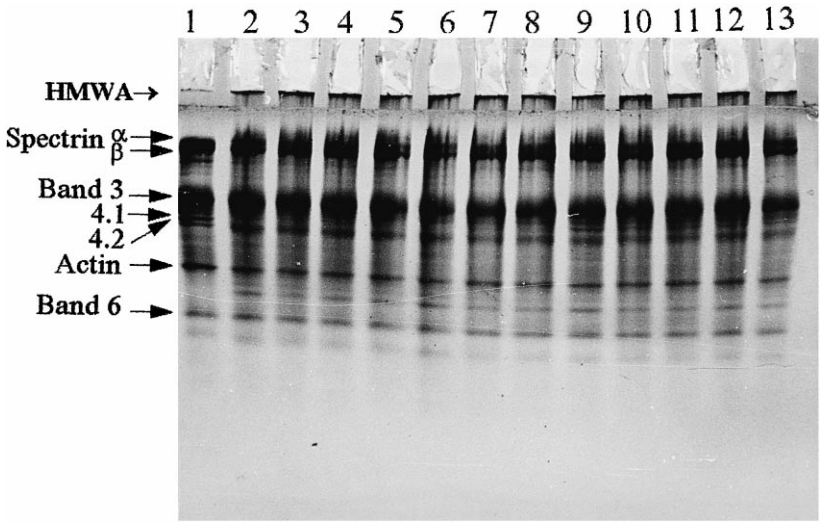

Fig. 2. The SDS-polyacrylamide electrophoresis of erythrocyte ghost membranes oxidized with Fe^{2+}/CHP. The slots 1–5 are just after oxidation, slots 6–13 are after triple washing in $310P_i7$. 1, Control (membranes without Fe^{2+} and CHP). The slots 2 and 8: Fe^{2+} 25 mmol/l and CHP 100 mmol/l (Fe^{2+}/CHP/membranes = 0.005:0.02:1). The slots 3 and 9: Fe^{2+} 50 mmol/l and CHP 100 mmol/l (Fe^{2+}/CHP/membranes = 0.01:0.02:1). The slots 4 and 10: Fe^{2+} 250 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1). The slots 5 and 11: Fe^{2+} 500 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.1:0.05:1). The slots 6 and 12: Fe^{2+} 250 mmol/l and CHP 500 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.1:1). The slots 7 and 13: membranes incubated with α-tocopherol (850 mmol/l) and then oxidized with Fe^{2+} 250 mmol/l CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1). Membrane samples were solubilized without SH-reagents.

formed through the homolysis of phospholipid hydroperoxides [9] in the presence of residual heme iron of membranes [40,43,44]. We used for experiments the membranes containing in the control not more than 200 nmol heme iron per 1 mg dry wt. of membranes ($\leq$ 100 nmol/mg membrane protein or $\leq$ 2 μmol in the incubation mixture of membranes) and containing TBA-RS at a mean level of 0.22 ± 0.13 (mean $\pm$ S.D.) μmol/l (Fig. 1C). Addition of more than 250 mmol/l Fe^{2+} or CHP caused formation of TBA-RS (Fig. 1B,C) that was also associated with the degradation of phospholipid hydroperoxides in the presence of Fe^{2+} and homolysis of CHP in the presence of heme [5,8,22]. Addition of Fe^{2+} to a suspension of ghosts with CHP led to a rapid increase in the quantity of TBA-RS during the first minutes (Fig. 1C). The quantity of TBA-RS was also dependent on the ratio Fe^{2+}/CHP/membranes. Fast accumulation of TBA-RS in the absence of kinetic events was associated with a rapid Fe^{2+} oxidation since the subsequent addition of Fe^{2+} (to the ratio Fe^{2+}/CHP/membranes < 0.02:0.05:1) induced a new 'oxidation burst' and accumulation of TBA-RS. Addition of Fe^{2+} above 100 mmol/l (Fe^{2+}/CHP/membranes > 0.02:0.05:1) was not conducive to a statistically significant rise of TBA-RS (Fig. 1B). After incubation of membranes with Fe^{2+} up to 100 mmol/l and CHP up to 250 mmol/l (Fe^{2+}/CHP/membranes < 0.02:0.05:1) the membranes were washed totally free from the formed TBA-RS to the initial level. After a strong oxidation (Fe^{2+}/CHP/membranes > 0.02:0.05:1) the membranes could not be washed free from TBA-RS to the initial level. The triple washing of such membranes in $310P_i7$ reduced the level of TBA-RS only 2.5–3 times (Fig. 1B, curve 5).

3.2. Electrophoresis

Incubation of membranes with Fe^{2+} or CHP added to a level of 500 mmol/l did not induce any statistically significant electrophoretic pattern of membranes. Oxidation of Fe^{2+}/CHP at 50 mmol/100 mmol/l (Fe^{2+}/CHP/membranes < 0.01:0.02:1) led to the disappearance of protein 4.1 band and the appearance of HMWA (Fig. 2, slot 3). Upon rise of Fe^{2+} and CHP concentrations up to 250 mmol/l (Fe^{2+}/CHP/membranes < 0.05:0.05:1; Fig. 2, slot 4) the oxidation induced a decrease in the intensity of bands of actin, protein 4.9, spectrin, ankyrin, Band 3 and the appearance of a new band at about 38 kDa (Fig. 2, slots 1–4). Further increase in the Fe^{2+} concentration above 250 mmol/l (at 250 mmol/l CHP) or in the CHP concentration above 250 mmol/l (at 250 mmol/l Fe^{2+}) (Fig. 2, slots 5 and 6) did not change the pattern of electrophoresis obtained at Fe^{2+}/CHP/membranes = 0.05:0.05:1. The electrophoretic pattern was independent of incubation time and did not change after centrifugation and triple washing of membranes for removing TBA-RS by centrifugation. After the first sedimentation of

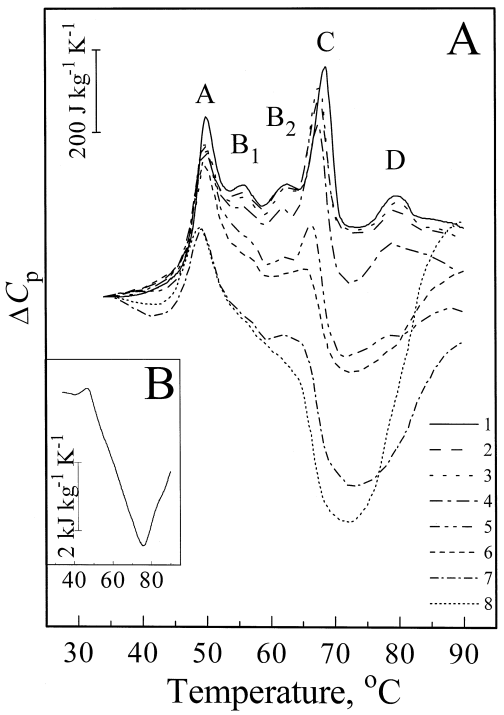

Fig. 3. (A) The temperature dependence of excess specific heat absorption by suspension of erythrocyte ghost membranes: 1, Control membranes without oxidation. Membranes oxidized with: 2, Fe^{2+} 500 mmol/l; 3, CHP 850 mmol/l; 4, Fe^{2+} 25 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.005:0.05:1); 5, Fe^{2+} 100 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.02:0.05:1); 6, Fe^{2+} 250 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1); 7, Fe^{2+} 250 mmol/l and CHP 500 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.1:1); 8, Fe^{2+} 500 mmol/l, CHP 500 mmol/l (Fe^{2+}/CHP/membranes = 0.1:0.1:1). (B) Membranes oxidized with Fe^{2+} 250 mmol/l, CHP 30 mol/l (Fe^{2+}/CHP/membranes = 0.05:5.8:1).

oxidized ghosts the supernatant did not contain any polypeptide bands and protein aggregates.

3.3. Microcalorimetry

Fig. 3 shows typical thermograms of suspensions of intact and oxidized membranes. High levels of Fe^{2+} and CHP, above 500 mmol/l, (oxidant/membranes > 0.1 mmol/mg dry wt. ghosts membranes) decreased the intensity of the A- and C-transitions by 10% on the average and lowered the T_{max} of C-transition by 0.7°C, while the ΔH value of membrane denaturation did not display any significant variation.

Oxidation of membranes by Fe^{2+}/CHP induced irreversible changes in the heat absorption by membranes (Figs. 3 and 4). Firstly, the oxidation diminished the ΔH of membranes. The curve of concentrational dependence formed a plateau at 50 mmol/l Fe^{2+} and then did not change significantly up to an Fe^{2+} level of 300 mmol/l (Fig. 4A). Besides ΔH lowering, the oxidation reduced the intensity of all transitions. The first to disappear was the visible

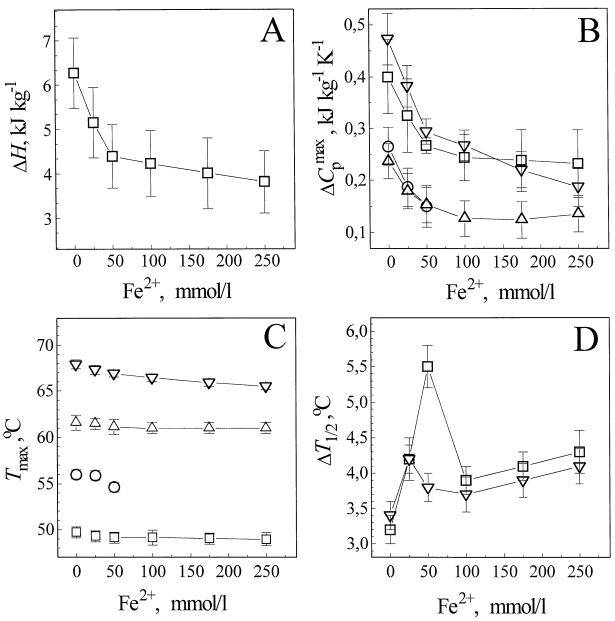

Fig. 4. The effect of increasing concentrations of Fe^{2+} on thermogram parameters (CHP 250 mmol/l, CHP/membranes = 0.05:1): (A) Enthalpy of denaturation (ΔH); (B) Intensities of transition (ΔC_p^{max}); (C) Temperatures of transitions maximum (T_{max}); (D) Transitions half-width ($\Delta T_{1/2}$). □, A-transition; ○, B_1-transition; △, B_2-transition; ▽, C-transition. The data are expressed as mean ± S.D. ($n = 4$).

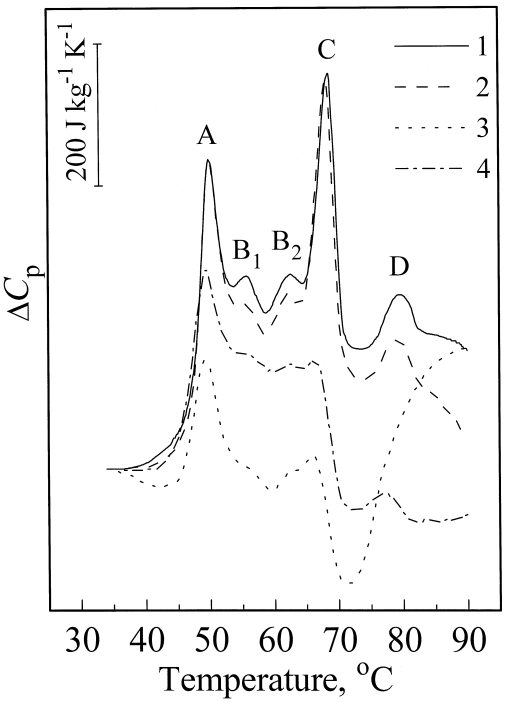

Fig. 5. The effect of α-tocopherol on heat absorption of membranes: 1, Control membranes without tocopherol and oxidation. 2, Membranes with α-tocopherol 850 mmol/l (0.1% ethanol) and without oxidants. 3, Membranes without α-tocopherol oxidized with Fe^{2+} 250 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1). 4, Membranes with α-tocopherol 850 mmol/l oxidized with Fe^{2+} 250 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1).

maximum of B_1-transition at $Fe^{2+} > 50$ mmol/l (Fe^{2+}/CHP/membranes > 0.01:0.5:1). Then at an Fe^{2+} level of about 250 mmol/l (Fe^{2+}/CHP/membranes ≈ 0.05:0.05:1) the maxima of B_2- and C-transitions disappeared (Fig. 3A, curve 6). The intensity of A-transition decreased with the growth of Fe^{2+} concentration to 50 mmol/l and more and then did not change significantly with further rise of Fe^{2+} and CHP concentrations above 500 mmol/l (Fig. 3B). Secondly, the oxidation induced insignificant lowering of T_{max} of visible transitions (Fig. 4C). The T_{max} of A-transition decreased only at the CHP concentration rise above 500 mmol/l (Fig. 3A, curves 7 and 8), though even at Fe^{2+}/CHP/membranes ≈ 0.05:0.05:1 the T_{max} decreased only by 3°C (Fig. 3B). Thirdly, the oxidation was found to increase the $\Delta T_{1/2}$ values of A- and C-transitions (Fig. 4D). A strong rise of $\Delta T_{1/2}$ of A-transition at 50 mmol/l

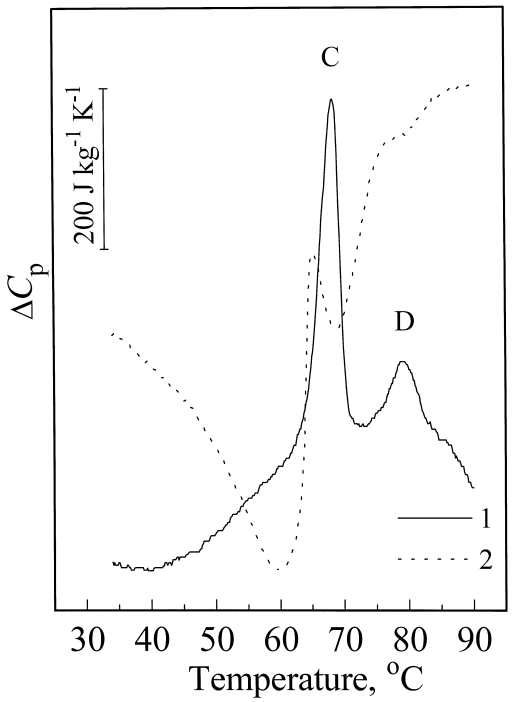

Fig. 6. The effect of oxidation on heat absorption of membranes with msdB3 (C-transition): 1, Control membranes without oxidation; 2, membranes oxidized with Fe^{2+} 250 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1).

Fe^{2+} was associated with the disappearance of the visible maximum of B_1-transition and its overlapping with the A-transition. Fourthly, the oxidation of membranes was conducive to changes in the slope of thermograms—reduction of heat absorption in the range of 55–72°C and increasing heat absorption above 72°C that resulted in the disappearance of D-transition (Fig. 3A, curves 7 and 8).

3.4. The effect of tocopherol

Preincubation of membranes with tocopherol (0.5–0.85 mol/l) averted the accumulation of TBA-RS (Fig. 1C), the negative slope of thermograms and suppressed totally the sharp rise in the heat absorption above 70°C (Fig. 5, curve 4). But even at tocopherol levels three times the CHP concentration, tocopherol prevented only partially the decrease in ΔH, did not restore the decrease in the intensity of transitions and did not alter the electrophoretic pattern of oxidized membranes (Fig. 2, slots 7 and 13).

3.5. The effect of oxidation on band 3 protein

To study the influence of oxidation on Band 3, we isolated the membrane domain of Band 3 by alkaline treatment as in [45]. Fig. 6 shows that a short-time oxidation of such ghosts affects strongly the character of heat absorption by membranes, reduces the T_{max}, intensity and enthalpy of the C-transition. Like in the case of thermograms of strongly oxidized membranes, the oxidation induced a dramatic increase in the heat absorption above 70°C.

3.6. Oxidation of erythrocytes

Oxidation of whole erythrocytes in the presence of 2.5 mmol/l Fe^{2+} and 50 mmol/l CHP led to the development of hemolysis. A thermogram of a suspension of ghost membranes isolated from oxidized red cells is shown in Fig. 7. The oxidation of erythrocytes accompanied by hemolysis is seen to be conducive to the result qualitatively analogous to the

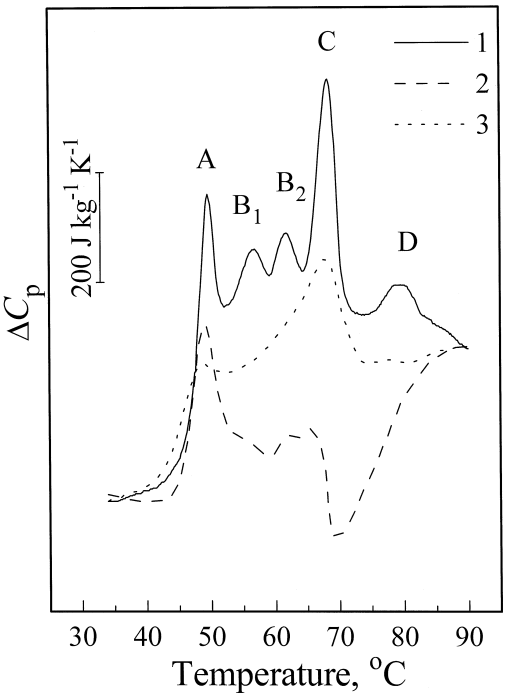

Fig. 7. Thermograms for: 1, Control ghost membranes (without oxidants); 2, Membranes oxidized with Fe^{2+} 250 mmol/l and CHP 250 mmol/l (Fe^{2+}/CHP/membranes = 0.05:0.05:1); 3, Ghost membranes isolated from oxidized erythrocytes (2.5 mmol/l Fe^{2+} and 50 mmol/l CHP).

oxidation of ghosts—decrease in the membrane ΔH, drop of the intensity of all transitions and the disappearance of the visible maxima of B_1- and B_2-transitions. However, in contrast to the oxidation of ghosts, that of membranes did not cause any significant decrease in T_{max} of C-transition, changes in the slope of thermograms and higher heat absorption above 70°C.

4. Discussion

The results presented in this paper indicate that homolysis of CHP catalyzed by Fe^{2+} induces three basic processes: (1) considerable accumulation of TBA-RS which is indicative of PUFA degradation; (2) decrease in the concentration of native protein in membrane due to aggregation into HMWA; (3) changes in heat absorption parameters for all thermal transitions of membranes. The earlier work showed that CHP induced complete degradation of PUFA of phospholipids [3,22]. Under the conditions of our experiments the maximal quantity of TBA-RS was produced at 100 mmol/l Fe^{2+} which suggests that virtually all PUFA degraded at Fe^{2+}/CHP/membranes > 0.02:0.5:1. The production of TBA-RS was totally blocked by tocopherol. According to the results of electrophoresis, homolysis of CHP induces the aggregation of proteins into HMWA that leads to the decrease in the intensity of bands of spectrin, ankyrin, Band 3, 4.1, 4.2 and 4.9 proteins, and actin. The aggregation is not blocked by α-tocopherol. Unfortunately, the conventional gradient SDS-electrophoresis of oxidized membranes does not have the resolution capacity, which would allow the establishment of the dependence of band intensity and HMWA on the concentration of oxidants and the level of TBA-RS. Therefore, we can make only qualitative comparison of the electrophoretic and calorimetric data. Nonetheless, our results and the available literature data [6,8,10,15,16,20,21,24,29] are sufficient to maintain that homolysis of CHP leads to a decrease in the native protein concentration in the membrane. Despite the fact that both the degradation of PUFA and the aggregation of proteins are extensively described in the literature [3,5,18,22,46], there is very scanty evidence that both processes induce global structural changes in the membrane skeleton. The microcalorimetric data presented in this paper support the existence of such interrelation. The major microcalorimetric consequences of CHP homolysis are: (a) decrease in the intensity of B_1-, B_2 and C-transitions and their eventual disappearance; (b) lower ΔH of oxidized membranes; (c) negative slope of thermograms; (d) increase in the heat absorption above 72°C and the disappearance of the D-transition. All these changes occur within the range of 5–100 mmol/l Fe^{2+} (Fe^{2+}/CHP/membranes < 0.02:0.05:1), i.e., before the formation of maximal quantity of TBA-RS (total degradation of PUFA), and form a plateau at Fe^{2+} levels higher than 100 μmol/l (Fig. 4A and B). Evidently, the decrease in the intensity of A-transition is associated with the aggregation of spectrin–actin, while that of B_1-transition—with the aggregation of 4.1 and 4.2 proteins and ankyrin, and finally the decrease in the intensity of B_2- and C-transitions —with the aggregation of Band 3 protein. However, the appearance of plateaus in the curves of concentrational dependence of the intensity A-, B_1- and B_2-transitions is due to different reasons. The plateau for the B_1- and B_2-transitions is associated primarily with the disappearance of the visible maxima of these transitions at Fe^{2+} levels above 100 mmol/l. In the case of A-transition its intensity went down with the increase in the Fe^{2+} level up to 50 mmol/l and then did not change significantly (as confirmed by the electrophoretic data). This allows us to suppose that the same portion of the spectrin–actin domain aggregates over a wide range of Fe^{2+} concentrations. In this sense the A-transition—the spectrin–actin domain—proved to be the most resistant to oxidation. This domain did not disappear even under a very strong oxidation (Fig. 3B). The increase in the CHP level above 500 mmol/l caused only a lowering of the T_{max} of A-transition but it did not induce any additional aggregation and decrease in the ΔC_p^{max} of A-transition. The following explanation of the plateau appearance seems to us to be reasonable for the A-transition. Since the A-transition includes denaturation of the spectrin–actin domain, which is in the aqueous phase and does not interact directly with the membrane [33,35], its aggregation must be due to the attack of radicals formed in the aqueous phase. The source of these radicals is the water-dissolved CHP, which has a limited, about 15 g/l (98 mmol/l), solubility in water. It is this portion of CHP which

serves as the source of the same quantity of cumyloxy radicals over a wide range of Fe^{2+} concentrations (5–500 mmol/l) and induces the aggregation of the same spectrin quantity.

That a portion of radicals is generated and attacks the proteins from the water phase is proven by the disappearance of B_1-transition, which is formed by ankyrin and proteins of 4.1 and 4.2 bands, and by the reduction of the intensity of A- and B_2-transitions formed by the spectrin–actin and the cdB3, respectively. All of these proteins are localized in the water phase [47]. Apparently, it is due namely to this reason that tocopherol does not block the aggregation and the decrease in the intensity of these transitions.

On the other hand, in addition to the aggregation into HMWA, the oxidation perturbs the structure of the nonaggregated portion of the spectrin–actin domain due the larger $\Delta T_{1/2}$ of the A-transition which is indicative of the decrease in the cooperativity of denaturation. This may be associated with both the weaker spectrin–actin interaction due to the aggregation of protein 4.1 and the oxidation of spectrin and actin by radicals.

The most vivid changes are characteristic of the heat absorption by the Band 3 protein which is denatured in the course of B_2- and C-transitions. Oxidation induces a decrease in the intensity, insignificant lowering of T_{max} and larger $\Delta T_{1/2}$ of the C-transition before and after reaching the Fe^{2+} level of 100 mmol/l. The visible maximum of C-transition disappears only at concentrations of oxidants above 500 mmol/l The lower intensity of B_2- and C-transitions is associated with the aggregation of the cytoplasmic and membrane domains of Band 3. Surprisingly, even strong oxidation has little effect on both the Tmax of C-transition in whole membranes and on the T_{max} and $\Delta T_{1/2}$ of C-transition of the isolated msdB3 after alkaline treatment. This resistance of msdB3 to oxidation may be due to a few reasons. In the first place, the msdB3 shows a preferential binding of long chain saturated lipids [48] and cholesterol [49]; consequently, the oxidative degradation of phospholipids reflects poorly the phase state of the surrounding and 'boundary' lipids and has little effect on the T_{max} and $\Delta T_{1/2}$ of the C-transition. Furthermore, if the decrease in the intensity of C-transition were mediated by the degradation of phospholipids surrounding msdB3, tocopherol would block changes in the intensity, T_{max} and $\Delta T_{1/2}$; this however is not the case in reality. On the other hand, this may mean that either radicals do not attack directly the msdB3 and do not affect strongly its structure or the attack by radicals results in the immediate aggregation of the msdB3 without formation of intermediate 'oxidized' conformations which denature at lower T_{max}. The idea of weak modification of the msdB3 by oxidation is supported by the data reported by Carpari et al. [30], who failed to reveal any changes in the distribution of intramembrane particles over the PF-fracture face of erythrocyte membranes oxidized by BHP. The high sensitivity of Band 3 protein to oxidation is most likely determined mainly by the aggregation of the cdB3 and not the membrane-spanning domain of Band 3 protein.

As noted above, the oxidation induces the appearance of the negative slope of thermograms and a dramatic increase in the heat absorption above 72°C. These two effects are interrelated since they are dependent on the oxidant concentration and the influence of tocopherol which suppresses both effects. Consequently, they are determined by thermotropic properties of the oxidized lipid membrane bilayer. This proves that the degradation of PUFA of phospholipids alters phasic properties of the lipid membrane bilayer. An analogous conclusion was drawn by Caprari et al. [30] who showed that the oxidation *t*-BHP modifies strongly the ordering of the lipid phase in lipid membrane. It is noteworthy that the processes of lipid phase degradation and changes in the structure of protein domains are relatively 'independent' of each other since tocopherol totally inhibited the formation of TBA-RS but did not block the lowering ΔH of membrane the decrease in the intensity of transitions and the aggregation of proteins in electrophoresis. Many other authors [6,50,51] drew analogous conclusions. However, there is no general consensus regarding the cause of this phenomenon. There is a view that different radicals mediate these processes: peroxidation—by peroxyl radicals, while aggregation—by alkoxyl and hydroxyl radicals [6,15,21,24,50,51]. Investigation of this problem is the subject of our further studies.

Thus, based on the general concept of the membrane skeleton organization [47], changes in the character of heat absorption by oxidized membranes may be interpreted as perturbations of the structural orga-

nization and interactions inside the spectrin–actin-protein 4.1 domains, the spectrin–protein 4.2 domain, as well as inside the domain of spectrin–ankyrin–cdB3 and the domain formed by the msdB3. These perturbations are determined by the decrease in the native protein concentration in the above domains due to oxidative aggregation of proteins and by changes in the protein conformation in these domains. It should be noted that the HMWA formed remain bound to the membrane since we have failed to reveal any proteins or aggregates in the supernatant of oxidized membranes. This is in keeping with the data of Carpari et al. [30] indicating that the oxidation induced by *t*-BHP reduces the extractability of oxidized spectrin from membranes.

The oxidation of intact erythrocytes accompanied by hemolysis is on the whole conducive to the same changes in the heat absorption by membranes as in the case of oxidation of ghosts: decrease in the intensity of transitions, ΔH of membranes, the disappearance of the maxima of B_1- and B_2-transitions and the removal of D-transition. However, the oxidation of erythrocytes does not induce any negative slope of thermograms, growth of heat absorption above 70°C and lowering of the T_{max} of C-transition. These differences are still difficult to interpret since we have not succeeded in establishing the dependence of the thermogram shape on the concentration of oxidants, the quantity of TBA-RS and the level of hemolysis, and these will be the topics of our subsequent studies. Nonetheless, our preliminary data indicate that the CHP homolysis destabilizes the membrane skeleton structure that in turn explains the development of erythrocyte hemolysis. It appears obvious that the changes in the membrane skeleton structure described in this paper underlie modifications in the membrane permeability to ions [21,25–27], and the decrease in the deformability and mechanical stability [10,28] of erythrocytes.

References

[1] R.P. Hebbel, J. Lab. Clin. Med. 107 (1986) 401–404.
[2] D.T.Y. Chiu, F.A. Kuypers, B.H. Lubin, Semin. Hematol. 26 (1989) 257–276.
[3] J.F. Koster, R.G. Slee, Biochim. Biophys. Acta 752 (1983) 233–239.
[4] M. Beppu, K. Murakami, K. Kikugawa, Biochim. Biophys. Acta 897 (1987) 169–179.
[5] J.J.M. Van den Berg, F.A. Kuypers, B. Roelofsen, J.A.F. Op den Kamp, Chem. Phys. Lipids 53 (1990) 309–320.
[6] J.J.M. Van den Berg, F.A. Kuypers, in: J.A.F. Op den Kamp (Ed.), Dynamics of Membrane Assembly, Springer-Verlag, Berlin, 1992, pp. 141–151.
[7] A.W. Girotti, J.P. Thoas, J.E. Jordan, Arch. Biochem. Biophys. 236 (1985) 238–251.
[8] M. Beppu, M. Nagoya, K. Kikugawa, Chem. Pharm. Bull. 34 (1986) 5063–5070.
[9] K. Ando, M. Beppu, K. Kikugawa, Biol. Pharm. Bull. 18 (1995) 659–663.
[10] W.D. Corry, H.J. Meiselman, P. Hochstein, Biochim. Biophys. Acta 597 (1980) 224–234.
[11] B.N. Ames, R. Cathcart, E. Schwiers, P. Hochstein, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 6858–6862.
[12] U. Benatti, A. Morelli, G. Damiani, A. De Flora, Biochem. Biophys. Res. Commun. 106 (1982) 1183–1190.
[13] R.J. Trotta, S.G. Sullivan, A. Stern, Biochem. J. 204 (1982) 405–415.
[14] R.J. Trotta, S.G. Sullivan, A. Stern, Biochem. J. 212 (1983) 759–772.
[15] B. Deuticke, P. Lutkemeier, M. Sistemich, Biochim. Biophys. Acta 899 (1987) 125–128.
[16] M.-J. Chen, M.P. Sorette, D.T.Y. Chiu, M.R. Clark, Biochim. Biophys. Acta 1066 (1991) 193–200.
[17] A. Yesilkaya, A. Yegin, G. Yucel, Y. Aliciguzel, T.A. Aksu, Int. J. Clin. Lab. Res. 26 (1996) 60–68.
[18] D.T.-Y. Chin, B.H. Lubin, Semin. Hematol. 26 (1989) 128–135.
[19] Y. Chancerelle, J. Mathieu, J.F. Kergonou, Biochem. Mol. Biol. Int. 34 (1994) 1259–1270.
[20] R.B. Moore, M.L. Brummitt, V.N. Mankad, Arch. Biochem. Biophys. 273 (1989) 527–534.
[21] J. Van der Zee, J. Van Steveninck, J.F. Koster, T.M.A.R. Dubbelman, Biochim. Biophys. Acta 980 (1989) 175–180.
[22] J.J.M. Van den Berg, F.A. Kuypers, J.H. Qju, B.H. Lubin, B. Roelofsen, J.A.F. Op den Kamp, Biochim. Biophys. Acta 944 (1988) 29–39.
[23] R. McKenna, F.J. Kezdy, D.E. Epps, Anal. Biochem. 196 (1991) 443–450.
[24] M. Beppu, K. Kikugawa, Lipids 22 (1987) 312–317.
[25] P.A. Ney, M.M. Christopher, R.P. Hebbel, Blood 75 (1990) 1192–1198.
[26] R.P. Hebbel, N. Mohandas, Biophys. J. 60 (3) (1991) 712–715.
[27] J.F.S. Dwight, B.M. Hendry, Clin. Chim. Acta 249 (1996) 167–181.
[28] D. Galaris, D. Yova, P. Korantzopoulos, S. Barbounaki, D. Koutsouris, Clin. Hemorheol. 15 (1995) 107–120.
[29] M. Beppu, A. Mizukami, M. Nagaya, K. Kikugawa, J. Biol. Chem. 265 (1990) 3226–3233.
[30] P. Caprari, A. Bozzi, W. Malorni, A. Bottini, F. Iosi, M.T. Santini, A.M. Salvati, Chem. Biol. Interact. 94 (1995) 243–258.

[31] Y. Sato, S. Kamo, T. Takahashi, Y. Suzuki, Biochemistry 34 (1995) 8940–8949.
[32] J.F. Brandts, K.A. Lysko, A.T. Schwartz, L. Eryckson, R.B. Carlson, J. Vincentelli, R.D. Taverna, Colloq. Int. C.R.S. 246 (1976) 169–175.
[33] K.A. Lysko, R.B. Carlson, R.D. Taverna, J.W. Snow, J.F. Brandts, Biochemistry 20 (1981) 5570–5576.
[34] G.G. Zhadan, E. Villar, V.L. Shnyrov, Biochem. Soc. Trans. 22 (1994) 368S.
[35] J.F. Brandts, L. Eryckson, K.A. Lysko, A.T. Shchwartz, R.D. Taverna, Biochemistry 16 (1977) 3450–3454.
[36] K.C. Appell, P.S. Low, Biochemistry 21 (1982) 2151–2157.
[37] S.R. Davio, P.S. Low, Biochemistry 21 (1982) 3585–3593.
[38] J.W. Snow, J. Vincentelli, J.F. Brandts, Biochim. Biophys. Acta 642 (1981) 418–428.
[39] V.L. Shnyrov, S. Orlov, G.G. Zhadan, N.I. Pokudin, Biomed. Biochim. Acta 49 (1990) 445–453.
[40] S.A. Kuross, B.H. Rank, R.P. Hebbel, Blood 71 (1988) 876–882.
[41] P.L. Privalov, V.V. Plotnikov, Thermochim. Acta 139 (1989) 257–277.
[42] H. Ohkawa, N. Ohishi, K. Yagi, Anal. Biochem. 95 (1979) 351–358.
[43] S.A. Kuross, R.P. Hebbel, Blood 72 (1988) 1278–1285.
[44] T. Repka, O. Shalev, R. Ratnammal, J. Yuan, A. Abrahamov, E.A. Rachmilewitz, P.S. Low, R.P. Hebbel, Blood 82 (1993) 3204–3210.
[45] T.L. Steck, J. Yu, J. Supramol. Struct. 1 (1973) 220–232.
[46] C.J. Dillard, A.L. Tappel, Lipids 8 (1973) 183–189.
[47] D.M. Gilligan, Vann Bennett, Semin. Hematol. 30 (1993) 74–83.
[48] L.R. Maneri, P.S. Low, Biochem. Biophys. Res. Commun. 159 (1989) 1012–1019.
[49] D. Schubert, K. Boss, FEBS Lett. 150 (1982) 4–8.
[50] P.J. Thornalley, R.J. Trotta, A. Stern, Biochim. Biophys. Acta 759 (1983) 16–22.
[51] M. Miki, H. Tamai, M. Mino, Y. Yamamoto, E. Niki, Arch. Biochem. Biophys. 258 (1987) 373–380.