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Inhibition of membrane erythrocyte (Ca^{2+} + Mg^{2+})-ATPase by hemin

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Red blood cell lysis is a common symptom following severe or prolonged oxidative stress. Oxidative processes occur commonly in sickle cells, probably mediated through denatured hemoglobin and the accumulation of ferric hemes in the membranes. Calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase from sickle red cell membranes is partially inactivated (Leclerc et al. (1987) Biochim. Biophys. Acta 897, 33–40). In this study (Ca^{2+} + Mg^{2+})-ATPase activity from normal adult erythrocyte membranes was measured in the presence of hemin. We report a time- and concentration-dependent inhibition of the activity of the enzyme by hemin due to a decrease in the maximum velocity. Only a mild inhibitory effect was observed in the presence of iron-free protoporphyrin IX, indicating the catalytic influence of the iron. Experiments carried out with hemin (ferric iron) liganded with imidazole or with reduced protoheme (ferrous iron) liganded with carbon monoxide, demonstrated that the inhibition requires that hemin be capable of binding additional ligands. The inhibition was not influenced by the absence of oxygen but was prevented by addition of bovine serum albumin. Addition of butylated hydroxytoluene, a protective agent of lipid peroxidation, failed to prevent the inhibition of calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase. As dithiothreitol partially restores the enzyme activity, we postulated that hemin interacts with the thiol groups of the enzyme.

Introduction

Hemolytic anemia is a common symptom in patients carrying an unstable hemoglobin (genetic variants or thalassemias) or a defect of the reducing capacity of the red cells such as glucose-6-phosphate dehydrogenase or vitamin E deficiencies. In these cells, by-products of oxidized hemoglobin bind to or accumulate in the cell membrane [1,2]. These may be oxidized tetramers, isolated subunits or ferric hemes [3]. The role of these ferric iron complexes in the membrane damage has been postulated but the mechanism(s) of their effects is still unknown [4–6]. Hemin, the oxidized form of the heme and a highly lipophilic compound, has been found abnormally abundant in sickle red blood cell membranes [7,8]. This powerful oxidant interacts also with spectrin which results in the destabilization of the red cell cytoskeleton [9].

We have previously reported that calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase (referred to hereafter as (Ca^{2+} + Mg^{2+})-ATPase) from sickle red blood cells was inhibited by approx. 50% compared to the value measured in normal red blood cell membranes [10,11]. We postulated that this partial inhibition observed in sickle cell dis-

Abbreviations: BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; BHT, butylated hydroxytoluene.

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ease was due to the susceptibility of the enzyme to oxidative damage caused by free radicals overproduced in sickle cells [12]. Actually, the $(Ca^{2+} + Mg^{2+})$-ATPase from normal red blood cells is very sensitive to oxidative stress and is inhibited to a varying extent by thiol reagents [10,13,14] or potent radiosensitizers [15] indicating the involvement of -SH groups in the inhibitory process. It was also shown that malondialdehyde, a by-product of phospholipid peroxidation, could inactivate the $(Ca^{2+} + Mg^{2+})$-ATPase indicating that cross-link products in the protein were another mechanism for the inhibition [10].

In the present report, we describe the effect of hemin on the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase from normal red cell membranes in vitro. A profound and rapid inhibition is observed in the presence of hemin, whereas protoporphyrin IX has only a mild effect. This points to the role of the ferric iron moiety in the inhibitory process. The oxidative and peroxidative properties of hemin were investigated as well as its protein-binding propensity. These results lead us to postulate that the initial step of the inhibitory process is probably due to the interaction between the ferric iron moiety and the -SH groups of the $(Ca^{2+} + Mg^{2+})$-ATPase.

Material and Methods

Chemicals

Hemin (Fe(III)-protoporphyrin IX-chloride) and bovine serum albumin were purchased from Serva (F.R.G.), protoporphyrin IX and butylated hydroxytoluene (BHT) from Sigma (U.S.A.), vanadium-free ATP, hog brain calmodulin (CaM) and dithiothreitol (DTT) from Boehringer (Mannheim, F.R.G.). All other chemicals were high purity grade. Electrophoresis of membrane proteins was performed on SDS-polyacrylamide gels.

Fresh blood was obtained from healthy volunteers and collected on heparin. Erythrocyte membranes were prepared on the same day as described previously [10]. After three washes in a 10 mM Hepes buffer (pH 7.4) containing 150 mM KCl and 11 mM glucose, red blood cells were hemolyzed in an isosmotic imidazole buffer (pH 7.4) (1 : 14, v/v) and the membranes spun down for 10 min at 20000 rpm. The pellet was washed twice with a 20 mM imidazole buffer and finally with a 40 mM histidine-imidazole buffer (pH 7.1). The resealed vesicles were stored at $-80\,^{\circ}C$.

Membrane preparations were incubated with hemin at varying concentrations from 10 to 100 μM and for different periods of time from 10 to 120 min. Saponine (1 mg per mg membrane protein) was added to membrane suspensions in order to insure full accessibility of the various reagents to the enzyme. Before treatment, membranes were rinsed with 50 mM Bistris buffer to eliminate the histidine and imidazole of the storage buffer for reasons explained below. Hemin was first dissolved in 0.1 M NaOH and brought to 1 mM in 50 mM Bistris buffer (pH 7.1). A fresh solution was prepared daily and the final concentration was obtained by direct dilution in the membrane suspension. In some experiments protoporphyrin IX was used without further purification. It was prepared and added to the incubation medium as indicated for hemin.

Typical membrane treatment with hemin was performed by incubating the red cell membranes at $37\,^{\circ}C$ for 60 min in 50 mM Bistris buffer (pH 7.1) in the presence of 40 μM hemin and various reagents as indicated below. At the end of the treatment period, the membranes were rinsed with the buffer and resuspended in the assay mixture. For all experimental procedures a control experiment was run in the absence of hemin.

The amount of hemin associated with the membranes at the end of the treatment period was measured by spectrophotometry (Cary 219 spectrophotometer, Varian, U.S.A.) after centrifugation and solubilization of the membrane pellet in 1% SDS Bistris buffer. Absorbances were read at 605 nm (ferric heme) or at 535 nm (imidazole-hemin complex). Concentrations were estimated from a calibration curve for pure hemin solutions added to the same SDS-buffer mixture. Recording of the Soret band revealed that SDS shifts the spectrum to higher wavelengths and increases the absorbance value likely due to a deaggregation of hemin dimers. Membrane-associated hemin is expressed as μmol hemin per mg membrane protein.

$(Ca^{2+} + Mg^{2+})$-ATPase activity was measured in a 50 mM Bistris-HCl buffer (pH 7.1) at $37\,^{\circ}C$. The typical assay mixture contained 2 mM vanadium-free ATP/3 mM $MgCl_2$/0.1 mM

ouabain and 0.1 μM calmodulin (CaM)/0.1 mM EGTA and 0.15 mM calcium chloride giving a calculated free Ca^{2+} concentration of 0.03 mM. Inorganic phosphate (P_i) released by the hydrolysis of ATP was measured according to Kallner [16]. ($Ca^{2+} + Mg^{2+}$)-ATPase activity is expressed in nmol P_i/min per mg membrane protein. The concentration of membrane protein was estimated by the Coomassie blue dye method described by Bradford [17]. In the conditions of our assay the activity of the ($Ca^{2+} + Mg^{2+}$)-ATPase is 114 ± 20 (1 S.D.) nmol P_i/mg protein per min ($n = 20$). Due to the small variations in the activity of the enzyme between the different preparations, the results were expressed in % activity of the control values.

In order to gain understanding of the mechanisms of the inhibitory effect of hemin on the activity of the red cell ($Ca^{2+} + Mg^{2+}$)-ATPase several experimental procedures have been followed:

Conditions of the assay. We tested the conditions of the assay for membranes pretreated with 40 μM hemin. This was performed by varying the concentrations of ATP from 0.1 to 4 mM, of CaM from 1 nM to 10 μM and of calcium from 0.015 to 0.3 mM.

Modifications of the state of ligation of the iron. This was obtained in two ways: (i) Hemin was added in membranes suspended in an 18 mM imidazole histidine buffer (pH 7.1) at 37°C and the ($Ca^{2+} + Mg^{2+}$)-ATPase assay was performed in the same buffer. Control experiments had demonstrated that the ($Ca^{2+} + Mg^{2+}$)-ATPase activity from native membranes was similar when measured in Bistris or histidine imidazole buffers. In the presence of imidazole, the ferric iron is transformed into a liganded complex, as indicated by the change in the visible spectrum of the solution (Table I). (ii) The ferric iron was reduced to its ferrous state upon addition of 2 molar excess sodium dithionite in the absence of oxygen. The solution was then equilibrated under 1 atm carbon monoxide. Full transformation into carbonmonoxy heme was checked by spectrophotometric criteria. Excess dithionite was removed by passing the solution on a Sephadex® G-25 resin column. The treatment period of the membranes in the presence of CO-heme was performed in sealed vessels under 1 atm CO.

Incubation in the presence of bovine serum albumin. Albumin binds hemin with a high affinity [18]. Membranes were treated with hemin in the presence of 0.4 mM albumin which was added either at the beginning of the 60 min membrane treatment with hemin or during the time-course of the treatment period.

Effects of reducing agents. Butylated hydroxytoluene was used as a phospholipid peroxidation protective agent and DTT as a disulphide bond reducing agent. Native membranes treated for 60 min with either compound resulted in enzyme activities similar to control membranes (control = 108.9 ± 7.4, DTT = 105.9 ± 4.6, BHT = 102.6 ± 4.7). BHT (1 mM) was added at the onset of a 30 min treatment period in the presence of hemin. To test the effect of DTT, membranes were treated with hemin for various periods of time then rinsed and reincubated for 60 min in the presence of 10 mM DTT.

Effect of oxygen. Experiments similar to those described above were performed under strict

TABLE I

THE AMOUNT OF HEMIN ASSOCIATED WITH THE RED CELL MEMBRANES UNDER VARIOUS SOLUTION CONDITIONS

Membrane-associated hemin was measured after a 60 min period of incubation with hemin by dissolving the rinsed membrane pellet in 1% SDS Bistris buffer as described in Materials and Methods.

Additions	Hemin (μM/mg protein) [c]	Absorbance peaks (nm)	
		visible	Soret
Hemin 100 μM	65	605	401.5
Hemin 40 μM	26	605	401.5
Hemin 40 μM + 40 mM imidazole	23	535	411
Hemin + albumin [a]	5	605	403
Hemin 40 μM + 0.4 mM albumin [b]	25 11	605 605	401.5 401.5

[a] 0.4 mM BSA was added to the 40 μM hemin treatment medium at the onset of the incubation period.

[b] Membranes were incubated first with 40 μM hemin for 30 min, then rinsed and incubated with 0.4 mM albumin buffer solution for 30 min.

[c] Mean of three determinations. Percent error was less than 10%.

anaerobic conditions. For these experiments the membrane suspensions and the hemin solution were degassed and equilibrated under high purity grade nitrogen (N60, CFPO France, $O_2 < 0.1$ ppm) in sealed vessels. The treatment and incubation periods were also made under 1 atm N_2.

Results

Membranes treated with hemin in Bistris buffer exhibited an inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity. The inhibition is concentration- and time-dependent (Fig. 1). The results indicate that incubating red cell membranes for 60 min at 37°C in the presence of 40 μM hemin induces a 50% inhibition of the enzyme. In these conditions the inhibition of the enzyme is linearly related to the duration of the incubation period. SDS-polyacrylamide gel electrophoresis of the membrane proteins treated with 100 μM hemin for 60 min did not show high-molecular-weight complexes, indicating that no aggregation of the membrane proteins had occurred under these conditions (not shown). Values given in Table I show that a large fraction of hemin was associated with the membranes at the end of the 60-min treatment period.

The influence of hemin on the kinetic properties of the enzyme was investigated. Assays of the enzyme activity were performed at varying concentrations of ATP (0.1–4 mM), Ca^{2+} (0.015–0.3 mM) and calmodulin (10^{-9} M and 10^{-5} M) for membrane suspensions treated with 40 μM hemin for 60 min. Under all conditions the inhiibtion of the enzyme activity was approximately 50%, indicating a decrease in the maximum velocity with an unmodified K_m for Ca^{2+} (control = $0.8 \cdot 10^{-6}$ M, plus hemin = $0.7 \cdot 10^{-6}$ M) and for Mg ATP (control = $50 \cdot 10^{-6}$ M, plus hemin = $55 \cdot 10^{-6}$ M). Treatment of the membrane with hemin in the dark led to the same level of inhibition. When membranes were incubated in the presence of 100 μM protoporphyrin IX for up to 60 min, in the dark, only a mild inhibition was noticed (Fig. 1). Thus, iron seems to play a major role in the hemin inhibitory process.

Results shown in Table II demonstrate that the inhibition of the enzyme requires that hemin be capable of binding additional ligands. When hemin was reacted with imidazole, or reduced to its ferrous state and liganded with carbon monoxide, no inhibition was observed. It is noticeable from Table I that the amount of hemin-imidazole complex associated with the membranes at the end of the treatment period was comparable to that of hemin. A similar conclusion was made after treatment with CO protoheme as the membranes were clearly pink.

Hemin is known to bind to many proteins

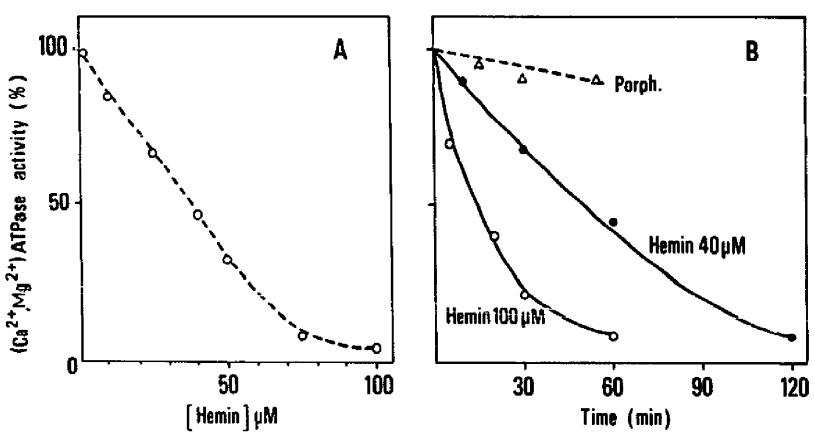

Fig. 1. Inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity by hemin. (A) Red cell membranes (1 mg protein/ml) from normal individuals were incubated for 60 min, at various concentrations of hemin. Then the membranes were rinsed and assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity as described in Materials and Methods. (B) Red cell membranes were treated with the concentration of hemin indicated for various periods of time. Open triangles illustrate the effect of treating the membranes with 100 μM protoporphyrin IX (Porph.) in the dark. Conditions for the treatment period were 0.1 M KCl, 50 mM Bistris buffer (pH 7.1), 37°C.

TABLE II

THE EFFECT OF HEMIN ON THE ACTIVITY OF THE $(Ca^{2+} + Mg^{2+})$-ATPase FROM HUMAN RED CELLS UNDER VARIOUS CONDITIONS

Experimental conditions are described in Materials and Methods.

Incubation condition	% activity [c]
Hemin [a]	42
Hemin + 18 mM imidazole	93
Hemin [a]	48
Hemin + 10 μM calmodulin	90
Hemin [a]	52
Heme (Fe(II)) + CO	86
Hemin (air) [b]	28
Hemin (N_2)	20
1 mM BHT	94
Hemin 100 μM	25
Hemin + BHT	19

[a] Isolated RBC membranes were incubated for 60 min in 0.1 M KCl, 50 mM Bistris buffer (pH 7.1) at 37°C in the presence of 40 μM hemin.

[b] Incubation with hemin lasted 70 min.

[c] Mean of at least three different preparations.

either through covalent linkage between the iron atom and amino-acid side chains and/or hydrophobic interactions between the porphyrin moiety and the polypeptide chains. When bovine serum albumin was added to the incubation mixture containing hemin, no inhibition of the enzyme was observed. The binding of hemin to albumin was demonstrated by recording the visible spectrum of the incubation solution showing the presence of hemichromes. Table I shows that only a small amount of the albumin-hemin complex was associated with the membranes, indicating that hemin was trapped by albumin thus preventing its interaction with the enzyme. In the experiment depicted in Fig. 2 the membranes were first incubated with 40 μM hemin for 60 min. Albumin was then added to the reaction mixture. The results show that the presence of albumin did not restore the activity of the enzyme, despite a lower amount of hemin associated with the membranes (Table I).

We checked whether the inhibition of the enzyme was related to the interaction of hemin with calmodulin, a 17 kDa calcium-binding protein. This interaction was demonstrated as a shift in the maximum absorbance peak of hemin, upon addition of calmodulin, similar to that observed in the presence of albumin. Further, when calmodulin was added at the onset of the incubation period with hemin, no inhibition of the enzyme was detected (Table II). It is unlikely that the inhibition by hemin described in Fig. 1 is due to an interaction of hemin with calmodulin during the assay as (i) the activity of the enzyme was measured in membrane suspensions which were thoroughly washed to remove the unbound hemin present in the solution and (ii) preliminary experiments have demonstrated that hemin inhibits to the same extent the activity of the Ca^{2+}-ATPase from the sarcoplasmic reticulum isolated from rabbit muscles which is not stimulated by calmodulin (results not shown).

$(Ca^{2+} + Mg^{2+})$-ATPase from the erythrocyte membranes is an integral protein embedded in the phospholipid bilayer. This suggested that the inhibition of the enzyme might be induced indirectly through the peroxidation of surrounding phospholipids [18]. BHT was added to the membrane suspension containing hemin at the onset of the treatment period. As shown in Table II, the high concentration of the reducing agent failed to prevent the inhibition of enzyme activity.

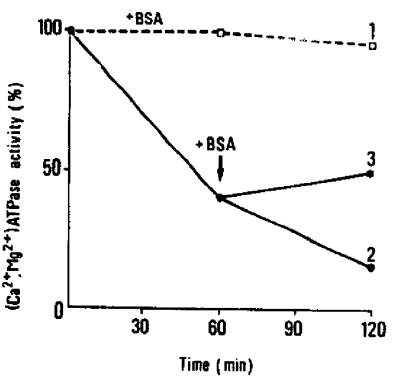

Fig. 2. The effect of bovine serum albumin (BSA) on the inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase induced by hemin. (1) Controls: 0.4 mM BSA was added to the buffer at the onset of the treatment period, in the absence of hemin. (2) Membrane treatment with 40 μM hemin in the absence of BSA. (3) Membranes were treated for 60 min with 40 μM hemin, then 0.4 mM BSA was added to the incubation mixture for a further 60-min period. Activities were assayed in the suspensions of the rinsed membranes, as described in the text.

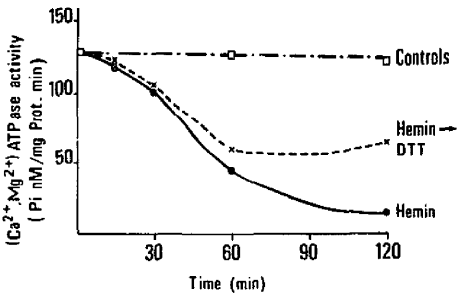

Fig. 3. The effect of DTT on the inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase activity induced by hemin. □, Control membranes incubated in the absence of hemin. ●, Control membranes incubated with hemin. ×, Membranes incubated with 40 μM hemin for various periods of time; then the membrane suspensions were rinsed with the hemin-free buffer and incubated for 60 min in the presence of 10 mM DTT before the assay.

Ferric iron may induce oxidation of -SH groups of cysteine residues through different mechanisms [19]. One of these is the formation of disulfide bonds which are reversible by DTT. It was shown in a previous study that the inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity induced by diamide was entirely reversible by this agent [10]. We tested the effect of DTT in membranes incubated with 40 μM hemin for periods of 15–120 min. After each incubation period, hemin was washed out of the suspensions and 10 mM DTT were added to the incubation media for a further 60-min period. Fig. 3 shows that in these conditions a partial restoration of the activity of the enzyme was obtained at the longer hemin incubation periods.

When membranes were treated with hemin in the absence of oxygen the inhibition of the enzyme was similar to that observed under normal atmospheric conditions (Table I), indicating that oxygen-activated species are probably not at the origin of the inhibitory process.

Discussion

Hemin is a lipophilic compound with oxidative properties. It may interact with the phospholipids of the membrane generating activated oxygen species [20] or with the -SH group residues leading to various derivatives such as disulfide bond formation [19,21]. The porphyrin moiety has also oxidative effects mostly under light activation [22]. Another important property of hemin is its propensity to bind to proteins via the formation of metal complexes or hydrophobic interactions through the porphyrin ring at hydrophobic clusters of the polypeptide chains. This was characterized years ago for globin chains whose tertiary structures are stabilized by hemin [23]. Binding of hemin to albumin has also been demonstrated [18]. More recently, hemin binding to spectrin has been reported, resulting in a less stable oligomer possibly at the origin of the membrane instability in certain blood diseases [9]. We report in the present study that hemin binds also to calmodulin; detailed studies on this binding will be published elsewhere (Marden et al., in preparation).

This study demonstrates that hemin inhibits the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase of the red cell membranes and accumulates in the lipid bilayer. The inhibitory effect appears to be a slow process compared to fast incorporation of hemin into membrane liposomes [24].

It has been suggested by Hebbel et al. [14] that the mechanism for the inhibition of the enzyme might not be unique and that different parts or domains in the protein may be susceptible to the oxidative stresses. Recent analyses by Davies et al. [5] indicate also that various oxygen-activated species may be generated by the ferric hemes leading to varying protein damage. We postulated first that the inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase by hemin was mediated by its peroxidative properties. This was supported by our finding that unliganded ferric iron was a requisite for the inhibition. When hemin was liganded with imidazole or transformed into its ferrous state and liganded with carbon monoxide, no inhibition was observed despite the presence of heme in the membrane. But the finding that BHT does not protect the activity of the enzyme during the hemin treatment indicates that by-products of lipid peroxidation are probably not responsible for the effect of hemin. The inhibition of the enzyme by hemin in the absence of oxygen renders also unlikely the possible role of oxygen-activated species at the origin of the inhibition. Several recent studies have shown that ferric iron ought to be in equilibrium with its ferrous form in order to pro-

duce oxidizing activated species [6]. In our conditions, only ferric iron appears to initiate the inhibitory effect.

When DTT was added, after incubating the membrane with hemin for short periods of time, the activity of the enzyme was not restored. However, Fig. 3 shows that DTT partially restores the enzyme activity when the membranes were treated with hemin for one to two hours. This indicated the formation of disulfide bonds as a secondary process. We postulate, therefore, that there is a direct interaction of hemin with the enzyme at -SH groups through iron-sulfur binding which are favored in non-aqueous solvents, according to the following reaction [19]:

$$2(\text{heme } Fe^{3+}) + 2\,RSH \rightarrow 2(\text{heme-}Fe^{3+}\text{-SR})$$

$$\rightarrow 2(\text{heme-}Fe^{2+}) + RSSR$$

in which ferric heme first binds to the sulfur atom of a cysteine residue then is reduced to its ferrous form and the formation of disulfide derivatives. However, we did not find spectral evidence for the appearance of ferrous hemes in the membrane, as predicted from the above reaction. This may not be contradictory with our hypothesis as the hemin $(Ca^{2+} + Mg^{2+})$-ATPase complexes should represent only a small fraction of all heme-membrane protein complexes present in the red cell membranes.

We have postulated above a direct interaction of the ferric heme to the protein leading primarily to its inactivation, possibly through the blockade of the conformational transition responsible for the normal function of the red cell calcium pump [25]. Ferric iron can also bind to the protein at various residues such as histidine, arginine or lysine [26] and these interactions should be stabilized by the multiple hydrophobic bonds with the porphyrin ring. Since the major functional domains of the enzyme contain such residues, they may be involved in the binding of hemin [25,27]. Studies, similar to those described in the present work and using the almost pure Ca^{2+}-ATPase from the sarcoplasmic reticulum of rabbit muscles, should allow a better understanding of the mechanism of the inhibition and more detailed information of the hemin binding site to the enzyme.

In conclusion, our results demonstrate that hemin inhibits the activity of the human red cell membrane $(Ca^{2+} + Mg^{2+})$-ATPase possibly via the formation of ferric iron-sulfur complexes. These observations may be relevant to the pathophysiology of hemolytic anemia observed in sickle cell disease and in other hematological disorders where ferric hemes accumulate in the red cell membrane. It is interesting to note that the deleterious effect of ferric hemes on the $(Ca^{2+} + Mg^{2+})$-ATPase activity may be easily prevented when transformed in hemichrome-like complexes in the presence of imidazole.

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