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Heme as an optical probe for studying the interactions between calmodulin and the Ca^{2+}-ATPase of the human erythrocyte membrane

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Abstract

The heme group was used as an optical probe to study the interactions between calmodulin and its targets: the peptide melittin and the enzyme Ca^{2+}-ATPase. As already reported, melittin when present in Tris buffer binds hemin-CN which quenches the tryptophan fluorescence. Addition of calmodulin restores the fluorescence significantly accompanied by a blue shift. We show here that the recovery of fluorescence is very slow and takes about 120 min to become constant. In a hydrophobic buffer, the fluorescence spectrum of melittin is already shifted with a peak at 335 nm and intensity almost 2-fold relative to a similar concentration of melittin in Tris buffer. The quenching of tryptophan fluorescence is lesser in this buffer and further addition of calmodulin fails to restore the fluorescence. This indicates the absence of binding of calmodulin to melittin in hydrophobic conditions. Under similar conditions of hydrophobicity, hemin-CN quenches about 35% of the tryptophan fluorescence of the Ca^{2+}-ATPase. The subsequent addition of calmodulin restores about half of the quenched fluorescence. The interaction of calmodulin with the Ca^{2+}-ATPase even under hydrophobic conditions suggests its high specificity for the enzyme which may be expected for a physiological target.

Keywords: Erythrocyte; Calmodulin; ATPase, Ca^{2+}-; Melittin; Heme

1. Introduction

The iron protoporphyrin IX, the prosthetic group of natural globins like the hemoglobin and myoglobin, interacts with them with nanomolar affinity [1]. There appears to be an entire class of proteins that bind heme at the micromolar range. Spectrin, protein 4.1 and actin are some of the red blood cell proteins which have been shown to bind heme [2,3]. Heme also binds to the calcium binding proteins calmodulin, parvalbumin and troponin C [4,5]. More recently, several amphiphilic calmodulin binding peptides like melittin, mastoparan, Baa17 etc. have been shown to bind heme with a very high affinity [6]. The binding can be followed by changes in the heme absorption spectra or by a decrease in the tryptophan fluorescence of the peptides due to energy transfer to the heme. Further addition of calmodulin to one of these peptide-heme complexes, is able to partially restore the tryptophan fluorescence [6]. This effect reflects the fact that in the presence of calmodulin, which has a greater affinity for the peptide, the heme is released from its complex with melittin. The heme group therefore serves as an excellent optical probe for studying the conformational and affinity changes between molecules and permits the study of protein–peptide or protein–protein interactions [4–6].

In the present study this technique has been applied to study a more complex system: the Ca^{2+}-ATPase of the human erythrocyte membrane which is the enzyme responsible for transporting calcium out of the erythrocytes and maintaining its low intracellular concentrations [7]. It is a large protein having a molecular weight of approx. 140 000 and is present in very small amounts in the red blood cell membrane (approx. 0.1% of the total membrane protein) [8]. The protein is embedded in the plasma membrane with 10 putative transmembrane segments connected towards the external side by short loops but 80% of the pump mass is believed to be exposed to the cytoplasm [9,10]. The Ca^{2+}-ATPase has a very strong affinity for calmodulin which is its physiological modulator and stimulates both ATP hydrolysis and calcium transport [11]. The calmodulin binding domain is present towards the C-terminal end of the enzyme which protrudes into the cytoplasm.

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In the present study we have reported the binding of heme to the Ca^{2+}-ATPase of the human erythrocyte membrane. We have also compared the interactions of calmodulin with two of its targets: the peptide melittin and the protein Ca^{2+}-ATPase under hydrophobic conditions using the heme group as an optical probe. The heme group is a hydrophobic molecule and tends to bind to the hydrophobic pockets of proteins especially in its monomeric form (heme-CO or hemin-CN). Earlier studies involving heme binding to peptides and proteins were conducted in an aqueous environment. Since the Ca^{2+}-ATPase is an integral membrane protein, it needs a special buffer containing hydrophobic reagents like phospholipids and glycerol to retain its physiological activity. Moreover, the ability of the peptide melittin to interact with and permeate artificial and native membranes has been proved [12]. Melittin has also been shown to interact with the erythrocyte membrane and to induce disorders in the lipid-protein matrix of the bilayer [13]. Therefore, it is of interest to investigate the interaction of calmodulin with these targets under hydrophobic conditions.

2. Materials and methods

All reagents were of the highest purity grade available. Hemin was a product of Serva, USA. The detergent $C_{12}E_8$ was from Nikko, Japan and ATP from Boehringer-Mannheim, Germany. Calmodulin-Sepharose was purchased from Pharmacia, Sweden and phosphatidylcholine from Sigma, USA. Calmodulin was purified from bovine brain acetone powder (Sigma) by methods described earlier [6]. The calmodulin stock concentration was determined by using the UV band at 276 nm with $\epsilon = 3740\ M^{-1}\ cm^{-1}$ [14].

2.1. Purification of the Ca^{2+}-ATPase

The Ca^{2+}-ATPase was purified from calmodulin depleted erythrocyte membranes by calmodulin-affinity chromatography in presence of the nonionic detergent $C_{12}E_8$ as described elsewhere [15]. Briefly, the supernatant obtained after solubilisation of the membranes with Triton X-100 (1 mg/mg protein) was stabilized with phosphatidylcholine and calcium (final concentration 0.5 mg/ml and 100 μM, respectively) and applied on a calmodulin-sepharose column. Elution was performed with a buffer containing 10 mM Hepes pH 7.2, 120 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 0.5 mg/ml phosphatidylcholine, 0.05% $C_{12}E_8$, 2 mM EDTA and 5% glycerol (buffer D). The fractions were checked for the activity of Ca^{2+}-ATPase using the spectrophotometric-coupled enzyme assay [15]. The active fractions were pooled and $MgCl_2$ and $CaCl_2$ added to a final concentration of 2 mM and 0.5 mM, respectively. The enzyme was stored at $-80°$ C with no loss of activity.

Protein measurement was performed by precipitating the Ca^{2+}-ATPase with trichloroacetic acid and deoxycholate [16] using bovine serum albumin as standard. The isolated Ca^{2+}-ATPase preparation was checked for purity by SDS-PAGE [17] on a 10% polyacrylamide gel.

Hemin, hemin-CN and heme-CO were prepared as described earlier [5,6].

2.2. Spectroscopic measurements

Static absorption spectra were measured on an SLM-Aminco DW 2000 spectrophotometer. Fluorescence spectra were made using an SLM-Aminco 8000 spectrofluorimeter. Excitation wavelength was at 290 nm to minimize the excitation of tyrosine. The spectra were corrected for the inner filter effects due to absorption by hemin-CN by using a solution of tryptophan with the same concentration of hemin-CN. Control experiments were done to correct the spectra for the solvent effects of buffer D and KCN (10 mM). The concentration of Ca^{2+}-ATPase was 1 μM, so that of melittin was adjusted to 10 μM to obtain the same level of fluorescence. The buffer D was prepared fresh before use. Other experimental conditions were kept constant. CO recombination kinetics were measured after dissociation from heme-CO by a 10 ns laser pulse at 532 nm (Quantel YG 585).

3. Results and discussion

In the present report, we have studied the interactions between calmodulin and the Ca^{2+}-ATPase of the red blood cell membrane using the heme group as an optical probe. Calmodulin lacks tryptophan residues making its UV dosage difficult. The heme group (ferrous form heme-CO and the ferric form hemin-CN) has recently been used as a chromophore to visualize the interactions between various molecules [4–6]. It has several distinct advantages to be able to serve this goal. The absorption spectra of heme-CO varies significantly depending on its environment. In addition, the ligand CO is photodissociable and the recombination kinetics gives useful information regarding the local heme environment. Thirdly, the heme group is a strong acceptor of fluorescence energy and quenches the tryptophan fluorescence.

3.1. Interaction of Ca^{2+}-ATPase with heme

The binding of heme to the Ca^{2+}-ATPase was monitored by changes in the visible absorption spectra of heme-CO which is very well characterized. The spectra for heme-CO (1 μM) alone and in the presence of the Ca^{2+}-ATPase (1 μM) is given in Fig. 1. In an aqueous environment (50 mM Tris-HCl pH 7.4) the Soret peak of heme-CO is at 407 nm but in buffer D, it is shifted to 412 nm. Addition of Ca^{2+}-ATPase shifts the peak further towards

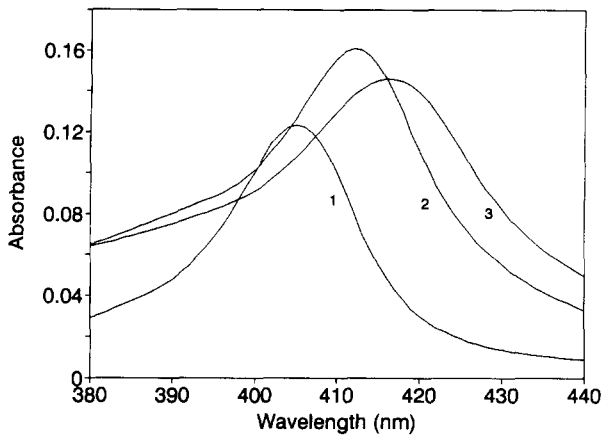

Fig. 1. Absorption spectra of heme-CO in the absence and presence of Ca^{2+}-ATPase. Heme-CO in 50 mM Tris-HCl pH 7.4 (1), in the hydrophobic buffer D (2) and in the presence of Ca^{2+}-ATPase in buffer D (3). The concentration of heme-CO and Ca^{2+}-ATPase is 1 μM each and of calcium is 1 mM.

the red side to 416 nm. The spectral shift of heme-CO can be attributed to the binding of the heme to the Ca^{2+}-ATPase. Further increase in the concentration of heme-CO beyond 1 heme per protein molecule increases the spectrum characteristics of the free form of heme-CO (data not shown), indicating the stoichiometry of binding to be 1:1. The binding is not temperature dependent as the results obtained are the same at 25° C or 37° C.

Further evidence of heme binding to the Ca^{2+}-ATPase was obtained by the flash photolysis technique. The recombination kinetics of heme-CO in buffer D is similar to that in an aqueous buffer although there was a significant spectral shift from 407 nm to 412 nm as shown in Fig. 1. However, in the presence of Ca^{2+}-ATPase, the recombination kinetics of heme-CO is much slower than in buffer D (Fig. 2). This indicates a binding of the heme to the enzyme and hence its lower accessibility to the ligand CO. The binding of heme to Ca^{2+}-ATPase is consistent with an earlier report [18] from this laboratory where a time- and concentration-dependent inhibition of the enzyme was observed in the presence of hemin.

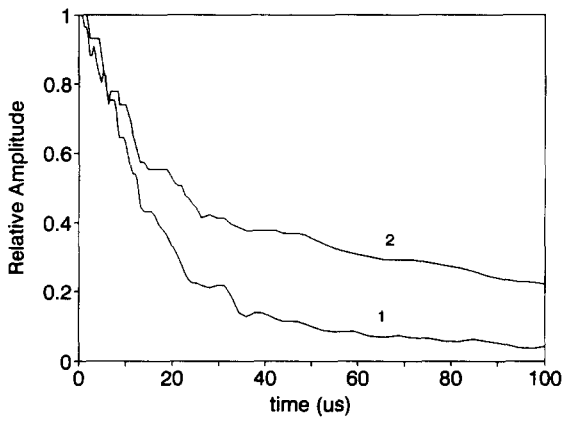

Fig. 2. Recombination kinetics of CO after photodissociation of heme-CO. Heme-CO in buffer D (1) and in the presence of Ca^{2+}-ATPase (2). The concentration of heme-CO and Ca^{2+}-ATPase is 1 μM each and of calcium is 1 mM.

3.2. *Interaction of calmodulin with its targets: melittin and Ca^{2+}-ATPase*

Calmodulin modulates a wide variety of calcium dependent events through interactions with many enzymes and cytoskeletal proteins of the cell. Calcium binding to calmodulin creates conformational changes within the protein which exposes hydrophobic surfaces [19] and calmodulin may then bind to its targets through these sites [20]. In an aqueous medium like 50 mM Tris-HCl pH 7.4, it has been reported that calmodulin binds heme (the reduced form, heme-CO only) in the presence of calcium [4]. Under these conditions the Soret absorption band of heme-CO is shifted to 420 nm. We investigated whether this binding could take place in a hydrophobic environment. When heme-CO is present in buffer D there is no spectral shift from 412 nm and calmodulin does not bind heme-CO under these conditions (Fig. 3). This lack of interaction indicates the binding of heme to calmodulin to be of a hydrophobic nature as observed earlier [4].

Calmodulin binds in a calcium dependent manner to the bee venom melittin [21]. In an earlier report it was shown that melittin is also capable of binding heme (heme-CO and hemin-CN) with a very high affinity [6]. Melittin has a single tryptophan residue which gives a fluorescence spectrum with a peak at 350 nm which is like that of free tryptophan in an aqueous environment. The fluorescence of the lone tryptophan residue is significantly quenched (about 70%) by the addition of hemin-CN at a stoichiometry of 1:1. Further increase in the concentration of heme completely quenches the tryptophan fluorescence. The addition of calmodulin to the melittin-hemin-CN complex restores significantly the fluorescence, accompanied by a

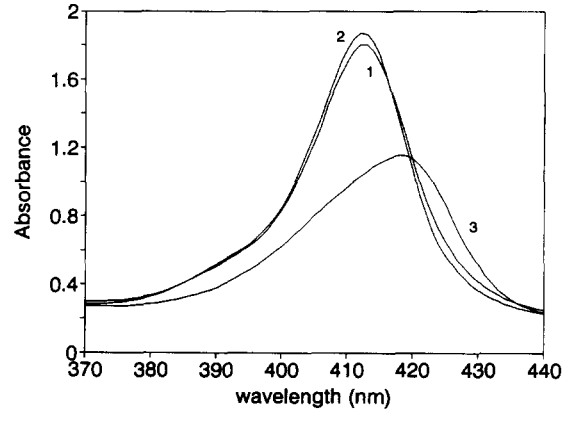

Fig. 3. Absorption spectra of heme-CO in the absence and presence of calmodulin. Heme-CO in buffer D (1), in the presence of calmodulin in buffer D (2) and in the presence of calmodulin in 50 mM Tris-HCl pH 7.4 (3). The concentration of heme-CO is 10 μM, calmodulin 5 μM and calcium 1 mM.

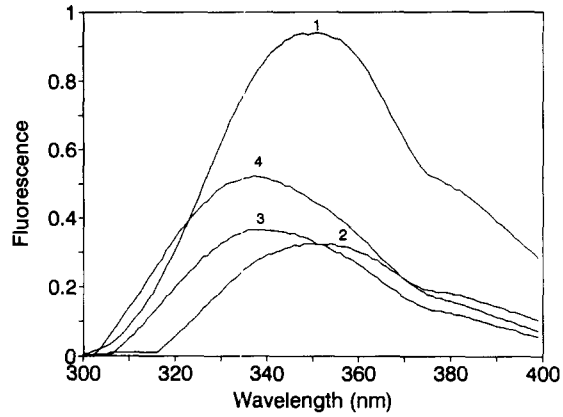

Fig. 4. Fluorescence emission spectra of melittin in 50 mM Tris-HCl pH 7.4. Melittin in presence of KCN and calcium (1), with added hemin-CN (2), just after the addition of calmodulin (3) and with calmodulin after 120 min (4). The concentration of melittin is 10 μM, KCN 10 mM, hemin-CN 10 μM, calmodulin 12 μM and calcium 1 mM. Excitation wavelength is 290 nm.

blue shift which means that tryptophan is exposed to a less polar environment when melittin is complexed with calmodulin. The restoration of the quenched fluorescence by calmodulin is shown in Fig. 4. The recovery of fluorescence is slow and it takes several minutes before hemin-CN can be displaced and fluorescence recovery becomes constant.

When 10 μM melittin is solubilized in buffer D, its fluorescence spectrum is very different with a peak at 335 nm and intensity of fluorescence almost 2-fold that of 10 μM melittin in 50 mM Tris-HCl pH 7.4 (Fig. 5). Melittin is known to assume an amphiphilic α-helical conformation upon interaction with hydrophobic surfaces like membranes and detergents [22,23] and this conformation appears to be mandatory for establishing hydrophobic interactions with calmodulin [24]. Addition of hemin-CN quenches about 50% of the tryptophan fluorescence. Further addition of calmodulin does not restore the fluorescence in this case even if the incubation is monitored for 2 h. This suggests that the affinity of calmodulin for melittin is greatly decreased in an hydrophobic environment.

The fluorescence spectrum of Ca^{2+}-ATPase (1 μM) exhibits a peak at 335 nm like melittin in buffer D but has a high intensity (Fig. 6). This may be expected as the Ca^{2+}-ATPase has 13 tryptophan residues out of which one is known to be present in the calmodulin binding domain. The addition of the hemin-CN quenches about 35% of the tryptophan fluorescence of the Ca^{2+}-ATPase in contrast to a much higher quenching obtained when melittin is the target. This interaction again shows no temperature dependence. Further addition of calmodulin to the Ca^{2+}-ATPase-hemin-CN complex restores about half of the quenched fluorescence. The restoration is immediate and confirms the high affinity of calmodulin for the Ca^{2+}-ATPase. By contrast, in the absence of hemin-CN, when calmodulin is added to the Ca^{2+}-ATPase there is a decrease in fluorescence. The restoration of fluorescence by calmodulin means that either hemin-CN binds to the calmodulin binding domain which possesses one tryptophan residue or alternatively, that the binding of calmodulin to the Ca^{2+}-ATPase creates conformational changes in the enzyme molecule thus releasing hemin-CN.

The recovery of fluorescence of the Ca^{2+}-ATPase by calmodulin although not large in magnitude is very significant if we consider that calmodulin does not interact any more with its targets, e.g., melittin and heme under mild hydrophobic conditions. Comparison between the results obtained with melittin and the Ca^{2+}-ATPase reflects the

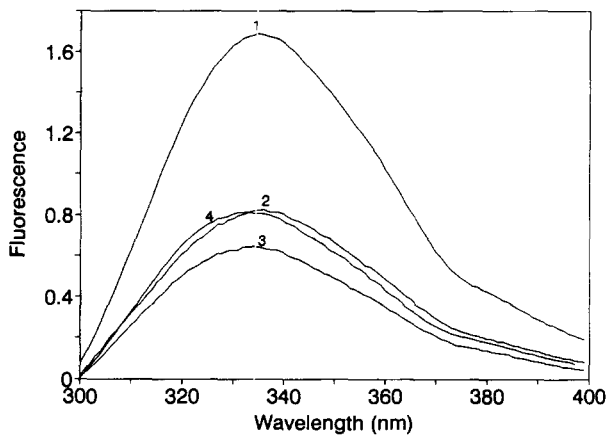

Fig. 5. Fluorescence emission spectrum of melittin in the hydrophobic buffer D. Melittin in presence of KCN and calcium (1), with added hemin-CN (2), just after the addition of calmodulin (3) and with calmodulin after 120 min (4). The concentration of melittin is 10 μM, KCN 10 mM, hemin-CN 10 μM, calmodulin 12 μM and calcium 1 mM. The excitation wavelength is 290 nm.

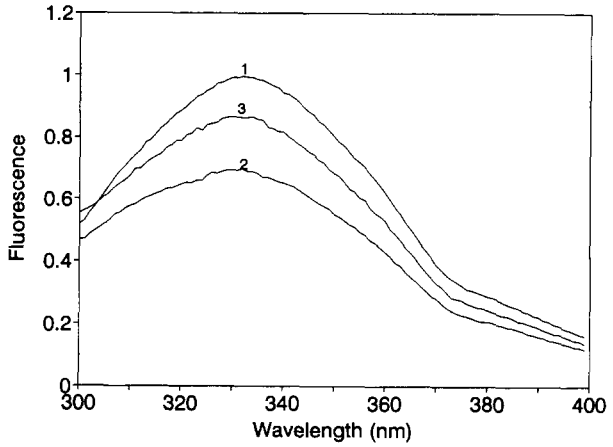

Fig. 6. Fluorescence emission spectra of Ca^{2+}-ATPase in the hydrophobic buffer D. Ca^{2+}-ATPase in presence of KCN and calcium (1), with added hemin-CN (2) and after the addition of calmodulin (3). The concentration of Ca^{2+}-ATPase, hemin-CN and calmodulin is 1 μM each and of calcium and KCN is 1 mM and 10 mM, respectively. The excitation wavelength is 290 nm.

higher specificity of interaction between calmodulin and the Ca^{2+}-ATPase as may be expected for a preferred physiological target.

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