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Conformational changes of (Ca^{2+}-Mg^{2+})-ATPase of erythrocyte plasma membrane caused by calmodulin and phosphatidylserine as revealed by circular dichroism and fluorescence studies *

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Two spectroscopic techniques, circular dichroism and steady-state fluorescence, were employed in order to study conformational changes of the purified, detergent-solubilized (Ca^{2+}-Mg^{2+})-ATPase of porcine erythrocyte ghost membranes. Circular dichroism (CD) spectra in the peptide region were obtained from the purified (Ca^{2+}-Mg^{2+})-ATPase of porcine erythrocyte ghost membranes with the aim to investigate the secondary structure of the enzyme in the presence of calmodulin (CaM) or phosphatidylserine (PS), as well as in the E_1 and E_2 states. The E_1 conformation was stabilized by 10 μM free Ca^{2+}, while the E_2 conformation was stabilized by 0.1 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). It was found that the E_1 and E_2 states of the enzyme strikingly differed in their secondary structure (66% and 46% of calculated α-helix content, respectively). In the presence of Ca^{2+}, PS decreased the helical content of the ATPase to 61%, while CaM to 55%. Quenching of intrinsic fluorescence of (Ca^{2+}-Mg^{2+})-ATPase by acrylamide, performed in the presence of Ca^{2+}, gave evidence for a single class of tryptophan residues with Stern-Volmer constant (K_{SV}) of 10 M^{-1}. Accessibility of tryptophan residues varied depending on the conformational status of the enzyme. Addition of PS and CaM decreased the K_{SV} value to 7.6 M^{-1} and 8.5 M^{-1}, respectively. In the absence of Ca^{2+}, K_{SV} was 7.0 M^{-1}. KI and CsCl were less effective as quenchers. The fluorescence energy transfer between (Ca^{2+}-Mg^{2+})-ATPase tryptophan residues and dansyl derivative of covalently labeled CaM occurred in the presence of EGTA, but was further promoted by Ca^{2+}. It is concluded that the interaction of CaM and PS with (Ca^{2+}-Mg^{2+})-ATPase results in different conformational states of the enzyme. CD and fluorescence spectroscopy allowed to distinguish these states from the E_1 and E_2 conformational forms of the ATPase.

Introduction

Erythrocyte plasma membrane (Ca^{2+}-Mg^{2+})-ATPase (EC 3.6.1.38) belongs to the class of P-type ATPases; it forms acyl phosphate intermediate during the reaction cycle [1,2]. It is a single polypeptide of M_r of about 138 000 [3], whose activity is stimulated by calmodulin, CaM-tryptic fragments, acidic phospholipids, polyunsaturated fatty acids, controlled proteolysis, and by cAMP-dependent phosphorylation of C-terminal domain of the enzyme [4]. On the other hand, the activity of (Ca^{2+}-Mg^{2+})-ATPase is inhibited by low concentrations of orthovanadate [5] and lanthanides [6].

The Ca^{2+} pumping CaM – stimulated ATPase accounts for less then 0.2% of total protein of erythro-

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Abbreviations: CD, circular dichroism; CaM, calmodulin; PS, phosphatidylserine; PC, phosphatidylcholine; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DDAO (Ammonyx LO), *N,N*-dimethyloctadecylamine-*N*-oxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; K_{SV}, Stern-Volmer constant for quenching of intrinsic fluorescence of tryptophan residue; F_0, fluorescence intensity without quencher; F, fluorescence intensity with quencher.

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cyte ghost membrane preparation [3]. Therefore, to study conformational changes of (Ca^{2+}-Mg^{2+})-ATPase, of particular importance are solubilization and purification of the active form of the enzyme, what requires the use of appropriate detergents.

The Ca^{2+} pump of erythrocyte ghost membranes undergoes E_1-E_2 conformational transitions during the catalytic cycle [7]. The equilibrium between these conformational forms is influenced by various ligands that preferentially react with the enzyme in either of the two states. The E_1 form of the enzyme is stabilized by calcium ions (10 μM free), while in the absence of Ca^{2+}, orthovanadate (20 μM) shifts the conformational equilibrium to the E_2 form [7]. Two major conformational states of the purified enzyme from pig erythrocytes have been detected by circular dichroism and fluorescence spectroscopy [7]. Also proteolysis studies provided evidence for the existence of conformational changes in the ATPase secondary structure [8], as striking differences in digestion pattern were observed, when the purified enzyme, in E_1 or E_2 states, was submitted to proteolysis.

Untill now, no studies have been done on conformational changes caused by interactions of the enzyme with CaM or PS. CaM and PS, each having the same but not additive effect on the activity of (Ca^{2+}-Mg^{2+})-ATPase in solubilized system, differ presumably by their mode of interaction with the enzyme. CaM binds to a domain of the ATPase molecule located in the C-terminal portion exposed to the cytoplasm [9], while the mechanism of PS influence still remains unclear. Therefore, it was interesting to determine whether it would be possible, using CD spectroscopy, to distinguish between the effects of CaM and PS on the conformational status of (Ca^{2+}-Mg^{2+})-ATPase. We also checked the influence of various quenchers on the intrinsic fluorescence of tryptophan residues of the purified enzyme in E_1 and E_2 conformations. To study a direct interaction of (Ca^{2+}-Mg^{2+})-ATPase with calmodulin we adopted the method of fluorescence energy transfer measurements. For this purpose, dansylated CaM was used and fluorescence energy transfer between tryptophan residues of the enzyme molecule and dansyl derivative of CaM, in the presence and absence of Ca^{2+}, was assessed.

A part of these results has already been presented in a preliminary form [10].

Materials and Methods

Chemicals

Triton X-100 was obtained from Merck (Darmstadt, F.R.G.), polydocanol from Calbiochem Behring Diagnostics (San Diego, CA, U.S.A.), and Ammonyx LO (DDAO) from Millmaster Onyx International (Fairfield, NJ, U.S.A.). Phosphatidylcholine (PC) was prepared from egg yolk as described by Sarzała and Michalak [11], and PS from bovine brain according to the method of Comfurius and Zwaal [12]. CaM from bovine brain was purified as described by Brzeska et al. [13] and dansylated according to the procedure reported for dansylation of troponin C [14]. Modified protein exhibited the characteristic fluorescence emission spectra for dansyl derivatized CaM obtained at excitation wavelength 340 nm.

The method employed in this study routinely yielded in 90–95% of labeled CaM and the molar ratio of dansyl derivative to CaM was 1:1. Dansylated CaM fully activated (Ca^{2+}-Mg^{2+})-ATPase when the enzyme to CaM molar ratio was 1:1. Dansyl chloride was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and CNBr-Sepharose 4B from Pharmacia Biotechnology International AB (Uppsala, Sweden). All other chemicals were of the highest purity commercially available.

Preparation of erythrocyte ghost membranes

Preparation of erythrocyte ghosts from fresh porcine blood provided by slaughter-house was performed according to Steck and Kant [15] with some modifications. Briefly, hemolysis of erythrocytes was carried out in 10 vols. of 20 mM Hepes-Tris (pH 7.4), and ghost membranes were spun down by centrifugation at 14000 rpm for 20 min at 2°C in Beckman JA 14 rotor. Then, membranous fragments were resuspended in 10 vols. of 20 mM Hepes-Tris (pH 7.4), 100 mM KCl, 0.1 mM $PhMeSO_2F$, 0.4 mM dithiothreitol, and 0.1 mM EGTA, and centrifugation was repeated. At the end of preparation, the collected material (3–6 mg protein/ml), in the same buffer as listed above, was quickly frozen in liquid nitrogen and stored at −70°C in polypropylene containers until use (2–3 weeks). The proteinase inhibitor, $PhMeSO_2F$, was present in all buffers used for membrane preparation.

Purification of (Ca^{2+}-Mg^{2+})-ATPase

Purification of (Ca^{2+}-Mg^{2+})-ATPase was carried out according to Niggli et al. [16] with modifications introduced by Nelson and Hanahan [17]. Briefly, erythrocyte ghosts (total 1.2 g protein) in 300 ml of 100 mM KCl, 20 mM Hepes-Tris (pH 7.4), 0.1 mM $PhMeSO_2F$, 0.4 mM dithiothreitol, 0.1 mM EGTA, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$, were solubilized by adding 4 mg Triton X-100 per ml of the medium. After 10 min of incubation at 2°C, the nonsolubilized material was removed by centrifugation at 30000 rpm for 60 min at 2°C in Beckman 30 type rotor. To the supernatant thus obtained PC was added to the final concentration of 1 mg/ml, and then the material was applied onto a 10-ml column of CNBr-activated Sepharose 4B with covalently linked CaM (1 mg CaM protein/ml). Prior to

use, the column was equilibrated with 50 mM KCl, 5 mM Tris-HCl (pH 7.4), 0.4 mM dithiothreitol, 0.2 mM $CaCl_2$, 0.2 mg Triton X-100/ml, and 0.25 mg PC/ml. Unbound proteins were removed and Triton X-100 was replaced by passing through the column with 10 vols. of 50 mM KCl, 5 mM Tris-HCl (pH 7.4), 0.4 mM dithiothreitol 0.1 mM $CaCl_2$, 0.1 mg polydocanol/ml, and 0.25 mg PC/ml. Finally, (Ca^{2+}-Mg^{2+})-ATPase bound to the column was collected by washing with the same buffer as above except that 0.1 mM $CaCl_2$ was replaced by 0.5 mM EGTA, and the medium was supplemented with 1 mM $MgCl_2$. The detergent-solubilized, lipid-stabilized (Ca^{2+}-Mg^{2+})-ATPase preparation (0.2–0.3 mg protein/ml) in 50 mM KCl, 5 mM Tris-HCl (pH 7.4), 0.4 mM dithiothreitol, 0.5 mM EGTA, 1 mM $MgCl_2$, 0.1 mg polydocanol/ml and 0.25 mg PC/ml was directly used for all measurements.

CD measurements

CD spectra were taken using a JASCO 500 A automatic recording spectropolarimeter (Japan). The instrument was equipped with DP-500N microprocessor unit for spectral smoothing (scanning speed and time constant were 5 nm/min and 4 s, respectively). CD spectra were recorded under conditions to minimize differential light-scattering in a quartz cuvette of 0.1 mm optical path length thermostated at $25 \pm 0.2°C$. The instrument was calibrated with d-camphorsulfonic acid at 192.5 and 290 nm. Usually six spectra were time-averaged and normalized. The basic medium contained 50 mM KCl, 5 mM Tris-HCl (pH 7.4), 0.4 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mg polydocanol/ml, 0.25 mg PC/ml, and 0.5 mM EGTA. Protein concentration in the cuvette was routinely 0.25 mg/ml. Corresponding baselines were obtained using protein-free standard buffer solution containing 0.1 mg polydocanol/ml with $CaCl_2$. EGTA, CaM, PS and/or phospholipids added as appropriate. Three independent repeats of each experiment were run on a single preparation of the detergent-solubilized, lipid-stabilized enzyme. All of them gave very similar results (±1–2%). No changes in the aggregation state of the protein as evaluated by UV absorption spectra (run from 200 to 400 nm on a Perkin-Elmer dual-wavelength spectrophotometer), and no significant differences in (Ca^{2+}-Mg^{2+})-ATPase activity could be detected before and after recording of CD spectra.

The α-helical content of erythrocyte (Ca^{2+}-Mg^{2+})-ATPase was evaluated on the basis of ellipticity measurements using the calculations of Siegel et al. [18].

Steady-state fluorescence measurements

Quenching of intrinsic fluorescence. Quenching of intrinsic fluorescence of tryptophan residues of (Ca^{2+}-Mg^{2+})ATPase was performed at $22 \pm 2°C$ in a cuvette of optical path length of 0.5 cm using a Perkin-Elmer LS 5B spectrofluorimeter (U.S.A.) equipped with a Perkin-Elmer 3700 data storage system. The basic medium of total 0.7 ml contained 100 mM KCl, 10 mM Hepes-Tris (pH 7.4), 0.4 mM dithiothreitol, 0.1 mM $PhMeSO_2F$, 1 mM $MgCl_2$, 0.1 mg polydocanol/ml, 0.25 mg PC/ml, 17 μg of (Ca^{2+}-Mg^{2+})-ATPase protein per assay and either $CaCl_2$ to adjust concentration of free Ca^{2+} to 10 μM or 0.1 mM EGTA. To minimize the effect of oxygen on the quenching of the intrinsic fluorescence of tryptophan residues all measurements were performed in an atmosphere of argon and buffer solutions were saturated with argon prior to use. Acrylamide, KI or CsCl at various concentrations, from 0 to 100 mM, were used as quenchers. In some experiments the effect of CaM (0.18 μM) and PS (66 μM) was checked, as indicated in the text. The F_0/F ratio was calculated from fluorescence intensities measured at the maximal value of emission (328 nm, 2.5 nm slit width), where F_0 indicates fluorescence intensity without quencher and F, fluorescence intensity in the presence of the quencher. The excitation wavelength was 292 nm (10.0 nm slit width). The data presented were averaged from 3–5 independent measurements which varied with a standard error (S.E.) of ±1–2%.

Fluorescence energy transfer. Fluorescence energy transfer between tryptophan residues of the enzyme and dansylated CaM was measured in the same medium as used for quenching experiments. Fluorescence emission spectra of (Ca^{2+}-Mg^{2+})-ATPase (24 μg protein/ml) and dansylated CaM (0.18 μM) were recorded within wavelength range of 300 to 550 nm (2.5 nm slit width). The excitation wavelength was 285 nm (10.0 nm slit width). Emission spectra of dansylated CaM were subtracted from those obtained for ATPase and dansylated CaM in the presence or absence of Ca^{2+}, respectively. This was done with the aid of Perkin-Elmer 3700 data storage and retrieval system. Fluorescence energy transfer was evaluated as a decrease of fluorescence intensity at 328 nm and a concomitant increase at 502 nm. The fluorescence intensity at 502 nm caused by the energy transfer was routinely twice as high as the value observed for dansylated CaM alone.

Other determinations

(Ca^{2+}-Mg^{2+})-ATPase activity was determined spectrophotometrically at 37°C using coupled enzyme assay system, essentially as described by Niggli et al. [16]. Protein was measured by the method of Lowry et al. [19]. The concentrations of free Ca^{2+} were calculated using a computer program on the basis of dissociation constants published by Fabiato and Fabiato [20]. All results presented in the tables are expressed as mean values ± S.E.

Results

Effect of CaM, Ca^{2+} and PS on CD spectra of (Ca^{2+}-Mg^{2+})-ATPase

CD spectra shown in Fig. 1 reflect the characteristic course for the proteins and peptides with a high degree of α-helical content, i.e., they reveal a double minimum at 222 nm and 208–210 nm, and a maximum at 191–193 nm [21]. The spectra cross the baseline from positive to negative values at around 201–202 nm. Typical CD spectra of (Ca^{2+}-Mg^{2+})-ATPase represented as the so-called smoothed curves are shown in Fig. 1. By removing Ca^{2+} from high-affinity Ca^{2+}-binding sites of the enzyme with 0.5 mM EGTA, one can observe a change of the spectra caused by a shift of the equilibrium between the E_1 and E_2 states (Fig. 1A). Inactivation of the enzyme with a zwitterionic detergent Ammonyx LO (Table I) was accompanied by a complete loss of the secondary structure characteristic for the native protein (Fig. 1A, dashed curve). The effect of CaM and PS on the CD spectrum of purified (Ca^{2+}-Mg^{2+})-ATPase is shown in Fig. 1B. In the presence of 10 μM free Ca^{2+} and either 1.8 μM CaM (Fig. 1B, solid curve) or 66 μM PS (Fig. 1B, dashed curve) CD spectra of purified ATPase turned out to be different from the spectrum of the enzyme incubated with calcium ions alone (Fig. 1A, dotted curve). The addition of PS to the assay medium containing both 10 μM free Ca^{2+} and 1.8 μM CaM also changed the shape of the CD spectrum of the enzyme (Fig. 1B, dotted curve), as compared to CaM or PS alone. Moreover, CaM and PS each stimulated the enzyme activity to the same extent (Table I). It is worth mentioning that phospholipids extracted from erythrocyte ghosts, the detergents used, and PS itself, gave no significant signal within the wavelength range of interest (not shown).

TABLE I

Effect of calmodulin (CaM) and phosphatidylserine (PS) on the activity of purified (Ca^{2+}-Mg^{2+})-ATPase

The enzyme activity was measured spectrophotometrically at 37°C using coupled enzyme assay system, as described by Niggli et al. [16], in the presence of 10 μM free Ca^{2+} with the additions listed below. Values are means ± S.E. for 7–10 independent experiments.

Additions	Enzyme activity (μmol P_i/mg protein per min)
None	2.01 ± 0.25
66 μM PS	8.50 ± 0.57
0.18 μM CaM	8.52 ± 0.50
66 μM PS + 0.18 μM CaM	8.72 ± 0.62
0.18 μM CaM + 0.4 mg Ammonyx LO/ml	0.00
66 μM PS + 0.4 mg Ammonyx LO/ml	0.00

From the spectra presented in Fig. 1 and using the method of Siegel et al. [18], we calculated the content of α-helix of purified (Ca^{2+}-Mg^{2+})-ATPase in the E_1 (+10 μM free Ca^{2+}) and E_2 (+0.5 mM EGTA) conformations (Table II). Our observations corroborate the results reported by Krebs et al. [7] that the E_1 form of the enzyme is characterized by higher content of α-helix (66%), while in the E_2 form this value is lower (46%). We also calculated the α-helix content of the enzyme in the presence of Ca^{2+} and either CaM or PS. It is interesting to note that PS lowered the α-helix content

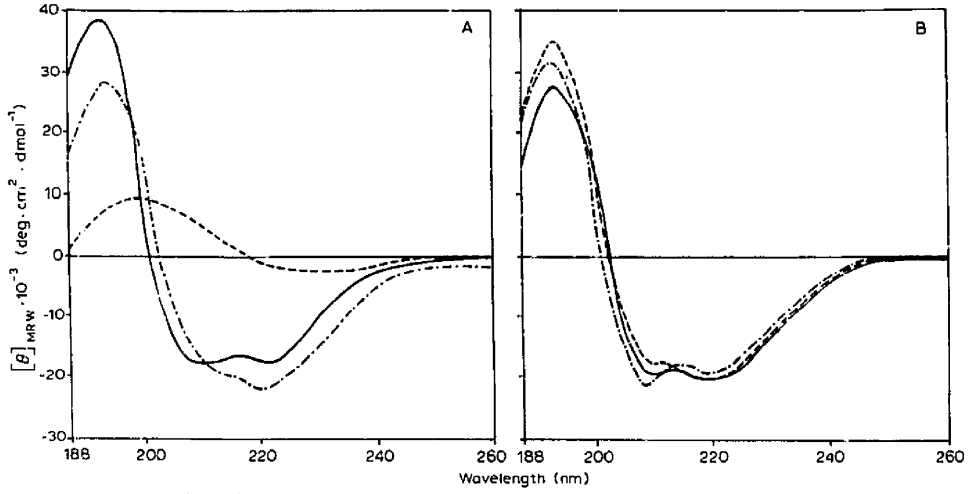

Fig. 1. CD spectra of purified (Ca^{2+}-Mg^{2+})-ATPase of porcine ghosts. The additions to the medium were as follows: Panel A: none (———), 10 μM free Ca^{2+} (–·–·–) or 10 μM free Ca^{2+} and 0.4 mg Ammonyx LO/ml (- - - - -). Ammonyx LO was introduced to the medium to completely inactivate the ATPase activity (see Table I). Panel B: 10 μM free Ca^{2+} and either 1.8 μM CaM (———), or 66 μM PS (- - - - -), or 1.8 μM CaM plus 66 μM PS (–·–·–).

TABLE II

α-Helix content of erythrocyte (Ca^{2+}-Mg^{2+})-ATPAse

Values were obtained by using calculations of Siegel et al. [18] on ellipticity measurements presented in Fig. 1. Data are expressed as means ± S.E. for three experiments.

Additions	α-Helix content (%)
None	46 ± 2
10 μM free Ca^{2+}	66 ± 3
10 μM free Ca^{2+} + 66 μM PS	61 ± 3
10 μM free Ca^{2+} + 1.8 μM CaM	55 ± 2
10 μM free Ca^{2+} + 66 μM PS + 1.8 μM CaM	57 ± 2

of the enzyme to 61% and CaM to 55%, as compared to the E_1 state (66%).

Influence of Ca^{2+}, CaM and PS on quenching of intrinsic fluorescence of tryptophan residues of purified (Ca^{2+}-Mg^{2+})-ATPase

Quenching of the intrinsic fluorescence of (Ca^{2+}-Mg^{2+})-ATPAse tryptophan residues is illustrated in Fig. 2. The fluorescence intensity of the purified enzyme was reduced by acrylamide, KI and CsCl in a concentration-dependent manner. At concentrations employed (up to 100 mM) neither of the three quenchers had significant effect on the CaM-stimulated (Ca^{2+}-Mg^{2+})-ATPase activity (not shown). Therefore, denaturation of the (Ca^{2+}-Mg^{2+})-ATPase is not likely to contribute to quenching of fluorescence by either of these agents.

TABLE III

Stern-Volmer constants (K_{SV}) for quenching of intrinsic fluorescence of tryptophan residues of purified (Ca^{2+}-Mg^{2+})-ATPase by different quenchers

Stern-Volmer constants for quenching of intrinsic fluorescence of tryptophan residues of purified (Ca^{2+}-Mg^{2+})-ATPase were calculated from the experimental data presented in Fig. 2 on the basis of transformed Stern-Volmer equation: $K_{SV} = (F_0/F - 1) \cdot [Q]^{-1}$, where F_0 is fluorescence intensity without quencher; F, fluorescence intensity with quencher; and [Q], concentration of a collisional quencher. F_0/F ratio was calculated at the maximal value of emission (328 nm). The values are means ± S.E. for 3–5 independent measurements. n.s., not studied.

Additions	K_{SV} (M^{-1}) for quenching of intrinsic fluorescence by		
	acrylamide	KI	CsCl
0.1 mM EGTA	7.0 ± 0.1	2.1 ± 0.1	0.50 ± 0.01
10 μM free Ca^{2+}	10.0 ± 0.3	4.1 ± 0.1	0.80 ± 0.02
10 μM free Ca^{2+} + 0.18 μM CaM	8.5 ± 0.2	n.s.	n.s.
10 μM free Ca^{2+} + 66 μM PS	7.6 ± 0.1	n.s.	n.s.

Acrylamide was the most potent quencher, but its effect, similarly to other compounds used, was ex-

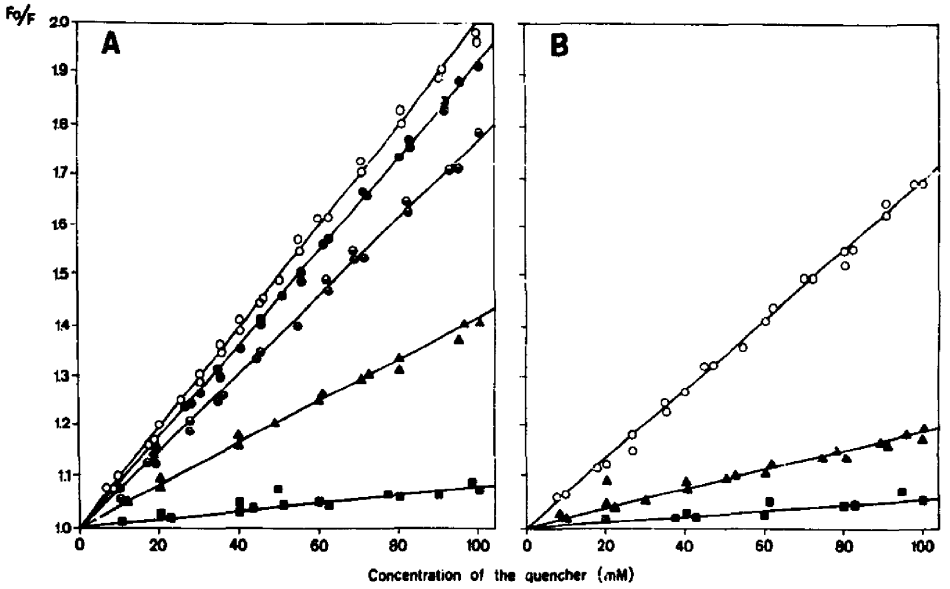

Fig. 2. Quenching of the intrinsic fluorescence of purified (Ca^{2+}-Mg^{2+})-ATPase in the presence Ca^{2+} or EGTA. Measurements were performed in the medium described in Materials and Methods containing either 10 μM free Ca^{2+} (panel A) or 0.1 mM EGTA (panel B). The assay medium was supplemented with the following quenchers: KI (triangles), CsCl (squares) or acrylamide (circles). In the latter case, ○, indicates, no other additions; ●, plus 0.18 μM CaM; and ◒, plus 66 μM PS. Additions were made from concentrated stock solutions to keep the dilution effect at a minimum.

tremely dependent on the conformational status of the enzyme. When 10 μM free Ca^{2+} was present in the incubation medium, 100 mM acrylamide decreased the fluorescence intensity of the purified enzyme by 50%, while in the presence of 0.1 mM EGTA by 41%. A similar effect of Ca^{2+} and EGTA could be seen for quenching by 100 mM KI (30% and 16%, respectively), and even by the less effective CsCi (100 mM) (8% and 5%, respectively). The calculated Stern-Volmer constants for quenching of intrinsic fluorescence of tryptophan residues of the ATPase by three quenchers used are summarized in Table III. We also studied the influence of CaM (0.18 μM) and PS (66 μM) on the quenching of tryptophan fluorescence at Ca^{2+} concentration sufficient to saturate high-affinity Ca^{2+}-binding sites of the enzyme. Under these conditions PS diminished the effect of acrylamide by 12%, while CaM was less effective (decreased the effect of acrylamide in the presence of Ca^{2+} by 4%) (Fig. 2A, Table III). These data clearly demonstrate that the accessibility of tryptophan residues for the quenchers varied mainly due to saturation of high-affinity Ca^{2+}-binding sites of (Ca^{2+}-Mg^{2+})-ATPase in the E_1 state or removal of Ca^{2+}, shifting the conformational equilibrium toward the E_2 state.

The influence of CaM and PS on the quenching of ATPase fluorescence by acrylamide was less pronounced than that of Ca^{2+} and EGTA. This corroborates changes in the CD spectra of the purified enzyme recorded in the presence of these effectors (Fig. 1).

Fluorescence energy transfer from tryptophan residues of (Ca^{2+}-Mg^{2+})-ATPase to dansylated calmodulin

As it was described earlier [7], the intrinsic fluorescence of tryptophan residues of (Ca^{2+}-Mg^{2+})-ATPase is influenced by Ca^{2+}. When 10 μM free Ca^{2+} was added to the reaction mixture the ATPase exhibited an increase in intrinsic fluorescence of tryptophan residues, while after removal of Ca^{2+} by EGTA, one could observe the reverse effect (not shown). The spectra presented in Fig. 3 indicate that the addition of equimolar amount of dansylated CaM (0.18 μM) to (Ca^{2+}-Mg^{2+})-ATPase (24 μg protein/ml, i.e., 0.18 μM) in the basic medium containing 0.1 mM EGTA resulted in a decrease of the intrinsic fluorescence of tryptophan residues by about 8% (calculated at maximal value of emission at 328 nm), compared to the spectrum of the enzyme alone, and appearance of an extra fluorescence emission peak (maximum at 502 nm) characteristic for the dansyl derivative of CaM. This new peak is associated with fluorescence energy transfer from tryptophan residues of the ATPase to dansylated CaM. When the basic medium was supplemented with $CaCl_2$ (10 μM free Ca^{2+}) an additional decrease (by about 16% at 328 nm) of intrinsic fluorescence was observed, accompanied by an increase of fluorescence emission of the

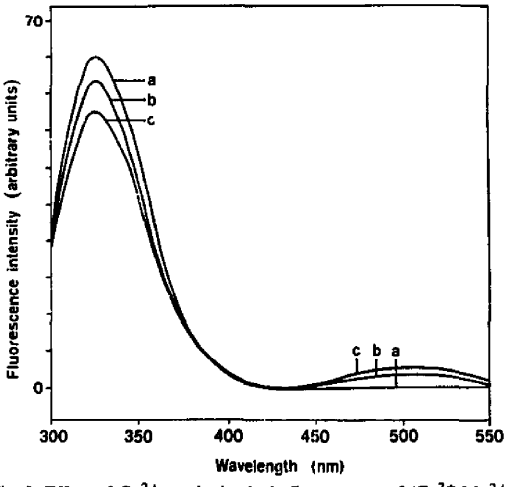

Fig. 3. Effect of Ca^{2+} on the intrinsic fluorescence of (Ca^{2+}-Mg^{2+})-ATPase in the presence of dansylated calmodulin. The spectra were recorded in the medium containing 0.1 mM EGTA (spectrum a), 0.1 mM EGTA and 0.18 μM dansylated CaM (spectrum b), and 10 μM free Ca^{2+} and 0.18 μM dansylated CaM (spectrum c). The spectra are representative for three independent experiments. The values were obtained after subtraction from the spectra of ATPase, the respective spectra of the basic medium and the medium with dansylated CaM, recorded in the presence or absence of Ca^{2+}.

dansyl derivative. Therefore, it is evident that fluorescence energy transfer between tryptophan residues of the enzyme and dansylated CaM could be detected either in the presence or absence of Ca^{2+}. It should be stressed that dansylated CaM was indistinguishable from the native compound in its ability to activate (Ca^{2+}-Mg^{2+})-ATPase, both in terms of the concentration required for half-maximal stimulation and the extent of maximal activation (not shown).

Discussion

Analysis of CD spectra of purified (Ca^{2+}-Mg^{2+})-ATPase of erythrocyte plasma membrane performed by Krebs et al. [7] provided information about conformational changes due to the E_1 and E_2 states of the enzyme. Surprisingly large differences in α-helical content of the enzyme in the E_1 and E_2 forms were observed. This may suggest that the E_1-E_2 transition is related to the refolding of the polypeptide backbone rather than only to local rearrangement of domains by a hinge-type or sliding motions within the ATPase molecule, which were postulated for Ca^{2+}-transporting ATPase of sarcoplasmic reticulum from fast-twitch muscle [22,23]. Such a difference between the two ATPases is unexpected, considering the similarities in their reaction mechanisms [1,2].

We have confirmed the observations of Krebs et al. [7] and found that the E_1 and E_2 forms of erythrocyte

(Ca^{2+}-Mg^{2+})-ATPase differ significantly with respect to their secondary structure (Fig. 1A, Table II). The discrepancy existing between observations made on Ca^{2+}-transporting ATPase of sarcoplasmic reticulum from fast-twitch muscle [22,23] and (Ca^{2+}-Mg^{2+})-ATPase of erythrocyte plasma membrane ([7] and this study) is probably associated with the content and distribution of tryptophan residues within the enzyme molecule. The isoenzymes of the Ca^{2+} pump of sarcoplasmic reticulum from fast-twitch and slow-twitch skeletal muscles contain 13 tryptophan residues [24,25]. Twelve of them are located in the hydrophobic fragment of the enzyme and assigned in the predicted secondary structure to membrane-spanning and stalk helices near the surface of lipid bilayer. One tryptophan residue (552) is assigned to the nucleotide-binding region of cytoplasmic domain. In case of (Ca^{2+}-Mg^{2+})-ATPase of erythrocyte plasma membrane, of total 7 tryptophan residues [3], one is located in the CaM-binding domain near C-terminus of the protein molecule [9]. This domain shares structural features common to other CaM-binding peptides and proteins [26]. The tryptophan residue in the ATP-binding domain of sarcoplasmic reticulum ATPase is, in erythrocyte plasma membrane ATPase, replaced by isoleucine [27].

Up to now, no studies have been done, using CD spectroscopy, on the conformational changes, which may occur upon interaction of CaM or PS with (Ca^{2+}-Mg^{2+})-ATPase of erythrocytes. By analysing the CD spectra, we have found that the addition of CaM in the presence of Ca^{2+} caused a decreased of α-helical content of (Ca^{2+}-Mg^{2+})-ATPase, as compared to the form of the enzyme in the presence of Ca^{2+} alone (Fig. 1B, Table II). Under these conditions (Ca^{2+}-Mg^{2+})-ATPase was fully active (Table I), what requires, according to Verma et al. [28], the binding of one CaM molecule per (Ca^{2+}-Mg^{2+})-ATPase molecule. In the presence of Ca^{2+} in the basic medium, PS also decreased the α-helix content of (Ca^{2+}-Mg^{2+})-ATPase, compared to the enzyme in the E_1 state (Fig. 1B, Table II). PS is known to be a potent activator of the solubilized ATPase of erythrocyte plasma membrane (Table I). On the other hand, binding of the enzyme to a CaM-Sepharose 4B-affinity column was observed in the presence of PS [16]. This leads to the suggestion that the mechanism of stimulation of the enzyme activity by CaM and PS is different. Measurements of CD spectra confirmed this presumption.

Unfortunately, CD measurements of plasma membrane (Ca^{2+}-Mg^{2+})-ATPase in the membrane-bound form are not possible. Thus, a comparison of the solubilized and the membranous forms of the plasma enzyme has not been provided so far. Moreover, the rearrangement of helical transmembrane segments of the (Na^+-K^+)-ATPase, upon transfer from the phospholipid bilayer into detergent micelles, has been reported by Gresalfi and Wallace [29]. The same observations were made for Ca^{2+}-transporting ATPase of sarcoplasmic reticulum [30]. This may suggest a similar behaviour of the erythrocyte enzyme.

Another approach to study conformational changes of (Ca^{2+}-Mg^{2+})-ATPase is provided by the method of quenching of the intrinsic fluorescence of tryptophan residues. In fact, studies on quenching of protein fluorescence by iodide ion, molecular oxygen or acrylamide have become quite popular in recent years (for review, see Ref. 31). Included in the large amount of information provided by this method are indications of the exposure and the microenvironment of tryptophan residues in the protein molecule [31]. Quenching, under the conditions employed in this study, did not affect the ATPase activity and did not show evidence of saturation, and it is therefore likely to be collisional [31]. We observed that the intensity of intrinsic fluorescence of (Ca^{2+}-Mg^{2+})-ATPase tryptophan residues was significantly reduced by acrylamide, KI and, to a much lesser extent, by CsCl (Fig. 2). These results were calculated according to the Stern-Volmer equation (Eqn. 1), which relates the drop in fluorescence (expressed as the F_0/F ratio) as a function of concentration of a collisional quencher (Q) as:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where K_{SV} is the Stern-Volmer constant for the collisional quenching. Note that the Stern-Volmer equation predicts a linear plot of F_0/F versus concentration of the quencher for homogenously emitting solutions [32]. The determined K_{SV} values for the quenching of intrinsic fluorescence (Table III) support the conclusion drawn from CD measurements that major changes in accessibility of tryptophan residues of (Ca^{2+}-Mg^{2+})-ATPase molecule are related to the shifting of the conformational equilibrium toward the E_1 or E_2 forms of the enzyme. The effect of CaM on quenching is probably associated with diminution of the accessibility for the quencher of the tryptophan residue located within the CaM-binding domain of the enzyme (which was identified as tryptophan 1107 in the Ca^{2+}-pumping enzymes homologous to erythrocyte ATPase such as isoform 1 from rat brain [33] and the ATPase from human teratoma [28]). In the case of PS, it probably affected the interaction of quenchers with other tryptophan residues located within the hydrophobic portion of erythrocyte (Ca^{2+}-Mg^{2+})-ATPase.

Fluorescence energy transfer studies permitted the assessment of the interaction of purified (Ca^{2+}-Mg^{2+})-ATPase with CaM. It was found, that transfer of energy between tryptophan residues of the enzyme and the dansyl derivative of CaM occurs both in the presence and absence of Ca^{2+} (Fig. 3). This may suggest that even in the presence of EGTA, CaM locates closely

to the tryptophan residue of the ATPase CaM-binding domain [34], and Ca^{2+} further promotes this interaction.

In summary, parallel CD and fluorescence studies have shown that the E_1-E_2 transition of purified (Ca^{2+}-Mg^{2+})-ATPase of erythrocyte plasma membrane involves a relatively major refolding of the polypeptide chain. The interaction of the enzyme with CaM or PS revealed the existence of additional conformational forms of Ca^{2+} pump which differ with respect to secondary structure from E_1 and E_2 states.

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