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Tryptophan fluorescence of sarcoplasmic reticulum ATPase. A fluorescence quench study

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The calcium-dependent change in the tryptophan fluorescence intensity of the sarcoplasmic reticulum Ca^{2+}- and Mg^{2+}-ATPase was investigated using different quenching reagents. It is demonstrated that only those compounds which are bound to the enzyme (i.e., 1-(9,10-dibromomyristoyl)-*sn*-2-glycerophosphorylcholine and 1-(9,10-dibromostearoyl)-*sn*-glycero-3-phosphorylcholine) are able to decrease the amplitude of the fluorescence decrement observed after removal of calcium ions. From the position of the bromine atom within the lysophosphatidylcholines, it is concluded that the tryptophan residues involved are located in the hydrophobic part of the ATPase molecule and are in contact with the hydrocarbon chains of the phospholipids.

Introduction

A decrease in the intrinsic tryptophan fluorescence intensity after removal of calcium ions from sarcoplasmic reticulum vesicles from fast skeletal muscle was first described by Dupont [1] and Dupont and Leigh [2]. Thereafter, several groups demonstrated that this fluorescence decrement is an intrinsic property of the calcium ATPase protein since it was also observed in completely solubilized and purified preparations [3–6]. In addition, a transient reduction of the fluorescence decrement at increasing amounts of detergents, such as dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$) or myristoyllyso-PC was found [4,5]. Using water-soluble and membrane-soluble quenching reagents, we now could characterize and localize the tryptophan residues involved in the calcium-dependent fluorescence decrement.

It is demonstrated that only quenching reagents which are bound to the ATPase protein are able to influence the fluorescence decrement and that the tryptophan residues involved are located in the hydrophobic part of the protein.

Materials and Methods

Br_2-myristoyllyso-PC and Br_2-stearoyllyso-PC were synthesized from *cis*-9-tetradecenoic acid and from oleic acid as described previously [7–10]. Both lysophosphatidylcholines gave a single spot on thin-layer chromatography. Sarcoplasmic reticulum vesicles from fast skeletal muscle were prepared according to Hasselbach and Makinose

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Abbreviations: myristoyllyso-PC, 1-myristoyl-*sn*-glycero-3-phosphorylcholine; Br_2-myristoyllyso-PC, 1-(9,10-dibromomyristoyl)-*sn*-glycero-3-phosphorylcholine; stearoyllyso-PC, 1-stearoyl-*sn*-glycero-3-phosphorylcholine; Br_2-stearoyllyso-PC, 1-(9,10-dibromostearoyl)-*sn*-glycero-3-phosphorylcholine; parinariclyso-PC, 1-((all *trans*)parinaric)-*sn*-glycero-3-phosphorylcholine; τ_ϕ, fluorescence lifetime measured by phase shift; τ_{mod}, fluorescence lifetime measured by modulation; Mops, 4-morpholinepropanesulfonic acid.

[11], as modified by De Meis and Hasselbach [12]. Protein concentration was determined by the Biuret method with Kjeldahl calibrated standards. The calcium-dependent ATPase activity was measured at room temperature in a buffer comprising 20 mM imidazole (pH 7.0)/40 mM NaCl/5 mM ATP/$2 \cdot 10^{-5}$ M X-537 A/5 mM $MgCl_2$/0.5 mM $CaCl_2$/0.45 mM EGTA/0.1 mg $\cdot$ ml^{-1} protein as described in Ref. 13, but ATP-splitting was stopped with an equal amount of 2% sodium dodecyl sulfate instead of 6% trichloroacetic acid [5]. Fluorescence measurements were carried out with a SLM 4800/A spectrofluorometer (SLM Instruments, Urbana, Il, U.S.A.). Excitation wavelength was 285 nm (slit 1 nm) and emission was detected using a WG 320 cut-off filter (Schott, Mainz, F.R.G.). The vigorously stirred reaction mixture comprised 20 mM Mops (pH 7.0)/80 mM KCl/5 mM $MgCl_2$/0.5 mM $CaCl_2$/0.45 mM EGTA/0.1 mg $\cdot$ ml^{-1} vesicular protein and the amount of quenching reagent given in the table and figures at 20°C. The decrease of fluorescence intensity on addition of 2 mM EGTA, which reduced the calcium concentration below 0.1 μM was recorded. In addition, the reversibility of the fluorescence decrement was checked by the readdition of calcium to give a final concentration of 2 mM [5,14]. Fluorescence lifetimes (τ_ϕ, lifetime measured by phase shift and τ_{mod}, fluorescence lifetime measured by modulation) were analyzed using SLM interface, programs and a 97S I/O Hewlett Packard calculator [15]. Parinariclyso-PC was a gift from Professor Dr. Egge, University of Bonn, F.R.G. All chemicals were pA grade and either from Serva, Heidelberg (F.R.G.), C. Roth, Karlsruhe (F.R.G.), E. Merck, Darmstadt (F.R.G.) or Sigma, St. Louis, MO (U.S.A.).

Results

The following idea was the basis for the experiments described below: If the tryptophan residues involved in the fluorescence decrement after removal of calcium ions from the calcium ATPase are located in the hydrophobic, water-inaccessible part of the enzyme, quenching of water-accessible tryptophan residues should increase the fluorescence decrement due to the decrease in total fluorescence intensity. Consequently, the fluorescence decrement should decrease if the hydrophobic tryptophan residues are quenched by membrane-soluble agents. An analogous reasoning would be valid if the tryptophan residues involved in the fluorescence decrement were accessible from the water phase. The results obtained with different water-soluble and membrane-soluble quenching reagents are summarized in Table I. It is evident that only Br_2-myristoyllyso-PC and Br_2-stearoyllyso-PC had an influence on the fluorescence decrement after removal of calcium ions. All other substances are ineffective, although in most cases the fluorescence is considerably quenched. This obviously is in contrast to the argument given above (see also Discussion). Therefore, the effects of brominated lysophosphatidylcholines were investigated in more detail and were compared with the results obtained with the analogous unbrominated lysophosphatidylcholines. Fig. 1 shows that increasing amounts of Br_2-stearoyllyso-PC reduce the fluorescence intensity to about 50%, whereas stearoyllyso-PC has no effect. But both Br_2-stearoyllyso-PC and stearoyllyso-PC abolished the fluorescence decrement at 1 mg/mg protein (Fig. 1B). A 50% reduction of the fluorescence decrement is obtained at 0.1 mg/mg protein of Br_2-stearoyllyso-PC and at 0.2 mg/mg protein of stearoyllyso-PC. Different results were obtained with Br_2-myristoyllyso-PC and myristoyllyso-PC (Fig. 2), which in contrast to stearoyllyso-PC are able to sustain ATPase activity (Refs. 5, 17 and Fig. 3). As previously described, myristoyllyso-PC shows a transient reduction of the fluorescence decrement after removal of calcium ions [4,5]. In contrast, using Br_2-myristoyllyso-PC the fluorescence decrement is not recovered at high amounts of lysophosphatidylcholine (> 0.6 mg/mg protein). This is not due to an inactivation of the enzyme, since ATPase activity of fully uncoupled sarcoplasmic reticulum vesicles (e.g., in the presence of ionophore X-537 A) exhibits the same dependence on Br_2-myristoyllyso-PC and on myristoyllyso-PC: i.e., a transient decrease at about 0.4 and 1 mg/mg protein, respectively, and a full reactivation at 2 mg/mg protein (Fig. 3).

The decrease in fluorescence intensity obtained with, e.g., Br_2-stearoyllyso-PC (50% at 2 mg/mg protein) is completely reversed by the addition of more than 5 mg/mg protein of myristoyllyso-PC.

TABLE I

DECREASE OF FLUORESCENCE INTENSITY AND CONCOMITANT INFLUENCE ON FLUORESCENCE DECREMENT AFTER REMOVAL OF CALCIUM IONS OF ALL QUENCHING REAGENTS TESTED

The following remarks describe some effects of the quenching agents used in this study which may be of general interest but will not be described in detail, because this would be beyond the scope of this study. [a] KJ has an adsorption at 285 nm which has to be taken into account for the determination of I_F/I_{F_0}. [b] Compare to Ref. 14. [c] After addition of the quenching agent, the fluorescence intensity first decreases and then slowly increases again to the upper level given in the table. No influence on the fluorescence decrement was observed during the period of increasing fluorescence intensity. [d] The fluorescence increase after the readdition of calcium (see Materials and Methods) is about twice as large as the fluorescence decrement after the addition of EGTA. [e] The same results were obtained at 35°C. [f] The effect of trypsin is included for comparison. During the incubation with trypsin, the ATPase protein is split in at least four fragments, but calcium still binds with high affinity [16].

Quenching reagent	Concentration	I_F/I_{F_0} *	ΔF(EGTA) ** (%)
None (control)	–	1.0	2.5 (4 ***)
KJ [a]	0.074 M	0.9	4.0 ***
KNO_3	0.074 M	0.5	4.0 ***
Acrylamide	0.6 M	0.2	2.5
Pyruvate	0.01 M	0.7	2.5
Trichloroacetic acid [b]	0.2 M	0.75	2.5
Bromobenzol [c]	0.02% (v/v)	0.6–0.8	2.5
Parinariclyso-PC [c]	0.25 mg/mg protein	0.3–0.5	2.5
Trifluoroacetamide [d]	0.5 M	0.7	2.5
Br_2-stearoyllyso-PC [e]	0.3 mg/mg protein	0.5	0
Br_2-myristoyllyso-PC	1.0 mg/mg protein	0.6	0.5
'Trypsin' [f]	1:10 (w/w), 1 h	–	2.5

* Relative fluorescence intensity before (I_{F_0}) and after (I_F) addition of the quenching agent.

** Relative fluorescence decrement after the addition of 2 mM EGTA to the reaction mixture containing 50 μM 'free' calcium and the given concentration of quenching reagent (see Materials and methods).

*** Fluorescence emission measured with a monochromator set at 330 nm instead of a cut-off filter.

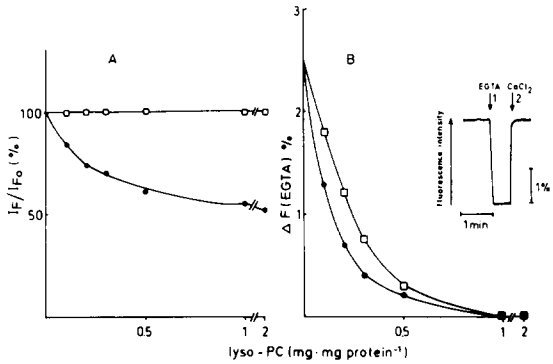

Fig. 1. Influence of stearoyllyso-PC and Br_2-stearoyllyso-PC on the fluorescence intensity and on the fluorescence decrement after removal of calcium ions from the sarcoplasmic reticulum membranes. (A) Increasing amounts of stearoyllyso-PC (□) or Br_2-stearoyllyso-PC (●) were added to a vesicle suspension (0.1 mg/ml protein) and the relative fluorescence intensity (I_F/I_{F0}, see Table I) was monitored as described in Materials and Methods. (B) Fluorescence decrement (ΔF(EGTA) after removal of calcium ions (final $pCa = 7.3$) was determined at increasing amounts of stearoyllyso-PC (□) or Br_2-stearoyllyso-PC (●), See Materials and Methods. Inset: Fluorescence intensity changes of native vesicles. At arrow 1, calcium ions were complexed by the addition of 2 mM EGTA and at arrow 2, $CaCl_2$ was readded to give a final concentration of 2 mM.

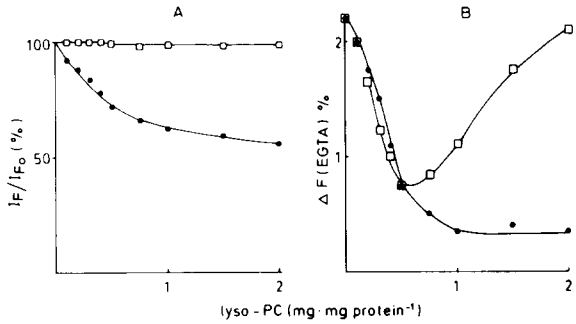

Fig. 2. Influence of myristoyllyso-PC and Br_2-myristoyllyso-PC on the fluorescence intensity and on the fluorescence decrement after removal of calcium ions. The experiment described in Fig. 1 was repeated using myristoyllyso-PC (□) and Br_2-myristoyllyso-PC (●).

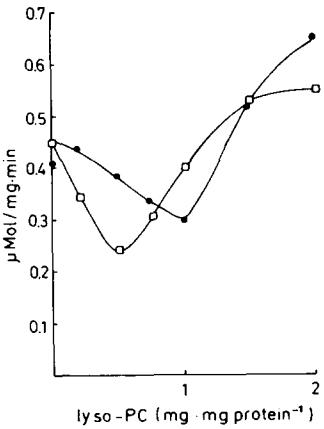

Fig. 3. Calcium-dependent ATPase activity determined at increasing amounts of myristoyllyso-PC and Br_2-myristoyllyso-PC. Calcium-dependent ATPase activity was measured as described in Materials and Methods. □, Myristoyllyso-PC; ●, Br_2-myristoyllyso-PC.

Unfortunately, this large amount of myristoyllyso-PC (as well as of all other commonly used detergents) abolish the fluorescence decrement obtained after the removal of calcium ions. Therefore, the reversibility of the effect of brominated lysophosphatidylcholines on the fluorescence decrement cannot be demonstrated, since there exists no other experimental approach to remove the brominated lysophosphatidylcholines from the protein.

To characterize further the difference between Br_2-myristoyllyso-PC and a quenching reagent which has no influence on the fluorescence decrement (i.e., KNO_3), the lifetime of tryptophan fluorescence of the ATPase protein was determined at increasing concentrations of quenching reagent. The results are shown in Fig. 4. τ_ϕ and τ_{mod} linearly decrease at increasing concentrations of KNO_3 (Fig. 4A) which is expected for dynamic quench. On the other hand, using Br_2-myristoyllyso-PC τ_ϕ and τ_{mod} first decrease with a slope of 0.7–0.5 ns/mg per mg protein until a concentration of 0.5 mg/mg protein is reached. Afterwards, τ_ϕ decreases with a slope of 0.3 ns/mg per mg protein whereas τ_{mod} remains constant. This may be interpreted as the result of a mixture of different types of quenching (see Refs. 18, 19 and Discussion).

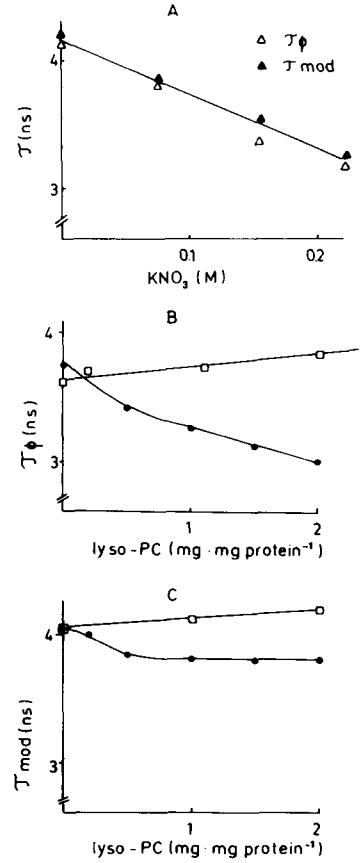

Fig. 4. Fluorescence lifetime at increasing amounts of quenching reagents. Fluorescence lifetimes were determined as described in Materials and Methods. All determinations have a standard deviation smaller than 0.1 ns. (A) Increasing amounts of KNO_3 were used. △, τ_ϕ; ▲, τ_{mod}. (B) τ_ϕ at increasing amounts of myristoyllyso-PC (□) or Br_2-myristoyllyso-PC (●). (C) τ_{mod} at increasing amounts of myristoyllyso-PC (□) or Br_2-myristoyllyso-PC (●).

Discussion

The results shown in Table I and Figs. 1–4 demonstrate that an influence of a quenching agent on the calcium-dependent fluorescence decrement is only observed if the reagent is able to bind to and to solubilize the ATPase protein, as it is the case for Br_2-myristoyllyso-PC and Br_2-stearoyllyso-PC. Therefore, the permanent close vicinity of the quenching reagent and the tryptophan residues involved is a prerequisite to decrease the fluorescence decrement. This is supported further by the

results shown in Fig. 4B and C, since the nonlinearity of the concentration dependence of τ is best explained by the existence of at least two populations of quenching molecules [18,19] differing in their quenching properties. It should be noted that in a recent fluorescence quench study by Simmonds et al. [20] two types of binding sites for brominated cholesterol and for oleic acid have been described. For an unknown reason, paranaiclyso-PC has no effect on the fluorescence decrement. But the fluorescence intensity after addition of this compound was not constant for more than 0.5 h and therefore no binding studies have been carried out (see legend to Table I). The fact that Br_2-stearoyllyso-PC and stearoyllyso-PC had about the same effect on the fluorescence decrement is best explained by the inability of stearoyllyso-PC to sustain ATPase activity [17]. The difference in the concentrations necessary for a 50% reduction of the fluorescence decrement using Br_2-stearoyllyso-PC and stearoyllyso-PC already indicated that the brominated lysophosphatidylcholine might decrease the fluorescence decrement. This effect is clearly demonstrated at high concentrations of Br_2-myristoyllyso-PC and myristoyllyso-PC, where full restoration of the fluorescence decrement was obtained with myristoyllyso-PC and a complete abolition using Br_2-myristoyllyso-PC. In contrast, both, myristoyllyso-PC and Br_2-myristoyllyso-PC were able to completely restore ATPase activity (Fig. 3). The minimal activity is not observed at the same concentration of myristoyllyso-PC and Br_2-myristoyllyso-PC if expressed as mg/mg protein. This is due to the difference in M_r and to differences in the solubilizing properties. Since lysophosphatidylcholines, as all other detergents, are tightly bound to the ATPase protein, the effect of the brominated lysophosphatidylcholines is most probably dependent on the lysophosphatidylcholine to protein and not to the lysophosphatidylcholine to lipid ratio. From the fact that only Br_2-stearoyllyso-PC and Br_2-myristoyllyso-PC have an influence on the fluorescence decrement after removal of calcium ions from the high-affinity binding sites, it is concluded that the tryptophan residues involved are located in the hydrophobic part of the enzyme and therefore are in contact with the phospholipid carbon chains of the membrane. This is in agreement with the conclusion of Pick and Racker [21] and Lüdi and Hasselbach [16] that the high-affinity binding sites for calcium are located in a hydrophobic part of the protein. On the other hand, Verjovski-Almeida [22] reported that the tryptophan residues quenched by the ionophore X-537 A are not involved in the fluorescence decrement after removal of calcium ions. This supports the interpretation given above, since it has been shown that in native vesicles, ionophore X-537 A is most probably not bound to the ATPase protein [23].

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