FLUOROMETRIC STUDY OF THE SOLUBILIZED Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM

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1. Introduction

The intensity of the tryptophan fluorescence of the Ca^{2+}-ATPase is modified by specific substrates of the protein (Ca^{2+}, ATP, P_i) [1–3]. In spite of the small amplitude of the fluorescence changes, relevant equilibrium and kinetic parameters were obtained from the study of the calcium–enzyme interaction. We have proposed a calcium binding mechanism which accounts for the rate of the fluorescence change [2]. A detailed study will appear in [4] for a larger pCa and pH range. The significance of these results is, however, questionned in [5]. The argument is based on the lack of effect of Ca^{2+} on the fluorescence of the Ca^{2+}-ATPase solubilized in detergent and on the observation of a very slow Ca^{2+}-induced modification of the UV spectrum. The results in [1–3] were all carried out with native sarcoplasmic reticulum (SR) vesicles. We have therefore repeated some of these measurements under very precise solubilization conditions and confirmed that the fluorescence change is an intrinsic property of the calcium pump protein in its soluble as well as in its membrane bound form.

2. Materials and methods

2.1. *Preparation of solubilized ATPase*

Sarcoplasmic reticulum is prepared from rabbit muscles and purified with a low concentration of deoxycholate as in [6]. Vesicle suspension, 0.5 ml (16 mg/ml) is clarified by mixing with 0.37 ml $C_{12}E_8$ (100 mg/ml) in 10 mM TES (pH 7.5), 100 mM KCl and 0.1 mM $CaCl_2$. The soluble fraction is applied to a Sepharose 6B column (1.5 × 90 cm). The elution solution contains 10 mM TES, 100 mM KCl and 0.1 mM $CaCl_2$ with or without 0.1 mg/ml Tween 80. Additional details of sample preparation are given in [7,8].

2.2. *ATPase activity measurement*

The Ca^{2+}-ATPase activity is measured with a Radiometer TT1 pH stat at 20°C in 1–2 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 5 mM Mg-ATP with 5–15 μg protein/ml. The total volume is 10 ml and the titrant 10 mM KOH.

2.3. *Fluorescence measurements*

The fluorescence is measured with a Durrum D 117 fluorimeter, modified to allow measurements in a standard 1 × 1 cm fluorescence cuvette. The solution is continuously stirred from the top of the cuvette and reagents are injected with Hamilton syringes in very small volumes (1–3 μl) to avoid dilution effects. The fluorescence is excited at 295 nm with a 75 W Xe lamp and the emitted light is filtered with a Corning 0.54 cut-off filter. Due to the wide band accepted in the photomultiplier the observed intensity change is slightly lower than the change measured with a monochromator at 325–330 nm [1]. This is, however, largely compensated by the higher light flux and the larger overall sensitivity of the apparatus.

Measurements are made in 50 mM MOPS (pH 7.2) 100 mM KCl, 5 mM Mg^{2+} at 20°C.

Abbreviations: $C_{12}E_8$, dodecyloctaoxyethylene glycol monoether; TES, *N*-tris(hydroxymethyl) methyl-2-aminomethane sulfonic acid; MOPS, morpholinopropane sulfonic acid; EGTA, ethylene glycol–bis (amino-2 ethylether), *N,N,N',N'*-tetraacetic acid; c.m.c., critical micellar concentration of detergents

Table 1
Effect of some non-ionic detergents on the amplitude of the Ca^{2+}-induced fluorescence change of the membrane form of the Ca^{2+}-ATPase

Detergent	SR protein conc. (mg/ml)	Detergent concentration for $[\Delta F/F] = 1/2([\Delta F/F]_o)$ $[C]_{1/2}$ mg/ml	(M)	Ca^{2+}-ATPase activity for $[C]_{1/2}$; 100 = native	Light scattering for $[C]_{1/2}$; 100 = native
C 12 E 8	0.20	0.026	(0.5×10^{-4})	100	90
	0.05	0.010	(0.2×10^{-4})	100	92
Amonix LO	0.17	0.050	(2×10^{-4})	65	100
Emulphogène	0.17	0.075	(1.2×10^{-4})	106	98
Tween 80	0.20	>5	$(>5 \times 10^{-3})$		

3. Results

3.1. *Effect of detergents on the fluorescence of the protein*

Effects of detergent on the Ca^{2+}-induced fluorescence change are shown in table 1. A very strong reduction of the fluorescence change is observed for relatively low concentrations of non-ionic detergents, well below the c.m.c. and before solubilization. The mild detergent Tween 80, however, only slightly affects the fluorescence change even at 5 mg/ml. This could be an indication of a weaker interaction with the protein and this result is of interest with regard to the solubilization technique used in [7]. In this procedure, Tween 80, which does not inactivate the Ca^{2+}-ATPase as other non-ionic detergents, was used to displace $C_{12}E_8$ from the solubilized protein. Indeed, results shown in table 2 clearly show that the Ca^{2+}-induced fluorescence change, which is completely quenched by $C_{12}E_8$ can be restored if $C_{12}E_8$ is displaced by high concentrations of Tween 80.

3.2. *Gel chromatography of $C_{12}E_8$ solubilized ATPase*

One conclusion of the above experiments is that, in the case of the solubilized form of the Ca^{2+}-ATPase, fluorescence measurements are possible only if the detergent $C_{12}E_8$ used for solubilization is eliminated during the chromatography or displaced by Tween 80. We have used the technique in [7,8] and the elution patterns obtained by chromatography with or without Tween 80 are shown in fig.1,2. In these two experiments $[^{14}C]C_{12}E_8$ is added to the solubilization medium so that the $C_{12}E_8$ concentration in the eluted fractions can be obtained precisely. The leading fractions of the protein peak are eluted in <10 μg/ml $C_{12}E_8$. The detergent is eliminated later, mostly as mixed phospholipid–detergent micelles.

The ATPase activity in the eluted fractions is measured with the techniques in section 2. The activity in the fractions eluted in the presence of 0.1 mg/ml Tween 80 are assayed in the same buffer and most of the activity is found in the leading edge of the protein peak (fig.2). The protein eluted in the absence of Tween 80 are essentially inactive if the ATPase activity is measured in the absence of added detergent. The activity is restored by the addition of $C_{12}E_8$ and with a much lower efficiency by Tween 80. Delipidated Ca^{2+}-pump has very little ATPase activity in the absence of added $C_{12}E_8$ [9]. Our data agree with [9] although the residual phospholipid content of our preparation is somewhat larger (0.15 g/g).

The ATPase activity of the proteins eluted in the

Table 2
Effect of C 12 E 8 on the fluorescence change and reactivation by Tween 80

SR proteins conc. (mg/ml)	C 12 E 8 conc. $[C]$ (μg/ml)	$\Delta F/F$ at $[C]$ (%)	$\Delta F/F$ after addition of 1 mg/ml Tween 80 (%)
0.2	0	4	3
0.2	50	1	3
0.2	125	<0.2	2.5

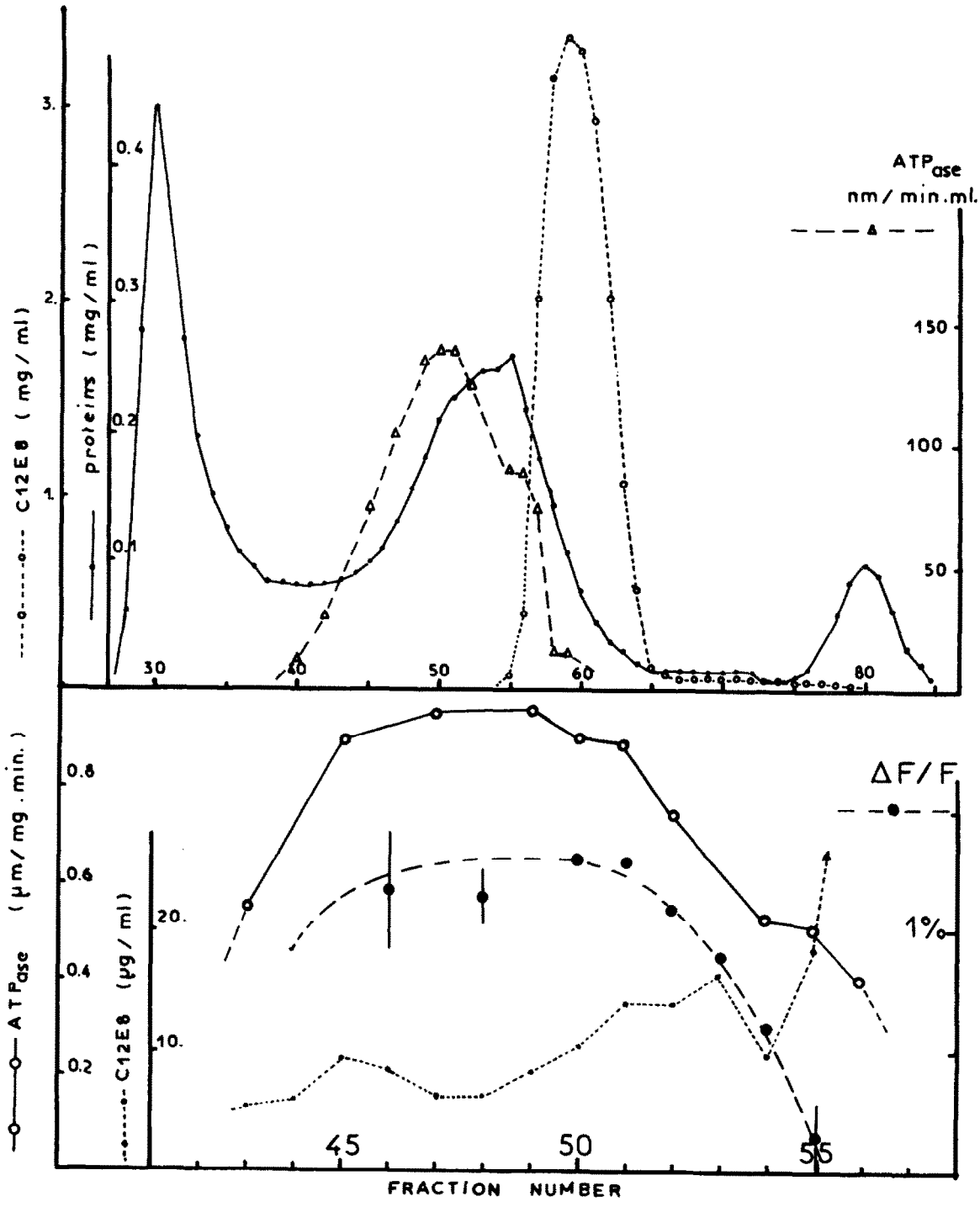

Fig.1. (Top) gel chromatography of $C_{12}E_8$ solubilized Ca^{2+}-ATPase in the absence of detergent in the elution buffer. (Bottom) An enlarged view of the protein elution peak. The average detergent to protein ratio in these fractions is 8 mol $C_{12}E_8$/mol ATPase (mol. wt 115 000). *Experimental details are given in section 2.*

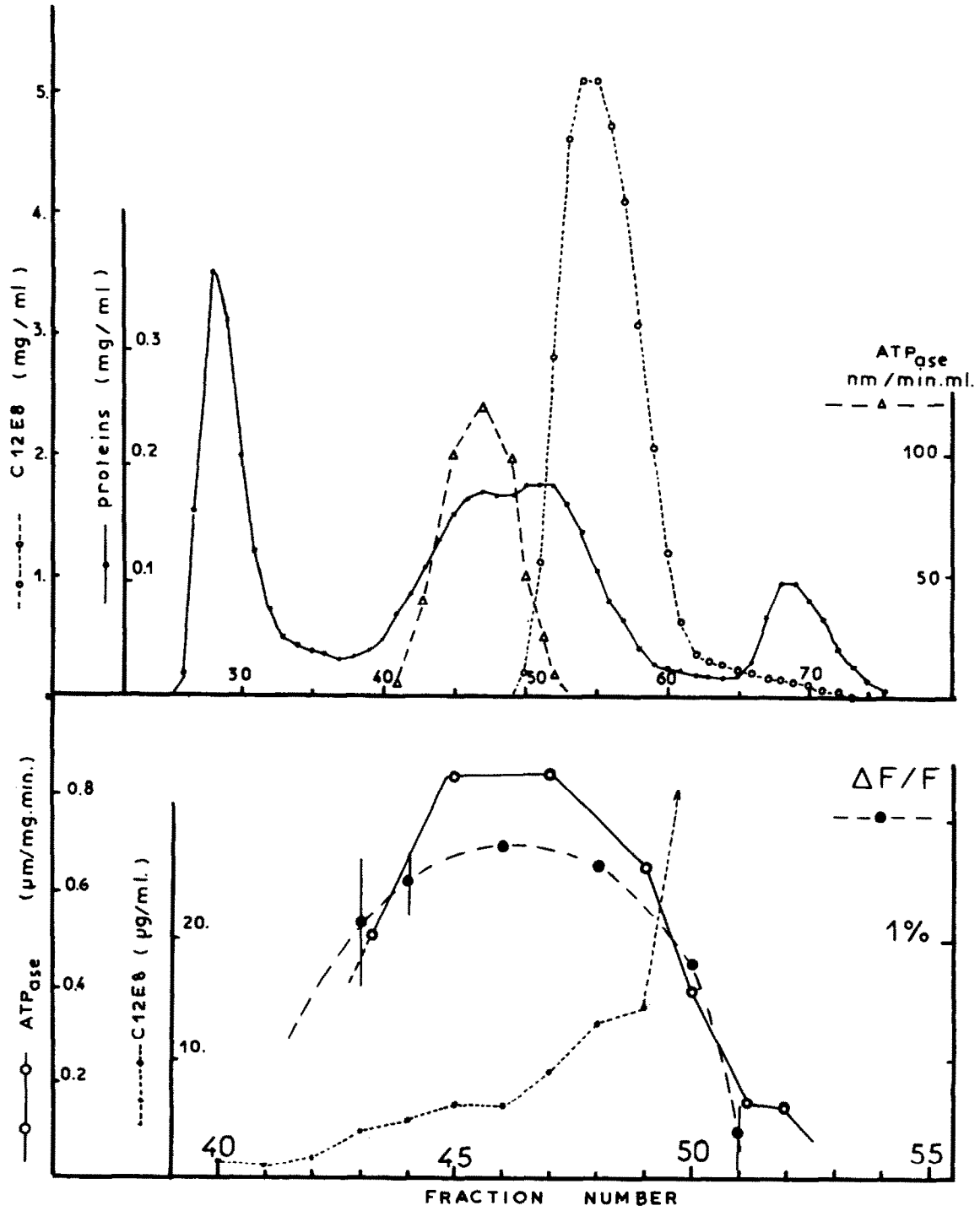

Fig.2. Same as fig.1 but with 0.1 mg Tween 80/ml in the elution buffer. In fraction 43–48 the average $C_{12}E_8$: protein ratio is 6.6 mol/mol.

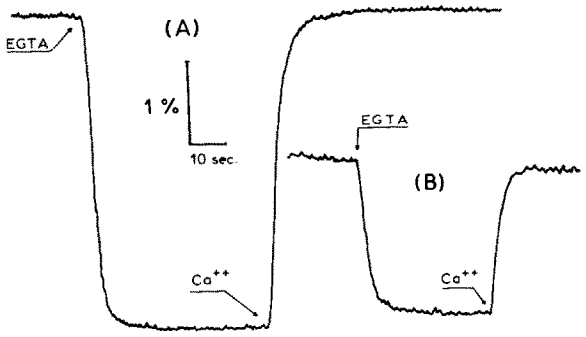

Fig.3. Fluorescence change of the Ca^{2+}-ATPase in vesicles (A) or of the solubilized form (B). Ca^{2+} before EGTA injection is 50 μM. Ca^{2+} and EGTA are injected as 3 μl sample to give final conc. 150 μM. The protein concentration is: 100 μg/ml (A) and 80 μg/ml (B). The integration time constant of the recorder is τ = 5 s.

absence of detergent is measured in the presence of 0.1 mg/ml $C_{12}E_8$ and results are shown in fig.1.

3.3. *Fluorescence change induced by calcium*

In membrane bound Ca^{2+}-ATPase binding of Ca^{2+} induces an intrinsic fluorescence change of ~4% (fig.3).

Under the conditions used for solubilization the protein fluorescence is completely insensitive to calcium but after elution through the column and elimination of most of the $C_{12}E_8$ the calcium sensitivity is partialy restored (fig.3).

Fluorescence changes of 1.2–1.8% have been obtained with no significant effect of the presence of Tween 80 in the elution buffer. One interesting result is the close correlation between the specific ATPase activity and the intensity of the fluorescence change of the soluble proteins (fig.1,2). ATPase activity and fluorescence change are found in the leading part of the protein peak. The trailing edge contains mainly inactive monomers [7,8] whose fluorescence is insensitive to calcium even when 1 mg/ml Tween 80 is added in an attempt to displace as much as possible the remaining $C_{12}E_8$.

4. Discussion

We have shown that the intrinsic fluorescence change induced by calcium in the Ca^{2+}-ATPase molecule is completely quenched by low concentrations of non-ionic detergents like $C_{12}E_8$. Therefore fluorometric studies of solubilized ATPase are only possible if the detergent used for solubilization is eliminated. The simplest interpretation of the calcium-induced fluorescence changes is that binding of Ca^{2+} induces a conformation change which exposes one or more tryptophan residue to environments of different polarity. A plausible explanation for the effect of detergents is that this change is completely screened by the binding of detergent molecules.

It is also not easy to give a precise meaning to the low value of the fluorescence change observed for the solubilized ATPase (1/3–1/2 of native). Very small amount of detergent bound to the solubilized protein may alter the fluorescence change. The observed effect of detergents cannot simply be extrapolated from the membrane bound protein to its soluble form because of the considerable difference in the amount of phospholipids per ATPase molecule in the two cases.

It is however a clear conclusion of this work that the tryptophan fluorescence change observed for membrane bound or solubilized Ca^{2+}-ATPase upon Ca^{2+} binding is an intrinsic property of the protein and is not due to a structural change induced by calcium on the membrane as proposed [5]. The main argument in [5], the absence of fluorescence change in solubilized form, is clearly disproved here.

We have shown that the concentration of detergent is very critical. Since no data on detergent binding are presented in [5], it is possible that $C_{12}E_8$ used for solubilization is not sufficiently eliminated during their solubilization and purification procedure [10] which is a modification of the technique in [7].

Ca^{2+} were shown to produce a change in UV absorbtion [5]; this change is interpreted as a protein conformation change induced by the binding of Ca^{2+} to the Ca^{2+}-ATPase. It was concluded [5], however, that it is not related to the activity of the pump since other substrates like P_i or ATP have no effect and since the absorbance change follows a very slow time course ($\simeq$0.1 min^{-1}).

The fluorescence change that we have described has certainly nothing to do with the UV absorbtion change observed in [5]. The rate of the fluorescence change induced by Ca^{2+} at 20°C is much higher: k_{on} = 5–10 s^{-1}, k_{off} = 15–30 s^{-1}, [2,4] and the transition can also be observed with other substrates like ATP (unpublished), P_i [3] or during the turnover of the protein [2].

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