SPIN LABELLED SARCOPLASMIC RETICULUM VESICLES: Ca^{2+}-INDUCED SPECTRAL CHANGES

Philippe CHAMPEIL, Françoise BASTIDE, Christiane TAUPIN* and Claude M. GARY-BOBO
Laboratoire de Physiologie Cellulaire, Collège de France, Paris, France and
**Laboratoire de Physique de la Matière Condensée, Collège de France, Paris, France*

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1. Introduction

A growing number of reports dealing with transport ATPases, either ($Na^+ + K^+$)-activated or Ca^{2+}-activated Mg^{2+}-dependent ATPase, support the hypothesis that reactivity of their active center for phosphorylation is controlled by the bound cations [1–5]. To investigate this hypothesis we prepared rabbit skeletal muscle sarcoplasmic reticulum fragments [6–8], since these membranes are easily isolated as vesicles in which a Ca^{2+}-dependent ATPase is the major protein. We labelled these vesicles with a paramagnetic iodoacetamide analog [9,10], and found that the bound label exhibits an e.s.r. spectrum which is insensitive to magnesium but sensitive to calcium concentration. The spectral change occurred between 1 and 3 μM at pH 8.0 and 4°C; when the same metal buffers were used to measure the K_M of formation of the phosphorylated intermediate, the value of 1.3 μM was found. We discuss the possibility that this Ca^{2+} binding-induced spectral change indeed reveals a conformational change of the ATPase molecule upon Ca binding.

2. Materials and methods

Sarcoplasmic reticulum vesicles were prepared and tested according to the method of Meissner and Fleischer [7]. They were incubated with the iodoacetamide spin label (ISL)** in the following medium: 0.25 M sucrose, 0.1 M KCl, 10 mM histidine buffer, pH 7, 4°C. They were extensively dialyzed afterwards against 0.1 M KCl, 5 mM Tris maleate buffer at pH 7, 4°C; then they were stored in liquid nitrogen. Spectra were recorded with a Varian E9 ESR spectrometer equipped with the variable temperature accessory.

** ISL: *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-iodoacetamide (Synvar).

3. Results and discussion

Various intensities of labelling can be obtained by a proper choice of incubation time (from 10 min up to 20 h) and initial label/protein ratio (from 3 up to 9 mol of ISL per 10^5 g protein). In these conditions however, no more than 1–2 SH groups per 10^5 g protein react with the label, and phosphoprotein formation is not affected.

The spectra that can be recorded after a thorough dialysis of the unbound label (see for instance fig.1), are typical of a number of protein bound spin labels [9–11]. They include so called 'strongly' (i) and 'weakly' (ii) (see arrows) immobilized signals. The relative proportion of component (ii) to component (i) increases as a function of incubation time or of initial label/protein ratio: we conclude that the label may react with more than one type of SH groups: type I SH groups react first and the labels bound to these sites are 'strongly immobilized', contributing to component (i); type II SH groups react slower with labels which then contribute to component (ii).

Fig.1 shows that the presence of 5 mM $CaCl_2$, in contrast to the condition in which all Ca^{2+} ions are chelated, slightly but definetely affects the spectrum

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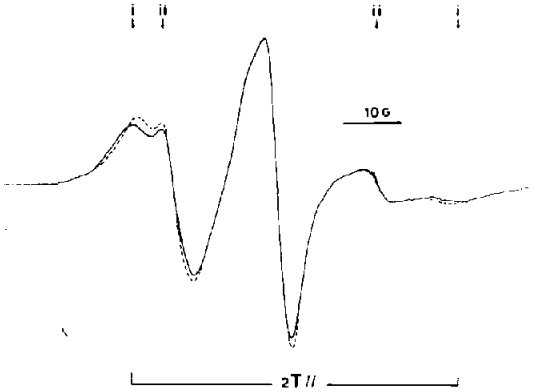

Fig.1. E.s.r. spectra of labelled sarcoplasmic reticulum vesicles. Conditions of labelling were 3 mol/10^5 g protein, incubation time 10 minutes (see methods). The labelled protein is 15 mg/ml in 0.1 M KCl, 50 mM Tris-maleate, 0°C, pH 8; $MgCl_2$ = 5 mM; total volume 50 µl; (———) EGTA = 20 mM. (—) $CaCl_2$ = 5 mM. The spectra were recorded with the following settings: 1.6 G modulation amplitude, 10 mW power, $4 \cdot 10^3$ gain, 1 s time constant, 100 G/8 min field scan rate. Arrows (i) and (ii) refer to 'strongly' and 'weakly' immobilized components. The splitting 2T// is measured after increasing the gain in the appropriate field regions (not shown).

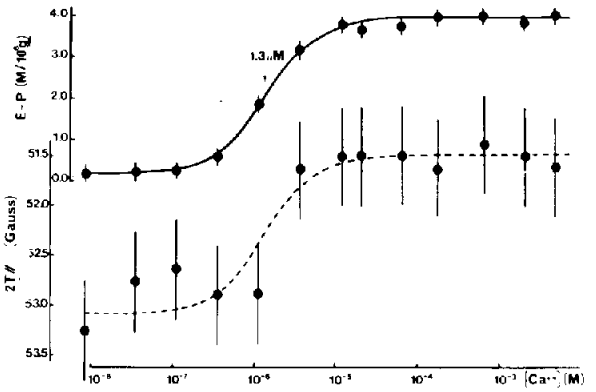

Fig.2. (Upper curve) Dependence of the amount of phosphorylated protein on Ca^{2+} ion concentration. Phosphoprotein was measured in 0.1 M KCl, 50 mM Tris-maleate, on ice, pH 8.0, 10 µM ATP, added $MgCl_2$, $CaCl_2$, and EGTA or EDTA calculated to give a free Mg^{2+} of 5 mM and the indicated free Ca^{2+} [13]. 15 s after adding the radioactive [γ-^{32}P]ATP the reaction was quenched with TCA and carrier ATP and phosphate; the mixture was filtered on a Millipore filter and the filter rinsed with 20 ml TCA 1%, P_i 25 mM, then counted. (Lower curve) Dependence on Ca^{2+} ion concentration of the measured splitting 2 T// of the bound label. Conditions were as depicted in fig.1. The same metal buffers as in the upper curve were used. The phosphorylation (upper curve) has been superimposed on the experimental points as a dotted line to allow comparison between the two sets of data.

of these spin labelled reticulum fragments. The presence of $MgCl_2$ (0–5 mM), on the contrary, does not affect either spectrum and the change in spectrum occurs equally in the presence of 5 mM $MgCl_2$ or with no added $MgCl_2$ (i.e. with 'endogenous' Mg^{2+} *). This spectral change occurs irrespective of the labelling conditions and final component (i)/component (ii) ratio, suggesting that this spectral change is a property of type I SH groups. Therefore, optimal labelling conditions for its precise characterization are attained by incubating ISL and protein for a short time at a low label/protein ratio, since this results in a spectrum in which component (ii) minimally interferes with the measurement of the component (i) splitting 2T// (see fig.1). This parameter, which is a much more significant index [12] than the commonly used ratio of the two low field peaks of the spectrum, changes from 53.1 ± 0.5 G (with all Ca^{2+} ions chelated) to 51.5 ± 0.5 G (5 mM Ca^{2+} in solution).

Fig.2 shows how the spectrum is modified as a function of free Ca^{2+} concentration, taking the splitting 2T// as an index. We prepared [13] EGTA and EDTA buffers to cover the whole Ca^{2+} range, with a constant free Mg^{2+} concentration of 5 mM. Total Ca^{2+} was fixed at 5 mM and high chelator concentrations were used, in order to buffer effectively the endogenous calcium, which is particularly important with the high protein concentrations used. The spectral change clearly occurs between 1 and 3 µM. A K_M however could not be deduced because the precision of peak position determination is limited to ± 0.5 G, due to a low label concentration and resulting background noise in the spectrum. However, these remarkably low free calcium concentrations suggest that the Ca^{2+}-sensitive label is indeed

* A set of Ca^{2+}-buffers were tested with and without added Mg^{2+} (endogenous Mg^{2+}, or free Mg^{2+} = 5 mM). A medium free from Ca^{2+} and Mg^{2+} was tested (i.e. with EDTA present, no added Mg^{2+} or Ca^{2+}). No test was done both in the presence of Ca^{2+} and in the absence of Mg^{2+}.

bound to a protein with a Ca^{2+} site of very high affinity.

Besides the ATPase, one of the acidic proteins of the reticulum vesicles has been described as also having a high affinity site, with a K_M of 3 μM [14]. This acidic protein however accounts for at most 10% of the vesicular proteins [8,15] and since it is considered [8,16] to be interior to the vesicles it should be labelled less rapidly than the outward facing ATPase part, (whereas Ca^{2+}-sensitive type I SH groups are labelled first). Furthermore, if the label were bound to the high affinity Ca^{2+} binding extrinsic protein, it should be extractable using suitable procedures. We treated labelled vesicles with the alcaline EDTA medium described by Duggan and Martonosi [17]. After centrifugation the supernatant contained 5% of the total proteins, but less than 1% of the original e.s.r. signal: this result precludes any preferential labelling of this fraction. The same conclusion was reached using the deoxycholate treatment of McLennan [14,18]. Thus it seems likely that the label is not bound to an extrinsic protein, but indeed to the Ca^{2+}-ATPase.

This conclusion is supported by an experiment where, in view of the discrepancies in Ca^{2+}-EGTA dissociation constants used by different authors – which make precise comparisons difficult – we decided to use the same set of metal buffers to determine a K_M for formation of the phosphorylated intermediate with the labelled enzyme. As shown in fig.2, reasonable agreement between the spectral and biochemical data was found. The spectral transition would therefore correspond to the binding of Ca^{2+} at its activating site on the enzyme.

Considering that Ca^{2+} binding on S.R. vesicles *decreases* ISL splitting and thus cannot act by a mere steric hindrance (which would increase label immobilization), it may appear as an attractive hypothesis that this Ca^{2+} binding-induced spectral change reveals a Ca^{2+} binding-induced protein conformational change (at least, in the neighbourhood of one SH group).

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