Calcium translocation mechanism in sarcoplasmic reticulum vesicles, deduced from location studies of protein-bound spin labels

(transport/adenosinetriphosphatase/spin exchange)

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ABSTRACT Sarcoplasmic reticulum vesicles were exposed to various thiol-directed spin labels, and the position of the label on the inner or outer vesicle surface was investigated as a function of the ATPase (adenosinetriphosphatase; ATP phosphohydrolase, EC 3.6.1.3) chemical state. Previous measurements of label accessibility to externally added ascorbate had been considered to suggest an external—internal transition of protein-bound labels, coupled with ion translocation [Tonomura, Y. & Morales, M. F. (1974) Proc. Natl. Acad. Sci. USA 71, 3857–3861]. We show that these ascorbate studies do not lead to convincing conclusions. We demonstrate, on the contrary, that transition ions (nickel and ferricyanide) can be used as selective line-broadening agents for the signals arising from external labels. No significant difference in nickel- or ferricyanide-label interaction can be attributed to a different orientation of the label in any of the enzyme chemical states tested. Our results therefore contradict the current interpretation of ascorbate quenching experiments in terms of calcium ATPase rotary motion; rather they are consistent with ion transport models involving only limited conformational rearrangements of the pump.

Two main classes of model for the mechanism of ion translocation through membranes by intrinsic proteins exist: (a) the rotary-carrier model, in which the ion binding site is carried from one side of the membrane to the other as a result of the general reorientation of the transporting protein (1), and (b) the fixed pore model, in which the enzyme forms a channel within which a cation binding site becomes alternately accessible to the inner and outer aqueous phases through minimal conformational changes in the polypeptide chain (2–4). Further studies with proton pumping systems have led to pump models in which a water-soluble catalytic unit is gating a hydrophobic channel-forming polypeptide. Although for thermodynamic reasons the first model has been judged less plausible (4) and is not easily reconciled with the results of immunology experiments (5–9), chemical modification experiments by Tonomura and coworkers (10–12) apparently support the hypothesis that in sarcoplasmic reticulum (SR) fragments Ca2+-ATPase (adenosinetriphosphatase; ATP phosphohydrolase, EC 3.6.1.3) functions by rotary molecular motion.

Tonomura and Morales (10) tried to reveal how the accessibility of the ATPase sulfhydryl groups to the external aqueous medium changed with the changing chemical state of the enzyme. They exposed the protein to a spin-labeled maleimide derivative and then added ascorbate externally to deduce the position of the label on the inner or outer vesicle surface from its measured reduction rate. Kinetic analysis of the quenching profiles of the bound label’s electron spin resonance (ESR) spectrum revealed two distinct classes: fast-reacting externally located labels and slow-reacting labels, believed to be internal. The ratio of slowly-to-rapidly quenched fractions changed when the spin-labeled enzyme-containing medium was altered.

A simple rotatory model was then designed to account for the changed distribution of the spin labels rotated internally after formation of the intermediate enzyme–ATP and phosphorylated enzyme complexes.

However, in the experiments of Tonomura and Morales, the vesicles were probably permeable to ascorbate (as recognized by the authors) due to lengthy incubation at pH 8.5 during labeling. Yet a crucial requirement of the original ascorbate quenching method (14) was that membranes had to be impermeable to the quenching agent to allow discrimination between internal and external signals. We attempted to meet this requirement by labeling the vesicles for a short time at neutral pH. Even under these conditions, we found that ascorbate still entered the vesicles at a significant rate. We therefore worked out another way of distinguishing internal from external signals by adding line-broadening transition ions like nickel or ferricyanide, which are considered nonpenetrating (15–19). We were then able to show that no internal rotation of labels can be detected on protein phosphorylation, the step coupled with calcium translocation (20–26).

MATERIALS AND METHODS

SR vesicles were prepared and tested as already described (27, 28). SR labeling was performed by vesicle incubation for 2–3 hr with the following maleimide derivatives: 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl (label I), 3-maleimido-2,2,5,5-tetramethyl-1-pyrroldinylnoxy (label II), 3-[2-maleimidoethyl]carbamoyl-2,2,5,5-tetramethyl-1-pyrroldinylnoxy (label III), and 3-[2-(maleimidoethyl)oxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrroldinylnoxy (label IV) (Syva, Palo Alto, CA). The medium contained 240 mM sucrose, 80 mM KC1, 25 mM morpholinoethanesulfonic acid (Mops) at pH 7 and 0°C, and was used throughout this study; incubation was followed by exhaustive dialysis against the same medium.

X-band ESR spectra were recorded initially with a Varian E9 (Laboratoire de Physique de la Matière Condensée, Collège de France, Paris) and later with a Bruker ER 200 tt ESR spectrometer equipped with a variable temperature accessory. Capillary tubes (50 μl) were positioned in the cavity as reproducibly as possible. Spectrum recording (200 sec) started 1–1.5 min after addition of quenching agent.

Permeability measurements were performed as described

Abbreviations: SR, sarcoplasmic reticulum; ESR, electron spin resonance; spin label I, 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl; spin label II, 3-maleimido-2,2,5,5-tetramethyl-1-pyrroldinylnoxy; spin label III, 3-[2-(maleimidoethyl)carbamoyl]-2,2,5,5-tetramethyl-1-pyrroldinylnoxy; spin label IV, 3-[2-(maleimidoethyl)oxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrroldinylnoxy; tempocholine, 4-[(N-[2-hydroxyethyl]-(N,N-dimethylammonium)-2,2,6,6-tetramethylpiperidine-1-oxyl].

* The authors did not actually state that their vesicles were labeled at pH 8.5, but this can be inferred from their reference to Nakamura's results (inhibition of ATPase but not of phosphorylated enzyme in the presence or absence of ATP) (13).
(29) by diluting 1:200 in unlabeled medium aliquots of the incubation or loading medium (standard sucrose buffer plus appropriate radioactive addition; 20 mg of protein per ml). Samples (1 ml) were filtered on a Millipore filter (0.45 μm pore size) and then quickly rinsed.

RESULTS

Vesicle Labeling. Vesicles were incubated with maleimide labels in our standard sucrose buffer (pH 7 and 0°C) at label and SR protein concentrations of 1.25 mM and 25–32 mg/ml, respectively—i.e., 4–5 mol of label per 10^9 g of protein. The extent of labeling was monitored by the drop in amplitude of the free label signal, as described earlier (28). Around 0.7 mol of SH groups per 10^9 g of protein reacted with label I during the first minutes, and 1–1.5 mol of slower groups were labeled in the next 2–3 hr. A smaller number of fast-acting groups was detected when 1 mol of N-ethylmaleimide per 10^9 g of protein was incubated with the vesicles for 1 hr before label addition; the resulting bound label then displayed a more strongly “impermeabilized” spectrum. Labeling decreased vesicle ATPase activity (by less than 50% for 2–3 hr of incubation); phosphoenzyme formation was even less affected than was hydrolytic activity or calcium transport (30). These results are in agreement with reported values (10, 13, 31, 32).

The various five-membered maleimide derivatives (labels II–IV) also reacted with both fast and slow reacting groups. The very similar labeling patterns observed (data not shown) suggested identical binding sites. The ATPase activity of the SR suspension modified with label III was 50% inhibited; inhibition after SR reaction with label II was not tested. On the other hand, activity was completely unaltered, within experimental errors, after SR reaction with label III was 50% inhibited; inhibition expressed as percentage of initial value, which corresponded to this experiment to 1.5 μl of internal volume per mg. Radioactivity retained by the filter alone was subtracted. (C) Vesicles (20 mg/ml) were incubated with 200 mM labeled nickel at 20°C in the sucrose buffer. Aliquots were diluted and filtered at various times and radioactivity remaining on the rinsed filter was assayed. Nickel retained by the filter in a vesicle-free sample was subtracted. Irreversible passive binding of nickel to vesicles (shaded area) was tested by similarly incubating the vesicles for 10 min in unlabeled nickel medium, and adding radioactive tracer 1 min before dilution and filtration, as in ref. 16. Nickel cpm were expressed as percentage of total cpm in the sample.

The slow protected signal decrease could either be due to broadening agent entry into the vesicles or to transition ion-induced tempocholine release, or to both. We examined the effect of nickel on vesicle permeability to sucrose (Fig. 1B). We found that addition of 200 mM nickel at 20°C did accelerate radioactive sucrose release from previously loaded vesicles. This nickel-induced faster sucrose release is probably related to the previously recognized fact that nickel treatment tends to promote vesicle clumping (16). Nevertheless, additional data suggested that virtually no nickel penetrated into the vesicles during incubation for 1–1.5 hr (16). We repeated the nickel entry experiment (Fig. 1C) and also found negligible radioactive nickel counts in the vesicles after a long incubation period, probably because of the nickel-induced modification of vesicle surface properties. Over shorter periods, on the other hand, we were able to record nickel radioactivity in the vesicles.

Nickel and probably ferricyanide are therefore not completely impermeable agents. However, our results make clear that they can be used to discriminate between external and internal signals in the first few minutes after their addition to the vesicle suspension. For instance, 3 min after the addition of 200 mM nickel at 20°C, 25% of the vesicles in the worst case were disrupted (Fig. 1B). Starting with an internal volume of
1.5 μl/mg of protein (i.e., 3% of total volume is internal at 20 mg/ml), the nickel concentration in the intravesicular space is at most 1/4 of the external concentration (Fig. 1C), and the line broadening of any internal signal is also only 1/4 of external signal line broadening. When nickel is added at 0°C, its internal concentration after 3 min must necessarily be still lower than that at 20°C, due to the lower nickel entry rate. For instance, Fig. 1A shows that more than 85% of the internal tempocholine signal is still apparent after 3 min.

Vesicle permeability to ascorbate at 0°C was demonstrated by the same techniques. When tempocholine-loaded vesicles (20 mg/ml) were exposed to an ascorbate concentration high enough to reduce all accessible labels during the first minute (20 mM), the vesicle-protected labels (unreduced signal at, say, 2 min minus the unreduced signal in a vesicle-free system or in a vesicle suspension previously solubilized with NaDodSO4 or Triton) comprised only 0.3–0.4% of the original tempocholine signal. Only when the ascorbate concentration was decreased to 2 mM could we demonstrate that the difference between the unreduced signal in the complete system (two compartments) and the unreduced signal in the reference system (one compartment) amounted to a few percent. This unreduced differential signal itself decreased by half in 3–5 min; independent permeability measurements with radioactive ascorbate showed that at 4°C ascorbate equilibrated across the vesicular membrane with a time constant around 0.4 min⁻¹.

Transition Ion-Induced Line Broadening. Collision-dependent line broadening of a given spin label by a transition ion decreases the amplitude of the label’s first derivative absorption line to an extent depending on the initial width of the resonance line. Further, in a multicompartiment system like our vesicle suspensions, line-broadening by nickel does not extend beyond the interface zone. As an example of this, we performed a series of experiments with N-oxyl-4,4'-dimethoxazolidine derivatives of stearic acid [I’(m, n)]. At 0°C, we found that addition of 0.2–0.7 M nickel to vesicles labeled with I(3, 12) decreased by 36–64% the height of this relatively water-accessible but highly immobilized label (central linewidth around 5 G). Under the same conditions, these high nickel concentrations almost completely removed the signal originating from a typical soluble label like tempocholine (linewidth around 1–1.5 G). On the other hand, the relatively narrow spectrum (central linewidth around 3G) of vesicles labeled with I(1, 14) was nearly unaffected by nickel addition, probably because the nitroxide group in this fatty acid derivative is located within the core of the lipid leaflet.

The ESR spectrum of SR labeled with label I is shown in Fig. 2, together with the effect of 200 mM nickel addition at 0°C. Under the same conditions, the addition of nickel decreased the signal amplitude of unbound maleimide spin label to less than 4% of its original value. The different quenching efficiencies of nickel for bound or free spin labels might be due either to the broad linewidth of bound label signal, or to the relative inaccessibility of the possibly deeply buried membrane label, or to both. However, in order to test the hypothetical inward reorientation of some of the labels during pump activity, it is highly desirable for apparent quenching of the external signal in the ground state to be as complete as possible. We therefore decided to prepare vesicles labeled with maleimide derivatives of increasing arm length between maleimide and nitroxide in order to increase both the motional freedom and water accessibility of the nitroxide group. Fig. 3 shows the ESR spectra for these SR-bound labels, together with the resulting spectra after addition of 200 mM nickel at 20°C. All three labels displayed composite signals, the "less strongly immobilized" one being more efficiently quenched than the "more immobilized" one. Due to a narrower resonance line and possibly to better water accessibility, the long-armed label IV was very efficiently quenched by nickel addition. The same results were obtained when ferricyanide was used as a line-broadening agent (data not shown).

Facing Direction of Bound Labels. From the above results, we can now turn to the experiments directly connected with our initial aim of locating the SR-bound labels on the inner or outer vesicle surface.

We began by investigating label positions in the ground state (absence of substrate) as follows: (i) In the event that some labels are on the inner vesicle surface, the permeability characteristics described above (Fig. 1) would allow most of their signals to remain visible on the first recordings, made 2–3 min after nickel addition, and then they would eventually disappear along with nickel penetration. However, in practice, the resulting signal
after nickel addition was perfectly stable for all four maleimide labels tested. For instance, the SR-bound label I residual signal was monitored from 2 min up to 1.5 hr after the addition of 0.2 M nickel at 0°C without showing any drop in amplitude; the same applied when the label II residual signal was monitored at 20°C for up to 1 hr. (ii) After the addition of 200 mM nickel at 0°C, vesicles labeled with label IV displayed a residual spectrum that was found insensitive to previous vesicle solubilization by Triton. (iii) The signal arising from this SR-bound label IV could be quenched to very low values (addition of 200 mM or 400 mM nickel at 20°C left only 12% or 8%, respectively, residual amplitude), suggesting that most of the label was accessible to the outer phase. From these three results, we conclude that labeled SR groups do not reside on the inner surface of the vesicles.

Next, we tested whether nickel or ferricyanide quenching was dependent on the ATPase chemical state. We concentrated on the phosphorylation step, and therefore added to our labeled vesicles an ATP-containing medium with a high calcium concentration in order to ensure full phosphorylation but a decreased dephosphorylation rate and hence minimum ATP hydrolysis (35). We checked that, at 0°C in 10 mM Mg2+/5 mM Ca2+/7.5 mM ATP at pH 7, the ATP turnover (<0.01 μmol per mg/min), was slow enough to allow recording of several ESR scans with minimal ATP hydrolysis, even at protein concentrations as high as 6 mg/ml. We also tested the effect of nickel or ferricyanide on the phosphorylation level in the same medium (except that the ATP concentration was 12.5 μM) and found that 50 mM ferricyanide had virtually no effect, nor had 200 mM nickel, when added after the ATP, as in our experiments. After checking these points, we compared the residual ESR quenched signals obtained when the medium contained either 5 mM Ca2+ plus 7.5 mM ATP, or 5 mM Ca2+ alone, or no exogenous calcium (endogenous calcium was, however, present). By using 200 mM nickel at 0°C (i.e., conditions allowing the first spectra to be recorded with minimum nickel penetration), we could not detect any modification in residual signal amplitude with any of our four types of SR-bound label. The same result was obtained by quenching label IV with 50 mM ferricyanide. Specific experimental conditions, namely less (50 mM) nickel added at 20°C, apparently produced a residual signal 9% higher when nickel was added in the presence of ATP. This can be most simply explained in terms of decreased quenching efficiency of the nickel–ATP complex versus nickel alone (see, for instance, decreased efficiency of Ni2+ and EDTA versus Ni2+ alone, in ref. 18). We therefore conclude that labels do not undergo any inward reorientation on phosphorylation of the Ca2+ pump.

We also tried to derive this conclusion from chemical quenching studies at 0°C. However, in the absence of vesicles, we found that 2 mM ascorbate reduced label I with a rate constant (0.3 min−1) comparable to the previously measured equilibration rate of ascorbate across vesicle membrane (around 0.4 min−1). From this result it appeared to us impossible to demonstrate that protein-bound labels quenched either at the same rate as free label or at a much lower rate, respectively, correspond to external and internal positions. We therefore decided not to pursue this line. We could, however, confirm that, as described by Tonomura and Morales (10), the bound label signal dropped to a non-zero value on addition of ascorbate.

**DISCUSSION**

The experiments reported were designed to test whether calcium translocation by SR ATPase resulted in an inward rotation of externally located spin labeled sulfhydryl groups. Prerequisites of such testing are of course that (i) labeled SH groups should be located on the ATPase polypeptide and (ii) the labeled ATPase molecules should still be active. As regards (i), it is now widely accepted that 95% of the SR SH groups belong to the Ca2+ pump. Although, as suggested by Hidalgo and Thomas (31), a 30,000-dalton glycoprotein might be responsible for part of our fast-reacting SH groups, we consider this possibility unlikely to be significant on the basis of our electrophoresis gels (28). In any case, it would not affect the validity of our conclusions. As regards (ii), the activity we observed was only partially inhibited under conditions in which the ATPase molecules were labeled with several maleimides (2–3 mol per 1.5 X 106 g of protein). Most of our labeled molecules were therefore active.

The medium conditions under which the possible inward protein reorientation was sought calls for brief comment. Established evidence shows that calcium initially bound to high-affinity sites is rapidly translocated across the membrane as a consequence of enzyme phosphorylation (20–26). To detect possible concomitant rotation of the whole polypeptide chain, we therefore decided to focus on the phosphorylation step and studied the labeled enzyme under minimal dephosphorylation rate conditions (high calcium concentration) to avoid problems due to substrate hydrolysis. In such a medium, the phosphoenzyme is considered the predominant chemical species.

Line-broadening transition ions, particularly nickel, which was most widely used in this study, offer definite advantages for discrimination between external and internal spin label signals compared to ascorbate. We showed that the relative impermeability of these broadening agents permitted at least one spectral line recording before perturbing penetration into the vesicles. One advantage of such transition ions is that all accessible labels in the external compartment are immediately line-broadened on mixing, whereas a given moderate ascorbate concentration removes the outer signal at a speed that complicates two-compartment data treatment. Furthermore, label–transition ion interaction is nondestructive (the opposite is true for label–ascorbate interaction), which allows examination of any stationary pump turnover. The result of line-broadening treatment will then reflect the ratio of the average label residence time on the outer surface to its average residence time on the inner surface. It should be pointed out that even the residual nickel permeability was turned to advantage in studying the facing direction of the labels in the ground state.

Our results do not show any inward label reorientation coupled with pump activity. On the basis of low-concentration ascorbate quenching studies in highly permeable vesicles, Tonomura and Morales (10) suggested the opposite conclusion. However, interpretation of their experimental results relies heavily on the hypothesis that labels quenched rapidly and slowly correspond to external and internal positions, respectively. Our results, which contradict this hypothesis, rule out the possibility that a significant part of the labeled SH groups resides on the inner surface of the vesicles in the ground or phosphorylated state. The following results also argue against the external–internal assignment of fast or slow SH-reacting labels: (i) the very asymmetrical binding of Hg-azoferitin to sonicated vesicle SH groups found by Hasselbach and Elvin (98), (ii) the unmodified biphase ascorbate quenching pattern measured by Nakamura at Triton X-100 or deoxycholate concentrations high enough to destroy the vesicular structure (97), and (iii) the high chloride SR permeability which makes unlikely the constitution of any transmembrane asorbate gradient due to an electrical potential difference suggested by Tonomura and Morales (10).

In addition, these last authors mention that, on ascorbate addition, the bound label ESR signal appears to decay to a
non-zero value. We confirmed this fact, which is probably due to the very deeply buried location of part of the labels. Consequently, what Tonomura and Morales should have plotted as a semilogarithmic analysis of their results is the difference between signal amplitude and the non-zero final value. When their data are fitted to a combination of exponentials plus a constant, no "gratifyingly simple" results are obtained. The difference in the quenching profile of the labels under the different conditions should rather be explained by other mechanisms, such as simple electrostatic screening of some labels on ATP binding to the vesicle surface or the influence of the different conformational states of the enzyme which are known to be induced by various mediums. These states, which are detectable by several techniques including fluorescence (28, 39), chemical reactivity towards SH reagents (28, 40–43), and even ESR spectroscopy with another label (27, 44), can give rise to different degrees of label accessibility to ascorbate. As evidenced in transfer saturation studies (45), the maleimide label spectrum itself might well be insensitive to these changes, because of the already highly constrained state which is apparent from its spectrum.

To return to the original question about translocation mechanisms, Tonomura and Morales' results can no longer be considered consistent with a rotatory hypothesis for calcium translocation, nor can the present results. The following conclusions may therefore be drawn: (i) Complete reorientation of the Ca\textsuperscript{2+} pump protein is not only unlikely but must be excluded, because experimental results contradict this hypothesis. (ii) This leaves us with pump models in which only limited transmembrane movement occurs. A limited portion of the polypeptide chain, remote from the protein region tested by the SH reagents, might experience complete transmembrane translocation; this is, however, unlikely. Rather, we have to consider the two following last models: (i) a pore with allostERIC properties, in which the cation binding site becomes alternately accessible to the inner and outer aqueous phases through conformational rearrangement of the whole polypeptide chain (or chains) (this would be best consistent with an oligomeric structure of the Ca\textsuperscript{2+} pump) or (ii) a pump system in which a passive channel-forming peptide structure is closed or opened by limited displacement or modification of a selective gate. The latter mechanism could be accommodated by a monomeric species and is actually the one now preferred by Tonomura and coworkers (46). Distinction between these various models is, however, mainly semantic, at least as judged on topological grounds (9, 46–49).

A final comment can be added. Although transmembrane translocation of an important portion of the polypeptide chain can now be excluded for SR Ca\textsuperscript{2+}-ATPase, such a definite conclusion has apparently not yet been reached for other transport systems (see, for instance, refs. 50 and 51). We suggest that in these cases the above-described nickel or ferricyanide method might prove to be a valuable tool.

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