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DETECTION, BY HYDROGEN EXCHANGE, OF A MODIFIED MEMBRANE CONFORMATION LINKED TO CALCIUM TRANSPORT BY SARCOPLASMATIC VESICLES

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SUMMARY

1. Sarcoplasmatic vesicles were isolated and equilibrated for a few hours at 24° in tritiated water in order to label their exchangeable hydrogens; no loss of transport activity by this treatment was observed.

2. Complete recovery and thorough purification of the vesicles from the incubation media and especially from the tritiated water were obtained by molecular sieve chromatography on short columns of Sephadex G-200.

3. When the rate of loss of vesicle-bound tritium was followed for 40 min, it was observed that a group of labelled hydrogens, immediately lost by exchange in vesicles at rest, was protected from exchange when vesicles were transporting Ca^{2+}.

4. When vesicles were not transporting, the presence of ATP did not protect this group from being rapidly exchanged.

5. These results indicate the existence of a conformational remodelling in the membrane, in the direction of an increase in stability during energy-coupled Ca^{2+} transport.

INTRODUCTION

The many models presented in the literature as a basis for energy-coupled transport through membranes may usually be classified into two main categories:

(a) The carrier is fixed. In this case the carrier has many sites, the substrate moves along the sites, displaced by a variation in affinity of the binding sites. Although relative displacements of the substrate to the carrier channel are necessary, the carrier unit is immobile and its conformation may not necessarily be changing during activity. If the fixed carrier has only one binding site, the latter has to travel across the membrane, and it must be pulled back and forth by a remodelling of at least a minimal region of the carrier unit.

(b) The carrier is mobile inside the membrane, binding the substrate on one side and discharging it on the other side of the membrane. Such a carrier, moving as one unit, would undergo no remodelling by itself. In pushing aside the neighbouring units, it could, however, initiate around itself a far-reaching reorganization, eventually translated into an observable conformational change of the membrane as a whole.

Such changes in conformation are commonly called upon in the models describing energy-coupled transport or oxidative phosphorylation, but direct demonstration is lacking; a recent report on a direct test by optical methods showed no observable change in the conformation of sarcoplasmatic vesicles during calcium transport[1].

We thought an investigation to test such a change in conformation could be fruitful if two conditions were fulfilled: the transport activity of the membrane should be very high and the method used to detect conformation changes very sensitive.

Accordingly, we have selected the sarcoplasmatic vesicles of skeletal muscles which are reported to be composed of the simplest and most active membrane available[2,3]. The method chosen to detect conformation changes in proteins was the hydrogen exchange method which has brought about much important data, and is reasonably simple in its modern version[4,5].

The following is a preliminary account of our results showing that some hydrogens are stabilized during Ca^{2+} accumulation, which may be interpreted as indicating a change in the membrane conformation coinciding with energy-coupled transport.

MATERIALS AND METHODS

Sarcoplasmatic vesicles were prepared from rabbit white skeletal muscle according to WEBER[2], but using 0.10 M KCl with 0.020 M imidazole at pH 6.90.

Membrane activity

Vesicles at a protein concentration of 0.10 mg/ml were incubated at 24° with constant stirring, in 4 ml of a medium containing: 0.100 M KCl; 0.020 M imidazole, pH 6.90; 0.55 mM ethyleneglycol-bis-(2-aminoethyl)-*N*,*N*,*N'*,*N'*-tetraacetic acid; 5 mM sodium oxalate; 2 mM phosphocreatine; 0.04 mg/ml phosphocreatine phosphokinase. The experiment was started by adding ATP and Mg^{2+} to final concentrations of 0.020 mM and 1.0 mM, respectively. Aliquots for basic ATPase (no Ca^{2+} present) were taken after 2, 3, 4 and 5 min. One aliquot for calcium content before accumulation was also taken. At the 6th min, $CaCl_2$ was introduced to a final concentration of 0.50 to 0.70 mM. Aliquots for total ATPase (with Ca^{2+} present) and aliquots for Ca^{2+} content in the vesicles were taken alternatively up to 15 min of incubation. One aliquot was also taken for protein estimation.

Basic and total ATPase were computed from the ATP utilization with time and the concentration of the membrane protein in the incubation mixture. The ATP utilized is equal to the creatine released and the latter was measured as described by WEBER[2].

Protein was estimated according to LOWRY *et al.*[6] against a standard of gelatin, and true values were recalculated from the automatic amino acid analysis of a hydrolyzed aliquot of vesicles.

Calcium accumulation was measured directly on the vesicles after isolation as described below. Two aliquots of 0.100 ml were used for protein estimation as described above. The remaining portion was used directly in the flame photometer (Eppendorf), and Ca^{2+} concentration read at 622 mμ against a blank made of the column equilibration buffer, and a standard of 0.10 mM $CaCl_2$ in the same buffer.

Isolation of vesicles

The method described by ENGLANDER[5] was adapted for the purification of particles and tested carefully. We used 10-ml columns at 2°, filled with Sephadex G-200

(Pharmacia, Uppsala, Sweden) equilibrated with 0.10 M KCl buffered with 0.020 M imidazole at pH 6.90. A vol. of 0.45 ml of incubation mixture was pipetted onto the column and eluted immediately with the equilibration buffer. Provided the vesicles are not aggregated, a 100% protein yield is recovered within 5 min. We used to collect a 1.0-ml fraction containing only the first 3/4 of the excluded peak, so that a purification factor of 10^7 could be secured. The method is well suited for quick separation of tritiated particles. It was also found to be very convenient for recovering (in view of direct Ca^{2+} estimation) vesicles having accumulated Ca^{2+} from the incubation mixture containing oxalate, since preliminary tests showed that the time required for the separation accounted for a loss of Ca^{2+} by outflow, of only 1 to 2% of the amount present into the vesicles.

Hydrogen exchange

Vesicles were first labelled (exchange-in) by incubation for 5–6 h, at 24° in the solution used for their preparation, to which tritiated water was added at a final concentration of 10–20 mC/ml. The specific activity of this solution was determined by counting a diluted aliquot.

The loss of tritium by labelled vesicles was measured in a solution differing somewhat from that used for optimal Ca^{2+} accumulation: the concentration of vesicles was raised here by a factor of 5 and oxalate was omitted in order to ensure Ca^{2+} transport for a prolonged period of time without a high Ca^{2+} concentration, which acts as an inhibitor.

For each hydrogen exchange experiment, one required incubation mixture was prepared at a time and stirred at 24°. An aliquot of labelled vesicles containing 1.0–2.0 mg protein was separated extemporaneously from the tritiated water as described above. The exchange-out was initiated by immediate mixing of the isolated vesicles with the incubation solution at 24°. At periods between 2 and 40 min, 0.45-ml aliquots of this suspension were pipetted on individual columns for isolation of the vesicles and these were kept in the cold. One control experiment was started just afterwards, or *vice versa*.

The specific activity of the vesicles after exchange-out was computed from the counts/min in a 0.50-ml aliquot and from the protein concentration estimated on duplicate aliquots of 0.100 ml. Radioactivity was measured in a liquid scintillation spectrometer (Packard Instr.), counting at least 40 000 counts and 10 times the blank values. Protein was estimated as described above.

The number of unexchanged hydrogens per chain of 1000 amino acid residues was calculated from the specific activity in counts/min per mg protein (1.00 mg equals 10.1 μmoles amino acid residue) in the vesicles separated after exchange-out, and from the specific activity in counts/min per (g) atom hydrogen in the labelling solution.

The ATP utilization in the conditions used for hydrogen exchanges was deduced from a creatine estimation in an aliquot taken at the end of the incubation period.

RESULTS

Calcium accumulation

We should like to stress one important point: the conditions used for hydrogen exchange have been chosen so that a high accuracy in the specific activity of the

isolated vesicles was obtained, and not the highest rate of Ca^{2+} transport. The latter was present, however, during hydrogen exchange as shown by comparison with the rate at which calcium is transported in optimal conditions. When tested in conditions optimized for Ca^{2+} accumulation the first 2-min rate amounted to 1.3 μmoles Ca^{2+}/min per mg protein. The basic ATPase (no Ca^{2+} present) was usually around 0.15 μmole/min per mg protein and the ATPase due to the Ca^{2+} transported followed closely the rate of Ca^{2+} accumulation, the ratio Ca^{2+} to ATP used being around 1.9. Moreover, when tested after tritiation, the activity was not decreased, which proved that no radiation damage had developed.

During the incubation for hydrogen exchange measurements, the vesicles were also accumulating Ca^{2+}:

(a) From the control level of 0.16, the ATP utilized when Ca^{2+} was present increased from 0.20 to 0.24 μmole/min per mg protein, as tested at the end of each hydrogen exchange experiment.

(b) Ca^{2-} accumulation could be directly demonstrated in the vesicles, although in smaller amounts, since it is not trapped by oxalate.

Rate of hydrogen exchange

The rate of hydrogen exchange in vesicles incubated in conditions such that they do, or do not, transport Ca^{2+}, is shown in Fig. 1. Vesicles equally labelled before mixing with the incubation media behaved differently when Ca^{2+} was present (A) than when Ca^{2+} was absent (B). The main point is that the curves had already diverged before the first measurement was made and then ran roughly parallel to each other between 2 and 40 min. More hydrogens were exchanged when vesicles were not transporting. Four such additional comparative experiments were carried out on other

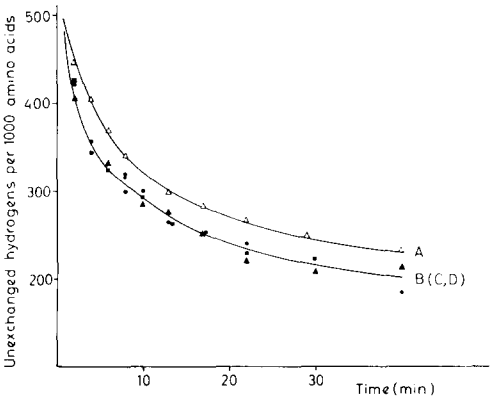

Fig. 1. The time-dependent hydrogen exchange of sarcoplasmatic vesicles in different activity conditions at 24°. Curve A (△) is obtained during Ca^{2+} transport with the following components in the incubation mixture: 0.10 M KCl; 20 mM imidazole chloride (pH 6.90); 0.4 mg/ml vesicles protein; 2 mM ATP; 10 mM Mg^{2+}; 4 mM phosphocreatine; 0.04 mg/ml phosphocreatine phosphokinase; 0.55 mM disodium salt of (ethyleneglycol-bis-(2-aminoethyl)-*N*, *N*, *N'*, *N'*-tetraacetic acid) (Fluka, Buchs, Switzerland); 0.55 mM $CaCl_2$. Control conditions (no Ca^{2+} transport) were obtained by the following modifications of the above complete medium: Curve B (▲), Ca^{2+} omitted and replaced by 10 mM ethyleneglycol-bis-(2-aminoethyl)-*N*, *N*, *N'*, *N'*-tetraacetic acid. Curve C (●), ATP and Mg^{2+} omitted. Curve D (■), ATP and phosphocreatine omitted. One single curve is drawn for all three controls, since the values in B, C or D do not differ significantly from each other.

preparations of sarcoplasmatic vesicles and similar sets of curves were obtained. The standard deviation from the best smooth curve drawn between 8 experimental values varied from 2 to 4% for different curves. For comparison of all experimental values on curve A with the values on curve B, without consideration of the time variable, the number of unexchanged hydrogens in transporting vesicles (A) was expressed in % of the number of unexchanged hydrogens in inactive vesicles (B) at the same time. The value corresponding to Fig. 1 is 112; the values for additional experiments are 111, 116, 116, 112.

To summarize, for each chain of 1000 amino acid residues, 300 hydrogens are still unexchanged after 10 min at 24° in inactive vesicles, but an extra 35 to 40 hydrogens are sequestered as soon as the vesicles are transporting and kept unexchangeable as long as the activity continues.

This stabilization of a group of hydrogens is linked to energy-coupled ion movement and not to independent ATPase activity. This is shown by the coincidence in Fig. 1 of values obtained in 3 different control conditions: one single exchange curve can fit values for conditions when ATP is absent (C, D), or present (B). The ATP consumption in the absence of Ca^{2+} (B), although higher than the ATP utilization for Ca^{2+} transport, does not coincide with a stabilization of that group of hydrogens; coupling only is linked to an immediate stabilization of a group of hydrogens in the sarcoplasmatic vesicles.

Kinetic analysis of the curves in Fig. 1 was carried out. The number of slowly exchangeable hydrogens H_3 (half-life $>$ 80 min) was determined by plotting the rate of loss per unit time $\Delta H_{(t)}/\Delta t$ *versus* the total number of unexchanged hydrogens at the same time $H_{(t)}$ and extrapolating to zero rate. After deduction of H_3 from the total number of hydrogens remaining at each time interval, the number of fast exchanging hydrogens is obtained; a plot of the logarithm of that number *versus* time gives, for conditions (A) and (B) in Fig. 1, broken straight lines, indicating that the hydrogen exchange proceeds by two first-order reactions. Table I shows the approximate values for the number of hydrogens in each class and corresponding half-times of exchange. As could already be deduced from Fig. 1, a group of 35 to 40 hydrogen shifts from very fast exchanging classes in inactive vesicles to a slowly exchangeable class in active vesicles. We are aware of the limitations imposed on that classification into discrete groups. The absolute numbers of each group and their half-lives in Table I must be considered to be very approximate, because we are dealing with rather fast-exchanging hydrogens and because the values may vary with the conditions used for tritiation.

TABLE I

COMPARISON OF CLASS SIZES (n) AND EXCHANGE HALF-TIMES ($t_{1/2}$) FOR EACH CLASS OF HYDROGENS IN ACTIVELY TRANSPORTING, AND IN INACTIVE SARCOPLASMATIC VESICLES.

The data are calculated, as explained in the text, on the two experimental curves of Fig. 1.

	Class 3		*Class 2*		*Class 1*	
	n	$t_{1/2}$ (*min*)	n	$t_{1/2}$ (*min*)	n	$t_{1/2}$ (*min*)
Ca^{2+} transport	226	>80	178	10	111	<3
No transport	189	>80	191	10	135	<3

While these uncertainties are pertinent to studies of the absolute conformation of these membranes, they do not seriously compromise the validity of the exchange method for detecting changes in stability and conformation. As the exchange-out is performed, for both sets of incubations, on the same tritiated sample, at the same temperature and pH, and as the shift in a reasonable fraction of the hydrogens is from a group with a short half-life to a group with a much longer half-life (Table I), we feel that the comparison of the groups in Table I is valid. The conclusion drawn from this calculation corresponds closely, and furthermore does not go beyond, the immediate interpretation of the set of curves of Fig. 1.

The present results can be accounted for by the existence of two conformations: one present during inactivity, the other present during energy-coupled ion translocation; the latter conformation is more stable as judged by hydrogen stability measurements. The present results do not support the existence of a continuous variation in the conformation, proportional to the amount of work performed by the membrane.

DISCUSSION

Our observation reported here of a conformation change accompanying the energy-dependent transport of Ca^{2+} by the sarcoplasmatic vesicles is different from the recent report of MOMMAERTS[1]. Using circular dichroism measurements on the same system and incubation conditions very close (except for the absence of an ATP-generating system) to the one we selected, the latter author could detect no change in conformation related to transport activity; in his special case, no change in the content of α-helical structure.

The comparison of our positive and his negative results seems to infer that the hydrogen-exchange method is much more sensitive than the optical method, or can detect changes which occur outside the α-helical region, or more exceptionally not even in the protein portion of the active unit, all changes which seem undetectable by the optical methods known so far. We agree with the very cautious interpretation of negative results by the author[1]. Our results show, however, that the active fraction of this kind of membrane is certainly not too small to be undetectable by a change in conformation.

We might assume that the higher stability measured and related to the transport activity could reside not in the membrane structure itself, but in a water fraction, gaining the labelled hydrogens lost by the membrane in any condition of incubation. This water, as a whole intravesicular aqueous phase, or as a cluster into the membrane itself, would be completely segregated only in the case when the membrane is actively transporting Ca^{2+}. This would consequently correspond to the very unlikely situation of a living membrane completely impermeable to water. A particular case in this situation would be the sequestration of hydrogens into the water of crystallization of the hydrated calcium phosphate which is probably precipitated after more calcium has been accumulated by the vesicles in the absence of oxalate. Segregation, and consequently the difference between the curves of Fig. 1, would then be proportional here, after a measurable delay, to the time of incubation, a situation which is not observed. We insist that if valid, these assumptions would, however, not infirm our contention that a conformational change appears during coupled transport, since sequestration of water would necessitate a remodelling of the membrane.

If the membrane itself carries these hydrogens involved in this remodelling, it is inescapable to locate them in the protein fraction.

According to MARTONOSI, DONLEY AND HALPIN[7], the other constituents of these membranes are phospholipids, in amounts of 0.3 μatom phospholipid phosphate per mg protein. We have estimated 1 mg of vesicular protein to contain 10.1 μatoms peptidic hydrogen. There are therefore about 30 phospholipid molecules per 1000 peptidic hydrogens. The splitting of 20 of them, with solubilization of phosphorylcholine, by phospholipase C, abolished the energy-coupled transport. Reintroduction of half, re-establishes the activity[7]. When these facts prove the necessity of phospholipids for the integrity of the structure, they do not minimize the equal importance of the proteins. We think that phospholipids do not obviously possess the kind of slowly exchangeable hydrogens we have detected, but confirmation from the literature is, to our knowledge, not available. If we decide to postulate that these stabilized hydrogens belong to phospholipids, we have to admit, from their amount deduced from our experiments and from the minimal amount of lecithin molecules necessary to the activity[7], that, if each phospholipid in the membrane is involved in coupled transport, it carries at least 2, and if only one out of 10 is involved, it carries at least 20 slowly exchangeable hydrogens. We feel that this situation is very unlikely.

Consequently the most likely localization for a modified hydrogen stability in the membrane would still reside in its protein fraction. It is known that labile hydrogens with measurable half-lives belong to the primary amide groups of the peptide bonds. A tight structure like the α-helix confers to these hydrogens a very high stability, and uncoiling makes them immeasurably rapidly exchangeable[4]. The exact correspondence between half-life for exchangeable hydrogens in discrete classes of known proteins and the specific conformation of the backbone as deduced from X-ray diffraction and optical rotatory dispersion is still to be delineated. We shall limit the interpretation of our results in suggesting that the detected shift of 35 hydrogens from one class to the other could involve hydrogens belonging to the polypeptide chains of the active units. Two conformations would be present: one in vesicles at rest, the other in vesicles activated by ATP and the ion transported, which would together increase the stability, in other words, decrease the entropy of the active unit, in accordance with an entropy contribution by the free energy of the ATP utilized. This situation seems to correspond rather closely to the qualitative evaluation by electron microscopic studies on the gross conformation of membranes in mitochondria and other systems; two conformations are emphasized: a non-energized configuration reversibly transformed into an energized configuration by ATP utilization or electron transfer[8].

In regard to the carrier hypotheses presented in INTRODUCTION, it seems that a continuous remodelling of a carrier unit, proportional to the amount of ions translocated and to the time the experiment is performed, would result, in the case of the remodelled region exposed to the solvent, in a continuous decrease in the number of unexchanged hydrogens with respect to the control. No such result was obtained. However, such a limited continuous remodelling might well be concealed by the more important initial shift in conformation and then escape detection. It appears that the present results would better describe one of these situations: (1) A fixed carrier unit with many sites: a de-energized conformation would shift to a more stable energized conformation for the operation of the carrier. (2) A mobile carrier, which would slip

into and move cyclically in a complete hydrophobic environment during operation, with a very localized contact of its carrier site with the two hydrophilic faces of the membrane.

We are aware of the illusiveness and inadequateness of these mechanistic models as well as of the difficulty in locating these stabilized hydrogens within the structural units of the membrane, but we are confident that further developments of this technique will clarify the picture.

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