Arrangement of Proteins and Lipids in the Sarcoplasmic Membrane

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The number of amino residues present in the proteins of the sarcoplasmic reticulum which can react with Fluram has been determined in native and sonicated SR vesicles. Sonication increases the number of amino groups accessible to Fluram from 0.57 to 0.87 μmol·mg prot.$^{-1}$. This increase indicates that 66% of the amino residues are present in the external and 34% in the internal membrane leaflet. The distribution of the amino phospholipids is computed from the distribution of Fluram in the membrane proteins in conjunction with the relative distribution of Fluram between protein and lipid in native and sonicated vesicles. The distribution of the calcium transport protein has been approximated under different assumptions concerning the distribution of the residual protein and taking into account that 15% of the membranes of the SR vesicles might have changed their sideness during preparation.

In a preceeding study[1] we have shown that Fluram – 4-phenylspiro[furan-2-2(3H), 1′phthalan]-3,3′-dione[2] – can be used to label membrane proteins and lipids located at the external surface of the vesicular fragments of the sarcoplasmic reticulum (SR). Under suitable conditions Fluram reacts in a fraction of a second quantitatively with the free amino groups of protein and lipids, forming highly fluorescent components. Therefore, the extent of labeling can easily be controlled and measured. This is an essential requirement since the SR membranes are destroyed when four amino residues per 10^5 g membrane protein are labeled. The extent of labeling is very different for the main membrane constituents, the calcium transport protein, the calcium precipitating protein and for phosphatidylethanolamine and changes when the internal membrane surface is exposed by solubilization or sonication. In closed sarcoplasmic membranes the specific fluorescence of the calcium precipitating protein is three times higher than that of the transport protein. When the vesicles are lysed, this ratio is shifted in favour of the transport protein. This result indicates an asymmetric distribution of the Fluram reactive amino residues in the membrane proteins. As discussed in the preceeding paper, the extent of labeling depends not only on the number of groups that can react with the reagent but also on the rate of labeling. The latter may differ considerably for the different membrane components and may be influenced by conditions used for exposing the internal surface to the reagent. The asymmetric distribution of the membrane protein and the reaction rates determine the labeling ratio (u) which is obtained by comparing the fluorescence intensity of the membrane protein of labeled lysed and closed sarcoplasmic vesicles as follows:

$$u\,\frac{r\,a_0}{r\,a_0 + s\,b_0} = \frac{r'\,a_0(1+\alpha)}{r'\,a_0(1+\alpha) + s'\,b_0(1+\beta)}\,. \quad (1)$$

Correspondingly, the labeling ratio of the lipids (v) is given by:

$$v\,\frac{s\,b_0}{r\,a_0 + s\,b_0} = \frac{s'\,b_0(1+\beta)}{r'\,a_0(1+\alpha) + s'\,b_0(1+\beta)}\,. \quad (2)$$

r, r' and s, s' are coefficients by which the different rates of labeling are taken into account. a_0 and b_0 are the number of external Fluram binding sites present in the membrane proteins and lipids, respectively. The number of the internal binding sites a_i and b_i are assumed to be fractions of the external binding sites. $a_i = \alpha \cdot a_0$ and $b_i = \beta \cdot b_0$. The asymmetric distribution of the membrane constituents can be expressed by α and β. The fractions of the membrane protein and lipids in the external membrane leaflet are $1/(1+\alpha)$ and $1/(1+\beta)$, respectively. The discussed relations show that due to the many unknown factors the measurable parameters u and v reflect rather indirectly the degree of asymmetry.

Not only the determination of the degree of asymmetry is impeded by the necessity to take into account reactivity changes but also the estimate of the absolute number of amino groups in the membrane protein that can react with Fluram is hindered. In principle, absolute figures can be obtained when the vesicles are labeled in the presence and in the absence of a protein (test protein) whose number (N) of Fluram reactive groups is known and the relative fluorescence intensities a and b are measured.

$$a = \frac{r\,L}{P}\;; \quad b = \frac{r\,L}{P + s\,N}\;; \quad P = \frac{b\,s\,N}{a-b}\,.$$

a is the observed ratio of fluorescence intensity of the lipid and protein fraction of SR vesicles. b is the same ratio observed in the presence of test protein. L and P represent the number of residues in the lipid and protein fraction, respectively. N is the number of residues in the test protein. r and s are the coefficients for the different reaction rates. However, these relations give absolute values only if the reactivities of the native protein and the test

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Table I. Fluram labeling was performed in the assay medium of 5 ml containing 0.1 M KCl, 0.05 M borate buffer pH 8.3 and 10 mg vesicular protein. 0.5 μmol and 1 μmol Fluram dissolved in acetone were added to the vigorously shaken assay. In parallel an assay was run containing in addition 7 – 15 mg test protein. Aliquots were taken for the determination of the total fluorescence. Subsequently the protein was precipitated and the lipids were extracted by adding 4 volumes of ethanol and ether 2:1. The precipitated protein was resuspended in acetone, centrifuged and subsequently dissolved in 0.05 M borate buffer and 0.1% SDS. All samples were identically diluted by 0.05 M borate buffer and 0.1 SDS. Fluorescence was exited at a wave length of 390 nm and light emission was measured at 480 nm with a spectrofluorometer Hitachi Perkin-Elmer MPF-2A. The test protein was prepared from SR vesicles. The lipids were extracted with ethanol-ether. After the treatment with ethanol-ether the protein was washed two times with ether and dried.. The protein powder was kept at -18 °C. The number of Fluram active residues in the test protein was determined by titrating the preparation with Fluram as described for native vesicles. Fluorescence intensity becomes constant at a degree of labeling of 0.7 – 0.8 residues · mg prot.$^{-1}$. The sensitivity of the spectrofluorometer was set with a solid fluorescence standard. The total fluorescence intensity obtained after labeling 10 mg SR vesicles with 0.05 – 0.1 μmol was used as calibration standard. Under the described conditions Fluram reacts quantitatively with the SR preparations. The SR vesicles were prepared as described by de Meis and Hasselbach [7].

(o) Values were calculated with the assumption that all nontransport protein reside in the external membrane leaflet.

(i) Values were calculated with the assumption that the calcium precipitating protein resides in the external and the residual proteins in the internal membrane leaflet.

Preparation		Number of amino residues in native vesicles (N_n) [μmol mg^{-1}]	Number of amino residues in sonicated vesicles (N_s) [μmol mg^{-1}]	Relative labeling ratio $\frac{u}{v} = \frac{1+\alpha}{1+\beta}$	Protein fraction in the external membrane leaflet $\frac{1}{1+\alpha} = \frac{N_n}{N_s}$	Amino lipid fraction in the external embrane leaflet $\frac{1}{1+\beta}$
Native vesicles		0.57 ± 0.029	0.87 ± 0.043	1.28 ± 0.056	0.66 ± 0.07	0.85 ± 0.09
Native vesicles (corrected for 15% membrane inversion)		0.62	0.87	1.28	0.71	0.92
Transport protein	(o)	0.39	0.69		0.56	
	(i)	0.49	0.69		0.71	
Transport protein (corrected for 15% membrane inversion)	(o)	0.45	0.69		0.66	
	(i)	0.56	0.69		0.81	

protein are identical. If this is not the case, apparent numbers are obtained. A comparison between closed and open membranes to determine the distribution of the membrane components between external and internal membrane surfaces is meaningful only if the opening procedures avoid changes in the reactivity in the test protein as compared to the vesicular protein. Considerable reactivity changes have been observed when cholate or Triton X-100 were used for solubilization of the SR vesicles [3]. The described difficulties are circumvented if the Fluram reactive groups were exposed by sonication. The Table presents the average values from 7 – 9 experiments in which native and sonicated vesicles were labeled with 0.05 – 0.1 μmol Fluram pro mg of protein. The number of amino groups in the protein which reacts with Fluram is considerably less than the total number of basic amino acids present [4]. From the figures obtained for closed and sonicated vesicles it results that 66% of the vesicular protein are located in the outer leaflet of the membranes. This figure obtained for the protein can be used to determine the distribution of the amino lipids if they are introduced together with the experimental determined value u/v into the relation $1/(1+\beta) = u/v(1+\alpha)$ which results from Eqs (1) and (2) when $r = r'$ and $s = s'$. The figure 0.85 ± 0.09 indicates that, presumably, only a small fraction of the reactive lipids resides in the internal membrane leaflet which is in agreement with the conclusion drawn from experiments in which the amino lipids in the closed SR vesicles were titrated with Fluram [1]. From the numbers obtained for the total membrane protein the distribution of the transport protein in the membrane can only be approximated. The assumptions are as follows. 1. 80% of the membrane protein are transport protein. 2. The calcium precipitating protein constituting 8% is located in the external surface [1]. 3. The residual proteins (12%) may reside inside, outside or at both sides of the membrane. If all nontransport protein is assumed to be in the external leaflet, the transport protein would be distributed nearly symmetrically, the most asymmetric distribution

would result if the residual proteins were located in the internal leaflet. An essential prerequisite for the validity of the discussed data is the assumption that the SR membranes do not change their sideness during isolation. This assumption is based on two findings showing a. that only the cytoplasmic surface of the SR membranes can be labeled with Hg-phenylazoferritin and b. Hg-phenylazoferritin decorates the surface of nearly all vesicles present in our preparations [5]. Most recently Chevallier *et al.* [6] have presented evidence that 10 – 15% of the vesicles may actually be inverted. If this inversion is taken into account the degree of asymmetry of the total membrane protein, the transport protein and the amino phospholipids increase slightly. However, the corrected values do not differ significantly from the uncorrected ones.

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