Reaction of 1-Fluoro-2,4-dinitrobenzene and 2,4,6-Trinitrobenzenesulphonic Acid with Membranes of Sarcoplasmic Reticulum

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Membranes of sarcoplasmic reticulum were labelled with 1-fluoro-2,4-dinitro[³H]benzene at pH 6.5 and with 2,4,6-trinitrobenzenesulphonate at pH 9.2. Conditions were chosen to restrict reaction to amino groups, and the effect of blockings of these groups by methyl acetimidate was determined. All proteins were labelled to some extent by both reagents, but, whereas the trinitrophenylation of both lipid and protein amino groups was almost completely blocked by methyl acetimidate, the dinitrophenylation of the ATPase at pH 6.5 was much less affected. The seven amino groups on the ATPase that were labelled under these conditions did not react with methyl acetimidate. This reagent can therefore be used to enhance the specificity of fluorodinitrobenzene for amino groups in a hydrophobic environment. The amino groups on the minor proteins and on the phospholipids that reacted with fluorodinitrobenzene at pH 6.5 were probably in an aqueous environment, since the reaction was blocked by methyl acetimidate.

Differential labelling of membrane proteins by small charged reagents has been extensively studied and the interpretation of the results has led to much controversy (reviewed by Carraway, 1975).

Problems arise because few membranes are completely impermeable to the reagents, so that the extent of labelling of a given protein depends not only on its location but also on its reactivity towards the reagent, on the period of labelling and on the concentration of the reagent used. One way of providing an internal control for these factors is to compare two reagents of similar reactivity but different lipid-solubility.

We applied this principle to study the location of the proteins in vesicles of sarcoplasmic reticulum, using the reagents 1-fluoro-2,4-dinitrobenzene and 2,4,6-trinitrobenzenesulphonate. Arrotti & Garvin (1972) applied a related pair of reagents, 1-chloro-2,4,6-trinitrobenzene and 2,4,6-trinitrobenzenesulphonate, to the proteins of erythrocyte membranes and showed that they labelled different proteins. From an analysis of the results of labelling whole erythrocytes with trinitrobenzenesulphonate they concluded that, although there was slight permeation of the reagent that labelled the internal

haemoglobin, only a single membrane protein was labelled. It therefore appeared that the labelling of a protein by trinitrobenzenesulphonate was a useful criterion for its location on the outer surface of the erythrocyte membrane. In contrast, since our first results showed no significant differences in the relative labelling of different proteins by the two reagents, it appeared that the method would not yield useful information about the location of particular proteins in sarcoplasmic reticulum, probably because of the high permeability of this membrane (Duggan & Martonosi, 1970). The objective was therefore modified to permit distinction between classes of amino groups located in predominantly aqueous or hydrophobic environments. Since the pK of the latter will be lowered they should be relatively more reactive at low pH. We therefore enhanced the specificity of fluorodinitrobenzene for such groups by performing the reaction at pH 6.5 but labelling with trinitrobenzenesulphonate at pH 9.2. The differential labelling was further increased by a preliminary blocking of the amino groups in the aqueous phase with methyl acetimidate.

Materials and Methods

Bovine serum albumin, ovalbumin and β -lactoglobulin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Acrylamide and methylene-

Abbreviations used: Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl.

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bisacrylamide were recrystallized (Loening, 1967) and their solutions were filtered before use. Fluorodinitro[^3H]benzene (10 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Unlabelled fluorodinitrobenzene and trinitrobenzenesulphonate were obtained from BDH Chemicals (Poole, Dorset, U.K.).

Sarcoplasmic reticulum was prepared by a modification of the method of Martonosi (1968), as previously described (Thorley-Lawson & Green, 1973).

Tnp-proteins were prepared from solutions of the appropriate protein (30 mg/ml in 0.1 M-sodium borate buffer, pH 9.2) to which was added trinitrobenzenesulphonate (1 M) to a final concentration of 5 mM. After 2 h *N*-acetylcysteine was added to a concentration of 0.1 M and the mixture was allowed to stand for 1 h. Low-molecular-weight reaction products were removed by passage through a short column of Dowex 2 (Cl^- form; 30–50 mesh). The concentration of Tnp groups in the product was determined spectrophotometrically at 372 nm, the isosbestic point for the Tnp-amino group and its complex with sulphite ($\epsilon = 10800 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Goldfarb, 1966).

Methyl acetimidate was prepared from acetonitrile by the method of Ludwig & Hunter (1967).

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate was performed by the method of Weber & Osborn (1969) in 0.5 cm-diameter tubes, as described by Thorley-Lawson & Green (1973). Stock solutions of the proteins (1 mg/ml) were prepared in 0.1 M-sodium borate and their concentrations were determined from the A_{280} values ($A_{1\text{cm}}^{1\%}$ values of standards: bovine serum albumin, 6.6; β -lactoglobulin, 9.4; ovalbumin, 7.0). Mixtures of these solutions were treated with sodium dodecyl sulphate and run on gels for calibration purposes.

Determination of protein and of Tnp groups on polyacrylamide gels

The gels for protein estimation were stained for 30 min at 50°C with Coomassie Blue (0.02% in acetic acid/ethanol/water, 2:9:9, by vol.) and destained for 15 h at 50°C in 7.5% (v/v) acetic acid (100 ml), the solvent being changed after 1 h. The gels (diameter 0.5 cm) were compressed between glass plates (0.3 cm apart) and scanned on a Joyce-Loebl microdensitometer fitted with the green filter (λ_{max} , 550 nm). The sections containing the protein peaks were cut out and weighed. Duplicate gels were run at each loading and the mean of three scans was used to estimate the protein.

The calibration curve for gels stained with Coomassie Blue showed a linear response only up to 2–3 μg of protein. Stray light originating from imperfect match between the dye and the green filter

led to a rapid fall off at higher absorbances, which was greater for the higher-molecular-weight proteins because of the decrease in bandwidth on the gel with migration distance. In later experiments a gel-scanning attachment for a Unicam SP.1800 spectrophotometer gave a response that was linear up to much high loading (Bennett & Scott, 1971).

Tnp groups were determined on unstained gels run at a higher loading (approx. 100 μg of total protein, <30 nmol of Tnp groups). They were soaked in 7% (v/v) acetic acid to decompose any residual sulphite complex of the Tnp group that may remain from the original reaction (Goldfarb, 1966), and then scanned as described above with the use of a Chance OX1 filter (λ_{max} , 350 nm). The method was calibrated with Tnp derivatives of bovine serum albumin, ovalbumin and β -lactoglobulin.

The calibration curve for Tnp-proteins showed a linear response up to 25 nmol of Tnp groups. By using protein loadings of up to 100 μg on these gels it was possible to detect 1–2 mol of Tnp groups/10⁵ g of protein.

Trinitrophenylation of sarcoplasmic reticulum

Trinitrobenzenesulphonate (1 M) (4 μl) was added to sarcoplasmic-reticulum vesicles (4 mg in 2 ml of 0.1 M-sodium borate buffer, pH 9.2), and after 5 min at 4°C the mixture was centrifuged (165 000 *g* for 30 min). The pellet was washed by resuspension in borate buffer (2 ml) and was centrifuged as before. It was resuspended again with the addition of *N*-acetylcysteine (0.1 M) to remove any *S*-Tnp groups. After 1 h at 4°C the vesicles were again centrifuged, and the pellet was dissolved in sodium dodecyl sulphate (2%) and heated briefly at 100°C. Appropriate samples were analysed for protein and Tnp groups on polyacrylamide gels as described above.

Dinitrophenylation of sarcoplasmic reticulum

Fluorodinitrobenzene (4 μmol in ethanol) was mixed with fluorodinitro[^3H]benzene (20 nmol, 200 μCi) and the solvent was removed. Vesicles of sarcoplasmic reticulum (4 mg in 2 ml of 0.1 M-sodium phosphate buffer, pH 6.5) were added and then allowed to react for 24 h at 4°C. The product was centrifuged as above, washed with 0.1 M-sodium phosphate buffer, pH 6.5, and treated as described for Tnp-labelled vesicles. The distribution of [^3H]-Dnp groups on the stained gels was measured by counting the radioactivity of the sliced frozen gels as previously described (Thorley-Lawson & Green, 1973). The counting efficiency was 25%.

Blocking of amino groups with methyl acetimidate (Ludwig & Hunter, 1967)

Methyl acetimidate hydrochloride (0.2 g) in water (1 ml) was adjusted to pH 9 with 2 M-NaOH and added to sarcoplasmic-reticulum vesicles (20 mg in

4 ml of 0.1 M-sodium borate buffer, pH 9.2). The pH of the mixture was maintained at 9.2 (with 1 M-HCl) during reaction for 1 h at 4°C. Three further additions of neutralized methyl acetimidate (0.2 g) were made at hourly intervals, to ensure complete formation of the derivative, and the pH was maintained at 9.2 as before. The vesicles were centrifuged (165 000g for 30 min), resuspended in 4 ml of 0.1 M-borate buffer, pH 9.2, and again pelleted, before further modification as described above.

Results

The labelling of the proteins and the lipids of sarcoplasmic reticulum by fluorodinitrobenzene and by trinitrobenzenesulphonate is shown in Figs. 1 and 2. The relative labelling of the different proteins by the two reagents was essentially the same in spite of difference in their polarity. There was no marked preferential labelling of any specific protein, as can be seen by comparison with gels stained with Coomassie Blue (Thorley-Lawson & Green, 1973). The water-soluble calsequestrin and the lipid-associated ATPase were labelled to similar extents by both re-

agents, which also labelled the lipid region of the gels.

The effects of acetimidate on the two reactions were markedly different. Almost all the labelling by trinitrobenzenesulphonate was blocked, confirming our tentative assumption that this compound reacts primarily with amino groups exposed to the aqueous solvent. The labelling of lipids and minor proteins by fluorodinitrobenzene was also greatly diminished, but that of the ATPase and of a high-molecular-weight component was not affected. It is likely that the latter is a dimer of the ATPase, since it runs in the appropriate position and our earlier work (Thorley-Lawson & Green, 1973) showed that it could be phosphorylated by ATP. We can conclude that the amino groups of the ATPase that react with fluorodinitrobenzene at pH 6.5 belong to a different class from those that react with trinitrobenzenesulphonate at pH 9.2 or with methyl acetimidate at pH 9.2. In contrast, the amino groups of the minor proteins and of the phospholipids cannot be classified so distinctly, since many of those that reacted with fluorodinitrobenzene also reacted with acetimidate.

The number of groups in the different classes on the ATPase and on phospholipid were estimated from calibration curves as described above (Table 1). Out of a total of 55 lysine residues in the ATPase molecule about 30 reacted with trinitrobenzenesulphonate but only 6 reacted with fluorodinitrobenzene.

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of sarcoplasmic reticulum labelled with fluorodinitro[³H]benzene at pH 6.5 with (---) or without (—) previous treatment with excess of methyl acetimidate

The Figure shows the results from two separate gels superimposed. Approx. 100 µg of protein labelled with fluorodinitro[³H]benzene either with or without previous treatment with excess of methyl acetimidate was run on sodium dodecyl sulphate/polyacrylamide gels for 3 h at 6 mA/gel. The gels were then frozen and sliced, and each slice was analysed for [³H]Dnp by scintillation counting of the radioactivity. Gel electrophoresis of 5 and 20 µg of each sample was also performed separately and the protein determined for each band by staining with Coomassie Blue and scanning.

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of sarcoplasmic reticulum labelled with trinitrobenzenesulphonate at pH 9.2 with (---) or without (—) previous treatment with excess of methyl acetimidate

The procedure was as described in the legend to Fig. 1 except that, instead of the freezing and slicing, the gels were soaked for 30 min in 7% (v/v) acetic acid and scanned at 350 nm for determination of Tnp.

Table 1. *Effect of acetimidation on the labelling of amino groups of the ATPase and phospholipid of sarcoplasmic reticulum*

The results are expressed as $\mu\text{mol}/150\text{ mg}$ of sarcoplasmic-reticulum protein. This quantity is equivalent to approx. $1\mu\text{mol}$ of ATPase (Thorley-Lawson & Green, 1973) or $25\mu\text{mol}$ of total phosphatidylserine and phosphatidylethanolamine (MacLennan *et al.*, 1971).

	Dnp labelling ($\mu\text{mol}/150\text{ mg}$)		Tnp labelling ($\mu\text{mol}/150\text{ mg}$)	
	Acetimidate-		Acetimidate-	
	Control	treated	Control	treated
ATPase	6	7	30	1
Phospholipid	8	2	25	1

In view of the arbitrary reaction conditions, these numbers have no absolute significance. The main point of interest is the differential blocking of different classes of amino groups by methyl acetimidate.

Discussion

The large excess of acetimidate that we used to block the amino groups in these experiments should have been sufficient to ensure complete reaction of all residues exposed to the solvent (Ludwig & Hunter, 1967). This is consistent with the almost complete blocking of the reaction with trinitrobenzenesulphonate. It is worth noting that this extensive modification of both proteins and lipids did not cause the vesicles to aggregate nor alter their appearance in the electron microscope. The noteworthy observation that none of the amino groups of ATPase that reacted with fluorodinitrobenzene at pH 6.5 was available to methyl acetimidate confirms that the latter reagent reacts mainly in the aqueous phase, in spite of its ability to penetrate erythrocyte plasma membranes (Whitely & Berg, 1974). This would be consistent with the kinetic evidence (Hand & Jencks, 1962), which shows that the cationic form of the imide is the predominant reactive species in aqueous solution.

The location of the amino groups that react with fluorodinitrobenzene at pH 6.5 but not with trinitrobenzenesulphonate or methyl acetimidate is of interest in relation to membrane structure. Almost all of the ϵ -amino groups of globular proteins are exposed to the solvent, since they have a normal pK and

can react completely with many reagents. This observation can be rationalized if one considers that at neutral pH, where the protein folds, less than 1% of any ϵ -amino group will be uncharged, so that each one that is buried would decrease the stability of the structure by about 12.5 kJ/mol . Burial of the amino groups of membrane proteins in the hydrocarbon phase of the lipid bilayer will also be thermodynamically unfavourable, but it is possible that the process can be driven by the favourable free-energy change from extensive hydrophobic interaction. Potentiometric titration of the proteins in the membrane of *Halobacterium halobium* (Brown, 1965) showed that about half of the amino groups did not titrate normally, but there is little evidence available to show if this happens commonly. The experiments described in the present paper provide a method for labelling such groups and determining their distribution. Although it is possible that some of the differential labelling observed with the two reagents may have resulted from a difference in conformation of the protein between pH 6.5 and 9.2, there is no independent evidence (e.g. from loss of catalytic activity) for conformational changes in this pH region.

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