Cross-Linking Experiments with the Adenosine Triphosphatase of Sarcoplasmic Reticulum

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The proteins of sarcoplasmic reticulum were cross-linked by rapid oxidation of thiol groups with I_2 . About two-thirds of the thiols were oxidized without any significant cross-linking, implying an extensive formation of intramolecular disulphide bonds. When the thiols were completely oxidized at room temperature a series of oligomers containing up to five molecules were observed, as well as large aggregates which were excluded from the gels. Complete oxidation at -10°C left most of the ATPase (adenosine triphosphatase) as monomer. Similar results were obtained when copper–phenanthroline complexes or dimethyl suberimide were used as cross-linking reagents. We conclude that most of the cross-linked species arise by linking of randomly colliding ATPase molecules which are present in the membrane at very high concentration.

Evidence from several independent experimental approaches has suggested an oligomeric structure for the ATPase of sarcoplasmic reticulum, but none of these is conclusive if taken independently. Electron micrographs of freeze-fractured preparations show approximately one particle for four molecules of ATPase (Jilka *et al.*, 1975; Scales & Inesi, 1976). However, the significance of such particles at the molecular level is not yet clear. In contrast, negative staining showed approximately one projection per molecule. Gel-filtration experiments in Tween 80 (Le Maire *et al.*, 1976a) showed that most of the ATPase activity was associated with an approximately tetrameric aggregate. In deoxycholate, in the presence of sucrose and phospholipid, the enzymic activity is associated with monomeric ATPase (Le Maire *et al.*, 1976b). These contrasting results suggest that detergent–lipid or detergent–protein as much as protein–protein interactions may determine the state of aggregation.

Cross-linking experiments have stabilized various aggregates, including dimers (Giotta, 1976), tetramers (Murphy, 1976) and a range of oligomers (Louis & Shooter, 1972; Louis *et al.*, 1977; Chyn & Martonosi, 1977). It is difficult to draw firm conclusions because of the high concentration of ATPase in the membrane. The mean distance between molecules of monomeric ATPase of about 7 nm is equivalent to a bulk

concentration of about 50 mg/ml. The time required to diffuse through this distance would be about $1\ \mu\text{s}$ at 25°C (Poo & Cone, 1974). Since the published cross-linking experiments involve relatively slow reactions, such as oxidation with copper–phenanthroline complexes or amidine formation, a large contribution from randomly colliding molecules to cross-linked species is likely. In aqueous solution intermolecular cross-linking of aldolase by dimethyl suberimide becomes significant at a protein concentration of 5 mg/ml (Davies & Stark, 1970). These problems have been analysed in detail (Hajdu *et al.*, 1976; Kiehm & Ji, 1977). Since there is no way to dilute the system without disrupting the membrane structure, we have attempted to minimize the effect of random collision by using rapid oxidation with I_2 and by lowering the temperature to -10°C to increase the viscosity of the lipid. We have also repeated some of the earlier experiments at low temperature.

Materials and Methods

Dimethyl suberimide was prepared from suberonitrile (Davies & Stark, 1970). Myosin cross-linked by dimethyl suberimide was a gift from Dr. W. B. Gratzer (Department of Biophysics, King's College, London). 1,10-Phenanthroline and suberonitrile were from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Sarcoplasmic reticulum was prepared as described by Thorley-Lawson & Green (1973). The preparation was stored at -70°C in the presence of $2\ \mu\text{g}$ of the antioxidant butylated hydroxytoluene/ml. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was carried out as described by Thorley-Lawson & Green (1973) at a polyacrylamide concen-

Abbreviation used: ATPase, adenosine triphosphatase.

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tration of 5%. Protein bands were stained with Coomassie Brilliant Blue and scanned in a Pye-Unicam SP.1800 spectrophotometer with gel-scanning attachment. The protein in each peak was estimated by tracing the curves and weighing the excised peaks. Since equal amounts of protein were applied to each gel, it was possible to determine the proportion of protein that did not enter the gel by summing the areas under the individual peaks and subtracting this from the total area found with a control gel. In other experiments the protein concentration was calculated from $A_{280}^{1\%} = 1.2$ (Thorley-Lawson & Green, 1977).

Cross-linking with dimethyl suberimidate

Sarcoplasmic reticulum was resuspended at 4.7mg/ml in 1M-sucrose/3mM-CaCl₂/0.3M-triethanolamine/HCl, pH 8.5. For experiments at room temperature (20°C) this solution was made 15mM in dimethyl suberimidate. For experiments at -10°C the suberimidate concentration was raised to 156mM. Control experiments showed that this concentration was sufficient to cross-link aldolase to dimers at -10°C. The temperature was maintained at $-10 \pm 1^\circ\text{C}$ in an ice/salt mixture. The reaction was terminated by adding sufficient 10% (w/v) sodium dodecyl sulphate to give a final concentration of 2% and the resulting solution was heated to boiling.

Cross-linking with iodine

Sarcoplasmic-reticulum vesicles were resuspended

at 4mg/ml in 1.0M-sucrose/0.1M-Tris adjusted to pH 7.0 with maleic acid. To this was added an equal volume of 1M-sucrose in 0.1M-KI. Small volumes of a solution of 50mM-I₂ in 0.1M-KI were injected rapidly from a Hamilton syringe to give final I₂ concentrations of 100, 200, 300 or 400 μM . The solutions were shaken vigorously after addition of I₂ until the colour was discharged (about 30s at -10°C). Residual thiol groups were determined by the method of Ellman (1959) in 0.3% sodium dodecyl sulphate/60mM-Tris/HCl, pH 8.4. For experiments involving phosphorylated ATPase the vesicles were resuspended at 4mg/ml in 1.0M-sucrose/0.1M-KI to which was added an equal volume of 1M-sucrose/0.5mM-CaCl₂/3mM-MgCl₂/0.5mM-ATP/0.1M-Tris/maleate, pH 7.0. The phosphorylation reaction was allowed to proceed at room temperature for 30s before addition of I₂. In the experiments at -10°C, the solution was cooled in an ice/salt bath before addition of I₂.

Cross-linking with copper-phenanthroline

This was carried out as described by Murphy (1976). To 1ml of sarcoplasmic reticulum (2mg/ml) in 1mM-CaCl₂/0.1M-morpholinopropanesulphonate buffer, pH 7.2, was added 5mM-CuSO₄ (0.02ml) and 15mM-1,10-phenanthroline (0.02ml). The mixture was incubated at either room temperature or 0°C. Samples were taken at intervals, the reaction was terminated by addition of an equal volume of *N*-ethylmaleimide (15mM in 2mM-EDTA/2% sodium dodecyl sulphate) and the products were separated by electrophoresis in 5% acrylamide gels. Parallel

Fig. 1. Sarcoplasmic reticulum oxidized by I₂

Sarcoplasmic-reticulum vesicles (4mg/ml) in 1M-sucrose/0.1M-KI at pH 7.0 (280 μM -thiol) were oxidized by injection of I₂ to the final concentrations indicated. For gels 6-9 the reaction was performed at -10°C. The iodine concentrations were: (1) 0, (2) 100 μM , (3) 200 μM , (4) 300 μM , (5) 400 μM , (6) 100 μM , (7) 200 μM , (8) 300 μM , (9) 400 μM . Markers for molecular weight are shown in Fig. 2.

samples were taken for determination of residual thiol groups as described above.

Results

In the first experiments the work of Louis & Shooter (1972) using dimethyl suberimidate was repeated and similar results were obtained. The gels showed oligomers decreasing in amount from dimer to hexamer. After long reaction times large polymers were found which did not enter the gel. Under all conditions there was more of the residual monomer than of any single oligomeric species. At -10°C the proportion of monomer was greater and the amount of large polymer decreased. Addition of Triton X-100 to a concentration of 1% prevented cross-linking by the dimethyl suberimidate.

The results of cross-linking by oxidation with I_2 are shown in Fig. 1 and Table 1. The smallest oligomeric species had the same mobility as monomeric myosin (see Fig. 2) (mol.wt. 210000) and was therefore a dimer. The dimer was preceded on the gels by progressively smaller amounts of trimer, tetramer and pentamer; large polymers accumulated at the surface of the gel. At -10°C less oligomer was present and no large polymer was formed. Table 1 shows that the extent of oxidation was dependent only on the I_2 concentration and not on the temperature. The first addition of I_2 oxidized more than 1 mol of thiol/mol of I_2 , indicating little consumption of I_2 in side reactions with tyrosine, histidine or unsaturated lipids.

At room temperature the extensive cross-linking of the ATPase to large polymer was correlated with the disappearance of the last two or three thiol groups. At -10°C all of the thiol groups could be

oxidized without extensive cross-linking. Repetition of the I_2 -oxidation experiments in the presence of ATP, Ca^{2+} and Mg^{2+} gave results which did not differ significantly from those in Fig. 1.

The results of oxidation with Cu^{2+} -phenanthroline (Fig. 2) showed proportions of oligomers similar to those observed with I_2 at room temperature, but there was less of the large polymer. Even after 3 h exposure to the reagent five thiol groups remained unoxidized. These correspond in number to the membrane-buried thiol groups previously observed (Thorley-Lawson & Green, 1977) and their resistance to oxidation may be correlated with the lower amount of large polymer. In none of our experiments did we see the preferential formation of the tetrameric species reported by Murphy (1976). We did not observe cross-linking of any protein other than the ATPase.

Cross-linking after tryptic cleavage of the ATPase

The observation that oxidation of about two-thirds of the thiol groups produced only small amounts of oligomer suggested that most of the disulphide bonds formed at this stage were intramolecular. It was of interest to know whether or not these bonds were also confined within the three major tryptic fragments of the molecule or whether they linked these fragments together. Digestion of sarcoplasmic reticulum with trypsin cleaves the ATPase to fragments A (mol.wt. 60000) and B (mol.wt. 55000), followed by slower cleavage of fragment A to subfragments A_1 (mol.wt. 33000) and A_2 (mol.wt. 24000). Each of these fragments contains several thiol groups (Thorley-Lawson & Green, 1977), so that a variety of cross-linked species is imaginable. The results of oxidation with I_2 after cleavage with trypsin are

Table 1. Effect of I_2 on the aggregation state and thiol content of sarcoplasmic-reticulum ATPase

Cross-linking was performed as described in Fig. 1. The products were dissolved in sodium dodecyl sulphate and separate samples were fractionated on 5% polyacrylamide gels and analysed for residual thiol content. The large polymers which did not enter the gel were estimated by scanning the areas under individual peaks and subtracting these from the total area found for the unoxidized sample. The results, expressed as a percentage of the total ATPase on the gel, are means of three experiments at 20°C and two experiments at -10°C . The range in percentage area was $\pm 10\%$ of the area for bands containing over 30% of the loaded protein, but was greater for the lower-intensity bands ($\pm 15\%$ at 20°C , $\pm 30\%$ at 5°C).

Temperature ($^{\circ}\text{C}$)	Concn. of I_2 (μM)	Thiol content (mol/ 10^5 g)	Oligomers, $n =$					Polymer
			1	2	3	4	5	
20	0	14	98	2	0	0	0	0
	100	6.4	61	14	9	5	2	10
	200	3.0	42	18	13	8	4	16
	300	1.8	14	10	9	7	5	55
	400	0	15	11	9	7	5	54
-10	100	5.6	68	17	9	4	2	0
	200	2.6	52	21	12	6	3	0
	300	n.d.	53	26	11	6	4	0
	400	0	55	25	10	5	4	0

Fig. 2. *Sarcoplasmic reticulum oxidized by copper-phenanthroline complex*

Vesicles were oxidized for the indicated temperatures and times. At 20°C: (1) zero time, (2) 1 min, (3) 5 min, (4) 20 min, (5) 40 min, (6) 60 min; at 0°C: (8) 10 min, (9) 40 min, (10) 60 min, (11) 180 min. Gel (7) shows monomers and dimers of myosin as markers for molecular weight.

Fig. 3. *Sarcoplasmic reticulum oxidized with I_2 after cleavage with trypsin*

Sarcoplasmic reticulum (5.3 mg/ml) in 1 M-sucrose/4 mM- $CaCl_2$ /20 mM-Tris/maleate, pH 7.0, was digested with 1 mg of trypsin for 10 min. The vesicles were pelleted at 165 000g (20 min), resuspended in 1 M-sucrose/0.1 M-Tris/maleate (pH 7.0)/0.1 M-KI to a concentration of 2 mg/ml and oxidized with I_2 as for Fig. 1 at 20°C. (a) Undigested control. (b) 100 μ M- I_2 . (c) 200 μ M- I_2 . (d) 300 μ M- I_2 . (e) 500 μ M- I_2 .

shown in Fig. 3. The digestion conditions used left no intact ATPase and partially cleaved fragment A to subfragments A_1 and A_2 . None of the fragments was cross-linked until sufficient I_2 was added to oxidize more than 80% of the thiol groups. Complete oxidation gave four high-molecular-weight bands at the expense of the fragments A, A_1 and B. Fragment A_2 appeared undiminished, but since it overlapped a band present in the undigested sarcoplasmic reticulum it was not possible to conclude with certainty that it was not cross-linked. The main conclusion to be drawn is that initially most of the disulphide bonds formed were between neighbouring cysteine residues within a fragment. Only the last few to be oxidized gave interfragment links. Since it was at this stage of the oxidation that dimers and trimers of the whole peptide chain appeared (Table 1), it is likely that the linked fragments were derived from different molecules.

Discussion

Over 95% of the thiol groups of sarcoplasmic reticulum are contributed by the Ca^{2+} -activated ATPase, containing about 20 thiol groups per molecule, of which one-third are unreactive (Murphy, 1976; Thorley-Lawson & Green, 1977). Our results showed relatively little cross-linking when the more reactive groups were oxidized, even at room temperature, showing that these thiol groups readily form intramolecular disulphide bonds. Furthermore, the experiment with sarcoplasmic reticulum in which

the ATPase had been cleaved with trypsin showed that these disulphides were confined within each major tryptic fragment. Only when the less-reactive thiol groups were oxidized were cross-linked molecules or fragments observed.

The progressively decreasing amounts observed of each successive oligomer suggested that random cross-linking of colliding monomers had occurred. The results of the experiments at room temperature were not significantly different from those obtained by other workers, except for those of Murphy (1976), which we and others (Chyn & Martonosi, 1977; Louis *et al.*, 1977) have been unable to repeat. However, all previous experiments showing aggregated species were taken to support an oligomeric structure for the ATPase, although a recent paper (Chyn & Martonosi, 1977) did consider the possibility that they might have arisen by random collisions during the cross-linking reaction. Since no specific oligomer has been shown to predominate, and, since our results show a marked decrease in cross-linking at a given extent of oxidation when the temperature was lowered to -10°C , we think that the latter explanation of the observed pattern of oligomers is the correct one.

We cannot exclude the possibility that there may be dimers present in equilibrium with monomers and that these dimers are the species required for active transport. However, we can say that the concentration of dimer was not increased in the presence of ATP, Ca^{2+} and Mg^{2+} , which phosphorylate the protein and bring about translocation of Ca^{2+} . We conclude that more convincing evidence is required before an oligomeric structure for the ATPase can be accepted.

It is clear from this discussion that firm conclusions from cross-linking experiments on membrane proteins are not possible when a broad spectrum of oligomers is formed. The use of I_2 as an oxidant in place of the more slowly reacting copper-phenanthroline complex did not significantly affect the overall pattern of the results. The extent of random cross-linking depends on the relative values of the rate of intramolecular reaction of the sulphenyl iodide intermediate and the rate of diffusion of ATPase molecules to appropriate orientation for intermolecular reaction. The rate at which the reactive intermediate is formed from I_3^- and $-\text{SH}$ is not relevant. Although it is not possible to evaluate all the rate

factors in such a complex system, it can be said that the second-order rate constants for reaction of sulphenyl iodide with thiols (Cunningham, 1964) are less than $50\text{M}^{-1}\cdot\text{s}^{-1}$, so that the lack of effect of using the more rapid reaction is not surprising. There are nevertheless advantages in using I_2 , since the reaction time is short and the extent of reaction is readily controlled by the amount of reagent added. The diminished cross-linking at -10°C is more likely to be an effect of high viscosity on diffusion than due to a decreased aggregation, since in other membranes low temperatures cause clustering of intramembraneous proteins rather than the reverse (e.g. Hochli & Hackenbrock, 1976).

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