The Reactivity of the Thiol Groups of the Adenosine Triphosphatase of Sarcoplasmic Reticulum and their Location on Tryptic Fragments of the Molecule

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The ATPase (adenosine triphosphatase) from sarcoplasmic reticulum contains 20 thiol groups/115000 daltons, measured by using either *N*-ethyl[¹⁴C]maleimide or 5,5'-dithio-bis-(2-nitrobenzoate) in sodium dodecyl sulphate. After reduction there were 26 thiol groups, in good agreement with 26.5 residues of cysteic acid found by amino acid analysis. The difference between this and the 20 residues measured before reduction implies the presence of three disulphide residues. The same number of disulphide residues was found by direct measurement. Three to six fewer thiol groups were found in preparations made in the absence of dithiothreitol. The missing residues were accounted for as cysteic acid. The distribution of disulphide bonds and of exposed and buried thiol groups among the tryptic fragments of the molecule was measured after labelling with *N*-ethyl[¹⁴C]-maleimide. The disulphides were confined to fragment B (mol.wt. 55000), whereas several thiol groups were present on each of the fragments (A, B, A₁ and A₂). The kinetics of the reaction of the ATPase with 5,5'-dithiobis-(2-nitrobenzoate) showed that four or five of the thiol groups were unreactive in the absence of detergent and that 13 of the remainder reacted with a single first-order rate constant. In the presence of ATP and Ca²⁺ the reaction rate of all but two groups of this class was uniformly decreased. In the presence or absence of ATP and Ca²⁺ the rate constant for inactivation was close to the rate constant for this class, but was not identical with it. No selective protection of a specific active-site-thiol group was observed. Parallel experiments with sarcoplasmic reticulum gave similar results, except that the reaction rates were a little lower and there were two more buried groups. Solution of ATPase of sarcoplasmic reticulum in detergent greatly increased the reactivity of all thiol groups. The effects of low concentrations of deoxycholate were reversible. EGTA or low concentrations (0.02 mM) of Ca²⁺ or Mg²⁺ had very little effect on the reactivity.

The Ca²⁺-transporting ATPase† of sarcoplasmic reticulum is a protein of mol.wt. 115000. Part of it projects from the membrane, giving a characteristic appearance in electron micrographs of negatively stained preparations (Hardwicke & Green, 1974; Stewart & MacLennan, 1974), and the remainder is buried in the membrane, giving rise to the intramembranous particles seen in freeze-fractured preparations (MacLennan *et al.*, 1971). Trypsin cleaves the molecule in two stages to give fragment A (mol.wt. 60000) and fragment B (mol.wt. 55000), and then fragment A is cleaved to give fragments A₁ (mol.wt. 33000) and A₂ (mol.wt. 24000), (Thorley-Lawson & Green, 1973; Migala *et al.*, 1973). We introduce this nomenclature for convenience of reference and because similar fragments obtained by Stewart *et al.* (1976) have been assigned different molecular

weights, so that nomenclature based on molecular weight is confusing. Both our experiments (Thorley-Lawson & Green, 1973) and those of Stewart & MacLennan (1974) suggested that the B fragment was the more extensively associated with the membrane. However, the difficulty that we experienced in releasing any of these fragments from the intact membrane suggests that each has a segment buried in the membrane (Thorley-Lawson & Green, 1975).

The ATPase was shown by amino acid analysis to contain about 26 cysteine residues (MacLennan, 1970; Thorley-Lawson & Green, 1973), which provide useful markers for our present work on the structure of the molecule (Allen & Green, 1976). In the present paper we have determined the distribution of cysteine and cystine residues between the tryptic fragments of the molecule. We have also measured their reactivity towards Nbs₂ under a variety of conditions.

Measurements of the free-thiol-group content of purified ATPase have not been reported. However,

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† Abbreviations: ATPase, adenosine triphosphatase; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoate).

since the ATPase accounts for about 70% of the protein of sarcoplasmic reticulum (Thorley-Lawson & Green, 1973; Meissner & Fleischer, 1974), the whole membrane should contain at least 16 thiol groups/ 10^5 daltons if there are no disulphide bonds. Previous work, in the absence of detergent (Hasselbach & Seraydarian, 1966), accounted for only one-half of these, but more recent work (Panet & Selinger, 1970; Louis & Shooter, 1972) has shown up to 14 thiol groups in the presence of sodium dodecyl sulphate. The results are still low and might indicate the presence of disulphide bonds in the ATPase. The difference might also arise in part from the different methods used to determine protein concentration and we have therefore compared some of these.

Materials and Methods

Sodium deoxycholate was from E. Merck (Darmstadt, Germany). *N*-Ethyl[14 C]maleimide was from The Radiochemical Centre (Amersham, Bucks., U.K.). Dithiothreitol, Nbs_2 , EGTA, disodium ATP (grade I), β -NADH (grade III), trisodium phosphoenolpyruvate, pyruvate kinase (type II) and lactate dehydrogenase (type X) were all from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Trypsin (twice-crystallized) was from Worthington Biochemicals (Cambrian Chemicals, Croydon CR9 3OL, U.K.). Bovine serum albumin (crystalline) was from Armour Laboratories (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.). The Ca^{2+} ionophore, A23187, was a gift from Dr. R. L. Hamill (Eli Lilly Research Laboratory, Indianapolis, IN, U.S.A.). Acrylamide and methylenebisacrylamide were recrystallized (Loening, 1967) and their solutions were filtered before use. Other reagents were of analytical grade.

Preparation of sarcoplasmic reticulum and of the ATPase

Sarcoplasmic reticulum was prepared by a modification of the method of Martonosi (1968) as previously described (Thorley-Lawson & Green, 1973). In most of the preparations, dithiothreitol (1 mM) was added to the buffers used, except for the final sucrose-gradient fractionation, which served to remove this reagent from the product.

The ATPase was purified by the method of MacLennan (1970) and reconstituted into vesicles (Hardwicke & Green, 1974), by using a coarse ion-exchange resin [Zerolit FF-1P (SRA 62), 2–3% cross-linked, 30–50 mesh (BDH, Poole, Dorset, U.K.)], previously equilibrated with MacLennan's (1970) buffer B [0.66 M-sucrose, 50 mM-Tris/HCl, pH 8.0, 1 mM-histidine, to which was added 0.1 vol. of ammonium acetate solution (20 g of ammonium acetate dissolved in 27 ml of water)]. The vesicles were con-

centrated by centrifugation (100000g, 30 min) after dilution of the effluent with an equal volume of water to decrease its density. Dithiothreitol (1 mM) was added to the last preparation and was removed during the revesiculation by the ion-exchange resin used to remove deoxycholate.

The ATPase [60 mg in 3 ml of MacLennan's (1970) buffer B containing 1% sodium deoxycholate] was delipidated by gel filtration on a column (1.5 cm $\times$ 90 cm) of Sephadex G-50 in 1% sodium deoxycholate (Hardwicke & Green, 1974). It proved necessary to work at a higher pH (20 mM-Tris/HCl buffer, pH 9.0) than that used previously to avoid precipitation after removal of deoxycholate, because of the higher protein concentration used in these experiments. The pooled fractions from the void volume were dialysed overnight against the same Tris buffer and run through a column (10 ml) of Zerolit FF-1P (50–100 mesh) equilibrated in the same buffer, to remove the remaining deoxycholate. The recovery of protein was 36 mg in 9 ml of buffer. For nitrogen analyses this solution was dialysed against 2 mM-Tris, and samples of the equilibrating buffer were used for control analyses.

Nitrogen determination

Protein nitrogen was determined in triplicate, after digestion in H_2SO_4 (conc.) by the ninhydrin method (Jacobs, 1962). Amide nitrogen was also measured on triplicate samples from two different preparations by the ninhydrin method after hydrolysis in conc. HCl for 10 days at 37°C (Wilcox, 1967).

Protein determination

ATPase protein was determined from the A_{280} in 1% sodium dodecyl sulphate ($A_{1\text{cm}}^{1\%} = 12.0$; Hardwicke & Green, 1974). Sarcoplasmic-reticulum protein was determined in the same way by using $A_{1\text{cm}}^{1\%} = 10$. These factors were correlated with colour yields from Lowry *et al.* (1951) and micro biuret reactions (Goa, 1953), determined from the slopes of the calibration curves. Sodium dodecyl sulphate (0.5%) was added to the alkaline component of the Lowry reagent (Dulley & Grieve, 1976). The biuret reagent was prepared from Benedict's reagent (Goa, 1953) (5 ml) and 2.5 M-NaOH (30 ml). The protein in 0.6 ml was added to the biuret reagent (0.3 ml). Sodium dodecyl sulphate [0.1 ml of a 5% (w/v) solution] was added after 15–20 min and the A_{333} measured. A calibration curve with bovine serum albumin was linear with respect to concentration up to at least 0.4 mg/ml ($A_{333} = 0.62$). The sodium dodecyl sulphate was added after colour development, rather than before, to minimize the risk of crystallization in the presence of 1 M-sodium salts. It did not affect the colour yield, and any crystallization could be reversed by warming.

Amino acid analyses

Amino acid analyses were carried out on a Bio-Cal BC 100 automatic analyser. To determine cysteic acid after reduction and treatment with an excess of *N*-ethylmaleimide, as described below, the samples were dialysed against water overnight to remove reactants and then freeze-dried in Pyrex-glass tubes (0.7 cm × 10 cm). To the solid was added 0.5 ml of constant-boiling HCl containing 0.1% phenol and the tubes were evacuated and sealed. The protein was then hydrolysed for 24 h at 110°C.

Analysis of labelled tryptic fragments

After digestion of the ATPase with trypsin under the appropriate conditions for single or double cleavage (Thorley-Lawson & Green, 1973), the vesicles were centrifuged and the pellet was dissolved in 2% sodium dodecyl sulphate/50 mM-Tris/HCl (pH 7.0)/1 mM-EDTA, to give a protein concentration of 4 mg/ml (about 0.8 mM in thiol groups). A fivefold excess of *N*-ethyl[¹⁴C]maleimide (4.8 mCi/mmol) was transferred to a reaction tube in pentane, the solvent was removed with a stream of N₂ and the solution of cleaved protein (0.1 ml) was immediately added. After 15 min a small volume of aq. 80% (w/v) sucrose was added and a sample containing 20–40 µg of protein was loaded into 7.5% polyacrylamide gels and subjected to electrophoresis as described below.

Disulphide bonds were labelled in the same way after alkylation of thiol groups with unlabelled *N*-ethylmaleimide under the conditions described above, followed by passage of the reaction mixture (1 ml) through a column (1 cm × 10 cm) of Sephadex G-10 in the same buffer. The protein in the void volume was reduced with 2 mM-dithiothreitol (30 min at 25°C) and a sample (0.1 ml) was alkylated with a fivefold excess of *N*-ethyl[¹⁴C]maleimide over total thiol groups.

Buried thiol groups were labelled by a modification of this procedure. The vesicles obtained after cleavage with trypsin (5 mg/ml in 0.1 M-Tris/HCl, pH 8.0) were treated with unlabelled *N*-ethylmaleimide (5 mM) for 30 min at 25°C. Excess of reagent was removed with *N*-acetylcysteine (10 mM) followed by passage through a column (0.5 cm × 3 cm) of Dowex 2 (Cl⁻ form; 30–50 mesh). The effluent, containing no free thiol groups was assayed for buried thiol groups with Nbs₂ and labelled with *N*-[¹⁴C]ethylmaleimide in sodium dodecyl sulphate as described above.

Gel electrophoresis

Proteolysis products were separated in sodium dodecyl sulphate on 7.5% polyacrylamide gels as previously described (Thorley-Lawson & Green, 1973), with a longer period (6 h) of electrophoresis

to increase the separation of fragments A and B. The initial pH of the gel buffer was 7.8 and that of the reservoir was 7.3. The gels were stained with Coomassie Blue, scanned on a Unicam SP.1800 spectrophotometer, sliced and counted for radioactivity as previously described for ³²P-labelled samples (Thorley-Lawson & Green, 1973). The relative yields of the fragments were determined from the area under the stained peaks and the thiol content was calculated from the radioactivity.

Determination of thiol groups with N-ethyl[¹⁴C]-maleimide

Reconstituted vesicular ATPase was dissolved at a concentration of 4 mg/ml in 2% sodium dodecyl sulphate/50 mM-Tris/HCl (pH 7.0)/1 mM-EDTA. If the protein was to be reduced before determination, 2 mM-dithiothreitol was also present and the solution was heated at 100°C for 5 min. The solution was then added to a fivefold molar excess of *N*-ethyl[¹⁴C]-maleimide (4.8 mCi/mmol) and was left for 15 min at 25°C. The solution was diluted with 4 vol. of water and dialysed for 48 h with two changes against a large excess of 0.1% sodium dodecyl sulphate in water. Protein-bound radioactivity was then measured in a Beckman LS-133 liquid-scintillation counter in 5 ml of scintillating fluid consisting of 750 ml of toluene, 250 ml of 2-methoxyethanol and 4.9 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen. The efficiency of counting, estimated from standards, was 82%.

Determination of thiol groups with Nbs₂

Reactive thiol groups were measured by the method of Ellman (1959) in samples of sarcoplasmic reticulum (0.5 mg) or vesicular ATPase (0.3 mg) in 50 mM-Tris/HCl, pH 8.4 (0.9 ml), by adding 10 mM-Nbs₂ (0.1 ml). The reference cell contained the same concentration of Nbs₂. The reaction was followed in a recording spectrophotometer (412 nm) and the thiol content was calculated from the steady reading, which was attained in about 30 min ($\epsilon_{412} = 13600$). Measurements of A_{500} showed that there were no changes of turbidity during the reaction. The total thiol groups were determined in the same way, by including sodium dodecyl sulphate (1%, w/v) in the reaction mixture. The protein concentration was calculated from the u.v. spectrum of a parallel sample in the absence of Nbs₂. The concentration of buried thiol groups was measured by difference.

The total cystine-plus-cysteine content was measured after reduction of the ATPase [2 mg/ml in 50 mM-Tris/HCl (pH 8.4)/1% sodium dodecyl sulphate] with dithiothreitol (2 mM) for 30 min at 25°C. The excess dithiothreitol was removed by rapid passage of a sample through a column (1 cm × 12 cm) of Sephadex

G-10, and the u.v. spectrum and thiol content of the pooled protein fractions were measured as described above.

The kinetics of the reaction with 0.5 mM-Nbs₂ at 25°C were followed at 420 nm in the same buffer system. Reactions were also performed in the presence of ATP (6 mM) and CaCl₂ (0.02 mM), added 1 min before the Nbs₂. No Mg²⁺ was added, so that hydrolysis of the ATP during the reaction was minimized without preventing phosphorylation of the protein (Inesi *et al.*, 1970). Since the Nbs₂ was in a large excess, each class of thiol groups should contribute an exponential term to the rate curve. The results were plotted semi-logarithmically and analysed in terms of two or three exponentials by subtraction of the extrapolated slow components from the overall reaction.

ATPase activity

This was determined by measuring oxidation of NADH in a coupled assay system (Neet & Green, 1977). To follow the rate of inactivation by Nbs₂, samples of the reaction mixtures described above containing about 20 µg of protein were added to the assay solution (0.9 ml) and the rate of decrease of A₃₄₀ was measured at 25°C. After an accurate rate had been measured, EGTA (0.01 ml; 0.1 M) was added to the cuvette. The resulting basal ATPase activity was subtracted from the total activity to give the Ca²⁺-dependent rate. In measurements on sarcoplasmic reticulum the Ca²⁺ ionophore A23187 (1 µg) was included in each assay to prevent accumulation of Ca²⁺ within the vesicles and thus avoid any complications caused by changes in permeability after reaction with Nbs₂.

Results

Determination of protein concentration

In previous papers we based protein concentrations on a value of 12.0 for A₂₈₀^{1%}, determined by dry-weight measurements on delipidated ATPase (Hardwicke &

Green, 1974). This procedure was laborious and subsequently gave erratic results. A more reliable value was therefore determined based on the amino acid composition of the ATPase, calculated from the amino acid composition (Thorley-Lawson & Green, 1975) and the amide-nitrogen content. A total of six determinations on two preparations of ATPase gave 0.78 ± 0.06% of amide nitrogen, equivalent to 64 ± 5 residues of amide/molecule. We also redetermined the amino acid composition and found a higher arginine content (45 residues/molecule) as observed by MacLennan (1970) and Meissner & Fleischer (1974). From these results we calculated the nitrogen content to be 16.8%. The value of A₂₈₀/mg of ATPase based on nitrogen analysis (Table 1) agreed well with our previous value. The biuret colour yield for the ATPase based on this factor agreed well with that for bovine serum albumin, whereas the Lowry colour yield was significantly higher. This is not unexpected, since the Lowry colour is a function of the content of tyrosine and tryptophan in the protein. Both the Lowry colour and the A₂₈₀ factor were lower for the mixed proteins of whole sarcoplasmic reticulum. For this mixture we used the biuret colour as the primary standard assuming the same factor as for bovine serum albumin and ATPase.

We conclude that different A₂₈₀ factors should be used for ATPase and sarcoplasmic reticulum and that the Lowry method slightly overestimates the protein content of solutions of ATPase.

The analytical values for cysteine and cystine content given below are based on an A₂₈₀^{1%} of 12.0 and a mol.wt. of 115000 for the ATPase (Thorley-Lawson & Green, 1973). Although this molecular weight is higher than the values obtained by other workers using gel electrophoresis, it agrees well with that determined by sedimentation equilibrium (Rizzolo *et al.*, 1976).

Cysteine and cystine content of ATPase

The ATPase preparations used in the early part of the present work were prepared without any added

Table 1. *Factors for determination of protein content of ATPase and sarcoplasmic reticulum*

The factors for sarcoplasmic-reticulum proteins were based on the calibration curves of the biuret reaction in 0.5% sodium dodecyl sulphate (measured at 333 nm relative to bovine serum albumin). The A₂₈₀ factors were determined on two separate ATPase preparations.

	ATPase	Sarcoplasmic reticulum
A ₂₈₀ /mg of N per ml	7.01 7.14	—
A ₂₈₀ /mg of protein per ml	1.18 1.20	0.99 ± 0.02 (four preparations)
$\frac{A_{330}/\text{mg of protein per ml}}{A_{330}/\text{mg of albumin per ml}}$ } (biuret)	1.02	1.00 (assumed)
$\frac{A_{750}/\text{mg of protein per ml}}{A_{750}/\text{mg of albumin per ml}}$ } (Lowry)	1.06	0.96

Table 2. *Cysteic acid and thiol content of the ATPase*

ATPase(O) was prepared in the absence of dithiothreitol. The cysteic acid analysis of the ATPase performed after oxidation with performic acid is taken from Thorley-Lawson & Green (1975). The corresponding analyses of preparations treated with *N*-ethylmaleimide were without performic acid oxidation and followed reduction with dithiothreitol in sodium dodecyl sulphate. All values are in groups/115000 daltons.

	ATPase			ATPase(O)			ATPase(O)*		
	Non-reduced	Reduced	Difference	Non-reduced	Reduced	Difference	Non-reduced	Reduced	Difference
<i>N</i> -Ethyl[¹⁴ C]maleimide	20.0	25.8	5.8	13.7	20.8	7.1	16.7	23.3	6.6
Nbs ₂	20.2	24.5	4.3	13.9	—	—	17.0	20.7	3.7
Cysteic acid									
(a) After performic acid				26.5					
(b) After <i>N</i> -ethylmaleimide		0.7			6.5			2.9	

* This preparation, containing an intermediate number of thiol groups, was obtained when the whole purification procedure was carried out in a single day.

thiol compound. Since it was found that the thiol content was raised by addition of dithiothreitol or *N*-acetylcysteine to the extraction buffer, the ATPase must have lost thiol groups by oxidation, and these preparations are referred to as 'ATPase(O)'. Their specific ATPase activity was only about 10% lower than that of preparations made in the presence of dithiothreitol (7 μ mol of ATP hydrolysed/min per mg at 25°C). Unless otherwise indicated the preparations of ATPase and sarcoplasmic reticulum were made with the addition of dithiothreitol, which was removed during the final stage of the preparation. Storage for up to 1 week at 4°C led to loss of less than 5% of the thiol content, showing that after purification the rate of loss of thiol groups was greatly diminished.

ATPase preparations were analysed for cysteine before and after reduction. Labelling with *N*-ethyl[¹⁴C]maleimide showed that the reduced ATPase contained 26 thiol groups/115000 daltons, whereas Nbs₂ gave a slightly lower result (Table 2). The non-reduced ATPase contained between four and seven fewer thiol groups.

Parallel sets of analyses on two samples of ATPase(O) showed about six fewer thiol groups in both reduced and non-reduced preparations. This shows that thiol groups disappeared during purification in the absence of dithiothreitol and that they were not regenerated by reduction. This suggests that they were oxidized to cysteic acid, since other partially oxidized intermediates would have regenerated cysteine when treated with dithiothreitol. A third preparation of ATPase(O), made rapidly (1 day instead of 2), had lost only three thiol groups.

The difference between the reduced and non-reduced forms of all the ATPase preparations confirmed the presence of either two or three disulphide

bonds. It is possible that the lower value of two disulphide bonds, obtained from analyses with Nbs₂, arose from losses of thiol groups by oxidation during separation of the reduced protein from excess dithiothreitol on columns of Sephadex G-10. This step was necessary only for the reduced proteins, and only these preparations showed significant discrepancies between the analyses with *N*-ethylmaleimide and those with Nbs₂. Taking the value of 26.5 cysteic acid residues as given by amino acid analysis as the most reliable value for total cysteine, we conclude that there are most probably three disulphide residues in the molecule.

Further evidence for the fate of the thiol groups that were lost during purification was obtained by analysis for cysteic acid in reduced preparations that had been treated with *N*-ethylmaleimide before hydrolysis in the presence of phenol to minimize oxidation. Under these conditions only ATPase(O) gave a significant amount of cysteic acid; there was sufficient to account for the loss of thiol groups during purification (Table 2).

Distribution of thiol groups and disulphide bonds between the tryptic fragments of the ATPase

The distribution of radioactivity on sodium dodecyl sulphate/polyacrylamide gels after labelling of thiol groups of singly and doubly cleaved ATPase is shown in Fig. 1. It should be noted that the second cleavage does not go to completion (Thorley-Lawson & Green, 1973), so that about one-third of the original fragment A remains in Fig. 1(b).

From these results combined with the protein distribution determined on the same gels we calculated the number of thiol and disulphide residues on each fragment. The results are compared in Table 3 with the cysteic acid contents of the performic acid-oxidized

fragments determined previously (Thorley-Lawson & Green, 1975). Only fragment B gave significantly more cysteic acid than free thiol groups. The difference of seven residues confirms the presence of three disulphide bonds in the ATPase and shows that all

three are located on fragment B. This conclusion was confirmed by the results from the preparation in which the thiol groups were blocked before reduction (Fig. 2).

The distribution of the thiol groups which disappeared during purification can be deduced from the difference between the results in Fig. 1 and those for ATPase(O) (Fig. 3). Most of these groups were located

Fig. 1. Distribution of thiol groups between the tryptic fragments of ATPase

ATPase, cleaved once (a) or twice (b) with trypsin, was dissolved in 2% sodium dodecyl sulphate/1 mM-EDTA/50 mM-Tris/HCl (pH 7.0) and treated with a fivefold excess of *N*-ethyl[¹⁴C]maleimide. After 15 min samples (20–50 μg of protein) were loaded on polyacrylamide gels and run for 6 h. The gels were stained with Coomassie Blue and scanned, then they were sliced and counted for radioactivity.

Fig. 2. Distribution of disulphide residues between the tryptic fragments of ATPase

ATPase, cleaved once with trypsin, was treated with unlabelled *N*-ethylmaleimide as described in Fig. 1 to block thiol groups. After removal of excess of reagent by gel filtration, the disulphide groups were reduced with 2 mM-dithiothreitol and a sample was labelled with *N*-ethyl[¹⁴C]maleimide. Samples were loaded on to polyacrylamide gels and analysed as described (Fig. 1). ----, Protein (scanned at 565 nm); —, radioactivity.

Table 3. Distribution of cysteic acid and thiol groups between the tryptic-fragments of ATPase

The results for cysteic acid are taken from Thorley-Lawson & Green (1975). The thiol contents were calculated from the relative areas under the radioactive peaks (Figs. 1 and 3) and the total number of thiol groups in the ATPase preparations. Scanning of the stained gels at 565 nm confirmed that equimolar amounts of fragments A and B were produced and that splitting of the A fragment gave equimolar amounts of the A₁ and A₂ fragments. The results are expressed as groups/115 000 daltons of original ATPase.

Fragments from		Fragment ...	A	B	A ₁	A ₂
ATPase (non-reduced)		Mol.wt. ...	60 000	55 000	33 000	24 000
	Total thiol groups		13.1	6.8	9.9	3.2
	Total cysteic acid		13.0	14.0	9	3.5
	Disulphide residues (by difference)		0.1	3.6	0.5	0
	Buried thiol groups		2	2	(1)	(1)
ATPase(O) (non-reduced)						
	Total thiol groups		7.8	6.2	6.6	1.4
	Thiol groups oxidized during isolation (by difference)		5.3	0.6	3.3	1.8

Fig. 3. Distribution of thiol groups between the tryptic fragments of ATPase(O) ATPase(O), cleaved twice with trypsin, was treated as described in Fig. 1.

Table 4. Reactivity of the thiol groups of whole sarcoplasmic reticulum

The basic reaction mixture (1.0ml) contained sarcoplasmic-reticulum vesicles (0.4mg of protein/ml; 50 μ M-thiol groups), 0.05M-Tris/HCl buffer, pH8.4, and 0.5mM-Nbs₂. The reaction was followed at 420nm (ϵ = 13200/mol of thiol groups) at 25°C. The results were analysed in terms of two or three exponentials, as illustrated in Fig. 4. Thiol groups reacting with a half-time <5s were classified as very fast, whereas those that had not reacted after 30–40min were classified as non-reactive. The number of residues (*n*) in each class is expressed per 150000 daltons of protein, equivalent to approx. 1 molecule of ATPase. The total thiol content of this preparation was 20.5 groups/150000 daltons. The pseudo-first-order rate constant (*k*) of each class of groups is given in parentheses.

Additions to reaction	Class of thiol group ...		Very fast		Fast		Medium fast		Slow		Non-reactive
			<i>n</i>		<i>n</i>	10 ³ × <i>k</i> (s ⁻¹)	<i>n</i>	10 ³ × <i>k</i> (s ⁻¹)	<i>n</i>	10 ³ × <i>k</i> (s ⁻¹)	
None							2.1	(33)	11	(1.9)	6.0
0.2mM-EGTA							3.7	(19)	10	(1.9)	5.5
0.02mM-Ca ²⁺							2.5	(27)	11	(1.8)	5.5
0.1% Deoxycholate							2.0	(19)	11	(2.1)	6.0
0.2% Deoxycholate			3.5				2.7	(40)	6.5	(2.2)	1
									5.5	(5.2)	
1% Deoxycholate			7.6	3.8	(120)		4.0	(23)	3.5	(3.2)	
1.0% Sodium dodecyl sulphate			1.4	5.5	(92)		7.9	(26)	4.2	(4.6)	

on fragment A, and less than one was on fragment B (Table 3).

Kinetics of the reaction of thiol groups of sarcoplasmic reticulum with Nbs₂

In the absence of detergents all the reaction mixtures were opalescent (*A*₄₂₀ < 0.5), but since the turbidity monitored at 550nm did not change during the reaction, this presented no problem. The effects of a variety of different conditions on the reactivity of the thiol groups of whole sarcoplasmic reticulum are shown in Table 4. The number of groups in each class and their first-order rate constants were calcu-

lated from semilogarithmic plots as shown in Fig. 4 (the results shown in this Figure are described in the next section). The *A*₄₂₀ reached a plateau and did not increase significantly during at least 30 min, except in the presence of EGTA, when a very slow further increase was observed, equivalent to about 1 thiol group. This very slow-reacting group was included in the class of unreactive groups, determined from the difference between the plateau reading and that obtained in 1% sodium dodecyl sulphate. The reactive groups were divided into four classes, the fastest being those which reacted before measurement could be started (10s after mixing). In the

Fig. 4. Reaction of purified ATPase with Nbs_2 in the presence and absence of substrate

○, ●, Rate of reaction of thiol groups (A_{420}); △, ▲, rate of inactivation. The composition of the reaction mixtures in the absence of substrate (●, ▲) is given in Table 4, with vesicles of purified ATPase in place of sarcoplasmic reticulum. For the other experiments (○, △), ATP (6mM) and $CaCl_2$ (0.02mM) were also present. The two curves (○, ●) should start at $\log A_{420} = -0.167$; they are arbitrarily lowered by 0.1 and 0.2 unit respectively. The inset shows a first-order plot of the faster-reacting thiol groups after subtraction of the contribution of the main set.

Table 5. Effect of substrate on the reactivity of the thiol groups of sarcoplasmic reticulum and of the purified ATPase. The reaction rates were determined and analysed as described in Fig. 4 and Table 4. The rate of loss of Ca^{2+} -dependent ATPase activity was measured on a parallel set of reaction mixtures as described in the text.

Enzyme preparation		Inactivation rate $10^3 \times k$ (s^{-1})	Class of thiol groups ... Medium fast		Slow		Non-reactive n
			n	$10^3 \times k$ (s^{-1})	n	$10^3 \times k$ (s^{-1})	
Sarcoplasmic reticulum (20.5 thiol groups/ 150000 daltons)	—	(2.1)	2.0	(30)	11.5	(1.8)	7.0
	+ATP/ Ca^{2+}	(0.22)	2.8	(20)	10.5	(0.45)	7.2
ATPase (19.5 thiol groups/115000 daltons)	—	(3.7)	1.8	(43)	13.2	(2.1)	4.5
	+ATP/ Ca^{2+}	(0.97)	3.8	(21)	10.9	(0.63)	4.5

absence of detergents there were no very fast groups, but only a large homogeneous class of about 11 slowly-reacting thiol groups and a small class of two groups which reacted about ten times more rapidly.

In the presence of EGTA the number of moderately reactive groups increased by one or two residues, but the rate constant of the main class was not significantly affected. Low concentrations of Ca^{2+} (0.02 mM) had no effect, though higher concentrations (>1 mM) of Ca^{2+} or Mg^{2+} led to a marked acceleration, which was not analysed because of increasingly turbid reaction mixtures. Although most of the present experiments were performed without addition of

Ca^{2+} , it is likely that there were sufficient Ca^{2+} ions present from the original preparation and from glassware to saturate the specific binding site of the ATPase, the dissociation constant of which is $2 \mu M$ (Meissner, 1973).

Low concentrations of sodium deoxycholate (0.1%), insufficient to clarify the vesicle suspension, had little effect on the kinetics, but clarification of the suspension (0.2% deoxycholate) was accompanied by greatly increased reactivity, including reaction of most of the buried thiol groups. At a concentration of 1%, sufficient to remove most of the lipid from the protein (Hardwicke & Green, 1974), there was a

further shift to higher reactivity. The results obtained in 1% sodium dodecyl sulphate were similar to those in deoxycholate, though the rates were lower, probably because of electrostatic repulsion by the large amounts of bound sodium dodecyl sulphate.

Effect of substrate on reactivity of thiol groups and on enzymic activity

In these experiments sarcoplasmic reticulum was compared with the purified ATPase. Results obtained with the ATPase are shown in Fig. 4 and their analysis is compared with a parallel experiment with sarcoplasmic reticulum in Table 5. The two sets of results were similar. In the absence of ATP/Ca²⁺, the only marked difference was the larger number of buried thiol groups in sarcoplasmic reticulum. The rate constants for the ATPase were somewhat higher, but the number of thiol groups in each class was similar. The total thiol content of the sarcoplasmic reticulum could be accounted for if the ATPase constituted 75% of the total protein, which is close to our previous estimate of 70% (Thorley-Lawson & Green, 1973).

In the presence of ATP/Ca²⁺, both preparations were similarly affected. The rate of reaction of the slow thiol groups was decreased by a factor of 3.5–4. One or two groups from the slow class became more reactive and appeared in the medium-fast class. The rate of loss of ATPase activity was also decreased, but with the ATPase the decrease was no greater than that of the rate constant of the slow class. With sarcoplasmic reticulum, protection against inactivation was significantly greater. In neither preparation was there any increase in the number of unreactive thiol groups. In none of these experiments was it possible to obtain more than 5–10% reversal of the inactivation by adding an excess of dithiothreitol (5 mM) either during the reaction or after its completion.

Location of the buried thiol groups

The exposed thiol groups of trypsin-cleaved ATPase were blocked with *N*-ethylmaleimide at pH 8.4. The product contained less than 0.5 residue of free thiol/molecule and 3.8–4.2 residues which reacted with Nbs₂ in sodium dodecyl sulphate. This material was labelled with *N*-ethyl[¹⁴C]maleimide at pH 7.0 in sodium dodecyl sulphate and immediately analysed by gel electrophoresis. Several preliminary experiments showed approximately equal radioactivity in fragments A and B of singly-cleaved ATPase. In doubly cleaved preparations both fragments A₁ and A₂ were labelled. Quantitative analysis of a singly cleaved preparation showed that the ratio of radioactivity in the A and B fragments was 1.05:1, and the ratio of protein was 1.06:1. Each fragment therefore contained two buried thiol groups. The presence of background radioactivity in the

neighbourhood of the A₁ and A₂ fragments from doubly cleaved preparations made it difficult to draw firm conclusions, although qualitatively it appeared that these smaller fragments were equally labelled.

Discussion

Our results show that the low values for thiol content of the ATPase arise partly from oxidation during isolation and partly from the presence of two or three disulphide bonds in the molecule. The irreversibility of this loss and the identification of the product as cysteic acid was unexpected, since oxidation of thiol groups usually yields disulphides. Cysteic acid has been identified as an oxidation product of protein thiol groups in a study of the effects of lipid hydroperoxides on proteins (Little & O'Brien, 1967), and it is possible that hydroperoxides formed from the highly unsaturated lipids of sarcoplasmic reticulum were responsible for our results. However, the low loss of thiol groups on subsequent storage of the ATPase at 4°C suggests that additional factors are involved. The location of the oxidizable groups predominantly on fragment A of the ATPase showed that the reaction was not a random process. These groups were not essential for ATPase activity, since the activity of ATPase(O) was not much decreased.

Our finding of three disulphide residues does not agree with results of Murphy (1976), who found none in sarcoplasmic reticulum. Although our results were obtained mainly with the purified ATPase, we have observed similar numbers of disulphide residues in experiments with sarcoplasmic reticulum. The disulphide bonds were not reducible in the native molecule, but they could be reduced slowly after delipidation with 1% deoxycholate (N. M. Green, unpublished work) as well as rapidly after heating in sodium dodecyl sulphate. The exclusive location of the disulphide bonds on fragment B is of considerable structural interest, since it provides useful markers for this part of the molecule.

Both exposed and buried thiol groups were found on each of the proteolytic fragments. The reversible exposure of the buried groups in deoxycholate suggests that they may be directly associated with the membrane, which would be consistent with the presence of a membrane-associated segment in each fragment. However, since deoxycholate also increases indirectly the reactivity of the exposed groups the effects of detergent on the buried groups may also be an indirect consequence of the dissolution of the membrane. The highest density of exposed groups was on fragment A₁. Three of the ten groups on fragment A₁ have been located in the 31-residue tryptic peptide, which includes the aspartyl phosphate of the active site (Allen & Green, 1976). Most of the thiol groups lost during purification were also on fragment A.

The observation that the overall kinetics of reaction and inactivation of the ATPase were similar to those of sarcoplasmic reticulum shows that the effects of dissolution in deoxycholate during purification were reversed by revesiculation. The purified ATPase could also be redissolved in 0.2% deoxycholate and revesiculated a second time without further change in the kinetics (N. M. Green, unpublished work).

It appears that in the intact membrane the thiol groups are subject to constraints that are removed on dispersal in detergent. It is particularly noteworthy that only in the intact membrane is there a large class which reacts with a common rate constant. This would be consistent with the presence of a critical thiol group the reaction of which triggers the remainder of the class to react. However, this group would have to differ from the thiol group that is critical for activity, since the rate constant for inactivation is not the same as the rate constant for the slow class. Studies (N. M. Green, unpublished work) have shown that this rate constant is not a linear function of the concentration of Nbs₂. Further study of these complexities and of the irreversible changes which accompany reaction of the thiol groups should provide information about conformational states of the ATPase. It would be of interest to know whether the constraints provided by the membrane, which decrease thiol reactivity, are essential to catalytic activity. Although the enzyme can be dissolved in minimal amounts of detergent without irreversible inactivation, this detergent is normally diluted out when samples are added to the assay mixture, so that no information is obtained about activity in the dispersed state. Measurements of activity at high concentration of solubilized enzyme, which avoid dilution of deoxycholate, have shown the activity to be very low (le Maire *et al.*, 1976), so there may be some correlation of ATPase activity with low reactivity of thiol groups.

The similarity of the rate constant for inactivation with that for the slow class of thiol groups is consistent with earlier kinetic evidence that modification of a single thiol group inactivates the ATPase (Hasselbach & Seraydarian, 1966; Panet & Selinger, 1970). However, since all but two members of the slow class are protected by substrate almost as efficiently as is the critical thiol group, the latter cannot be labelled specifically without some more selective reagent. This agrees with results of Martonosi (1976) which show that ATP protects at least seven different cysteine peptides from reaction with *N*-ethylmaleimide. These observations imply that attempts to label the active site after blocking thiol groups in the presence of substrate will give heterogeneous products.

Our kinetic results with sarcoplasmic reticulum are in good general agreement with those of Murphy (1976), bearing in mind that his were obtained at pH 7.8 in 4 mM-Nbs₂. The biggest difference from our

results was his observation of a slower sub-class of groups in the presence of EDTA. We found no evidence for this with 0.5 mM-Nbs₂, but we have observed such a class in 4 mM-Nbs₂. This difference is probably correlated with the non-linear dependence of the rate constants on concentration of Nbs₂. Similar kinetics have also been observed by Anderson & Moller (1977).

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