Reaction of Fluorescein Isothiocyanate with Thiol and Amino Groups of Sarcoplasmic ATPase

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Several model compounds containing thiol and/or amino groups (mercaptoethanol, glutathione, cysteine, ethanolamine, glycine) were studied with respect to their reactivity towards fluorescein isothiocyanate (followed spectrophotometrically at 504 and 412 nm), stability of product and long-wave absorption maximum of the fluorescein residue attached. Thiol groups reacted by far more readily than amino groups. A specific effect was observed with cysteine, indicating an intramolecular transfer of the fluorescein residue from SH to NH_2.

With sarcoplasmic vesicles both types of reactions were observed. The ratio of products, which can be distinguished by their different stabilities and absorption spectra, depended on the absence or presence of detergents. While with native vesicles the NH_2 reaction predominated, with vesicles solubilized with sodium dodecylsulfate, octaethyleneglycol mono-n-dodecyl ether or 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine the SH reaction became prevailing. Already 0.35 mg sodium dodecylsulfate per mg protein were sufficient to give rise to dithiourethane formation exclusively. Excess fluorescein isothiocyanate reacted with several thiol groups of dodecylsulfate-solubilized vesicles. In the presence of ATP binding of fluorescein isothiocyanate to native vesicles was significantly reduced.

Total blockage of the vesicular SH groups with N-ethyl-maleimide led to preparations that reacted with fluorescein isothiocyanate much more slowly, compared to native vesicles. Octaethyleneglycol mono-n-dodecyl ether or 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine in the assay accelerated the thioureide formation from N-ethylmaleimide modified vesicles, whereas sodium dodecylsulfate prevented it almost completely.

Our results support the suggestion that one or several thiol groups in vicinity of the highly reactive lysyl residue might play a role in the fast specific reaction, which is only observed with intact native vesicles.

Introduction

Fluorescein isothiocyanate (FITC) has been demonstrated to act as a potent inhibitor of the Ca^{2+} transport ATPase [1–3]. One mol of FITC bound per mol of ATPase protein already caused a complete inhibition of Ca^{2+} uptake, Ca^{2+}-dependent ATPase activity and phosphorylation from ATP, while phosphoenzyme formation from inorganic phosphate was not impaired. ATP had a specific protective effect, although relatively high concentrations of ATP were required. It has been postulated that FITC selectively blocks the ATP binding site without affecting the phosphorylation site [3–5].

It has been generally assumed that isothiocyanates react primarily with amino groups of proteins [6, 7]. In fact, a single small peptide containing a FITC-labelled lysyl residue could be isolated from modified sarcoplasmic vesicles [8]. Yet, there are indications that thiol groups, as well, could be involved in the reaction. Thus dithioerythritol (DTE) prevents binding of and inhibition by FITC (unpublished observations). Dithiothreitol has been used to stop the reaction between FITC and sarcoplasmic vesicles [4]. Furthermore, the titration of FITC with mercaptoethanol was reported [9]. The kinetics of the reaction of simple thiols with some isothiocyanates, *e.g.* phenylisothiocyanate, has been studied in detail [10], demonstrating that SH compounds are by several orders of magnitude more reactive than the NH_2 group of amino acids.

In sarcoplasmic ATPase, although the final attachment of the fluorescein residue on a lysine has been demonstrated, a thiol-isothiocyanate binding might still play a transitory role. We were interested in the

Abbreviations: FITC, fluorescein isothiocyanate; SDS, sodium dodecylsulfate; $C_{12}E_8$, octaethyleneglycol mono-n-dodecyl ether; DTE, dithioerythritol; NEM, N-ethylmaleimide; DTNB, 5,5′-dithiobis (2-nitrobenzoate). Enzyme: Ca^{2+}-ATPase (EC 3.6.1.3).

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question whether possibly SH groups were involved in the primary interaction between FITC and the ATPase protein, or at least, whether vesicular thiol groups could be shown to react with FITC under certain conditions. In order to test this possibility we were looking for a simple way of distinguishing between a fluorescein residue bound to SH and NH_2, respectively. Our approach was: a) comparison with model compounds, especially those containing both SH and NH_2, like glutathione and cysteine. – b) Following the reaction of sarcoplasmic vesicles with FITC under modified conditions, *e.g.* in the presence of detergents. – c) Studying the effect of blockage of the SH groups of vesicles on the FITC reaction.

Materials and Methods

Sarcoplasmic vesicles from rabbit skeletal muscle were prepared according to Hasselbach and Makinose [11] as modified by de Meis and Hasselbach [12].

FITC (isomer I), NEM, SDS and Dowex (type 2×4, 20–50 mesh, Cl^- form) were obtained from Serva (Heidelberg, FRG). FITC was dissolved in dimethylformamide (to 10 mM) shortly before use and stored at −20 °C. The reagent, which was reported to contain about 70–85% of active material [9], was used directly. L(+)-Cysteine hydrochloride, DTE and dimethylformamide (spectroscopical grade) were from Merck (Darmstadt, FRG), ATP was from Pharma Waldhof GmbH (Düsseldorf, FRG), DTNB from EGA-Chemie (Steinheim, FRG), reduced glutathione from Boehringer Mannheim (Mannheim, FRG) and glycine from Roth KG (Karlsruhe, FRG). Mercaptoethanol and ethanolamine (both p.a. grade) were obtained from Fluka AG (Buchs, Switzerland). $C_{12}E_8$ was purchased from Nikko Chemicals (Tokyo, Japan). It is important to use fresh aqueous solutions of $C_{12}E_8$, otherwise the number of vesicular SH groups, titrated with DTNB, is drastically decreased, presumably due to peroxide formation. 1-0-Tetradecyl-propanediol-(1,3)-3-phosphorylcholine was synthesized in our laboratories according to refs. [13, 14].

Protein concentration was determined by the Biuret method, standardized by Kjeldahl, or from the absorbance at 280 nm in 1% SDS [15]. Since the fluorescein residue contributes to the absorbance at 280 nm, usually a control without FITC was included in every set of experiments to check the protein concentration after Dowex treatment or other procedures.

FITC binding was determined in 1% SDS and 0.1 N NaOH according to refs. [4, 8], using ε_{max} = 80000 [8, 16]. Because of varying absorption maxima generally a spectrum was run in the range between 485 and 500 nm. The E_{max} values of free FITC and differently bound FITC were approximately the same.

DTNB reactions were performed according to Ellman [17] with a high excess of reagent. 1% SDS was included when vesicles were reacted.

Reaction of SH and NH_2 model compounds (mercaptoethanol, glutathione, cysteine; ethanolamine, glycine) with FITC

The reaction was followed spectrophotometrically at 504 or 412 nm. The standard medium consisted of 50 mM sodium borate (final pH 8.8), 5 mM EGTA, 0.15 M NaCl and 0.3 M sucrose. The concentrations of SH/NH_2 compound and FITC were varied depending on the compound and wavelength used.

When following the reaction of FITC with thiols at 504 nm an excess of SH compound (mercaptoethanol, glutathione, cysteine; 20–200 μM) was added directly in the cuvette to FITC (11.3 μM ~ $E_{494} \approx$ 0.9 with ε_{494} = 80000) in the standard medium and the decrease of absorbance was recorded during about 10–30 min. The reference cuvette contained the same amount of thiol (no FITC), so that on addition of DTNB (1–2 mM) to both cuvettes the contribution of the thiophenolate anion to E_{504} was approximately compensated. For following the reaction of FITC with thiols at 412 nm higher FITC concentrations (40–360 μM) could be used.

For the modification of amino compounds (ethanolamine, glycine, Tris base) 0.5 mM FITC and 2 mM NH_2 compound were incubated in the medium given above for 3–4 hours or over night in the dark and diluted 40-fold for spectrophotometrical evaluation (at 504 nm and at the maximum).

Modification of sarcoplasmic vesicles with FITC

The standard reaction medium contained 0.3 M sucrose, 0.15 M NaCl, generally EGTA (1 or 5 mM, sometimes omitted, no significant effect noticeable) and 50 mM buffer (usually sodium borate, pH 8.8 or 8.0, in some experiments imidazole, pH 7, or

Tris.Cl, pH 7.2–9.5; the free NH_2 of Tris buffer did not interfere with the vesicle / FITC reaction within the normal reaction time). Sarcoplasmic vesicles (4 mg/ml) were incubated in this medium (total volume 2 ml) with 0.1–8 mol of FITC/10^5 g protein (corresponding to 4–320 μM FITC) for 20–30 min (at pH 7 for 1–2 hrs) in the dark. In some experiments ATP (1–20 mM) and/or $MgCl_2$ (1–2 mM) or $CaCl_2$ (0.1 mM) were included. Several series of experiments were performed in the presence of detergents (SDS, $C_{12}E_8$ or 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine) in different concentrations as given in the Tables and Figs. Excess reagent was usually removed by gentle stirring with Dowex 2×4 [8] (0.1–0.15 g/ml) for 30–50 min and filtering after dilution to 3.6 ml with water or buffer. A control without FITC was included in each set of experiments. The filtrates were used for the determination of protein concentration and bound FITC or of free SH groups with DTNB (about 0.5 mg protein/ml, 1.3 mM DTNB, 1% SDS, pH adjusted to 7.2–7.3 by addition of 0.5 M imidazole buffer, pH 7).

Another method employed for the removal of excess FITC consisted of passing 1 ml of the reaction mixture through a small column of Sephadex G-15 (1 × 6–7 cm), equilibrated with the same buffer. When up to 2 equivalents of FITC had been added, separation of vesicle-bound FITC from unreacted FITC was practically complete within a few min. Yet the protein concentration of SDS-solubilized eluates had to be estimated indirectly, as varying contents of fluorescein gave different contributions to the 280 nm absorbance. Other methods of protein determination, *e.g.* of Bradford [18], did not work either in the presence of detergents.

For the isolation of thioureides from mixtures of SH and NH_2/FITC products samples were dialysed against solutions containing the components of the standard medium (buffer generally Tris.Cl, pH 8) + 1 mM DTE and 0.04% SDS for at least 15 up to 40 hours in the dark (change of solutions after the first 2–4 hours).

For following the time course separate assays of 1 ml each, containing all components of the reaction mixture given above except FITC, were prepared. The reactions were started with FITC (1 equiv.) and stopped after the desired time by addition of 200 μl of 0.1 M DTE + 5% SDS. If the Sephadex column method was employed for further analysis the samples had to be put on the column immediately after stopping, in order to avoid partial dissociation of the dithiourethane fraction.

Spetrophotometrical methods for following the reaction between sarcoplasmic vesicles and FITC

a) At 412 nm in the presence of SDS with excess FITC. 3 ml of standard medium (buffer: sodium borate, pH 8.8, or imidazole, pH 7) containing additionally 1% SDS were reacted with FITC (0.5–8 mol/10^5 g protein, 20–320 μM) in a cuvette (ves. protein concentration 4 mg/ml). The increase of absorbance at 412 nm was recorded and evaluated after subtraction of E_{412} of free FITC.

b) At 494 nm with or without SDS at a low degree of FITC labelling. To avoid turbidity problems with native vesicles lower protein concentrations (1–1.5 mg/ml) had to be employed. Only 0.3–0.5 equivalents of FITC were added to the vesicles in the standard medium (buffer: sodium borate, pH 8.8, or Tris.Cl, pH 8, or imidazole, sodium phosphate, resp. MOPS, pH 7). The reference cuvette contained the same amount of protein. The addition of FITC to the vesicle suspension and the progress of the reaction were not accompanied by significant changes of turbidity, measured at 550 nm. The change of absorbance at 494 nm during the reaction was quite small, therefore the incubation was continued until the position of the long-wave maximum of the fluorescein residue was found constant. Then SDS was added (to 1%) and the position of the maximum recorded. Finally NaOH was added (to 0.1 N) and the procedure repeated.

Analogous experiments were performed with SDS-solubilized vesicles (1% SDS at 1.5 mg protein/ml, as well as 0.1–0.5 mg SDS/mg protein) and with free FITC (with and without SDS; no protein).

Modification of sarcoplasmic vesicles with NEM

The vesicles (4–5 mg/ml) were incubated for 2–3 hours in a medium consisting of 0.3 M sucrose, 0.15 M NaCl, 1 mM EGTA, 0.1 M Tris.Cl or sodium borate, pH 8–8.1, usually SDS (0.5–1%) and NEM (dissolved in ethanol, added to 1–1.5 mM). Unreacted NEM was removed by dialysis whereby the buffer was adapted to the subsequently required conditions. A control (vesicles without NEM) was included in most preparations. In one experiment SDS and NEM were removed by first passing the reaction mixture through a Dowex 2×4 column and pre-

cipitating the eluted protein in the ultracentrifuge after dilution with 0.1 M NaCl (30 min at 40000 rpm). The pellet was then homogenized in the medium used for FITC modification. In other experiments the centrifugation step was omitted without any effect on the FITC binding results. Instead of SDS in some cases $C_{12}E_8$ or 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine was used (1%). If no detergent was added precipitation occurred.

Treatment of NEM-modified vesicles with FITC

For direct spectrophotometrical measurements at 412 nm the dialysates (NEM-modified and control vesicles containing 1% SDS) were used directly (ves. protein concentration 3.5–4 mg/ml, determined at 280 nm; 2 equiv. of FITC added).

For the determination of FITC binding the conditions were as described with native vesicles. In some assays SDS, $C_{12}E_8$ or 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine were included. The concentrations of the detergents were not very critical in the range employed. Preparations that contained 2 mg detergent per mg protein during NEM modification and were dialysed for 1–3 hours to remove excess NEM could be reacted with FITC directly or after addition of more detergent (+1 mg/mg protein) without any significant change of the results.

In the absence of detergent NEM- and FITC-modified vesicles tended to precipitate, especially after Dowex treatment, so that care had to be taken when removing the Dowex by filtration. Glasswool was then used instead of filter paper and the protein concentration in the filtrate was controlled. For studying the time course aliquots of the NEM-modified preparations were treated with FITC (1 equiv.) and stopped as described for unmodified vesicles.

Results

Reaction of SH and NH_2 model compounds with FITC

In search of a quick method to distinguish between a fluorescein residue attached to a thiol group and an amino group, respectively, we studied the reaction of FITC with several simple compounds containing SH (mercaptoethanol) or NH_2 (ethanolamine, glycine) or both (reduced glutathione, cysteine). The conditions were chosen similar to those required for the modification of sarcoplasmic vesicles. The titration of FITC (and some other isothiocyanates) with mercaptoethanol has been reported by Wilderspin and Green [9], making use of the decrease of the fluorescein absorbance at 504 nm. We employed a similar method also to glutathione and cysteine. As can be seen from Fig. 1 the thiol reaction was complete within 1–2 min. Thereby the long-wave absorption maximum of fluorescein (494.5 nm) was slightly shifted towards shorter wavelengths (492 and 491.5 for mercaptoethanol and glutathione, respectively). On addition of excess DTNB the dithiourethanes were cleaved again, free FITC being regenerated, as shown by a rise of E_{504} and λ_{max} to their original values. Thus reduced glutathione behaves like mercaptoethanol, as the amino group does not react under normal conditions (*cf.* legend to Fig. 1 and Materials and Methods). Ethanolamine and glycine did not show any reaction either within 30 minutes to 1 hour.

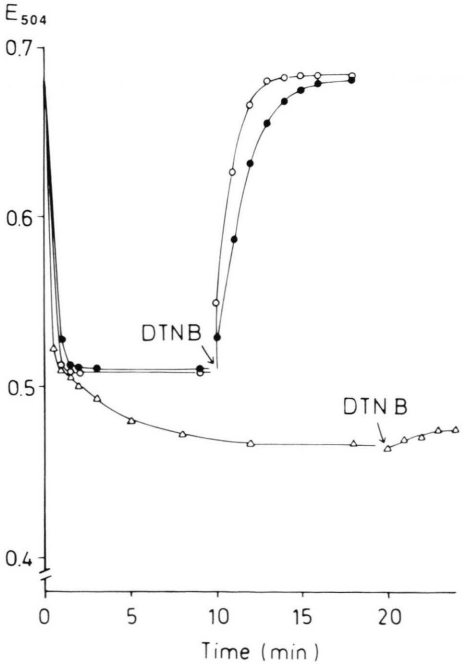

Fig. 1. Reaction of FITC with excess SH compound (mercaptoethanol, glutathione, cysteine). 0.2 mM SH compound were added to 11.3 μM FITC in 0.3 M sucrose, 0.15 M NaCl, 5 mM EGTA and 50 mM sodium borate, pH 8.8, in a cuvette and the absorbance at 504 nm was recorded. DTNB was added to 1.25 mM. The reference cuvette contained the same concentrations of DTNB and thiol in the above medium (no FITC). (●) Mercaptoethanol, (○) glutathione, (△) cysteine.

Cysteine, on the other hand, behaved differently. The initial steep drop of the 504 nm absorbance, corresponding to the fast reaction of FITC with SH, was followed by a slower decline, which presumably corresponds to an intramolecular transfer of the fluorescein residue from SH to NH_2 (compare also ref. [19]). Concomitantly λ_{max} was shifted to 490 nm. DTNB had only a minor effect on the product (see Fig. 1), indicating that most of the FITC was bound to NH_2. Similar observations were described by Wilderspin and Green [9] with mercaptoethylamine and FITC, on addition of NEM. It seems plausible that such a transfer could be facilitated by the special sterical arrangements of SH and NH_2 in cysteine and cysteamine. In both molecules a transfer of the fluorescein moiety can proceed via a five-membered ring.

Fig. 2 shows the dependence of the extent of the FITC reaction on the concentration of the SH compound, demonstrating that the reaction is incomplete in this concentration range (≈ 20–200 μM SH, 11μM FITC). This is in line with dissociation constants in the range of 5 to 10 μM as reported in ref. [9]. The $\triangle E_{504}$ values for the cysteine SH reaction were estimated from the sharp break in the time curves like the example shown in Fig. 1. All SH compounds tested gave essentially the same curve, whereas the NH_2 reaction of cysteine was practically independent of its concentration.

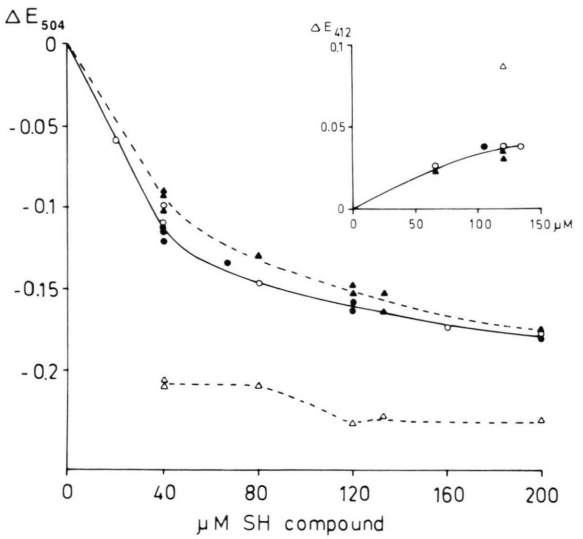

Fig. 2. Titration of FITC with thiol compounds. The procedure was the same as for Fig. 1. The points at the curves represent separate experiments at different concentrations of thiols. The ΔE_{504} values were obtained by subtracting the absorbance of free FITC from the final absorbance at 504 nm after completion of the reaction. – Inset: The same procedure was performed with 42 μM FITC and the increase of the absorbance at 412 nm was recorded. (●——●) Mercaptoethanol, (○——○) glutathione, (▲---▲) cysteine (SH reaction ~ break in the curve E_{504} *vs.* time, *cf.* Fig. 1); (△---△) cysteine (total reaction).

For the modification of amino groups more drastical conditions were required (higher concentrations and several hours). When 0.5 mM FITC were reacted with 2 mM ethanolamine or glycine for 3–4 hours, after 40-fold dilution a decrease of the 504 nm absorbance and a slight shift of λ_{max} (to 492 nm) similar to the effect of the SH reaction could be seen. The thiourea derivatives formed were stable, while on employing the same high concentrations to a thiol compound, dilution caused partial dissociation (reincrease of E_{504}). Tris.Cl, which is widely used as buffer for sarcoplasmic vesicle reactions even with FITC, also showed some reaction at pH 8.8, while the amino group of ATP did not.

Since the change in the fluorescein absorbance at 504 nm (very close to the maximum in the steep part of the absorption spectrum of FITC) appeared not well suitable for following the reaction of sarcoplasmic vesicles spectrophotometrically, we also tested the range around 400 nm, where free FITC has only a very low absorbance which is somewhat raised during the reaction with a thiol (*cf.* Fig. 1 of ref. [9]). Besides in this range higher FITC concentrations can be used and measured directly in the cuvette. The corresponding "titration curve" of FITC with mercaptoethanol, glutathione and cysteine, followed at 412 nm, is shown in Fig. 2 – inset. The absorbance of the N-cysteine derivative was about 2–3 times higher compared to the SH products. During the reaction of FITC with ethanolamine or glycine, as well, the 412 nm absorbance was slightly increased (not shown). Since the E_{412} values of free FITC, SH-bound and NH_2-bound FITC show different dependencies on the pH, care has to be taken to maintain the same pH value in the experiments compared. Generally the absorbance at 412 nm increases with decreasing pH.

Finally glutathione (0.2 mM) was titrated with excess FITC at 412 nm. At the equivalence point the deviation of the titration curve ($\triangle E_{412}$ *vs.* FITC concentration, not shown) from linearity corresponded to approximately 80% completion of the reaction.

Binding of FITC to sarcoplasmic vesicles

The binding curve obtained when sarcoplasmic vesicles were modified by increasing concentrations of FITC at pH 8 to 9, and excess reagent was removed by treatment with Dowex 2×4, is shown in Fig. 3. The amount of FITC that remained bound to native vesicles became quasi constant when about 0.5 mol of fluorescein were attached to 1 mol of protein, which corresponds to approximately 0.7 mol per mol ATPase, assuming 75% ATPase in the sarcoplasmic vesicles. This number is in line with the results of Andersen, Møller and Jørgensen [5]. The small further increase at higher FITC/protein ratios probably reflects nonspecific labelling. The product had a long-wave absorption maximum at 494 nm, when measured in 0.1 N NaOH + 1% SDS, the conditions of determining FITC binding [8]. The position of the maximum corresponds to FITC mainly bound to NH_2, as will be shown below.

In the presence of Ca^{2+}ions (0.1 mM $CaCl_2$, pH 7 or 9) FITC binding was not affected, which is in contrast to the report of Pick (*cf.* Fig. 7 of ref. [3]).

If ATP was present in the reaction mixture (5–10 mM ATP at a protein concentration of 4 mg/ml, pH not higher than about 8.2) FITC binding was drastically reduced and the binding curve became linear. No saturation was observed in the range tested (Fig. 3). The presence of Mg^{2+} (1–5 mM) and EGTA or Ca^{2+} (0.1 mM) in addition to ATP did not significantly alter the ATP effect. Competition between FITC and ATP was already reported by Pick and Bassilian [2].

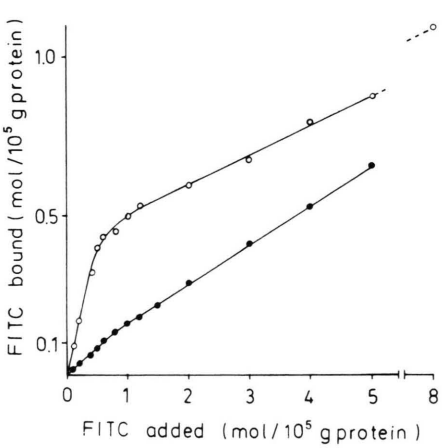

Fig. 3. Binding of FITC to native sarcoplasmic vesicles. The vesicles (4 mg/ml) were reacted for 20–30 min with different FITC concentrations in a medium containing 0.3 M sucrose, 0.15 M NaCl and 50 mM sodium borate (final pH of the solution 8.8 or 8.0). Excess FITC was removed by Dowex treatment. The amount of vesicle-bound fluorescein was determined in 0.1 N NaOH + 1% SDS. (○) No addition, (●) with 10 mM ATP at pH 8.

Addition of 5 mM DTE to the reaction mixture prevented the modification of vesicles almost completely (0.06 equivalents of FITC bound of 1 added). Obviously the reaction of FITC with excess thiol is very fast compared to the specific modification of sarcoplasmic vesicles. In some experiments DTE or ATP (15–20 mM) were used to stop the vesicle modification by FITC after different time intervals (3 seconds to 10 minutes, 2 equivalents of FITC added). It turned out that the FITC reaction was almost complete within 5 minutes at pH 8 (half-life approximately 1 min). The rate of reaction under different conditions will be discussed below.

When excess FITC was removed by passing the reaction mixture through a Sephadex column, somewhat more FITC remained bound (0.74 equiv. of 2 added, compared to 0.56 after stirring with Dowex, see Table I). At the same time the absorption maximum was slightly shifted towards shorter wavelengths (492.5–493 nm), later assigned to a mixture of NH_2 and SH product. Treatment with Dowex probably had removed part of the FITC reversibly bound to vesicular SH groups, so that the proportion of NH_2-bound fluorescein in the mixture was raised.

Effect of detergents on the modification of sarcoplasmic vesicles by FITC

When solubilizing amounts of SDS were included in the FITC reaction mixture, no thioureide was obtained. Instead, only dithiourethanes were formed. After Dowex or Sephadex treatment the residual vesicle-bound fluorescein, dissolved in NaOH/SDS, showed an absorption maximum at 491 nm, which was assigned to the FITC-SH product. Within about 30–40 min at alkaline pH the maximum was further shifted to 490 nm, reflecting dissociation of the dithiourethane(s), followed by alkaline hydrolysis (*cf.* [20]). The same 490 nm maximum was measured with free FITC after some minutes in 0.1 N NaOH with or without SDS. ATP had no effect on the FITC reaction in the presence of SDS.

At low SDS/protein ratios mixtures of both types of compounds were formed. Attempts to separate

Table I. Comparison of FITC binding under different conditions.
Sarcoplasmic vesicles (4 mg/ml) were reacted for 30 min with FITC (1 or 2 equivalents) in a medium containing 0.3 M sucrose, 0.15 M NaCl, 5 mM EGTA and 50 mM sodium borate (final pH of the mixture 8.0–8.1) without detergent or with the amounts of different detergents as given in the Table. Excess FITC was removed by either stirring with Dowex 2 × 4 or on a Sephadex G-15 column, equilibrated with the reaction buffer including the corresponding detergent concentration. Dialysis was performed for about 15–40 hrs in the dark against a solution containing 0.3 M sucrose, 0.15 M NaCl, 1 mM EGTA, 50 mM Tris.Cl, pH 8, 0.04% SDS and 1 mM DTE. FITC binding was determined in 1% SDS + 0.1 N NaOH. λ_{max} was measured after at least 30 min in the alkaline solution. The accuracy of the λ_{max} values was approximately ± 0.5 nm. Mean values are given ± S.E. (n in parentheses).

FITC added (equiv.)	Addition to FITC reaction		Bound fluorescein [mol/10^5g protein] after further treatment				
	Detergent	[mg/mg protein]	Dowex stirring	λ_{max} [nm]	Sephadex col.	λ_{max} [nm]	Dialysis[a]
2	–	(–)	0.56 ± 0.01 (12)	494	0.74 ± 0.02 (10)	493	0.43 ± 0.02 (8)
	SDS	(0.2)	0.59 ± 0.04 (7)	492.5	0.91 ± 0.06 (6)	491	0.29 ± 0.01 (12)
		(0.3)	0.50 ± 0.07 (4)	491	–		0.08 ± 0.01 (4)
		(0.5)	0.45 ± 0.07 (4)	490.5	0.92 ± 0.05 (2)	490	0.025
		(2.5)	1.0 ± 0.15 (6)	490	1.1 ± 0.06 (3)	490	0.02
1	–	(–)	0.47	494	0.54	493.5	0.39
	$C_{12}E_8$	(2.0)	0.15 – 0.30[b]	491.5	0.56	490.5	0.12
	1-0-Tetradecyl-propanediol-(1,3)-3-phosphorylcholine	(2.0)	0.16 – 0.26[b]	490.5	–	–	0.05

[a] All λ_{max} values 494 – 494.5 nm.
[b] Depending on the time with Dowex.

these mixtures by incubation with either excess DTE or NaOH, followed by precipitation of the protein by perchloric acid and measurement of the proportions of fluorescein absorbance in the supernatant and in the redissolved pellet, did not lead to reproducible results.

Satisfactory results were finally obtained from dialysis experiments. It turned out, that SH-bound FITC could be quantitatively removed by prolonged dialysis (15–40 hours in the dark at room temperature) in the presence of 1 mM DTE. Thioureides, on the other hand, were stable towards dialysis. This could be expected and was also confirmed by dialysis experiments with FITC-modified SH-blocked vesicles (see below), which could only represent a thioureide.

FITC bound to	Addition	λ_{max} [nm]	Type of product
–	–	494.5	–
	1% SDS	494.5	–
	1% SDS + 0.1 N NaOH	490[a]	monothiocarbamate (hydrolysis product)
Native vesicles	–	500	thioureide
	1% SDS[b]	495	
	1% SDS + 0.1 N NaOH	494.5	
SDS-solubilized vesicles (1% SDS)	–	494–494.5	dithiourethane
	0.1 N NaOH[b]	491 → 490[c]	dithiourethane → monothiocarbamate

[a] Measured at least 5 min after addition of NaOH.
[b] Added after completion of the reaction.
[c] Complete hydrolysis took about 30–40 min.

Table II. Dependence of the long-wave absorption maximum of the fluorescein residue on the state of binding.
The FITC reaction was performed directly in a cuvette: 0.3 equivalents of FITC were added to sarcoplasmic vesicles (1.5 mg/ml) in 0.3 M sucrose, 0.15 M NaCl, 5 mM EGTA and 50 mM sodium borate (pH 8.8). The reference cuvette contained all components except FITC. The λ_{max} values listed in the Table (±0.5 nm) refer to a complete reaction under the conditions. The corresponding E_{max} values remained more or less constant under all conditions tested.

Table II summarizes the assignments of the various long-wave absorption maxima of the fluorescein residue to different states of binding. Spectrophotometric titration at a low FITC/protein ratio (0.3 equivalents of FITC) was chosen, since according to Fig. 3 in this range a relatively high percentage of the FITC added is bound. During the reaction with native vesicles a long-wave shift was observed (λ_{max} = 500 nm at pH 8.8, mainly NH_2-bound FITC). FITC binding was usually measured at alkaline pH, however, to make sure the fluorescein carboxyl group was completely dissociated. Besides the different types of products can be well distinguished under these conditions.

If excess FITC was employed in the presence of SDS several vesicular SH groups could be shown to react. Fig. 4 demonstrates the results of spectrophotometric titrations at 412 nm. The reactions were complete within a few minutes at pH 8.8 and took about 5 times as long at pH 7. The corresponding absorbance increase observed was higher at lower pH, reflecting the dependence of the absorption spectrum of FITC (free and bound) on the pH. As under these conditions (2.5 mg SDS per mg vesicular protein) only SH groups are reacting, the absorbance increase, which is linear up to about 4–6 equivalents of FITC added, corresponds to the formation of

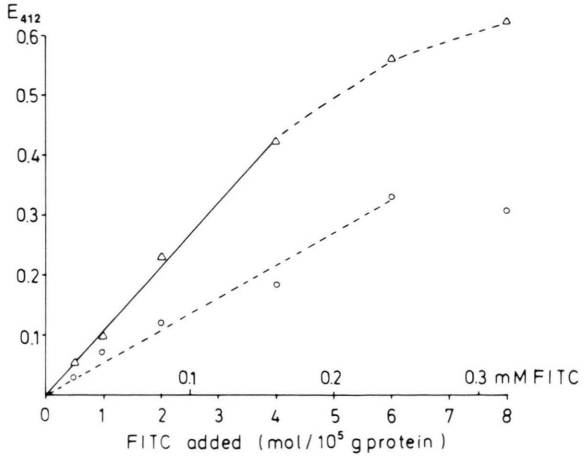

Fig. 4. Spectrophotometric titration of SH groups of SDS-solubilized sarcoplasmic vesicles with FITC. The FITC reactions were performed as described in Materials and Methods directly in cuvettes with 4 mg protein per ml and 2.5 mg SDS per mg protein. Each symbol represents the final absorbance at 412 nm after completion of the reaction and subtraction of the contribution of free FITC added. (○) pH 8.8 (sodium borate), (△) pH 7 (imidazole).

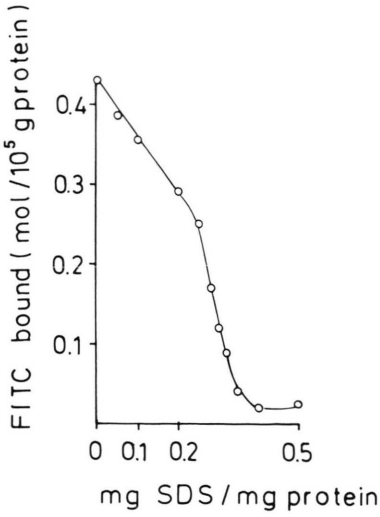

Fig. 5. Effect of SDS on the amount of thioureides formed during FITC modification of sarcoplasmic vesicles. The vesicles (4 mg/ml) were reacted for 30 min at pH 8 (sodium borate) with 2 equiv. of FITC in the presence of varying concentrations of SDS, as described in Materials and Methods (Dowex method). SH-bound fluorescein was removed by dialysis and the residual vesicle-bound fluorescein determined as described.

dithiourethanes. Assuming that the linear portion of the curve reflects complete binding of the FITC added, 1 equivalent of FITC bound would correspond to $\triangle E_{412}$ values of about 0.06 at pH 8.8 and 0.1 at pH 7. Attempts to determine the number of FITC-modified SH groups indirectly by measuring the free thiol content with DTNB were not successful, as could already be expected from the behaviour of simple thiol compounds. With mercaptoethanol and glutathione DTNB had been shown to displace the reversibly bound FITC (*cf.* Fig. 1).

In a series of assays containing varying SDS concentrations and 2 equivalents of FITC, the products were analysed by dialysis, as described above. The values listed in Table I and the curve shown in Fig. 5 demonstrate that already very low SDS/protein ratios have a drastic effect on the course of the reaction. At concentrations around 0.3 mg SDS per mg protein a very steep decline of the curve, representing the residual = NH_2-bound fluorescein, was observed. This is just the range where the viscosity of vesicle – SDS mixtures passes through a transient maximum [21]. No differences were observed whether the samples had been treated with Dowex, Sephadex or not at all before dialysis.

According to Table I the amount of FITC bound by SDS-containing vesicles is somewhat higher compared to native vesicles, when a Sephadex column, equilibrated with the reaction buffer (SDS in the corresponding concentration) has been employed for product isolation. Treatment with Dowex, on the other hand, usually led to lower values, which is plausible, considering the prolonged contact with the anion exchange resin, in view of the reversibility of the FITC-SH reaction. Furthermore, Dowex 2×4 binds the dodecylsulfate anion as well. In fact, the value of bound fluorescein found after Dowex treatment is significantly dependent on the amount of Dowex material employed and on the time of contact.

The variety of results so far can be best explained by assuming the formation of mixtures of thioureides and dithiourethanes, the ratio of which depends on the concentration of the detergent during FITC modification. The fast procedure of passing the reaction mixture through a Sephadex column removes essentially free FITC and low molecular fluorescein derivatives, but leaves the composition of the modified vesicle products virtually unchanged. During the prolonged contact with Dowex, however, FITC is continuously removed from equilibrium, thus promoting partial dissociation of the dithiourethanes. The differences between the values found by the two procedures will therefore concern the SH-bound fluorescein solely.

Similar to SDS the non-ionic detergent $C_{12}E_8$ caused a preferential reaction of FITC with vesicular SH groups. The differences between the reaction of native and detergent-solubilized vesicles with FITC are demonstrated in Fig. 6A, which illustrates the rates of the reactions under various conditions. The upper curves (broken lines) represent the total amounts of products formed, as determined by stopping the FITC reaction after different time intervals (with DTE + SDS) and immediately afterwards passing the reaction mixture through a Sephadex column. The lower curves (full lines) were obtained after dialysis of the column eluates and represent the proportions of thioureides. If Dowex stirring was employed, values in between the two sets of curves were obtained, which is plausible, as has been discussed above.

As can be seen from Fig. 6A and Table I, after 30 minutes of incubation with 1 equivalent of FITC native vesicles contain about 0.54 equivalents of fluorescein, 73% of which are NH_2-bound, while with $C_{12}E_8$-solubilized vesicles only 20% of the totally attached fluorescein (0.56 equiv.) are a thioureide. In the presence of the lysolecithin-related compound 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine the proportion of NH_2 product was even lower (Fig. 6A, bottom curve) and with SDS only 0.02 equivalents were found after incubation with 2 equivalents of FITC for 30 minutes and subsequent dialysis (Table I).

The detergents tested obviously act by increasing the reactivity of the vesicular SH groups towards FITC. In addition to the exposure of membrane-buried thiol groups by detergent solubilization charge effects appear to have an influence on the relative reactivities SH/NH_2. This would explain the increasing effects of the detergents in the order $C_{12}E_8$ (non-ionic) – 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine, an ether – deoxy lysolecithin (zwitterionic) – dodecylsulfate (charged). The fast specific reaction of native vesicles with FITC will be discussed in the following chapter.

Effect of SH modification by NEM on the FITC reaction

Additional information about the pathway of the FITC reaction could be expected if sarcoplasmic vesicles, totally blocked with NEM, were incubated with FITC. Fluorescein binding should be completely abolished, if FITC reacted only with SH or if the SH reaction were a requisite intermediate step to a NH_2 product. Fluorescein bound comparable to the value obtained with native vesicles should be expected, however, if FITC reacted directly with NH_2.

In fact, both possibilities could be verified, depending on the conditions of the subsequent FITC reaction. If the NEM and FITC modification assays contained SDS (1–2 mg per mg protein) no more reaction with FITC occurred. Practically no fluorescein was bound after 20 minutes (*cf.* Fig. 6B and Table III). Only prolonged treatment (15 hrs) led to some nonspecific binding to NH_2 (0.6 equiv. of FITC of 4 added), under conditions comparable to those required for the reaction of ethanolamine or glycine with FITC. As expected, spectrophotometric titration of SH-blocked vesicles with FITC in the presence of SDS gave practically no increase of the absorbance at 412 nm within 30 minutes, in contrast to the

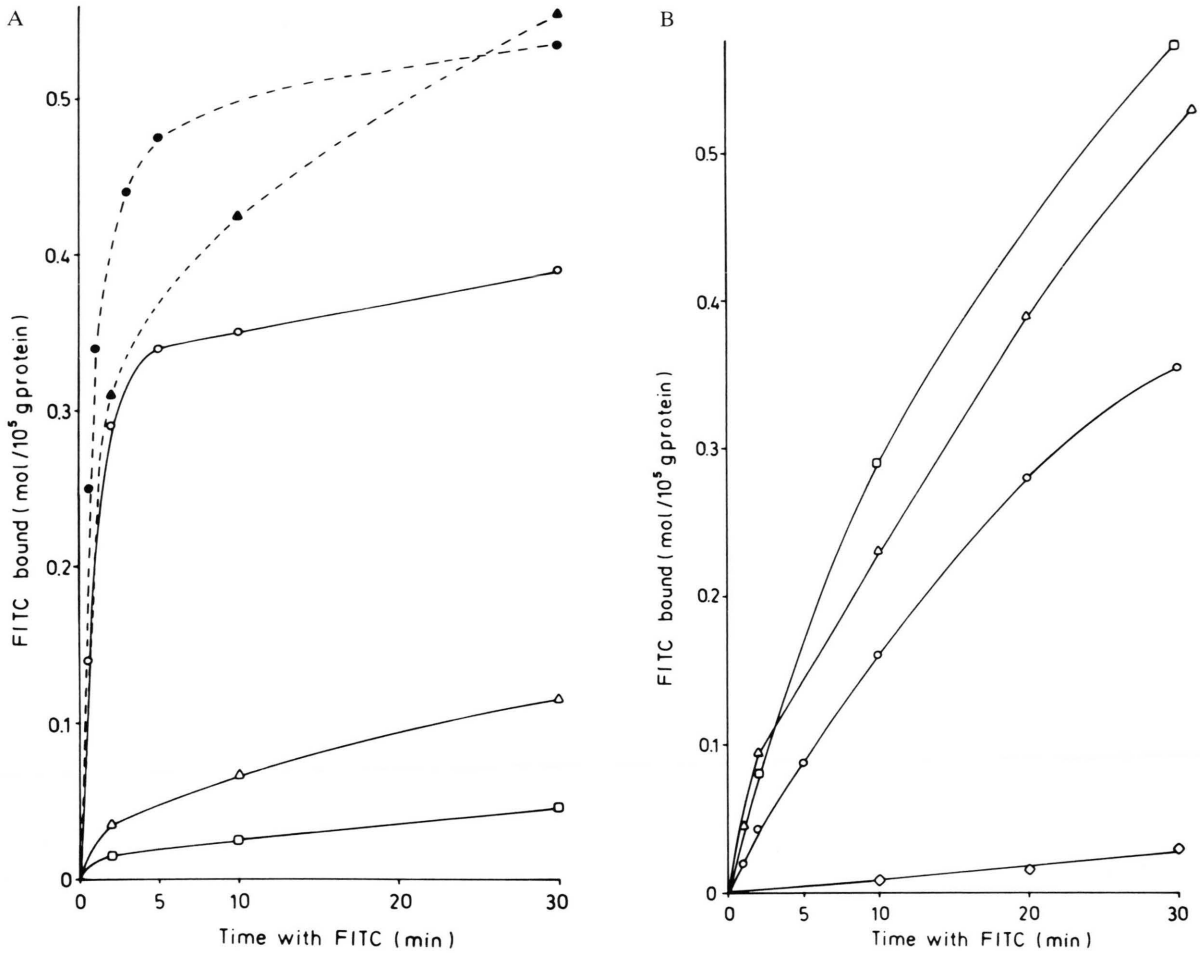

Fig. 6. Time course of the reaction of FITC with sarcoplasmic vesicles, (A) unmodified, (B) after SH modification by NEM. For the conditions of the NEM modification see Materials and Methods. Detergents were included or omitted as indicated below. The unmodified vesicles were incubated in the same medium without NEM. The FITC reactions (1 equiv.) were stopped after different time intervals by addition of 200 µl 0.1 M DTE + 5% SDS to 1 ml assay. Low molecular FITC products were removed by either a Sephadex G-15 column or stirring with Dowex 2×4. SH-bound fluorescein in the controls was removed by dialysis. (A) (●, ○) Native vesicles, (▲, △) $C_{12}E_8$-solubilized vesicles (2 mg $C_{12}E_8$/mg protein); (□) vesicles, solubilized with 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine (2 mg/mg protein); filled symbols with broken line: total amount of FITC bound to NH_2 and SH (Sephadex column method); empty symbols with full line: FITC bound to NH_2 (after dialysis). – (B) NEM and FITC reaction (○) without detergent, (△) with $C_{12}E_8$ (2 mg/mg protein), (□) as in (A), (◇) with SDS (1–2 mg/mg protein).

corresponding control without NEM. Furthermore DTNB, added before or after FITC treatment of the NEM-modified vesicles did not produce any significant change of the 412 nm absorbance.

On the other hand, if no detergent was added or if NEM poisoning was performed in the presence of SDS, followed by its removal on a Dowex anion exchange column, FITC reacted readily with vesicular NH_2 groups according to the corresponding curve shown in Fig. 6B. After 30 minutes almost the same amount of fluorescein was bound to NH_2 as with native vesicles, but the initial rate of the reaction was significantly lower (*cf.* Figs. 6A and B).

Generally the FITC reactions were stopped after 30 minutes. When 2 resp. 4 equivalents of FITC were added, even some more fluorescein was bound, compared to native vesicles (*cf.* Table III with Table I and Fig. 3). This could be explained by a somewhat higher FITC concentration available for NH_2 groups as no SH groups are present.

Table III. FITC binding of totally NEM-modified sarcoplasmic vesicles. NEM modification was performed as described in Materials and Methods in a medium containing 0.3 M sucrose, 0.15 M NaCl, 1 mM EGTA, 100 mM sodium borate (pH of the mixture 8.1), 0.5% SDS (if not otherwise stated) and 1.5 mM NEM (ves. concn. 5 mg/ml). Subsequently SDS was removed on a Dowex column. FITC modification, removal of FITC (Dowex, dialysis) and measurement of bound fluorescein were done as described for Table I. All λ_{max} values were about 494.5–495 nm (± 0.5 nm) in NaOH/SDS. Mean values are given ± S.E. (n in parentheses).

Addition to FITC reaction		Bound fluorescein [mol/10^5g protein] Equivalents of FITC added (reaction time)			
Detergent	[mg/mg protein]	1 (30 min)	2 (30 min)	4 (30 min)	4 (15 hrs)
–	(–)	0.33 0.35[a]	0.68 ± 0.03 (2)	1.1	3.1 $\xrightarrow{\text{dialysis}}$ 3.1
SDS	(2.5)	0.03	0.04 ± 0.01 (2)	0.06	0.6
$C_{12}E_8$[b]	(2.0)	0.52	0.92		
1-0-Tetradecyl-propanediol-(1,3)-3-phosphorylcholine[b]	(2.0)	0.57			

[a] In this experiment the NEM modification assay contained no detergent and precipitation occurred.
[b] This detergent was also included in the NEM reaction instead of SDS.

Prolonged reaction times (15 hrs, without detergent) led to a very high binding (3 equivalents out of 4 added) which remained unchanged after 15 hours of dialysis (Table III). All FITC products of NEM-modified vesicles showed a long-wave absorption maximum at 494.5–495 nm in NaOH/SDS. This confirms the previously mentioned assignment of the 494 nm maximum to mainly NH_2-bound fluorescein.

Readdition of SDS (2.5 mg/mg protein) to the FITC assay yielded again only a negligible amount of FITC bound (0.04 of 2 equivalents added), while addition of $C_{12}E_8$ (2 mg/mg protein) led to even elevated values (Table III). The detergent effects on the time course of the FITC reaction of SH-blocked vesicles (Fig. 6B) are in agreement with these observations. $C_{12}E_8$ and the lysolecithin-like compound enhance the reactivity of the vesicular amino groups, compared to the assay without detergent, while SDS abolishes it completely. The finding that SDS suppresses the reaction of vesicular amino groups with FITC can only be explained by charge effects. Presumably the dodecylsulfate anion prevents the interaction of NH_2 with the fluorescein moiety, containing a carboxyl group that is also negatively charged at pH 8–9.

When comparing the corresponding curves of the Figs. 6A and B it seems plausible to explain the effects of $C_{12}E_8$ and 1-0-tetradecyl-propanediol-(1,3)-3-phosphorylcholine just by detergent solubilization leading to increased reaction rates of SH, resp. NH_2 where no SH is present. The rapid NH_2 reaction of native vesicles, however, cannot be explained satisfactorily with these factors alone. If two independent reactions of FITC with NH_2 and SH groups of native vesicles occurred, it would not be understandable why the amino group reactivity, after abolishing the thiol groups, should be reduced to such a degree. The assumption that the proximity of one or several thiol groups to the highly reactive amino group in the native conformation does play a role in the specific FITC reaction, appears very plausible.

Discussion

We have shown that fluorescein isothiocyanate, widely referred to as an amino reagent, actually reacts with both amino and thiol groups of sarcoplasmic ATPase. Although the reactivity of SH groups is much higher compared to NH_2 groups in simple model compounds, FITC incubation of native sarcoplasmic vesicles results in the preferential labelling of a single lysine amino group out of over 40 per mol ATPase [22], besides some reversible thiol modification. According to Mitchinson *et al.* [8] it is the Lys_{190}

localized in the tryptic fragment B that contains practically all of the fluorescein bound. On the basis of our results it can be expected that an SH bound fraction is no longer detectable after the complicated procedures applied to isolate the peptides [8]. We have tried to explain this high specific NH_2 reactivity by suggesting a transient involvement of thiol groups. Yet, this postulate is very difficult to prove strictly because of the reversibility of the dithiourethane formation which, on the other hand, is the prerequisite for the proposed group transfer reaction.

Our explanation is supported by the following findings: The fast reaction with NH_2 is only observed in the presence of intact vesicular SH groups. Thiol blockage by NEM decreases the rate of NH_2 modification by FITC significantly. Furthermore, detergent solubilization leads to a loss of the specificity in so far as the pathway of the reaction is changed. When sarcoplasmic vesicles, solubilized with SDS, $C_{12}E_8$ or 1-0-tetradecyl-propanediol-(1,3)-3-phosphoryl-choline are incubated with FITC, the SH reaction becomes predominant, producing mainly dithiourethanes. The vesicles under these conditions behave like the model compounds: In both cases the SH groups are by far more reactive than the NH_2 groups, as long as they react independently. With cysteine, on the other hand, NH_2 modification by FITC occurs quite rapidly; it is preceded though by a reversible addition of FITC to SH. This effect can be explained by intramolecular sterical factors. From these observations it seems very probable that the specific reaction of sarcoplasmic vesicles, as well, requires special sterical conditions that are only given in the native conformation. The tertiary structure of the ATPase protein most likely contains one or several SH groups in a position favourable for a transfer of the fluorescein moiety from a cysteine to a lysine residue, forming the stable thioureide. All procedures that disturb the tertiary structure of the ATPase protein would then be expected to abolish the specificity. A transfer of fluorescein from SH to NH_2 is no longer possible if the two groups have been further separated by a conformational change.

Of all conditions studied only the FITC reaction with native vesicles is accompanied by a long-wave shift of the absorption maximum from 494 to 500 nm. This must correspond to energetically favourable interactions between the FITC molecule and the specific binding site at the ATPase protein. Hydrophobic as well as electrostatic interactions could be involved. All other FITC reactions investigated lead to a slight short-wave shift (*cf.* Table II), which would rather be expected in view of the loss of a conjugated double bond during the reaction.

SDS is already effective at very low detergent / protein ratios that are not assumed to cause unfolding of the ATPase protein yet [21]. Under these conditions charge effects could already be involved. If the polar head groups of the zwitterionic fluorescein residue play some role during the incorporation of the FITC molecule into the ATPase protein one would expect the dodecylsulfate anion to interfere with FITC binding.

The fact that SDS inhibits the FITC modification of SH-blocked sarcoplasmic vesicles is probably also due to charge effects. In contrast, the non-ionic detergent $C_{12}E_8$ and the ether-deoxy lysolecithin even accelerate the FITC reaction with the amino groups of NEM-modified vesicles, compared to preparations without detergent. Still the initial rates are considerably lower than of the specific NH_2 labelling of native vesicles. We assume that all reactions of FITC with amino groups of SH-blocked sarcoplasmic vesicles are nonspecific.

The protective effect of ATP regarding FITC binding and inactivation has been explained earlier by a competition of FITC and ATP for a common binding site [2]. According to ref. [4] and [5] FITC is bound to the high-affinity ATP binding site, as with FITC-labelled ATPase no high-affinity binding of ATP was detected. In view of our model it would also be conceivable that this binding site also contains SH groups, since ATP has been shown to counteract thiol group labelling by NEM or DTNB (see *e.g.* [23–25]). Inactivation produced by NEM is prevented by ATP at concentrations saturating the high-affinity site (*cf.* [24], p. 160). For the protection of sarcoplasmic vesicles from FITC modification ATP concentrations in the mM range were required, however. This would suggest that also low-affinity ATP binding sites are involved in the protection. The apparent simultaneous involvement of high and low-affinity sites would be plausible, if high and low-affinity binding of ATP occurs at identical sites, the binding properties of which are determined by the enzyme conformation (see *e.g.* [26]). If ATP binding is accompanied by a conformational change the specific FITC reaction would be expected to be abolished for the reasons already discussed. Or the

reduced FITC binding could simply be a consequence of a decreased accessibility of thiol groups essential for the formation of the specific thioureide as well as for the enzymatic functions of the ATPase.

It is interesting to note that other "NH_2 reagents" obviously preferentially react with different sarcoplasmic amino groups. Murphy [27] described the inactivation of sarcoplasmic ATPase by pyridoxal-5′-phosphate, leading to the specific modification of a lysyl residue that was located in the A_1 subfragment after tryptic digestion. Fluorescamine, on the other hand, preferentially reacts with lipid NH_2 groups [28, 29], possibly because the fluorescamine molecule is more hydrophobic. Yet phosphatidylethanolamine labelling has no effect on ATPase activity or phosphoenzyme formation and only a weak effect on Ca^{2+} transport at high degrees of lipid modification. The inhibition of distinct functions of the enzyme by fluorescamine could be correlated with different extents of the concomitantly occurring labelling of amino groups of the ATPase protein.

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