Biochimica et Biophysica Acta, 422 (1976) 357—364

BBA 67710

BINDING OF ANTIBODY TO THE ACTIVE SITE OF THE ADENOSINE TRIPHOSPHATASE OF SARCOPLASMIC RETICULUM

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(Received August 25th, 1975)

Summary

1 The thiol group of fragmented sarcoplasmic reticulum that is protected from reaction with *N*-ethylmaleimide by 1 mM ATP was labelled with *N*-ethyl-[2,3-$^{14}C_2$]maleimide. Autoradiography after electrophoresis of this material on dodecylsulphate/polyacrylamide gels showed that this group is located on the polypeptide chain of the ATPase.

2 The ATP-protected thiol group of fragmented sarcoplasmic reticulum has been labelled by treatment with either 1-(2,4-dinitrophenylamino), 6-(*N*-maleimido)hexane or *N*,*N'*-bis(2,4-dinitrophenyl)-L-cystine. The total dinitrophenyl contents of the dinitrophenyl-vesicle conjugates found by spectrophotometry were in good agreement with the ATP-protected thiol content, especially in the case of the *N*,*N'*-bis(2,4-dinitrophenyl)-L-cystine-treated vesicles. Fluorescence-quenching titrations of anti-dinitrophenyl-antibody tryptophyl fluorescence with the dinitrophenyl-vesicle conjugates showed that not all the dinitrophenyl groups were available for combination with antibody.

3. Phospholipase C(EC 3.1.4.3) digestion of ATP-protected, *N*-ethylmaleimide-treated vesicles, labelled with dinitrophenyl groups using *N*,*N'*-bis(2,4-dinitrophenyl)-L-cystine, caused the dinitrophenyl groups to become completely inaccessible to anti-dinitrophenyl-antibody, although no dinitrophenyl groups were lost during the incubation. This indicates a possible crowding together of the ATPase molecules as the effective membrane area was reduced

Introduction

The thiol groups of the sarcoplasmic reticulum were first examined in detail by Hasselbach and Seraydarian [1], who reported that one of the ten thiol

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Abbreviations dinitrophenyl-maleimide, 1-(2,4-dinitrophenylamino), 6-(*N*-maleimido) hexane, bis-dinitrophenyl-cystine, *N*,*N'*-bis(2,4-dinitrophenyl)-L-cystine

groups per 10^5 g total membrane protein of intact sarcoplasmic reticulum vesicles which are alkylated by *N*-ethylmaleimide could be protected from reaction with the reagent by either ATP or ADP, that this thiol belonged to a class of thiol group (four per 10^5 g total protein) which when alkylated led to loss of the Ca^{2+}-activated ATPase activity and Ca^{2+}-transporting ability of the vesicles, and that when the integrity of this thiol group alone was preserved, the sarcoplasmic reticulum vesicles retained Ca^{2+}-ATPase activity and the ability to transport Ca^{2+}. Hasselbach and Elfvin [2] further demonstrated that this substrate-protected thiol group was located on the outer surface of the sarcoplasmic reticulum vesicles by using mercury-phenylazo-ferritin to label the group for examination in the electron microscope Panet and Selinger [3] confirmed the previous work, using 5,5′-dithio-bis(2-nitrobenzoic acid) instead of *N*-ethylmaleimide and showed that an extra four thiol groups per 10^5 g membrane protein became available for reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) in the presence of dodecyl sulphate and that only one band was heavily labelled with *N*-ethyl[1-^{14}C]maleimide when gel electrophoresis of vesicles treated with *N*-ethylmaleimide in the presence of ATP followed by *N*-ethyl[^{14}C]maleimide in the absence of ATP was carried out by a modification of the procedure of Takayama [4] Migala et al [5] have shown that the major protein component of the vesicles is labelled with *N*-ethyl[^{14}C]maleimide

In order to further elucidate the nature of the "essential" thiol group, the work reported here examines the identity of the protein with which it is associated using the now standard dodecyl sulphate/polyacrylamide gel electrophoresis system The thiol content of the purified ATPase is estimated The binding of anti-dinitrophenyl-antibody to a dinitrophenyl moiety coupled to the "essential" thiol group by two different methods is investigated by the fluorescence quenching technique

Materials and Methods

N-ethyl[2,3-$^{14}C_2$]maleimide (2 Ci/mol) was obtained from the Radiochemical Centre (Amersham, Bucks , England) Bis-dinitrophenyl-cystine was from B D H All other reagents were of analytical grade Phospholipase C (EC 3 1 4 3) was from Koch-Light Ltd

Synthesis of dinitrophenyl-maleimide This was prepared according to C.G Knight and N M Green (unpublished results)

Preparation of anti-dinitrophenyl-antibody Purification was by the method of N M. Green and E J Toms (unpublished results)

Fragmented sarcoplasmic reticulum This was prepared essentially according to the methods of Hasselbach and Makinose [6] and Holland and Perry [7] Before centrifugation on a linear sucrose gradient (0 32—1 3 M) for 1 h at 75 000 × *g* (average), the preparation was washed with 0 6 M KCl according to Martonosi [8] The dodecyl sulphate gel electrophoresis pattern was very similar to those published by other workers (e g see Migala et al [5] and MacLennan et al [9])

Purified ATPase of the sarcoplasmic reticulum This was prepared according to the method of MacLennan [10], and stored as described previously [11]

ATP-protected, N-ethylmaleimide-treated sarcoplasmic reticulum vesicles

Alkylation of vesicles with *N*-ethylmaleimide in the presence of ATP was carried out as described by Panet and Selinger [3]. Unreacted *N*-ethylmaleimide was then removed by adding excess 100 mM mercaptoethylamine to 4 mM concentration The mixture was left for 5 min at room temperature The reaction product of *N*-ethylmaleimide with mercaptoethylamine and excess mercaptoethylamine were then removed by slowly passing the mixture through a short column of Dowex 50-X8 equilibrated against 10 mM Tris HCl, pH 8 5 ATP and ADP were then removed by slowly running the suspension through two volumes of Dowex 2-X8 equilibrated against 10 mM Tris HCl, pH 8 5

N-ethyl[^{14}C] maleimide-labelled vesicles These were prepared essentially according to the method of Panet and Selinger [3].

Reaction of ATP-protected, N-ethylmaleimide-treated vesicles with dinitrophenyl-maleimide 40 mM dinitrophenyl-maleimide, dissolved in dry dimethylformamide, was added to give a 4—5 molar excess over the theoretically available thiol groups of ATP-protected, *N*-ethylmaleimide treated vesicles in 10 mM Tris HCl, pH 8.5 The mixture was left for 10 min at room temperature. Mercaptoethylamine was then added in a 2—4-fold excess over the dinitrophenyl-maleimide and the mixture was left for 5 min at room temperature before being passed through Dowex 50-X8 equilibrated against 10 mM Tris HCl, pH 8 5, to remove the reaction product of dinitrophenyl-maleimide and mercaptoethylamine and excess mercaptoethylamine Apart from reaction of the reagent with thiol groups, coupling to very reactive histidine and lysine residues has to be anticipated [12]

Reaction of ATP-protected, N-ethylmaleimide-treated vesicles with bis-dinitrophenyl-cystine 100 mM Tris HCl, pH 8 5, was added to the treated vesicles in unbuffered 0.3 M sucrose to a final concentration of 10 mM 32 mM bis-dinitrophenyl dissolved 100 mM Tris HCl, pH 8 5, was added to give a 10-fold molar excess over the theoretical thiol content of the ATP-protected, *N*-ethylmaleimide-treated, vesicles and the mixture left for 1 h at room temperature Excess bis-dinitrophenyl-cystine and dinitrophenyl-cysteine formed during the reaction were then removed on a short column of Dowex 2X-8 equilibrated against 10 mM Tris · HCl, pH 8.5

Phospholipase C treatment ATP-protected, *N*-ethylmaleimide-treated vesicles were reacted with bis-dinitrophenyl-cystine as described above The treated suspension was then digested in 10 mM $CaCl_2$, 0 1 M KCl and 10 mM histidine, pH 7.0, with 0 1 mg phospholipase C per mg of vesicle protein for 30 min before washing off the enzyme by ultracentrifugation and resuspension in 0 3 M sucrose

Analytical methods The thiol content of the various preparations was measured by the method of Ellman [13] It was found that the presence of 0 34 or 1.0 mM sodium deoxycholate together with 0 5 M sucrose allowed the thiol determinations on the solubilised ATPase to be carried out without any significant aggregation of the material, as judged by turbidity

Dodecyl sulphate gel electrophoresis was carried out according to Weber and Osborn [14]. The gels contained 7.5% total acrylamide, and were calibrated as described previously [11] and stained for protein with Coomassie brilliant blue When autoradiography was carried out *N*-ethyl[$^{14}C_2$]maleimide-labelled vesicle samples containing 250 and 350 μg of protein (with 5 000 and 7 000

cpm, respectively) were denatured in sodium dodecyl sulphate in the absence of 2-mercaptoethanol Duplicate gels for each loading were run

Protein was determined by the method of Lowry et al [15], or spectrophotometrically in 1 or 2% (w/v) sodium dodecyl sulphate, using an $E^{\%}_{280\ nm,\ 1cm}$ value of 10 for whole vesicles based on Lowry determinations with bovine serum albumin as standard An $E^{\%}_{280\ nm,\ 1cm}$ of 12 was used for purified ATPase [11]

In spectrophotometric measurements, scattering at 280 nm was corrected for by a linear extrapolation from 330 to 360 nm Spectrophotometric estimations of dinitrophenyl content were based on a value of $\epsilon = 17\ 800\ M^{-1}\ cm^{-1}$ at the absorption maximum (359 7 nm for the dinitrophenyl-maleimide and 354 6 nm for the bis-dinitrophenyl-cystine conjugates, respectively)

Fluorescence quenching titrations were carried out in an apparatus described elsewhere [16], thermostated at 25°C 1 μM *N*-acetyltryptophanamide in water was used as the reference The excitation wavelength was 266 nm All fluorescence values were corrected for volume changes, and for trivial quenching from dinitrophenyl present (using a correction curve from the titration of *N*-acetyltryptophanamide with dinitrophenyl-ϵ-aminocarpoate) Anti-dinitrophenyl-antibody was stored frozen in small aliquots and clarified by centrifugation when required for use Prior to use its binding capacity was estimated by fluorimetric titration with 25 μM dinitrophenyl-ϵ-aminocaproate The medium for titration was either 50 mM borate, pH 8 5, or 0 1 M KCl, 50 mM borate, pH 8 5 (clarified by filtration through a No. 4 sinter) A blank experiment was carried out with the dinitrophenyl-vesicle conjugate added to the buffer alone in identical quantities to those added when the antibody was present to enable a correction to be made for the fluorescence of the dinitrophenyl-vesicle conjugate The ratio of the antibody fluorescence (F) to the initial antibody fluorescence (F_0) was then plotted against dinitrophenyl or protein added

Results and Discussion

Thiol contents The data are presented in Table I. When denaturation of both the whole vesicles and ATP-protected, *N*-ethylmaleimide-treated vesicles was carried out in 5 25 M guanidinium chloride, the number of extra thiol groups appearing was slightly greater than with dodecyl sulphate as the denaturant

1 mM ATP markedly reduces the rate of reaction of 5,5′-dithio-bis(2-nitrobenzoic acid) with the purified ATPase

Autoradiography of sodium dodecyl sulphate gels The *N*-ethyl[$^{14}C_2$]maleimide label attached to the substrate-protected thiol group of the sarcoplasmic reticulum vesicles was found to be associated only with the major band of 105 000 molecular weight and its dimer, i e with the ATPase polypeptide chain

Treatment of ATP-protected, N-ethylmaleimide-treated vesicles with dinitrophenyl-maleimide and bis-dinitrophenyl-cystine The dinitrophenyl contents of the vesicle preparations as determined by spectrophotometry and fluorimetric titration with anti-dinitrophenyl-antibody are shown in Table II Titration curves of anti-dinitrophenyl-antibody with the two kinds of dinitrophenyl-vesicle conjugate and dinitrophenyl-ϵ-aminocaproate are illustrated in Fig 1 Al-

TABLE I

THIOL GROUP CONTENT USING 5,5′-DITHIO-BIS(2-NITROBENZOIC ACID)

The reactions were carried out as described in Materials and Methods Sodium dodecyl sulphate was added to 0 67 or 1 0% (w/v), and guanidinium chloride to 5 25 M The thiol contents of the purified ATPase in the presence of ATP are based on the A_{412nm} values after 10 min reaction, by which time the rate of increase of the A_{412nm} was extremely low Average value in the absence of ATP 8 4 and 11 5 mol thiol per 10^5 g protein in the absence and presence of sodium dodecyl sulphate, respectively Average value in the presence of ATP 5 9 and 11 5 mol thiol per 10^5 g protein in the absence and presence of sodium dodecyl sulphate, respectively

Preparation	Mol 5,5′-dithio-bis(2-nitrobenzoic acid)-reactable groups per 10^5 g protein		
	No sodium dodecyl sulphate	+ Sodium dodecyl sulphate	+ Guanidinium chloride
Sarcoplasmic reticulum vesicles			
Preparation 1	10 9	14 1	15 6
Preparation 2	9 8	13 1	—
Average	10 35	13 6	
ATP-protected, *N*-ethylmaleimide-treated vesicles	1 1	4 6	5 7
Purified ATPase			
(1) With 1 mM deoxycholate + 0 5 M sucrose			
(a) No ATP	8 4	11 5	—
(b) With 1 mM ATP	5 6	11 7	—
(2) With 0 34 mM deoxycholate + 0 5 M sucrose			
(a) No ATP	8 4	11 4	—
(b) with 1 mM ATP	6 1	11 2	—

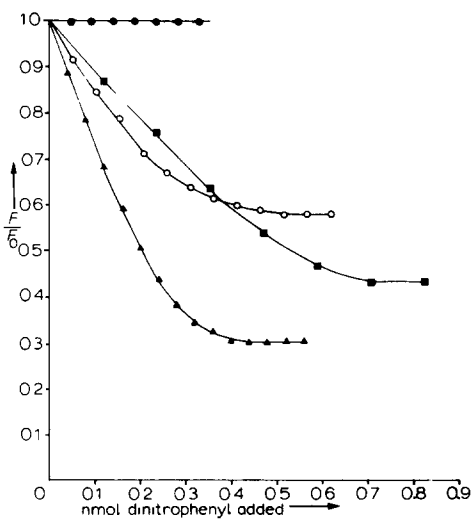

Fig 1 Fluorimetric titrations These were carried out as described in Materials and Mthods 10 μl of antidinitrophenyl-antibody (3 53 mg/ml, 0 262 nmol binding sites) were first added to 2 ml 0 1 M KCl, 50 mM borate, pH 8 5 ■, addition of 1-μl aliquots of ATP-protected, *N*-ethylmaleimide-treated vesicles after treatment with dinitrophenyl-maleimide (7 98 mg/ml, 1 48 mol dinitrophenyl per 10^5 g protein by spectrophotometry), ○, addition of 2-μl aliquots of ATP-protected, *N*-ethylmaleimide-treated vesicles after treatment with bis-dinitrophenyl-cystine (2 28 mg/ml, 1 13 mol dinitrophenyl per 10^5 g protein by spectrophotometry), ●, addition of 2-μl aliquots of phospholipase C-digested, bis-dinitrophenyl-cystine-treated, vesicles (2 08 mg/ml, 1 13 mol dinitrophenyl per 10^5 g protein by spectrophotometry), ▲, addition of 2-μl aliquots of 25 μM dinitrophenyl-ε-aminocaproate

TABLE II

COMPARISON OF THE DINITROPHENYL CONTENTS OF ATP-PROTFCTED, *N*-ETHYLMALEIMIDE-TREATED SACROPLASMIC RETICULUM VESICLES TREATED WITH DINITROPHENYL MALEIMIDE AND BIS-DINITROPHENYL-CYSTINE

Spectrophotometry was carried out as described in Materials and Methods Several of the fluorimetric titrations are described in the legend to Fig 1 The details of the remainder are as follows The concentration of deoxycholate when added to the dinitrophenyl-maleimide treated vesicles in a 1 1 weight ratio to the protein was 21 5 mM, the protein concentration being 9 28 mg/ml The concentration of deoxycholate in the bis-dinitrophenyl-cystine-treated vesicles was 6 6 mM, the protein concentration being 2 22 mg/ml The deoxycholate incubation was for 30 min on ice immediately prior to the titration

Preparation	Dinitrophenyl groups per 10^5 g protein		
	By spectrophotometry	By antibody titration	
		No deoxycholate preincubation	With deoxycholate preincubation
Dinitrophenyl-maleimide conjugate	1 48	0 62	0 69
Bis-dinitrophenyl-cystine conjugate			
(a) Before phospholipase C treatment	1 13	0 68	0 63
(b) After phospholipase C treatment	1 13	0	—

though short, the initial portions of the quenching curves obtained with the dinitrophenyl-vesicle conjugates were sufficiently linear to allow extrapolation to be made to find an equivalence point

Titrations of both types of dinitrophenyl-vesicle conjugate with anti-dinitrophenyl-antibody were also carried out after preincubation of the dinitrophenyl vesicles with a 1 1 weight ratio of sodium deoxycholate on ice for 30 min before the titration. This quantity of deoxycholate is sufficient to dissolve the sarcoplasmic reticulum membrane [10], the dinitrophenyl moiety now being present mainly on the solubilised ATPase, at least in the case of the bis-dinitrophenyl-cystine-treated material (see below) A blank titration where deoxycholate was present in dinitrophenyl-ε-aminocaproate added to anti-dinitrophenyl-antibody showed that this detergent had no significant effect on the binding of dinitrophenyl to antibody

The total percentage quenching of the antibody fluorescence by the dinitrophenyl-vesicle conjugates was always less than that found in titrations of the antibody with dinitrophenyl-ε-aminocaproate (see Table III) Thus, not all of the antibody binding sites were able to combine with dinitrophenyl groups on the vesicles This behaviour is possibly a consequence of the bi-functional nature of antibody molecules Thus, the effective antibody binding site concentration present was less than that based on the dinitrophenyl-ε-aminocaproate titration In the calculation of the equivalence points this was corrected for by comparing the maximum quenching of antibody fluorescence produced by the dinitrophenyl-vesicle conjugate with that produced by dinitrophenyl-ε-aminocaproate and reducing the assumed value for the available binding site concentration accordingly

Detailed interpretation of the results is difficult, but it is possible to make the following observations

TABLE III

THE DEGREE OF QUENCHING OF ANTIBODY FLUORESCENCE BY DINITROPHENYL-VESICLE CONJUGATES, AND THE FRACTION OF VESICLE-BOUND DINITROPHENYL GROUPS COMBINING WITH ANTIBODY

The experimental details are given in the legends to Fig 1 and Table II The fraction of vesicle-bound dinitrophenyl groups combining with antibody has been taken as the ratio of the antibodv-titratable dinitrophenyl content to that obtained by spectrophotometry, using the data of Table II

Preparation	Maximum quenching of antibody fluorescence (%)	Maximum quenching of antibody sample by dinitrophenyl-aminocaproate (%)	Fraction of dinitrophenyl groups combining with antibody (%)
Dinitrophenyl-maleimide conjugate			
(a) No deoxycholate	56	70	42
(b) With deoxycholate	57	71	47
Bis-dinitrophenyl-cystine conjugate			
(a) No deoxycholate	41	70	61
(b) With deoxycholate	53	70	56
After phospholipase C treatment, no deoxycholate	0	70	0

Firstly, the bis-dinitrophenyl-cystine procedure produced a conjugate having, by spectrophotometry, a dinitrophenyl content the same as that expected from the 5,5′-dithio-bis(2-nitrobenzoic acid)-reactable thiol content of ATP-protected, *N*-ethylmaleimide-treated vesicles (1 1 mol per 10^5 g protein in both cases) The closeness of the dinitrophenyl content of this conjugate to the unreacted thiol group content indicates that the dinitrophenyl label was probably attached to the unreacted thiol group, especially as this reagent is unlikely to react with residues other than cysteine or cystine. As anticipated, the dinitrophenyl-maleimide conjugate had a higher dinitrophenyl content (1.5 mol per 10^5 g protein) than the theoretical ATP-protected thiol content, possibly due to non-specific reaction of the reagent with histidine or lysine residues [12]

Secondly, in the case of both the conjugates, some of the antibody dinitrophenyl binding sites remain uncombined at the end of the titration, particularly in the case of the bis-dinitrophenyl-cystine conjugate Presumably some of the antibody molecules have only one Fab arm combined with dinitrophenyl, the other remaining unattached because of the distance between the membrane-bound dinitrophenyl groups, or because of steric hindrance This effect was further investigated by digesting the dinitrophenyl-vesicle conjugate with phospholipase C, as described below

Thirdly, the fraction of the total dinitrophenyl groups of the dinitrophenyl-vesicle conjugates able to combine with antibody binding sites was higher in the case of the bis-dinitrophenyl-cystine conjugate (61%) than with the dinitrophenyl-maleimide conjugate (42%)

Effect of phospholipase C treatment on the binding of anti-dinitrophenyl-antibody by ATP-protected, N-ethylmaleimide-treated vesicles treated with bis-dinitrophenyl-cystine When the effect of phospholipase C digestion on the bis-

dinitrophenyl-cystine-treated preparation of Table II was examined, it was found that after 30 min digestion none of the dinitrophenyl groups on the vesicles were now accessible to the antibody, i e the vesicles no longer quenched the antibody fluorescence (see Fig 1), although spectrophotometry in 2% (w/v) sodium dodecyl sulphate showed that no dinitrophenyl had been lost from the preparation during the digestion

Adding phospholipase C (under the usual digestion conditions 0 1 M KCl, 10 mM $CaCl_2$, 10 mM histidine, pH 7 0) directly to either the intact vesicles or to the dinitrophenyl-cystine conjugate, in the fluorimeter cuvette, showed little or no change in the tryptophyl fluorescence of the sarcoplasmic reticulum membranes occurred during the digestion process

Phospholipase C treatment of sarcoplasmic reticulum vesicles leads to a decrease in the surface area of the membranes [17] Thus, the explanation of the effect of phospholipase C treatment on the binding of antibody to the dinitrophenyl groups attached to the active site thiol groups of the vesicles is probably a steric one as the surface area of the membrane is reduced by the action of the enzyme, the ATPase molecules embedded in it [9,18], become crowded together, and the dinitrophenyl moieties are no longer accessible to the antibody

Acknowledgements

I would like to thank Dr N M Green for encouragement and advice and Mr D J Julian for technical assistance

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