LOCALIZATION OF THE REACTIVE TRINITROPHENYLATED LYSYL RESIDUE OF MYOSIN ATPase SITE IN THE NH_2-TERMINAL (27 k DOMAIN) OF S1 HEAVY CHAIN

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1. Introduction

Trinitrophenylation by 2,4,6-trinitrobenzene sulfonate of 1 highly reactive ϵ-NH_2 lysine residue/myosin subunit induces a remarkable change in the ATPase activity of the enzyme; the Mg^{2+}-ATPase is significantly accelerated, the maximal activity not being enhanced by actin, while concomitantly the K^+-ATPase is inhibited; these enzymatic properties are much less affected when the modification is performed in the presence of ATP [1,2]. The trinitrophenylated lysine is reported to reside on S1 heavy chain [2–4] in the large hydrophobic crevice of the ATPase site [5]. Confusing results were first obtained amino acid sequencing around this critical lysine was attempted [6] as different TNP-peptides were isolated from the starting modified myosin and prevented a conclusive structural identification of the trinitrophenylated site. It was concluded that the modified lysine resides in one of the previously identified TNP-peptides the structure of which is Asn–Pro–Pro–Lys [7].

As part of our work on the structure–function relationships in myosin heads, this study was directed at definition of the location of the functional lysine in the three-domain structure of chymotryptic S1 heavy chain [8]. The procedures used demonstrate the presence of the active lysine in the NH_2-terminal M_r 27 000 (27 k) domain; they provide also an efficient experimental approach for isolation of specifically modified 27 k segment allowing unambiguous characterization of the chemical structure around this residue.

2. Experimental

2,4,6-Trinitrobenzene sulfonate (TNBS) was from Serva; α-chymotrypsin and TPCK-trypsin were from Worthington. Rabbit myosin was prepared according to [9]. Subfragment-1 (S1) was prepared by digestion of myosin filaments with chymotrypsin [10]; it was purified on Sephacryl S-200 and stored at 4°C in 0.05 M Tris–HCl buffer (pH 7.5); enzyme preparations were used in <1 week. Actin was purified according to [11]. Protein concentrations for native and TNP-S1 were estimated using $E^{1\%,\,1\ cm}_{280\ nm} = 7.5$ [2]; the absorbance value was corrected for the small contribution of the TNP group. Concentration of TNP-lysine was estimated using $\epsilon_{345\ nm} = 1.45 \times 10^4\ cm^{-1}$ [2]. Concentration of 27 k and 50 k peptides was measured by amino acid analysis. Calculations were based on S1 M_r 115 k [10]. Determination of K^+-EDTA-, Ca^{2+}-, Mg^{2+}- and actin-stimulated ATPase activities was performed as in [12]. SDS–10% polyacrylamide slab gel electrophoresis was done as in [8].

2.1. *Trinitrophenylation of chymotryptic S1*

S1 (10 mg/ml) was treated with 10-fold molar excess TNBS in 50 mM Tris–HCl buffer (pH 7.5) at 25°C. Another enzyme sample was treated under identical conditions in the presence of 10 mM ATP or Mg^{2+}-ATP. At suitable time intervals 5–10 μl aliquots were withdrawn from the reaction mixtures and assayed for K^+-, Mg^{2+}- and actin-dependent ATPase activities. After 5 min labeling, excess TNBS was eliminated by quick gel filtration over Sephadex G-25 (coarse) in 0.025 M Tris–HCl (pH 7.5) at 25°C; this procedure was very efficient in stopping the chemical modification as assessed by enzymatic activity measurements before and following the gel filtration step.

2.2. *Limited proteolysis of modified S1 and separation of the trinitrophenylated heavy chain tryptic fragments*

TNP-S1 (1–2 mg/ml) and TNP-S1 complexed to

F-actin (molar ratio actin:enzyme = 2) were converted to (27 k–50 k–20 k)–S1 and (27 k–70 k)–S1 derivatives, respectively, by limited proteolysis of the heavy chain with a trypsin to enzyme ratio 1:100 (w/w) for 30 min, at 25°C, in 100 mM CO_3HK (pH 8.0) [8]. Proteolysis was stopped by addition of soybean trypsin inhibitor (twice the amount of trypsin) and the digestion medium was lyophilized. The digest (12–15 mg protein) was dissolved in 1–1.5 ml solution of 1% SDS, 2 mM EDTA, 1 mM dithiotreitol, 50 mM Tris–HCl buffer (pH 7.5) then heated for 5 min at 100°C. The separation of the different tryptic heavy chain fragments was achieved by gel filtration on two columns in series (each 130 X 1.1 cm) of Sephacryl S-200 eluted at room temperature with the same buffer. The effluent was monitored by spectrophotometry (at 230 nm and 345 nm) and by 0.1% SDS–10% polyacrylamide slab-gel electrophoresis.

3. Results and discussion

When the reaction of TNBS with chymotryptic S1 was monitored at 345 nm, it showed the kinetics expected for the preferential trinitrophenylation of ~1 lysine/mol S1 (fig.1, curve 1). When the modification was performed in the presence of ATP, the trinitrophenylation of this fast reacting ϵ-NH_2 group was retarded but this effect was much more prononced with Mg^{2+}-ATP (fig.1, curve 2). Because TNBS can

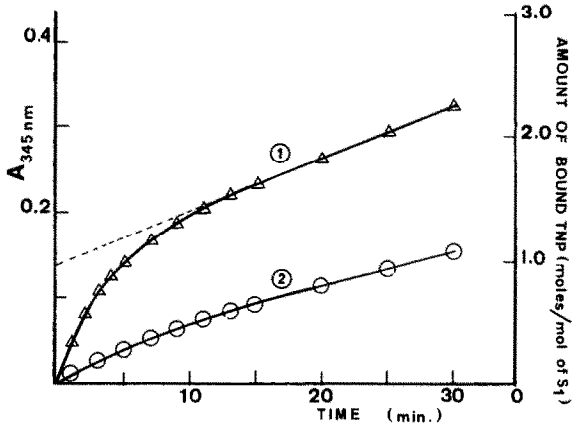


Fig.1. Kinetics of trinitrophenylation of chymotryptic S1 in the absence and presence of Mg^{2+}-ATP. S1, 10 nM in 0.05 M Tris–HCl (pH 7.5) was mixed with 100 nM TNBS. The reaction was monitored at 345 nm in a Cary 118 spectrophotometer at 25°C. The experiment was done in the absence (△) and presence (○) of 10 mM Mg^{2+}-ATP.

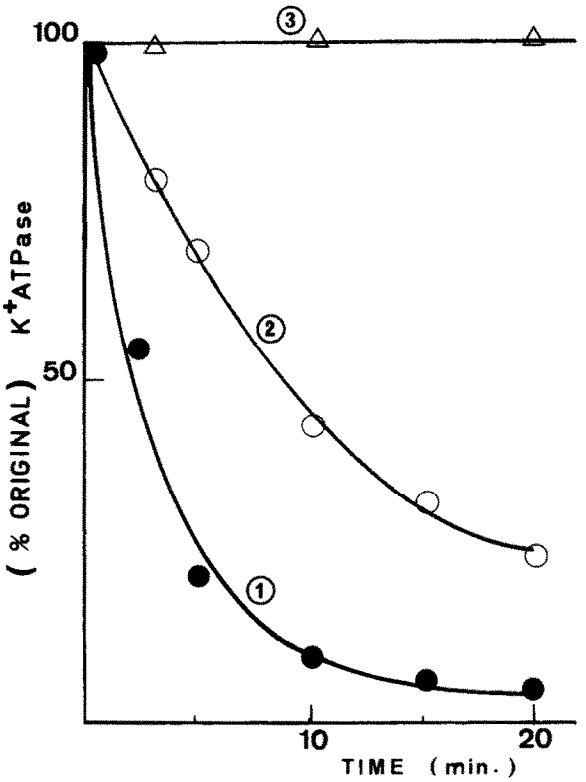


Fig.2. Time course of changes in K^+-ATPase of S1 upon trinitrophenylation in the absence and presence of ATP. S1 was reacted with TNBS as in fig.1. At the time indicated 50 μl aliquots were assayed for K^+-ATPase. Reaction mixtures contained 10 mM ATP (○), 10 mM Mg^{2+} ATP (△), no nucleotide (●).

react with the adenosine moiety of ATP [13] a control experiment with a mixture of ATP and TNBS was run in parallel but gave no evidence for consumption of the reagent by ATP under the experimental conditions employed. When the enzymatic activities of S1 were measured during the course of the reaction, the K^+-ATPase was found to decrease with increasing degree of trinitrophenylation (fig.2, curve 1); ATP and Mg^{2+}-ATP afforded partial and complete protection respectively (fig.2, curves 2,3). Moreover the Mg^{2+}-ATPase activity was 7-fold accelarated while the actin-dependent Mg^{2+}-ATPase concomitantly decreased reaching a value similar to that measured in the absence of actin (fig.3); the rate of Mg^{2+}-ATPase activation was essentially similar to that related to K^+-ATPase inactivation (fig.3, inset). Together these results clearly indicate that all the criteria reported earlier for specific modification of the TNBS-sensitive

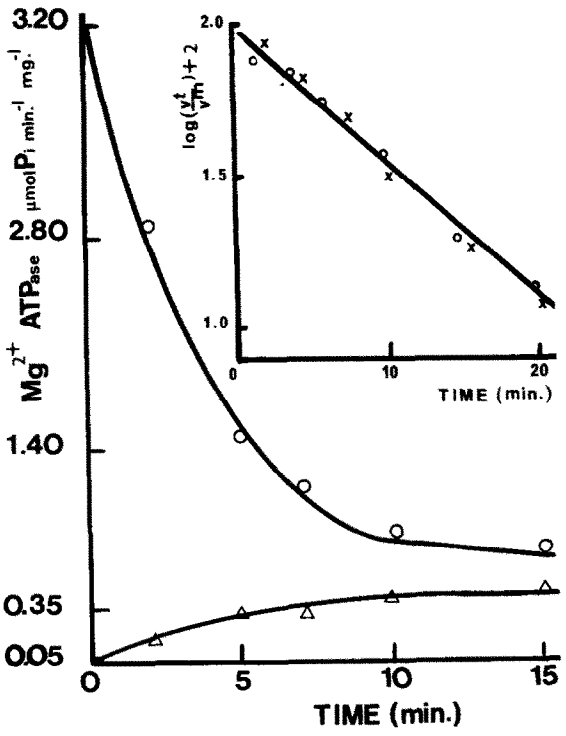


Fig.3. Effect of trinitrophenylation of S1 on the Mg^{2+}-ATPase activity measured in the presence (○) and in the absence (△) of actin. The experimental conditions were as in fig.1. Inset: determination of the pseudo-first order rate constant (K_i) for K^+-ATPase inactivation (×) and Mg^{2+}-ATPase activation (○).

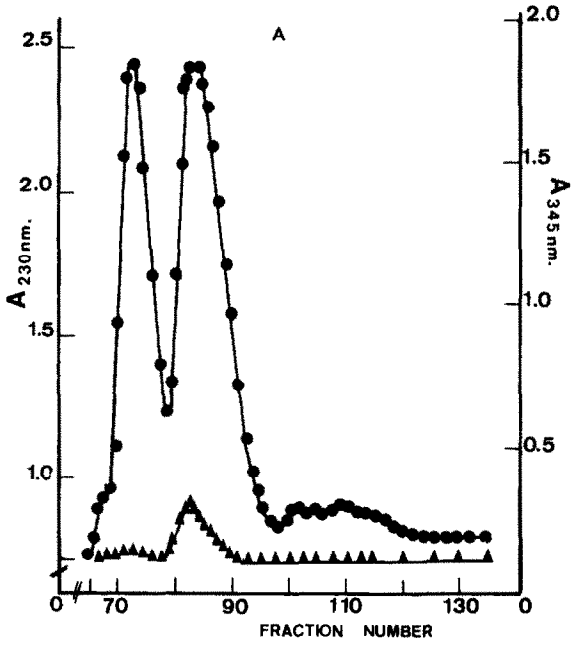
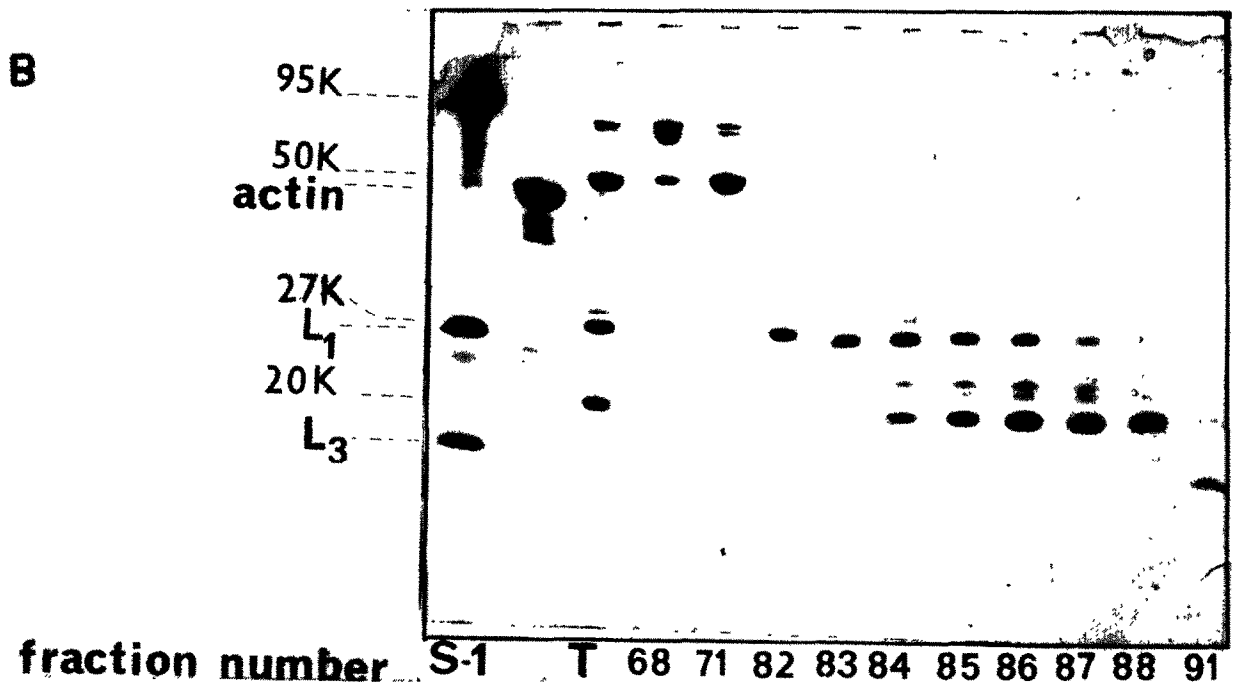


Fig.4. Fractionation of the tryptic fragments derived from the heavy chain of trinitrophenylated (27 k–50 k–20 k)–S1. The experimental conditions were as in section 2. The columns of Sephacryl S-200 were eluted at 7 ml/h and the effluent (1.1 ml) was monitored at 230 nm (●) and 345 nm (▲). (A) Elution profile of S1 labeled in the absence of Mg^{2+}-ATP. (B) 0.1% SDS–10% polyacrylamide gel-slab electrophoresis of the fractions eluted in (A). S1, native enzyme; T, initial TNP–(27 k–50 k–20 k)–S1 derivative.



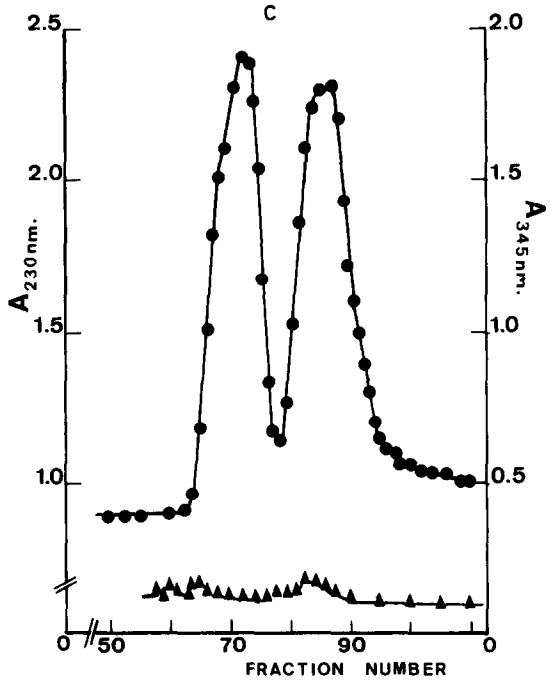


Fig.4. (C) Elution of S1 labeled in presence of Mg^{2+}-ATP.

ϵ-NH_2 lysine of myosin head [2] have been fulfilled under our experimental conditions.

After purification of labeled S1 over Sephadex G-25, the actual amount of TNP group incorporated in the absence and presence of Mg^{2+}-ATP was estimated to be, respectively, 1.5 mol and 0.4 mol/mol S1 (table 1); as observed in [1] TNBS was still incorporated into S1 in the presence of nucleotide but without significant effect on the enzymatic properties. Both the inhibited and active TNP-S1 samples were then submitted to a limited tryptic proteolysis which is known to convert the monomeric 95 k heavy chain of native S1 into a three-fragment complex of M_r 27 k–50 k–20 k [8]; no apparent effect of the chemical modification on this fragmentation pattern was noted after analysis of the digestion medium with gel electrophoresis. The digests could be fractionated on Sephacryl S-200 in a dissociating medium (fig.4A). Two well-separated peptide peaks were obtained and analyzed by slab-gel electrophoresis (fig.4B). The first peak (fractions 72–79) was pure 50 k fragment. The second peak contained the 27 k peptide in the leading edge (fractions 81–85) and the 20 k fragment as well as light chain 3 and the breakdown products of light chain 1 [8] in the trailing edge (fractions 85–91); a trace amount of the transient NH_2-terminal 75 k fragment was present in the initial 66–69 fractions

Table 1
Amount of TNP groups incorporated into S1 and its heavy chain tryptic fragments in the absence and presence of Mg^{2+}-ATP

Protein prep.	TNP group incorporation/mol S1		K^+-ATP-ase
	Mg^{2+}-ATP absent	Mg^{2+}-ATP present	
S1	1.3–1.5	0.3–0.4	2% 90%
27 k fragment	0.9–1.2	0.2	
50 k fragment	0.2	0.1	

of the effluent. About 1.2 mol TNP group/mol S1 (80% of the total A_{345} in the material put on the column) were associated with the 27 k peptide containing fractions and ~0.2 mol TNP group/mol S1 were within the 50 k peptide peak (fig.4A, table 1). The fractionation of the ATP-protected S1 showed the small TNP absorbance of the sample to be evenly distributed over 27 k and 50 k fragments (fig.4C, table 1).

To ascertain that 27 k peptide represents the major site for binding of the TNP group, it was necessary to isolate this peptide free of 20 k fragment. A decisive separation of the 27 k element from the contaminating 20 k peptide was achieved by first digesting with trypsin the complex actin-inhibited TNP-S1; because actin binding to S1 affords complete protection of the 50 k–20 k junction against proteolysis, the heavy chain is split into only 2 fragments of M_r 27 k and 70 k [8]. After Sephacryl gel filtration (fig.5A), the 27 k unit (fractions 81–88) was obtained in almost pure state as assessed by gel electrophoresis of the column effluent (fig.5B) and by amino acid analysis. It was found to contain 0.90 mol TNP/mol S1 corresponding to 70% of the total TNP absorbance of the sample; the remaining material absorbing at 345 nm was eluted as 70 k peptide (fractions 68–71). Thus the amount of TNP group bound to 27 k peptide closely accounts for the observed K^+-ATPase inhibition.

Our results are in contrast with [14] which suggests, without providing experimental evidence, that the TNP-lysine is located in either the 20 k or 50 k peptides but not in the 27 k unit. This proposal is unlikely. Here, we did not observe appreciable labeling of the 20 k material; also, the covalent structure of

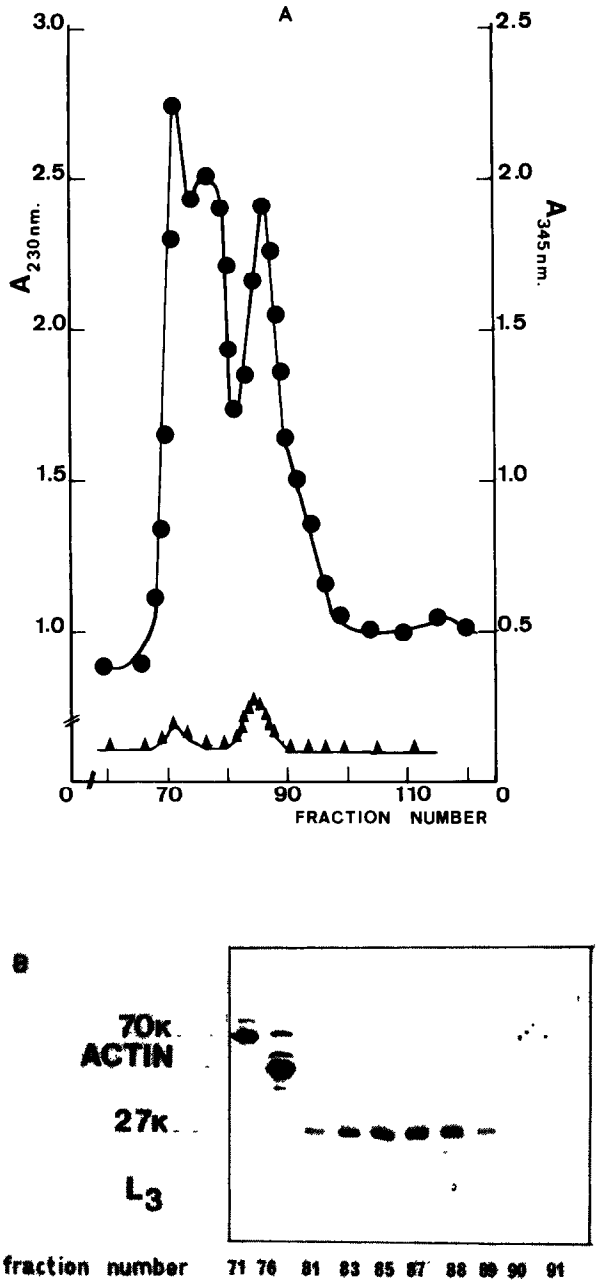


Fig.5. (A) Fractionation of the tryptic fragments derived from the heavy chain of trinitrophenylated (27 k–70 k)–S1. Native S1 was labeled in the absence of Mg^{2+}-ATP, cleaved with trypsin in the presence of F-actin (as specified in section 2) and fractionated on Sephacryl S-200 as in fig.4; the effluent was monitored at 230 nm (•) and 345 nm (▲). (B) Slab-gel electrophoretic pattern of the different column fractions.

the 20 k peptide [15] does not show the 4-residue sequence containing the modified lysine [7]. As TNBS is reacting preferentially but not exclusively with the 27 k segment, the fractional amount of TNP group incorporated into the 50 k peptide can be, on the contrary, a potential source of misleading results; trinitrophenylation of 50 k unit significantly increases under non-controlled conditions (use of aged S1 preparations or long reaction time); Mg^{2+}-ATP decreased TNP group incorporation into 27 k material only. TNP-lysine in the 27 k peptide was advocated in [16] but without experimental support.

The actin-modulated fragmentation of S1 heavy chain into 27 k and 70 k fragments provides a specific way for simple isolation of the native or modified NH_2-terminal component which may be used for sequence studies. Binding of nucleotides to TNP-S1 changes the environment of the functional lysyl residue [17]. Recently, the 27 k fragment was found to be involved in the covalent attachment of an inhibitory arylazido analogue of ATP [18]. These findings together with this investigation, which illustrates the localization of the reactive lysine within the 27 k fragment, suggest that the NH_2-terminal domain of S1 heavy chain may be part of myosin ATPase site.

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