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**3-(BROMOACETAMIDO) - PROPYLAMINE HYDROCHLORIDE:
A NOVEL SULFHYDRYL REAGENT AND ITS FUTURE POTENTIAL IN
THE CONFIGURATIONAL STUDY OF S1-MYOSIN**

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ABSTRACT

Configurational study of S1-Myosin is an important step towards understanding force generation in muscle contraction. Previously reported NMR studies have been corroborated by us. We have synthesized a novel compound, 3-(Bromoacetamido) - Propylamine Hydrochloride, and its potential as a sulfhydryl reagent provides an indirect but elegant approach towards future structural elucidation of S1-Myosin. Our preliminary investigation has shown that this compound, BAAP, reacted with S1 in the absence of MgADP and the modified enzyme had a 2-fold increase in Ca-ATPase activity and no detectable K-EDTA ATPase activity. Reaction of BAAP with S1 in the presence of MgADP resulted in a modified enzyme which retained a Ca-ATPase activity that was about 60% of unmodified S1 and had essentially zero K-EDTA ATPase activity. Sulfhydryl titration indicated that about 1.5 and 3.5 -SH groups per S1 molecule were blocked by BAAP in the absence and presence of MgADP, respectively. When coupled to a carboxyl group of EDTA, the resulting reagent could become a useful -SH reagent in which chelated paramagnetic or luminescent lanthanide ions can be exploited to probe S1 conformation.

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Introduction

Myosin Subfragment 1 is the globular head of myosin and contains two sets of functional sites that are essential for force generation in muscle contraction. These are the two actin-binding sites and the single nucleotide-binding site at which ATP is hydrolyzed. One current model of contraction¹ proposes that force generation results from structural changes occurring in the S1 region of myosin while myosin is still attached to actin. Considerable attention has been paid to the structure of S1 in intact myosin or myofibrils as well as isolated S1 and structural changes that occur in S1 resulting from interaction with actin and nucleotides.

Among the various tools used in the study of proteins, NMR has been used successfully for proteins of molecular weights less than 20K. For larger macromolecules, however, the spectra are accompanied by broad, poorly-resolved overlapping NMR signals. Nevertheless, recent high-resolution NMR has provided valuable information on structural changes that proteins undergo under varied conditions.²

Within the context of structural studies of S1 in solution, in the present work ¹H NMR spectra were observed both with and without the addition of chemical denaturants. The spectra were then set against a computer-simulated random-coil spectrum³ (Fig.1) and the derived assignments compared against previously published results⁴. The present NMR data seem to be in general agreement with those previously reported.

S1 contains two reactive thiols (SH₁ and SH₂) located at Cys 707 and Cys 697 of the heavy chain which are readily alkylated by a number of sulfhydryl reagents⁵. The chemically-modified protein provides a model in which structural information can be obtained. Modification of one -SH group (SH₁) of S1 typically activates the Ca-ATPase by a factor of 2-3 and suppresses the K-EDTA ATPase activity⁶. When both SH₁ and SH₂ are blocked, both types of ATPase activity are abolished⁷. Early studies indicated that the two -SH groups can be crosslinked or chelated by several bifunctional alkylating agents⁸ suggesting considerable flexibility of the polypeptide backbone between Cys 697 and Cys 707. The equilibrium separation between SH₁ and SH₂ was determined by measuring the extent of fluorescence resonance energy transfer between probes covalently attached to the two thiols⁹. The measured distance was found to be sensitive to MgADP. Other workers have also investigated S1 conformation by using several extrinsic fluorescent probes¹⁰.

A different approach to the use of fluorescent probes for distance measurements was provided by Mears and coworkers¹¹, who synthesized para-substituted derivatives of 1-phenyl ethylenedinitrilotetraacetic acids in which the substituents are NO₂, NH₂, N₂⁺, HNCOCH₂Br, etc. Once attached to a protein, the tetraacetate groups can be exploited to chelate cation possessing suitable spectroscopic or radioactive properties for structural studies of the host protein. In particular, lanthanide ions can be used for this purpose. In preliminary studies we were unable to selectively modify SH₁ of subfragment 1 and to obtain the desired degree of ATPase activation. This lack of success led us to synthesize an aliphatic EDTA analog with sulfhydryl reactivity. In this report we describe the synthesis of a precursor of such an analog, 3-(Bromoacetamido)-propylamine hydrochloride, and its reaction with SH₁ and SH₂ of S1.

Experimental

Synthesis of BAAP.

An indirect method for synthesis of bromoacetamidoalkylamines has been reported¹². Though direct monoacylation of diamines has proved to be difficult¹³, in our present work we have obtained the product, the reaction being carried out at a relatively low temperature. To 2.5 mL of 1,3 diaminopropane (0.03 mol), in a small volume of dry chloroform, was added dropwise 2.47 mL bromoacetylchloride (0.03 mol) while stirring in an atmosphere of nitrogen. The reaction was carried out in the presence of dry ice/ acetone. The sticky white precipitate that formed was separated and extracted overnight with chloroform under ice-cold conditions. On evaporating to dryness, the chloroform extract yielded a white crystalline solid (m.p. 113-115°C). TLC tests with ninhydrin and 4-(4-Nitrobenzyl) pyridine established the purity of the product. ¹H NMR profile corresponded to four methylene resonances (4.1-1.7 ppm) and a downfield component (7.1 ppm) resulting from the exchange of the amide proton.

Microanalysis data (Table 1) correspond to the empirical formula C₅H₁₂BrClON₂ (FW 231.6). The molar extinction coefficient of a sample of BAAP in dimethyl sulfoxide was determined (E₂₇₇ = 4.4 x 10³).

Table 1. Microanalysis of BAAP.

	% C	% H	% N	% Halogen
Calculated	25.93	5.22	12.10	49.81
Microanalysis	26.03	5.11	11.96	50.16

Protein Preparation.

Myosin was obtained from rabbit skeletal muscle by a method described by Flamig and Cusanovich¹⁴. The procedure involved extraction of freshly-ground back and leg muscles with a phosphate buffer, pH 7.5 containing 0.3 M KCl for 1 hr. The precipitate was dissolved in 0.5 M KCl and subsequently reprecipitated. Subfragment 1 was obtained by digestion of myosin with α -chymotrypsin, followed by purification on a DEAE-cellulose (DE-52) column¹⁵. The S1 concentration was estimated from a molecular weight of 115,000 and an extinction coefficient of E₂₈₀^{1%} = 7.5 cm⁻¹¹⁶.

Reaction of S1 with BAAP.

S1 was labeled at SH₁ with a 1.5-fold molar excess of BAAP in a buffer containing 60 mM KCl and 50 mM Tris at pH 7.8 (Buffer A). Prior to labeling, the protein solution (130 μ M) was dialyzed in Buffer A in the presence of 1 mM DTT. Any residual DTT was subsequently removed by extensive dialysis with Buffer A. Appropriate quantities of 5 mM BAAP (dissolved in Buffer A) were added to S1 followed by incubation in the dark under ice-cold conditions for 18-20 hr. Any unreacted probe was subsequently removed by exhaustive dialysis in Buffer A. The percentage of protein sites labeled was calculated by measuring the absorbance of a sample of labeled protein at 280 nm and subtracting the optical density derived for an equivalent concentration of protein at this wavelength. The Lowry method¹⁷ was used to determine protein concentration in labeled S1 to avoid optical interference from the label.

For SH₂ modification, SH₁-modified S1 was incubated with a 4- and 50-fold molar excess of BAAP (3 mM) and MgADP, respectively, under ice-cold conditions in the dark for 38-40 hr. Ca and K-EDTA ATPase activities were determined by measuring the release of inorganic phosphate using a modification of the Fiske-Subbarow method as previously described⁵. The thiol content was determined using Ellman's method¹⁸. S1 (0.1 mL of 30-50 μM) was added to 2.6 mL of a freshly-prepared urea solution (9.0 M Urea, 10 mM EDTA, 50 mM Tris, 0.1 M KCl, pH 8.0) and the absorbance was measured at 412 nm. After approximately 15 minutes 0.3 mL of freshly-prepared DTNB (1 mM DTNB, 0.1 M KCl, 10 mM phosphate, pH 7.0) was added and the absorbance at 412 nm was followed until there was no further increase in absorbance. A molar extinction coefficient of 13600 at 412 nm was used for DTNB.

NMR

Sample buffer containing 50 mM KCl, 12 mM phosphate, 1 mM NaN₃ in ²H₂O at p²H 7.0 was passed through a column packed with chelex. S1 in sample buffer was next dialyzed against 330 mM EDTA in buffer followed up by dialysis against several changes of buffer at 4°C. Denatured samples were prepared by adding 8M Urea (²H). The ¹H NMR spectra were obtained on a Bruker WH-400 spectrometer operating in the FT mode. A typical sample consisted of 0.4 ml S1 (90 μM) in the presence of TSP, an internal standard. The H²O resonance was saturated with a homonuclear decoupling phase. All spectra were recorded at 4°C.

The ¹H NMR spectra of BAAP in CDCl₃ were recorded in a Nicolet -300-W.B. FT NMR spectrometer.

Results and Discussion

The relative ATPase activities of native and modified S1 and their sulfhydryl content are reported in Table 2. Modification of the SH₁ site was evidenced by a two-fold increase in Ca-ATPase while the corresponding K-EDTA ATPase activity was completely abolished. Comparison of the data on the third row of Table 2 indicated that the second site (SH₂) was labeled.

Table 2. Comparison of relative ATPase activities between native and modified S1 and the corresponding loss in the number of free sulfhydryl groups.

Sample	Relative ATPase activities ^(a)		Loss of -SH Groups per mole of S1 ^(a)
	Ca(%) ^(b)	K-EDTA(%) ^(c)	
1. S1-unmod	100	100	-
2. S1-(1) BAAP	207	0	1.48
3. S1-(1,2) BAAP	61	4	3.43

(a) The data are obtained by averaging over two samples.

(b), (c) 100% Ca-ATPase and K-EDTA ATPase activities correspond to 1.07 and 2.68 moles Pi mg⁻¹ min⁻¹, respectively.

Overall, preliminary biochemical tests indicate that BAAP is a monofunctional SH-coupling reagent. It has a relatively short span ($10.80 \pm 0.03 \text{ \AA}$)¹⁹ and appears reasonably specific for myosin SH₁ and SH₂. It should be possible to link BAAP via its terminal amino group to a carboxyl group of EDTA. Such a compound should still possess sulfhydryl reactivity and is expected to chelate paramagnetic or luminescent lanthanide ions such as Tb (III). The chelated cation provides a spectroscopic signal that may be useful for elucidating the structural properties of the protein by luminescence or NMR methods.

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Abbreviations: S1, chymotryptic subfragment-1 of myosin; BAAP, [3-(Bromoacetamido)- Propylamine hydrochloride]; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TSP, [3-(Trimethylsilyl)propionic acid, Na salt]; S1-(1) BAAP, subfragment-1 modified with BAAP at SH₁; S1-(1,2) BAAP, subfragment-1 modified with BAAP at SH₁ and SH₂.