ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF THE INHIBITORY PROTEIN OF THE TROPONIN SYSTEM

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1. Introduction

The protein complex troponin, which in combination with tropomyosin constitutes the regulatory protein system of skeletal muscle, has been shown to contain several protein components of different biological activities [1–3].

One component, troponin A or "calcium sensitizing factor" had no influence on desensitized actomyocin (DAM)* alone, but in combination with troponin B or "inhibitory factor" caused the ATPase activity of DAM to become dependent on calcium concentration.

Troponin B inhibited the ATPase activity of synthetic actomyosin, and tropomyosin enhanced this inhibition. SDS acrylamide gels showed troponin B to be composed of two major components of approximate molecular weights 39,000 and 26,000 daltons, respectively [4].

Wilkinson et al. [5] showed that inibitory factor preparations contained proteins of molecular weight 37,000 and 23,000 daltons, and depending on the preparative conditions, a third component of molecular weight 14,000 daltons. The precise role of the "37,000 component" is obscure as yet, but there are indications that it may have a special affinity for tropomyosin. The "14,000 component" possessed inhibitory activity to a limited degree which was not always enhanced by tropomyosin. It is possible that this component arises from the "23,000 dalton" one by proteolytic attack during acid isoelectric precipitations. Certainly it could not be detected in SDS acrylamide gels of whole fresh myofibrils. The "23,000 component" was implicated to be the major active fraction and its inhibitory activity was enhanced by tropomyosin.

The present investigation represents the first physico-chemical characterization of homogeneous inhibitory protein prepared by the method of Wilkinson et al. [5]. The measurements include low speed sedimentation equilibrium, circular dichroism in the presence of Mg^{2+}, SDS polyacrylamide gel electrophoresis, amino acid analysis and parallel biological activity studies.

2. Materials and methods

Crude troponin B and B supernatant prepared from rabbit skeletal muscle, using the method of Hartshorne and Mueller [1], were further purified by chromatography on a column of CM cellulose at room temp. according to Wilkinson et al. [5].

SDS acrylamide gel electrophoresis and molecular weight determinations were carried out according to Murray and Kay [6].

ATPase was assayed essentially as described by Schaub and Perry [3]. The reaction mixture consisted of 1.5 mg of DAM at 25°, containing 2.5 mM Tris ATP, 25 mM Tris-HCl buffer, pH 7.6, 2.5 mM $MgCl_2$, 1 mM EGTA and 150 μg tropomyosin. The total volume of the reaction mixture was 3 ml. Actomyosin was desensitized by trypsin treatment [7].

Circular dichroism measurements were made on a

* *Abbreviations*:
DAM, desensitized actomyosin; SDS, sodium dodecyl sulphate; sulphate; CM cellulose, carboxymethyl cellulose; ATPase, adenosine triphosphatase; DTT, dithiothreitol; G-HCl, guanidine hydrochloride.

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Cary model 6001 CD attachment to a Cary 60 recording spectropolarimeter, in accordance with the methodology of McCubbin and Kay [8]. The mean residue molecular weight was taken as 115 for the protein. Protein concentrations were determined by the Lowry method [9]. CD measurements were made in 0.5 M NaCl, 0.05 M Tris, pH 8.0. The protein in the above solvent was dialysed in the cold in the presence of Chelex-100 to remove any trace of Mg^{2+} and Ca^{2+} which might be present in the solvent. This Mg^{2+} and Ca^{2+} free protein was used in CD studies designed to investigate the effect of Mg^{2+} and Ca^{2+} on this system.

Amino acid analysis of the protein was affected in a Beckman model 121 analyzer on samples hydrolyzed for 24, 48, and 72 hr. The values reported are averaged values for the three hydrolysis times. Threonine and serine values were obtained by extrapolating to zero time. For valine and isoleucine the 72 hr value was used.

Ultracentrifugal studies were carried out at 20° in a Beckman Spinco model E ultracentrifuge, equipped with a photoelectric scanner, multiplex accessory and high intensity light source. Double sector charcoal-filled Epon cells with wide aperture window holders were used. Low speed sedimentation equilibrium experiments were carried out in 0.5 M NaCl, 0.05 M Tris, 1 mM DTT at pH 8.0 according to the methodology described by Chervenka [10]. The partial specific volume calculated from the amino acid analysis of our preparation was found to be 0.74 ml/gm and a value of 0.73 ml/gm was assumed in G-HCl medium [11].

3. Results and discussion

CM cellulose column chromatography of crude Troponin B and B supernatant resulted in 3 peaks (fig. 1). The protein in the first peak was not retarded on the column and was mainly troponin A and the 14,000 component. Peaks II and III corresponded to the inhibitory protein and the 37,000 component, respectively. SDS acrylamide gel electrophoresis (fig. 2) revealed a doublet band pattern for the inhibitory protein suggesting that it consists of two proteins of very similar mobility and molecular weight. The doublet band pattern was more pronounced when

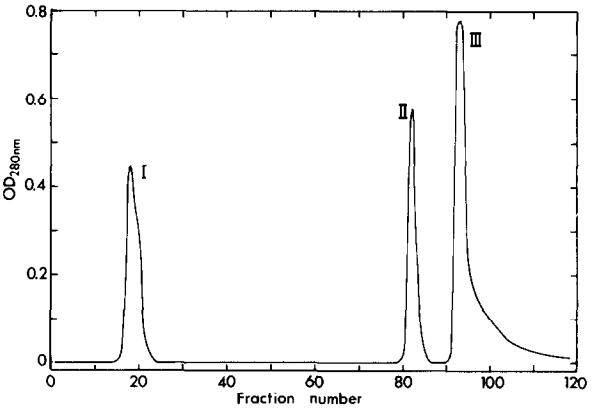

Fig. 1. Crude tropinin inhibitory protein (400 mg) was applied to a column of CM-cellulose in aqueous 70% (v/v) ethanol–2.6 M formic acid–0.1 M sodium formate. The column was eluted with a linearly decreasing gradient to aqueous 45% (v/v) ethanol. Total volume of the gradient was 900 ml. Each fraction contained 6 ml.

the gels were not overloaded and when the run was carried out in 10% gel for a longer time. The average molecular weight computed for the two components from SDS acrylamide gel electrophoresis was 23,000 ± 500. When the SDS acrylamide gels of inhibitory protein were scanned in a densitometer, there was no indication of any minor contaminants in our preparations. Also included in the figure are the gel patterns of crude Troponin B and B supernatant.

The 23,000 component was found to inhibit the Mg^{2+} stimulated ATPase activity of DAM in the presence of EGTA. The presence of tropomyosin was essential for the full inhibitory effect. 100 μg of inhibitory protein was required per mg of DAM for maximum inhibition in the presence of optimum concentration of tropomyosin (fig. 3). The maximum inhibition obtained was nearly 85% of the Mg^{2+} stimulated ATPase activity of DAM. In the bioassay, tropomyosin was present to the same extent in the control as well.

Since the inhibitory protein inhibited only the Mg^{2+} activated ATPase and not the Ca^{2+} stimulated one [12], it was decided to study the effect of Mg^{2+} and Ca^{2+} on the inhibitory protein by circular dichroism measurements in order to discern if the addition of these bivalent cations brought about any change in the conformation of the inhibitory protein. Representative

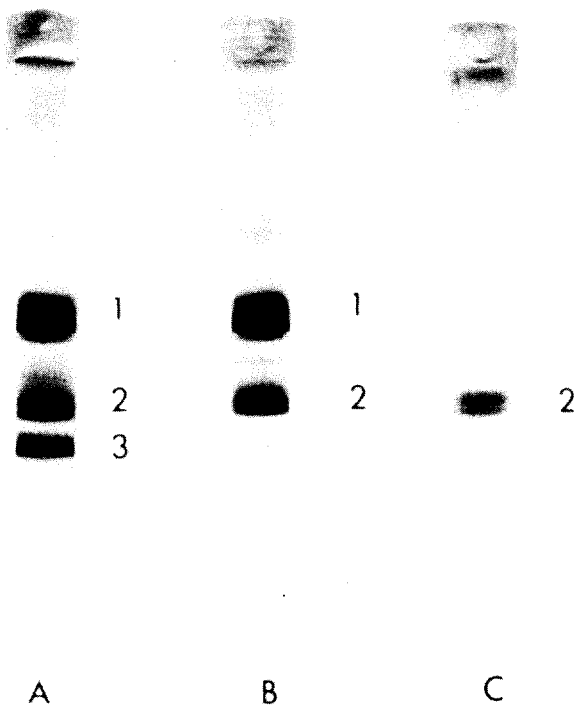

Fig. 2. SDS acrylamide gels of Troponin B supernatant (gel A), troponin B (gel B) and Troponin inhibitory protein (gel C). The numbers 1, 2, and 3 correspond to the 37,000 component, the inhibitory protein and Troponin A, respectively.

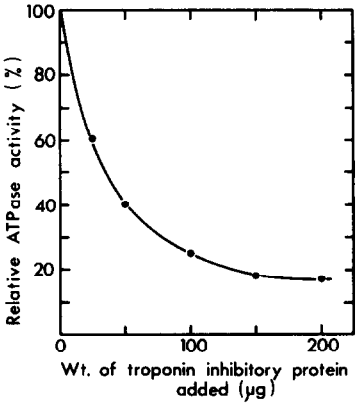

Fig. 3. Effect of the inhibitory protein component of troponin on the Mg^{2+} stimulated ATPase activity of DAM.

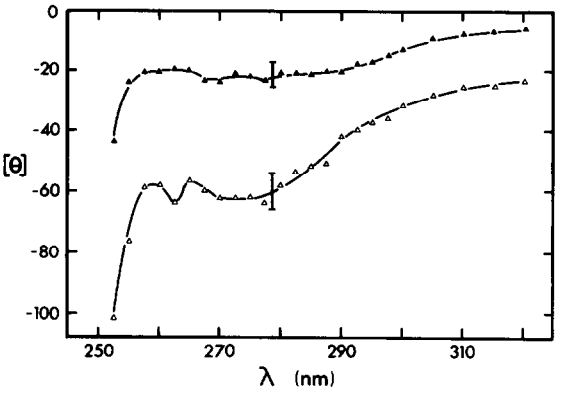

Fig. 4. Near-ultraviolet circular dichroism spectra of troponin inhibitory protein in 0.5 M NaCl, 0.05 M Tris (pH 8.0) (▲) and in 0.5 M NaCl, 0.5 M Tris (pH 8.0)–2.5×10^{-3} M $MgCl_2$ (△).

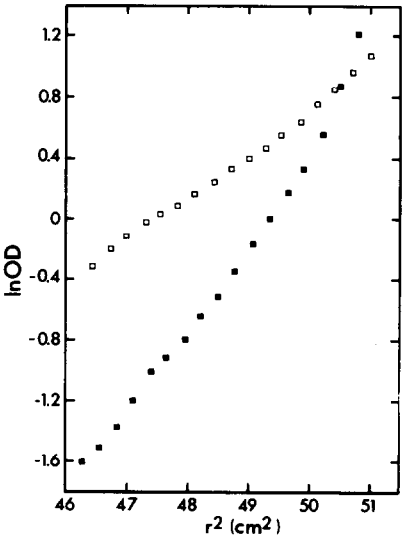

Fig. 5. Plot of the natural log of the concentration (optical density) as a function of the square of the distance from the axis of rotation for the inhibitory protein in 0.5 M NaCl, 0.05 M Tris (pH 8.0), 1 mM DTT (■) and in 6 M G-HCl, 0.2 M NaCl, 0.02 M NaAc, 0.5 mM DTT (pH 5.2) (□). The rotor speed was 18,000 rpm in each case.

near-ultraviolet circular dichroic spectra of the protein are presented in fig. 4. Upon the addition of 2.5×10^{-3} M Mg^{2+} to the protein, the whole spectrum becomes more negative, implying that the process may involve small alterations in asymmetric side chain interactions. No attempt was made to identify the chromophores

in view of the fact that the band intensities were very weak. However, when Ca^{2+} was added to the protein alone, there was no significant change in the spectrum. Also, when Mg^{2+} was added after the initial addition of Ca^{2+} to the protein, there was no significant change in the protein spectrum, suggesting that Ca^{2+} is able to counteract the effect of Mg^{2+}. In the far UV region the CD spectrum of the protein possesses two negative dichroic peaks, located at 222 and 205 nm, band positions typical of a protein containing α-helix. The θ values at these two wavelengths were $-11{,}000°$ and $-16{,}000°$, respectively. Addition of Mg^{2+} to the protein did not result in any significant alteration in the far UV CD spectrum suggesting that the process involves small alterations in side chain interactions, without affecting the overall protein secondary structure.

The amino acid content of the inhibitory protein given in table 1 is very similar to that reported by Wilkinson et al. [5]. However, small discrepancies do arise, the most notable of which is the lower aspartic acid content in our inhibitory protein preparation.

Low speed sedimentation equilibrium studies gave a molecular weight of 24,500 ± 500 for the inhibitory protein in 0.5 M NaCl, 0.05 M Tris, 1 mM DTT at pH 8.0. This value was derived from the limiting slope of ln O.D. versus r^2 plot (fig. 5). The initial loading concentration for the particular run shown in the figure was 0.65 mg per ml. It should also be noted that there is an indication of aggregation in this solvent system even in the presence of DTT. The molecular weight of 24,000 obtained is in agreement with the value deduced from SDS acrylamide gel electrophoresis. Schaub and Perry [12] have also reported a value of 22,900 ± 800 by gel filtration in guanidine hydrochloride. Our low speed sedimentation equilibrium studies under denaturing conditions in 6 M G-HCl, 0.2 M NaCl, 0.02 M NaAc at pH 5.2 gave a limiting value for the protein of 24,000 ± 500. Even in 6 M G-HCl medium there was an indication of some aggregation at the cell bottom and the molecular weight was in the vicinity of 45,000. However, when DTT was incorporated into the solvent system, the aggregation was minimized and the moelcular weight obtained from the limiting slope was 24,000 ± 500 (fig. 5).

Table 1
Amino acid composition of the inhibitory protein.

Amino acid	This study	Wilkinson et al. [5]
Lys	27.7	24.2
His	4.6	3.9
Arg	17.8	15.2
Asp	10.0	18.8
Thr	3.8	4.0
Ser	11.9	10.4
Glu	38.6	37.6
Pro	5.9	5.6
Gly	9.4	9.7
Ala	16.0	17.1
Val	8.4	8.7
Met	9.4	9.2
Ileu	5.5	5.2
Leu	20.6	20.0
Tyr	2.2	2.5
Phe	3.2	3.3
Cys	–	3.2

Content: (Mol/23,000 g).

Acknowledgements

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