HYDRODYNAMIC PROPERTIES OF BOVINE CARDIAC TROPONIN

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1. Introduction

A refined knowledge of muscle regulation at the level of the thin filaments can be obtained from a hydrodynamic study of the troponin complex and its subunits. This approach has already been used to determine the nature of the Ca^{2+}-induced conformational change in skeletal TN-C* [1,2] and its interaction with other subunits [2,3]. However, little or no work has been reported on the hydrodynamic properties of intact 'native' troponin, probably due to the difficulties in achieving clean preparations without resorting to denaturing conditions.

Preparative methodology has advanced to the point where such studies can now be carried out for both skeletal and cardiac troponin. Lovell and Winzor [4] have demonstrated that ox skeletal troponin undergoes a concentration-dependent association–dissociation equilibrium which is also affected by pH, ionic strength and temperature. In this study, bovine cardiac troponin has been examined by sedimentation velocity, sedimentation equilibrium and analytical gel filtration. The results indicate that cardiac troponin also aggregates as a function of protein concentration, although in this case there is little evidence for dissociation into subunits. Gel filtration studies, extrapolated to low protein concentration, suggest that the troponin complex may be asymmetric in solution. There appears to be no significant effect of Ca^{2+} on the hydrodynamic properties of the isolated troponin molecule.

* The abbreviations used are: TN-C, TN-I and TN-T represent the calcium-binding, inhibitory and tropomyosin-binding subunits of troponin, respectively; DTT, dithiothreitol; EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N'*-tetraacetic acid; MOPS, morpholino-propane sulfonic acid; KMED buffer, KCl/50 mM MOPS/1 mM EGTA/1 mM DTT

2. Materials and methods

Crude troponin was prepared by the method of Tsukui and Ebashi [5] as modified by Burtnick [6]. This material was dialyzed vs. 20 mM Tris–Cl, 0.5 mM DTT (pH 7.8) and applied to a DEAE-Sephacel column equilibrated with the same buffer. Troponin was eluted with a 0–0.4 KCl gradient in 20 mM Tris–Cl, 0.5 mM DTT (pH 7.8) (see fig.1). All preparative operations were performed at 4°C.

For all analytical experiments, troponin was dissolved in 0.2 M KMED buffer (0.2 M KCl, 50 mM MOPS, 1 mM EGTA, 1 mM DTT) and dialyzed at least 20 h (48 h for sedimentation equilibrium studies) at 4°C. Protein concentrations were determined spectrophotometrically at 280 nm on a Gilford 240 instrument employing an extinction coefficient ($A^{1\%}_{1\,cm}$) of 4.3, obtained refractometrically.

Ultracentrifugal studies were perfomed at 20°C on a Beckman Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner accessory. Sedimentation coefficients were calculated by the maximum ordinate method from Schlieren photos taken at 16 min intervals. Sedimentation equilibrium [7] and meniscus depletion [8] experiments were recorded as photoelectric scans at 280 nm or as Rayleigh interferograms, taken at 48–72 h. Rotor speeds for these experiments were 3600–16 000 rev./min. The partial specific volume ($\bar{v}$) of troponin used in calculations was 0.73 ml/g, determined from the amino acid composition [6].

Analytical gel filtration experiments were done at room temperature (21 ± 2°C) in a 60 × 0.9 cm column packed with Biogel A 1.5 m. Protein concentrations in the eluant were determined either spectrophotometrically at 280 nm or by the dye-binding assay of Bradford [9]. Peak positions were estimated by interpolation [10] and partition coefficients (σ) were calculated as in Siegel and Monty [11]. Void volume (12.2 ml) and included volume (22.4 ml) were measured with Blue dextran and nitrotyrosine, respectively.

3. Results

3.1. *Preparation of cardiac troponin*

Figure 1 illustrates the fractionation of crude troponin on DEAE-Sephacel: troponin eluted from the column at 0.30 M KCl. SDS polyacrylamide gels [12] of this material (fig.1, insert) show essentially three major components, corresponding to TN-T (M_r = 36 300), TN-I (M_r = 23 500) and TN-C (M_r = 18 500) [6]. A minor band of apparent molecular weight 23 000 was usually present as well; this band has been attributed to an intramolecular disulfide-linked TN-I [13] or to a degradation product of TN-T [14]. Our preparations did not show any low molecular weight protein material on either Biogel A 0.5 m or Sephacryl S-200 columns. Purified troponin conferred full Ca^{2+}-sensitivity to a Mg^{2+}-activated synthetic cardiac actomyosin adenosine triphosphatase assay system [6].

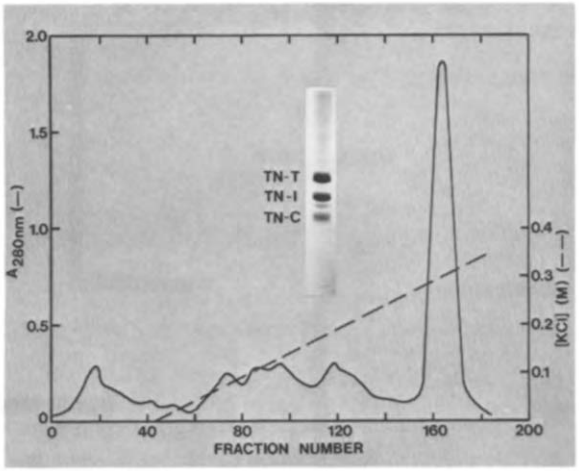

Fig.1. Purification of troponin on DEAE-Sephacel. The column (30 × 3 cm) was eluted with a linear 0–0.4 M KCl gradient (1 liter total) as described in the text. The flow rate was 50 ml/h and 6 ml fractions were collected. Insert: SDS polyacrylamide gel of the major fraction.

3.2. *Sedimentation velocity*

Schlieren photographs of troponin in 0.2 M KMED (pH 7.2) did not exhibit a single, symmetrical boundary but rather a major leading peak with a trailing smaller peak or shoulder (fig.2). The relative magnitude of this slower-moving component was dependent on pH and ionic strength, becoming more prominent at high KCl concentrations. Moreover, the size of the major peak increased with total protein concentration, while that of the slower component remained relatively constant. This behavior is characteristic of protein association of the Gilbert type [15].

The sedimendation coefficient ($S_{20,w}$) of the major peak was measured as a function of pH, ionic strength and protein concentration. Increasing the pH or ionic strength significantly reduced $S_{20,w}$ indicating a reduced molecular weight or an increased frictional coefficient (table 1). The effect of varying protein concentration is shown in fig.2. $S_{20,w}$ decreased dramatically at troponin concentrations below 2 mg/ml, indicative of protein dissociation. Addition of 2 mM $CaCl_2$ (about 1 mM free Ca^{2+}) to troponin in 0.2 M KMED resulted in a small but significant decrease in $S_{20,w}$. The sedimentation coefficient of the trailing

Table 1
Effects of pH, ionic strength and temperature on the sedimentation velocity of troponin[a]

Buffer (–Ca^{2+})	$S_{20,w}$ (S)
0.2 M KMED (pH 7.2)	7.06
0.2 M KMED (pH 6.7)	7.65
0.2 M KMED (pH 7.7)	6.77
0 M KMED (pH 7.2)[b]	7.58
0.5 M KMED (pH 7.2)	5.30
1.0 M KMED (pH 7.2)	5.30
0.2 M KMED (pH 7.2)[c]	6.53

[a] Experiments were done as described in text and in the legend to fig.2. All protein concentrations were 4.3–4.5 mg/ml
[b] Concentration refers to KCl, all other buffer components being constant
[c] Experiment performed at 4°C

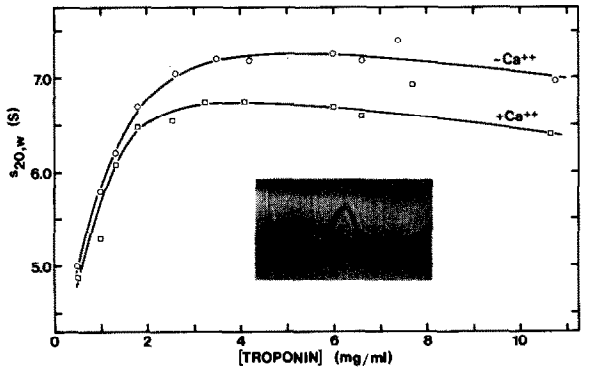

Fig.2. Effect of protein concentration on the sedimentation coefficient of troponin in the absence (○) or presence (□) of 2 mM Ca^{2+}. All samples were run at 60 000 rev./min using 12 mm double-sector cells, except for the two lowest concentrations (less than 1 mg/ml) for which 30 mm cells were used. Insert: Schlieren photograph of troponin (4.1 mg/ml, $-Ca^{2+}$) taken at 50 min.

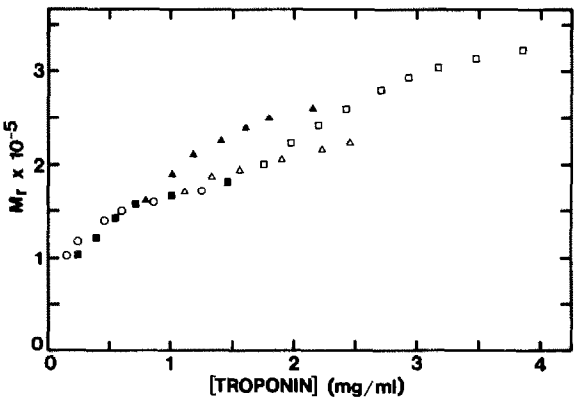

Fig.3. Effect of protein concentration on the molecular weight of troponin in 0.2 M KMED (pH 7.2). Conventional sedimentation equilibrium experiments were recorded either as (□) Rayleigh interferograms or (▲,△) photoelectric scans at 280 nm. The Rayleigh optical system was used for meniscus deplection experiments (○,■). Loading concentrations and equilibrium rotor speeds were: □, 2.53 mg/ml, 3600 rev./min; ▲, 1.35 mg/ml, 4800 rev./min; △, 2.0 mg/ml, 4800 rev./min; ○, 0.58 mg/ml, 16 000 rev./min; ■, 0.85 mg/ml, 16 000 rev./min.

boundary was difficult to measure accurately, but least squares treatment of the data gave $S_{20,w}$ values of 3.5–4.0 S in the presence and absence of Ca^{2+} (data not shown).

3.3. *Sedimentation equilibrium*

The concentration dependence of the molecular weight of cardiac troponin in the absence of Ca^{2+} is illustrated in fig.3. Slight sensitivity of results to rotor speed and loading concentration was noted, probably due to the aggregating nature of troponin. The apparent molecular weight of troponin ranged from about 80 000, a value obtained by extrapolation to zero protein concentration, to over 300 000 at high concentrations (fig.3). The limiting value of 80 000 daltons is indistinguishable from the combined molecular weight of the cardiac troponin subunits (78 000) as reproted [6]. The presence of Ca^{2+} had no significant effect on the molecular weight behavior of troponin over the concentration range studied (data not shown).

3.4. *Analytical gel filtration*

In order to study the hydrodynamic properties of troponin at low protein concentrations, analytical gel chromatography experiments were performed with and without Ca^{2+}. The results are shown in fig.4. All elution volumes were reproducible to within

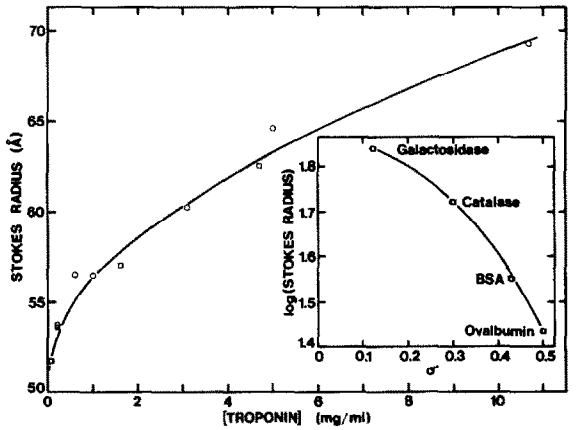

Fig.4. Effect of protein concentration on the apparent Stokes radius of troponin. Samples (300 μl) at the concentrations indicated were applied to Biogel A 1.5 m (100/200 mesh) column. The column was eluted with 0.2 M KMED (pH 7.2) containing (○) no or (□) 2 mM Ca^{2+}. The flow rate was 6 ml/h and 0.38 ml fractions were collected. Samples with initial protein concentrations less than 1 mg/ml were monitored using Bradford's dye assay (see text). Gel standards (insert) were β-galactosidase, 69 Å [20]; catalase, 52 Å [11]; bovine serum albumin (BSA), 35 Å [11], and ovalbumin, 27.3 Å [16].

± 0.2 ml and addition of $CaCl_2$ resulted in a negligible change in the elution volumes of the standards.

As expected, the elution position of troponin was sensitive to protein concentration (fig.4). Extrapolation of Stokes radius to zero troponin concentration yields a value of 52 Å, identical to that of catalase, a globular protein of 250 000 daltons [11]. The results were essentially identical in the presence of 2 mM $CaCl_2$ (fig.3).

4. Discussion

All the methods used in this study clearly indicate that bovine cardiac troponin, like its skeletal counterpart [4], aggregates in aqueous solution at neutral pH. In contrast to the skeletal protein, however, the minimum detectable molecular weight of cardiac troponin at low concentration does not suggest appreciable dissociation into subunits. However, it is possible that dissociation occurs at concentrations too low to be monitored by the methods used here. The aggregation of cardiac troponin is affected by pH, ionic strength and possibly also by Ca^{2+} (fig.2). It is interesting to note that the effect of Ca^{2+} on the sedimendation velocity of native troponin is opposite to that found for skeletal TN-C, which compacts upon binding this ion [1,2]. The significance of the Ca^{2+} effect on the sedimentation of cardiac troponin is not clear at this time.

The limiting molecular weight of troponin obtained by meniscus depletion ultracentrifugation (80 000) can be combined with the Stokes radius determined by gel filtration (52 Å) to calculate the apparent frictional ratio, f/f_{min} [11]:

$$f/f_{min} = \frac{Rs}{(3M\bar{v}/4\pi N)^{1/3}}$$

where Rs is the experimentally determined Stokes radius in cm, M is the molecular weight, $\bar{v}$ is the partial specific volume and N is Avogadro's number. The resulting value of 1.82 is substantially larger than is normally found for typical globular proteins (1.15–1.30, see Edsall [16]). Using this value of f/f_{min}, the calculated sedimentation coefficient of the troponin complex is 3.65 S, which is close to that observed for the slower-moving component in the Schlieren profiles. Thus troponin, long thought of as a globular protein on the thin filament, may in fact be somewhat elongated. This possibility is supported by studies with the tropomyosin-binding component of troponin, TN-T. Hincke et al. [17] have reported unusual elution properties of cardiac TN-T on Sephacryl S-200 gels. Moreover, binding [18] and electron microscopic [19] investigations have suggested that skeletal TN-T may interact with tropomyosin over an extended region of the tropomyosin molecule on the thin filament.

Use of the present data to compare troponin to any detailed hydrodynamic model is not justified. To begin with, the physical parameters reported here have been obtained by extrapolation through a concentration range in which a chemical equilibrium (i.e., aggregation) is occurring. These parameters will be weight-averages at those concentrations. Also, troponin is composed of three non-identical subunits; as such, an ellipsoidal model is probably not a valid representation. It is likely that any irregularities in the shape of the troponin complex will increase the value of f/f_{min} above that due to asymmetry or hydration alone.

Aside from the effect on sedimentation velocity at high protein concentration, we have been unable to demonstrate any significant changes in the hydrodynamic properties of cardiac troponin upon addition of Ca^{2+}. This finding is consistent with sedimentation studies on cardiac TN-C [6] and reconstituted skeletal troponin [2] which also failed to indicate changes in $S_{20,w}$ with Ca^{2+}. Thus, it is concluded that either the molecular changes accompanying Ca^{2+} binding to cardiac troponin are of a local nature or that the bulk hydrodynamic properties of troponin are dominated by one subunit (i.e., TN-T) and are insensitive to changes in the other subunits.

Acknowledgements

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