Bovine cardiac troponin subunits: Binary complexes and reconstitution of whole troponin

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The reconstitution of bovine cardiac troponin from its subunits has been investigated using hydrodynamic techniques. Gel filtration (Sephacryl S-300) and sedimentation velocity experiments indicate that troponin-C and troponin-I form a stable binary complex (1:1 mole ratio) with an apparent Stokes' radius of 36 Å (frictional ratio = 1.6). Troponin-C and troponin-T do not interact significantly while troponin-I and troponin-T undergo partial complex formation. The effect of subunit ratio on the reconstitution of whole troponin has been examined by SDS–polyacrylamide gel electrophoresis and gel filtration and the results suggest that native troponin contains the subunits in an equimolar ratio.

Troponin *Cardiac muscle* *Hydrodynamics* *Gel filtration* *Ultracentrifugation*

1. INTRODUCTION

The contraction of striated muscle is regulated on the thin filament by the binding of Ca^{2+} to the TN-C subunit of troponin. A Ca^{2+}-induced conformational change in TN-C is propagated through the other troponin subunits (TN-I and TN-T) and tropomyosin, ultimately resulting in activation of the actomyosin ATPase [1–3]. Although skeletal and cardiac troponin are similar in many respects, significant structural and functional differences exist which undoubtedly reflect the specific regulatory requirements of each tissue [3–5]. Since subunit interactions within the troponin complex appear to be central to the mechanism of regulation, there has been much interest in comparing the skeletal and cardiac proteins in this regard [6–10].

We have investigated the physical properties of bovine cardiac troponin [11] and TN-C [12] in solution using hydrodynamic methods. Here, this approach has been used to examine the interactions between cardiac troponin subunits. The results indicate that only TN-C and TN-I can combine to form a stable bimolecular complex and the hydrodynamic properties suggest that this complex is moderately asymmetric. Since the subunit stoichiometry of skeletal troponin is in some doubt [13], we have also used gel filtration to examine the effect of subunit ratio on the reconstitution of whole cardiac troponin. We conclude that the subunit stoichiometry of cardiac troponin is 1:1:1.

Abbreviations: TN-C, TN-T and TN-I, Ca^{2+}-binding, tropomyosin-binding and inhibitory subunits of troponin, respectively; DTT, dithiothreitol; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NMED buffer, NaCl, 50 mM Mops, 1 mM EGTA and 2 mM DTT.

2. EXPERIMENTAL

Bovine cardiac troponin [11] and TN-C [12] were prepared as described. The purification of TN-I and TN-T (ms. in preparation) is based on earlier work from our laboratory [14,15]. Analytical experiments were performed in either 0.2 M or 0.5 M NMED buffer (0.2 M or 0.5 M NaCl, 50 mM Mops, 1 mM EGTA, 2 mM DTT) at pH 7.2. Protein complexes in 0.5 M NMED were formed by dissolving the subunits in 6 M urea/0.5 M NMED and dialyzing for 48 h at 4°C *vs* the analytical buffer. The subunits were then mixed in

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the appropriate ratio. Protein complexes in 0.2 M NMED were prepared by mixing the subunits prior to the removal of urea by dialysis. Native troponin was dissolved directly in 0.5 M NMED (pH 7.2) and all analytical samples were clarified by centrifugation. Protein concentrations were determined spectrophotometrically (Cary 118C) at 276 nm using the following parameters: TN-C ($E^{1\%}$ = 2.3, M_r 18 500) [12], TN-I ($E^{1\%}$ = 5.2, M_r 23 000) and TN-T ($E^{1\%}$ = 4.4, M_r 36 000) (ms. in preparation).

Analytical gel chromatography studies were performed at room temperature (21 ± 2°C) on a Sephacryl S-300 column (Pharmacia) calibrated with proteins of known Stokes' radius. Protein concentration in the eluant was determined by absorbance at 276 nm. The detail of our column standardization procedure and the calculation of apparent Stokes' radius ($r_{S,gel}$) has been published in [12]. Pooled eluant fractions were analyzed by SDS–polyacrylamide gel electrophoresis [16] after precipitation of the protein with 20% trichloroacetic acid. Polyacrylamide gels were stained with Coomassie blue and quantitatively scanned on a Gilford densitometer.

Ultracentrifugal studies were carried out at 20°C on a Beckman Spinco Model E analytical ultracentrifuge. M_r-values were measured by the meniscus depletion equilibrium technique [17] using Rayleigh interference optics. Sedimentation coefficients were calculated from Schlieren photos taken at 60 000 rev./min and were adjusted to the standard conditions of water at 20°C ($s_{20,w}$) according to [18]. The Stokes' radius ($r_{S,sed}$) was obtained from the extrapolated value of $s_{20,w}$ at infinite dilution ($s^\circ_{20,w}$) by:

$$r_{S,sed} = M_r (1-\bar{v}\rho)/(6 N \pi \eta_o s^\circ_{20,w}) \quad (1)$$

where
M_r = the relative molecular mass
$\bar{v}$ = is the partial specific volume
ρ = the solvent density
N = Avogadro's number
η_o = the solvent viscosity in poise

The $\bar{v}$ values for troponin complexes were calculated from the subunit values: TN-C (0.70 ml/g) [12]; TN-T (0.72 ml/g); and TN-I (0.73 ml/g) (ms. in preparation).

Viscosity experiments were performed at 20 ± 0.01°C with a Cannon-Manning viscometer (water flow time ~ 530 s). The intrinsic viscosity was calculated from the solvent (t_o) and solution (t) flow times and the protein concentration (c) as in [12].

3. RESULTS AND DISCUSSION

3.1. *TN-IC*

TN-I and TN-C can be mixed in a 1:1 mol ratio in 0.5 M NMED (pH 7.2) to form a stable bimolecular complex (TN-IC), as demonstrated by gel filtration and sedimentation equilibrium. The TN-IC complex eluted from the Sephacryl S-300 column in a single symmetrical peak (fig.1A) with an apparent Stokes' radius ($r_{S,gel}$) of 36 Å, a value significantly larger than that for either TN-C (24 Å) or TN-I (30 Å) alone. Moreover, the weight av. M_r of TN-IC in the absence of Ca^{2+} (fig.2) was close to the value expected for the complex (M_r 41 500) except at the meniscus, where the effects of dissociation or subunit excess are undoubtedly present. Similar results were obtained in 0.2 M NMED (pH 7.2), where the normally insoluble TN-I was induced into solution by its interaction with TN-C. Evidence for a stable TN-IC complex is also provided from sedimentation velocity experiments in 0.2 M NMED (fig.3A) since only a single component was observed with a sedimentation coefficient (3 S) larger than that of either TN-C (1.9 S) [12] or TN-I (2 S) (in preparation). Our findings are consistent with demonstrations of cardiac TN-I–TN-C interaction by circular dichroism [6], electrophoresis [14], calorimetry [10] and Ca^{2+}-binding studies [19].

Hydrodynamic studies of cardiac TN-C [12] and TN-I (in preparation) have indicated that both molecules are moderately asymmetric with translational frictional ratios of ~ 1.5. To perform similar calculations for the TN-IC complex, it is necessary to assume that the measured physical parameters correspond to the bimolecular unit of M_r = 41 500. The frictional ratio can then be calculated by:

$$f/f_{min} = r_S/r_o = r_S/[3 M_r \bar{v}/4\pi N]^{1/3}$$

where
r_o (22.7 Å) = the Stokes' radius of the equivalent unhydrated sphere

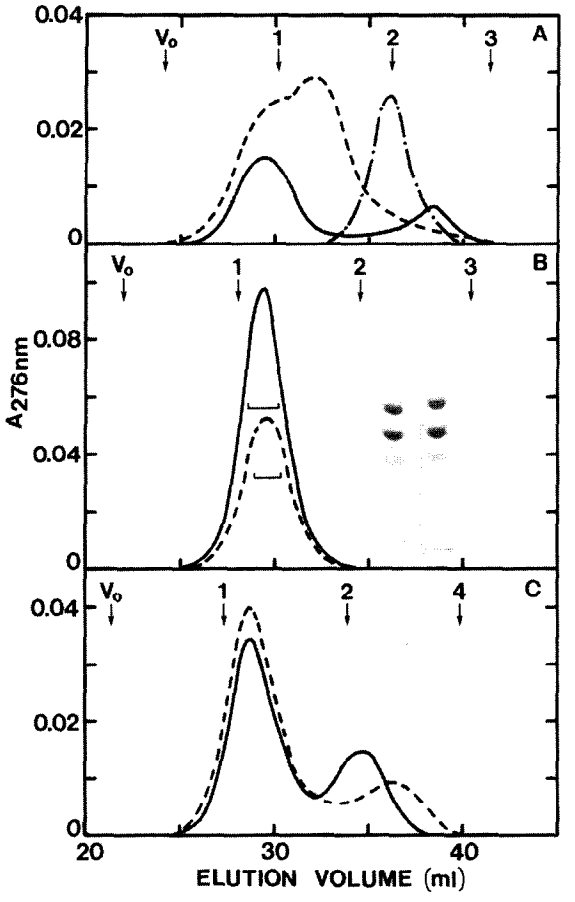

Fig.1. Analytical gel chromatography of cardiac troponin complexes. Samples (0.4–0.8 mg in 300 μl) were applied to a Sephacryl S-300 column (59 × 1.1 cm) in 0.5 M NMED (pH 7.2). The flow rate was 13 ml/h and 0.4 ml fractions were collected. The void volume (V_0) was measured with blue dextran. Protein standards and Stokes' radii (r_S) [12] were: 1 β-galactosidase (69 Å); 2 bovine serum albumin (35 Å); 3 myoglobin (19.8 Å); 4 cytochrome *c* (17.2 Å). (A) Binary complexes: TN-IC (---), TN-CT (——) and TN-IT (---). (B) Native troponin (——) and TN-ICT (---). Insert: SDS–polyacrylamide gels of the indicated native (left) and reconstituted troponin fractions. (C) TN-I_2CT (——) and TN-IC_2T (---).

The value of $r_{S,gel}$ is 36 Å (fig.1A) which is identical to the value of $r_{S,sed}$ calculated from eq. (1) where $s°_{20,w}$ = 2.9 S (fig.3A). The resulting value of f/f_{min} for TN-IC is 1.58, suggesting that TN-C and TN-I combine in a manner that does not substantially alter the hydrodynamic properties relative to the individual subunits. Asymmetry of the

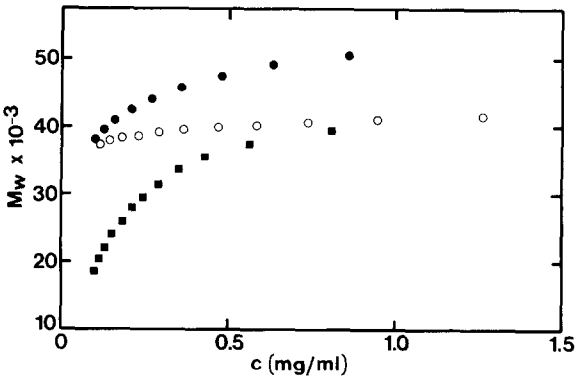

Fig.2. Effect of [protein] on the M_r-value of cardiac TN-IC and TN-CT mixtures. Meniscus depletion sedimentation equilibrium experiments were performed on 100 μl samples of TN-IC (○,●) and TN-CT (▪) in 0.5 M NMED (pH 7.2). The rotor speed for these runs was 28 000 rev./min and [Ca^{2+}] was: (○) no Ca^{2+}; (●,▪) 2 mM Ca^{2+} initial [protein] was: (○) 0.85 mg/ml; (●,▪) 0.68 mg/ml.

TN-IC complex is also indicated by the intrinsic viscosity of the protein (8.7 ml/g, fig.3B), which is significantly larger than the values of 3–5 ml/g normally expected for globular proteins [20].

The effect of Ca^{2+} on the hydrodynamic behaviour of TN-IC is complicated by its apparent influence on the degree of protein association. Although addition of 2 mM Ca^{2+} resulted in only minor changes in $s_{20,w}$ (fig.3A) and $r_{S,gel}$ (not shown), the apparent M_r-value of the complex was increased (fig.2), indicating that further aggregation of TN-IC occurs in the presence of this cation. Thus, the Ca^{2+}-induced decrease in the viscosity of TN-IC (fig.3B) should not be taken as proof of a conformational change to a more compact structure, even though such an event has been shown to occur with cardiac TN-C alone [12].

3.2. *TN-CT*

In contrast to TN-IC, no interaction between cardiac TN-C and TN-T was observed when these proteins were mixed in a 1:1 mol ratio. Both gel filtration (fig.1A) and sedimentation velocity experiments in 0.5 M NMED (pH 7.2) exhibited bimodal profiles with elution and Schlieren peaks corresponding to the isolated subunits. Addition of Ca^{2+} had no significant effect on these results. The observed M_r of TN-CT in 0.5 M NMED

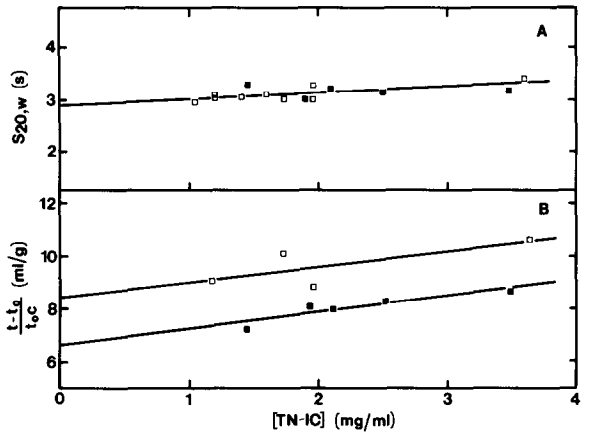

Fig.3. (A) Effect of protein concentration on the sedimentation coefficient ($s_{20,w}$) of cardiac TN-IC in 0.2 M NMED (pH 7.2). The value of $s_{20,w}$ (2.90 S) was calculated using the $-Ca^{2+}$ data only. (B) Viscosity of cardiac TN-IC in 0.2 M NMED (pH 7.2). The density-corrected intrinsic viscosity values [20] are 8.7 ml/g ($-Ca^{2+}$) and 6.9 ml/g ($+Ca^{2+}$). Calcium concentrations: (□) no Ca^{2+}; (■) 2 mM Ca^{2+}.

($+ Ca^{2+}$); measured by sedimentation equilibrium (fig.2), was well below the expected value for the bimolecular complex (M_r 54 500), further indicating that the 2 proteins do not interact strongly. Dialysis of TN-C and TN-T mixed in 6 M urea against 0.2 M NMED resulted in the precipitation of much of the TN-T, indicating that this subunit, unlike TN-I, is not solubilized by TN-C at the lower ionic strength.

These data would appear to be in conflict with various reports of cardiac TN-C–TN-T interaction, based on circular dichroism [6], gel electrophoresis [15] and calorimetry [10]. However, the transport methods employed in this investigation are intended to examine the overall hydrodynamic properties of the proteins involved and might not be expected to detect weak subunit interactions. Indeed, gel electrophoresis studies have indicated that only a small fraction of the total TN-C and TN-T mixed is involved in complex formation [15].

3.3. *TN-IT*

Interaction between cardiac TN-I and TN-T has already been demonstrated by gel filtration [9] and is not examined in detail here. Partial formation of a TN-IT complex in 0.5 M NMED (pH 7.2) is indicated by the appearance of a protein peak eluting between the individual subunits on Sephacryl S-300 (fig.1A). The intermediate Stokes' radius of this new material (50–60 Å) suggests that TN-T is dissociated from its normal aggregated state (in preparation) by the TN-I subunit.

3.4. *Reconstituted troponin*

Cardiac troponin can be fully reconstituted by mixing the 3 subunits in an equimolar ratio as illustrated by gel filtration in 0.5 M NMED (pH 7.2) (fig.1B). This ternary TN-ICT complex and native undissociated troponin both eluted in a single peak which contained all the subunits as determined by SDS–polyacrylamide gel electrophoresis. This observation supports our conclusion [11] that native troponin does not appreciably dissociate into its constituent subunits in non-denaturing buffers. These results are also in agreement with a thermodynamic study which suggested that stabilization of cardiac troponin occurs by mutual interaction of all 3 subunits [10]. TN-I would appear to have a special significance in the reconstitution of troponin since it is the only subunit which interacts strongly with each of the other 2 subunits in a bimolecular fashion.

There is presently some controversy regarding the subunit stoichiometry of skeletal troponin. Earlier evidence which suggested that the subunits are present in an equimolar ratio [21] has been challenged in [13] where skeletal troponin was reported to contain 2 TN-I subunits/molecule. It was claimed that the observed variability of subunit composition is due to proteolytic degradation during troponin purification [13]. Although no similar conflict has developed regarding cardiac troponin, the importance of determining the subunit stoichiometry of this protein is obvious by analogy. The subunit ratio of native cardiac troponin prepared by our methodology [11] was found to be 1:1:1, established by comparison to reconstituted TN-ICT using densitometric scans of SDS–polyacrylamide gels (see fig.1B). Furthermore, when troponin was reconstituted with an extra mole of TN-I (TN-I_2CT) or TN-C (TN-IC_2T), this excess subunit did not appear to associate with the troponin complex on Sephacryl S-300, but rather eluted in a separate peak (fig.1C). Although it is still possible that a second TN-I subunit could be much more weakly bound to the troponin complex in

vivo, the simplest explanation of these results is that the 3 cardiac subunits are present in the complex in an equimolar ratio. There is evidence for a precursor pool of unassembled TN-I in rat cardiac myofibrils [22], but there is no reason to believe that this extra protein has an active role in the function of troponin.

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