Self-Association of Troponin

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Ox muscle troponin was shown by equilibrium- and velocity-sedimentation studies to undergo concentration-dependent dissociation into its constituent subunits as well as self-association in imidazole buffers, pH 6.9. The extent of troponin association was found to be strongly dependent on ionic strength and also to exhibit a dependence on pH and temperature; under conditions physiological in regard to pH, temperature and ionic strength the extent of polymerization of troponin is considerable in 2 mg/ml solutions. The ability of polymeric troponin to bind to tropomyosin has been inferred from studies of mixtures containing actin–tropomyosin and an excess of troponin over the amount required for the normal 7:1:1 actin–tropomyosin–troponin complex. These findings should be relevant to studies of reconstituted actin–tropomyosin–troponin preparations, since they signify possible chemical as well as physical differences between the gel, paracrystalline and filamentous states of the complex that result from adoption of different preparative procedures for analogues of the native thin filament.

Troponin is widely recognized as a protein that is associated with tropomyosin and actin in the thin filaments of vertebrate striated muscle, and also as the protein responsible for imparting Ca^{2+} -sensitivity to the ATPase† of actomyosin (Ebashi & Endo, 1968). However, very little attention has been given to the physical properties of troponin, which has been envisaged (Hartshorne & Dreizen, 1973) as a globular protein with a mol.wt. of 85000. This value is obtained by summing the approximate molecular weights of the troponin T (tropomyosin-binding), troponin C (calcium-binding) and troponin I (inhibitory) subunits that are obtained by SDS/polyacrylamide-gel electrophoresis (Greaser & Gergely, 1971). In the present investigation of troponin by velocity and equilibrium centrifugation we demonstrate not only the dissociation of troponin into its subunits under conditions approaching physiological, but also its polymerization well beyond the 85000-dalton stage. Possible consequences of this polymerization in relation to the interaction of troponin, tropomyosin and actin in the formation of reconstituted thin filaments are also considered. Part of the present investigation has already appeared in thesis form (Lovell, 1976).

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† Abbreviations: ATPase, adenosine triphosphatase; SDS, sodium dodecyl sulphate.

Experimental

Materials

Troponin was prepared from freshly excised ox skeletal muscle by the method described previously (Clarke *et al.*, 1976). Preparations were examined routinely by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) to check that the troponin T, I and C subunits were present in the molar proportions 1:1:1 (Potter, 1974). Each preparation was also checked for its ability to confer Ca^{2+} -sensitivity on the ATPase activity of actomyosin (Table 1). Solutions of troponin in the appropriate medium were obtained by dialysis against buffer at 4°C for 16 h; all buffer solutions contained CaCl_2 (0.1 mM), MgCl_2 (1 mM) and β -mercaptoethanol (5 mM) unless otherwise specified. Concentrations of these dialysed solutions were determined either refractometrically at 546 nm by assuming a specific refractive increment of 1820×10^{-6} dl/g (Doty & Edsall, 1951) or spectrophotometrically at 280 nm on the basis of an absorption coefficient ($A_{1\%}^{1\text{cm}}$) of 4.7, a value obtained by combining the absorbances of troponin solutions with concentrations determined refractometrically.

Acetone-dried powders (Briskey & Fukazawa, 1971) of ox muscle were used for the preparation of tropomyosin (Clarke *et al.*, 1976), myosin (Briskey & Fukazawa, 1971) and G-actin (Spudich & Watt, 1971), the concentrations of which were determined refractometrically as above. F-actin was prepared from G-actin solutions by addition of 3 M-KCl to

Table 1. Test of the ability of a typical ox muscle troponin preparation to confer Ca^{2+} -sensitivity on actomyosin

ATPase activity

Assay mixtures (1.0ml) containing actomyosin (200 μg), tropomyosin (30 μg) and the indicated amount of troponin in 0.03M-imidazole buffer (pH6.8), 0.06M-KCl, 1.0mM-MgCl₂ and either CaCl₂ (0.1 mM) or EGTA (0.1 mM) were incubated for 15 min at 25°C (Clarke & Masters, 1974). Activities are expressed relative to those observed in the presence of Ca²⁺, which were unaffected by troponin addition.

Amount of troponin added (μg)	...	0	10	25	50	75	100
Relative ATPase activity (%)	...	100	80	54	38	32	30

Fig. 1. SDS/polyacrylamide-gel electrophoresis of ox muscle troponin

Troponin (100 μg) was subjected to SDS/polyacrylamide-gel electrophoresis as described by Weber & Osborn (1969), a current of 5 mA per gel being applied for 6 h. (a) Freshly purified troponin; (b) troponin sample affected by proteolysis (14000, 14000-dalton region); (c) sample examined on completion of an equilibrium-sedimentation experiment. The lighter intensity of the troponin-C band reflects a relatively lower staining capacity of this subunit (Potter, 1974). Abbreviations: TN-C, troponin C; TN-I, troponin I; TN-T, troponin T.

adjust the ionic strength to that of the buffer against which the F-actin was to be dialysed subsequently. Concentrations of F-actin suspensions were determined colorimetrically (Lowry *et al.*, 1951) on the basis of standard curves obtained with G-actin (Houk & Ue, 1974). Synthetic actomyosin was formed by mixing myosin and F-actin in the ratio 3:1 (w/w).

Methods

Sedimentation studies were performed in a Spinco model-E ultracentrifuge, the rotor of which was maintained at 20°C unless otherwise specified. Speeds in the range 6800–17000 rev./min were used in equilibrium experiments, and velocity runs were performed at either 52000 or 60000 rev./min. The schlieren optical system was used for velocity experiments, whereas the equilibrium runs were recorded as Rayleigh interferograms. In instances where the equilibrium experiments were of the low-speed (Van Holde & Baldwin, 1958) design, fluorocarbon FC43 was used to permit observation of the entire equilibrium distribution and hence determination of the meniscus concentration by considering solute conservation (Richards & Schachman, 1959). Buffer and solution densities were measured by means of an Anton Paar precision density meter; values of 0.715 and 0.680 ml/g were obtained for the partial specific volumes ($\bar{v}$) of troponin in 0.01 M-imidazole buffers (pH 6.9) containing 0.1 M- and 1.0 M-KCl respectively, in addition to the usual concentrations of CaCl₂, MgCl₂ and mercaptoethanol (see above).

Because of the susceptibility of troponin preparations to proteolysis (Drabikowski *et al.*, 1971b; Wilkinson *et al.*, 1972; Clarke *et al.*, 1976), the purity of each sample was checked before and after equilibrium sedimentation experiments by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). Detection of the faintest protein stain (with Coomassie Brilliant Blue R) in the 14000-dalton region of the gel (see Fig. 1) was considered sufficient evidence of proteolysis to warrant the discard of a sedimentation run. The formation of fragments of this size after storage of some troponin preparations for prolonged periods (2–4 days) has been studied in detail by Clarke *et al.* (1976), who also report the existence of two forms of troponin T in ox muscle.

Results

Fig. 2 summarizes the effect of ionic strength on the sedimentation behaviour of troponin (2 mg/ml) in imidazole buffers at pH 6.9. At low ionic strength (~ 0.025) pronounced aggregation and polydispersity of troponin solutions are evident (Fig. 2a, lower pattern). Increasing the concentration of KCl from 0.02 to 0.10 M decreases the extent of association (Figs. 2a and 2b, upper patterns), but bimodal boundaries are still observed. Inclusion of 0.22 M-KCl gives rise to a single, spread boundary (Fig. 2b, lower pattern). The reported patterns refer to ox muscle troponin, but similar results were obtained in experiments with preparations derived from rabbit muscle.

In the presence of 1.0 M-KCl, troponin is still in a state of reversible association equilibrium, as is

Fig. 2. Effect of KCl concentration on the sedimentation-velocity behaviour of ox muscle troponin

Troponin solutions (2mg/ml) used in the velocity-sedimentation experiments were prepared in 10mM-imidazole buffer (pH 6.9) containing the appropriate amount of KCl: (a) 0.02M (lower pattern), 0.10M (upper pattern); (b) 0.22M (lower pattern), 0.10M (upper pattern). All solutions contained CaCl_2 (0.1mM), MgCl_2 (1mM) and β -mercaptoethanol (5mM), except that corresponding to the upper pattern in (b); this solution contained EGTA (1mM) in place of the CaCl_2 and MgCl_2 .

evident from the concentration-dependence of the weight-average sedimentation coefficient ($\bar{s}_{20,w}$) (Goldberg, 1953) obtained in this medium (Fig. 3); patterns were bimodal for troponin concentrations greater than 3 mg/ml. The following observations also testify to the reversibility of the association. Firstly, troponin is purified in the presence of 1M-KCl (Clarke *et al.*, 1976) and hence its state of aggregation during purification presumably approximates to that observed in Fig. 3; from Fig. 2 it is evident that troponin freely associates on lowering of the ionic strength. Secondly, the fact that the solutions pertinent to Fig. 3 were obtained by direct addition of solid KCl to solutions of troponin in 0.1M-KCl only 1–3h before ultracentrifugation establishes the ability of aggregated troponin to dissociate.

To gain some insight into the likely nature of the species involved in troponin association at pH 6.9, solutions were subjected to equilibrium sedimentation after prior dialysis (Casassa & Eisenberg, 1964). Fig. 4 presents a preliminary analysis of experiments in terms of apparent weight-average mol.wt. [$\bar{M}_w(r)$].

Fig. 3. Concentration-dependence of the sedimentation coefficient of ox muscle troponin in 10 mM-imidazole buffer (pH 6.9) containing 1.0M-KCl

Weight-average sedimentation coefficients ($\bar{s}_{20,w}$) were obtained from five experiments with different concentrations of troponin in 10mM-imidazole buffer (pH 6.9) containing 1.0M-KCl in addition to CaCl_2 (0.1mM), MgCl_2 (1mM) and mercaptoethanol (5mM). The inset shows the schlieren records for solutions of troponin containing 4mg/ml (lower pattern) and 6.3mg/ml (upper pattern) in this medium; sedimentation is from left to right.

Values of $\bar{M}_w(r)$ were calculated from estimates of $d \log \bar{c}(r)/dr^2$ obtained by least-squares calculations on unsmoothed experimental [$r^2, \log \bar{c}(r)$] values in sets of three; $\bar{c}(r)$ denotes the total concentration of solute at radial position r . Despite the consequent scatter of $\bar{M}_w(r)$ values there can be no reconciliation of the results with a minimum mol.wt. of 85000 (Fig. 4, arrow A) for troponin in the presence of either 0.1M- or 1.0M-KCl; instead a value of 30000 (arrow B) seems more likely. In this connexion it seems relevant that such a value corresponds to the weight-average molecular weight of an equimolar mixture of troponin-T, -I and -C subunits, the respective sizes of which in ox muscle are approx. 40000, 25000 and 20000 daltons (Clarke *et al.*, 1976). An extrapolated value of 43000, the corresponding weight-average molecular weight of an equimolar mixture of troponin T and troponin C–troponin I complex (Head & Perry, 1974) cannot be excluded unequivocally, but the data are considered to favour the lower estimate. Fig. 4 also establishes that the association of troponin subunits proceeds well beyond the 85000-mol.wt. species. Some form of indefinite association seems indicated by the extreme dependence of $\bar{M}_w(r)$ on $\bar{c}(r)$, particularly in 0.1M-KCl (Fig. 4), and also by the evidence of even greater association at lower ionic strength (Fig. 2a).

To determine whether the observed association of troponin is appropriate to the biological environment, the effects of a number of possible metabolic modifiers of the association have been investigated. The absence

Fig. 4. Concentration-dependence of the apparent weight-average molecular weight $[M_w(r)]$ of ox muscle troponin. Before ultracentrifugation, troponin was dialysed for 24 h at 4°C against 10mM-imidazole buffer (pH 6.9) containing either 0.10M- (closed symbols) or 1.0M- (open symbols) KCl in addition to CaCl_2 (0.1 mM), MgCl_2 (1 mM) and mercaptoethanol (5 mM). Loading concentrations of troponin and rotational speeds at sedimentation equilibrium were as follows: $\square$, 1.05 mg/ml, 12000 rev./min; $\circ$, 1.06 mg/ml, 11000 rev./min; $\blacksquare$, 1.12 mg/ml, 6800 rev./min; $\bullet$, 0.4 mg/ml, 16000 rev./min; $\blacktriangle$, 0.4 mg/ml, 17000 rev./min. The final two experiments were of the meniscus-depletion (Yphantis, 1964) design.

of Ca^{2+} and Mg^{2+} had no noticeable effect on the sedimentation behaviour, as is evident from comparison of the upper patterns of Figs. 2(a) and 2(b), which reflect their presence and absence respectively. ATP (10mM), ADP (10mM) and potassium phosphate (10mM) similarly were without effect. Fig. 5 illustrates a dependence of the association on pH (Fig. 5a) and temperature (Fig. 5b). However, at 37°C the association of troponin still persists, and hence it seems appropriate to query current concepts of the nature of the troponin species that is involved with tropomyosin and actin in the formation of reconstituted thin filaments. Previously a 1:1 stoichiometry has been assumed for the troponin-tropomyosin interaction, but clearly this matter requires further scrutiny.

Fig. 5. Effect of pH and temperature on the sedimentation-velocity behaviour of ox muscle troponin

Solutions of ox muscle troponin (2mg/ml) for these experiments contained buffer (10mM-cacodylate, -Tris/HCl or -imidazole), KCl (0.1 M) and the usual concentrations of CaCl_2 (0.1 mM), MgCl_2 (1 mM) and mercaptoethanol (5 mM); (a) pH 6.1 (lower pattern) and pH 8.2 (upper pattern) and a temperature of 20°C; (b) pH 6.9 and 37°C.

Preliminary studies have been made of the interaction of troponin with the actin-tropomyosin complex (Drabikowski & Gergely, 1962). Muscle filaments are believed to contain actin, tropomyosin and troponin in the molar proportions 7:1:1, the major evidence for the troponin value having been obtained from electron-microscopy studies (Ohtsuki *et al.*, 1967; Spudich *et al.*, 1972; Hanson, 1973). In a typical experiment a preparation of actin-tropomyosin was prepared by incubating for 30 min at 48°C a mixture consisting of 2.97 mg of F-actin/ml and 0.74 mg of tropomyosin/ml (7:1 on a molar basis) in imidazole buffer (pH 6.9) containing 0.1 M-KCl and the concentrations of Ca^{2+} (0.1 mM), Mg^{2+} (1 mM) and mercaptoethanol (5 mM); the solution was then cooled to 20°C. This procedure proved satisfactory for converting the initial actin-tropomyosin gel into a filamentous form (Ishiwata, 1973). Addition of a fivefold molar excess of troponin (total troponin concentration of 4.92 mg/ml in the mixture) at 20°C caused pronounced gelation, presumably owing to cross-linking of the actin-tropomyosin filaments by troponin (Ishiwata, 1973). The concentration of free troponin in the supernatant was found to be 2.31 mg/ml, which implied involvement of

2.61 mg of troponin/ml in the actin-tropomyosin-troponin complex. The apparent molar proportions of 7:1:2.9 so obtained for the average composition of this complex was essentially unchanged, as was the nature of the gel, by incubating the actin-tropomyosin and troponin mixture at 48°C for 30 min before analysis at 20°C; this modification of the experiment was prompted by the method used by Ishiwata (1973) for the preparation of reconstituted filaments from gels deficient in troponin (mixing proportions 7:1:0.5).

Discussion

The concept of troponin as a stable, globular protein with a mol.wt. of 85000 is clearly untenable in the light of the results shown in Figs. 2, 3 and 4. The present report of troponin association is not the first, since similar velocity-sedimentation patterns were obtained by Wakabayashi & Ebashi (1968) for preparations from rabbit muscle. However, their findings seem to have been overlooked, by themselves as well as by others, in the development of the current view of the troponin molecule. Further, the only previous equilibrium study (Hartshorne & Dreizen, 1973) also detected substantial amounts of material with mol.wt. less than 85000, which were, however, attributed to the presence of proteolytic fragments in the troponin sample under study; in view of the control experiments performed in the present investigation (Fig. 1), the results presented in Fig. 4 can only reflect dissociation. We also note that the extreme influence of environmental factors such as pH, temperature and ionic strength on the extent of association presumably accounts at least in part for the very wide scatter (35000–150000) of molecular-weight values that have been reported for troponin (Arai & Watanabe, 1968; Ebashi *et al.*, 1968; Schaub & Perry, 1969; Drabikowski *et al.*, 1971a, 1973).

As mentioned in the Results section, the equilibrium-sedimentation data on troponin have only been subjected to a preliminary analysis. No attempt has been made either to refine the molecular-weight data by more elaborate curve-fitting of the $[r^2, \log \tilde{c}(r)]$ results (reviewed by Teller, 1973) or to take advantage of the $\Omega(r)$ function (Milthorpe *et al.*, 1975; Nichol *et al.*, 1976), because the complexity of the system would still preclude its quantitative assessment in terms of stoichiometries and association constants for the various equilibria. The simplest model to describe troponin association would entail a system such as $A+B+C \rightleftharpoons D$, with reversible polymerization of D; self association of troponin-T and -I subunits (Greaser & Gergely, 1971) may also need to be incorporated into the model. Interpretation of the equilibrium-sedimentation experiments on troponin is therefore restricted to the qualitative observation

that association of troponin subunits proceeds well beyond the proposed monomeric unit of 85000 daltons.

The finding of an average troponin/tropomyosin stoichiometry that is greater than unity in actin-tropomyosin-troponin complexes signifies either the binding of monomeric troponin to actin as well as to tropomyosin (Drabikowski *et al.*, 1973; Potter & Gergely, 1974) or the binding of polymeric troponin species to tropomyosin. The failure to observe additional striations in electron-microscope studies of actin-tropomyosin-troponin paracrystals containing excess troponin (Ohtsuki & Wakabayashi, 1972) favours the latter alternative, particularly in view of the greater intensities of the bands compared with 7:1:1 actin-tropomyosin-troponin mixtures. Also of interest in this regard are the studies of Hitchcock (1975), who observed not only a stoichiometry in excess of unity for the troponin-tropomyosin interaction in reconstituted actin-tropomyosin-troponin complexes, but also a dependence of the stoichiometry on the ionic strength of the mixture. Although Hitchcock (1975) interpreted these results in terms of decreased binding of troponin to actin at higher ionic strengths, the observations are also consistent with the alternative explanation that the nature of the troponin species binding to tropomyosin is governed to some degree by the extent to which the troponin is polymerized under the conditions (ionic strength) of the experiment.

These results do not comment on the nature of the actin-tropomyosin-troponin complex in native thin filaments, for which there is considerable evidence of the 7:1:1 stoichiometry (e.g. Ohtsuki *et al.*, 1967; Hanson, 1973; Head & Perry, 1974; Potter, 1974). Instead they signify possible chemical (stoichiometric) as well as physical differences between reconstituted actin-tropomyosin-troponin complex in gel, paracrystalline and filamentous forms. Since troponin and tropomyosin (Kay & Bailey, 1960; Woods, 1967, 1969) both exhibit strong tendencies to self-associate, it is clear that reconstitution of synthetic actin-tropomyosin-troponin filaments having direct relevance to their natural counterparts requires strict attention to be paid to conditions such as pH, temperature, ionic strength, ratios and concentrations of reactants that prevail *in vivo*.

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