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Binding of Aldolase to Actin-Containing Filaments

EVIDENCE OF INTERACTION WITH THE REGULATORY PROTEINS OF SKELETAL MUSCLE

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The interactions of aldolase with regulatory proteins of rabbit skeletal muscle were investigated by moving-boundary electrophoresis. A salt-dependent interaction of troponin, tropomyosin and the tropomyosin–troponin complex with aldolase was detected, the tropomyosin–troponin complex displaying a greater affinity for the enzyme than did either regulatory protein alone. The results indicate that aldolase possesses multiple binding sites (three or more) for these muscle proteins. Quantitative studies of the binding of aldolase to actin-containing filaments showed the interaction to be influenced markedly by the presence of these muscle regulatory proteins on the filaments. In imidazole/HCl buffer, I 0.088, pH 6.8, aldolase binds to F-actin with an affinity constant of $2 \times 10^5 M^{-1}$ and a stoicheiometry of one tetrameric aldolase molecule per 14 monomeric actin units. Use of F-actin–tropomyosin as adsorbent results in a doubling of the stoicheiometry without significant change in the intrinsic association constant. With F-actin–tropomyosin–troponin a lower binding constant ($6 \times 10^4 M^{-1}$) but even greater stoicheiometry (4:14 actin units) are observed. The presence of Ca^{2+} (0.1 mM) decreases this stoicheiometry to 3:14 without affecting significantly the magnitude of the intrinsic binding constant.

The three major protein constituents of the isotropic band (I-band) of muscle, namely F-actin, tropomyosin and troponin, all seem to possess the capacity to bind several glycolytic enzymes (reviewed by Clarke & Masters, 1976). In the initial studies of such interactions (Arnold & Pette, 1968; Arnold *et al.*, 1971) the binding of aldolase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase to F-actin was demonstrated under conditions of low ionic strength. Subsequently, Clarke *et al.* (1974) reported significant binding of aldolase to a muscle extract consisting principally of the tropomyosin–troponin complex. Support for the existence of such interactions in muscle is provided by the histochemical demonstration that several glycolytic enzymes are localized in the region of the I-band (Sigel & Pette, 1969; Dolken *et al.*, 1975).

The possible co-participation of all three structural proteins in the interaction of glycolytic enzymes with muscle filaments is indicated by the finding (Clarke & Masters, 1975) that the presence of tropomyosin and troponin on F-actin filaments increases their binding capacity for aldolase to such an extent that the interaction persists at physiological salt concentrations. Not only is this binding of aldolase dependent on the concentration of Ca^{2+} ions, but also there is a Ca^{2+}-sensitive modulation of enzymic activity for aldolase bound to F-actin–tropomyosin–troponin filaments (Walsh *et al.*, 1977).

Taken in conjunction with electron-microscopic studies that have indicated an interaction between aldolase and troponin on reconstituted thin filaments (Clarke & Morton, 1976; Morton *et al.*, 1977), there seems to be a potentially important role for troponin in the binding of at least some glycolytic enzymes to muscle filaments. However, no direct evidence of any such interactions has been reported previously. This paper presents electrophoretic studies of the interactions between the glycolytic enzyme aldolase and the muscle proteins troponin (the equimolar mixture of T, C and I subunits), tropomyosin and the tropomyosin–troponin complex. Quantitative studies of the binding of aldolase to actin-containing filaments are then presented to show that the interaction is influenced greatly by the presence of tropomyosin and troponin on the filaments.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Protein preparations

Rabbit muscle aldolase was either obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., or purified from rabbit skeletal muscle by chromatography on phosphocellulose (Penhoet *et al.*, 1969). Tropomyosin and troponin were also prepared from freshly excised back and leg muscles of rabbits by the procedures described previously (Clarke *et al.*, 1976). For electrophoretic studies of the interaction of aldolase with the tropomyosin–troponin complex, the latter was prepared by mixing equal molar amounts of the two purified regulatory proteins. The molecular weight of tropomyosin was taken as 68000 (Woods, 1967) and that of troponin as 70000, the value obtained from the sequences of the three subunits comprising this structural protein (Wilkinson & Grand, 1975; Collins *et al.*, 1977; Pearlstone *et al.*, 1977).

Actin was prepared from acetone-dried powders of rabbit skeletal muscle (Briskey & Fukazawa, 1971) by the procedure of Spudich & Watt (1971), except that the actin was freed of tropomyosin contamination by washing with 0.8M-KCl instead of 0.6M-KCl. Because of the larger amounts of tropomyosin–troponin complex required for the reconstitution of F-actin–tropomyosin–troponin filaments, this complex was also obtained from the residue of the acetone-dried powder after removal of actin, the residue being further extracted with 10mM-Tris/HCl/1mM-EGTA/5mM-mercaptoethanol, pH8.3, for 3h at room temperature. After removal of the insoluble material, the tropomyosin–troponin complex was collected in a 40–60%-satd.-$(NH_4)_2SO_4$ fraction and extensively dialysed against the above buffer. Inclusion of EGTA in this buffer decreased significantly the extent of proteolysis of the preparation as judged by SDS/polyacrylamide-gel electrophoresis.

F-actin–tropomyosin and F-actin–tropomyosin–troponin were prepared by mixing G-actin (in 2mM-Tris/HCl/0.2mM-$CaCl_2$/0.5mM-ATP/1mM-dithiothreitol, pH8.0) with either tropomyosin or the tropomyosin–troponin complex (in the 10mM-Tris/HCl/EGTA/mercaptoethanol buffer) in the appropriate ratio (Ishiwata, 1973). The actin was then polymerized by addition of an equal volume of 20mM-Tris/HCl/300mM-KCl/1mM-ATP/2mM-dithiothreitol, pH8.0, to the mixture. Sufficient $CaCl_2$ was added to the F-actin–tropomyosin–troponin to give an excess of 0.05mM-Ca^{2+} over the EGTA concentration in the mixture (Ishiwata, 1973). The mixtures were then incubated at room temperature for 1h to allow complete polymerization to take place, the F-actin–tropomyosin–troponin being subsequently incubated at 45°C for 10min, as recommended by Ishiwata (1973). Filament preparations were then centrifuged at 40000 rev./min for 90min in a 60 Ti rotor of a Beckman ultracentrifuge. The pellets were resuspended in 10mM-imidazole/HCl/50mM-KCl/1mM-$MgCl_2$/1mM-dithiothreitol, pH6.8. These stock filament preparations, which had a protein concentration of 10–15mg/ml, were stored on ice, in the presence of a crystal of thymol, for a maximum of 3 days; after that time deterioration in filament structure could be observed by electron-microscopic examination.

Myosin was prepared from rabbit skeletal muscle as described by Briskey & Fukazawa (1971). Synthetic actomyosin was then made by mixing myosin and actin filaments in a ratio of 3:1 (w/w).

Assays

Aldolase activity was measured by the procedure of Richards & Rutter (1961). The Ca^{2+}-sensitivity of the ATPase activity of synthetic actomyosins prepared with the reconstituted thin filaments was tested by measuring the ATPase activity (Clarke & Masters, 1974) in the presence of either 0.1mM-$CaCl_2$ or 0.2mM-EGTA.

Concentrations of purified proteins were determined spectrophotometrically at 280nm on the basis of specific absorption coefficients ($A^{1\%}_{1cm}$) of 9.1 for aldolase (Kawahara & Tanford, 1966), 4.5 for troponin and 3.0 for tropomyosin. Values for the two regulatory proteins, which were obtained by combining absorbance measurements with concentrations determined refractometrically at 546nm (Doty & Edsall, 1951), are in good agreement with the reported coefficients of 4.7 for bovine muscle troponin (Lovell & Winzor, 1977) and 3.2 at 277nm for rabbit muscle tropomyosin (Holtzer *et al.*, 1965; Woods, 1967). Concentrations of filament preparations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis

Proteins were checked for purity by polyacrylamide-gel electrophoresis in the presence of SDS (Laemmli, 1970). Because of the susceptibility of troponin to proteolysis (Drabikowski *et al.*, 1971; Wilkinson *et al.*, 1972; Clarke *et al.*, 1976), any mixtures containing this protein were also analysed on completion of the experiment. No proteolysis was detected in the current series of experiments, which were of shorter duration and/or conducted at a lower temperature than those reported previously (Lovell & Winzor, 1977).

Characterization of F-actin–tropomyosin–troponin filaments

In addition to analysis by SDS/polyacrylamide-gel electrophoresis, two further criteria were used to judge the successful preparation of F-actin–tropomyosin–troponin filaments. The first was the obser-

vation of adequate Ca^{2+}-sensitivity of the ATPase activity in synthetic actomyosin prepared from these filaments: the binding studies reported below used only those preparations in which the actin-stimulated ATPase activity was inhibited by 80–90% in the absence of Ca^{2+}. The second criterion was the ability of F-actin–tropomyosin–troponin filaments to form characteristic ordered aggregates in the presence of aldolase as judged by electron-microscopic examination of negatively stained preparations. As discussed elsewhere (Clarke & Morton, 1976; Stewart *et al.*, 1980), these highly characteristic structures are only formed when troponin is correctly positioned on the filament. Observation of this ordered structure correlated well with that of maximum Ca^{2+}-sensitivity of ATPase activity. In a successfully reconstituted preparation very few filaments are not incorporated into the bundles. These additional criteria were used because difficulties are experienced in the reconstitution of fully active F-actin–tropomyosin–troponin filaments by the method described. Some possible reasons for the difficulties encountered in the preparation of thin filaments have been considered elsewhere (Lovell & Winzor, 1977). Only those F-actin–tropomyosin–troponin preparations that satisfied all three criteria were used in the following binding studies.

Filament-binding studies

Rabbit muscle aldolase was first equilibrated with imidazole/HCl buffer (10mM-imidazole/HCl/80mM-KCl/1mM-$MgCl_2$/2mM-dithiothreitol), I 0.088, pH 6.8, by extensive dialysis. Reaction mixtures were then prepared by adding 0.1ml of the appropriate stock filament preparation (10mg/ml) to 0.9ml of dialysed enzyme solution that had been suitably diluted with the final diffusate to give the required aldolase concentration: if required, EGTA (0.2mM) or $CaCl_2$ (0.1mM) was included in the dialysis medium. After incubation for 15min at 25°C, reaction mixtures were centrifuged for 60min at 40000 rev./min in a 50 Ti rotor of a Beckman ultracentrifuge operated at 25°C. The supernatants were collected and assayed immediately for aldolase activity. To establish that quantitative recovery of enzyme was being obtained, the pellets were resuspended in 1ml of 0.1M-potassium phosphate buffer, pH7.5, and the enzymic activity was again determined. Results were interpreted in terms of a weight-based binding function r, defined (Nichol *et al.*, 1967) as the amount (g) of aldolase bound per g of filament preparation.

Moving-boundary electrophoresis

Before use the proteins were dissolved directly in the appropriate buffer and dialysed for 16h at 5°C against more (2 × 500ml) of the same buffer, which comprised 10mM-imidazole/HCl/1mM-$MgCl_2$/2mM-dithiothreitol, pH6.8, and either 40mM- or 80mM-KCl. Electrophoresis was conducted in the 15-mm cell assembly of a Perkin Elmer model 238 apparatus with an applied potential gradient of 4.3–4.8V/cm. These values were based on conductivities of diffusate measured at 0°C, and consequently all mobilities refer to migration at 0°C. However, the values of equilibrium constants determined from these mobilities refer to the reactions at 5°C, the temperature at which the electrophoresis experiments were performed.

In electrophoresis of a mixture of two reactants A and B the velocity of any complex is almost certainly intermediate between those of A and B. For this particular combination of velocities the electrophoretic patterns are of the form represented schematically in Fig. 1 (Gilbert & Jenkins, 1959), where the ordinate is expressed in terms of constituent concentrations ($\bar{m}_A + \bar{m}_B$): for a system in which A (aldolase in this instance) possesses p sites for B these are given by

$$\bar{m}_A = m_A + \sum_{i=1}^{p} m_{AB_i} \qquad (1a)$$

$$\bar{m}_B = m_B + \sum_{i=1}^{p} i m_{AB_i} \qquad (1b)$$

where m_X denotes the free molar concentration of X (= A or B). In terms of anodic migration each of the three structural proteins (troponin, tropomyosin, tropomyosin–troponin complex) migrates faster than aldolase, and accordingly the ascending side comprises a boundary corresponding to free B (at $v_B t$, the distance migrated in time t) plus a reaction

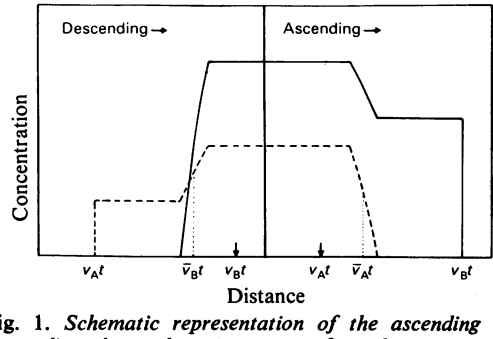

Fig. 1. *Schematic representation of the ascending and descending electrophoresis patterns for a heterogeneously associating system in which the velocities of all complexes are intermediate between those of the reactants A and B*

$v_A t$ and $v_B t$ denote the distance migrated by boundaries of pure components A and B respectively after electrophoresis for time t. $\bar{v}_A t$ and $\bar{v}_B t$ refer to the positions of reaction boundaries across which constituents A and B, respectively, disappear (see the text).

boundary, whereas the descending side consists of a reaction boundary plus a boundary corresponding to free aldolase (A). Somewhere within the region of each reaction boundary there is a position corresponding to the constituent velocity of the reactant that does not separate from the mixture ($\bar{v}_A t$ and $\bar{v}_B t$ in the ascending and descending sides respectively). Exact determination of these constituent velocities requires separate experimental records of the concentrations (or concentration gradients) of the two individual constituents. However, provided that the electrolyte distribution across the original boundary positions has a negligible effect on either the velocities of species or the equilibrium constants, estimates of $\bar{v}_A$ and $\bar{v}_B$ may be obtained from the positions of the reaction boundaries (Smith & Briggs, 1950; Longsworth, 1959), which represent the sum of both constituent boundaries.

Results of electrophoretic experiments were analysed quantitatively by means of eqn. (2), in which v_A and v_B denote the velocities (electrophoretic mobilities) of aldolase and structural protein respectively. This expression,

$$m_B = \frac{\bar{m}_B[\bar{v}_A - \bar{v}_B - (1/p)(\bar{v}_A - v_A)] + \bar{m}_A[\bar{v}_A - v_A]}{[\bar{v}_A - v_B - (1/p)(\bar{v}_A - v_A)]} \quad (2)$$

derived initially by Smith & Briggs (1950), is based on the assumptions (i) that A (aldolase) possesses p equivalent and independent sites for B with an intrinsic association constant K (Klotz, 1946) and (ii) that there is a constant incremental change Δ in v_A induced by the successive addition of each molecule of B, i.e. that $v_{AB_i} = v_A + i\Delta$; $1 \leqslant i \leqslant p$. If the value of p is known, eqn. (2) provides a means of determining m_B experimentally, and hence of defining the Klotz (1946) binding function r_M and the intrinsic association constant K (eqn. 3):

$$r_M = (\bar{m}_B - m_B)/\bar{m}_A = pKm_B/(1 + Km_B) \quad (3)$$

In the present study the stoicheiometries of the interactions with aldolase were not known, and accordingly apparent values of m_B were calculated for a range of values of p. The resulting sets of ($r_M^{app.}$, $m_B^{app.}$) from experiments with different constituent concentrations of A and B were then tested for conformity with eqn. (3) by calculating the apparent equilibrium constant $K^{app.}$: appropriate values of p and K were then selected on the basis of constancy of the $K^{app.}$ so determined.

Results

Initial studies of the interactions of aldolase with the three structural proteins were carried out in 10mM-imidazole/HCl containing 1mM-$MgCl_2$, 2mM-dithiothreitol and 20mM-KCl, pH6.8, I 0.028, to permit comparisons to be made with earlier studies (Clarke *et al.*, 1974). However, marked precipitation occurred on mixing aldolase with troponin, with tropomyosin or with the tropomyosin–troponin complex. This precipitation, which was observed over a wide range of molar mixing proportions, could be reversed by adding KCl. Analysis of the insoluble material by SDS/polyacrylamide-gel electrophoresis showed it to contain aldolase and structural protein. Although the observed precipitation lends qualitative and unequivocal support to the earlier ultracentrifugal evidence (Clarke *et al.*, 1974) of an interaction between aldolase and tropomyosin–troponin complex, the reasons for the quantitative differences remain uncertain. Greater purity of the reactants used in the present study, or species specificity (rabbit versus ox), could account for the differences in solubility.

Increasing the KCl concentration in the mixtures to 40mM sufficed to prevent precipitation, and accordingly quantitative studies of the various interactions were carried out in this medium (10mM-imidazole / HCl / 1mM-$MgCl_2$ / 2mM-dithiothreitol / 40mM-KCl, pH6.8, I 0.048). Preliminary velocity-sedimentation experiments revealed the presence of Ca^{2+}-insensitive interactions in mixtures of aldolase and the muscle proteins troponin and tropomyosin. However, attempts to use velocity sedimentation for quantitative studies were discontinued for three reasons. Firstly, an extremely narrow range of molar mixing ratios could be used with tropomyosin–troponin complex because of gel formation outside this range. Secondly, poor resolution of the troponin boundary was achieved with aldolase–troponin mixtures. Thirdly, the concentration-dependence of the sedimentation coefficient for the self-associating troponin molecule (Lovell & Winzor, 1977) makes quantitative interpretation of the sedimentation patterns for heterogeneously associating mixtures prohibitively difficult. This third problem prompted the decision to investigate the possible study of these interactions by moving-boundary electrophoresis, a technique in which a polymerizing protein can migrate essentially as a single species provided that charge is conserved on polymerization (Creeth & Nichol, 1960).

Electrophoretic patterns for the four reactants in the I 0.048 buffer (pH6.8) are shown in Fig. 2, from which it is evident that the ascending and descending boundaries exhibit the degree of enantiography that is observed with non-interacting solutes at this relatively low ionic strength. Under these conditions aldolase migrates very slowly in the cathodic direction, whereas the three structural proteins migrate anodically. The migration of an equimolar mixture of troponin and tropomyosin as a single symmetrical peak in the ascending and descending limbs confirms the stoicheiometric interaction of these two proteins and also justifies consideration of the tropomyosin–

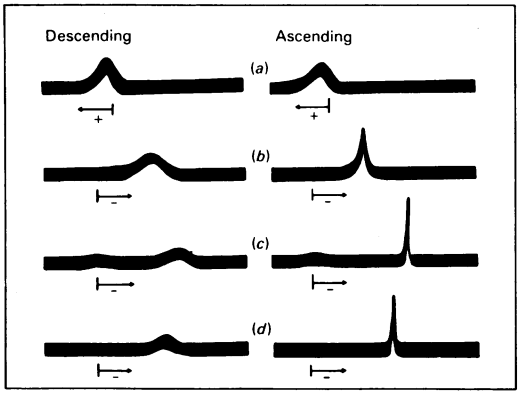

Fig. 2. *Moving-boundary electrophoresis of aldolase and muscle regulatory proteins in imidazole/HCl buffer, pH* 6.8, *I* 0.048

Electrophoresis was carried out in 10mM-imidazole/HCl / 40mM-KCl / 1mM-$MgCl_2$ / 2mM-dithiothreitol, pH6.8. Positions of initial boundaries and direction of migration are indicated below each pattern: (*a*) aldolase (3.5mg/ml); (*b*) troponin (4.6mg/ml); (*c*) tropomyosin (2.7mg/ml); (*d*) tropomyosin–troponin complex (2.0mg/ml). Exposures were taken approx. 3h after application of a potential gradient of 4.8 V/cm.

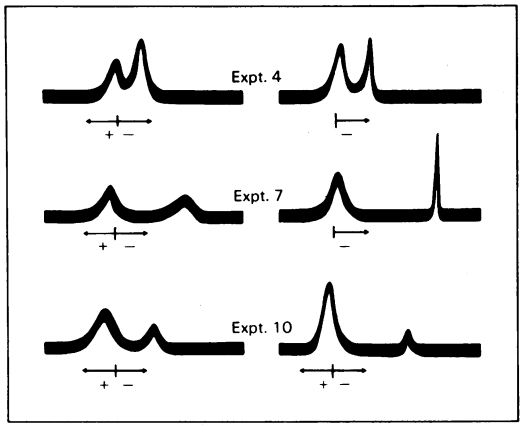

Fig. 3. *Electrophoresis of mixtures of aldolase and regulatory proteins in imidazole/HCl buffer, pH* 6.8, *I* 0.048

Mixtures of aldolase and (*a*) troponin, (*b*) tropomyosin and (*c*) tropomyosin–troponin complex were subjected to electrophoresis under the conditions described in Fig. 2. Experiment numbers refer to Table 1, which lists the compositions of the various mixtures.

troponin complex as a single species. In the application of eqn. (2) to mixtures of aldolase and each of the structural proteins, electrophoretic mobilities were substituted for velocities, the following reactant mobilities being used: aldolase, $+0.44\times10^{-5}cm^2\cdot s^{-1}\cdot V^{-1}$; tropomyosin, $-5.70\times10^{-5}cm^2\cdot s^{-1}\cdot V^{-1}$; troponin, $-3.15\times10^{-5}cm^2\cdot s^{-1}\cdot V^{-1}$; tropomyosin–troponin complex, $-4.41\times10^{-5}cm^2\cdot s^{-1}\cdot V^{-1}$. These values refer to migration in the descending limb. Mobilities of the reactants were routinely slightly faster in the ascending limb, and accordingly values of $\bar{v}_A$, the constituent velocity of aldolase that must be obtained from that limb, have been decreased proportionately to account for this phenomenon: because of the very small absolute magnitude of $\bar{v}_A$ the consequences of any quantitative error in this correction factor are minimal.

Typical electrophoretic patterns obtained with mixtures of aldolase and the three individual structural proteins in *I* 0.048 buffer, pH6.8, are shown in Fig. 3. In each case the ascending and descending patterns conform qualitatively with those predicted (Fig. 1) for a system in which complexes have velocities intermediate between those of the two reactants. The evaluation of the apparent intrinsic association constant via eqns. (2) and (3) from experiments with different molar mixing ratios of reactants are summarized for various values of *p* in Table 1, together with the mean values of $K^{app\cdot}$ for each interaction. Since constancy of $K^{app\cdot}$ over a wide range of mixing ratios is the criterion for identifying appropriate *p* values, relative errors in the mean $K^{app\cdot}$ are included in parentheses. For each of the interactions the relative error in $K^{app\cdot}$ decreases as *p* increases from 1 to 3, but thereafter remains essentially constant. Consequently, the results signify the existence of at least three sites on aldolase for the binding of troponin, of tropomyosin, and of the tropomyosin–troponin complex. To this end, a value of 4 may well be pertinent, in view of (*a*) the tetrameric nature of aldolase and (*b*) the evidence of competition between the structural protein and substrate (fructose bisphosphate) for the aldolase binding sites (Arnold & Pette, 1970; Clarke *et al.*, 1974). The interaction with aldolase seems to be marginally stronger with the tropomyosin–troponin complex than with either ligand singly, troponin and tropomyosin having very similar affinities for the enzyme.

Further increase in the ionic strength caused a marked decrease in the extent of interaction between aldolase and the muscle regulatory proteins. The finding is illustrated in Table 2, which summarizes results of electrophoresis experiments on mixtures of aldolase and tropomyosin–troponin complex in the imidazole/HCl medium made 80mM with respect to KCl (*I* 0.088, pH6.8). Three points are noted. Firstly, under the conditions of higher ionic strength aldolase migrates anodically, the changeover from cathodic to anodic migration presumably being a consequence of ion (chloride) binding (Brown &

Table 1. *Evaluation of apparent association constants for the interaction of aldolase (A) with various regulatory muscle proteins (B) in imidazole/HCl buffer, pH 6.8, I 0.048*

K values are calculated from eqns. (2) and (3) (see the text). Values of $\bar{m}_A$ are based on a mol.wt. of 160000 for aldolase (Kawahara & Tanford, 1966).

Expt. no. B = troponin	$10^5 \times$ Constituent concn. (M)		$10^5 \times$ Constituent mobility ($cm^2 \cdot s^{-1} \cdot V^{-1}$)		$10^{-3} \times$ Apparent association constant, K (M^{-1})					
	$\bar{m}_A$	$\bar{m}_B$	$\bar{v}_A$	$\bar{v}_B$	$p=1$	$p=2$	$p=3$	$p=4$	$p=5$	$p=6$
1	5.00	2.94	+0.08	−1.83	20.7	9.8	6.5	4.8	3.9	3.2
2	4.19	4.86	−0.07	−2.24	24.9	10.4	6.6	4.8	3.8	3.2
3	4.12	4.69	−0.14	−2.47	18.9	8.3	5.4	4.0	3.2	2.6
4	3.18	7.22	−0.21	−2.24	63.8	15.3	8.9	6.3	4.8	3.9
5	2.05	9.57	−0.39	−2.60	231.0	15.2	7.9	5.4	4.1	3.3
			Mean		71.9	11.8	7.1	5.1	4.0	3.2
			Relative error (%)		(126)	(27)	(19)	(16)	(15)	(15)
B = tropomyosin										
6	4.31	3.29	−0.12	−4.28	17.3	7.7	5.0	3.7	2.9	2.4
7	3.07	6.24	−0.03	−4.53	18.1	6.8	4.2	3.0	2.3	1.9
8	2.20	7.84	−0.11	−3.73	6.8	6.7	6.7	6.7	6.7	6.7
9	1.25	9.89	0.00	−4.68	−44.2	31.8	11.7	7.2	5.2	4.0
			Mean		1.3	12.8	6.1	4.0	3.1	2.5
			Relative error (%)		(2300)	(99)	(63)	(54)	(49)	(51)
B = tropomyosin–troponin										
10	6.16	2.14	+0.33	−2.32	20.1	9.2	6.1	4.5	3.6	3.0
11	3.75	2.12	0.00	−3.30	21.5	10.3	6.7	5.0	4.0	3.3
12	2.56	2.97	−0.12	−2.47	94.1	32.1	19.5	14.1	11.1	9.1
13	1.87	4.29	−0.92	−3.66	56.5	20.4	12.6	9.2	7.3	6.0
			Mean		48.0	18.0	11.2	8.2	6.5	5.3
			Relative error (%)		(73)	(59)	(56)	(54)	(54)	(54)

Table 2. *Evaluation of the apparent association constant for the interaction of aldolase (A) with tropomyosin–troponin complex (B) in imidazole/HCl buffer, pH 6.8, I 0.088*

K values are calculated from eqns. (2) and (3) by assuming $p = 4$ (see the text). Values of $\bar{m}_A$ are based on a mol.wt. of 160000 for aldolase (Kawahara & Tanford, 1966).

$10^5 \times$ Constituent concn. (M)		$10^5 \times$ Constituent mobility* ($cm^2 \cdot s^{-1} \cdot V^{-1}$)		$10^{-3} \times$ Apparent association constant, K (M^{-1})
$\bar{m}_A$	$\bar{m}_B$	$\bar{v}_A$	$\bar{v}_B$	
4.97	0.71	−0.45	−5.47	0.36
2.49	1.40	−0.73	−5.52	0.12
1.41	1.59	−0.68	−5.66	1.34
1.24	2.14	−1.21	−5.81	1.01
				Mean 0.71 (80%)

* Reactant velocities were as follows: $v_A = -0.35 \times 10^{-5} cm^2 \cdot s^{-1} \cdot V^{-1}$; $v_B = -5.66 \times 10^{-5} cm^2 \cdot s^{-1} \cdot V^{-1}$.

Timasheff, 1959; Drewe & Winzor, 1976). Secondly, since the results are to be used for comparative purposes, only values of m_B and $K^{app.}$ for $p = 4$ are tabulated: calculations based on other values of p exhibited similar trends to those evident in Table 1. Thirdly, the mean $K^{app.}$ of $700 M^{-1}$ is significantly lower than the corresponding value of $8200 M^{-1}$ for the same interaction at the lower ionic strength (Table 1): a qualitatively similar decrease in the extent of binding with increasing ionic strength was inferred previously (Clarke *et al.*, 1974).

The binding of aldolase to F-actin-containing filaments was much stronger, as is evident from Fig. 4, which presents adsorption isotherms for the binding of enzyme to each of the three filament preparations in imidazole/HCl, *I* 0.088, pH 6.8 (10mM-imidazole/HCl/1mM-$MgCl_2$/2mM-dithiothreitol/80mM-KCl). The adsorption of aldolase to F-actin (●) and F-

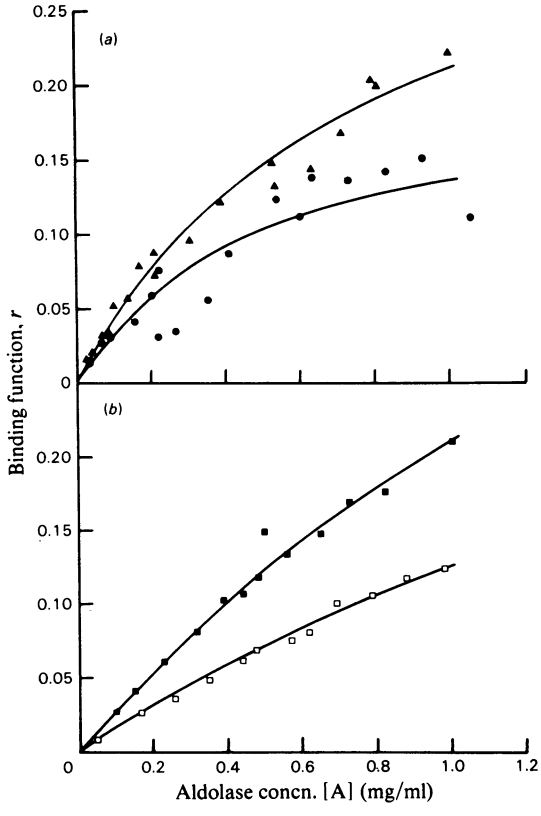

Fig. 4. *Binding isotherms for the interaction of aldolase with F-actin-containing filaments in imidazole/HCl buffer, pH* 6.8, *I* 0.088, *at* 25 °C

Aldolase (0.05–1.25 mg/ml) was incubated with filaments (1 mg/ml) in 10 mM-imidazole/HCl/80 mM-KCl/1 mM-$MgCl_2$/1 mM-dithiothreitol, pH 6.8. [A] denotes the concentration of free enzyme and *r* the binding function expressed on a weight basis. ●, F-actin filaments; ▲, F-actin–tropomyosin filaments; ■, F-actin–tropomyosin–troponin filaments in the presence of EGTA (0.2 mM); □, F-actin–tropomyosin–troponin filaments in the presence of $CaCl_2$ (0.1 mM). Solid lines represent the theoretical binding curves obtained with the parameters listed in Table 3.

actin–tropomyosin (▲) was Ca^{2+}-insensitive and found to be described adequately by an expression of the form $r = r_{max.}K[A]/(1+K[A])$, where [A] denotes the concentration of free aldolase, *K* the association equilibrium constant for the enzyme–filament interaction and $r_{max.}$ the value of *r* corresponding to saturation of the filaments with aldolase. Such analyses of the results in terms of aldolase binding at equivalent and independent filament sites (Klotz, 1946) are summarized in Table 3, in which the values of $r_{max.}$ and *K*, and also of their uncertainties, have been obtained by regression analysis of the results in Scatchard (1949) format. Addition of tropomyosin to the actin filaments has a minimal effect on the affinity for aldolase, but possibly results in an increase in $r_{max.}$, the binding capacity (see the Discussion section).

The adsorption of aldolase to F-actin–tropomyosin–troponin filaments is Ca^{2+}-sensitive, the adsorption isotherms obtained in the presence (□) and absence (■) of Ca^{2+} ions being also presented in Fig. 4, and the corresponding values of $r_{max.}$ and *K* shown in Table 3. As for F-actin and F-actin–tropomyosin, the binding curves followed simple Langmuir isotherms consistent with binding at equivalent and independent sites. Clearly, the presence of Ca^{2+} influences the amount of enzyme that can be bound to the F-actin–tropomyosin–troponin filaments, the maximal binding capacity being lower in the presence of Ca^{2+}: the difference between the binding affinities is not significant. Comparison of these results with those obtained for F-actin and F-actin–tropomyosin filaments as adsorbents reveals

Table 3. *Binding parameters for the interaction of aldolase with actin-containing filaments in imidazole/HCl buffer, pH* 6.8, *I* 0.088

The intrinsic binding constant, *K*, and maximal enzyme-binding capacity of the filaments, $r_{max.}$, were obtained by least-squares analysis of Scatchard (1949) plots of the experimental results shown in Fig. 4. Numbers in parentheses denote the numbers of filament preparations used to obtain the results.

Filament	$10^{-5} \times K$ (M^{-1})	Maximal enzyme-binding capacity (mg/mg of filament)	Maximal enzyme-binding capacity (sites/14 actin units)
F-actin	2.01 ± 0.40	0.27 ± 0.13 (3)	1.0
F-actin–tropomyosin	1.63 ± 0.12	0.40 ± 0.08 (3)	1.8
F-actin–tropomyosin–troponin*	0.63 ± 0.10	0.74 ± 0.14 (6)	4.0
F-actin–tropomyosin–troponin†	0.51 ± 0.06	0.52 ± 0.09 (6)	2.8

* Result obtained in the presence of EGTA (0.2 mM).
† Result obtained in the presence of $CaCl_2$ (0.1 mM).

that the presence of troponin on the filaments decreases their affinity for aldolase by a factor of 3–4, but increases the maximal binding capacity of the filaments, this latter parameter being sensitive to the concentration of Ca^{2+} ions.

To express the maximal amounts of enzyme bound to filaments in molar proportions, the amounts of aldolase bound to each type of filament at saturation have been determined as the number of mol of enzyme bound per 14mol of actin monomer, since there are approx. 14 actin monomers per turn of the actin helix (Hanson & Lowy, 1963). These ratios, presented in the final column of Table 3, have been calculated on the basis of the following molecular weights: actin, 42000 (Elzinga & Collins, 1972); tropomyosin, 68000 (Woods, 1967); troponin, 70000 (cited above); and aldolase, 160000 (Kawahara & Tanford, 1966). The proportions 7:1:1 for actin:tropomyosin:troponin (Spudich & Watt, 1971) have also been assumed. These calculations reveal an integral relationship between the amount of enzyme bound and the repeating unit of the actin filament. With pure F-actin there is one aldolase site per 14 actin monomer units, and this ratio increases to 2:14 when tropomyosin is present on the filament. Addition of troponin to the filament leads to a further increase in the number of aldolase sites, the stoicheiometry being 3:14 and 4:14 in the presence and absence, respectively, of Ca^{2+}.

Discussion

Aldolase has previously been shown to interact with actin in both G and F forms under conditions of low (*I* 0.02) ionic strength (Arnold & Pette, 1968, 1970; Clarke & Masters, 1976). The present study provides definitive evidence that the two regulatory proteins tropomyosin and troponin also interact with the enzyme under similar conditions. These findings, which support our earlier conclusion (Clarke *et al.*, 1974) based on studies with a crude tropomyosin–troponin preparation, possibly explain the enhanced binding of aldolase to actin filaments that is induced by tropomyosin–troponin (Clarke & Masters, 1975, 1976; Table 3 above).

A significant finding of the present investigation is the detection of multiple binding sites on aldolase for both regulatory proteins, the evidence (Table 1) being far more compelling than that obtained earlier (Clarke *et al.*, 1974). The abolition of such interactions by fructose bisphosphate (Arnold & Pette, 1970; Clarke *et al.*, 1974) and the quantitative conclusion (Table 1) that there are at least three sites are certainly compatible with the presence of one binding site per aldolase subunit, but the precise number of binding sites has not been established. As discussed fully in an accompanying paper (Stewart *et al.*, 1980), the existence of multiple binding sites on aldolase for the structural proteins allows the enzyme to cross-link actin-containing filaments, thereby giving rise to the highly ordered filament bundles that have been observed by electron microscopy (Clarke & Morton, 1976).

From the results obtained for the binding of aldolase to filaments it is evident that quantitative differences exist between the adsorption of enzyme to the three filament systems. Adsorption of aldolase to F-actin in imidazole/HCl, *I* 0.088, pH 6.8, occurs at a single class of binding site and with a maximal binding capacity of 0.27mg/mg of actin. This behaviour contrasts with the situation at low ionic strength (Arnold *et al.*, 1971; Clarke & Masters, 1976), where adsorption to F-actin occurs at two classes of binding sites up to a maximum of 2mg/mg of actin, the affinity also being much greater. Much of the adsorption under conditions of low ionic strength presumably reflects relatively non-specific electrostatic interactions between the enzyme and filament, whereas the adsorption observed in the present study is confined to more specific interactions of enzyme with actin. Although the binding of aldolase to F-actin is governed by the largest association constant under the present conditions (*I* 0.088, pH 6.8), that to F-actin–tropomyosin–troponin presumably has the greatest biological significance inasmuch as it predominates at physiological ionic strengths (Clarke & Masters, 1975).

The major effect of tropomyosin and troponin on the adsorption of aldolase to actin-containing filaments is the increased stoicheiometry of binding (Table 3). The two muscle regulatory proteins could achieve this effect either by providing additional binding sites themselves or by altering the filament structure to make possible the changed stoicheiometry. Electron-microscope studies (Clarke & Morton, 1976) have revealed that the binding of aldolase to actin-containing filaments cross-links them in a regular fashion to produce highly ordered filament bundles that are readily seen by the negative-staining technique. These filament bundles consist of parallel arrays of actin-containing filaments cross-linked at regular intervals along their length. With F-actin and F-actin–tropomyosin the enzyme cross-linking occurs at a regular repeat distance of 38nm along the filament bundles. However, the suggested presence of an additional aldolase molecule per cross-link in F-actin–tropomyosin filaments (Table 3) is supported by the computer image analysis of F-actin–aldolase and F-actin–tropomyosin–aldolase paracrystals that is presented in the accompanying paper (Stewart *et al.*, 1980). In view of the evidence (Table 1) that tropomyosin interacts with aldolase, the additional binding site on F-actin–tropomyosin filaments may be provided by the tropomyosin itself.

Regularly cross-linked filament bundles are also observed in electron microscopy of F-actin–tropo-

myosin–troponin–aldolase (Clarke & Morton, 1976), but their appearance differs from those formed by the binding of aldolase to either F-actin or F-actin–tropomyosin. Interpretation of this observation to indicate an interaction of aldolase with troponin molecules on the filaments (Clarke & Morton, 1976) is supported by the demonstration (Table 1) that the enzyme interacts with this muscle regulatory protein. Thus troponin may well contribute the additional binding sites detected on F-actin–tropomyosin–troponin (Table 3).

The other major feature of aldolase absorption to F-actin–tropomyosin–troponin filaments is its Ca^{2+}-sensitivity (Table 3), which is expressed mainly by a change in the stoicheiometry of binding in the presence of Ca^{2+}: in the absence of Ca^{2+} there are four binding sites per repeat of the filament structure, but only three in its presence. A Ca^{2+}-sensitive modulation of enzymic activity for aldolase bound to F-actin–tropomyosin–troponin filaments has been reported previously (Walsh *et al.*, 1977). The accompanying paper (Stewart *et al.*, 1980) presents a detailed structural analysis of enzyme–filament interactions based on electron microscopy and computer image processing, and structural models that correlate with the observed (Table 3) stoicheiometries of binding.

Although the evidence presented in this and the accompanying paper (Stewart *et al.*, 1980) indicates that aldolase interacts with tropomyosin and troponin in the intact filament systems, the interaction of enzyme with the individual proteins is much weaker, as is evident from a comparison of Tables 1 and 2 with Table 3. One factor contributing to the increased affinity of filaments may be the regular location of binding sites along the filament structure, leading to an effective increase in the availability of binding sites that are suitably placed to participate in the aldolase cross-linking reaction and thus facilitate the formation of the ordered filament bundles. Irrespective of the reasons, it is clear that the complexes formed between enzyme and filaments are inherently more stable than those formed with tropomyosin and troponin, a factor that underlines the subtlety of the interactions between actin, tropomyosin, troponin and aldolase.

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