Interaction of Aldolase with the Troponin–Tropomyosin Complex of Bovine Muscle

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Ultracentrifugal studies of mixtures of aldolase and the troponin–tropomyosin complex from bovine muscle showed the existence of a labile interaction between these two myofibrillar constituents in imidazole buffers, pH 6.8, I 0.02–0.10 (mol/l), and the suppression of the reaction by fructose 1,6-diphosphate. Analysis of the sedimentation-velocity patterns suggests the binding of more than 2 molecules of troponin–tropomyosin/molecule of aldolase. The results illustrate the necessity of considering additional or alternative sites to F-actin to account for the observed binding of aldolase to the thin filaments of skeletal muscle.

In keeping with the behaviour of a number of glycolytic enzymes, aldolase can be bound reversibly *in vitro* to the cellular fraction of muscle (Starlinger, 1967; Clarke *et al.*, 1970). From a comparison of such adsorption to selected structure proteins and from histochemical localization of glycolytic enzymes in skeletal muscle, Arnold & Pette (1968) concluded that F-actin was the most probable cellular constituent involved in the binding of aldolase, and this conviction persisted through subsequent investigations (Sigel & Pette, 1969; Arnold & Pette, 1970; Arnold *et al.*, 1971). The techniques of these authors excluded troponin and tropomyosin from the comparison of structural proteins, however, even though these proteins are known to be intimately involved in the contractile process and to be associated with F-actin in the thin filament of skeletal muscle. The present investigation was designed to test the possibility of an interaction between aldolase and the troponin–tropomyosin complex (or EGTA*-sensitizing factor) of bovine muscle, and thereby to consider such additional or alternative sites for the binding of aldolase to myofibrils.

Materials and methods

Bovine muscle from freshly slaughtered animals was used for the preparation of both aldolase and the troponin–tropomyosin complex by procedures developed for isolation of the corresponding components from rabbit muscle. Aldolase was prepared essentially by the method of Penhoet *et al.* (1969), and the troponin–tropomyosin complex by the procedure described by Spudich & Watt (1971). Solutions of the two reactants were prepared for sedimentation analysis by exhaustive dialysis at 4°C against a very weak imidazole buffer (5 mM-imidazole–HCl, pH 6.8) containing 1 mM-MgCl₂ and 2 mM-dithiothreitol,

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid.

with KCl (20 mM unless specified otherwise) as the major contributor to the ionic strength. Concentrations of these dialysed stock solutions were determined colorimetrically by the procedure of Lowry *et al.* (1951), and the reaction mixtures prepared from them just before centrifugal analysis.

Sedimentation-velocity experiments were performed at 20°C and 60000 rev./min in a Spinco model E ultracentrifuge, and the resulting schlieren patterns measured on a Nikon two-dimensional comparator fitted with projection screen and accurate to 2 μ m. Quoted sedimentation coefficients refer to migration in the particular medium used, but approximate very closely to $s_{20,w}$ values because of the low relative viscosities of KCl solutions.

Results and discussion

Typical sedimentation-velocity patterns for the separate reactants in the imidazole–HCl buffer ($I = 0.024$) are shown in Fig. 1(a), the upper and lower patterns referring to aldolase and troponin–tropomyosin respectively. The sedimentation coefficient of 7.8S for aldolase (4.0 mg/ml) agrees well with published values for the rabbit muscle enzyme (Kawahara & Tanford, 1966), and the sedimentation behaviour of the troponin–tropomyosin complex (3.7 mg/ml) also finds parallel in earlier studies (Hartshorne & Mueller, 1969), both with respect to sedimentation coefficient (5.0S) and the presence of a slower-migrating impurity.

Fig. 1(b) illustrates the results obtained on centrifuging mixtures of aldolase and troponin–tropomyosin in the imidazole–HCl buffer ($I = 0.024$), the upper pattern referring to a sample made 1 mM with respect to fructose 1,6-diphosphate. Several points in relation to Fig. 1(b) require comment. (i) The lower pattern provides definitive evidence of complex formation, since the sedimentation coefficient of the fastest boundary (13.8S) is larger than that of

either reactant. (ii) The interaction is suppressed by fructose 1,6-diphosphate (upper pattern in Fig. 1*b*), the schlieren profile representing the sum of those for the two reactant species: this suppressive effect of substrate on the present system is also observed with the interaction between aldolase and F-actin (Arnold & Pette, 1970). (iii) Complex-formation occurs only between aldolase and the major component of the

EGTA-sensitizing factor, since the pattern for the slow-migrating component remains unchanged. (iv) From the magnitude of its s_{20} the slower boundary (7.7S) corresponds to aldolase, there being no sign of a boundary corresponding to pure troponin-tropomyosin. Such a result implies either stoichiometric complexing of all of the latter component or the coexistence of a complex and the reactants in a state of rapid dynamic equilibrium (Gilbert & Jenkins, 1959). (v) Analysis of additional mixtures (Table 1) establishes the latter as the likely explanation because of the variation in the magnitude of s_{20} for the fast-moving peak (11.4–13.8S). The fast-moving peak is therefore considered to correspond to a reaction boundary, and not to represent the migration of the aldolase-troponin-tropomyosin complex, which would have an even larger s_{20} value.

Table 1. *Sedimentation of mixtures of aldolase (A) and troponin-tropomyosin (B) in imidazole-HCl buffer, pH 6.8, $I=0.02$*

Mixtures were prepared from dialysed solutions of the reactants immediately before centrifugation at 60000 rev./min and 20°C. The concentration listed in the final column denotes the concentration obtained from the slower peak (see the text).

Concn. of reactants (mg/ml)		s_{20} (S)		c_A^B (mg/ml)
A	B	Fast peak	Slow peak	
2.5	2.8	11.4	7.8	1.2
2.7	2.4	12.4	8.0	0.8
3.3	2.5	12.8	8.0	1.6
2.5	2.5	13.1	7.6	0.8
3.0	2.6	13.8	7.7	1.0

Fig. 1. *Typical sedimentation-velocity patterns for aldolase and troponin-tropomyosin in imidazole-HCl buffers, pH 6.8*

(a) Aldolase (upper trace) and troponin-tropomyosin (lower trace) centrifuged separately in imidazole-HCl buffer, pH 6.8, $I=0.024$. (b) A mixture of aldolase (3.0 mg/ml) and troponin-tropomyosin (2.6 mg/ml) in imidazole-HCl buffer, $I=0.024$ (lower pattern) and a mixture of aldolase (1.7 mg/ml) and troponin-tropomyosin (3.1 mg/ml) in the imidazole-HCl buffer made 1 mM with respect to fructose 1,6-diphosphate (upper trace). (c) Mixtures of aldolase (2.5 mg/ml) and troponin-tropomyosin (2.5 mg/ml) in imidazole-HCl buffers of $I=0.054$ (upper pattern) and 0.104 (lower pattern). In these exposures, taken after centrifugation for 45 min at 60000 rev./min, the values adjacent to the peaks indicate the measured sedimentation coefficients. Centrifugation is from left to right.

Since the velocity of the aldolase-troponin-tropomyosin complex is greater than that of either aldolase (A) or troponin-tropomyosin (B), the concentration of aldolase separating as the slower boundary (c_A^B) does not represent its free concentration in the equilibrium mixture (α phase). However, the equilibrium concentration of troponin-tropomyosin (c_B^A) can in principle be obtained from eqn. (1):

$$c_B^A = (\bar{c}_B^A - \bar{c}_A^A + c_A^B)(s_B - s_A)/(s_B - s_A) \quad (1)$$

where $\bar{c}_B^A$ and $\bar{c}_A^A$ denote the initial concentrations of troponin-tropomyosin and aldolase respectively, s_B and s_A the respective sedimentation coefficients, and s_B that of the reaction boundary. As noted by Nichol & Ogston (1965), eqn. (1) assumes identity of the median bisectors of the concentration gradients of aldolase and troponin-tropomyosin across the reaction boundary. With the present system $s_B > s_A$ and $s_B < s_A$, whereupon the velocity term in eqn. (1) is negative. It is thus mandatory that the concentration term on the right-hand side of eqn. (1) also be negative for a positive value of c_B^A , the equilibrium concentration of troponin-tropomyosin, to be obtained. Although eqn. (1) refers to concentrations on a molar scale, qualitatively similar conclusions can be drawn from weight-concentrations because of the essential identity of the molecular weights of aldolase (160000; Kawahara & Tanford, 1966) and troponin-tropomyosin (156000): the latter value is obtained by summing values of 68000 for tropomyosin (Woods, 1967) and 88000 for troponin (Hartshorne & Dreizen, 1972). Consideration of the results in Table 1 shows that in all experiments the term $(\bar{c}_B^A - \bar{c}_A^A + c_A^B)$ is positive instead of negative, and thus the interaction does not result in the formation of a labile 1:1 complex.

Description of systems with multiple binding to equivalent and independent sites merely requires redefinition of the concentration terms in eqn. (1) on a base-molar scale (Nichol & Winzor, 1964). With the interaction of two macromolecular reactants it is necessary to consider both possible cases of multiple binding, i.e. multiple binding of aldolase to troponin-tropomyosin and of troponin-tropomyosin to aldolase: the former will be examined first.

If we consider the reaction to be $pA + B \rightleftharpoons A_pB$, the conversion to base-molar concentrations leaves $\bar{c}_A^A$ and c_A^B unchanged, but increases $\bar{c}_B^A$ p -fold. We can therefore eliminate multiple binding of aldolase to troponin-tropomyosin, since selection of p values larger than unity leads to prediction of values for c_B^A that are even more negative than the corresponding estimates with $p = 1$. There remains only the situation that complex-formation involves the binding of several molecules of troponin-tropomyosin/molecule of aldolase ($A + qB \rightleftharpoons AB_q$). With q greater than 2 the concentration term on the right-hand side of eqn. (1)

assumes the required negative sign for all experiments, but there is no unanimity as to the magnitude of the equilibrium constant for any single value of q . Indeed, there are many instances where the selected value of q gives estimates of c_B^A in excess of $\bar{c}_B^A$, which also represents a physically unreal situation. However, since the equilibrium concentration of B obtained from eqn. (1) is sensitive to minor changes in c_A^B and becomes increasingly so with larger values of q , the physically unreal predictions are considered more likely to reflect limitations in the accuracy of concentration and velocity measurements than invalidation of the particular case of multiple binding (value of q). In this connexion it should be noted that the 'rectangular approximation' is implicit in eqn. (1) which also assumes equivalence and independence of sites when base-molar concentration scales are used. [Consequences of the Johnston & Ogston (1946) effect have also been neglected.] Thus the limit to quantitative interpretation of the results presented in Table 1 seems to be the conclusion that the interaction involves rapidly reversible multiple binding of troponin-tropomyosin to aldolase.

To gain further insight into the possible number of troponin-tropomyosin molecules bound/molecule of aldolase, the likely magnitudes of $s_{20,w}$ for various aldolase-troponin-tropomyosin complexes were estimated from eqn. (2) on the basis of a value of 0.74 for the partial specific volume ($\bar{v}$) of all components and a frictional ratio (f/f_0) that was the weighted average of those for the constituent components. Some support for this empirical approach to assigning values of f/f_0 is provided by the relatively good agreement between the value of 1.94 obtained by this procedure for the troponin-tropomyosin complex and the value of 1.92 that is obtained from eqn. (2):

$$s_{20,w} = \frac{M(1 - \bar{v}\rho_{20,w})}{N6\pi\eta_{20,w}(f/f_0)} \left(\frac{3\bar{v}M}{4\pi N} \right)^{-1/2} \quad (2)$$

where M and N are the molecular weight and Avogadro's number respectively. On such a basis values of 10.0, 12.2, 14.8 and 16.4S are obtained for $s_{20,w}$ of complexes with $q = 1, 2, 3$ and 4 respectively. Since the sedimentation coefficient must be greater than that of the observed reaction boundary (fast-moving peak in Table 1), these calculations also implicate the binding of more than two molecules of troponin tropomyosin/molecule of aldolase. In this connexion a value of $q = 4$ may be pertinent in the light of the suppressive effect of fructose 1,6-diphosphate, for which there are four active sites on aldolase.

Although the occurrence of aldolase binding to the thin filaments of skeletal muscle is indicated as biologically significant by other lines of investigation (Starlinger, 1967; Sigel & Pette, 1969; Arnold & Pette, 1970; Clarke & Masters, 1973a,b), it is

recognized that the binding studies described to this point, and also those of Arnold & Pette (1968, 1970) were carried out under conditions of very low ionic strength *in vitro*. It was therefore of interest to examine the influence of variation in ionic strength. In the presence of 0.05M-KCl, the aldolase and reaction boundaries are still clearly evident (upper pattern in Fig. 1c), but the smaller sedimentation coefficient of the latter (10.2S) signifies a lesser extent of complex-formation. On increasing *I* to 0.104 the velocity of the reaction boundary is decreased still further, to the extent that it merges with the aldolase boundary (lower pattern in Fig. 1c). That this pattern does still reflect reversible complex-formation is evident not only from the magnitude of the weight-average sedimentation coefficient (8.4S) compared with that of 6.4S for the corresponding non-interacting mixture, but also from the difference in shapes of the upper schlieren profile in Fig. 1(b) and the lower one in Fig. 1(c): as mentioned above the former reflects suppression of the interaction.

Since the effect of ionic strength on the interaction of aldolase with troponin-tropomyosin is qualitatively similar to that observed for its interaction with F-actin (Arnold & Pette, 1968, 1970), either of these structural proteins may be considered as plausible sites for reversible binding of the enzyme to muscle myofibrils. However, whereas the present results signify a rapid reversible equilibrium with troponin-tropomyosin as ligand, Arnold & Pette (1970) have suggested that differences in the effects of ionic strength on adsorption and desorption curves for the aldolase-F-actin system reflect lack of equilibrium after a 15 min time-interval. In the event that these interactions do occur *in vivo* and do have significance

in relation to metabolic control of glycolysis, rapid attainment of equilibrium is clearly desirable.

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