Rabbit Muscle Myogen

INTERACTIONS WITH PHOSPHATE AS THE SOURCE OF NON-ENANTIOTROPY IN MOVING-BOUNDARY ELECTROPHORESIS

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Rabbit muscle myogen has been subjected to moving-boundary electrophoresis and velocity sedimentation in 0.0187M-potassium phosphate buffer, pH 7.7, $I = 0.05$. The ascending and descending electrophoretic patterns are sufficiently non-enantiographic to suggest the existence of rapid, reversible interactions in the myogen solutions. However, no evidence of pronounced macromolecular association was obtained in velocity-sedimentation experiments. The source of the non-enantiography in electrophoresis has been traced to interactions of phosphate with components of myogen, which should therefore be considered as a mixture, rather than a complex, of glycolytic enzymes.

Experimental support for the existence of interactions between different glycolytic enzymes has been inferred from kinetic (Garfinkel & Hess, 1964; Reed & Cox, 1966), velocity sedimentation (Clarke & Masters, 1973), gel chromatography (Földi *et al.*, 1973) and electrophoretic (Amberson *et al.*, 1964) studies. From the biological viewpoint this concept of myogen itself as a multi-enzyme complex has been made largely redundant by the detection of interactions between most of the individual glycolytic enzymes and the fibrous components of muscle (Arnold & Pette, 1968; Amberson & Bauer, 1971; Clarke & Masters, 1975). Indeed, Clarke & Masters (1974) have attributed their earlier ultracentrifugal evidence of complex-formation in myogen (Clarke & Masters, 1973) to the presence of myosin in the preparation. At this stage the evidence of reversible association derived from moving-boundary electrophoresis of myogen (Amberson *et al.*, 1964) remains unquestioned.

In the present investigation of myosin-free myogen from rabbit muscle we confirm the earlier result (Amberson *et al.*, 1964) that the ascending and descending electrophoretic patterns are decidedly non-enantiographic, a characteristic of reversibly associating systems (Gilbert & Jenkins, 1959). However, the phenomenon is shown to result from reversible phosphate binding to, and not from intermolecular association of, myogen components.

Materials and Methods

Preparation of myogen

Myogen preparations were obtained from freshly excised white muscle of adult rabbits, essentially by procedures described previously (Arnold & Pette, 1968; Clarke & Masters, 1973, 1974), except that 0.3M-sucrose/10mM-triethanolamine/3mM-EDTA (pH 7.2 at 4°C) (Czok & Bücher, 1960) was used as the extracting medium. This change of procedure proved effective in eliminating the traces of myosin that were present in earlier myogen preparations made in this laboratory (Clarke & Masters, 1973, 1974). Samples were checked for possible myosin contamination by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). Myogen was stored at 4°C as a suspension in 3.2M-(NH₄)₂SO₄.

Before use in either electrophoresis or sedimentation-velocity experiments, the myogen (approx. 200mg in 5ml) was dialysed exhaustively against the appropriate buffer (4 × 500ml). Concentrations were determined spectrophotometrically at 280nm on the basis of an extinction coefficient ($E_{1\%}^{1\text{cm}}$) of 9.6, which was deduced refractometrically at 20°C by assuming a specific refractive increment of 1820×10^{-6} at 546nm (Doty & Edsall, 1951).

Moving-boundary electrophoresis

Electrophoresis experiments were performed in either the 9mm or 15mm cell of a Perkin-Elmer model 238 electrophoresis apparatus, the bath of

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which was maintained at 5°C by circulation of water from an external, thermostatically controlled cooling system. However, mobility measurements refer to 0°C, the temperature at which the resistivities of the dialysed protein solutions were measured with a Leeds and Northrup precision a.c. resistance bridge and a Shedlovsky-type conductivity cell (2ml capacity).

Ultracentrifugation

Myogen preparations were subjected to velocity sedimentation at 5°C and 60000 rev./min in a Spinco model E ultracentrifuge. Estimates of sedimentation coefficients ($S_{20,w}$) were obtained from the rates of migration of the maximum ordinates of the peaks observed with the schlieren optical system. No allowances were thus made for boundary asymmetry (Goldberg, 1953) or for effects of concentration-dependence. The latter would have been considerable, but presumably of similar magnitudes, in the various experiments, all of which were performed in 3mm cells on 4–4.5% (w/v) myogen solutions.

Results and Discussion

In the previous electrophoretic study (Amberson *et al.*, 1964) decidedly non-enantiographic and complex patterns were obtained from myogen in 0.0187M-potassium phosphate, pH 7.7 (0.0164M- K_2HPO_4 /0.0023M- KH_2PO_4). This non-enantiography and also the complexity of the patterns were absent from experiments performed with myogen in 0.15M-phosphate, pH 7.7 (0.134M- K_2HPO_4 /0.0186M- KH_2PO_4). Similar results were obtained in the present investigation of myosin-free myogen (Figs. 1a and 1b), and hence the effects observed by Amberson *et al.* (1964) cannot be ascribed to interactions of myogen components with myosin contaminants present in the preparation (Clarke & Masters, 1973, 1974).

On the basis of such results, Amberson *et al.* (1964) proposed that phosphate prompted intermolecular association of myogen constituents and that a phosphate concentration of 0.15M was sufficient for stoichiometric formation of this macromolecular complex; the non-enantiography of patterns obtained with 0.0187M-phosphate was considered to reflect co-existence of this complex in rapid association equilibrium with its constituent myogen reactants. To test these proposals, samples of the solutions used for electrophoresis (Figs. 1a and 1b) were also subjected to velocity sedimentation (Figs. 2a and 2b). The qualitative similarity of these sedimentation patterns for myogen in the presence of low (0.0187M) and high (0.15M) phosphate concentrations clearly precludes the Amberson *et al.* (1964) interpretation of their electrophoresis results. Minor changes in the detailed shapes of the patterns are observed, but the

overall migration rate is slightly faster (by approx. 0.2S) for myogen in the lower phosphate concentration. Phosphate-induced interactions between the macromolecular components of myogen would thus seem to be either essentially non-existent or stoichiometric at both concentrations of phosphate.

The likely explanation of the Amberson *et al.* (1964) results follows from electrophoresis and sedimentation runs in 0.037M-NaCl/0.015M-Tris/HCl buffer, pH 7.7 (Figs. 1c and 2c respectively). Substitution of Tris/HCl for phosphate buffer at the lower ionic strength (0.05) has not greatly affected the sedimentation pattern for myogen but has a pronounced effect on its electrophoretic behaviour. Not only are the latter patterns essentially enantiographic, but there is also a change from anodic to largely cathodic migration (see Figs. 1a and 1c): the small amount of material migrating with the faster negative mobility in Fig. 1(c) is almost certainly due to plasma-albumin impurity (Amberson *et al.*, 1964). The slightly lower

Fig. 1. Moving-boundary electrophoresis of myogen at pH 7.7

The schlieren patterns refer to electrophoresis of 4% (w/v) myogen solutions in (a) 0.0187M-phosphate ($I = 0.05$) for 320 min at an applied potential gradient of 0.9 V/cm, (b) 0.15M-phosphate ($I = 0.40$) for 480 min at an applied potential gradient of 0.8 V/cm, and (c) 0.037M-NaCl/0.015M-Tris/HCl buffer ($I = 0.05$) for 540 min at an applied potential gradient of 1.0 V/cm. Positions of the initial boundaries and the nature of migration (anodic or cathodic) are indicated below each pattern. Greek letters refer to different phases in the ascending boundary system (see the text).

Fig. 2. Velocity sedimentation of myogen at pH 7.7

The schlieren patterns refer to exposures taken after centrifugation of 4–4.5% (w/v) myogen solutions in (a) 0.0187M-phosphate, (b) 0.15M-phosphate and (c) Tris/HCl buffer ($I=0.05$) for approx. 160min at 60000rev./min and 5°C.

sedimentation rate (by about 2–4%) for myogen in the Tris/HCl medium can probably be attributed to greater physical interactions at the higher protein concentration (4.5, cf. 4.0%) used in this experiment,

rather than to dissociation of a macromolecular complex. Although these results cannot be taken to imply necessarily the complete absence of phosphate-induced macromolecular interactions in myogen, interactions of phosphate with the constituent enzymes of myogen must be responsible for much of the complexity observed in moving-boundary electrophoresis of myogen.

Closer inspection of Fig. 1(a) shows a substantial source of the non-enantiography to be the very large boundary with virtually zero mobility in the ascending pattern. In the event that this boundary should represent the δ -boundary anomaly, considerable differences would exist between the phosphate concentrations in the α (original-solution) and β (created-plateau) phases (Fig. 1a). The extent of dilution that occurs across the stationary boundary has been established by the Charlwood (1950) procedure, which entails estimation of the supernatant concentration of buffer required as γ phase (Fig. 1a) to eliminate the δ boundary. Results of a series of electrophoresis experiments with different concentrations of supernatant buffer ($[P_1]^*$) are summarized in Fig. 3, which presents the size of the stationary boundary as a function of the ratio $[P_1]^*/[P_1]^d$, where $[P_1]^d$ is the concentration of phosphate buffer (0.0187M) used for dialysis of the 4.6% myogen solution before electrophoresis. Linear extrapolation of Fig. 3 to zero stationary boundary yields a value of 1.60 for the dilution of buffer that occurs across the stationary ($\alpha\beta$) boundary. A similar estimate (1.5–1.6) is obtained for the corresponding dilution of protein, as judged from the relative total areas under the migrating peaks in the descending and ascending sides. Hence we can reasonably attribute the entire $\alpha\beta$ boundary to the re-adjustment of concentrations of all species that occurs to satisfy the requirement (Kohlrausch, 1897) that the regulating function ω at a given position in the cell be time-independent.

By coupling the condition of electroneutrality, the expression for the Donnan redistribution of ions across a semipermeable membrane, and the definition of the regulating function, i.e. $\omega = \sum c_i/u_i$, where c_i denotes the electrochemical concentration (product of valence and molarity) of species i with mobility u_i , it is possible (Charlwood, 1950; Creeth & Winzor, 1962) to calculate the concentrations of all ions in the α phase, including the mean electrochemical concentration $\bar{c}_{Pr}$ of protein. With $u_{K^+} = +40 \times 10^{-5}$, $u_{HPO_4^{2-}} = -22 \times 10^{-5}$, $u_{H_2PO_4^-} = -12 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ (Charlwood, 1950; Nichol, 1950) and a value of $-0.8 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ for $\bar{u}_{Pr}$, the constituent mobility of myogen in the α phase, $[P_1]^*$, the concentration of phosphate referring to descending migration in Fig. 1(a), is calculated to be 0.0183M; taken in conjunction with the dilution factor of 1.60 across the $\alpha\beta$ boundary (Fig. 3), this signifies a value of 0.0114M for $[P_1]^d$, the concentration of phosphate appropriate

Fig. 3. Elimination of the δ boundary in electrophoresis of myogen in phosphate buffer, pH 7.7, $I = 0.05$

Experimental points denote the size of the δ boundary observed with various supernatant concentrations $[P_i]^*$ in the electrophoresis of 4.6% (w/v) myogen solutions dialysed against 0.0187M-phosphate ($[P_i]^\delta$) buffer, pH 7.7.

to ascending migration in Fig. 1(a). The electrochemical concentration ($\bar{c}_{Pr}$) of myogen is -1.24 mM, which corresponds to a mean net charge of -2.7 per 10^5 g of protein. If we assume that no change in the molecular dimensions of the myogen macromolecules accompanies the changeover from 0.0187M-phosphate to the Tris/HCl buffer of the same pH (Figs. 2a and 2c), the value of $+0.3 \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$ for $\bar{u}_{Pr}$ in the non-phosphate buffer (Fig. 1c) implies an average net charge of $+1.0$ per 10^5 g of protein. By virtue of the estimated difference between the charge on myogen in the two media, it would appear that on average two to four phosphate ions per 10^5 g of protein are bound to myogen dialysed against 0.0187M-phosphate buffer, pH 7.7, $I = 0.05$; such interpretation does, of course, neglect consideration of any chloride binding.

Although the dramatic differences between the phosphate concentrations in the ascending and descending limbs explains qualitatively the faster electrophoretic migration observed in the ascending pattern of Fig. 1(a), comparison with patterns obtained for ovalbumin at a similar ionic strength

Fig. 4. Effect of decreasing the δ -boundary size on the electrophoresis of myogen in phosphate buffer, pH 7.7, $I = 0.05$

Myogen solutions (4.6%, w/v) dialysed against 0.0187M-phosphate buffer, pH 7.7, were subjected to moving-boundary electrophoresis with (a) 0.0187M-, (b) 0.0224M- and (c) 0.0282M-phosphate buffer, pH 7.7, as the supernatant buffer. The position of the initial boundary is indicated below each pattern.

(Fig. 1c of Cann, 1949) shows that the present profiles are not those expected of a non-interacting heterogeneous system. For such systems the ascending and descending patterns have similar general shape, but differences between the applied potential gradients to the two limbs result in faster ascending migration of all components. There is little correspondence between the peaks observed in the two segments of Fig. 1(a). Closer correspondence is observed as the difference between $[P_i]^*$ and $[P_i]^\delta$ is diminished, as is evident from Fig. 4, which presents the electrophoretic patterns obtained in three of the experiments used for estimating the dilution factor across the $\alpha\beta$ boundary. However, in Fig. 4(c), where the δ boundary is decreased substantially, there is still considerable non-enantiography. Migration of the slower boundary is comparable in the two limbs, but

Table 1. *Effect of phosphate concentration on the electrophoretic mobility of glyceraldehyde 3-phosphate dehydrogenase at pH 7.7*

Results refer to experiments on 0.3% solutions of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (product of Sigma Chemical Co., St. Louis, MO, U.S.A.) dialysed against the various buffers (pH 7.7, $I=0.10$); Tris/HCl buffer, pH 7.7, was used to supply any additional ionic strength to that contributed by phosphate.

Phosphate composition of buffer			$10^5 \times$ Electrophoretic mobility ($\text{cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$)
K_2HPO_4 (M)	KH_2PO_4 (M)	$[\text{P}_i]^d$ (M)	
0.0082	0.00115	0.00935	1.14
0.0164	0.0023	0.0187	1.21
0.0328	0.0046	0.0374	1.36

the patterns differ with regard to the relative distribution of solute between the two boundaries (the slow boundary comprising approx. 60% and 45% of the descending and ascending patterns respectively), and to the appearance of a very sharp peak within the faster-migrating ascending boundary; these phenomena may be accounted for partly by the operation of a Johnston-Ogston (1946) effect. The supernatant concentration $[\text{P}_i]^d$ is essentially that required to equate the buffer concentrations in the α and β phases, where the protein concentration is then 4.6% (w/v). In the leading edge of the ascending boundary, where the protein concentration is lower, the redistribution of ions to maintain constant ω results in phosphate concentrations higher than $[\text{P}_i]^d$ and hence slower migration because of the increased conductivity. Some sharpening of the faster-migrating ascending boundary, and underestimation of the size of the slower ascending boundary by about 20–30% are thus predicted as Johnston-Ogston effects, but it is questionable whether all of the non-enantiography can be attributed to this cause. In particular, the persistence of the 'spike' within the faster-migrating ascending boundary may well reflect a reaction boundary caused by shifts in the positions of phosphate-protein equilibria due to the gradient in phosphate concentration across the $\beta\gamma$ boundary.

The feasibility of differences in the extents of phosphate binding to myogen in the ascending and descending limbs of an electrophoresis experiment is demonstrated with glyceraldehyde 3-phosphate dehydrogenase, one of the major constituents of myogen (Czok & Bücher, 1960). Table 1 summarizes the experimental details and results of experiments performed at constant ionic strength (0.10), but with varying phosphate concentrations; any additional ionic strength was supplied by inclusion of Tris/HCl

buffer of the same pH, namely 7.7. The reported mobilities, which refer to migration of the centroid (Longworth, 1943) of the descending boundary, clearly establish the equilibrium nature of the phosphate interaction with glyceraldehyde 3-phosphate dehydrogenase by virtue of the progressive change in mobility with increasing phosphate concentration. In regard to electrophoresis of myogen under conditions pertinent to Fig. 1(a), where the phosphate concentrations are 0.0183 and 0.0114M for descending and ascending migration respectively, a greater electrophoretic mobility for glyceraldehyde 3-phosphate dehydrogenase in the descending limb is thus implicated, although this effect would be countered to some extent by the normal ionic strength-dependence of mobility (Henry, 1931). From the results of Velick & Hayes (1953) for the mobility of this enzyme in phosphate buffers of different ionic strengths, the change in mobility resulting from increased phosphate binding at higher concentrations of phosphate outweighs the ionic-strength effect.

Although glyceraldehyde 3-phosphate dehydrogenase is thus characterized by a smaller electrophoretic mobility in the ascending limb of the moving-boundary cell under conditions pertinent to Fig. 1(a), the actual velocity of ascending migration is almost certainly greater than that of descending migration because of the lower conductivity and hence greater applied potential gradient in that limb. However, there are myogen components that exhibit even larger disparities between velocities of migration in the two limbs by virtue of a greater ascending mobility superimposed on the greater velocity due to conductivity effects. Components that do not bind phosphate would be included in the latter category, as would solutes for which phosphate binding was essentially stoichiometric at phosphate concentrations greater than 0.01 M. As an example we may cite another major component of myogen, namely aldolase (Czok & Bücher, 1960), for which the association constant describing the weakest phosphate binding has been reported as 1050 M^{-1} (Ginsburg & Mehler, 1966), 300 M^{-1} (Crowder *et al.*, 1973a) and 400 M^{-1} (Crowder *et al.*, 1973b) under conditions similar to those used in the present investigation. A situation in which a mixture of solutes is subjected to electrophoresis under conditions where the mobility of some of the components is greater in the ascending limb (those for which there is either stoichiometric or no phosphate binding) and of others is smaller (those for which phosphate binding is dependent on phosphate concentration) can obviously lead to the type of non-enantiography observed in Fig. 1(a) for myogen dialysed against 0.0187M-phosphate, pH 7.7.

In summary, the present study of myogen confirms the earlier electrophoretic results of Amberson *et al.* (1964) but negates their interpretation of results in terms of macromolecular interactions between glyco-

lytic enzymes. Instead, the non-enantiography of electrophoretic patterns observed with myogen in phosphate buffer, pH 7.7, $I = 0.05$, is attributed to the existence of phosphate interactions, some stoichiometric and some in a state of equilibrium, with individual components of myogen, which should therefore be considered as a mixture of glycolytic enzymes.

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