ELECTROPHORETIC EXAMINATION OF NATIVE MYOSIN

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1. Introduction

Whereas it is firmly established that the light chains of myosins from different types of muscle within one species are different [1–3], the evidence regarding the heavy chains is more tenuous. Examination of published amino acid compositions shows that the variation between data for different myosins is of the same order as those for the same material investigated in different laboratories. The only strong evidence for differences appears to be that of Huszar and Elzinga [4], who have reported substitutions in the sequences of corresponding methylhistidine-containing peptides isolated from skeletal and cardiac muscle myosins. This approach is arduous and is of course limited to the detection of substitutions in small, highly localised parts of the chains, and gives little idea of the total extent of the overall differences. A related question is whether the myosin in a given muscle is homogeneous, and several, so far inconclusive, attempts have been made to determine whether heterogeneity exists, either between the two heavy chains of a single myosin molecule, or within the population of myosin molecules in one type of muscle (for discussion see Lowey [5]). The problem has also now acquired a particular interest in regard to the biosynthesis of muscle proteins in embryos [6], and to the reported change in the nature of the myosin, so far only in terms of the light chains, after cross-innervation of fast and slow twitch muscles [7].

The simplest approach to the examination of heterogeneity is in general zone electrophoresis. In the case of myosin the electrophoretic studies so far described have involved either myosin fragments, such as heavy meromyosin or subfragment-1 [5], or the denatured chains. Such experiments are not very informative in the present context, for proteolytically generated fragments are not expected to be homogeneous [5], and are apt to contain differently truncated chains, as well as internal breaks, and consequently additional α-amino and carboxyl groups. As to the denatured state, myosin is strongly aggregated in all denaturing media so far tried, with the possible exception of 11 M urea at high temperature [8], the use of which introduces physical and chemical problems of its own, and of sodium dodecyl sulphate, in the presence of which the electrophoretic mobility is insensitive to the charge on the protein, and leads to separation only on the basis of differences in molecular weight [9].

The difficulties in examining native myosin by gel electrophoresis are compounded of the high molecular weight and dissymmetry, which necessitate the use of very weak gels if there is to be appreciable migration, and of the high ionic strengths required to maintain solubility and prevent filament formation [10]. We describe here how these difficulties can be overcome, and show that sizeable electrophoretic differences exist between the native myosins of different types of muscles of the chicken, which appear to be interpretable only in terms of differences in the heavy chains.

2. Materials and methods

Myosin from chicken breast and heart muscle and from dissected posterior and anterior latissimus dorsi were prepared according to Lowey and Risby [2], except that the salt gradient in the column

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purification was replaced by stepwise buffers changes, from 0 to 0.1 to 1 M KCl, 0.02 M pyrophosphate being present throughout. The native myosin elutes at the second step, being completely adsorbed in the first. Rabbit skeletal muscle myosin was prepared by the method of Perry [11]. Samples in different states of purification [12] were generously provided by R.L. Starr and G.W. Offer.

For electrophoresis, the myosin samples were diluted to a concentration of 0.1 mg/ml with the running buffer, sucrose and a trace of bromophenol blue were added, and 5 μg were routinely applied to the gels, though for tests of concentration effects total quantities in the range 2–75 μg were run. The electrophoresis was usually performed in vertical glass tubes, 6 × 0.65 cm. The electrophoresis buffer contained 0.175 M potassium chloride, 0.0875 M tetrasodium pyrophosphate, 1 mM EDTA and 0.5% β-mercaptoethanol, pH 8.5. Other buffers were also successfully used, with more potassium chloride and less pyrophosphate, for example. Gels in the concentration range 2.2–3.2% could be used, 2.8% being convenient for reasonable migration and relative ease of handling. The gel was prepared in running buffer in the usual way [13]; a layer of surgical gauze was tied to the bottom ends of the tubes because the gels otherwise tended to slide out. Gels were prepared in the cold room at 4°, using 2.8% acrylamide, 0.1%, *N, N'*-methylene*bis*acrylamide (both from British Drug Houses), 0.5% tetramethylethylene diamine (TEMED), with 0.2% ammonium persulphate, added immediately before pouring. After polymerisation, the gels were subjected to a preelectrophoresis for about an hour to eliminate persulphate ions, and allow the mercaptoethanol to enter. The protein samples were layered onto the tops of the tubes, and the electrophoresis was allowed to proceed for 24–60 hr (most commonly about 48 hr) in the cold room, at a current of 8 mA/tube, corresponding to a potential drop across the tubes of only about 1.5 V/cm, in consequence of the high conductivity of the buffer. For added cooling, an electric fan was set up to circulate air past the tubes. To avoid contamination with electrode products, it was found desirable to use external mercury–calomel reversible electrodes, making contact with the buffer reservoirs by way of polyacrylamide–KCl bridges (5% acrylamide, 1 M KCl). These were reversed once during a typical run.

After electrophoresis the gels were carefully rimmed with a needle, and allowed to slide into tubes containing 0.25% Coomassie Brilliant Blue in methanol:acetic acid:water (4.5 : 1 : 4.5, by vol.), left overnight, and destained by washing with a mixture of the same solvents in the ratio 0.5 : 0.75 : 8.75, v/v/v. Migration distances of myosin could not be expressed in terms of the mobility of the tracker dye, which left the gels after some hours, but could conveniently be measured relative to glutamate dehydrogenase, which has a molecular weight of 330,000, and migrates much more rapidly than myosin. SDS-polyacrylamide gels were run following Weber and Osborne [14]. Zones of myosin (75 μg samples) were cut out with a razor blade from gels. The slices were soaked in 0.02 M sodium phosphate buffer, containing 0.1% SDS and 0.1% β-mercaptoethanol, immersed in boiling water for 5 min, and then applied to the tops of 10% SDS-acrylamide gels. Myosin preparations were screened for associated species by analytical ultracentrifugation in a Spinco model E instrument, using schlieren optics.

3. Results and discussion

The success of the electrophoresis of native myosin depends on the use of acrylamide gels of low concentration, otherwise the protein does not migrate any appreciable distance. The low diffusion coefficient is an advantage in this regard, and also in relation to the length of the runs. In our experience, the upper limit of acrylamide concentration at which useful results can be obtained is about 3%. Each myosin examined displays an essentially linear change of mobility with gel concentration up to this limit. The high ionic strengths which we have used are necessary to prevent aggregation. In the chloride–pyrophosphate medium there is not expected to be any appreciable dimerisation [10, 15]. The formation of dimers in rapid equilibrium with monomers [10] would not be apparent as a resolved electrophoretic zone, but would manifest itself only as a change in mobility with protein concentration between the limits corresponding to pure monomer and pure dimer [16]. In our conditions no such change of mobility with protein concentration is observed. The myosins are evidently stable in the gels in the cold, since there is no

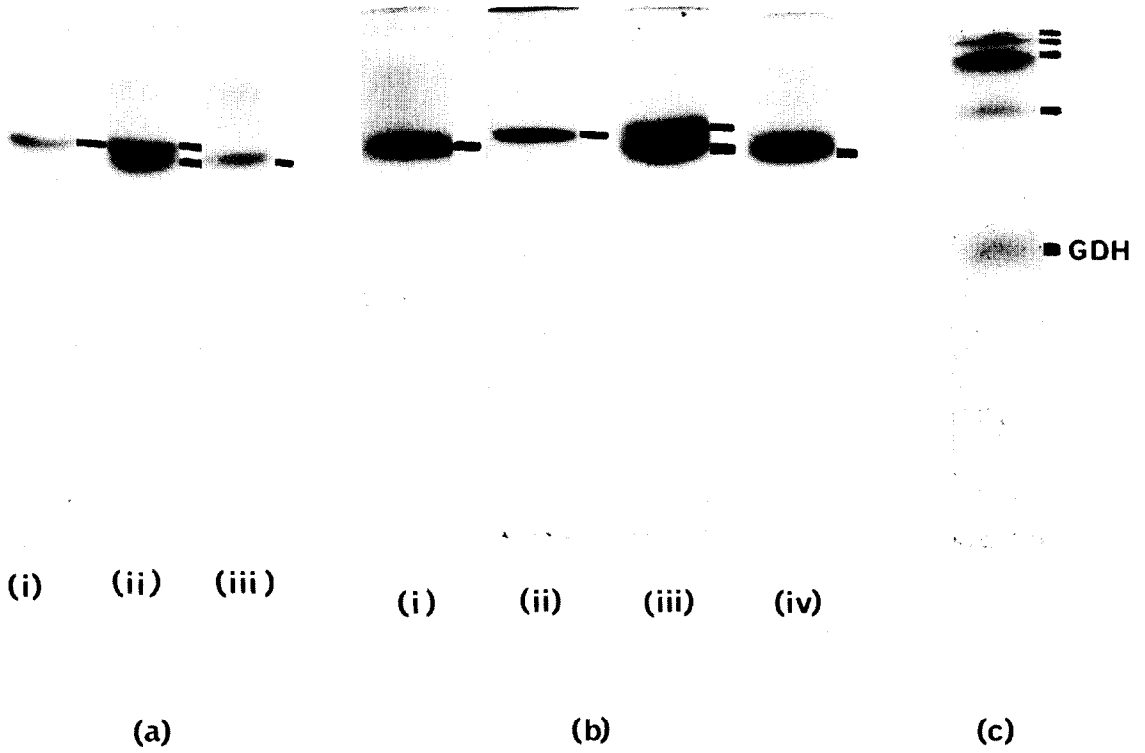

Fig. 1. Typical polyacrylamide gel electrophoresis experiments on myosins from different muscles. (a) Comparison of chicken anterior and posterior latissimus dorsi muscle myosins: (i) anterior, (ii) mixture, (iii) posterior. Conditions were: 2.8% acrylamide, 60 hr, protein loading 5 μg. (b) Comparison of myosins from fast, slow and cardiac muscles of the chicken: (i) breast, (ii) heart, (iii) mixture of anterior latissimus dorsi and breast, showing separation, (iv) mixture of posterior latissimus dorsi and breast, showing no separation. Conditions as in (a) except for the length of the run, which was 48 hr. (c) Appearance of additional components with aging: posterior latissimus dorsi myosin after one week at 4°. Glutamate dehydrogenase was included as an electrophoretic marker. Conditions as in (a), except for length of the run, which was 38 hr. All other conditions as given in text.

appreciable trailing or precipitation, and the migration distance is linear with time. In the cold room at 4°, the temperature measured inside the gel with a thermistor probe is about 10°, in our running conditions. Results for a set of myosins from different dissected muscles of the chicken are shown in fig. 1a and b. It can be seen in the first place that the preparations are of good purity, contaminating proteins appearing only as faint bands (though small molecules could well have migrated out of the gels). Similarly, in fresh preparations no aggregated material remains at the origin. Several of the chicken myosins that we have examined appear to be different from each other, and can be resolved in pairwise mixtures. Thus anterior latissimus dorsi, and heart muscle myosin are different from each other, and from posterior latissimus dorsi and breast muscle myosin, the latter two being indistinguishable from each other, and from rabbit skeletal myosin. On this basis it appears that fast twitch muscle myosins, of which the last three are examples, may well be distinguishable by the electrophoretic criterion from those of other muscle types. The technique then provides a simple means of distinguishing between such molecular species. The differences in the whole myosin thus accompany the differences that have been reported between the light chains [1–3]. We can exclude, however, the possibility that the latter are the cause of the different migration rates of the whole molecules. From an examination of amino acid compositions [5] one finds that about 3% of the net charge of the myosin molecule arises from the light chains, so that even gross differences in light chain compositions (which judging from the fragmentary data available [17, 18]

certainly do not exist) could affect the overall charge only by the order of 1%. Even such an overestimated difference would make no noticeable impression in terms of the relative mobility differences that we observe in the native intact molecules. Electrophoretic mobilities in polyacrylamide gels cannot of course be analytically related to those in free solution, or therefore to the net electrostatic charge of the molecule, but the simplest interpretation of electrophoretic mobilities different by as much as 10–15% is that they reflect differences in the balance of charged amino acids. There is at present no direct evidence of such large compositional differences as this conclusion would imply. An alternative explanation is that relatively small differences in composition, involving even conservative substitutions, could appreciably affect the rigidity and thus dissymmetry of the molecule. In principle, shape effects on electrophoretic mobility can be distinguished from pure charge effects by comparison of migration rates in polyacrylamide gels of different concentrations. We cannot at this stage rule out small differences in the mobility–gel concentration relations of the different myosins, but with such slow migration rates it is difficult to achieve the precision needed to allow a definite conclusion. Attempts are being made to compare sedimentation rates by differential procedures. At all events our results would seem to establish that compositional differences exist between the different myosins, but we are not yet able to determine whether they affect the mobility in consequence of changing the net charge, the shape, or a combination of both.

Since the myosins from several different sources are all distinctly different, and each shows only one zone, we conclude that, unless there are components in each of them, differing in charge from one another by much less than they differ from the myosin of any of the other muscles, each myosin is homogeneous. This does not of course exclude the possibility that each molecule is a heterodimer of heavy chains [5]. We may note that the zones in a mixture may be applied individually to SDS-acrylamide gels and give characteristic, identifiable, light chain patterns.

Myosin samples after storage in solution at 4° for some days showed on the gels the formation of dimers and higher oligomers (fig. 1c). A thiol reagent was present throughout, and these are not therefore disulphide dimers, neither are they the dimers known to exist in rapid equilibrium with monomer at lower salt concentrations [10, 15], for, as we have noted, in such a case a discrete new zone would not be formed. The nature of these species is at present unknown, though it is likely that to show such a large electrophoretic difference their dissymmetry must be larger than that of native myosin, and they therefore do not presumably have a simple parallel structure. They could have an overlapping structure of the type deduced by Harrington and Burke [19] for equilibrium dimers. In many preparations of rabbit skeletal muscle and chicken posterior latissimus dorsi muscle myosins we observed the presence of a second, slower zone, with a concentration comparable to that of the first, even in the freshest preparations. The electrophoresis pattern was never significantly different when rabbit muscle preparations purified by ion-exchange chromatography on DEAE-cellulose were used. Moreover the same samples, examined in the analytical ultracentrifuge using schlieren optics, showed only one boundary. It must of course be recognized that concentration conditions in the acrylamide gel are very different from those in the ultracentrifuge, and that very high concentrations probably occur at the gel–buffer interface at the beginning of an experiment. We are at present unable to state how, and under what conditions the second zone is generated. This question is now being investigated.

We anticipate that the electrophoretic technique may be useful to workers in a number of areas, particularly perhaps in regard to myosin biosynthesis in embryos or in cross-innervated tissue.

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