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Changes Associated with Glycolytic-Enzyme Binding in the Equatorial X-Ray-Diffraction Pattern of Glycerinated Rabbit Psoas Muscle

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The binding of fructose bisphosphate aldolase to the thin filaments of glycerinated rabbit psoas muscle produces a significant change in its low-angle X-ray-diffraction pattern. The intensity of the (11) reflection relative to that of the (10) reflection increases by $26 \pm 3\%$ (mean ± S.E.M.), which is consistent with the increase in the mass of the thin filaments produced by enzyme binding. A similar effect is found with a mixture of aldolase and glyceraldehyde 3-phosphate dehydrogenase. The significance of the change in intensity is considered with reference to the interpretation of the equatorial patterns obtained from muscles in different physiological states. The magnitude of the increase in the relative intensity of the (11) reflection is lower than that observed between relaxed and contracting muscle and does not bring into question the interpretation linking changes in these patterns to cross-bridge movement. However, the effect due to enzyme binding may be important when making detailed interpretations of these changes. It may also be related to an unusual pattern sometimes observed in cardiac muscle.

Vertebrate skeletal muscle is made up of interdigitating arrays of thick and thin filaments which slide past one another when the muscle contracts (Huxley & Hanson, 1954; Huxley & Niedergerke, 1954). The major constituent of the thick filaments is myosin, whereas the thin filaments contain actin and the regulatory proteins troponin and tropomyosin, with seven G-actin units being associated with one tropomyosin and one troponin molecule (Weber & Murray, 1973). The filament arrays are arranged on a hexagonal lattice, which gives rise to a characteristic series of equatorial reflections in X-ray-diffraction patterns of whole muscle (reviewed by Huxley, 1971). The first two orders of this lattice, namely the (10) and (11) reflections, have been studied extensively because their relative intensity changes with the state of the muscle: the intensity of the (10) reflection decreases relative to that of the (11) reflection as one progresses from relaxed to contracting to rigor muscle. This change in intensity has been interpreted as resulting from a transfer of mass from the vicinity of the thick filaments to that of the thin filaments [for a detailed discussion of this point, see Huxley (1971)]. This mass transfer has generally been associated with a movement of myosin heads from the vicinity of the thick filaments to form cross-bridges by attaching to the actin of the thin filaments, but some details of the motion are still uncertain (Haselgrove *et al.*, 1976).

Recent studies have indicated that myosin is not the only protein present in muscle which can bind to the proteins of the thin filaments. A number of glycolytic enzymes, including aldolase and glyceraldehyde 3-phosphate dehydrogenase, also bind, particularly to troponin but also to tropomyosin and actin (Clarke & Masters, 1976; Walsh *et al.*, 1979). Although the physiological significance of the association is unclear, the amount of enzyme bound has been shown to vary considerably and also to be significantly influenced by electrical stimulation and the concentration of Ca^{2+} (Starlinger, 1969; Walsh *et al.*, 1979; Clarke *et al.*, 1979). Because it would result in an increase in their mass relative to that of the thick filaments, it is possible that the binding of glycolytic enzymes to the thin filaments could influence the relative intensities of the (10) and (11) reflections. It is technically very difficult to investigate this possibility in contracting or relaxed muscle, but the task is much easier in muscle in which rigor has been induced by glycerination, because the disruption of membranes produced by this treatment allows the diffusion of enzymes in and out of the muscle to proceed readily. Accordingly, we have used this system to investigate the effect of glycolytic-enzyme binding on the equatorial X-ray-diffraction pattern and, in this paper, demonstrate a significant increase

Abbreviation used: SDS, sodium dodecyl sulphate.

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in the relative intensity of the (11) reflection of rigor muscle associated with aldolase binding.

Materials and Methods

Muscle specimens

Glycerinated rabbit psoas muscle was prepared as described by Lymn (1975*a*) and stored below −20°C in 50% glycerol/standard salt solution (0.1M-KCl, 2mM-$MgCl_2$, 6.7mM-potassium phosphate buffer, pH7.0). Before examination by X-ray diffraction, small specimens were washed in 30% glycerol/standard salt solution and then standard salt solution at 4°C for approx. 30min each. Residual enzymes were removed by soaking in an extraction buffer (0.1M-potassium phosphate/1mM-EDTA/1mM-dithiothreitol/0.1mM-fructose bisphosphate/0.1mM-ATP, pH7.0) for at least 2h at 4°C, after which the muscle was equilibrated in IKMDN buffer (10mM-imidazole/HCl, 20mM-KCl/1mM-$MgCl_2$/1mM-dithiothreitol/1mM-NADH, pH7.0) by soaking at 4°C for several hours. Aldolase was bound to the muscle by soaking in 1mg of enzyme/ml of IKMDN buffer at 4°C.

If required, muscles were fixed in 2% glutaraldehyde/5mM-potassium phosphate buffer, pH7.0 at 4°C, for at least 2h while being held under tension.

Sarcomere lengths were measured by optical diffraction as described by Haselgrove & Huxley (1973).

X-ray diffraction

Low-angle equatorial patterns were obtained by using copper K_{α_1} radiation from an Elliott GX-13 rotating anode generator and a camera of the mirror-monochrometer type described by Huxley & Brown (1967), with a specimen-to-film distance of approx. 80cm and an approx. 0.1mm × 15mm line focus. Patterns were usually recorded on Ilford Industrial G film, although occasional use was also made of a position-sensitive detector of the type described by Faruqi (1975).

To obtain estimates of the intensities of the (10) and (11) reflections, density traces of the films were detained on a Joyce–Loebl IIIc densitometer and analysed as described by Haselgrove & Huxley (1973).

The patterns are discussed in terms of the intensity of the (11) reflection relative to that of the (10) reflection. Because the aldolase treatment could not be satisfactorily performed in the X-ray cell, it was not possible to ensure that exactly the same area of the specimen was examined before and after binding. Consequently, the thickness of the specimen and amount of scattering material in the beam could not be considered constant (as it is, for example, when contracting and relaxed patterns from the same muscle are compared), and this, combined with possible fluctuations in X-ray output, the small magnitude of the changes observed and the lack of an internal standard, precluded the determination of absolute intensity changes.

Enzyme assays

After incubation as described above, muscle samples were homogenized for 30s in an Ultraturrax homogenizer. Then 1ml of the homogenate was immediately centrifuged at 32000*g* for 4min in an Eppendorf microcentrifuge and the supernatant removed and kept for assay. The pellet was twice extracted with 1vol. of stabilization buffer (0.1M-potassium phosphate, 1mM-EDTA, 1mM-dithiothreitol, 0.1mM-fructose bisphosphate and 0.1mM-ATP, pH7.5). The extracts obtained from the pellet were pooled and assayed as 'bound' enzyme, since this treatment is sufficient to ensure that all the enzyme activity was extracted from the pellet (Clarke *et al.*, 1979). Aldolase was assayed in a mixture containing 50mM-Tris/HCl, pH7.5, 1.6μg of α-glycerophosphate dehydrogenase/triose phosphate isomerase mixture/ml, 0.1mg of NADH/ml and 3.2mM-fructose bisphosphate. Glyceraldehyde 3-phosphate dehydrogenase was assayed by the back-reaction by the method of Bass *et al.* (1969). The assay system consisted of 50mM-Tris/HCl, pH7.5, 0.1mM-NADH, 3.3mM-Mg_2SO_4, 1.5mM-ATP, 7mM-3-phosphoglyceric acid, 5mM-EDTA and 0.6μg of 3-phosphoglycerate kinase/ml. The actin content of the pellet was determined by densitometry of SDS/polyacrylamide electrophoresis gels (Laemmli, 1970), with actin purified by the method of Spudich & Watt (1971) as standard.

Results

To assess the influence of aldolase binding on the equatorial X-ray-diffraction pattern of glycerinated rabbit psoas muscle, we measured the change in the relative intensities of the (10) and (11) reflections produced by soaking a sample in a 1mg/ml solution of the enzyme under conditions known to favour its binding to the thin filaments (Walsh *et al.*, 1979). Because the glycolytic enzyme content of glycerinated muscle is somewhat variable and depends to an extent on the history of the muscle before glycerination (Clarke *et al.*, 1979), any residual enzymes were first removed by soaking the samples in an extraction buffer. Assays for glycolytic enzyme activity (aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphofructokinase, lactate dehydrogenase) indicated that 0.10 ± 0.01 (mean ± S.E.M., $n = 11$) enzyme molecules remained per seven actin subunits after this treatment. The equatorial X-ray-

diffraction patterns of these specimens were recorded after equilibration in IKMDN buffer. The samples were then soaked in aldolase in IKMDN buffer, followed by washing with IKMDN buffer to remove any non-specifically bound enzyme. Assays indicated that this treatment resulted in the binding of 1.04 ± 0.18 ($n = 5$) aldolase molecules per seven actin molecules (that is to say, about 1 per tropomyosin–troponin complex). The X-ray-diffraction pattern was again recorded and compared with that obtained in the absence of enzyme, to detect any change in the equatorial intensities. Because the extent of filament overlap influences the relative intensities of the equatorial reflections (Elliott *et al.*, 1963; Haselgrove & Huxley, 1973), the sarcomere length of the specimens was measured at each stage. Although there was some variation between specimens, presumed to be mainly due to some samples not being held exactly at rest length during the glycerination procedure, the sarcomere length of individual specimens did not appear to change greatly and, when the second pattern was recorded, was always either the same or slightly longer (generally by less than 0.1 μm) than the initial value.

As shown in Table 1, in all the samples measured there was a significant increase in the intensity of the (11) reflection relative to that of the (10) reflection when aldolase was bound to the thin filaments. The mean increase observed in the relative (11) reflection intensity was 25.9%, with a standard error of 2.65% ($n = 6$). This change is significant at the 0.1% level (t test) and cannot be attributed to the slight increase in sarcomere length sometimes observed after enzyme treatment, because the relative intensity of the (11) reflection is known to decrease with increased sarcomere length (Elliott *et al.*, 1963; Haselgrove & Huxley, 1973). Over the range measured, we did not observe a marked influence of sarcomere length on the magnitude of the increase in relative (11) reflection intensity (correlation coefficient 0.43). However, it would be necessary to measure many more samples before a definitive answer could be given to this question.

Table 1. *Effect of aldolase binding on the intensity of the* (10) *reflection relative to that of the* (11) *reflection in glycerinated rabbit psoas muscle*

The average increase was 25.9% with a standard error of 2.65% and was relatively insensitive to sarcomere length. See the text for experimental details. Results are means ± S.E.M. for four to six determinations.

Sarcomere length (μm)	Intensity of (11) reflection relative to (10)		Increase (%)
	Without aldolase	With aldolase	
2.3	3.64 ± 0.07	4.60 ± 0.41	26.4
2.3	4.33 ± 0.16	5.03 ± 0.35	16.2
2.5	3.55 ± 0.18	4.49 ± 0.23	26.5
2.5	3.00 ± 0.13	3.84 ± 0.34	28.0
2.75	1.95 ± 0.17	2.65 ± 0.09	35.9
2.75	1.91 ± 0.20	2.34 ± 0.22	22.5

Although this experimental procedure had the marked advantage of measuring the same sample before and after enzyme binding, and thereby greatly reducing the spread of results due to variations between specimens, it was open to the possible objection that the muscle could have deteriorated during the sequence of treatments. Consequently, it could be argued that the changes that we observed simply reflected the alteration of the structure of the muscle with time. We thought this unlikely, because the change most usually observed with time is a decrease in the crystalline order of the lattice. This would be expected to broaden the reflections and also to attenuate the higher orders, which would be expected to reduce the relative intensity of the (11) reflection. Because the reflections did not appear broader after the second exposure and also because we observed an increase in the relative intensity of the (11) reflection rather than a decrease, we considered it unlikely that the change observed could be ascribed to such an effect. However, we also investigated this point experimentally by taking a number of samples of glycerinated rabbit psoas muscle and, after any residual enzyme had been removed by extraction as before, they were divided randomly into two groups. Samples from one group were soaked in IKMDN buffer containing 1 mg of aldolase/ml, sometimes with the addition of 1 mg of glyceraldehyde 3-phosphate dehydrogenase/ml, and samples from the other group were soaked, under identical conditions, in IKMDN buffer alone. After approx. 2 h, both groups of specimens were washed in IKMDN buffer and then fixed in an identical manner with glutaraldehyde, before the recording of their equatorial X-ray-diffraction patterns. Fig. 1 shows the intensity ratios observed from these specimens and, although there is more scatter in the results compared with those obtained previously, there is clearly an increased (11) reflection intensity associated with enzyme binding. All of the values for the relative intensity of the (11) reflection obtained when enzyme was bound are higher than those obtained in their absence, with the average relative (11) reflection intensity being 2.66 (standard error = 0.16, $n = 6$) in the former case and 1.58 (standard error = 0.13, $n = 5$) in the latter. This corresponds to a 63% increase in relative (11) reflection intensity and is significant at the 0.1% level (t test).

These results would appear to indicate a greater increase in the relative (11) reflection intensity associated with enzyme binding than was seen in the first series of experiments. However, the higher scatter

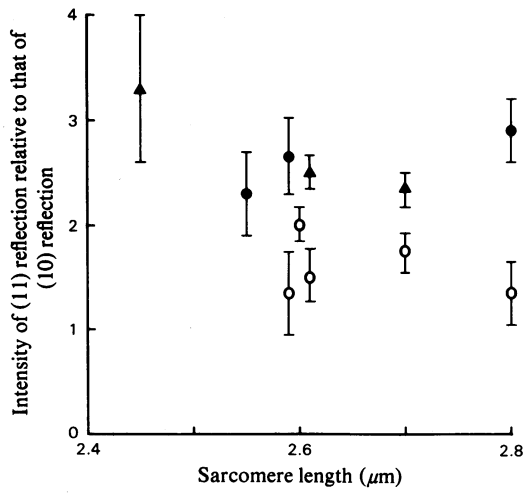

Fig. 1. *Intensity of the* (11) *reflection relative to that of the* (10) *reflection in samples fixed in glutaraldehyde under tension*

The sarcomere lengths of these specimens are slightly longer than rest length, presumably as a result of stretching during fixation. The relative intensity of the (11) reflection is clearly increased in those specimens soaked in either aldolase alone (▲) or a mixture of aldolase and glyceraldehyde 3-phosphate dehydrogenase (●), compared with the control (○). Results are means ± S.E.M. for between four and six densitometer traces of different areas of the pattern.

observed in the second series and the possibility of systematic errors associated with the lack of pairing of treated and untreated specimens, would incline us to place greater reliance on the earlier value of about 25%. Be that as it may, the results of the second experiment certainly confirm the trend observed in the first series and clearly associate an increased relative (11) reflection intensity with enzyme binding to the thin filaments. Essentially the same result was obtained when glyceraldehyde 3-phosphate dehydrogenase was also present. Since this enzyme is known also to bind to thin filaments to a similar extent to aldolase (Clarke & Masters, 1976) and enzyme assays indicated that both enzymes bound under the conditions employed (a total of 1.01 mol of enzyme per 7 mol of actin, being made up of 0.51 mol of aldolase and 0.5 mol of glyceraldehyde 3-phosphate dehydrogenase), it would appear that its influence is similar to that of aldolase.

Discussion

These results establish clearly that the binding of aldolase to the thin filaments of glycerinated rabbit skeletal muscle produces a significant change in the relative intensities of the (10) and (11) equatorial X-ray reflections. The exact magnitude of the change is a little uncertain, but it would certainly appear to be of the order of a 30% increase in the intensity of the (11) reflection relative to that of the (10) reflection. Simple computer modelling experiments, in which the thick and thin filaments were approximated by cylinders of the appropriate dimensions and density, indicated a change of similar magnitude when the density of the thin filaments was increased by an amount corresponding to 1 aldolase molecule binding per tropomyosin–troponin complex (that is to say, per 7 G-actin subunits). As a number of glycolytic enzymes bind to thin filaments in a manner analogous to that of aldolase (Clarke & Masters, 1976), it would appear reasonable to conclude that this increase in relative (11) reflection intensity is a quite general effect and that the binding of any of these enzymes would produce a similar change in the equatorial X-ray-diffraction pattern. Certainly the change produced by a mixture of aldolase and glyceraldehyde 3-phosphate dehydrogenase [both of which are known to bind to thin filaments to a similar extent (see Clarke & Masters, 1976)] appeared to be the same as that observed with aldolase alone.

Because our results demonstrate that glycolytic-enzyme binding influences the intensity distribution in muscle equatorial diffraction patterns, it is pertinent to consider what implications this could have for the interpretation of the changes observed between contracting and relaxed muscle. When compared with relaxed muscle, the relative intensity of the (11) reflection in contracting muscle is higher, as a consequence of both a decrease in the (10) reflection intensity and an increase in the (11) reflection intensity (Haselgrove *et al.*, 1976; Podolski *et al.*, 1976). This change has been interpreted as being associated with the movement of cross-bridges to the vicinity of the thin filament in contracting muscle and so increasing their mass relative to the thick filaments (Huxley, 1969, 1971). Glycolytic-enzyme binding could also produce an increase in thin-filament mass, but a number of considerations indicate that alone this binding would be unlikely to account for the changes observed. For glycolytic-enzyme binding to influence the intensity change between contracting and relaxed muscle, it would be necessary for the extent of binding to vary according to the state of the muscle. There is, unfortunately, no direct evidence available on this point and one can imagine formidable technical difficulties associated with its acquisition. However, the binding of aldolase to thin filaments has been studied *in vitro* and been shown to be sensitive to physiological concentrations of Ca^{2+} (Walsh *et al.*, 1979). Interestingly, the amount of enzyme bound to thin filaments *in vitro* is less in 0.1 mM-Ca^{2+} (corresponding to contracting muscle) than in the absence of this ion (corresponding to relaxed muscle). One would therefore expect that any

change in enzyme binding associated with contraction would serve to reduce the relative intensity of the (11) reflection. In other words, if such an effect were to be present *in vivo*, it would produce a change opposite to that observed, and so cause the difference between relaxed and contracting muscles to be underestimated. Of course, in a living muscle a large number of factors, such as steric effects due to myosin heads and the actual concentration of the enzymes in the sarcoplasm, could complicate the situation. Also, the data obtained *in vitro* only refer to equilibrium binding and it is possible that the rate of aldolase binding is too slow to influence the X-ray-diffraction pattern significantly. Furthermore, aldolase binding would only be expected to increase the thin-filament mass by about 35% and to cause no decrease to thick-filament mass. This binding could not therefore account for the large transfer of mass found to accompany contraction (Haselgrove & Huxley, 1973). Consequently, our results should not be taken to refute in any way the hypothesis linking changes in the equatorial diffraction pattern to cross-bridge movement during contraction (Huxley, 1969, 1971). However, our results do suggest that one should consider the possibility that these changes may not be due solely to cross-bridge movement. Such a consideration would be particularly relevant when undertaking detailed modelling studies which relied heavily on the values taken for the equatorial intensities (see, for example, Lymn, 1975*b*; Haselgrove *et al.*, 1976).

The observation that the degree of enzyme binding is related to the history of the muscle may also be relevant, particularly when one considers that the usual procedure for recording X-ray-diffraction patterns from living muscles is to subject them to a series of electrical stimulations and to record the 'contracting' pattern during the tetanus so produced and the 'relaxed' pattern between the tetani. Similar electrical-stimulation treatments have been associated with a general increase in the binding of glycolytic enzymes to thin filaments (Starlinger, 1969; Clarke *et al.*, 1979), presumed to be related to the altered metabolic state of the tissue. If such an effect were to occur in the X-ray-diffraction experiments, then the (11) reflection intensity would be overestimated in both the contracting and relaxed state. The differences between the two states, however, would be much less sensitive to these changes. In this context, it is interesting to consider an observation of Matsubara *et al.* (1977) with respect to the low-angle X-ray-diffraction pattern of cardiac muscle. In addition to the patterns due to contracting and relaxed muscle, these workers observed a third type of pattern which was often found when, after a series of contraction/relaxation cycles, the muscle was allowed to relax for a period. This third or 'quiescent' state was characterized by a decrease in the relative intensity of the (11) reflection compared with the 'relaxed' pattern. This observation can be easily reconciled with our results if one were to propose that, during the electrically induced cycles of contraction and relaxation, the binding of glycolytic enzymes to the thin filaments increased. This would elevate the relative (11) reflection intensity in both contracting and relaxed patterns, but would otherwise not be expected to greatly alter the differences between them. However, as the muscle was allowed to relax for a considerable period without further electrical stimulation, the binding of enzymes to the thin filaments would decline, which would result in a decrease in the (11) reflection intensity and so produce the quiescent pattern.

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