Effect of Electrical Stimulation *post mortem* of Bovine Muscle on the Binding of Glycolytic Enzymes

FUNCTIONAL AND STRUCTURAL IMPLICATIONS

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(Received 12 July 1979)

The extent of binding of glycolytic enzymes to the particulate fraction of homogenates was measured in bovine psoas muscle before and after electrical stimulation. In association with an accelerated glycolytic rate on stimulation, there was a significant increase in the binding of certain glycolytic enzymes, the most notable of which were phosphofructokinase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase. From the known association of glycolytic enzymes with the I-band of muscle it is proposed that electrical stimulation of anaerobic muscle increases enzyme binding to actin filaments. Calculations of the extent of enzyme binding suggest that significant amounts of enzyme protein, particularly aldolase and glyceraldehyde 3-phosphate dehydrogenase, are associated with the actin filaments. The results also imply that kinetic parameters derived from considerations of the enzyme activity in the soluble state may not have direct application to the situation in the muscle fibre, particularly during accelerated glycolysis.

The effect of electrical stimulation of carcasses is to increase the rate of glycolysis *post mortem* (Hallund & Bendall, 1965; Forrest *et al.*, 1966; Chrystall & Hagyard, 1976; Bendall *et al.*, 1976; Davey *et al.*, 1976). Rabbit, sheep, ox and pig muscle all respond to electrical stimulation with increased glycolysis and an accelerated onset of rigor. The behaviour of the anaerobic glycolytic system in response to simulated tetanic contraction has been studied by Scopes (1974) in a system reconstituted from purified soluble enzymes and a highly active ATPase. Scopes (1974) suggested that the metabolic events observed in the soluble simulated system are basically the same as occur in muscle during heavy work. On the other hand, there have been numerous reports of the binding of glycolytic enzymes to the particulate fraction of muscle (Starlinger, 1967; Arnold & Pette, 1968, 1970; Amberson & Bauer, 1971; Arnold *et al.*, 1971; Melnick & Hultin, 1973; Ratner *et al.*, 1974; Dagher & Hultin, 1975; Clarke & Masters, 1976). Generally, the adsorption of the glycolytic enzymes has been found to be reversible and dependent on pH, ionic strength and specific metabolites. Arnold & Pette (1968) examined the binding of purified muscle fructose biphosphate aldolase (EC 4.1.2.13) to the structural proteins F-actin, myosin, actomyosin

and stroma protein isolated from rabbit skeletal muscle. F-actin has the greatest affinity and binding capacity for aldolase, and this is consistent with the histochemical demonstration that a range of glycolytic enzymes, including aldolase, are localized in the I-band of muscle (Sigel & Pette, 1969; Arnold *et al.*, 1969; Dolken *et al.*, 1975). More recent studies (Clarke & Masters, 1975) have demonstrated that adsorption of glycolytic enzymes to actin-containing filaments is possible under conditions approximating to those found within the muscle fibre and that the regulatory proteins of the I-band filaments, tropomyosin and troponin, markedly influence the adsorption of enzymes to these filaments (Clarke & Morton, 1976; Morton *et al.*, 1977), with significant consequences for the kinetic properties of the enzymes (Walsh *et al.*, 1977).

There is evidence from the work of Starlinger (1967) that electrical stimulation of rat muscle in the live anaesthetized animal leads to an increased binding of aldolase to the particulate fraction of the muscle homogenate. This enhanced binding decreased rapidly after electrical stimulation ceased. The purpose of the present experiments was to establish whether, under the conditions of accelerated glycolysis in electrical stimulation *post mortem*, there are changes in the binding of the glycolytic enzymes which may influence glycolytic activity.

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Materials and Methods

Animals and stimulation techniques

Six Hereford bullocks, 2–3 years old, of carcass weight 230–250 kg were used. The animals were stunned, bled, dressed and split into sides. Stimulation was applied to six sides; the remaining six sides served as controls. One side from each of four animals was stimulated while the other served as a control; for one animal neither side was stimulated, and for the remaining animal both sides were stimulated. Stimulation commenced immediately after the dressed carcasses were split into sides (30–40 min after stunning). Two multi-point electrodes were used. One was inserted at the distal end of the junction of the biceps femoris muscle with the semitendinosus muscle, and the other was inserted into the brachio-cephalicus muscle. The side was suspended by the Achilles tendon during stimulation. A pulse generator supplying 40 pulses/s (pulse width 2 ms) direct current was used. The voltage was increased in a series of steps as follows: 0–10 s, 10 V; 10–30 s, 50 V; 30–60 s, 75 V; 60–90 s, 110 V.

Treatment of samples

Immediately after stimulation, 6 g samples of the psoas muscle were homogenized with a Buchler homogenizer for 30 s in 3 vol. of ice-cold 0.25 M-sucrose which contained 0.001 M-dithiothreitol. Then 1 ml of the homogenate was immediately centrifuged at 32000 g for 5 min in an Eppendorf 3200 micro-centrifuge. The resulting supernatant was removed and diluted with 4 vol. of a stabilization buffer, which contained 0.1 M-potassium phosphate, 0.001 M-EDTA, 0.002 M-dithiothreitol, 0.1 M-fructose 1,6-bisphosphate and 0.1 M-ATP, pH 7.5. Soluble enzyme activity was then measured. Dilution in the stabilization buffer was necessary to preserve labile enzyme activities such as phosphofructokinase. The pellet from the centrifugation was extracted with 2 × 1 ml of stabilization buffer, and the resulting extracts were pooled. Bound enzyme activity was then measured. Preliminary experiments established that this procedure was sufficient for the extraction of all the bound enzyme activity from the pellet.

Immediately after homogenization a second 1 ml sample of the homogenate was diluted with 4 ml of stabilization buffer and total enzyme activity in the homogenate was measured. This served as a control to check the recovery of activity in the soluble and pellet fractions.

Enzyme assays

Glucose 6-phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase and lactate dehydrogenase were assayed by procedures based on those of Wu &

Racker (1958). Hexokinase was assayed as described by Joshi & Jagannathan (1966), and glyceraldehyde 3-phosphate dehydrogenase was assayed as described by Bass *et al.* (1969). The enzyme rates were followed at 340 nm or 240 nm where appropriate in a Cary 14 spectrophotometer at 30°C, with all fractions being assayed in triplicate. The enzyme activity recovered in the supernatant plus pellet ranged from 90 to 105% of that measured in the control homogenates.

Lactate and glycogen determinations

Samples of control and stimulated psoas muscles were rapidly frozen by immersion in liquid nitrogen immediately after stimulation. Extracts of the frozen tissues were made by powdering them under liquid nitrogen and extraction with ice-cold 5% (w/v) HClO₄ (10 ml/g). After centrifugation (10000 g, 10 min) the extracts were neutralized with KOH and the KClO₄ precipitate was removed by a further centrifugation. Residual glycogen was released from the residue by digestion with 30% (w/v) KOH in a boiling-water bath for 1 h. Glycogen was precipitated with ethanol, redissolved, and combined with the neutralized HClO₄ extract. The glycogen content of these extracts was measured by the method of Krisman (1962) and lactate was determined by the method described by Gutmann & Wahlefeld (1974).

Results and Discussion

Electrical stimulation resulted in a significant decrease ($P < 0.005$ by Student's *t*-test) of tissue glycogen from 26.5 ± 1.4 mg/g in control muscle to 21.9 ± 2.5 mg/g in stimulated muscle. Concomitant with this decrease in glycogen content there was a significant increase ($P < 0.005$) in tissue lactate from 24.8 ± 3.6 μ mol/g in control muscle to 41.9 ± 5.1 μ mol/g in stimulated muscle, these values being means $\pm$ s.d. of four determinations. These results show that stimulation resulted in a significant depletion of glycogen stores, which was accompanied by a marked elevation of the muscle lactate content, indicative of accelerated glycolysis during stimulation. During the 90 s stimulation period the average rate of lactate production was 11 μ mol/min per g, compared with 0.1 μ mol/min per g in psoas muscle not stimulated *post mortem*.

The data in Fig. 1 demonstrate that concurrent with this increased glycolytic rate during stimulation there was a significant increase in the binding of phosphofructokinase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase, and to a lesser extent glucose 6-phosphate isomerase, phosphoglycerate kinase and lactate dehydrogenase. The other enzymes of the pathway showed no significant change in their binding on stimulation of the muscle. In general, those enzymes that showed the largest increase in binding were also those that were

Fig. 1. Binding of glycolytic enzymes in control and stimulated muscles

Values represent means $\pm$ s.d. from four determinations in the control (□) and from six determinations in the stimulated (■) muscles. * $P < 0.005$ by Student's t test. Abbreviations: HK, hexokinase; GPI, glucose 6-phosphate isomerase; PFK, phosphofructokinase; ALD, aldolase; TPI, triose phosphate isomerase; GPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENOL, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase.

bound most extensively in the unstimulated control muscle.

It is doubtful whether the values in the muscle not stimulated *post mortem* reflect the true extent of enzyme binding in the unstimulated resting animal, because the act of removal and other attendant procedures may cause an elevation of the resting values. The principal concern of the present paper, however, is not the absolute values of enzyme binding, but the demonstration of a significant and reproducible elevation of the adsorption of individual enzymes in association with an increased glycolytic rate as the result of electrical stimulation. This is consistent with the previous demonstration by Starlinger (1967) of increased binding of aldolase on stimulation of rat muscle. In the experiments of Starlinger (1967), live anaesthetized animals were stimulated, and under these circumstances the increased binding of aldolase was shown to be reversible. If the muscle was allowed to recover *in situ* after stimulation, the aldolase binding returned to resting values within 10 min. However, no similar recovery was observed in muscle stimulated *post mortem*, as the enzyme binding up to

1 h after stimulation was only slightly lower than that measured immediately after stimulation.

The direct cause of increased enzyme binding in stimulated muscle is not yet known. During electrical stimulation of muscle *post mortem*, lactate accumulates, with a consequent lowering of intracellular pH. The average pH difference between the control and stimulated muscles immediately after stimulation was 0.2 pH unit (from approx. pH 6.5 to 6.3), which reflects the different lactate contents, 24 and 41 $\mu\text{mol/g}$ respectively. In studies on the adsorption of glycolytic enzymes to F-actin, Arnold *et al.* (1971) demonstrated a pH-dependency of adsorption, but only lactate dehydrogenase had a marked dependence in the pH range below 7.0. Although extrapolation to the intracellular situation is difficult, it appears doubtful if the decline in pH could completely account for the increased binding of glycolytic enzymes in stimulated muscle. A contributory role for pH in association with more specific influences such as variations in metabolite concentrations is possible, particularly in view of the well-established specific influence of glycolytic metabolites on the

Table 1. *Amounts of bound enzymes in control and stimulated muscle*
 Values in the last two columns are calculated from the data in Fig. 1.

Enzyme	Mol.wt.*	Amount (mg/g of muscle)†	Total (nmol/g of muscle)	Amount bound (nmol/g)	
				Control	Stimulated
Glucose 6-phosphate isomerase	134000	0.8	6.0	1.0	1.4
Phosphofructokinase	360000	0.35	0.97	0.38	0.66
Aldolase	160000	6.5	40.6	19.2	28.6
Triose phosphate isomerase	53000	2.0	46.5	6.2	7.1
Glyceraldehyde 3-phosphate dehydrogenase	144000	11.8	81.5	23.5	33.0
Phosphoglycerate kinase	56000	1.4	41.1	7.6	9.8
Phosphoglycerate mutase	66000	0.8	13.3	1.7	2.7
Enolase	82000	2.4	29.2	5.1	5.3
Pyruvate kinase	240000	3.2	13.3	2.2	3.7
Lactate dehydrogenase	140000	3.2	22.8	3.6	4.8

* Data compiled by Darnall & Klotz (1975).

† Data of Scopes (1973).

adsorption of individual glycolytic enzymes (Arnold & Pette, 1970; Knull *et al.*, 1973). The ability of muscle in the live animal to recover to normal extents of enzyme binding some minutes after very extensive stimulation (Starlinger, 1967), whereas no similar recovery occurs in muscle stimulated *post mortem*, suggests, at least tentatively, that oxidation or removal of some accumulated intermediate(s) controlling binding normally takes place. Similarly, the involvement of other mechanisms such as reversible phosphorylation-dephosphorylation of enzyme or adsorbent cannot be ruled out, and further studies are required to test these possibilities.

Although these experiments do not establish whether the increased enzyme binding is a cause or an effect of the increased glycolytic rate, it is clear that enzyme binding could exert important influences on the function of the pathway, particularly when it is recalled that the kinetic properties of several key glycolytic enzymes, such as aldolase (Walsh *et al.*, 1977), glyceraldehyde 3-phosphate dehydrogenase (Dagher & Hultin, 1975), lactate dehydrogenase (Ehmann & Hultin, 1973) and phosphofructokinase (Karadsheh & Uyeda, 1977), are now known to be significantly modified on binding to particulate material. In addition, studies in model systems have established that organized, particulate pathways have quite different properties from their counterparts in free solution. Sreer *et al.* (1973), for example, synthesized a particle that contained three enzymes catalysing sequential reactions, and demonstrated the complex to be more catalytically efficient than the free enzymes because the intermediates are held at higher concentrations within the microenvironment of the particle than they would be in free solution. In studies of glycolysis in isolated stimulated frog sartorius muscle, Helmreich & Cori (1965)

concluded that the glycolytic system operated as a well-integrated unit over a wide range of flux rates, and they invoked a specific micro-localization and organization of the system to account for their results. The role of compartmentation and the microenvironment in the control of glycolysis was extensively reviewed by Ottaway & Mowbray (1977), and the present results suggest that these factors should in future be given serious consideration when the operation of the glycolytic system in stimulated muscle is analysed.

In view of the fact that glycolytic enzymes make up approx. 20% of the muscle proteins (Czok & Bucher, 1960), it is important to consider not only the functional implications but also the structural implications of enzyme binding. In Table 1 the amount of each enzyme bound in control muscle and muscle stimulated *post mortem* has been calculated in molar quantities from a knowledge of the amount of each enzyme present per g of muscle (Scopes, 1973) and the binding data presented in Fig. 1. Although these values should be considered only as approximations, they do illustrate a number of significant features. Firstly, the actual quantity of enzyme bound in terms of actual protein varies markedly between the individual enzymes. For example, although there is a large difference in the phosphofructokinase activities bound in the control and stimulated muscles, a situation which no doubt has important functional implications in view of the prime regulatory role commonly attributed to this enzyme, there are actually only very small amounts of this protein bound in either case. At the other extreme there are particularly large amounts of the proteins aldolase and glyceraldehyde 3-phosphate dehydrogenase bound, to the extent that two-thirds of all bound glycolytic protein in the control muscle is made up

by these two enzymes alone. Moreover, on stimulation they account for greater than 70% of the increased protein binding. Secondly, in both control and stimulated muscle the molar ratio of bound aldolase to bound glyceraldehyde 3-phosphate dehydrogenase is very close to unity, i.e. they are bound in approximately equimolar quantities. These calculations also raise considerations of the amount of adsorbent available to accommodate the proposed amounts of bound enzymes, bearing in mind that all evidence (see the introduction) indicates that it is the actin-containing filaments of the I-band that are responsible for enzyme adsorption. Taking a median value of 20mg/g for the actin content of skeletal muscle (Murphy *et al.*, 1974) and a molecular weight of 42000 for G-actin, there would be approx. 470nmol of actin monomers/g of muscle. As there are approx. 30nmol each of aldolase and glyceraldehyde 3-phosphate dehydrogenase bound per g in the stimulated muscle (Table 1), then the molar ratio of each of these bound enzymes to actin would be about 1:15, which means that there would be one of each of these enzymes per turn of the actin helix, assuming the enzymes are equally distributed throughout the I-band. Thus there would seem to be ample adsorbent to accommodate the binding of enzymes. Clearly, future studies must seek the reasons why there are such large amounts of these two enzymes bound to the I-band filaments with such an interesting relationship to the underlying structure of the I-band filaments themselves.

We thank Ms. S. Tucek for skilled assistance. This work was supported in part by the Australian Meat Research Committee.

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