Biochimica et Biophysica Acta, 977 (1989) 87–90
Elsevier

BBABIO 43065

The effect of fatigue on the binding of glycolytic enzymes in the isolated gastrocnemius of *Rana pipiens*

Helga Guderley, Christine Jean and Mario Blouin

Département de biologie, Université Laval, Québec (Canada)

(Received 2 May 1989)

Key words: Enzyme binding; Muscle fatigue; Phosphofructokinase; Glycolysis; (Gastrocnemius muscle); (*R. pipiens*)

Fatigue of isolated gastrocnemius muscles from *R. pipiens* leads to a marked increase in the proportion of phosphofructokinase bound to the particulate fraction and a decrease in the binding of lactate dehydrogenase, pyruvate kinase, creatine phosphokinase and glyceraldehyde-3-phosphate dehydrogenase. Only the proportion of aldolase bound to the particulate fraction was unaffected by fatigue. This pattern was unchanged when fatigued muscles were extracted at pH 6.5 rather than 7.5. Thus, muscle fatigue leads to opposite changes in the binding of the glycolytic enzymes.

Introduction

Immunofluorescent techniques indicate that many of the cytosolic forms of the enzymes of energy metabolism are associated with the I band in relaxed striated muscle [1]. Furthermore, in vitro studies demonstrate that glycolytic enzymes can reversibly bind to structural proteins from muscle, heart and other tissues [2–11]. In vivo studies with trout muscle indicate that glycolytic enzyme binding generally increases with duration of exercise, although phosphofructokinase is the major enzyme which consistently changes its binding according to the energy demands of muscle [12,13]. The correlation of increased enzyme binding with increased glycolytic activity has led to the suggestion that changes in enzyme localization are important in the control of glycolysis [7,8]. Glycolysis would be activated not only through covalent modification of enzymes and changes in the levels of regulatory metabolites [14,15], but also by increased association of the enzymes with structural proteins. Such increased binding could facilitate glycolytic flux by channeling metabolites between consecutive enzymes or by activating individual enzymes through conformational changes during binding [10].

Mammalian skeletal muscles generally contain a mixture of fast glycolytic, fast oxidative and glycolytic, fast oxidative, and slow oxidative fibers which differ in their reliance on anaerobic glycolysis [16,17]. For the last three fiber types, aerobic metabolism is considered to be the major source of ATP for muscle contraction. While in vitro stimulation of ischemic mammalian muscle should lead to lactate accumulation in all fibers, only fast glycolytic fibers are designed for reliance on anaerobic glycolysis. Frog skeletal muscle contains large fast glycolytic fibers, small oxidative fast fibers and a low proportion of slow tonic fibers [18,19]. Interspecific differences in aerobic capacities of amphibian muscles are not accompanied by shifts in fiber type proportions, but seem due to changes in the metabolic properties of the fibers themselves [19]. The inter-fiber differences in the metabolic capacities of amphibian fibers differ from those generally found for mammalian fibers [16,17]; small oxidative phasic fibers have the highest glycolytic and oxidative capacities, large phasic fibers are intermediate and tonic fibers have the lowest glycolytic and aerobic capacities [20]. This suggests that frogs prefer to use the same fibers whether performing aerobic or anaerobic exercise and that these fibers are designed for reliance on anaerobic glycolysis.

Forced exercise leads the leopard frog, *Rana pipiens*, to fatigue rapidly and to accumulate high levels of lactate in the muscles, blood and whole animal [21]. Most of the energy used to support contraction in the isolated perfused gastrocnemius from *Xenopus laevis* comes from anaerobic metabolism [22]. As the gastrocnemius of *X. laevis* shows less glycogen depletion with fatigue than that from *R. pipiens* [21], the gastrocnemius of *R. pipiens* must rely even more upon anaerobic glycolysis than that of *X. laevis*. Given this marked dependence on glycolysis to sustain contractile

Abbreviation: PFK, phosphofructokinase (EC 2.7.1.11).

Correspondence: H. Guderley, Département de biologie, Université Laval, Québec, P.Q., Canada, G1K 7P4.

activity and the low proportion of tonic fibers in this muscle [18,19], the gastrocnemius of *R. pipiens* should be an ideal tissue with which to attain reasonably uniform metabolic responses during stimulation of the isolated muscle. Numerous stimulation protocols can activate glycolysis, in particular, fatiguing isolated muscles will stimulate extensive use of anaerobic glycolysis throughout the muscle. While fish white muscle shares many of the metabolic attributes of frog skeletal muscle, isolated fish muscles are considerably more refractory to experimental study. Thus, we examined the effect of fatigue on the intracellular localization of phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase and creatine phosphokinase in isolated gastrocnemius from *R. pipiens*.

Methods

In vitro fatigue of isolated gastrocnemius

Adult *R. pipiens* were pithed and weighed and their gastrocnemius muscles were isolated. Each muscle was suspended from a clamp and attached to a transducer for isotonic contractions (Harvard Apparatus Model 357). One of the muscles was stimulated at 15 V at a frequency of 15 Hz until no more contractions could be recorded (Harvard Apparatus Stimulator Model 340; Physiograph Model 600-850), while the other muscle was left unstimulated. Both muscles were periodically rinsed with Frog Ringer's solution. The stimulation times necessary for fatigue generally ranged from 7 min to 9 min, with one extreme value of 13 min 12 s.

Separation of soluble and bound enzymes

After fatigue of the experimental muscle, the muscles were weighed and the 'soluble' enzymes were separated from those which are associated with the particulate fraction by extracting with an approximately isosmotic, low-ionic-strength buffer (0.25 M sucrose/1 mM dithioerythritol (pH 7.5)) and centrifuging at $23000 \times g$ at 4°C for 4 min (following Clarke et al. [8]). Subsequently, the enzymes in these fractions were stabilized in 0.1 M potassium phosphate/1.0 mM EDTA/2 mM dithioerythritol/0.1 mM fructose 1,6-bisphosphate/0.1 mM ATP (pH 7.5). The pellet was extracted three times with the stabilization buffer to ensure complete removal of the enzymes. This method has the disadvantage of markedly decreasing the protein concentration and the ionic strength in the vicinity of the enzymes during the initial extraction. Nonetheless, when this method for separating bound and soluble enzymes is compared with techniques which do not share these disadvantages, similar tendencies are obtained [11], but see Ref. 13. Furthermore, recent studies using poly(ethylene glycol) to increase the effective concentration of muscle enzymes indicate that the glycolytic enzymes show similar trends in their tendency to pellet (equivalent to binding) with F-actin in the presence and absence of poly(ethylene glycol) [23]. However, their study clearly indicates that low ionic strength generally favors binding of enzymes to F-actin. Thus, the technique of Clarke et al. [8] probably overestimates physiological binding.

Enzyme activity measurements

To determine enzyme localization and the recovery of enzyme activity, enzyme activities were measured in the particulate and unbound fractions as well as in the initial homogenate. Activity measurements were made in triplicate on a Varian Cary UV/Vis spectrophotometer. For aldolase, lactate dehydrogenase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase and creatine phosphokinase, the recovery of enzyme activity was $100 \pm 15\%$, but PFK consistently showed a lower recovery for fatigued muscles. For control muscles, the recovery of PFK activity in the particulate and soluble fractions was $93 \pm 11.3\%$ ($n = 6$) while fatigue significantly decreased this recovery to $73.6 \pm 10.9\%$ ($n = 7$) (Mann-Whitney U-test, $P < 0.02$). Given this decreased recovery of PFK, we express the binding data as the ratio of the activity in the supernatant to that in the pellet.

Assay conditions

Optimal assay conditions were determined for the enzymes from control muscles and are as follows:
Phosphofructokinase (PFK): 50 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 0.16 mM NADH, 2.5 mM ATP, 2.5 mM glutathione, 50 mM fructose-6-phosphate (pH 8.0) with excess levels of aldolase, triose-phosphate isomerase and 3-glycerolphosphate dehydrogenase.
Creatine phosphokinase: 80 mM glycine, 4.0 mM $MgCl_2$, 0.15 mM NADH, 5.0 mM ATP, 0.8 mM phospho*enol*pyruvate, 40 mM creatine (pH 9.0) with excess levels of pyruvate kinase and lactate dehydrogenase.
Pyruvate kinase: 50 mM Imidazole-HCl, 10 mM $MgCl_2$, 100 mM KCl, 0.16 mM NADH, 2.0 mM phospho*enol*-pyruvate, 6.0 mM ADP (pH 7.0) with excess levels of lactate dehydrogenase.
Aldolase: 50 mM triethanolamine-HCl, 0.15 mM NADH, 20 mM fructose 1,6-bisphosphate (pH 7.6), excess levels of triose phosphate isomerase and 3-glycerolphosphate dehydrogenase.
Lactate dehydrogenase: 100 mM potassium phosphate, 0.2 mM NADH and 20 mM pyruvate (pH 7.0).
Glyceraldehyde-3-phosphate dehydrogenase: 100 mM Tris-HCl, 3.3 mM cysteine, 20 mM NaF, 25 mM Na_2HAsO_4, 0.7 mM NAD and 3.0 mM glyceraldehyde-3-phosphate (pH 8.5).

Biochemicals and chemicals were from Sigma (St. Louis, MO). Statistical comparisons were carried out using the Mann-Whitney U-test.

Results and Discussion

In *R. pipiens* gastrocnemius, in vitro fatigue markedly changes the localization of several glycolytic enzymes. Fatigue leads to a 10-fold decrease in the proportion of soluble phosphofructokinase, while for the other glycolytic enzymes except aldolase, it increases the proportion of soluble enzyme (Fig. 1). Time required to fatigue did not affect the changes in enzyme localization. Since fatigue in frog muscles is accompanied by a decrease in intracellular pH [24], extraction at a relatively alkaline pH could have altered the distribution of the enzymes in the fatigued muscles. Thus, we compared the localization of enzymes in control muscles extracted at pH 7.5 and in fatigued muscles extracted at pH 6.5. Even under these conditions, fatigue increases the binding of PFK while tending to decrease that of the other glycolytic enzymes except aldolase (Fig. 2).

These results can be interpreted in two ways, either in terms of the activation of glycolysis which preceded the fatigue, or in terms of events causing the fatigue. In the first perspective, the increased binding of PFK corresponds to the pattern observed in mammalian and fish muscle during activation of muscle glycolysis [10,12,13]. However, the decreased binding of the other glycolytic enzymes does not correspond to this pattern. Given that the technique we used generally overestimates binding of the glycolytic enzymes [13], the decreases in binding which we observed are probably significant. Thus, we conclude that fatigue leads to

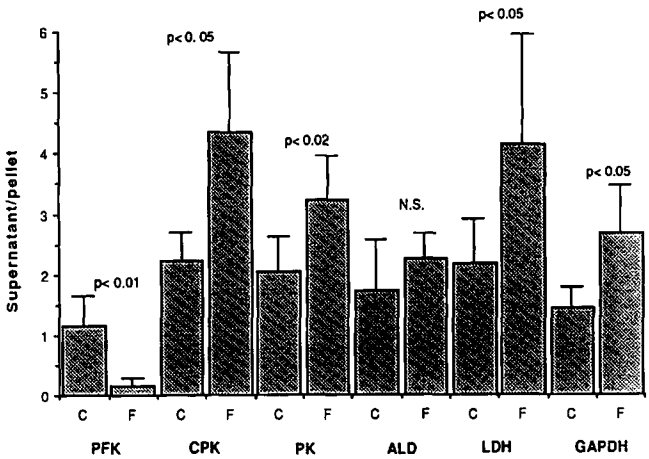

Fig. 1. Changes in enzyme localization with fatigue in isolated *R. pipiens* gastrocnemius muscle. The muscles were stimulated and extracted as described in Methods. The ratio of the enzyme activity in the supernatant and that in the pellet is plotted for each enzyme under study. C denotes the control muscle, while F indicates the fatigued muscle. Phosphofructokinase is PFK, creatine phosphokinase is CPK, pyruvate kinase is PK, aldolase is ALD, lactate dehydrogenase is LDH and glyceraldehyde-3-phosphate dehydrogenase is GAPDH. The probability values indicate whether differences between adjacent columns are significant according to the Mann-Whitney *U*-test.

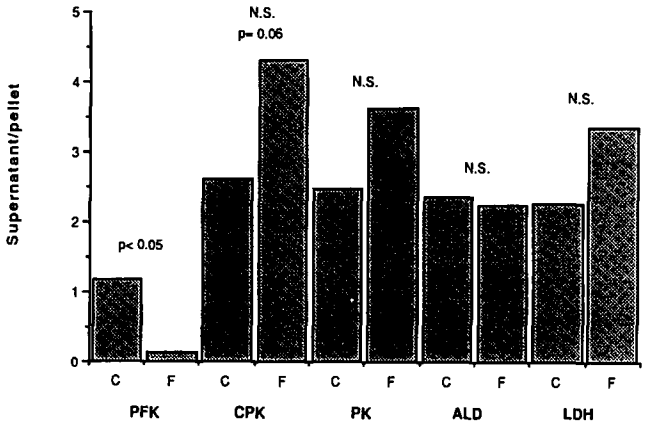

Fig. 2. The effect of fatigue on the localization of glycolytic enzymes in the isolated gastrocnemius muscle of *R. pipiens*. Muscles were isolated and stimulated as in Fig. 1, but the extraction was carried out at pH 6.5. Ratios of enzyme activity, enzyme names, muscle status and the probability of a significant effect of fatigue are indicated as in Fig. 1.

opposite changes in the localization of PFK and the other glycolytic enzymes.

A likely cause of these shifts in enzyme localization is declining pH. Effectively, PFK increases its binding to actin as pH is decreased from 7.5 to 6.7 [25]. Exercise increases the phosphorylation of PFK [26], thus increasing its affinity for actin [27,28]. Maintenance of the phosphorylation of PFK in fatigued frog muscle could explain its high binding. However, as the decline in tension development with fatigue is correlated with several metabolic variables [24], changes in other metabolites may also regulate these binding phenomena [13] as well as directly regulating the activity of PFK and other enzymes [15]. The intermediate changes in the binding of aldolase may reflect its association both with PFK [29], which remains bound in fatigued muscle, and with glyceraldehyde-3-phosphate dehydrogenase [30], which decreases its binding with fatigue.

As indicated in Methods, the recovery of PFK in the supernatant and pellet from the fatigued muscle was 20% lower than in the control muscle. If this decreased yield occurred in one fraction, expressing the localization data as a ratio could distort the changes. When the data are expressed as the percent of the activity recovered, fatigue increases the percent bound from $48.83 \pm 9.75\%$ ($n = 7$) to $88.44 \pm 7.15\%$ ($n = 7$; $P < 0.005$; Mann-Whitney *U*-test). Expressing the data in terms of the activity in the homogenate increases the variability, but the increase in binding of PFK with fatigue is still significant (from $46.12 \pm 14.37\%$ in the control to $68.52 \pm 10.13\%$ in the fatigued muscle) ($P < 0.05$; Mann-Whitney *U* test). Thus, the increased binding of PFK with fatigue remains significant with all methods used for expressing the data.

The fatigued muscles from individual frogs had 76.7 $\pm$ 23.8% of the PFK activity of the corresponding con-

trol muscles. Fatigue did not alter the specific activities of any of the other enzymes we examined. Since the levels of PFK in the gastrocnemius varied considerably among individual frogs, this tendency is apparent only when the PFK activity in the fatigued muscle is expressed as a percent of that in the corresponding control muscle. We found no difference in the activity of PFK in the two, non-exercised, gastrocnemius muscles of any frog. A fatigue-induced decrease in pH [31] may have led to dissociation and inactivation of PFK as occurs during in vitro acidification of rabbit skeletal muscle PFK [32] and of rat heart PFK [33]. In rat heart, ischemia leads to a similar loss of PFK activity and alkalinization of the extracts of ischemic rat heart recovers only 86% of the activity [34]. In our experiments, the extracts were consistently placed in the alkaline stabilization buffer before activity measurements. The decrease in PFK activity in our extracts of fatigued frog muscles may be due to such a partial recovery following realkalinization. If inactive dimers of PFK were more tightly bound to the particulate fraction than tetramers, the decreased recovery of PFK from fatigued muscles could also be explained.

In summary, our results clearly indicate that fatigue increases the binding of PFK to the particulate fraction while decreasing that of the other glycolytic enzymes. In contrast, as metabolic conditions which activate muscle glycolysis increase the binding of several glycolytic enzymes (PFK and aldolase) and do not change the binding of the others, activation of muscle glycolysis is considered to increase the binding of the glycolytic enzymes [7,8,10,12]. The metabolic depression occurring with anoxia in the whelk, *Busycon canaliculatum*, is accompanied by decreased binding of all the glycolytic enzymes [11]. These results are consistent with the concept that control of glycolysis involves changes in enzyme localization. Our results indicate that during muscle fatigue, the concerted binding of glycolytic enzymes to structural proteins is disrupted.

Acknowledgements

This study was supported by a grant from NSERC to H.G. and benefited from constructive advice from A. Demers.

References

1 Dölken, G., Leisner, E. and Pette, D. (1975) Histochemistry 43, 113–121.
2 Clarke, F.M. and Masters, C.J. (1975) Biochim. Biophys. Acta 481, 37–46.
3 Clarke, F.M. and Morton, D.J. (1976) Biochem. J. 159, 797–798.
4 Knull, H.R. (1978) Biochim. Biophys. Acta 522, 1–9.
5 Clarke, F.M., Shaw, F.D. and Morton, D.J. (1980) Biochem. J. 186, 105–109.
6 Bronstein, W.W. and Knull, H.R. (1981) Can. J. Biochem. 59, 494–499.
7 Walsh, T.P., Masters, C.J., Morton, D.J. and Clarke, F.M. (1981) Biochim. Biophys. Acta 675, 29–39.
8 Clarke, F.M., Stephan, P., Huxham, G., Hamilton, D. and Morton, D.J. (1984) Eur. J. Biochem. 138, 643–649.
9 Masters, C. (1984) J. Cell Biol. 99, 222s–225s.
10 Clarke, F., Stephan, P., Morton, D. and Weidemann, J. (1985) Regulation of Carbohydrate Metabolism, Vol. II (Beitner, R., ed.), pp. 1–31, CRC Press, Boca Raton.
11 Plaxton, W.C. and Storey, K.B. (1986) J. Comp. Physiol. B 156, 634–640.
12 Brooks, S.P.J. and Storey, K.B. (1988) Am. J. Physiol. 255, R289–R294.
13 Brooks, S.P.J. and Storey, K.B. (1988) Arch. Biochem. Biophys. 267, 13–22.
14 Yamada, T. and Sugi, H. (1987) Biochim. Biophys. Acta 931, 170–174.
15 Spriet, L.L., Söderlund, K., Bergström, M. and Hultman, E. (1987) J. Appl. Physiol. 62, 616–621.
16 Spamer, C. and Pette, D. (1979) Histochemistry 60, 9–19.
17 Spurway, N.C. (1981) Histochem. J. 13, 269–317.
18 Engel, W.K. and Irwin, R.L. (1967) Am. J. Physiol. 213, 511–518.
19 Putnam, R.W. and Bennett, A.F. (1983) Am. J. Physiol. 244, R558–R567.
20 Spurway, N.C. (1985) IRCS Med. Sci. 13, 78–79.
21 Putnam, R.W. (1979) J. Exp. Biol. 82, 35–51.
22 Boutilier, R.G., Emilio, M.G. and Shelton, G. (1986) J. Exp. Biol. 122, 223–235.
23 Walsh, J.L. and Knull, H.R. (1988) Biochim. Biophys. Acta 952, 83–91.
24 Mainwood, G.W. and Renaud, J.M. (1985) Can. J. Physiol. Pharmacol. 63, 403–416.
25 Roberts, S.J. and Somero, G.N. (1987) Biochemistry 26, 3437–3442.
26 Hofer, H.W. and Sörensen-Ziganke, B. (1979) Biochem. Biophys. Res. Commun. 90, 199–203.
27 Luther, M.A. and Lee, J.C. (1986) J. Biol. Chem. 261, 1753–1759.
28 Kuo, H.J., Malencik, D.A., Liou, R.-S. and Anderson, S.R. (1986) Biochemistry 25, 1278–1285.
29 Orosz, F., Christova, T.Y. and Ovadi, J. (1987) Biochem. Biophys. Res. Commun. 147, 1121–1128.
30 Ovádi, J., Salerno, C., Keleti, T. and Fasella, P. (1978) Eur. J. Biochem. 90, 499–503.
31 Hill, A.V. (1955) Proc. R. Soc. Lond. B. Biol. Sci. 144, 1–22.
32 Bock, P.E. and Frieden, C. (1974) Biochemistry 13, 4191–4196.
33 Hand, S.C. and Carpenter, J.F. (1986) Am. J. Physiol. 250, R505–R511.
34 Carpenter, J.F. and Hand, S.C. (1986) Am. J. Physiol. 250, R512–R518.