Soluble and Insoluble Phosphofructokinase in Rat Muscle

By W. M. POON and T. WOOD

Department of Biochemistry, McGill University, Montreal, Que., Canada

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When a clear supernatant, prepared by centrifuging a homogenate of rat skeletal muscle for 20 min. at 74 000*g*, was incubated with D-glucose 6-phosphate, ATP and NAD⁺ in the presence of nicotinamide, usually lactic acid was formed and fructose diphosphate and other intermediates accumulated. On some occasions, however, no lactic acid was produced and paper chromatography (Wood, 1961) showed the presence of glucose 1-phosphate and fructose 6-phosphate, but fructose diphosphate or any other intermediates lower in the glycolysis pathway could not be detected. These latter results suggested that phosphofructokinase was absent from the extract and to investigate this a series of assays of this enzyme was performed on the whole homogenate and on the fluid obtained after centrifugation and filtration through Millipore membranes.

Methods. Adult male Wistar rats (250–300*g*.) were starved overnight and lightly anaesthetized by intraperitoneal injection of sodium pentothal (5 mg./100*g*.). A few minutes later, Myanesin (mephensin, 3-*o*-toloxypropane-1,2-diol, 15–50 mg./100*g*.) was injected intraperitoneally until the limbs were completely relaxed and flaccid. After 10 min. the rat was killed by cervical dislocation. These rats are referred to as 'non-stimulated'. If kicking of the hind legs was observed at death, the rat was rejected. The treatment with the muscle-relaxant Myanesin was omitted with a second group of animals, which kicked and convulsed when killed. These rats are referred to as 'stimulated'. Muscle (6.0*g*.) was dissected from the hind limbs and homogenized in the 50 ml. stainless-steel container of a Lourdes Multi-Mixer (Lourdes Instrument Corp., Old Bethpage, N.Y., U.S.A.) for 2 min. at 0° in 25 ml. of 40 mM-nicotinamide or other extraction medium. This part of the procedure was completed 30 min. after the death of the animal. The homogenate was centrifuged at 3600*g* for 20 min. at 2°. The supernatant was ready 1 hr. after the death of the animal and was filtered through two Millipore filters (a 220 m μ followed by one of 100 m μ pore diameter) to give a perfectly clear fluid. The filtrate was available 1½ hr. after the death of the animal and assays of the homogenate, supernatant and filtrate were begun ½ hr. later. The assay system was similar to that described by Boxer & Shonk (1964).

Results and discussion. The results (Table 1) show that from 7 to 49% of the activity of a nicotinamide homogenate may appear in a soluble form in the filtrate. Nicotinamide solution was used as a homogenizing medium because its presence was required as an inhibitor of NAD breakdown in subsequent glycolysis experiments. However, replacement by imidazole buffer at pH 6.6 or 7.0 gave similar results. With a medium of pH 8.5 as much as 150% of the activity in the homogenate was recovered in the filtrate. No special significance was attached to recoveries greater than 100% since Paetkau & Lardy (1967) have clearly demonstrated the strong dependence of the activity of pure rabbit muscle phosphofructokinase on its precise physico-chemical environment. Nevertheless it is clear that at pH 8.2 no appreciable fraction of the enzyme was in an insoluble form.

Our results at pH 6.5 bear a superficial resemblance to those reported by Ohlmeyer & Grill (1962), who found that for 3 hr.-old ox muscle and for fresh frog muscle variable amounts of the total phosphofructokinase of a water homogenate could be sedimented at 1200*g* for 20 min. and that when the muscle was more than 1 day old all the enzyme was sedimented. Both Opie & Newsholme (1967) and Margreth, Catani & Schiaffino (1967) found a considerable part of the phosphofructokinase activity of a homogenate of frog muscle prepared at pH 8.0–8.2 to be in an insoluble form, whereas in rat muscle we find all the activity in the filtrate at this pH.

In Table 1 a general trend can be distinguished for the homogenates prepared at the lower pH values to have a lower measurable phosphofructokinase activity than those prepared at a higher pH. Mansour (1965) showed that incubation for 10 min. at pH values below 6.5 caused a reversible inactivation of partially purified phosphofructokinase from guinea-pig heart. Both he and Paetkau & Lardy (1967) concluded that at a low pH the enzyme became dissociated into inactive sub-units, which reaggregated to an active form when the pH was raised in the presence of adenine nucleotides. It is unlikely that the presence of insoluble phosphofructokinase in homogenates prepared at pH 6.6–7.0 can be attributed to an effect of pH alone, in view of the nearness of the pH values to the physiological pH and the large variability in the percentage of

Table 1. *Phosphofructokinase in homogenates of rat skeletal muscle before and after centrifugation and filtration*

Muscle was homogenized in: (a) 40 mM-nicotinamide, pH 6.8; (b) 50 mM-imidazole buffer, pH 6.6; (c) 50 mM-imidazole buffer, pH 7.0; (d) 50 mM-tris-chloride, pH 8.5. The assay mixture contained (final concentrations) 100 mM-imidazole buffer, pH 7.5, 10 mM-EDTA, 13 mM-MgCl₂, 130 mM-KCl, 2.5 mM-ATP, 3.5 mM-D-fructose 6-phosphate, 0.13 mM-NADH, 0.9 unit of aldolase, 0.7 unit of glycerol 1-phosphate dehydrogenase and 3.9 units of triose phosphate isomerase. The reaction was started by adding 0.01 ml. of the sample diluted five- or 25-fold with bovine serum albumin (1 mg./ml.) and the rate of change in extinction at 340 mμ was measured. n.d., Not determined.

Physiological state	Enzyme activity (μmoles/min./g. of muscle)			Soluble enzyme (% of total) $\left(\frac{F}{H} \times 100\right)$
	Homogenate (H)	3600g Supernatant (S)	Filtrate (F)	
(a) Non-stimulated	10.1	4.0	1.1	10
Stimulated	11.8	3.2	1.0	8.5
Non-stimulated	25.8 (pH 6.6)	n.d.	1.7 (pH 6.7)	7.0
Stimulated	27.4 (pH 6.6)	n.d.	6.2 (pH 6.7)	23
Stimulated	29.2	n.d.	11.2	38
Non-stimulated	32.2 (pH 6.8)	40.3 (pH 6.5)	15.7 (pH 6.5)	49
(b) Stimulated	10.7 (pH 6.9)	6.9 (pH 6.8)	2.2 (pH 6.8)	21
Non-stimulated	25.2 (pH 6.9)	4.0 (pH 6.9)	5.0 (pH 6.8)	20
Non-stimulated	60.3 (pH 6.8)	13.3 (pH 6.5)	18.1 (pH 6.5)	30
(c) Stimulated*	40.4 (pH 7.1)	16.1 (pH 6.9)	16.1 (pH 6.9)	40
Non-stimulated	46.7 (pH 7.4)	35.0 (pH 7.0)	16.9 (pH 6.7)	36
Non-stimulated	49.0 (pH 7.2)	15.3 (pH 7.0)	23.7 (pH 6.9)	48
(d) Stimulated	62.5 (pH 8.2)	97.5	92.5 (pH 8.2)	148
Non-stimulated	80.1 (pH 8.2)	105.0	90.5 (pH 8.2)	110
Stimulated	84.0 (pH 8.2)	80.0	96.0 (pH 8.2)	112

* Rat exercised for 45 min. on a treadmill before death.

soluble enzyme in homogenates of almost the same pH value. Although the pH₁ of the extraction medium appears to be important, there must be other factors that determine whether the enzyme is in a soluble or an insoluble form. An attempt was made to find a correlation with the physiological state of the muscle at death, but no relation could be observed between the percentage of enzyme in the filtrate and whether the muscle was 'stimulated' or 'non-stimulated'. It is possible that the results at pH 6.6–7.0 represent different speeds of change of physical state of phosphofructokinase, which was initially in either a completely soluble or in a completely insoluble form, during the 1½ hr. period of experimental manipulation. However, the few time-dependent changes of this type that have been reported take place over periods of 5–10 hr. (Lardy, 1965; Pette & Hofer, 1965).

The solubility of phosphofructokinase at pH 8.0 in homogenates of contractile tissues has been investigated by Mansour and his co-workers (Mansour, 1965; Mansour, Wakid & Sprouse, 1966), who concluded that the enzyme could exist in an insoluble inactive form, in a soluble active form, or as a mixture of both forms. They showed that incubation with adenine nucleotides, fructose diphosphate and Mg²⁺ could activate and solubilize the inactive insoluble form of phosphofructokinase. We

made a number of attempts to reactivate the enzyme by incubating the unwashed 3600g sediment with 50 mM-tris-chloride buffer, pH 8.5, containing MgSO₄ (50 mM), ATP (0.5 mM) and dithiothreitol (5 mM) (Mansour *et al.* 1966). No evidence of reactivation was obtained and in eight experiments the activity of the residue remained constant in the range 10.1–13.6 μmoles/min./g.

The precise significance of the above findings is not clear, but it is suggested that the results are due to the interruption of a process occurring at physiological pH in the intact muscle in which an insoluble form of phosphofructokinase of low activity is reversibly converted into a highly active soluble form. Some evidence for this hypothesis can be drawn from Table 1, since at a given pH a high percentage of soluble enzyme appears to be accompanied by a high activity of the original homogenate. The factors controlling this process are not known, but the activity of the muscle just before death does not appear to be one of them.

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