Studies with a Reconstituted Muscle Glycolytic System

THE ANAEROBIC GLYCOLYTIC RESPONSE TO SIMULATED TETANIC CONTRACTION

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By using a reconstituted glycolytic system and a highly active adenosine triphosphatase (ATPase), the metabolism during muscular tetanic contraction was simulated and observed. With an ATPase activity somewhat greater than can be maintained in muscle tissue, phosphocreatine was rapidly and completely utilized, lactate production commenced about 5 s after the ATPase was added and after 15 s adenine nucleotides were lost through deamination to IMP. By 40 s, all metabolism ceased because of complete loss of adenine mononucleotides. With a lower ATPase activity, glycolytic regeneration of ATP was capable of maintaining the ATP concentration at its initial value and even by 80 s, only one-half of the phosphocreatine had been utilized. No deamination occurred in this time. It is suggested that the metabolic events observed in the simulated system are basically the same as occur in muscle doing heavy work.

A previous publication (Scopes, 1973) described a glycolytic system reconstituted from purified enzymes which mimicked glycolytic metabolism in muscle. It was capable of establishing 'resting state' concentrations of metabolites, in particular phosphocreatine, comparable with those observed *in vivo*. Glycolysis ceased after maximum phosphocreatine concentrations had been established provided there was no ADP-producing system present; in the presence of low concentrations of ATPase† it was demonstrated that steady-state concentrations of phosphocreatine were lower.

There is a great variety of experiments that can be carried out with this reconstituted glycolytic system. For instance, it can be used to investigate the glycolytic response to various stimuli, such as the splitting of ATP as in contracting muscle, the effect of known natural inhibitors such as citrate, and the behaviour of the glycolytic complex at different temperatures and pH values. The present paper illustrates the response of the system to sudden very high ATPase activities, such as occur in muscle in tetanic contraction, or in conditions of maximum work output. It is well known that the first response is regeneration of ATP by phosphocreatine, which is so fast that a loss in ATP is undetectable *in vivo*, unless creatine kinase (EC 2.7.3.2) is first inactivated (Maréchal & Mommaerts, 1963; Infante *et al.*, 1964). After a substantial proportion of the phosphocreatine has been lost, glycolytic and oxidative regeneration of ATP can sustain the ATP concentration for a considerable

time (Hohorst *et al.*, 1962; Wilson *et al.*, 1967). Quantitative estimates of ATP turnover are difficult to obtain in these circumstances as the bloodstream carries away metabolic products. Eventually adenine nucleotides are lost through deamination by AMP deaminase (EC 3.5.4.6) to IMP (Lange, 1955; Jones & Murray, 1957; Lowenstein, 1972). Whether or not any deamination occurs depends on the intensity and duration of the ATP turnover.

The two experiments described here demonstrate the response both to a very high ATPase activity, which was beyond the capabilities of the system to keep up with, and a somewhat lower activity which was within the system's capabilities at least for the period investigated. The first case simulated development of 'exhaustion' or 'muscle fatigue', the second was a fair approximation to a burst of heavy work output, except for the absence of any oxidative phosphorylation or removal of metabolic products which occur *in vivo*.

Materials and Methods

The sources of chemicals and enzymes were as described previously (Scopes, 1973). The only additional enzyme used in these experiments was AMP deaminase, prepared from rabbit muscle by the method of Smiley *et al.* (1967), with minor modifications. These were that all buffers subsequent to the extracting solution contained 10 mM-potassium phosphate, pH 6.5, and 10 mM-mercaptoethanol. Recovery was as high as 80% and the specific activity, measured in the presence of a cacodylate buffer, pH 6.5, containing 0.15 M-KCl and 1 mM-AMP, was up to

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† Abbreviation: ATPase, adenosine triphosphatase.

1500 $\mu\text{mol}/\text{min}$ per mg at 30°C. AMP deaminase was included in the mixture at an activity (after mixing with all reagents) of 70 units/ml. The enzyme solutions and other reagents were prepared, stored and mixed as described previously. However, to cope with the high rates of glycolysis, the amounts of phosphorylase and phosphofructokinase were doubled to 100 units/ml (measured at 30°C).

In addition to the compounds listed previously, the final glycolysing mixture contained 30mM-histidine and 40mM extra acetate as additional buffer. Preparation and analysis of extracts of the glycolysing mixtures were carried out as described previously (Scopes, 1972a,b). However, as potato ATPase is not completely removed or inactivated by HClO_4 precipitation (Traverso-Cori *et al.*, 1965; Wood, 1971), extracts after neutralization rapidly lost any ATP and ADP that was in them, and so gave spuriously high AMP and P_i values. It was found that if the extracts were adjusted with 0.5M-KOH containing 20mM- K_2CO_3 to a pH around 10.5, with Nile Blue as indicator, together with EDTA at a final concentration of 1mM, any surviving ATPase was inactivated. Nevertheless despite adopting this procedure, in the first experiment where there were 120 units of ATPase/ml the estimates of AMP were somewhat variable and so have not been recorded.

The percentage of inosine nucleotide in the extracts was calculated as described previously from u.v. measurements (Newbold & Scopes, 1967). Samples were diluted automatically with the aid of the Technicon AutoAnalyzer, and passed through a 5mm path-length silica flow-cell positioned in a Unicam SP.800 spectrophotometer equipped with scale-expansion apparatus and recorder.

Accumulated ATPase activity was calculated from the analysis of the metabolites, nucleotides and phosphocreatine by using the following expression:

$$\text{'ATPase'} = 1\frac{1}{2} (\text{lactate} + \text{pyruvate}) + \frac{1}{2} (\text{phosphoglycerates}) - (\text{fructose diphosphate} + \text{ADP} + \text{phosphocreatine}) - \frac{1}{2} (\text{glycerol 3-phosphate}) - 2 \text{ATP}$$

and at time t , accumulated ATPase = 'ATPase' at time t minus 'ATPase' at time zero.

In these calculations only the phosphocreatine, ATP and lactate concentrations made major contributions. Similar calculations have been made in previous work (Newbold & Scopes, 1971a,b).

Results

In the first experiment to be described, before adding the ATPase, an 'initial steady state' was established with approximately 85% of the creatine phosphorylated. During muscular activity, phosphorylase α appears rapidly, reaching a maximum of about 40% of the total phosphorylase (Stull & Mayer, 1971). Accordingly, the phosphorylase used in this experiment was 40% in the α form. Since it was of

interest to note closely when lactate began to appear after adding the ATPase, the initial lactate concentration was minimized by starting with a high concentration of phosphocreatine. The starting conditions were as before with 30mM of both creatine and P_i , except that 24mM of each was in the form of phosphocreatine (disodium salt). In addition, the buffer pH was somewhat lower, so that at time zero the pH was 7.25.

Before adding the ATPase, the complete mixture was incubated at 37°C for 5 min, then placed in ice. Samples (200 μl) were transferred to small test tubes pre-warmed at 37°C, incubated again for exactly 5 min, then 2 μl of the ATPase solution (13000 units/ml, activity measured at 37°C) was rapidly mixed in. After a suitable interval the reaction was stopped with HClO_4 as described previously (Scopes, 1973). Three separate batches were investigated during the course of 1 day, covering a range of times after addition of ATPase from 3 to 60s. There was only very slight variation between batches, and results presented in Figs. 1 and 2 are means of results from the three batches.

Fig. 1(a) shows the changes in nucleotides during the first 60s and Fig. 1(b) the changes in phosphocreatine, P_i and lactate. For the first 8s, there were few

Fig. 1. Changes in nucleotides and metabolites in response to an ATP turnover of 130 $\mu\text{mol}/\text{ml}$ per min

Temperature was 37°C and phosphorylase was 40% in the α form. (a) $\circ$, ATP; $\triangle$, ADP; $\square$, IMP. (b) $\bullet$, Phosphocreatine; $\blacktriangle$, lactate; $\blacksquare$, P_i .

changes other than the decrease in phosphocreatine and increase in P_i ; in fact, the average rate of decrease of phosphocreatine was 110 mm/min, compared with 130 units of ATPase activity present/ml. Between 8 and 15 s, the depletion of phosphocreatine led to significant rises in ADP and AMP (AMP apparently reached a value of about 0.3 mm, but the results have not been included as they may be over-estimated; see the Materials and Methods section). These stimulated the glycolytic resynthesis of ATP, with lactate appearing; after 15 s, this was the sole source of regenerating ATP. However, because of the presence of AMP deaminase and the relatively high concentrations of AMP, IMP rapidly increased during this phase, with a parallel decrease in ATP. At about 40 s, virtually all adenine mononucleotides had been lost, and in the absence of ATP, ATPase and glycolytic activity ceased.

The calculated ATPase activity is shown in Fig. 2; it was linear up to 40 s, and the calculated value of 128 units/ml is in agreement with the known amount added. The fact that the ATPase activity did not decrease until the ATP concentration was well below 1 mm is explained by the low K_m of potato apyrase (0.12 mm for CaATP^{2-} ; the K_m for MgATP^{2-} is similar; Traverso-Cori *et al.*, 1965).

Changes in glycolytic intermediates not illustrated were variable during the metabolism. Hexose monophosphates increased to a maximum near 3 mm, then decreased; fructose diphosphate fluctuated around 1 mm. Pyruvate, phosphoglycerates and glycerol 3-P were in the range 1–2 mm and their values were taken into account when calculating ATPase activity. From the buffering capacity of the system it was calculated that the pH fell from 7.25 to about 6.5 during the glycolytic burst.

A normal muscle would not be able to sustain such a high ATPase activity for so long as in this first experiment. Even in tetanic contraction the ATPase

is probably little more than one-half this value (Wilson *et al.*, 1967; see below). To attempt a more realistic simulation, a second experiment was carried out. In this case, the hexose monophosphate production was at first through yeast hexokinase and glucose instead of phosphorylase, at the same time effectively acting as additional ATPase. When all added glucose had been consumed, phosphorylase (20% in the α form) and glycogen took over the hexose monophosphate production, and only the true ATPase remained. Unfortunately owing to an error in making up the hexokinase solution, the amount used was somewhat lower than intended, only 11 units/ml compared with 30 units of ATPase/ml. And so, starting with 8 mm-glucose, it took 45 s for all the glucose to be consumed (Fig. 3b); samples were not taken beyond 80 s. Nevertheless, this experiment demonstrated how at the relatively lower ATPase activity ($\frac{1}{2}$ that of

Fig. 2. Accumulated ATPase activity calculated from changes in metabolites

The amount of ATPase added at time zero was 130 units/ml. The accumulated ATPase activity, calculated as described in the Materials and Methods section, indicates 128 units/ml.

Fig. 3. Changes in metabolites in response to an ATP turnover of between 30 and 41 $\mu\text{mol/ml per min}$

Temperature was 37°C and phosphorylase was 20% in the α form. For other details see the text and the legend to Fig. 4. (a) ○, ATP; ●, phosphocreatine; ▲, lactate. (b) ▼, Hexose monophosphates; ▽, fructose diphosphate; ●, glucose.

the first experiment) anaerobic glycolysis was capable of maintaining the phosphocreatine concentration quite high while dealing with resynthesis of ATP (see Fig. 3a). Thus ADP and AMP concentrations were kept very low and no deamination had occurred by 80s, nor would it be expected to occur for some considerable time after. Hexose phosphate concentrations (Fig. 3b) were much lower than in the first experiment, a point which will be discussed below.

Calculated ATPase agreed with a rate of 41 units/ml up to 40s, followed by a slightly slower rate which fits the theoretical line in Fig. 4 for 30 units/ml (since the 11 units/ml ATP loss due to hexokinase action stopped after 45s).

Discussion

Studies of the metabolic response to tetanic contractions or otherwise heavily stimulated muscle have been made *in vivo* in cats, rats and frogs (Wilson *et al.*, 1967; Hohorst *et al.*, 1962; Wilkie, 1967). The frog experiments were mostly carried out close to 0°C, and so the metabolic responses were much slower. The overall results were that phosphocreatine was greatly depleted, lactate was produced and eventually adenine nucleotides were lost through deamination to IMP. Because of the variable nature of the experimental material and the obvious problems in taking a large number of samples, the precise sequence of the events has not been determined, but can be deduced by extrapolation. With the reconstituted glycolytic system these problems are overcome and a large number of samples from one experiment are possible, as well as reproducibility from repeated experiments.

Fig. 4. Accumulated ATPase activity calculated from changes in metabolites

The amount of ATPase added at time zero was 30 units/ml; additional 'ATPase' was acting in the first 40s owing to 11 units of hexokinase/ml. Accumulated ATPase activity, calculated as described in the Materials and Methods section, agrees with these values, namely 41 units/ml for the first 40s and 30 units/ml thereafter.

Moreover, the rate of ATP turnover can be controlled precisely by the addition of a known amount of ATPase; unfortunately it is not at present possible to stop the ATP splitting so easily. Of purified ATPases only potato 'apyrase' has a sufficiently high specific activity to enable simulation of heavy work in muscle; the natural muscle ATPases would obviously have to be added at a concentration comparable with their occurrence *in vivo* which, if it could be done, would make the mixture solid.

The results of the first experiment, in which the ATPase activity was approximately one-half the maximum sustainable rate of myofibrils, related to myofibrillar concentration in muscle (Bendall, 1961), demonstrated clearly and in more detail the behaviour found in studies *in vivo*. The enzyme creatine kinase was able to maintain the ATP concentration close to its initial value for a considerable period despite rapid ATP turnover. Lactate production commenced at about 5s, indicating that glycolytic resynthesis of ATP was under way by this time. Finally, loss of adenine nucleotides occurred, which would represent 'exhaustion' *in vivo*; the muscle would in fact go into rigor after depletion of ATP. Recovery would be possible *in vivo* through regeneration of adenine nucleotides by the purine cycle (Lowenstein, 1972) in the absence of further ATPase activity.

The rapid depletion of ATP resulted from a continuous ATP turnover of 130 μmol/min per ml, probably higher than occurs even in tetanic contraction. Wilson *et al.* (1967) give values which, not allowing for loss of lactate in the blood flow or any oxidative phosphorylation, can be used to calculate a minimum ATP turnover in tetanic contraction of 60 μmol/min per g. In the reconstituted system the maximum theoretical rate of glycolytic synthesis of ATP (from the contents of phosphorylase and phosphofructokinase present) is about 450 μmol/min per ml, but this could not occur without a substantial increase in AMP concentration and maintenance of a high pH. The AMP deaminase prevents the former, and the acidification owing to the ATP turnover prevents the latter. It is perhaps interesting to speculate on the reason for the presence of so much AMP deaminase in muscle. Apart from its functions as discussed by Lowenstein (1972), it would appear that in times of extreme muscular activity it prevents over-exertion. In the absence of deaminase, ADP and AMP concentrations would increase, enabling glycolysis to keep up with ATP demand. But because the bloodstream is relatively ineffective at high metabolic rates, the accumulating acidity in the muscle would probably cause irreversible damage to the muscle tissue. Thus an animal lacking AMP deaminase would be able to over-exert itself, and then not be able to recover. The presence of the enzyme leads to earlier exhaustion, but exhaustion at a metabolic state from which the muscle can recover.

In the second experiment the ATPase activity was three times lower. In this case the phosphocreatine again fell initially at the rate of the ATP splitting, but soon glycolytic resynthesis of ATP lessened the demand for phosphocreatine, and even at 80s there was still over one-half of the original phosphocreatine left. This result may have been influenced by the fact that most of the glycolytic throughput was due to hexokinase, which removed the requirements of AMP for the first step. Indeed, while glucose was still present, phosphorylase may have been partially inactivated (Wang *et al.*, 1965), as the low concentration of hexose monophosphates would suggest. Despite these low concentrations of hexose monophosphates and the presumed low concentration of AMP, phosphofructokinase was apparently not greatly restricted, for fructose diphosphate concentrations were not insignificant (0.2–0.3 mM), and phosphoglycerates were at similar concentrations. This suggests that the limitation on lactate production was the rate of availability of ADP, not the phosphofructokinase step; there is no clear explanation why phosphofructokinase was not inhibited more; possibly the presence of free glucose activates under these conditions.

In conclusion, the reconstituted system behaves overall in the same fashion as glycolysis *in vivo* as far as its response to high ATP turnover is concerned. Assuming that the details are also representative of the living system, then these details can now be demonstrated. For instance there was an approximately 4–5 s delay before lactate production commenced; the initial rate of decrease in phosphocreatine concentration was rapid, but in the example where the ATP turnover was lower, glycolysis could maintain the ATP concentration without all the phosphocreatine

supply being utilized. Further experiments are suggested from the results, such as the response of the system containing different percentages of phosphorylase α , and the behaviour of the hypothetical 'AMP deaminase-deficient' animal described above.

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