ON LATENT HEXOKINASE ACTIVITY IN SKELETAL MUSCLE MITOCHONDRIA

James H. SOUTHARD
Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. 53706, USA

and

H.O. HULTIN
Department of Food Science and Technology, University of Massachusetts, Amherst, Mass. 01002, USA

Received 27 September 1971

1. Introduction

Hexokinase is associated with mitochondria in many tissues. A characteristic of the enzyme in some brain mitochondrial preparations is that total activity can only be assayed after treatments known to disrupt mitochondria, such as detergents, freeze-thaw procedures, osmotic shock, or ultrasonic energy [1–3]. The increase in activity after such treatment compared to that without treatment has been termed "latent" activity [2]. We report some experiments concerning latent hexokinase activity in skeletal muscle mitochondria. It is concluded that the latent activity may be developed by either fatty acid complexing agents or mitochondrial disruption, but that in either case the mechanism is related to the fact that the availability of substrate to the enzyme is limited in the native state.

2. Experimental

2.1. *Preparation of mitochondria*

Mitochondria were prepared by homogenizing 20 g of finely-chopped chicken Pectoralis major muscle in 150 ml of cold 0.25 M sucrose – 15 mM histidine (pH 7.0) solution for 25–30 sec at maximal speed. An additional 150 ml of the sucrose–histidine solution was added, and the homogenate was strained through cheesecloth. All operations were carried out at 0–4°. The filtrate was centrifuged for 15 min at 1600 rpm in the No. 269 rotor of an IEC model PR-6 centrifuge. The supernatant fraction was saved. The residue was resuspended twice in the homogenizing medium and centrifuged for 15 min as above but at 2100 rpm and 3000 rpm, and the supernatant fractions saved. The combined supernatant fractions were centrifuged for 1 hr at 10,000 rpm in a GSA rotor in a Sorvall RC-2 centrifuge. The residue was resuspended in 80 ml of the sucrose–histidine solution and centrifuged for 5 min at 2250 rpm in the SS-34 rotor in the Sorvall centrifuge. This residue was discarded. The supernatant fraction was centrifuged as above for 30 min at 11,000 rpm. The final reddish-brown pellet was resuspended in a volume of the sucrose–histidine solution to give a final concentration of 15–20 mg protein per ml. The control sample was, therefore, in isotonic (0.25 M) sucrose and 15 mM histidine. All substances added to develop latent hexokinase activity were in addition to the sucrose–histidine.

2.2. *Development of latent hexokinase activity*

The mitochondrial suspension was diluted 1:5 in the sucrose–histidine solution (control) or the solution to which the indicated agents were added, and then assayed. The mitochondrial suspension treated with ultrasonic oscillations was also in 0.25 M sucrose – 15 mM histidine solution. Energy was supplied with a Branson Sonifier Model S-75 at maximal power (2–4 amps) without frothing for 3 ten sec intervals with 30 sec pauses to eliminate temperature increase. The sample was kept in an ice-water bath during this treatment and was immediately assayed.

2.3. *Inhibition of hexokinase by fatty acids*

Fatty acids were dissolved in 0.25 M sucrose–15 mM histidine at an alkaline pH with the aid of ultrasonic oscillations, and the pH was then adjusted to 7.0. The quantities of fatty acids used were such as to give the final concentrations indicated. The mitochondrial suspension was diluted 1:5 either with this fatty acid solution or sucrose–histidine (control). They were then assayed for hexokinase. To study the prevention of fatty acid inhibition, the appropriate agents were incorporated into the diluting solutions, or ultrasonic energy was applied to the diluted mitochondrial suspension.

2.4. *Hexokinase assay*

Hexokinase activity was determined spectrophotometrically at 340 nm by coupling to the reduction of $NADP^+$ by glucose-6-phosphate dehydrogenase at 30° in a Beckman DU-2 spectrophotometer. The reaction medium contained 0.1 M glucose, 20 mM $MgCl_2$, 5 mM ATP, 0.1 mM $NADP^+$, 0.2 M Tris buffer, pH 7.4; and 0.5 units of glucose-6-phosphate dehydrogenase in a final volume of 1.0 ml.

3. Results

The summary of our results on the effect of various agents and conditions on the development of latent activity is presented in table 1. All samples were processed in isotonic sucrose (0.25 M); therefore the effects observed are not due to osmotic shock. The latent activity developed by the BSA was less than the other treatments. This difference was statistically significant ($p < 0.02$ compared to the Triton X-100 sample, the next highest one). With the skeletal muscle mitochondrial system, BSA has a positive effect on the development of hexokinase latency that is not simply due to osmotic lysis. The combination of Triton X-100, BSA, and treatment with ultrasonic oscillations did not increase the latent activity development over that of Triton X-100 or ultrasonic treatment alone, which is consistent with the results of Craven et al. [4] with brain mitochondria.

To test the hypothesis that this effect was due to prevention of fatty acid inhibition, we diluted the mitochondria in isotonic sucrose–histidine, pH 7.0, in the presence of oleate and octanoate prior to assay.

Table 1
Development of latent hexokinase activity in skeletal muscle mitochondria.

Treatment	Latent hexokinase activity developed
Control	100
1.3% Tween 80 in 0.1 N KCl	133 (8)
0.5% Triton X-100	118 (8)
Ultrasonics	123 (10)
1% BSA	111 (8)
0.5% Triton X-100 + 1% BSA + Ultrasonics	120 (3)

The activities are expressed as percentages of the control. The number in parentheses refers to the number of preparations assayed.

As shown in table 2, approximately 50% of the mitochondrial hexokinase activity could be inhibited by oleate. Octanoate had little effect; in fact at a concentration of 50 mM in the diluting medium, octanoate produced a slight stimulation. Triton X-100 was able

Table 2
Effect of fatty acid on mitochondrial hexokinase and reversal of inhibition by Triton X-100 and BSA.

Diluted in	Relative hexokinase activity
Control	100
Oleate (mM)	
1.0	80
5.0	66
10.0	52
50.0	53
Oleate (50 mM)	
+ addition of 0.5% Triton X-100	90
+ addition of 1.0% Triton X-100	100
+ addition of 1.0% BSA	73
+ ultrasonic treatment	40
Octanoate (mM)	
10.0	100
50.0	114

The control mitochondria were suspended in 0.25 M sucrose–15 mM histidine, pH 7.0, and all additions or treatment indicated were in this solution. The figures represent the averages of 2–4 preparations.

to completely reverse the inhibitory effect of oleate while BSA at a concentration of 1% was partially effective. Treatment with ultrasonic energy in the presence of oleate caused a small increase in inhibition over that produced by the oleate alone. If the excess oleate was removed by centrifugation and the mitochondria then treated with ultrasonic energy, still no release of the inhibition was observed (not shown in table).

4. Discussion

Craven et al. [4] diluted beef brain mitochondria in various reagents and found that dilution in the detergent Triton X-100 or 1% serum albumin developed the same amount of latent activity. They also observed that dilution with BSA had no further effect on development of latent hexokinase activity after the mitochondria had been treated by ultrasonic energy. On the basis of their results, they suggested that the development of latent hexokinase activity might be due to removal of the inhibition caused by free fatty acids released during the preparation of the mitochondria. However, these authors were not able to demonstrate fatty acid inhibition of their mitochondrial hexokinase. Wilson [2] had previously suggested that development of hexokinase activity required disruption of the mitochondria, and in a latter paper Kropp and Wilson [5] questioned the interpretation of Craven et al. They explained that a 1% BSA solution would be hypotonic and would, therefore, disrupt the mitochondria by an osmotic effect. They did, in fact, show that dilution in water exposed the same amount of hexokinase activity as did a 1% BSA solution.

We found an effect of BSA in our system that did not depend on osmotic lysis of the mitochondria. We were also able to demonstrate that oleate inhibited mitochondrial hexokinase activity and further that BSA could partially reverse the inhibition. On the basis of these results and the fact that BSA should not affect the structural integrity of mitochondria [4], it is reasonable to suggest that the development of latency by BSA is due to its known affinity for fatty acids.

Our results also strongly support the suggestion of Wilson (1967) that disruption of the mitochondria leads to the development of latent hexokinase activity, since treatment with ultrasonic energy released the latent hexokinase activity. This procedure is known to disrupt mitochondria [6], but it probably has no effect on a fatty acid-protein complex and in fact, we observed no release of inhibition on exposure of our fatty-acid-treated samples to ultrasonic oscillations.

We suggest that the development of latent hexokinase in skeletal muscle mitochondria can be achieved by different means, but that they are related to the fact that the availability of substrate to mitochondrial hexokinase is limited in the native state. This may be due to the location of hexokinase in the mitochondria [4, 5, 7, 8]. Structural alterations of the mitochondria allow substrate to reach the enzyme and thus "latent activity" in observed. BSA works by a different means, presumably by binding fatty acids, since it probably does not disrupt mitochondria. It seems likely that the BSA-mediated reversal of inhibition is not caused by an effect on hexokinase *per se* since disruption of the mitochondria completely removes the necessity of adding BSA. This indicates that the fatty acids are not directly inhibiting hexokinase, but that BSA removes some restriction on the movement of substrate in intact mitochondria caused by the fatty acids.

Acknowledgement

This work was supported in part by U.S. Public Health Service Grant GM-12064 from the National Institute of General Medical Sciences.

References

[1] D. Biesold and P. Teichgraber, Biochem. J. 103 (1967) 13C.
[2] J.E. Wilson, Biochem. Biophys. Res. Commun. 28 (1967) 123.
[3] J.E. Wilson, J. Biol. Chem. 243 (1967) 3640.
[4] P.A. Craven, P J. Goldblatt and R.E. Basford, Biochemistry 8 (1969) 3525.
[5] E.S. Kropp and J.E. Wilson, Biochem. Biophys. Res. Commun. 38 (1970) 74.
[6] A.N. Malviya, B. Parsa, R.E. Yodaiken and W.B. Elliot, Biochim. Biophys. Acta 162 (1968) 195.
[7] O. Spydevold and B. Borrebaek, Biochim. Biophys. Acta 167 (1968) 291.
[8] C.G. Vallejo, R. Marco and J. Sebastian, European J. Biochem. 14 (1970) 478.